Metabolic variations in canine overweight

Aspects of lipid metabolism in spontaneously overweight Labrador Retriever dogs

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Cover: Lean Labrador Retriever dogs, Specs and Wille, owner Anna Wistedt.
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Abstract

The prevalence of overweight in dogs is increasing. Canine overweight is associated with reduced quality of life, shorter life expectancy and metabolic variations such as insulin resistance and postprandial hyperlipidaemia. Previous metabolic research on overweight dogs has been performed mainly in fasting condition and studies on spontaneously overweight dogs using dynamic metabolic tests are scarce. This thesis therefore evaluated metabolic variations in spontaneously overweight dogs using a feed-challenge test.

Lean and overweight healthy Labrador Retriever dogs underwent a feed-challenge test. Blood and urine samples collected at fasting and after food intake were analysed with serum biochemistry, ELISA and metabolomics techniques. Multivariate and mixed model repeated measurements analyses were used to evaluate responses between body condition groups and/or between time points in the feed-challenge test.

Postprandial serum triglycerides were higher in prominently overweight dogs compared with lean, while no differences between groups were found at fasting. Only one fasted plasma phosphatidylcholine showed higher concentration in prominently overweight compared with lean dogs. Postprandial urine metabolomes, but not fasting metabolomes, distinguished between lean and overweight groups of dogs. Prominently overweight dogs had higher fasting urine cortisol/creatinine ratio than lean dogs, and overweight dogs showed signs of amino acid catabolism in postprandial urine. The acetylcarnitine response in overweight dogs indicated low fatty acid oxidation at fasting and metabolic inflexibility to food intake. Overweight dogs also showed lower carnitine and taurine status than lean dogs, potentially representing an interrelated insufficiency that could theoretically slow down lipid metabolism.

In conclusion, spontaneously overweight Labrador Retriever dogs displayed variations in metabolic parameters compared with lean dogs. Use of a feed-challenge test allowed detection of subtle metabolic variations not noticeable in fasted condition, emphasising the importance of using dynamic tests in metabolic research on canine overweight. Six parameters, all directly or indirectly associated with lipid metabolism, differed between overweight and lean dogs. In this thesis, the complexity of lipid metabolism in canine overweight was revealed by identifying previously known and new metabolic variations in spontaneously overweight Labrador Retriever dogs.

Keywords: Acetylcarnitine, feed-challenge test, LC-TOFMS, lipid metabolism, metabolic inflexibility, metabolomics, NMR, obesity, postprandial response.

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Dedication

To my dear family and friends <3

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Söder J.*, Wernersson S., Hagman R., Karlsson I., Malmlöf K., Höglund K. (2016). Metabolic and hormonal response to a feed-challenge test in lean and overweight dogs. *Journal of Veterinary Internal Medicine,* 30, pp. 574-582.

II Söder J.*, Hagman R., Dicksved J., Lindåse S., Malmlöf K., Agback P., Moazzami A.A., Höglund K., Wernersson S. (2017). The urine metabolome differs between lean and overweight Labrador Retriever dogs during a feed-challenge. *PloS one,* 12, pp. 0180086.

III Söder J.*, Höglund K., Dicksved J., Hagman R., Röhnisch HE., Moazzami A.A., Wernersson S. Plasma metabolomics reveals lower carnitine concentrations in overweight Labrador Retriever dogs. (Submitted).

IV Söder J., Wernersson S., Dicksved J., Hagman R., Östman J., Moazzami A.A., Höglund K. Indication of metabolic inflexibility to food intake in spontaneously overweight Labrador Retriever dogs. (Manuscript).

* Corresponding author

Related publications not included in the thesis

Hillström A., Hagman R., Söder J., Häggström J., Ljungvall I., Kjelgaard-Hansen M. (2015). Validation and application of a canine-specific automated high-sensitivity C-reactive protein assay. *Journal of Veterinary Diagnostic Investigation,* 27, pp. 182-190.

Raffan E., Dennis R.J., O'Donovan C.J., Becker J.M., Scott R.A., Smith S.P., Withers D.J., Wood C.J., Conci E., Clements D.N., Summers K.M., German A.J., Mellersh C.S., Arendt M..L., Iyemere VP., Withers E., Söder J., Wernersson S., Andersson G., Lindblad-Toh K., Yeo G.S.H., O'Rahilly S. (2016). A deletion in the canine *POMC* gene is associated with weight and appetite in obesity-prone Labrador Retriever dogs. *Cell Metabolism,* 23(5), pp. 893-900.

Muñoz-Prieto A., Nielsen L.R., Dąbrowski R., Bjørnvad C.R., Söder J., Lamy E., Monkeviciene I., Ljubić B.B., Vasiu I., Savich S., Busato F., Yilmaz Z., Bravo-Cantero A.F., Öhlund M., Lucena S., Zelvyte R., Aladrovic J., Lopez-Jornet P., Caldin M., Lavrador C., Karveliene B., Mrljak V., Mazeikiene J., Tvarijonaviciute A. (2018). European dog-owner perceptions of obesity and factors associated with human and canine obesity. *Scientific Reports,* 8, pp. 13353.

Abbreviations

ANOVA	Analysis of variance
AQuA	Automated quantification algorithm
BCS	Body condition score
BFI	Body fat index
BMI	Body mass index
CI	Confidence interval
CT	Computed tomography scan
CV-ANOVA	Cross-validated analysis of variance
DER	Daily energy requirements
DEXA	Dual energy X-ray absorptiometry
ELISA	Enzyme-linked immunosorbent assay
HDL	High-density lipoprotein
$HOMA_{IS}$	Homeostasis model of assessment for basal insulin sensitivity
LC-TOFMS	Liquid chromatography–time of flight mass spectrometry
LDL	Low-density lipoprotein
MR	Magnetic resonance
MS	Metabolic syndrome
NMR	Nuclear magnetic resonance
OPLS-DA	Orthogonal partial least-squares discriminance analysis
OPLS-EP	Orthogonal partial least-squares effect projection analysis
ORMD	Obesity-related metabolic dysfunctions
PCA	Principal component analysis
PCaa C38:4	Phosphatidylcholine with two ester bonds, 38 carbons and four double bonds
PLS-DA	Partial least-squares discriminant analysis
POMC gene	Pro-opiomelanocortin gene
SD	Standard deviation
SEM	Standard error of mean

VIP	Variable importance of projection
VLDL	Very high density lipoprotein
WHO	World Health Organization

1 Introduction

1.1 Increasing overweight in humans and in dogs

During the past 25 years, the prevalence of human obesity has doubled in many countries (Collaborators, 2017). Daily work has become less laborious and spare time more sedentary, in combination with intake of high-calorie dense food that has become more readily available (Kopelman, 2000). The current lifestyle probably makes a profound contribution to the obesity epidemic that is spreading among both children and adults (Abarca-Gómez *et al.*, 2017). Obesity is defined as excess body fat (body mass index (BMI) $\geq$30 kg/m^2) with negative health effects and it develops when energy intake exceeds energy expenditure (Grundy, 2004).

According to data from the World Health Organization (WHO), in 2016 the prevalence of overweight (BMI $\geq$25 kg/m^2) reached 40-50% in Europe and $\geq$50% in the USA. Worldwide, the WHO reports that on average, 39% of adults are overweight and 13% are obese (WHO, 2016) (Figure 1).

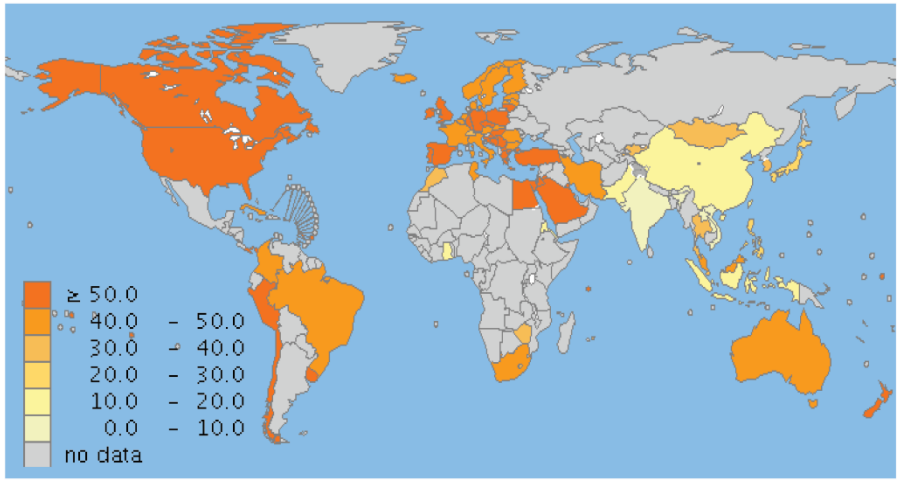

Figure 1. Overweight adults as a percentage of total adult population per country in 2016, where overweight is defined as body mass index ≥25 kg/m^2 (WHO, 2016). Diagram from http://apps.who.int/bmi/index.jsp.

Two prominent co-morbidities of human overweight are increased risk of cardiovascular disease and type 2 diabetes mellitus (Chandler *et al.*, 2017; Collaborators, 2017; Kopelman, 2007). In fact, type 2 diabetes is suspected to increase with overweight and with an older population, and is predicted to affect about 360 million people by 2030 (Wild *et al.*, 2004). Human obesity is classified as a disease and is one of the most feared threats to our future health (Kopelman, 2000).

Pet dogs are often seen as family members and largely share the lifestyle of their owners. The overweight dog population has increased in recent years, with a prevalence of up to 60% now being reported for some countries (German *et al.*, 2018; Corbee, 2013; Courcier *et al.*, 2010). In annual reports from Banifeld Pet Hospitals in the USA, the prevalence of overweight in dogs in general practice increased by almost 40% over the five years preceding 2016 and overweight was diagnosed in one-third of adult animal patients in 2016 (Banifeld, 2016).

Being overweight is the most common nutritional disorder in dogs. At a meeting of the World Small Animal Veterinary Association One Health (Atlanta, USA 2016), obesity in dogs was officially classified as a disease, as it is in humans (Kopelman, 2000). Canine overweight is a serious condition associated with severe health complications such as increased risk of chronic diseases early in life, a shortened life span and reduced quality of life (Adams *et al.*, 2018; Yam *et al.*, 2016; German *et al.*, 2012a; Kealy *et al.*, 2002).

While owner-dog relationships may be complicated, owners and dogs clearly influence each other's health. Overweight dog owners have been shown to be more likely to have overweight dogs (Nijland *et al.*, 2010; Kienzle *et al.*, 1998). On the other hand, dog owners may have a higher physical activity level than the general population (Christian *et al.*, 2013). In addition, dog owners have been shown to have a reduced risk of death in general, and death due to cardiovascular disease in particular, compared with people who do not own a dog (Mubanga *et al.*, 2017). A one-health perspective to combat human and dog obesity has been proposed by several research groups (Chandler *et al.*, 2017; Sandøe *et al.*, 2014; Day, 2010). In a survey-based study in 10 European countries, dog owners were asked for their opinions on obesity counselling in a one-health perspective, and almost 70% of around 3000 respondents were positive to collaborations between human and animal healthcare disciplines (Muñoz-Prieto *et al.*, 2018).

The true prevalence of canine overweight is difficult to determine. Previous studies have used various approaches, such as data from veterinary records, general veterinary estimations, body condition scoring at dog shows or dog shelters and dog-owner estimated body condition scoring (German *et al.*, 2018; Muñoz-Prieto *et al.*, 2018; Royal-Canine, 2017; Banifeld, 2016; Ricci *et al.*, 2007). These studies all suffer from potential selection bias and the results could also be influenced by the use of different scoring scales and personal scoring skills, as dog owners might underestimate the body condition score compared with veterinarians (Courcier *et al.*, 2011; White *et al.*, 2011). The prevalence of overweight dogs in Sweden is probably increasing, as reported in other countries. A study in 1999 reported 16% dog owner-estimated overweight in Swedish dogs (Sallander *et al.*, 2010), but the prevalence today is believed to be 30-50%, according to an online survey of dog owners and to general veterinary estimations, both studies performed in 2017 (Muñoz-Prieto *et al.*, 2018; Royal-Canine, 2017).

1.1.1 Evaluation of body composition in dogs

High body weight may correlate to high percentage body fat, but possibly also to large stature or large muscle mass. The actual body weight of an individual dog therefore needs to be assessed together with body condition score (BCS) and preferably in conjunction with a muscle mass score, especially in obese, ill or elderly dogs that are at risk of losing lean body mass (Baldwin *et al.*, 2010).

Morphometric assessments of body composition, such as dimensional evaluation and body condition scoring, are semi-quantitative techniques for assessing body fat content in dogs that are frequently used by healthcare professionals and by researchers. Dimensional evaluation involves

measurements of the animal's length and girth (Hawthorne *et al.*, 2005), but large breed differences need to be accounted for in dogs (Jeusette *et al.*, 2010). Different body condition scoring systems have been developed for dogs, using 1-5 and 1-9 point scoring scales. The 9-point body condition scale, where a score of 6-9 is defined as overweight, has been validated in dogs and the body fat percentage increases by approximately 5% with each additional point on the scale (Laflamme, 1997). The 5-point scale may be easier to use than the 9-point scale for non-health-care professionals such as dog owners. However, there is a risk of the body fat percentage estimated by this scale being inaccurate, or of dogs with slight overweight being classified as normal weight (Muñoz-Prieto *et al.*, 2018; Witzel *et al.*, 2014). Unfortunately, grossly obese dogs (>45% body fat) exist today and these dogs exceed the 9-point scoring scale. Therefore a body fat index (BFI) scoring system has been developed for assessment of normal weight to gross obesity in dogs (Witzel *et al.*, 2014). The 1-9 body condition scoring scale and body fat index both involve assessment of visual and palpable body characteristics known to correlate with total body fat percentage measured with a quantitative method (Witzel *et al.*, 2014; Laflamme, 1997).

Quantitative measurements of body composition, other than body weight, can be made using *e.g.* dual energy x-ray absorptiometry (DEXA), magnetic resonance (MR) or computed tomography (CT) scanning techniques. These techniques have the advantage of determining the exact composition of lean and adipose tissues in the body, but are quite costly and require sedatives or general anaesthesia to be performed accurately. The fat-cell hormone leptin has been analysed as an alternative of quantitative measurement of body composition in dogs (Ishioka *et al.*, 2007). This hormone has been shown to have a significant positive association with body fat content in dogs, does not seem to be influenced by gender or age and might be useful as a complement in the assessment of body composition in dogs (Ishioka *et al.*, 2007).

1.1.2 Risk factors for canine overweight

Risk factors for overweight in dogs can be divided into dog-related factors and owner-related factors. Dog-related factors such as breed, neutering and increasing age have been described as important drivers for overweight in several studies (Muñoz-Prieto *et al.*, 2018; Gossellin *et al.*, 2007; Colliard *et al.*, 2006; Lund *et al.*, 2006). The Labrador Retriever dog has often been described as greedy, with a great food interest, and this claim is supported by the recent discovery of a mutation in the pro-opiomelanocortin (POMC) gene (associated with greediness and increased adiposity), which is present in 22% of Labrador Retriever dogs (Raffan *et al.*, 2016).

Owner-related factors include low family income, increasing owner age and the owner's attitude to physical activity and healthy foods (Muñoz-Prieto *et al.*, 2018). The owner's perception of the dog's attitude to physical activity has also been shown to be important (Westgarth *et al.*, 2017). It has been shown that overweight dog owners are more likely than normal weight people to have overweight dogs (Nijland *et al.*, 2010). In a recent survey-based study in Europe that included Sweden, overweight in dog owners was not associated with overweight in their dogs, but owners' lifestyle and habits still clearly influenced the body composition of their dogs (Muñoz-Prieto *et al.*, 2018). That survey found that owners who spent little time with their dog, shared food with their dog, were smokers or did not believe obesity to be a disease were more likely to have overweight dogs. Interestingly, a high value of owner-estimated body fat in dogs was positively associated with a perception by owners that their dog became ill easily and was inversely associated with a perception that their dog was happy.

1.1.3 Treatment of canine overweight

Weight reduction in dogs is often challenging and involves energy restriction by using a diet designed for weight loss (German, 2016). Such diets are energy-restricted by having an increased fibre, water and air content, while still containing all the key nutritional factors in sufficient amounts (Hand *et al.*, 2010). The ideal weight of a particular dog can be estimated based on the obese weight in combination with the assigned BCS (German *et al.*, 2009). Dogs are then fed individually adjusted amounts of resting energy requirements according to the calculated ideal weight (Hand *et al.*, 2010).

Re-assessment and monitoring is important, as energy requirements are individual. Energy restriction aims for a 1-2% weight loss per week, which is considered safe for dogs (Laflamme *et al.*, 1997). Increased exercise is preferably included in the weight loss programme, to avoid a weight loss plateau and to preserve lean body mass (Vitger *et al.*, 2016). However, as many dogs suffer from other health problems, this is not always possible (Lund *et al.*, 2006). Weight loss in dogs is often challenging for pet dogs and for their owners. Owner commitment and good communication between veterinarian and dog owner are crucial factors for success in weight reduction. In a long-term study, it was shown that only 60% of overweight dogs reached their target ideal weight and of these, half re-gained weight (German *et al.*, 2012a; German *et al.*, 2012b). A large part of the dog population is suffering from overweight and this condition could be considered the most important metabolic disease of dogs today.

1.2 General metabolic parameters

Blood glucose increases after food intake, but the meal composition has been shown to affect the time to peak and the exact concentration of peak postprandial glucose concentration in dogs (Nguyen *et al.*, 1994). Insulin is an anabolic hormone that is secreted from β-cells in the pancreas under stimulation of glucose in the blood stream. The presence and binding of insulin mediates the incorporation of glucose transporters in the cell surface and glucose is then eliminated from the blood (Frayn, 2009). Glucagon is a catabolic hormone secreted from α-cells in the pancreas and is present in high concentrations in blood under fasting conditions. It mediates gluconeogenesis, lipid catabolism and hunger and through those actions keeps blood glucose and energy levels stable when no food is present (Frayn, 2009). Postprandial glucagon concentration in the blood stream decreases after intake of glucose, but may rise after intake of amino acids or lipids (Carr *et al.*, 2010).

Leptin is a fat-cell hormone that is released by adipocytes into the blood stream. Leptin has a role in the long-term regulation of energy intake, body weight and hunger (Frayn, 2009). Higher concentrations in overweight subjects compared with lean have been reported in humans and in dogs (Ishioka *et al.*, 2007; Friedman & Halaas, 1998). Adiponectin, another hormone derived from adipocytes, is involved in glucose homeostasis, fatty acid catabolism and energy homeostasis (Goldstein & Scalia, 2004). However, in contrast to leptin, adiponectin has been shown to decrease in obese humans (Matsubara *et al.*, 2002).

1.2.1 Lipid metabolism in dogs

Upon intake of dietary fat, triglycerides are hydrolysed by pancreatic lipase in the gut lumen and micelles are formed with gall salts and diffuse into the intestinal epithelial cells, *i.e*. enterocytes. Inside the enterocytes, triglycerides are reformed and, together with phospholipids and cholesterol, chylomicrons are created. Chylomicrons constitute one of several classes of lipoproteins and are responsible for transport of dietary lipids to adipose and muscle tissues, by secretion first into the lymphatic circulation and later into the blood circulation (Frayn, 2009). Triglycerides measured postprandially are mainly transported in chylomicrons and reflect dietary fat intake (Xenoulis & Steiner, 2010). Lipoprotein lipase in the capillaries of adipose tissue is activated by insulin and triglycerides are eliminated from plasma by incorporation into adipocytes (Frayn, 2009). Under situations of withheld food, such as in overnight fasting, lipid catabolism provides the main source of energy and free fatty acids are released from adipose tissue and enter the blood stream. Free fatty acid

concentrations in serum decline after insulin stimulation and food intake (Frayn, 2009).

Endogenously synthesised lipids are transported by other lipoprotein classes in the blood, namely very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Of these classes, VLDL is the lipoprotein that has triglycerides as the main lipid component in humans. In dogs, VLDL and LDL contribute equally to the plasma triglyceride content (Maldonado *et al.*, 2001). The content of the remaining lipoprotein classes consists mainly of different phospholipids or cholesterol esters. In dogs, the most abundant lipoprotein is HDL, which is the main carrier of phospholipids, cholesterol esters and free cholesterol, followed by LDL and VLDL. In contrast to humans, who have cholesterol-ester rich plasma, dogs have phospatidylcholine-rich plasma. In dog plasma, HDL carries more than 80% of the total lipid content, while this function is exerted by LDL in humans (Maldonado *et al.*, 2001).

Due to the absence of an enzyme (cholesteryl-ester transfer-protein enzyme) present in humans (Guyard-Dangremont *et al.*, 1998), HDL_2 molecules in dogs constantly acquire cholesterol esters and form HDL_1 molecules, which are unique to dogs. This lack of enzyme probably explains why cholesterol esters are not evenly distributed among lipoprotein fractions and why, unlike humans, dogs have much more efficient reverse cholesterol transport from peripheral tissue to the liver (Xenoulis & Steiner, 2010; Maldonado *et al.*, 2001).

In normal weight dogs, plasma phospholipid composition has been shown to be equal between all lipoprotein classes (Maldonado *et al.*, 2001), while data on lipoprotein changes in overweight dogs are inconsistent. In some studies, increased VLDL, LDL and HDL concentrations have been reported in overweight dogs, whereas overweight humans may instead show decreased HDL cholesterol concentration (Usui *et al.*, 2015; Xenoulis & Steiner, 2010; Yamka *et al.*, 2006; Jeusette *et al.*, 2005; Bailhache *et al.*, 2003).

1.2.2 Current knowledge of metabolic variations in canine overweight

In canine metabolic research, two main approaches are commonly used to study metabolic variations in overweight. In the first approach, dogs are exposed to either acute overfeeding, mostly not exceeding three months, or to chronic overfeeding (>3 months). Experimental set-ups are then often used and one dog breed is commonly included in the experiment. In the second approach, privately owned lean or overweight/obese dogs of various breeds are used for sample collection, but these dogs are seldom followed over longer periods unless undergoing weight reduction interventions (German, 2006) (Table 1).

The metabolism works in constant dynamic shifts between fasted and postprandial states and what is seen at fasting does not necessarily reflect what will appear in a challenge, such as a feed-challenge test or an oral lipid or sugar test (Morris *et al.*, 2015; Krug *et al.*, 2012; Pellis *et al.*, 2012) (Table 1). A meal challenge represents a dynamic test of the metabolism, a somewhat realistic situation that gives information on how the metabolism reacts to a meal. The background diet may influence metabolic parameters, but the postprandial plasma metabolome (*e.g.* the composition of different metabolites in plasma) in humans has been shown to be more stable and less dependent on the regular diet than the fasted metabolome (Karimpour *et al.*, 2016). In the past, researchers have mainly collected samples from dogs in fasted condition, but postprandial interventions have also been performed (Table 1).

Metabolic variations in canine overweight and obesity that have been commonly described are increased or higher glucose, insulin, triglyceride, cholesterol and leptin concentrations (Table 1). Decreased adiponectin concentrations, increased systolic blood pressure and variations in metabolites related to lipid metabolism have also been described in overweight and obese dogs (Table 1). Many of these metabolic variations of overweight dogs resemble features of human metabolic syndrome (Alberti *et al.*, 2006), and therefore attempts have been made to perform similar classifications for dogs (Kawasumi *et al.*, 2012; Tvarijonaviciute *et al.*, 2012). The classifications proposed for canine metabolic syndrome differ in the suggested inclusion parameters and their reference limits, as well as the cut-off for overweight (José Lahm Cardoso *et al.*, 2016). Moreover, the definitions have the drawback of being based only on parameters measured in fasted condition (Table 1). In addition, neutering has been shown to be a factor associated with metabolic syndrome in dogs (Table 1). The relevance of metabolic syndrome classification in dogs has been questioned (Verkest, 2014), as dogs do not seem to progress to type 2 diabetes due to the metabolic syndrome criteria (Chandler *et al.*, 2017; Verkest *et al.*, 2011) and reports on cardiovascular disease other than hypertension are scarce (Table 1). Nevertheless, overweight dogs have an increased risk of short life expectancy (Adams *et al.*, 2018; Kealy *et al.*, 2002), motivating research within the field.

Table 1. *Reported metabolic variations in canine overweight with relevance to this thesis*

Publication/s	Study design	Body-condition assessment	Dogs included	Samplings	Metabolic variations in overweight dogs
(Verkest *et al.*, 2011)	Spontaneously obese and lean dogs. Feed-challenge test	BCS	9 obese, 9 lean. Various breeds	F, PP serum	Fasting insulin and triglycerides were higher in obese dogs. Postprandial glucose, insulin and triglycerides were higher in obese dogs
(Tvarijonaviciute *et al.*, 2016; Tvarijonaviciute *et al.*, 2012)	Weight loss intervention in spontaneously obese dogs/spontaneously obese dogs with or without ORMD	BCS, DEXA/ BCS, DEXA	35 obese. Various breeds/8 neutered obese. Various breeds	F plasma/ F plasma	Systolic blood pressure, cholesterol, triglycerides and insulin decreased after weight loss and adiponectin increased. MS was present in 20% of overweight dogs/variations in proteins related to lipid metabolism were found in dogs with ORMD
(Adolphe *et al.*, 2014)	Acute and chronic overfeeding and weight loss intervention in dogs. OST	BCS, CT	8 neutered beagles	F, PP plasma	Fasting glucose, heart rate and systolic left ventricular thickness increased in acute overweight and obesity and adiponectin decreased. Fasting insulin was higher in chronic obesity. Not all parameters were normalised after weight loss
(RC de Godoy *et al.*, 2015)	Acute and chronic overfeeding in dogs	BCS	5 beagles	F plasma	Amino acid- and lipid metabolites showed a bi-phasic pattern with an increase in acute phase and normalisation or decrease in chronic phase. Leptin, triglycerides and insulin were higher in obesity
(José Lahm Cardoso *et al.*, 2016)	Spontaneously obese, overweight and lean dogs	BCS	170 overweight or obese, 101 lean. Various breeds	F plasma	Glucose, cholesterol, triglycerides and systolic blood pressure were higher in obese than in lean dogs. MS was present in 36% of overweight or obese dogs and those had more pronounced metabolic variations. Neutering was positively associated with MS
(Forster *et al.*, 2018)	Spontaneously obese, overweight and lean dogs	BCS	22 obese, 27 overweight, 17 lean. Various breeds	F plasma and urine	Various lipid- and protein metabolites were increased in overweight and obese dogs compared with lean dogs

BCS, body condition score; CT, computed tomography scan; DEXA, Dual energy x-ray absorptiometry; F, fasting; MS, metabolic syndrome; ORMD, obesity-related metabolic dysfunctions; OST, oral-sugar test; PP, postprandial

1.2.3 Taurine and carnitine in dogs

Taurine is a sulphur-amino acid involved in a variety of body functions, including fat metabolism, reproduction and the nervous system. Taurine has an essential role in lipid metabolism as a bile acid conjugator, aiding fat absorption in the small intestine (Xie *et al.*, 2012). Taurine deficiency in dogs may cause dilated cardiomyopathy in some breeds and treatment with taurine supplement for this condition has been suggested (Sanderson, 2006; Kittleson *et al.*, 1997). Dogs absorb taurine from their diet, but also have the capacity for taurine biosynthesis from cysteine and methionine in the liver (Ko *et al.*, 2007). Taurine is freely filtered in the kidneys (Hayes & Sturman, 1981). No compensatory kidney re-absorption during prolonged fasting has been detected in dogs (Gray *et al.*, 2015) and plasma taurine concentration is therefore crudely reflected in urine (Hayes & Sturman, 1981). Taurine is not required in commercial dog foods and is usually not added (Hand *et al.*, 2010).

Carnitine, a component of all animal cells, is often described as a vitamin-like metabolite (Rebouche & Paulson, 1986). Carnitine is essential for fatty acid oxidation and energy production, as it transports long-chain and medium-chain fatty acids into the mitochondria for beta-oxidation (Hand *et al.*, 2010). Dogs absorb carnitine from dietary animal proteins, but can also produce it endogenously. Biosynthetic production of carnitine requires several steps, the last of which takes place in the liver and is rate-limiting (Bremer, 1983). Carnitine is derived from lysine and methionine, but other co-factors such as vitamin B_6 are also required (Borum & Bennett, 1986). Carnitine deficiency in humans may cause muscle weakness, hypoglycaemia and cardiomyopathy, but patients can also be asymptomatic (Stanley, 1987).

Although low carnitine status in overweight dogs has not been firmly established, dietary carnitine supplementation has been proposed for weight loss in dogs (Floerchinger *et al.*, 2015; Roudebush *et al.*, 2008). The suggested mechanisms are that carnitine preserves lean body mass, decreases fat mass and to some extent increases weight loss (Floerchinger *et al.*, 2015; Shoveller *et al.*, 2014; Roudebush *et al.*, 2008; Ibrahim *et al.*, 2003). Carnitine supplementation has been suggested to improve fatty acid oxidation and insulin sensitivity (Schooneman *et al.*, 2013), but studies in rodent models of obesity using carnitine supplementation aimed to improve fatty acid oxidation have not yielded consistent results (Schooneman *et al.*, 2016; Vigerust *et al.*, 2012). In two studies of short-term overweight in dogs, no differences in plasma carnitine concentrations between lean and overweight dogs were found (RC de Godoy *et al.*, 2015; Diez *et al.*, 2004). L-carnitine addition (300 ppm dry matter) to

complete dog foods aimed for weight loss or maintenance is currently recommended, but not always followed (Hand *et al.*, 2010).

1.3 Metabolic inflexibility in overweight humans

A flexible metabolism is able to switch from fatty acid oxidation during fasting to suppression of fatty acid oxidation and a shift to glucose uptake, oxidation and storage under conditions with insulin stimulation (Kelly 2000). The term "metabolic inflexibility", first introduced by Kelley *et al*. (1999), refers to lowered capacity of the mitochondria to switch freely between carbon fuels derived from different substrates (fatty acids, glucose or amino acids) (Muoio, 2014; Kelley *et al.*, 1999). Metabolic inflexibility is considered a link between overweight and insulin resistance and is included as part of human metabolic syndrome (Collaborators, 2017; Bergouignan *et al.*, 2011; Kopelman, 2007). Obesity-related metabolic dysfunctions such as metabolic inflexibility have been speculated to contribute to energy dysregulation in obesity, and metabolic inflexibility has been proposed as a possible driver for overweight (Muoio, 2014). Use of acetylcarnitine concentration as a measure of metabolic inflexibility has been suggested in humans and in rodents (Prior *et al.*, 2014; Muoio *et al.*, 2012; Noland *et al.*, 2009), as this metabolite is commonly derived from fatty acids but can be formed from all substrates used for mitochondrial oxidation (Randle, 1998).

In humans, different treatments for metabolic inflexibility, *i.e*. overloaded mitochondrion or generally decreased oxidation rates, have been discussed in terms of different weight reduction diets. All treatments involve relieving the workload and gridlock for the mitochondria, *e.g*. increased physical exercise, reduced intake of carbohydrates (high-fat low-carb diets) or intermittent fasting (five-two diets) (Muoio, 2014). Recent studies on intermittent fasting methods for dogs showed promising results, with no adverse metabolic effects in the parameters investigated and maintained energy requirements post weight loss (Leung *et al.*, 2018; Pan *et al.*, 2018).

Figure 2. The Labrador Retriever dogs Specs and Wille in action. Photo: Lena Holm.

2 Aims of the thesis

The overall aim of this thesis was to evaluate metabolic variations in spontaneously overweight dogs in fasted and postprandial condition, using the Labrador Retriever as a model. The hypotheses tested were i) that overweight dogs display variations in metabolic parameters compared with lean dogs and ii) that feed-challenge tests permit detection of subtle metabolic variations not noticeable in fasted condition.

Specific objectives were:

- To study serum responses of metabolic hormones and biochemistry parameters in lean and overweight dogs during a feed-challenge test (Paper I).
- To study metabolic profiles in fasted and postprandial urine in lean and overweight dogs (Paper II).
- To study plasma metabolite responses in lean and overweight dogs during a feed-challenge test (Paper III).
- To study plasma acylcarnitines and phospholipid profiles in lean and overweight dogs during a feed-challenge test (Paper IV).

3 Comments on material and methods

This chapter describes the cohort of dogs used in the thesis and provides a summary of sample collection and the different methods used in Papers I-IV. More detailed descriptions of the methods and statistics can be found in the individual papers.

3.1 Recruitment procedure and dogs recruited

Data collection for all papers (I-IV) was performed during one year at the University Animal Hospital and at the Swedish University of Agricultural Sciences, Uppsala, Sweden. All dogs were sampled once, at the same time of day, according to a pre-designed study protocol. The experiment was approved by the Ethics Committee for Animal Experiments, Uppsala, Sweden, and owner consent was obtained for each dog.

The study population consisted of 28 privately-owned healthy intact male show-type Labrador Retriever dogs. The Labrador Retriever breed is the most popular and common breed in Sweden and is used as a pet and as a utility dog (https://www.skk.se), which was the main reason for selection of this particular breed. To qualify for inclusion in the study, each dog had to be considered healthy by its owner, pass a health examination including haematology and serum biochemistry and have had stable body weight for at least three months. Exclusion criteria included previous or current systemic or organ-related disease and treatment with antibiotics, non-steroid anti-inflammatory drugs, steroids, deworming drugs or proton pump inhibitors within three months prior to the examination day. All dogs were tested for hypothyroidism and diabetes mellitus, and basal cortisol/creatinine ratio in morning urine was tested to exclude hyperadrenocorticism. Dogs were also excluded if vital parameters, haematology or serum biochemistry were outside reference ranges for healthy dogs on the day of examination.

Dogs were recruited by personal letters to 715 owners (mostly located within 100 km of Uppsala) of potentially eligible male Labrador Retrievers registered by the Swedish Kennel Club. Sixty owners replied and their dogs were examined for eligibility by an on-line survey of dog health status and feeding and exercise routines. Thirty-two dogs were not invited for further data collection, based on information stated in the online surveys that met the exclusion criteria. The remaining 28 dogs were invited to participate in data collection in the feed-challenge test. All dogs proved to be healthy, there were no missing data in the collected samples (with the exception of two urine samples) and none of these 28 dogs was excluded.

As breed, gender, age and neutering are factors shown to influence body condition in dogs (Gossellin *et al.*, 2007; Colliard *et al.*, 2006; Lund *et al.*, 2006), only intact dogs of one breed and gender were included in the study. The dogs were allocated to lean, slightly overweight and prominently overweight groups and all had similar mean age (Paper I) (Table 2). The strict inclusion criteria probably minimised inter-dog variation and made it easier to detect subtle metabolic variations depending on body condition score, despite quite a small dog cohort. However, the strict inclusion criteria also reduced the total number of eligible dogs living within a reasonable distance from the University Animal Hospital in Uppsala.

Table 2. *Descriptive statistics for the 28 Labrador Retriever dogs studied in Papers I-IV and the amount of test food given in the feed-challenge test*. Table modified from Paper I*

	Lean	**Slight overweight**	**Pronounced overweight**
	(BCS 4-5) n=12	(BCS 6) n=10	(BCS >6) n=6
Age *(year)*	5.3 ± 1.4^{a}	4.6 ± 1.4^{a}	6.2 ± 1.6^{a}
Body weight *(kg)*	34.8 ± 2.5^{a}	36.9 ± 2.3^{ab}	43.9 ± 4.2^{b}
Ideal body weight***(kg)*	34.8 ± 2.5^{a}	34.4 ± 2.2^{a}	39.2 ± 2.7^{b}
Test-meal size*** *(g)*	222 ± 12^{a}	220 ± 11^{a}	243 ± 12^{b}

*Variables are expressed as mean ± SD. Within each row, values with different superscript letter (a or b) differ significantly ($P<0.05$).

**Ideal body weight of overweight dogs was calculated as previously described (Verkest *et al.*, 2011; Laflamme, 1997).

***Hills Science Plan™ Canine Adult Performance.

It would have been desirable to include a larger number of dogs that were obese, rather than overweight, but unfortunately such dogs proved extremely difficult to enrol. Possible reasons for this are that the owners of obese dogs did not realise their dogs were obese, that they somehow felt guilty for the overweight or that their dogs did not meet the criteria due to health issues.

Our intention was to create a 'natural' model of spontaneous overweight that had preferably persisted for at least three months prior to participation in the study, even though most of the overweight dogs had been overweight for longer than three months according to their owners. Acute experimental studies tend to be dominating on overweight dogs and there is a need for data on spontaneously overweight or obese dogs (German, 2006).

3.2 General study design

The dogs were fasted in the home environment from 6 pm on the day before clinical sampling. In the morning of the examination day, water was withheld and a voided urine sample was taken from each dog by the owner. On arrival at the University Animal Hospital (between 8 and 9:30 am), the dogs were examined by the same veterinarian (Josefin Söder) and fasting blood samples were taken, followed by intake of a test meal. Postprandially, blood samples were collected hourly from one to four hours and voided urine was sampled at three hours (Figure 3).

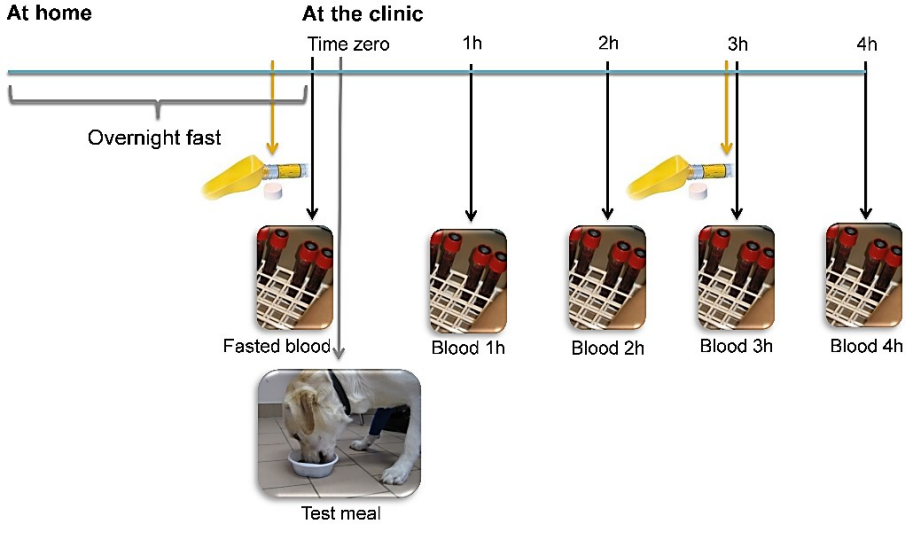

Figure 3. Overview of the sampling procedure conducted in the same way on each participating Labrador Retriever dog. Voided urine was sampled twice, at home after an overnight fast and 3 hours postprandially in the feed-challenge test. The postprandial period stared at the first bite of the test meal.

3.2.1 Diet in the home environment

No adjustments were made to the dogs' regular home diet of complete commercial dog foods and treats prior to participation in the study, presumably lowering the risk of changes in body weight preceding sample collection. Dietary history was acquired by daily food diaries provided by the dog owners during two weeks preceding the study. According to the food diaries, all dogs had their main energy supply from dry or wet (one dog) complete commercial diets and the most common protein source in the complete diets was chicken. A limited number of dogs of different body conditions were fed a low-fat, calorie-restricted diet or a diet containing carnitine additives, and it is therefore unlikely that this would have had an impact on the total group differences in measured parameters. The frequency with which the dogs were awarded table scraps, treats or dog chews did not differ between body condition groups (Paper III).

3.2.2 Assessment of health status and body condition

Each dog underwent a standard physical examination (assessment of general condition, skin condition, rectal temperature, visible mucus membranes, palpable lymph nodes, heart and lung auscultation, abdominal palpation and gait) and were weighed (Table 2) and photographed (Figure 4). Routine haematology and serum biochemistry analyses (alanine aminotransferase, alkaline phosphatase, fasting bile acids, creatinine, urea, glucose, fructosamine, total protein, albumin, C-reactive protein, total thyroxine, thyroid stimulating hormone, sodium, potassium and chloride) were performed on fasting blood samples. Urine was analysed by a standard dipstick chemistry test and by refractometry for urine specific density. Some minor health problems (slightly stiff gait and mild lameness, signs of mild periodontitis, palpable peri-articular osteophyte formation and skin furunculosis) were detected in 11 dogs. None of these dogs was excluded, as vital parameters, haematology, serum biochemistry and urine analysis were within the reference ranges for healthy dogs.

The dogs were assigned a clinical body condition score (BCS) on a 9-point scale (Laflamme, 1997) by the same veterinarian (Josefin Söder) and the cut-off for overweight (BCS $\geq$6) as suggested by the scoring scale was applied. Two dogs did not fit perfectly into the criteria of the scoring scale and were classified as BCS 6.5 (defined as a dog fulfilling all criteria of BCS 7 but with less well-defined fat deposits). In the feed-challenge test, these two dogs were fed according to lean body weight of BCS 6.

Based on BCS, the 28 dogs were assigned to one of three groups: a lean group (BCS 4-5), which consisted of 12 dogs; a slightly overweight group (BCS 6), which consisted of 10 dogs and a prominently overweight group (BCS >6) which consisted of six dogs (Paper I). Only one dog in the prominently overweigt group was obese (BCS 8). In Papers II-IV, where multivariate models were used, the dogs were assigned to two groups in order to get sufficient numbers of dogs in each group in the multivariate models. These were: a lean group (BCS 4-5), which consisted of 12 dogs, and an overweight group (BCS ≥6), which consisted of 16 dogs.

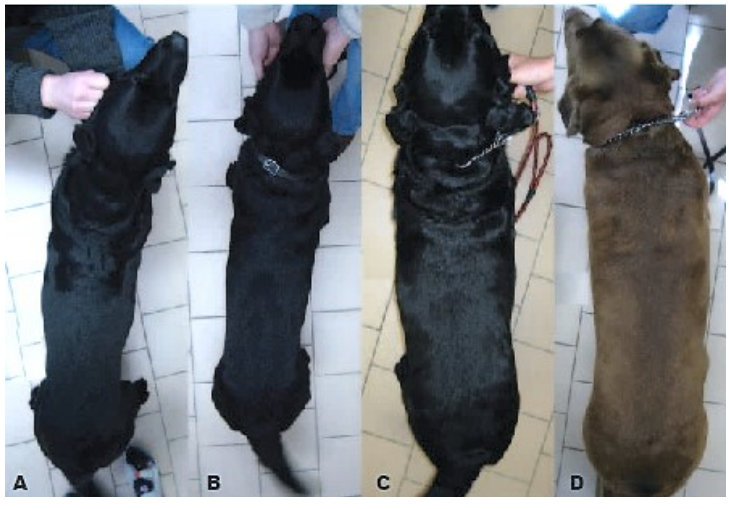

Figure 4. Photos of participating Labrador Retriever dogs showing increasing body condition score (BCS). A) BCS 5, B) BCS 6, C) BCS 7 and D) BCS 8. Photos: Josefin Söder.

The fat-cell hormone leptin was analysed in fasting serum samples and leptin concentration was found to be positively associated with BCS (Figure 5). Slightly overweight dogs (BCS 6) did not differ significantly from lean dogs (BCS 4-5) in fasting leptin concentration (one-way analysis of variance (ANOVA) with Tukey-Kramer adjustment), while prominently overweight dogs had higher leptin concentration than both lean and slightly overweight dogs ($P<0.05$).

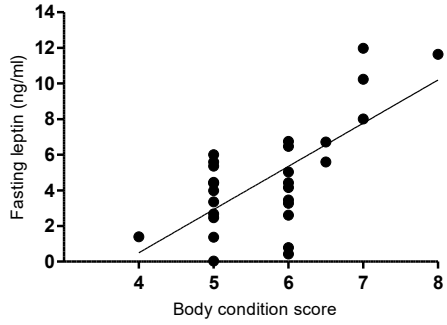

Figure 5. Leptin concentration measured in fasting serum samples as a function of assigned body condition score (significant association, linear regression $R^2 = 0.41$, $P<0.0001$). Re-analysis based on leptin data presented in Papers I-IV.

Slightly overweight dogs (BCS 6) were included in the study mainly for two reasons. First, the dogs scored BCS 6 were judged clinically to be slightly overweight, with palpable slight excess fat covering the ribs and a discernible waist, but not as prominent and well defined as the waist of a dog scored BCS 5 (Figure 4). Second, in-house clinical experience is that these slightly overweight dogs represent a great proportion of the dog population. Many pet dogs suffer from overweight today but, at least in Sweden, the majority of these overweight dogs could be described as slightly to moderately overweight. Inclusion of slightly overweight dogs in metabolic research is therefore motivated, as it is of interest to determine whether these dogs display the metabolic variations seen in more prominently overweight dogs.

According to a previous validation of the scoring scale used (Laflamme, 1997), total body fat percentage increases by 5% per unit higher body condition score. An objective measurement of body fat percentage, *e.g.* DEXA scan, would have been a good complement to the body condition scoring, but was not available at the University Animal Hospital. Although other objective techniques (*i.e.* MR or CT scanning) could have been useful in the body composition evaluation, it should be noted that the study design did not allow sedation without another day of sampling, which would have been difficult to achieve when dealing with privately owned dogs.

3.2.3 Feed-challenge test

The intention with the study design was to attain a natural model of spontaneous overweight with no intervention other than the feed-challenge test. A meal challenge represents a dynamic test of the metabolism, a physiological situation that reflects how the metabolism reacts to a daily meal and with the potential to

detect subtle metabolic changes in the postprandial state (Badoud *et al.*, 2015; Krug *et al.*, 2012; Pellis *et al.*, 2012).

All dogs were exposed to the same feed-challenge test, in which they were given half their daily energy requirement as a high-fat mixed meal. The equation used to compute daily energy requirement (DER) (131 kcal x body weight $_{kg}^{0.75}$) is designed specifically for adult intact Labrador Retriever dogs (Kienzle & Rainbird, 1991). To minimise the risk of giving overweight dogs too much food in comparison with lean dogs, the lean body weight of all overweight dogs (BCS ≥6) was calculated by subtracting the weight of the average fat content according to the estimated BCS and gender (Verkest *et al.*, 2011; Laflamme, 1997). The dogs were then fed according to the DER of ideal weight, using the actual body weight in lean dogs and the calculated lean body weight in overweight dogs (BCS ≥6). The calculated lean body weight of prominently overweight dogs (BCS >6) was higher than the actual body weight of dogs with BCS 4-5 and the calculated lean body weight of dogs with BCS 6 (Table 2). A possible explanation is that these dogs were not only fatter, but also larger in overall body stature (which was supported by visual observations).

The test meal was weighed and served with water added (same amount in grams as in the individual test meals). The test feed (Science Plan™ Canine Adult Performance, Hills, Etten Leur, the Netherlands) provided 4230 kcal/kg, with 51% of the metabolisable energy as fat, 26% as carbohydrate and 23% as protein (taurine, omega-3 and omega-6 fatty acids, betacarotene and vitamin A, C, D and E were added, according to the manufacturer). The idea behind choosing a high-fat meal was to challenge lipid metabolism, as overweight dogs have previously been reported to show variations in lipid parameters (see Table 1). Nutrient composition and energy content of the batch of test feed used were confirmed by an independent authorised laboratory (Paper III). The postprandial period started at the first bite and all 28 dogs voluntarily consumed all food and water within 10 minutes of being served. The dogs were given nothing further to eat or drink and were kept indoors until completion of the postprandial samplings.

3.2.4 Blood- and urine sample collections

A catheter was placed in the distal cephalic vein (Figure 6) and blood samples were collected 15 minutes before (fasting), and then hourly for four hours after the test meal (postprandial period). Fasting serum and plasma EDTA were used for health verification biochemistry and haematology analyses. At fasting and all postprandial time points, both serum and lithium-heparinised plasma were collected for analyses in Papers I, III and IV.

Prior to the examination day, the dog owners had accustomed their dogs to the urine sampling procedure for a minimum of three times. On the examination day, naturally voided morning urine was collected at home using a free-catch sampling device and was kept chilled on ice during transport. Total emptying of the bladder after morning urination could not be confirmed, as the dogs were still at home at that time, and there is a small but existing risk of a mix of fasting and postprandial urine being present in the postprandial samples obtained from some dogs. Fasting morning urine was used for the health verification analyses. Three-hour postprandial urine samples were collected by the owner as described, and both fasting and postprandial urine samples were used for analyses in Papers I and II. Full details of sample collection can be found in the individual papers.

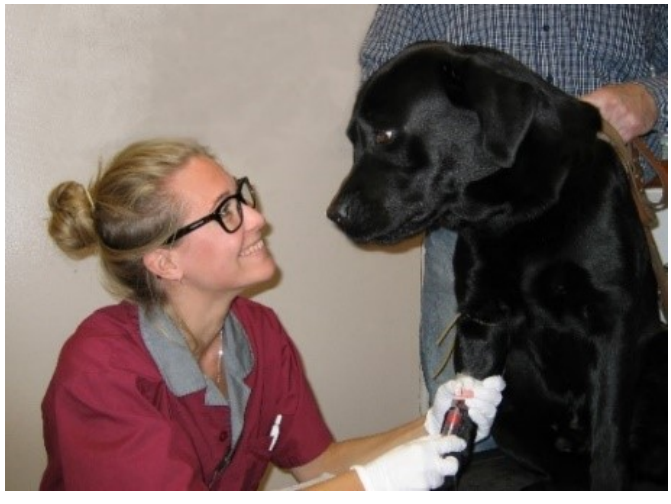

Figure 6. Blood sample collection from a Labrador Retriever dog by the veterinarian (Josefin Söder). Photo: Sanna Truelsen Lindåse.

3.3 Serum-, plasma- and urine analyses

ELISA assays and serum biochemistry analyses

Personnel blinded to dog identity performed enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' instructions. Serum leptin concentration was analysed in fasting samples, whereas serum insulin and glucagon were analysed in fasting and postprandial samples. Cortisol and creatinine were analysed in fasting and 3-hour postprandial urine. Commercially available canine ELISA kits were used to analyse serum leptin (Canine Leptin ELISA, Millipore, Missouri, USA) and insulin (Canine Insulin ELISA,

Mercodia, Uppsala, Sweden) and urine creatinine (Canine Urinary Creatinine ELISA, Arbor, Michigan, USA) concentrations. The ELISA kits for urine cortisol (Cortisol Urine ELISA, IBL, Hamburg, Germany) and serum glucagon (Human Glucagon ELISA (25 µL), Mercodia, Uppsala, Sweden) were validated for use in dog urine and serum (Paper I) before the analyses were performed. Urine creatinine concentrations were used for normalisation of urine cortisol. All samples were analysed in duplicate, and the mean of the two values was used in statistical analyses.

Serum glucose, triglycerides, free fatty acids (Free Fatty Acid Reagent, Wako NEFA-HR(2), Neuss, Germany) and total cholesterol were analysed by routine automatic methods (Architect c4000, Abbott Park, IL, USA) at the Clinical Pathology Laboratory, University Animal Hospital, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Nuclear magnetic resonance (NMR)

A total of 47 metabolites were identified in urine using each spectrum and the ChenomX database (https://www.chenomx.com/software/libraries) and their concentrations were quantified in mM relative to the internal standard added after accounting for overlapping signals. As creatinine and urea were present at much higher concentrations than other metabolites, any small change in their concentrations would cause a large change in the relative ratio of other metabolites and influence the robustness of the model (Favé *et al.*, 2011). These two metabolites were therefore excluded from the datasets, after which 45 metabolites remained.

Normalisation of urine data is crucial for comparisons of metabolite concentrations between different time points or studies. Normalisation to creatinine is generally used in veterinary medicine to account for differences in urine concentrations (Adamko *et al*., 2007; Braun *et al*., 2003) and that approach was used in Paper I. However, the suitability of creatinine as a general normalisation factor in urine metabolomics has been questioned, especially in analyses of postprandial urine, as meat-based food intake has been found to increase creatinine excretion (Xu *et al.*, 2013; Favé *et al.*, 2011). To account for different concentrations in the urine samples, the data from both fasting and postprandial time points were transformed to relative concentrations (% of total mM) using the following formula: [(mM of metabolite)/(sum (mM) of all 45 metabolites)] x100. Relative metabolite concentrations cannot easily be used for comparisons between studies using other normalisation approaches. However, in Paper II this normalisation approach was crucial to enable comparison between fasting and postprandial sampling time points and between lean and overweight dogs, while at the same time accounting for differences in urine

specific density. For details of nuclear magnetic resonance (NMR) spectral acquisition, identification and quantification of urine metabolites, see Paper II.

A set of 55 plasma metabolites was selected for quantification and the concentrations of these metabolites were determined in all experimental spectra using a previously described slightly modified strategy for automated quantification algorithm (AQuA), which accounts for complex overlap of experimentally observed signals (Röhnisch *et al.*, 2018). The AQuA was modified for the experimental spectra from the dog plasma samples in this thesis and estimated the concentrations (μM) of all 55 metabolites in all experimental spectra. It proved possible to quantify 43 of these 55 metabolites with acceptable quality using AQuA (quality indicators: coefficient of variation (CV) ≤20%, metabolite occurrence ≥50% of all samples, and no target signal positional deviation between the experimental spectra). Of these 43 metabolites, 41 were included in the multivariate and univariate statistical analyses. For details of NMR spectral acquisition, identification and quantification of plasma metabolites, see Paper III.

Liquid chromatography-time of flight mass spectrometry (LC-TOFMS)

Based on previous findings of variations in the lipid metabolism of overweight dogs (Söder *et al.*, 2017; RC de Godoy *et al.*, 2015), a list of interesting plasma metabolites related to lipid metabolism (*i.e.* acylcarnitines and taurine) was created and the metabolites included (n=61) were searched for in plasma based on compound accurate masses using the generated liquid chromatography-time of flight mass spectrometry (LC-TOFMS) spectra. The chromatographic peak of each metabolite was detected and relative intensity was determined. Six metabolites were found to be above the limit of quantification and were used in mixed model repeated measures analyses (Paper IV).

The presence of 317 phospholipids in plasma was determined by LC-TOFMS. Quantification of the detected phospholipids (nM) was carried out using four phospholipid internal standards. Phospholipids that had zero values in more than 50% of the observations were excluded. To handle the remaining zero values, all observations in the dataset for the remaining 118 metabolites had 0.01 nM added to the measured concentration (Paper IV).

3.4 Univariate statistical analyses

One-way ANOVA, t-test and non-parametric test

One-way analysis of variance and Kruskal-Wallis tests were used for normally and non-normally distributed comparisons, respectively, of data for lean (BCS 4-5), slightly overweight (BCS 6) and prominently overweight dogs (BSC >6) (GraphPad Prism 5.0, San Diego, California). Paired and unpaired t-tests, the Wilcoxon signed-rank test and the Mann-Whitney U test were used to compare normally and non-normally distributed data between time points and between lean (BCS 4-5) and overweight groups (BCS ≥6). A value of $P<0.05$ was considered significant in univariate comparisons. Univariate statistics were preceded by multivariate selection as described below, with the exception of the descriptive dog statistics in Paper I-IV (Table 2) and the hypothesis-driven selection of phospholipids (Paper IV).

Mixed model repeated measures analysis

Responses to the feed-challenge test were evaluated by mixed model repeated measures analysis in SAS (Cary, 2015; Fitzmaurice *et al.*, 2012; Littell *et al.*, 2007). In the statistical model, body condition group was defined as an independent variable and the fasting value was included as a time point. Multiple comparisons within the model were corrected for by Tukey-Kramer adjustment. The model analysed the overall response over time and the overall differences between the lean (BCS 4-5), slightly overweight (BCS 6) and prominently overweight dogs (BCS >6) (Paper I) and between lean (BCS 4-5) and overweight dogs (BCS ≥6) (Papers III and IV). Thus, the model was capable of overall and pair-wise comparisons within time points. Logarithmic transformation of raw data was performed to correct for non-normality when necessary for a parameter (based on the distribution of residuals). In Paper I, all biochemistry and hormonal parameters were tested with this model. In Paper III ketone bodies were tested, in Paper IV all metabolites related to lipid metabolism were tested (based on physiological importance for all). Otherwise, only discriminant metabolites identified by multivariate models were tested in mixed model repeated measures analysis (Papers III and IV). Bonferroni correction for multiple comparisons was applied to the results from the repeated measures model in all papers except in Paper I.

Homeostasis model of assessment of basal insulin sensitivity

Basal insulin sensitivity was estimated using the homeostasis model assessment ($HOMA_{IS}$) (Levy *et al.*, 1998) for fasting glucose (mmol/L) and insulin (μU/mL) concentrations. The calculations were made using the non-linear HOMA Calculator (version 2.2.3; Diabetes Trial Unit, University of Oxford, UK). Fasting serum insulin concentrations less than 2.9 μU/mL (the minimum concentration accepted in the calculation) were entered as 2.9 μU/mL. Kruskal-Wallis tests were used for non-normally distributed comparisons between body condition groups (Paper I).

3.5 Multivariate statistical analyses

Metabolomics analyses such as NMR and LC-TOFMS often generate extensive datasets in comparison with conventional biochemical analyses or ELISA. Using multivariate approaches, large datasets, *i.e.* metabolomic profiles, can be compared between groups and/or before and after different interventions. Various multivariate models were therefore used as a starting point for identification of important and discriminant metabolites or phospholipids that varied over time in the feed-challenge test or were related to the lean and overweight groups of dogs. Discriminant metabolites were thereafter further investigated by t-tests or non-parametric tests or by mixed model repeated measures analyses, as described above. Multivariate analyses were performed in the SIMCA program (SIMCA-P + 13.0 Umetrics, Umeå, Sweden). Randomisation of raw data, pareto-scaling and step-wise removal of up to five outliers in each multivariate model were implemented. Metabolite and phospholipid concentrations were used as x-variables in all comparisons.

Principal component analysis (PCA)

Principal component analysis (PCA) was used to identify and remove outliers and the ellipse was set at 95% confidence interval. Gaussian distribution of the whole dataset was then tested by normal probability plotting. The PCA model was used to visualise any unconstrained clustering of fasting and postprandial time points (including all 28 dogs), as well as any clustering of lean and overweight dogs within each time point. In an unconstrained multivariate model, as in a PCA, the computer program is unaware of any pre-designed grouping of the samples, which enables an unconditional and exploratory approach to the dataset. Any observed clustering was thereafter tested for significance with constrained models.

Partial least-squares discriminant analysis (PLS-DA)

In a constrained multivariate model, such as partial least-squares discriminant analysis (PLS-DA), the computer program is aware of the grouping and/or clustering of the samples and can evaluate whether there are any differences between treatments. The PLS-DA models were used to find differences in metabolite profiles between time points, or between lean and overweight groups of dogs, in Papers II and III. In the model, R^2Y represents the percentage of variation in the dataset explained by the model (a measure of fitness) and Q^2Y represents the percentage of variation in the dataset predicted by the model. A model with a $Q^2Y>0.3$ was considered significant and the significance of each model was further confirmed using cross-validated analysis of variance (CV-ANOVA) (see below).

I am aware that this multivariate model does not take into account the pairing of samples between different time-points. At the time of analyses, it was not known that such a multivariate model (orthogonal partial least-squares effect projection analysis (OPLS-EP)) existed, and it is possible that pairing the samples could have generated even stronger multivariate models in Papers II and III.

Orthogonal partial least-squares discriminance analysis (OPLS-DA)

Orthogonal partial least-squares discriminance analysis (OPLS-DA) is a type of constrained multivariate model similar to PLS-DA. However, in addition to expressing the covariation between the metabolite data and different classes (lean and overweight groups of dogs), it also expresses the orthogonal variation that is not related to classes. The reason for using OPLS-DA models in Paper IV (instead of PLS-DA as used in Papers II and III) was that a paired orthogonal model (OPLS-EP) was used for analysis of the time effects, and thus an orthogonal model was chosen for the comparison between body condition groups.

Orthogonal partial least-squares effect projection analysis (OPLS-EP)

A paired multivariate model, OPLS-EP, was used to compare each postprandial time point with fasting (Paper IV). This paired model compares responses (with the fasting concentration subtracted from each postprandial time point) as x-variables to a target value of y=1 (Jonsson *et al.*, 2015). The OPLS-EP model expresses the structure of the data and can identify individuals with a deviating metabolic response to an intervention, *i.e.* the feed challenge. In Paper IV, four OPLS-EP models were constructed (with the fasting concentration subtracted from each postprandial time point) using unit variance scaling (UVN) for x-

variables and no scaling for the y-variable, as recommended by the model inventors (Jonsson *et al.*, 2015).

Cross-validated analysis of variance (CV-ANOVA)

The discriminance models including OPLS-EP were verified for significance using CV-ANOVA (Eriksson *et al.*, 2008), where $P<0.05$ was considered significant.

Variable importance of projection (VIP)

Variable importance of projection (VIP) is a type of P-value that indicates how conservatively multivariate metabolite selection has been performed. Discriminative metabolites were identified using VIP in a PLS-DA, OPLS-DA or OPLS-EP model. Metabolites with VIP >1 (Papers II and III) and VIP >1.5 (Paper IV) and for which the corresponding jackknife-based 95% confidence interval were not close to or including zero were considered discriminative and significant for the observed separations. A more conservative VIP value was used in Paper IV (compared with Papers II and III) because of the many phospholipids identified in the LC-TOFMS dataset.

Stepwise logistic regression analysis

For each time point, binary (*i.e.* lean and overweight groups of dogs) stepwise logistic regression (Olsson, 2002) was used to identify metabolites that were related to overweight (BCS ≥6), (Paper III). The Logistic procedure in the SAS package (2014, 9.4 Institute Inc., Cary, NC) was used for this purpose.

3.6 Statistical analyses performed specifically for this thesis

Originally, in Papers I-IV dogs were divided into two or three body condition groups depending on the statistical procedures performed. In Paper I, mixed model repeated analysis was performed on three body condition groups for the parameters studied. In Papers II-IV, where a multivariate selection approach was used as a starting point, dogs were divided into two groups to get sufficient observations within each dog group in the multivariate models. To facilitate comparison between results from different papers and to give insights into the parameters for which slightly overweight dogs displayed metabolic variations, in addition to the original grouping (in Paper II-IV), the results are also presented here in groups of lean (BCS 4-5), slightly overweight (BCS 6) and prominently

overweight dogs (BCS >6). To make comparisons between these three groups of dogs over time in the feed-challenge test, the mixed model repeated measurements approach (adjusted for multiple comparisons with Tukey-Kramer adjustment) constructed for Paper I was used to test the six identified metabolic parameters that differed between body condition groups (Paper I-IV). Where re-analysis of original data was performed by mixed model repeated measurements analysis including the three groups, this is stated in the Results section and within the legend to each figure.

4 Results

This chapter summarises the main results from Papers I-IV, focusing on metabolic variations identified between body condition groups and/or time-dependent metabolic variations in the feed-challenge test, using mixed model repeated measures and multivariate analyses as main tools. When re-analyses were performed on data from Paper I-IV, this is clearly stated.

Section 4.1 provides a general metabolic description of the dog cohort. The main results from Papers I-IV are described in section 4.2 and summarised in Table 3. Section 4.3 describes the main metabolic variations observed, divided into:

- Time-dependent variations between body condition groups
- Variations between body condition groups
- Variations between sampling time points

4.1 General metabolic description of the dog cohort

In Paper I, analyses of fasted serum from lean, slightly overweight and prominently overweight dogs revealed no differences between body condition groups in terms of fructosamine, TSH and tT4 concentrations (data analysed as part of the general health examination), or in basal fasting insulin sensitivity measured by $HOMA_{IS}$. Glucose, insulin, glucagon, free fatty acids and total cholesterol concentrations did not differ between body condition groups at fasting or in the postprandial response. Fasting triglyceride concentrations did not differ between body condition groups, but postprandial triglycerides showed time-dependent variations between body condition groups (Paper I) (Figure 7). Prominently overweight dogs (BCS >6) showed higher fasting urinary cortisol/creatinine ratio than lean dogs (Paper I) (Figure 8).

4.2 Main results from Papers I-IV

In Paper I, serum biochemistry variables and hormones of importance for basic canine metabolism were analysed (Table 3). In Papers II and III, explorative approaches were used and a broad spectrum of urine and plasma metabolites were identified with NMR analyses (Table 3). In Paper IV, findings from Papers I-III, together with previously reported metabolic variations in overweight dogs and humans, formed a base for LC-TOFMS analysis directed towards metabolites related to lipid metabolism and phospholipids (Table 3).

Table 3. Main results from Papers I-IV, based on the study cohort of spontaneously lean and overweight Labrador Retriever dogs (n=28)

Paper	Study design	Main results	Conclusions
I	12 lean (BCS 4-5), 10 slightly overweight (BCS 6) and six prominently overweight dogs (BCS>6). Serum samples in the feed-challenge test and urine samples at fasting and 3 hours after food intake. Serum biochemistry and ELISA	Serum concentrations of insulin, glucagon, triglycerides, glucose and urine cortisol/creatinine ratio increased postprandially in all dogs. Prominently overweight dogs had higher postprandial triglyceride peak and higher overnight cortisol excretion than lean dogs	The higher postprandial triglyceride response and higher cortisol excretion in prominently overweight dogs might be early signs of metabolic imbalance
II	12 lean (BCS 4-5) and 16 overweight dogs (BCS ≥6). Urine samples at fasting and 3 hours after food intake. 45 metabolites were quantified with NMR	Fasting and postprandial urinary metabolomes differed in all dogs. Lean and overweight dogs were separated by their postprandial urinary metabolomes, but not by their fasting urinary metabolomes. Overweight dogs had lower postprandial urinary taurine excretion than lean dogs	Postprandial urinary metabolomes might be more useful than fasting metabolomes in detecting metabolic variations in canine overweight. The lower urinary taurine excretion in overweight dogs could indicate alterations in lipid metabolism
III	12 lean (BCS 4-5) and 16 overweight dogs (BCS ≥6). Plasma samples in the feed-challenge test. 41 metabolites were quantified with NMR	All postprandial plasma metabolomes differed from the fasting plasma metabolome in all dogs and 11 amino acids contributed to the separations. Carnitine was related to overweight at all time points and overweight dogs had overall lower carnitine response than lean dogs in the feed-challenge test	A postprandial amino acid response was detected in all dogs but no time-dependent variations between body condition groups were found. The lower carnitine status in overweight dogs could indicate an insufficiency related to spontaneous adiposity and altered lipid metabolism
IV	12 lean (BCS 4-5) and 16 overweight dogs (BCS ≥6). Plasma samples in the feed-challenge test. Six metabolites (acylcarnitines and taurine) and 118 phospholipids were quantified with LC-TOFMS	Propionylcarnitine, stearoylcarnitine and nine phospholipids increased in response to food intake, while vaccenylcarnitine decreased. At fasting, acetylcarnitine status was lower in overweight dogs than in lean and it did not decrease in response to food intake as it did in lean dogs. One fasting phosphatidylcholine was higher in prominently overweight (BCS >6) than in lean dogs	The acetylcarnitine pattern in overweight dogs indicated decreased fatty acid oxidation at fasting and metabolic inflexibility to food intake. The potential role of metabolic inflexibility in the metabolism of overweight dogs merits further investigation

BCS, body condition score; ELISA, enzyme-linked immunosorbent-assay; LC-TOFMS, liquid chromatography-time of flight mass spectrometry; NMR, nuclear magnetic resonance.

4.3 Metabolic variations

4.3.1 Time-dependent variations between body condition groups

In Paper I, serum concentrations of insulin, glucagon, triglycerides, glucose and urine cortisol/creatinine ratio increased postprandially in all dogs. Of these parameters, serum triglycerides and urine cortisol were found to show time-dependent variations between body condition groups (Paper I).

The overall triglyceride response in the feed-challenge test was higher in prominently overweight dogs (BCS >6, n=6) than in lean dogs (BCS 4-5, n=12) and slightly overweight dogs (BCS 6, n=10) (P=0.001 and P=0.02, respectively), whereas slightly overweight and lean dogs did not differ significantly in overall triglyceride response (Figure 7). Pairwise comparisons between body condition groups at peak postprandial time point demonstrated that triglyceride concentration was almost two-fold higher in prominently overweight than in lean dogs at 3 hours postprandially (P<0.001) (Figure 7).

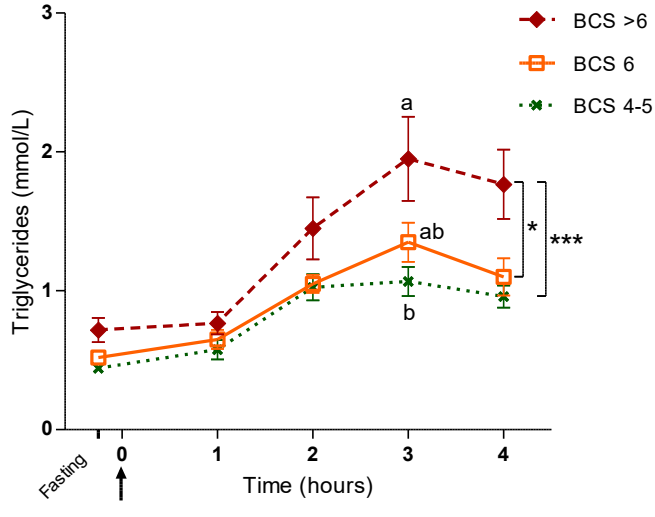

Figure 7. Triglyceride concentrations in the feed-challenge test. Mixed model repeated measures analysis was applied and values are expressed as mean ± SEM. Fasting serum samples were taken 15 minutes before serving of a test meal at time 0 (arrow) and triglyceride concentrations are shown as response curves from fasting to 4 hours after feeding. Significant differences in overall responses between body condition groups are indicated by asterisks (*P<0.05, ***P<0.001). Different letters (a and b) indicate significant differences between body condition groups within time point (P<0.001). Diagram modified from Paper I.

Re-analysis of cortisol data from Paper I based on the three body condition groups showed that slightly overweight dogs (BCS 6, n=8 due to missing data) and prominently overweight dogs did not differ significantly in cortisol/creatinine ratio between the fasting and postprandial time points (P>0.05). Lean dogs showed time-dependent variation, with an increase in urinary cortisol/creatinine ratio from fasting to 3 hours postprandially (P=0.01) in the feed-challenge test (Figure 8). Prominently overweight dogs (BCS >6, n=6) had higher fasting urinary cortisol/creatinine ratio than lean dogs (BCS 4-5, n=12) (Figure 8).

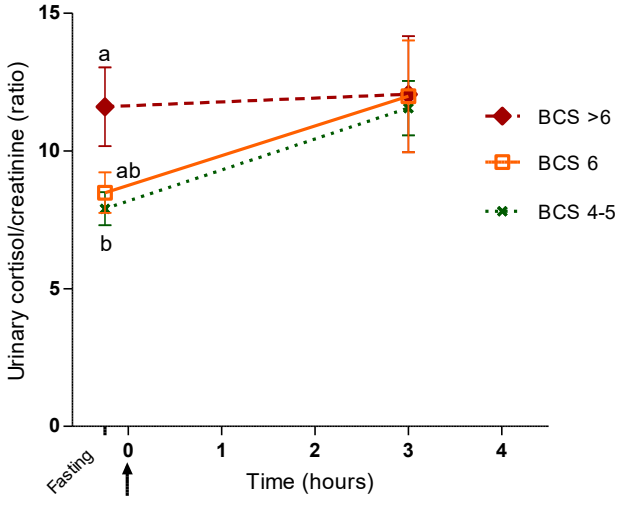

Figure 8. Overnight and postprandial excretion of urinary cortisol measured in voided urine. Cortisol was normalised to urinary creatinine concentration. Mixed model repeated measures analysis (log-transformed) was applied and values are expressed as mean ± SEM. Fasting urine was collected at home, a test meal was served at time 0 (arrow) and postprandial urine was collected at the clinic at 3 hours postprandially. Different letters (a and b) indicate significant differences between body condition groups within time point (P<0.05). Note: the large SEM in slightly overweight dogs (BCS 6) postprandially might partly explain the lack of time-dependent response in that group. Re-analysis and new diagram based on data from Paper I.

In Paper II, 45 urine metabolites were quantified with NMR and in the 3-hour postprandial urinary metabolomes a clear multivariate separation between lean and overweight dogs was shown in PCA (Paper II) (Figure 9A). Using VIP analyses based on PLS-DA (1 comp: R^2Y=0.5, Q^2Y=0.36; CV-ANOVA: P=0.005), discriminant postprandial metabolites separating lean and overweight dogs were identified as taurine, allantoin and guanidoacetate (Paper II) (Figure 9B). The most discriminant metabolite was taurine and overweight dogs excreted about half the amount in urine compared with lean dogs at the postprandial time point (Paper II). However, fasting urine metabolomes showed

no multivariate separation between overweight (BCS ≥6, n=16) and lean dogs (BCS 4-5, n=11). At fasting time point, mean urinary taurine/creatinine ratio was calculated to be 0.099 (95% CI: 0.06, 0.14) for all dogs (n=28) (Paper II).

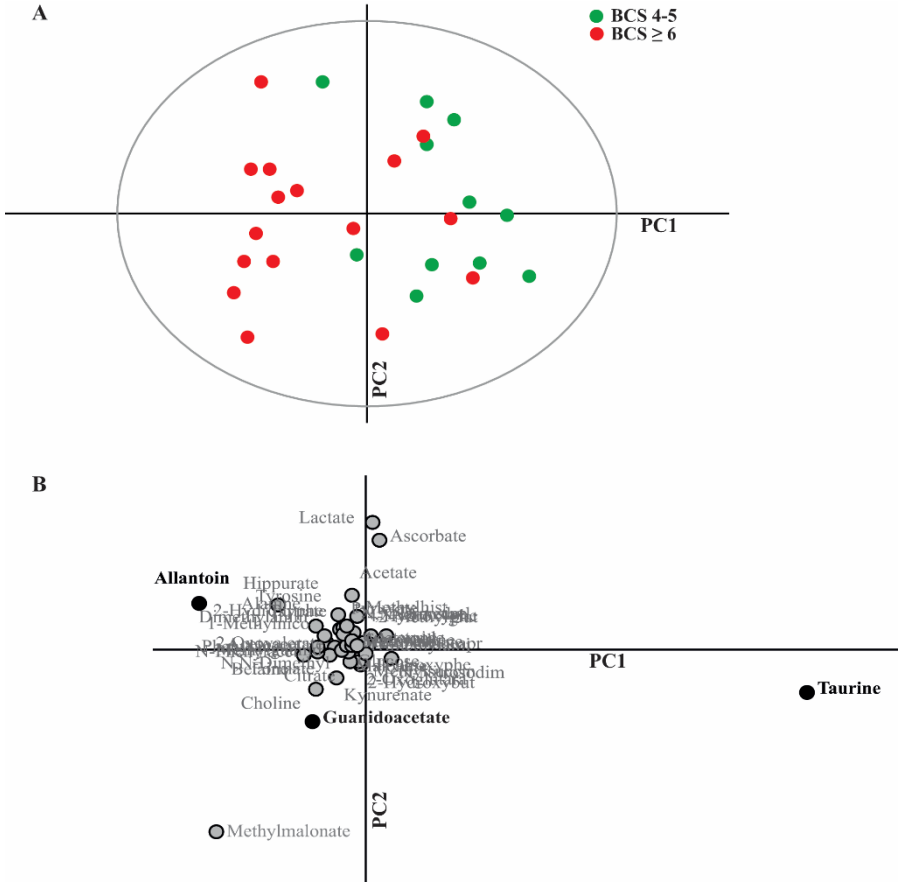

Figure 9. A) Lean (body condition score (BSC) 4-5, n=11 due to removal of one outlier, in green) and overweight dogs (BCS ≥6, n=16 in red) showed a clear visual separation in principal component analysis (PCA) score plot of the postprandial urine metabolome. A total of 45 metabolites quantified by nuclear magnetic resonance were included in this unconstrained model. Principal component (PC) 1 explained 8% of the total variance and PC2 4%. Partial least-squares discriminant analysis verified the separation between body condition groups and was used for variable importance of projection (VIP) analyses. B) Taurine, allantoin and guanidoacetate (highlighted with black dots in loading plot corresponding to the PCA) were significant VIPs separating lean and overweight dogs. Diagram modified from Paper II.

Re-analysis of taurine data from Paper II based on the three body condition groups showed that urinary taurine excretion in prominently overweight (BCS

>6, n=6) and slightly overweight dogs (BCS 6, n=10) was not affected by food intake. In lean dogs, taurine excretion showed time-dependent variation, as it increased (P=0.001) from fasting to 3 hours postprandially (BCS 4-5, n=12) (Figure 10). Postprandially, prominently overweight and slightly overweight dogs showed lower urinary taurine excretion compared with lean dogs ($P \leq 0.03$) (Figure 10).

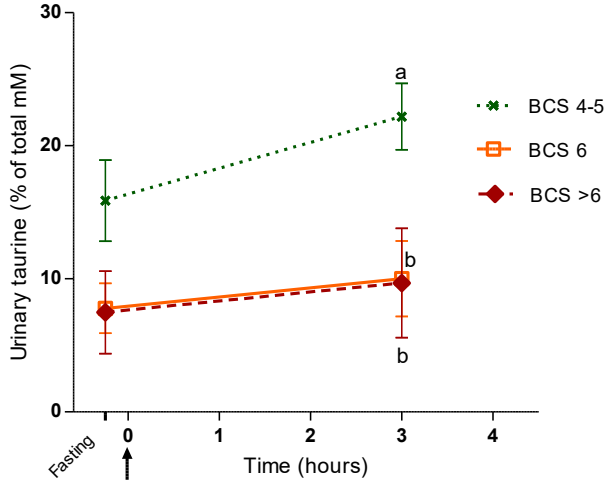

Figure 10. Urinary taurine excretion measured by nuclear magnetic resonance and normalised to total metabolite concentration of each dog. Mixed model repeated measures analysis was applied and values are expressed as mean ± SEM. Fasting urine was collected at home, a test meal was served at time 0 (arrow) and postprandial urine was collected at the clinic at three hours postprandially. Different letters (a and b) indicate body condition groups that differed significantly within time point (P<0.05). Re-analysis and new diagram based on data from Paper II.

In Paper IV, six plasma metabolites were quantified with LC-TOFMS analysis. Propionylcarnitine and stearoylcarnitine increased in response to food intake in all dogs (n=28), while vaccenylcarnitine and acetylcarnitine decreased (Paper IV). Re-analysis of acetylcarnitine data from Paper IV based on the three body condition groups showed that the overall acetylcarnitine response in the feed-challenge test was lower in prominently overweight dogs (BCS >6, n=6) and slightly overweight dogs (BCS 6, n=10) than in lean dogs (BCS 4-5, n=12) ($P \leq 0.02$ for both). Slightly overweight and prominently overweight dogs did not differ in overall acetylcarnitine response (Figure 11). At fasting, prominently overweight and slightly overweight dogs showed lower acetylcarnitine signal area than lean dogs ($P \leq 0.005$). In prominently overweight and slightly overweight dogs, the acetylcarnitine signal area was not affected by food intake,

while lean dogs showed time-dependent variation and the acetylcarnitine response decreased from fasting to one hour after feeding (P<0.0001) (Figure 11).

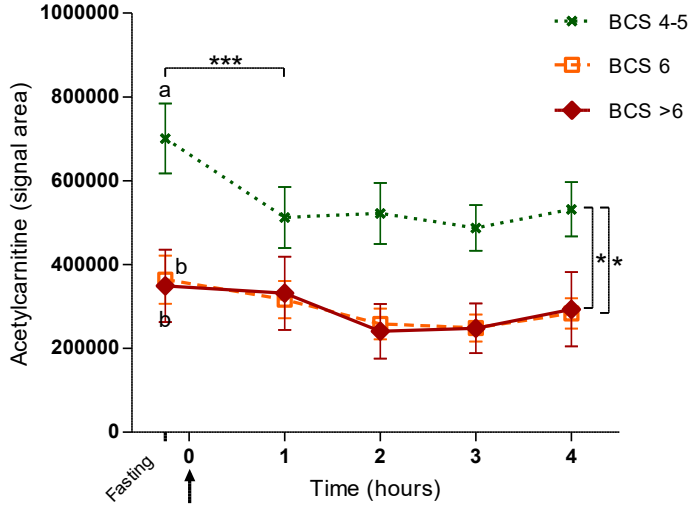

Figure 11. Acetylcarnitine signal areas measured by liquid chromatography-time of flight mass spectrometry in plasma. Mixed model repeated measures analysis (log-transformed) was applied and values are expressed as mean ± SEM. Fasting plasma samples were taken 15 minutes before serving of a test meal at time 0 (arrow) and acetylcarnitine signal areas are shown as response curves from fasting to 4 hours after feeding. Significant differences in overall responses between and within body condition groups are indicated by asterisks (*P<0.05, ***P<0.001). Different letters (a and b) indicate significant differences between body condition groups within time point (P<0.01). Re-analysis and new diagram based on data from Paper IV.

4.3.2 Variations between body condition groups

In Paper III, plasma metabolite profiles generated by NMR analysis showed no visual separation between overweight (BCS ≥6, n=16) and lean dogs (BCS 4-5, n=12) in PCA models, either at fasting time point or at postprandial time points. This was confirmed by the finding that none of the PLS-DA models could be fitted using lean and overweight dogs as pre-defined groups. In logistic regression analyses using the plasma metabolite dataset (n=41) generated by NMR, the metabolite carnitine was shown to be related to overweight (BCS ≥6) at fasting and all postprandial time points (P≤0.03 for all), while no other metabolites were identified by this statistical model (Paper III). At fasting, overweight dogs (BCS ≥6, n=16) showed about two-thirds of the carnitine concentration measured in lean dogs (BCS 4-5, n=12). No time-dependent

variations between body condition groups were found in the NMR metabolite dataset, which besides carnitine mostly contained amino acids (Paper III).

Re-analysis of carnitine data from Paper III based on the three body condition groups showed that prominently overweight dogs (BCS >6) had an overall lower carnitine response (P=0.03) in the feed-challenge test than lean dogs (BCS 4-5) (Figure 12). Slightly overweight dogs (BCS 6) did not differ significantly from lean or from prominently overweight dogs, although there was a trend for an overall lower carnitine response in slightly overweight dogs compared with lean (P=0.06). Consequently, the carnitine concentrations in the feed-challenge test showed variations between body condition groups, but the responses were not time-dependent (Figure 12).

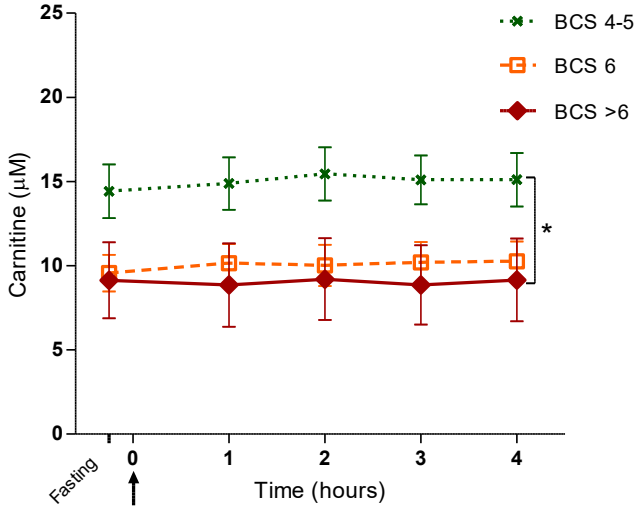

Figure 12. Carnitine concentrations measured by nuclear magnetic resonance in plasma. Mixed model repeated measures analysis (log-transformed) was applied and values are expressed as mean ± SEM. Fasting plasma samples were taken 15 minutes before serving of a test meal at time 0 (arrow) and carnitine concentrations are shown as response curves from fasting to 4 hours after feeding. Significant differences in overall responses between body condition groups are indicated by asterisks (*P<0.05). Re-analysis and diagram based on data from Paper III.

In Paper IV, plasma phospholipid profiles generated by LC-TOFMS analysis showed no multivariate separation in OPLS-DA models between overweight (BCS ≥6, n=16) and lean dogs (BCS 4-5, n=12) at fasting or at postprandial time points (Paper IV). A total of 118 phospholipids were quantified and nine phospholipids were found to increase in response to food intake (Paper IV). One fasting phosphatidylcholine (PCaa C38:4) was higher in prominently overweight (BCS >6, n=6) than in slightly overweight (BCS 6, n=10) and lean dogs (BCS 4-5, n=12). Only fasting plasma concentrations according to the original

hypothesis-driven approach are shown in Paper IV and Figure 13. Re-analysis of phosphatidylcholine (PCaa C38:4) by mixed model repeated measurements showed no time-dependent variations between body condition groups (data not shown), although fasting concentrations were different between the three body condition groups (Figure 13).

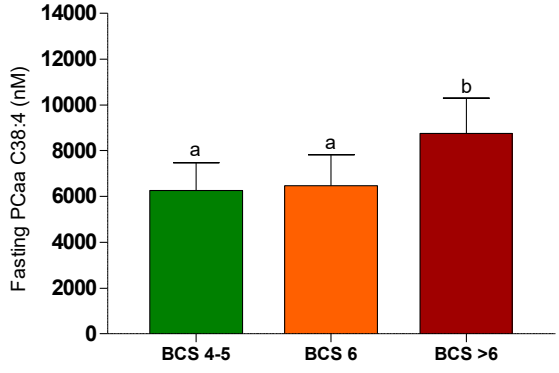

Figure 13. Phosphatidylcholine (PCaa) C38:4 concentrations measured in plasma at fasting by liquid chromatography-time of flight mass spectrometry. Data were analysed by one-way ANOVA with Tukey Kramer adjustment (P=0.003). Values presented are mean ± SD. Different letters (a and b) indicate significant differences between body condition groups (P<0.05). Diagram modified from Paper IV.

4.3.3 Variations between sampling time points

In Paper II, urinary metabolomes generated by NMR analyses differed between fasting and 3-hour postprandial time points, all dogs included (n=28; PLS-DA Q^2Y 0.32, CV-ANOVA $P=6\times10^{-5}$). Using VIP analyses, discriminant metabolites separating fasting and 3-hour postprandial time points were identified as taurine, allantoin, citrate and malonate (Paper II).

In Paper III, plasma metabolomes generated by NMR analyses differed between fasting and all postprandial time points, all dogs included (n=28; PLS-DA Q^2Y 0.31-0.63, CV-ANOVA $P\leq1.4\times10^{-4}$). Using VIP analyses, 11 discriminative amino acids were identified, of which nine showed increasing concentrations postprandially and one showed decreasing concentrations. None of the amino acids identified showed time-dependent variations between body condition groups (Paper III).

In Paper IV, plasma phospholipid profiles generated by LC-TOFMS showed significant models in three paired multivariate OPLS-EP comparisons between time points (*i.e.* fasting concentration subtracted from 2-, 3- or 4-hour postprandial time point; $P\leq2.8\times10^{-5}$ for all), all dogs included (n=28). Visual

interpretation of the significant OPLS-EP models could not detect any cluster/groups of dogs and no time-dependent phospholipid profile response related to body condition was therefore present. The original grouping of overweight (BCS ≥ 6, n=16) and lean dogs (BCS 4-5, n=12) could neither be visually separated in the model. These visual interpretations were in line with the finding that no OPLS-DA could separate lean and overweight dogs at any time point using their phospholipid profiles.

The most predictive OPLS-EP model was the 4-hour postprandial time point minus fasting (one predictive component and two orthogonal components; Q^2 0.91, CV-ANOVA $P=4.9\times10^{-9}$). Based on this OPLS-EP model, 12 significant phospholipids discriminating between fasting and the 4-hour time points were identified (Figure 14A). Mixed model repeated measures analysis of the 12 discriminant phospholipids showed that nine had overall increasing responses after food intake ($P\leq0.003$ for all), but none showed time-dependent responses between body condition groups. The two most discriminating phospholipids (based on VIP values) in the postprandial response are displayed in Figures 14B and 14C.

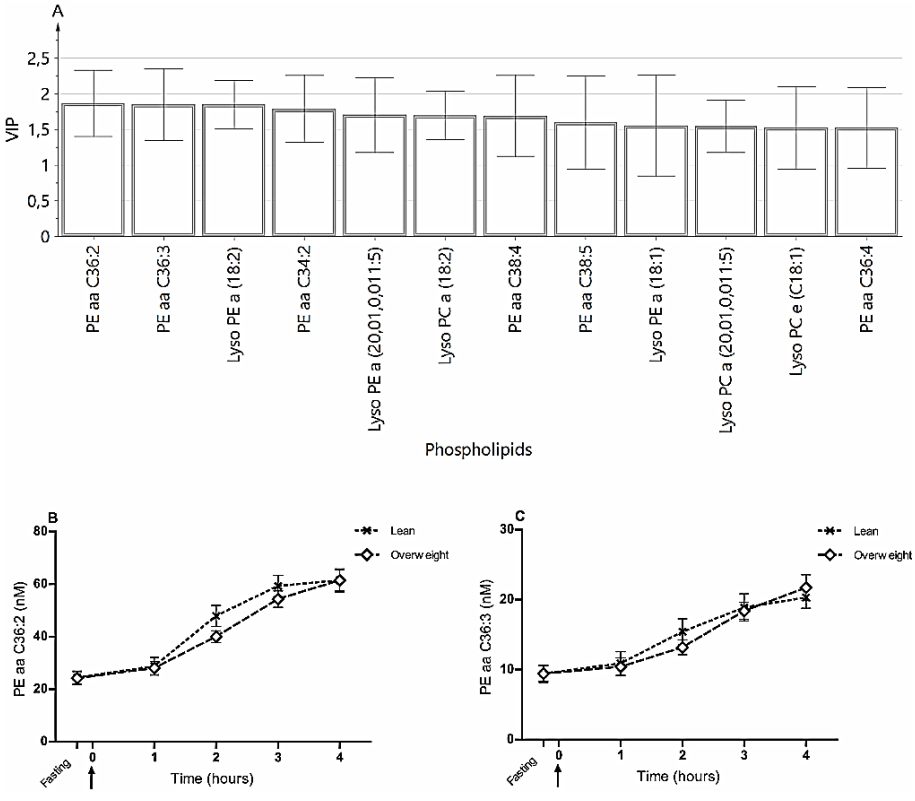

Figure 14. A) Variable importance of projection (VIP) values, based on orthogonal partial least-squares effect projection analysis (4-hour time point minus fasting) of the 118 plasma phospholipid concentrations quantified by liquid chromatography-time of flight mass spectrometry. Values are displayed as VIP and confidence interval (CI). Discriminant phospholipids adding significant structure to the model are displayed (VIP >1.5 and for which the corresponding jackknife-based 95% CI were not close to or including zero). B, C) Phosphatidylethanolamine (PEaa) C36:2 and C36:3 concentrations, presented as mean ± SEM. Mixed model repeated measures analyses were applied. Fasting plasma samples were taken 15 minutes before serving of a test meal at time 0 (arrow) and the phosphatidylethanolamine concentrations are shown as response curves from fasting to 4 hours after feeding. PEaa C36:2 and PEaa C36:3 concentrations were significant between time points (P<0.0001 for both), but the overall response did not differ between lean (BCS 4-5, n=12) and overweight dogs (BCS ≥6, n=16). Diagram from Paper IV.

5 General discussion and future perspectives

The work presented in this thesis was based upon analysis of a cohort of well-defined healthy Labrador Retriever dogs with a clinical body condition score of 4-8. The original hypotheses tested were i) that spontaneously overweight dogs display variations in metabolic parameters in comparison with lean dogs and ii) that the use of a feed-challenge test permits detection of subtle metabolic variations not noticeable in fasted condition.

Of the measured parameters, six were shown to differ between lean and overweight dogs and all of these were directly or indirectly associated with lipid metabolism. Concentrations of urinary cortisol, one plasma phosphatidylcholine (PCaa C38:4) and peak postprandial triglycerides were higher in prominently overweight dogs (BCS >6) than in lean dogs. Plasma carnitine concentration was lower in prominently overweight (BCS >6) than lean dogs and acetylcarnitine and urinary taurine concentration were both lower in slightly overweight (BCS 6) and prominently overweight dogs (BCS >6) than in lean dogs. Triglycerides, acetylcarnitine, taurine and cortisol showed time-dependent variations related to body condition status in the feed-challenge test.

5.1 Metabolic inflexibility in overweight dogs

In the dog cohort studied in this thesis, metabolic inflexibility may have been present already in slightly overweight dogs. At fasting, both slightly overweight and prominently overweight dogs showed signs of decreased fatty acid oxidation, as demonstrated by lower acetylcarnitine signal area in plasma compared with lean dogs. Most importantly, slightly overweight and prominently overweight dogs did not show the postprandial decrease in acetylcarnitine response after food intake seen in lean dogs. The lack of postprandial acetylcarnitine response is an example of metabolic inflexibility,

which has been proposed as a link between obesity and insulin resistance in other species (Miyata & Shimomura, 2013; Randle, 1998). Even though overweight dogs often become insulin resistant, to my knowledge acetylcarnitine concentrations in a feed-challenge test have not been investigated previously.

It has been proposed that overweight subjects oxidise more than one substrate at a time and thereby decrease the total mitochondrial oxidation rate (Muoio, 2014; Ramos-Roman *et al.*, 2012). This is similar to what probably occurred in overweight dogs in the cohort studied in this thesis. In lean dogs, the fuel switch from using endogenously stored lipids to recently absorbed nutrients from the test meal was evident by their postprandial acetylcarnitine decrease, representing a flexible metabolic response to food intake. The transition from catabolic to anabolic state was thus evident in lean dogs, but not in overweight dogs. These results indicate a need for further research on energy-restricting methods for overweight dogs that are directed towards alleviating the workload of the mitochondria to promote more flexible oxidation (*e.g*. intermittent fasting, as has been recently described in dogs) (Leung *et al.*, 2018; Pan *et al.*, 2018).

Signs of metabolic inflexibility in overweight dogs were also found in postprandial urinary metabolomes. The observed trend for higher allantoin and guanidoacetate concentrations in urine after food intake in overweight dogs, but not in lean, could be associated with a postprandial metabolism directed towards amino acids (Shestopalov *et al.*, 2006; Wyss & Kaddurah-Daouk, 2000). In addition, overweight dogs showed lower urinary taurine excretion postprandially compared with lean dogs, which might be interpreted as a non-physiological reaction, as all dogs had recently eaten a high-fat meal that also contained taurine supplements. These findings should be interpreted with caution, but could indicate that overweight dogs performed protein catabolism postprandially and were slower in shifting to using recently absorbed nutrients. Other studies have found higher plasma and urine lipid and protein metabolites in overweight compared with lean dogs (Forster *et al.*, 2018) and changes in proteins related to lipid metabolism in dogs with metabolic syndrome (Tvarijonaviciute *et al.*, 2016). However, substrate oxidation studies of mitochondria from both lean and overweight dogs in fasted and postprandial state are needed to confirm the results and to gain a deeper knowledge of substrate switching and metabolic inflexibility in overweight dogs.

In this thesis, cortisol excretion overnight was higher in overweight compared with lean dogs, a trait also reported in obese humans (Pasquali *et al.*, 2006). Increased glucocorticoid level due to increased metabolic stress in human obesity has been suggested (Muoio, 2014; Pasquali *et al.*, 2006) but the exact role and mechanisms are debated (Abraham *et al.*, 2013). One study has shown increased cortisol concentrations in plasma after a stimulation test in overweight

dogs (Martin *et al.*, 2006), but to my knowledge increased overnight urinary cortisol excretion has not been shown previously in spontaneously overweight dogs. A link to metabolic stress due to metabolic inflexibility is possible. Apart from metabolic functions, cortisol is a hormone involved in physiological stress reactions. Interestingly, overweight dogs were incapable of reacting with increased cortisol excretions postprandially in the manner observed in lean dogs, and instead showed equally high cortisol ratios at fasting and postprandial time points. Thus, prominently overweight dogs showed higher stress levels and signs of metabolic inflexibility. The potential relationship between cortisol excretion, overweight and normal response to stressful stimuli is intriguing. The cohort examined in this thesis only contained a few dogs (n=6) with pronounced overweight and the findings need to be interpreted with caution and confirmed using larger cohorts.

To summarise, this thesis is the first study to report signs of decreased fatty acid oxidation and metabolic inflexibility in spontaneously overweight dogs, as demonstrated by acetylcarnitine and cortisol responses and by postprandial metabolites in urine. Interestingly, slightly overweight dogs displayed the same acetylcarnitine pattern as dogs with more pronounced overweight.

5.2 Metabolic variations in slightly overweight dogs

One of the hypotheses tested was that overweight dogs (BCS ≥6) show variations in metabolic parameters compared with lean dogs (BCS 4-5). This hypothesis was confirmed by the data, but division of dogs coherently into lean (BCS 4-5) slightly overweight (BCS 6) and prominently overweight dogs (BCS >6) in the analyses of all six differentiating parameters showed two distinct patterns, where overweight dogs had lower or higher levels than lean dogs. Fasting urinary cortisol, one phospholipid in plasma (fasting PCaa C38:4) and serum peak postprandial triglyceride concentrations were higher in prominently overweight dogs (BCS >6), while plasma carnitine concentration was lower in prominently overweight dogs (BCS >6) than in lean dogs and acetylcarnitine and urinary taurine concentrations were lower in both slightly overweight (BCS 6) and prominently overweight dogs (BCS >6) than in lean dogs. All parameters that showed variations in overweight dogs compared with lean dogs were directly or indirectly associated with lipid metabolism. The lower carnitine and taurine status could potentially slow down lipid metabolism in overweight dogs and the higher triglyceride levels and cortisol ratio could be early signs of metabolic imbalance (Table 3). Most importantly, based on their acetylcarnitine response, dogs with only slight overweight (BCS 6) displayed similar signs of decreased

fatty acid oxidation at fasting and metabolic inflexibility to food intake as observed in dogs with more pronounced overweight (BCS >6).

In humans, metabolic inflexibility is considered to be a link between overweight and insulin resistance and is included as part of human metabolic syndrome, which predisposes for cardiovascular disease and type 2 diabetes (Collaborators, 2017; Bergouignan *et al.*, 2011; Kopelman, 2007). Being overweight significantly decreases life expectancy in dogs, but whether slight overweight affects life expectancy has not been investigated in life-long dog studies, to my knowledge. Whether metabolic inflexibility should be considered a potential health risk in dogs, as it is in humans, is not known. Metabolic inflexibility in dogs, as in humans, might be reversible by lifestyle interventions such as diet or exercise modifications, but this needs to be specifically tested in dogs. In humans, it has been shown that metabolic inflexibility becomes more severe with lower physical activity (Bergouignan *et al.*, 2011), which indicates that physical inactivity negatively affects metabolic flexibility in the same way as energy surplus and mitochondrion overload (Muoio, 2014). Occurrence of metabolic syndrome in dogs has been indicated in a few studies showing that metabolic features recognised in human metabolic syndrome may also be found in overweight and obese dogs (José Lahm Cardoso *et al.*, 2016; Kawasumi *et al.*, 2012; Tvarijonaviciute *et al.*, 2012). The major difference between species probably lies in the co-morbidities, as there is little evidence at the moment that overweight dogs develop cardiovascular diseases other than hypertension (Adolphe *et al.*, 2014) and currently no evidence of development of type 2 diabetes (Davison *et al.*, 2017; Verkest *et al.*, 2011) as consequences of meeting metabolic syndrome inclusion criteria. A hypothetical acyclic graph showing possible relationships between overweight and metabolic inflexibility in dogs and humans is shown in Figure 15. As metabolic inflexibility was found already in slightly overweight dogs in the cohort studied in this thesis, overweight can be a possible confounder, a mediator or both in the development of metabolic inflexibility in the graph. Once developed, metabolic inflexibility is taken as a possible driver for overweight (Figure 15).

Figure 15. A hypothetical acyclic graph illustrating possible relationships between different factors in the development of metabolic inflexibility in people and dogs. Illustration Josefin Söder.

Healthcare professionals should preferably bring up slight overweight early with dog owners. A slightly overweight dog has the advantage compared with an

obese individual that there is not too much extra fat mass to lose before reaching lean weight. Animal healthcare professionals should inform dog owners of the importance of keeping their dogs lean, and good arguments in this discussion are essential. This thesis adds to current knowledge by showing that even slightly overweight dogs may demonstrate variations in metabolic parameters associated with lipid metabolism and may be at risk of developing metabolic inflexibility. The occurrence of metabolic inflexibility to food intake in overweight dogs and the relevance of the finding needs to be confirmed in other cohorts of overweight dogs, but metabolic inflexibility might potentially lead to insulin resistance, metabolic syndrome or more pronounced overweight (Figure 15).

5.3 Carnitine status in overweight dogs

In Papers III and IV, the results for spontaneously overweight (BCS ≥6) Labrador Retriever dogs indicated that being overweight might be associated with lower plasma free carnitine concentrations. This in turn might slow down lipid metabolism, as carnitine mediates transport of fatty acids to the inside of mitochondria for oxidation (Hand *et al.*, 2010). Lower carnitine status may thus contribute to increased adiposity, which makes this an important finding. Notably, carnitine concentration in overweight dogs (9.4±4.2 μM) was at the lower range of the proposed reference interval (8-36 μM) for normal fasting plasma carnitine concentrations in dogs (Sanderson, 2006), and could therefore be referred to as potential carnitine insufficiency.

With the study design used, it was not possible to evaluate whether the lower carnitine concentration was a cause and/or a consequence of overweight in the dog cohort studied. A few scenarios are possible, *e.g.* free carnitine could have been depleted, there may be higher demand for carnitine in overweight dogs, carnitine status could be diet-dependent or endogenous production could be lower in overweight dogs.

In the first of these scenarios, carnitine could be depleted due to excessive acylcarnitine formation and buffering in obesity, as has been suggested in a previous review (Harmeyer, 2002). This scenario cannot completely be ruled out in the present case, but the fact that two long-chain acylcarnitines were not present in higher concentrations in overweight dogs makes it unlikely (Paper IV). Higher carnitine demand in obesity has been proposed due to higher metabolic stress (Muoio, 2014; Vigerust *et al.*, 2012). Overweight dogs in this thesis did in fact have higher overnight urinary cortisol excretion than lean dogs, but it is not known whether this leads to increased carnitine demand in overweight dogs. Carnitine status could be dependent on the fat content of the regular diet, as suggested in overweight humans and rodents (Noland *et al.*,

2009; Cederblad, 1987). Differences in total fat intake between lean and overweight dogs in this thesis cannot be excluded, as the fat content and exact amount of table scraps and treats fed were not possible to assess from the food diaries. However, the frequency with which dogs were given table scraps and treats did not differ between the lean and overweight groups. Dogs obtain carnitine from dietary protein and from biosynthetic production, so the type and amount of protein fed could have influenced plasma carnitine status, as suggested in humans (Lombard *et al*., 1989). Although complete commercial dog foods may vary in their concentration of available free carnitine, healthy dogs fed a variety of solely complete commercial diets (Shug & Keene, 1991) have been found to be well within recommended reference ranges for plasma free carnitine at fasting (Sanderson, 2006). In addition, lean and obese dogs fed high- or low-protein diets over a three-week period in a cross-over design study did not show changes in their plasma carnitine concentrations (Xu *et al.*, 2017). All dogs in the present cohort were fed complete diets based on animal proteins, and therefore they are unlikely to have suffered from protein-related carnitine insufficiency. Whether endogenous production is lower in overweight dogs is not known. It can therefore be suggested that the adiposity and possible metabolic stress of overweight dogs might had a greater impact on carnitine metabolism than potential differences in fat or protein intake alone.

5.4 Taurine status in overweight dogs

In Paper II, the results for spontaneously overweight (BCS ≥6) Labrador Retriever dogs indicated that being overweight might be associated with lower postprandial urinary taurine excretion. In Paper IV, overweight dogs showed a trend for lower overall plasma taurine response in the feed-challenge test (*P*-value not significant after correction for multiple comparisons). Lean dogs in Paper II showed a time-dependent urinary taurine response, with increased taurine excretion in postprandial compared with fasting urine, while slightly overweight and overweight dogs showed no response to feeding.

Increased urinary taurine excretion postprandially, as found in lean dogs, has been reported previously (Gray *et al.*, 2015). However, compared with findings in healthy Labrador Retriever dogs by Gray *et al.* (2015), there was a much lower relative increase in lean dogs in this thesis (40% and 286%, respectively) (Gray *et al.*, 2015). These discrepancies between study results could be related to use of different normalisation approaches for urine concentrations or to different fat contents in test diets. In previous tests in cats, cooked and raw diets resulted in different taurine losses to faeces and taurine and its metabolites were also metabolised by microbes in the gut (Backus *et al.*, 1994; Hickman *et al.*, 1992).

A high-fat test diet was used in this thesis, which could have resulted in greater gastrointestinal taurine losses in faeces compared with in urine as taurine aids bile-acid conjugation (Hardison, 1978). In support of this suggestion, it has been proposed that obesity induced by a high-fat diet results in taurine depletion in rodents, although that study proposed reduced taurine biosynthesis as the main reason for low taurine status in overweight rodents (Tsuboyama-Kasaoka *et al.*, 2006).

The findings presented in this thesis suggest that reduced taurine status could be linked to adiposity in dogs and such a connection is in line with findings in humans and rodents (Murakami, 2015; Xu *et al.*, 2013; Waldram *et al.*, 2009; Schirra *et al.*, 2008; Tsuboyama-Kasaoka *et al.*, 2006; Lee *et al.*, 2003). It has been suggested that taurine supplementation could alleviate adipose tissue inflammation and increase beta oxidation. Conversely, taurine insufficiency could promote obesity and obesity-related disorders in a vicious cycle (Murakami, 2015; Tsuboyama-Kasaoka *et al.*, 2006). Consequently, the lower taurine status in overweight dogs could be linked to less efficient lipid turnover, as could low carnitine status. Evidence of the effects of dietary taurine supplementation on reduced body weight and serum lipid concentrations has been found in rodents, but evidence from human studies is more uncertain (Tsuboyama-Kasaoka *et al.*, 2006; Zhang *et al.*, 2004; Nakaya *et al.*, 2000).

5.5 Possible interrelation of carnitine and taurine status in overweight dogs

Both taurine and carnitine concentrations were found to be lower in overweight dogs in the present cohort, findings which might be inter-related. Linear regression analysis of fasting plasma carnitine and taurine concentrations (re-analysis of data from Paper IV) showed a relatively weak but significant positive association (P=0.03, R^2 0.17) between the two plasma metabolites, with mutual decreasing concentrations in overweight dogs. Taurine and acylcarnitines (*i.e.* carnitine bound to fatty acids) are secreted into bile (Charles & Hermann, 1998; Rashed *et al.*, 1995; Huxtable, 1992) and potential long-term high fat intake causing subsequent increased losses in faeces is possible for both metabolites in overweight dogs. A general lower intake of foods containing carnitine and taurine in overweight dogs or, as proposed earlier, increased demand for these metabolites in comparison with lean dogs are also plausible. In addition, both taurine and carnitine require the amino acid methionine for biosynthetic production (Krajcovicova-Kudlackova *et al.*, 2000), so synergistic lower endogenous production in overweight dogs due to low methionine status could be suggested. Taurine biosynthesis rate has been shown to be lower in dogs with

low maintenance energy requirements (Ko *et al.*, 2007). Overweight compared with lean dogs could have lower energy requirements, especially at a weight-loss plateau. In certain breeds of dogs, taurine or combined taurine and carnitine deficiency has been suggested as a underlying cause of dilated cardiomyopathy (Kittleson *et al.*, 1997) and a genetic predisposition or low maintenance energy requirements have been suggested (Fox *et al.*, 1999). Unfortunately, taurine and carnitine concentrations in faeces or carnitine concentrations in urine were not measured in this thesis. However, the lower urinary taurine excretion of overweight compared with lean dogs could be interpreted as a compensatory sparing mechanism in the suspected generally low taurine status.

Many questions still need to be answered about whether, and by what mechanisms, spontaneous overweight dogs might reach insufficient carnitine and taurine status. Further studies that measure physiological carnitine and taurine concentrations in dogs with spontaneous overweight and examine the effects of carnitine and taurine supplementation in weight loss trials are warranted. Possible influences of a long-term, high-fat diet on carnitine and taurine metabolism in dogs also need further investigation. There may be room for advances in therapy for spontaneously overweight dogs, *e.g.* by dietary changes or individually adjusted taurine or carnitine supplementation.

5.6 Acute and chronic models of canine overweight

In relation to acute or spontaneous models of canine overweight, it is of interest to discuss two different types of abnormal fatty acid oxidation: 1) Decreased and complete, where the fatty acid oxidation reaches two carbon units but at a slow rate, and 2) increased and incomplete, where fatty acid oxidation exceeds the capacity of the tricarboxylic acid cycle and accumulation of acylcarnitines of different lengths may occur (Baker *et al.*, 2015; Schooneman *et al.*, 2013). In acutely overfed dogs, increased concentrations of long-chain acylcarnitines have been found in plasma (RC de Godoy *et al.*, 2015), which could be interpreted as a sign of increased and incomplete fatty acid oxidation. In the present cohort of spontaneous, more chronically overweight dogs, the short-chain *acetyl*carnitine and carnitine were present in lower levels in overweight dogs than in lean, and there was no increase in long-chain acylcarnitines in overweight dogs, which could be a sign of decreased complete fatty acid oxidation. Thus, discrepancies between acylcarnitine patterns in plasma might be attributable to different types of abnormal fatty acid oxidation in acute and chronic canine overweight. Variants of abnormal fatty acid oxidation, as described above, have been reported in humans and rodent models, where associations between acute and

spontaneous overweight and the presence of different acylcarnitines in muscle and plasma have been shown (Schooneman *et al.*, 2013; Noland *et al.*, 2009). While increased levels of long-chain acylcarnitines with acute weight gain were observed in a study by Godoy *et al.* (2015), free plasma carnitine concentrations were found to be unaffected during a 12-week weight gain period. Another study using short-term weight reduction found no changes in plasma carnitine concentrations (Diez *et al.*, 2004). In both studies, a limited number of laboratory dogs of different sexes and neutering status underwent short-term interventions. The reason for the discrepancies in carnitine results between those acute models and the spontaneous model of chronic overweight reported in this thesis is not known, but possible explanations could be differences between analytical techniques or that carnitine metabolism, as shown by the acylcarnitine patterns, may differ in acute experimental and spontaneous overweight in dogs.

The differences in results from different studies highlight the need for both acute and chronic models of overweight in canine metabolism research, as carnitine metabolism and fatty acid oxidation might be affected differently under these circumstances. Both models are important and have different advantages and drawbacks, such as different costs and time requirements, controlled diets versus a natural environment, breed diversity, concurrent diseases *etc*. In future dog studies, carnitine and acylcarnitines in both muscle and plasma should be assessed and the use of metabolomics analyses capable of detecting short-chain, medium-chain and long-chain acylcarnitines would be an advantage. It is of interest to test acute and chronic experimental set-ups and spontaneous overweight, in order to gain more insights into fatty acid oxidation and general oxidation rates, and how this might fit into the proposed theory of metabolic inflexibility of canine overweight.

5.7 The importance of dynamic metabolic tests

Evaluation of single metabolic parameters revealed four time-dependent variations related to body condition status in the feed-challenge test. These results emphasise the importance of including a dynamic test in metabolic research on canine overweight. If only fasting samples had been used, important information such as the novel finding of metabolic inflexibility in overweight dogs of the present cohort would have been missed.

Metabolite and phospholipid profiles also showed time-dependent responses in the feed-challenge test, but with no variations between lean and overweight groups of dogs, with the exception of the postprandial urinary metabolome. Likewise, human studies have found significant changes in metabolite profiles after feed-challenge tests compared with fasting conditions and in some

publications specific group responses have been identified (Rådjursöga *et al.*, 2018; de Toro-Martín *et al.*, 2017; Moazzami *et al.*, 2014). Differences in metabolite profiles between lean and overweight groups of dogs were seen in postprandial urine, but not in any other metabolite or phospholipid profile, in the study cohort in this thesis. These results indicate that multivariate variations in metabolic profiles in overweight dogs might be more prominent in postprandial than in fasting events, but further studies are needed to confirm this suggestion.

Future research should aim at including dogs with a wider range of body condition, different breeds and also different types of feed-challenges (such as low-fat, high-protein or oral sugar tests). The composition of the test feed, length of fasting period and length of postprandial sampling period should be carefully considered. It would also be of interest to perform exercise tests on lean and overweight dogs, to investigate whether the metabolism of overweight dogs is inflexible to physical challenge.

5.8 Lipid metabolism in overweight dogs

Prominently overweight dogs (BCS >6) showed postprandial hyper-triglyceridaemia compared with lean dogs, although no difference was found between body condition groups at fasting. The postprandial triglyceride concentrations in lean and slightly overweight dogs were within the range previously reported for healthy lean dogs of mixed breeds (Elliott *et al.*, 2011). A slight postprandial increase in plasma triglycerides, especially after a high-fat meal, is considered a physiological response in dogs (Downs *et al.*, 1997). The postprandial triglyceride concentration at or above which postprandial hypertriglyceridaemia occurs, and which might have negative health effects in dogs, has not been established, but attempts have been made to create reference ranges for dogs (Elliott *et al.*, 2008).

It should be noted that triglycerides measured postprandially reflect the sum of recently absorbed dietary fat transported in chylomicrons and fat in endogenously produced very high density lipoprotein (VLDL) (Frayn, 2009). Higher concentrations of serum triglycerides in overweight could be due to increased adipose tissue lipolysis, decreased plasma clearance or both. As free fatty acids did not differ between lean and overweight dogs, this suggests that reduced triglyceride clearance in serum of overweight dogs was the main reason for the postprandial triglyceride increase, as also suggested in obese humans (Couillard *et al.*, 1998). Unfortunately lipoproteinlipase activity, as a measure of triglyceride clearance in serum, was not possible to assess. Reduced inhibition of VLDL release into the circulation under insulin stimulation in overweight subjects is also possible (Frayn, 2009). In a study on bed-rested humans, the fate

of dietary lipids was recorded and it was demonstrated that reduced lipid clearance accounted for the increase in postprandial triglycerides, although the study suggested inactivity as the main reason and not the surplus in energy intake (Bergouignan *et al.*, 2011).

Although prominently overweight dogs had twice as high peak postprandial triglyceride concentrations as lean dogs, the values measured were only approximately half those reported in a study of obese dogs (Verkest *et al.*, 2012). Another study of obese dogs found increased triglyceride concentrations also at fasting (Tvarijonaviciute *et al.*, 2012). Taken together, these results support a positive association between serum triglyceride concentrations and body fat content, as previously reported in both humans and dogs (Miller *et al.*, 2011; Peña *et al.*, 2008). However, more pronounced overweight or obesity probably needs to be reached before fasting triglyceride values become altered in dogs, as indicated by the results in this thesis.

In the cohort studied here, lean and overweight dogs had similar free fatty acid concentrations at fasting and showed a similar declining response following food intake. This indicates comparable hydrolysis of triglycerides and release of free fatty acids into the blood stream during overnight starvation. However, the mitochondrion oxidation rate overnight differed between the groups, based on their fasting acetylcarnitine status. It can be speculated that a general lower oxidation rate of all substrates, rather than only decreased fatty acid oxidation, might be the reason for this pattern, as the overweight dogs did not accumulate free fatty acids during overnight starvation.

Although postprandial triglyceride concentrations differed between lean and overweight dogs, the much more extensive phospholipid dataset showed no multivariate separation between body condition groups at any time point. This might be attributable to distinction of exogenous and endogenous lipid pathways. The exogenous pathway handles dietary lipids, mainly triglycerides in chylomicrons, and the endogenous pathway handles HDL, LDL and VLDL, which are mainly composed of phospholipids in dogs (Xenoulis & Steiner, 2010; Maldonado *et al.*, 2001). Whether the phospholipid composition of lipoprotein fractions is different in overweight compared with lean dogs is not known. The results in this thesis do not address the distribution of lipoprotein fractions in overweight, as unfortunately it was not possible to measure those. However, it was found that overweight compared with lean dogs displayed signs of impaired exogenous handling of exogenous lipids, although no differences existed between groups in phospholipid profiles mainly representing the endogenous pathway.

In the phospholipid datasets, some interesting findings previously detected in humans were revealed in the dog cohort by the hypothesis-driven analyses. First,

fasting phosphatidylcholine PCaa C38:4 was found to be positively associated with overweight. The prominently overweight dogs (BCS >6) had significantly higher concentrations than lean and slightly overweight dogs. Associations of the same phosphatidylcholine to body mass index and waist circumference have been found in humans, even when the effect of lipoprotein fractions is accounted for (Bachlechner *et al.*, 2016; Lacruz *et al.*, 2016). In overweight humans, elevated phosphatidylcholines have been shown to be associated with insulin resistance and lipotoxicity (Rauschert *et al.*, 2016) or pro-inflammatory properties of the compounds have been proposed as underlying causes (Pietiläinen *et al.*, 2007). In this thesis, overweight dogs did not differ from lean dogs in terms of insulin sensitivity assessed by a fasting homeostasis model of assessment or in high sensitivity C-reactive protein concentrations (Hillström *et al.*, 2015). This is possibly explained by the quite moderate and spontaneous overweight in the cohort, or by species differences between dogs and humans. Further studies including heavily obese dogs and using more sensitive measures of insulin sensitivity are needed for better interpretation of the importance of elevated phosphatidylcholines in lipid metabolism of overweight dogs.

Second, the multivariate models of the phospholipid datasets showed a clear distinction between fasting and all but the one-hour postprandial time points in the dog cohort studied in this thesis. Time-dependent responses were mainly attributable to postprandial increases in phosphatidylethanolamines, which could be related to recent dietary fat intake. In an oral lipid challenge in humans, PEaa C36:2 and C36:3 showed an almost two-fold increase at two hours postprandially (Morris *et al.*, 2015). Interestingly, the dogs in this thesis displayed a comparable increase in the same phosphatidylethanolamines in response to the high-fat feed-challenge test. This implies that recent fat intake could also be measured by postprandial phosphatidylethanolamines in dogs. Future studies of lipid parameters in overweight dogs should aim at including measurements of different lipoprotein fractions, lipoprotein-lipase activity, in addition to phospholipid profiles using feed-challenge tests, to further deepen understanding of variations in lipid metabolism in overweight dogs.

6 Concluding remarks

It was found in this thesis that spontaneously overweight Labrador Retriever dogs displayed variations in metabolic parameters compared with lean dogs and that the use of a feed-challenge test allowed detection of subtle metabolic variations not noticeable in fasting conditions. Six parameters differed between spontaneously overweight and lean dogs, and all those parameters were directly or indirectly associated with lipid metabolism. The results presented in this thesis highlight the complexity of lipid metabolism in canine overweight by identifying previously known and new metabolic variations in spontaneously overweight Labrador Retriever dogs. These were:

- Triglycerides, acetylcarnitine, taurine and cortisol showed time-dependent variations related to body condition status, emphasising the importance of dynamic tests, such as feed-challenge tests, in metabolic research on canine overweight.

- Metabolic variations in general and metabolic inflexibility in particular may develop early in canine overweight, potentially already in slightly overweight dogs, as suggested by their acetylcarnitine response. Slightly overweight and prominently overweight dogs showed signs of low fatty acid oxidation at fasting and metabolic inflexibility to food intake without being profoundly insulin-resistant.

- Slightly overweight and prominently overweight dogs showed compromised carnitine and taurine status, potentially representing an interrelated insufficiency that could theoretically slow their lipid metabolism in comparison with lean dogs.

- Postprandial urine metabolomes distinguished between lean and overweight dogs, but no other metabolite or phospholipid profile was able to separate body condition groups. Metabolite and phospholipid profiles distinguished effectively between sampling time points in the feed-challenge test, but whether postprandial metabolomes might be more useful than fasting metabolomes in differentiating between lean and overweight dogs merits further investigation.

- Prominently overweight dogs showed postprandial hypertriglyceridaemia without having hyperlipidaemia at fasting, but had higher concentrations of only one plasma phosphatidylcholine than slightly overweight and lean dogs. Prominently overweight dogs also showed high overnight cortisol excretion in urine, which together with the postprandial hypertriglyceridaemia might be early signs of metabolic imbalance.

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Populärvetenskaplig sammanfattning

Under de senaste 25 åren har förekomsten av fetma hos människor fördubblats i många länder. Detta reflekterar sannolikt att vi blivit allt mer stillasittande samtidigt som kalorität snabbmat har blivit mer lättillgänglig. Våra hundar räknas idag ofta som familjemedlemmar och de delar i stor utsträckning vår livsstil. Övervikt hos hundar är också ett ökande problem. Det rapporteras att så stor andel som uppemot 60% av alla hundar i vissa länder är överviktiga. Det är visat att överviktiga hundar i större utsträckning riskerar att drabbas av kroniska sjukdomar, få sänkt livskvalité samt kortare livslängd. Vid övervikt påverkas hundens ämnesomsättning (metabolism) och hundar liksom människor drabbas av minskad känslighet för insulin (resistens) samt förhöjda blodfetter (bl.a. triglycerider) efter födointag. Vilka övriga förändringar som kan ske i ämnesomsättningen vid övervikt hos hundar är inte tillräckligt utrett. Det övergripande målet med denna avhandling var att undersöka variationer i ämnesomsättningen vid spontant uppkommen övervikt hos hundar vid fasta samt efter födointag. Teorierna var att överviktiga hundar uppvisar metabola variationer jämför med normalviktiga hundar samt att användningen av ett foderprovokationstest avslöjar variationer som varit svåra eller omöjliga att upptäcka vid analys av prover endast vid fasta. Tjugoåtta friska, okastrerade hanhundar av rasen Labrador retriever inkluderades och provtogs. Deltagande i studien innebar inte någon förändring av vad eller hur mycket hundarna åt normalt i sin hemmiljö men foderdagböcker fylldes i av hundägarna under två veckor innan provtagningen utfördes. Efter att hunden fastat över natten samlade djurägarna ett spontankastat urinprov i hemmiljön och reste sedan tillsammans med sin hund in till kliniken (Universitetsdjursjukhuset, Sveriges lantbruksuniversitet, Uppsala). Alla hundar hullbedömdes av samma veterinär enligt en hullbedömningsskala (Body condition score, BCS) som sträcker sig från ett till nio. Tolv hundar bedömdes som normalviktiga (BCS 4-5), tio som lindrigt överviktiga (BCS 6) och sex som tydligt överviktiga (BCS >6). På kliniken lades en permanent infart (venkateter) i ett blodkärl på frambenet

varefter fastande blodprover togs. Hundarna utfodrades sedan med en portion högfettsfoder som motsvarade halva deras dagliga energibehov justerat till deras normalvikt. Efter födointaget (postprandiellt) samlades blodprover varje timme under totalt fyra timmar. Vid tre timmar efter måltiden insamlades ytterligare ett urinprov. Blod- och urinproverna analyserades och en stor mängd data erhölls. I delarbete I analyserades vanliga biokemiska- och hormonella parametrar för att få viktig baskunskap om de normalviktiga och överviktiga hundarnas ämnesomsättning. I delarbete II och III användes mer utforskande analysmetoder och en mängd nedbrytningsprodukter från ämnesomsättningen (metaboliter) identifierades i urin och i blod. I delarbete IV analyserades metaboliter och en stor grupp av fetter (fosfolipider). Urvalet av analyser till delarbete IV baserades på resultaten från delarbete I-III samt från tidigare studier av andra överviktiga hundar och människor. De statistiska analyser som gjordes i samtliga delarbeten inriktades mot skillnader mellan olika hullgrupper och mot förändringar som uppstår mellan fasta och efter födointag. Sex av de analyserade parametrarna skilde sig mellan normalviktiga och överviktiga hundar och alla dessa parametrar var direkt eller indirekt kopplade till fettmetabolismen. Kortisol (stresshormon) i urinen, en fosfolipid och triglycerider i blodet efter födointag var högre hos de mest överviktiga hundarna. Metaboliterna carnitin och acetylcarnitin i blodet samt metaboliten taurin i urinen var istället lägre hos överviktiga hundar jämfört med normalviktiga. Fyra parametrar (triglycerider, acetylcarnitin, taurin och kortisol) skiljde sig mellan normalviktiga och överviktiga hundar i hur de utvecklades över tid från fasta till efter födointag. Dessa resultat visar på vikten av att använda sig av dynamiska tester såsom en foderprovokation i forskning om ämnesomsättning vid övervikt hos hund. Dessutom uppvisade de överviktiga hundarna tecken på en minskad fettnedbrytning vid fasta och svårigheter för ämnesomsättningen att växla mellan fasta och födointag (s.k. metabol inflexibilitet). Att det förekom svårigheter att växla i ämnesomsättningen hos överviktiga hundar i denna studie stöddes också av deras kortisolsvar och förekomsten av vissa metaboliter i urinen efter födointag. Hos de mest överviktiga hundarna var blodnivåerna av triglycerider efter födointag förhöjda, vilket överensstämmer med resultaten av tidigare studier. Intressant nog kunde dock inte några förhöjningar påvisas i fasta och endast en av de identifierade fosfolipiderna visade sig vara högre hos de mest överviktiga hundarna. Överviktiga hundar visade tecken på låga nivåer av metaboliterna carnitin och taurin vilket möjligen skulle kunna vara brister som i teorin skulle kunna göra fettomsättningen långsammare i denna grupp. Resultaten från denna avhandling belyser komplexiteten i fettmetabilismen hos överviktiga hundar genom att identifiera tidigare kända samt nya variationer i ämnesomsättningen hos spontant överviktiga Labrador retrievers.

Popular science summary

Over the past 25 years, the prevalence of overweight and obesity in humans has doubled in many countries. People have become more sedentary and calorie-dense food has become more readily available. Pet dogs are often seen as family members today and they largely share their owner's lifestyle. Problems with overweight in dogs are also increasing, *e.g.* in some countries up to 60% of the dog population is reported to be overweight. Overweight dogs are at risk of developing chronic illnesses early in life, suffering reduced quality of life and having shorter life expectancy. In overweight dogs, the metabolism is affected, *e.g.* overweight dogs have been shown to have resistance to insulin and elevated blood fats (*e.g.* triglycerides) after food intake, but knowledge of any other changes that may occur in the metabolism of overweight dogs is insufficient.

The overall aim of this thesis was to evaluate variations in the metabolism of spontaneously overweight dogs under fasting conditions and after food intake. It examined whether overweight dogs display differences in metabolic parameters compared with lean dogs and whether a feed-challenge test allows detection of subtle metabolic variations not noticeable in fasting conditions.

Twenty-eight healthy, intact privately-owned male Labrador Retriever dogs underwent sampling during one year. No changes were made to the diets in the home environment, but food diaries were filled in by the dog owners during two weeks before sample collection. After an overnight fast, the owners collected a urine sample at home and then travelled with their dog to the clinic (University Animal Hospital, Uppsala). At the clinic, a permanent infusion catheter was inserted into the blood vessel on the forelimb and fasting blood samples were taken. All dogs were evaluated by the same veterinarian using a 1-9 body condition scoring (BCS) scale. Twelve dogs were considered to be lean (BCS 4-5), 10 slightly overweight (BCS 6) and six prominently overweight (BCS >6). The dogs were fed a high-fat meal that corresponded to half their daily energy requirement, adjusted to their lean weight. After food intake (postprandially),

blood samples were collected every hour for four hours. At three hours after the meal, another urine sample was taken.

Blood and urine samples were analysed for common biochemical and hormonal parameters, to gain basic knowledge on the metabolism of lean and overweight dogs in the cohort. More exploratory analytical methods were also used and a large number of small particles from the metabolism (metabolites) were identified in urine and in blood. The results of these analyses and existing data on overweight dogs and humans were used to select target metabolites and a large group of fats (phospholipids) for analysis. The statistical analyses in all cases focused on differences between lean and overweight dogs and changes between fasted condition and after food intake.

Six of the parameters analysed differed between lean and overweight dogs, and all of these parameters were directly or indirectly linked to fat metabolism. Concentration of cortisol (stress hormone) in urine, one phospholipid and triglycerides in the blood after food intake were higher in prominently overweight dogs than in lean dogs, while the metabolites carnitine and acetylcarnitine in blood and taurine in urine were lower. Four metabolic parameters (triglycerides, acetylcarnitine, taurine and cortisol) differed between lean and overweight dogs in terms of the changes over time from fasting to after food intake. These results show the benefit and importance of using dynamic tests (such as the feed-challenge test) in metabolic research on overweight dogs. In addition, overweight dogs showed signs of reduced fat degradation at fasting and difficulties in switching the metabolism between fasted and postprandial state (so-called metabolic inflexibility). This was supported by their cortisol response and the presence of certain urinary metabolites after food intake.

Prominently overweight dogs had elevated triglycerides after food intake but, interestingly, no increase was observed at fasting and only one of the identified phospholipids proved to be present in higher concentrations in prominently overweight dogs. Overweight dogs showed compromised carnitine and taurine status, which could theoretically slow down fat metabolism in this group of dogs. This thesis revealed more about the complexity of fat metabolism in overweight dogs by identifying previously known and new variations in the metabolism of spontaneously overweight Labrador Retriever dogs.

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