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# The $^{13}$C-bicarbonate technique as a tool for measurement of energy expenditure in overweight dogs undergoing body weight reduction and the effect of different dietary composition

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## Abstract

Changes in body size and composition, i.e., body weight (**BW**) gain or loss, affect the daily energy expenditure (**EE**). To ensure an appropriate BW reduction and to find an efficient strategy to reduce and maintain a target BW, regular evaluations and adjustments of energy allowance are important. This study aimed to provide a detailed knowledge about the possible changes in resting EE using the oral $^{13}$C-bicarbonate technique (**o$^{13}$CBT**) as a research tool in 16 overweight pet dogs undergoing BW reduction. Dietary composition (i.e., in % of dry matter [DM] being a high protein [33.3], low fat [9.6], and high crude fiber [18.0] diet [$LFH_{Fibre}$], and a high protein [37.9], high fat [52.0], carbohydrate-free diet [$H_{Fat}$]) during 16 wk of energy restriction were evaluated regarding effects on resting EE, rate of BW reduction, body composition, and plasma concentrations of metabolic hormones involved in energy metabolism and appetite regulation. The mean BW loss was higher ($P < 0.05$) for the dogs fed the $LFH_{Fibre}$ diet (1.1%/wk) than that for dogs fed the $H_{Fat}$ diet (0.8%/wk), but the total BW reduction of 14.6% and 12.0% of initial BW did not differ significantly ($P > 0.05$). Resting EE was lower ($P < 0.02$) after the BW reduction; 414 kJ (99 kcal)/kg $BW^{0.75}$/d at the start (week 0) and 326 kJ (78 kcal)/kg $BW^{0.75}$/d at the end (week 16) of the study. The BW reduction in both groups ($P > 0.05$) consisted of both fat mass (**FM**) and fat-free mass (**FFM**). Energy expenditure, calculated in relation to amount of FFM, was not significantly ($P > 0.05$) affected by BW reduction. Dietary composition did not significantly affect ($P > 0.05$) plasma concentrations of insulin, leptin, and ghrelin, and no effect ($P > 0.05$) of BW reduction was observed on hormone concentrations. In conclusion, the o$^{13}$CBT proved to be a useful research method for studying short-term EE in overweight dogs. Even though all dogs lost BW, most dogs were still overweight at the end of the study. Due to a high individual variation among dogs, a longer experimental period with a larger sample size would be desirable.

## Lay Summary

The most common nutritional disorder in dogs is overweight, and knowledge about dogs' energy requirement is therefore important to adjust daily feed allowance. Changes in body weight may affect energy expenditure (EE) and, thereby, energy requirement. This study aimed to measure such potential changes under resting conditions in overweight dogs. It was found that the minimally invasive $^{13}$C-bicarbonate technique was a useful research method for studies regarding EE during weight loss (WL) in dogs. EE decreased when the dogs lost weight, and energy allowance needed to be reduced to maintain WL. The second objective of this study was to evaluate the effects of feeding diets with different macronutrient compositions on EE, rate of WL, body composition, and plasma concentrations of hormones involved in energy metabolism and appetite regulation. The mean WL rate was slightly higher for dogs fed a diet with high protein, low fat, and high crude fiber contents than those fed a carbohydrate-free diet with a high protein and fat contents. However, diet did not affect the resting EE, measured plasma hormone concentrations, or the total WL at the end of the study.

**Key words:** dogs, energy requirement, energy expenditure, overweight, stable isotopes

**Abbreviations:** $^{13}$CBT, $^{13}$C-bicarbonate technique; AUC, area under curve; BCS, body condition score; BMR, basal metabolic rate; BPM, beats per minute; CI, confidence intervals; CV, coefficients of variation;D, dose; DLW, doubly labeled water; DM, dry matter; EE, energy expenditure; EI, energy intake; FEDIAF, European Pet Food Industry Federation; FFM, fat-free mass; FM, fat mass; HR, heart rate; IC, indirect calorimetry; LSM, least squares means; ME, metabolizable energy; MEm, maintenance energy requirement; N, nitrogen; NRC, National Research Council; PDIFF, *P*-values for DIFFerences between LSM;ppm, parts per million; $RCO_2$, carbon dioxide production rate; RF, $^{13}$C recovery factor; RIA, radioimmunoassay;RMR, resting metabolic rate; RQ, respiratory quotient; RR, square root of residuals; SD, standard deviations;TBW, total body water

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## Introduction

The most common nutritional disorder in dogs is being overweight, and is primarily caused by the energy intake (**EI**) exceeding the energy expenditure (**EE**) over time (German, 2006; Nagoka et al., 2010). A successful body weight (**BW**) reduction can not only increase the median life span of the dogs, but may also prevent the manifestation of secondary diseases such as orthopedic disorders and cardiovascular diseases (German, 2006; Lawler et al., 2008). The maintenance energy requirement (**MEm**) of dogs is determined by their daily EE. Different components make up the daily EE, which can be partitioned into the EE related to the basal metabolic rate (**BMR**), the thermic effect of food, thermoregulation, and the activity induced EE (Speakman, 2013). The contribution from each component may differ greatly among individuals, and is affected by factors such as body size, body composition, gender, age, breed, reproductive status, and level of physical activity (NRC, 2006; Bermingham et al., 2014; Thes et al., 2016). A key factor for reducing BW is restricted energy provision (German et al., 2007; Flanagan et al., 2017). However, after a successful BW reduction program, many dogs regain BW. Although the reasons for weight regain may still be unclear, a likely cause is a decline in the MEm as a result of the BW reduction (Laflamme and Kuhlman, 1995; Nagoka et al., 2010; German et al., 2011). For most dogs, their MEm is mainly determined by the resting metabolic rate (**RMR**), which is strongly correlated with the amount of fat-free mass (**FFM**) in the body. The FFM includes vital organs and tissue components (e.g., nonlipid components of skeletal muscles) that are more metabolically active and have higher energy requirements than fat tissue (Elia, 1992). Owing to the relationship between FFM and RMR, reduced FFM after BW reduction may be an important contributing factor to the reduction of MEm.

Conventional weight loss (WL) diets for dogs usually have a high protein content, a reduced level of fat to reduce the energy density, and inclusion of dietary fiber in an attempt to make the feed more satiating. Besides being advantageous by inducing a higher thermic effect, i.e., diet-induced thermogenesis, than fat and carbohydrates (Westerterp, 2004), high protein diets can also contribute to maintaining FFM and decreasing hunger (Blanchard et al., 2004; Weber et al., 2007; German et al., 2010) and have, in some studies, been found to be more efficient in sustaining weight reduction. Provided sufficient substrates for gluconeogenesis (e.g., glucogenic amino acids), dogs have no actual requirement for dietary carbohydrates (i.e., starch) as a glucose precursor (Kienzle and Meyer, 1989; NRC, 2006). In humans, evidence suggests that high protein diets with a very low carbohydrate content result in a lower composite hunger score when compared with high protein diets with medium carbohydrate content. Despite a 4-fold greater whole-body ketone flux and a 5% lower glucose supply to the brain, this diet was not associated with the use of alternative fuels (Lobley et al., 2014). However, dogs are known to be more resistant in the development of ketosis during periods of fasting compared with humans due to differences in ketone body metabolism (De Bruijne and van den Brom, 1986). These findings suggest that even though dogs may not benefit from this proposed metabolic advantage of ketogenic diets (i.e., carbohydrate-free diets), with high protein and fat contents, it could provide a viable alternative to conventional WL diets for dogs. Also, high protein and fat contents, including high amounts of water, such as in wet diets, might enhance the feeling of satiety as water contributes to increase the bulk and gastric distension (Pappas et al., 1989; Holt et al., 1995).

To ensure appropriate weight reduction and to maintain the target BW after a successful WL program, regular evaluations and adjustments of energy allowance for the individual dog are important. EE can be measured by various methods; of which, indirect calorimetry (**IC**) is considered the "gold standard" and is based on the measurement of respiratory gases (Blaxter, 1989). Alternatively, EE can be measured by using stable isotope techniques such as the doubly labeled water (**DLW**) method (Westerterp, 2017) or the $^{13}$C-bicarbonate technique ($^{13}$CBT) when used under standardized measurement conditions (Elia, 1991; Junghans et al., 2007, 2015; Larsson et al., 2014a, 2014b; Marcussen et al., 2021). Both DLW and the $^{13}$CBT are based on the measurement of the $CO_2$ production rate ($RCO_2$) to estimate the EE. While the DLW method is based on long-term measurements (i.e., days to weeks depending on the size of the animal), the $^{13}$CBT can be used to assess short-term EE (i.e., hours) and, therefore, may be an advantageous method when measuring the change in EE and BW over time during WL.

The hypothesis of this study was that the EE of dogs changes during WL. The primary objective was to provide more knowledge on the level of possible changes in EE of privately owned overweight pet dogs undergoing BW reduction, knowledge that is important when determining an appropriate energy allowance for dogs undergoing WL programs. The second objective was to evaluate the effects of two diets with different macronutrient compositions on resting EE, rate of BW reduction, body composition, and plasma concentrations of metabolic hormones involved in energy metabolism and appetite regulation.

## Materials and Methods

The research protocol complied with the guidelines of The Animal Experiments Inspectorate, Ministry of Environment and Food, Copenhagen, Denmark, regarding animal experimentation and care (permit number 2015-15-0201-00753) and was approved by the Royal Canin ethical review committee. The study was performed during the period December to August, at the Faculty of Health and Medical Sciences, University of Copenhagen, Denmark, and as field studies in the homes of dog owners in Denmark and Sweden. The dog owners were carefully informed about the study procedure and methods, and gave a written consent before the study started.

### Animals and diets

Sixteen privately owned naturally overweight pet dogs of various breeds were included in the study. The criteria for participating were that the dog was adult (>2 years old), healthy, not on medication or a therapeutic diet, but with a body condition score (**BCS**) of 7 on a 9-point scale, representing 20% overweight, or with a BCS of 8 to 9, representing 30% and 40% excess weight, respectively (Laflamme, 1997). The majority of the dogs were mixed breeds ($n = 10$), and also the purebred breeds Pug ($n = 1$), Beagle ($n = 1$), and Labrador Retrievers ($n = 4$) were included. The dogs were divided into two dietary treatments groups: one group ($n = 8$) was fed a commercial dry WL diet (Satiety Weight Management, Royal Canin, Aimargues, France) with low fat content and high protein and fiber contents ($LFH_{Fibre}$). The other group

($n$ = 8) was fed a commercial quality assessed wet raw meat-based diet (Active/Salmon, Vom og Hundemad, Trøgstad, Norway) with high protein and fat contents, but without carbohydrates ($H_{Fat}$), and with a nutrient content that exceeded the minimum recommended nutrient levels as stated by European Pet Food Industry Federation (FEDIAF). The high fat content of the diet ($H_{Fat}$) consisted of sources rich in omega-3 fatty acids and antioxidants were added to prevent oxidation. BWs, BCS, and age of the dogs were first prioritized to select balanced pair of dogs. The next priority was the owner preference for dietary treatment. Descriptive characteristics of the two groups of dogs are presented in Table 1 and chemical composition, calculated metabolizable energy (ME) content, and protein–fat–carbohydrate ratios of the diets are listed in Table 2.

## Experimental design

The total experimental period lasted for 16 wk. At start (week 0), week 12 (w12), and week 16 (w16), dogs were weighed on the same platform scale (Kern EOS 150K50XL, Kern & Sohn GmbH, Germany) and BCS was determined independently. Target BW was determined for each dog by dividing their initial BW with the constants 1.2, 1.3, and 1.4 for dogs with BCS of 7, 8, and 9, respectively. The initial energy allowance was calculated as 315 kJ (75 kcal) ME/kg target $BW^{0.75}$/d and represented an energy restriction to approximately 80% of the estimated MEm of 398 kJ (95 kcal) ME/kg target $BW^{0.75}$/d (FEDIAF, 2018). The daily EI was calculated from food intake, recorded by the owner. The owners were instructed to feed only the allocated experimental diet to the dogs during the study, i.e., no table scraps or other treats. If needed, however, a part of the daily ration could be given as treats. In the period between w0 and w12, dogs were weighed weekly and the energy allowance was evaluated and adjusted to induce a WL rate (**WLR**) of 1% to 2% per wk. If the WL% was <1% per wk, the energy allowance was further reduced by 10%, although not below 65% of estimated MEm. Dogs were fed restrictedly between w0 and w12, or until w16 if target BW was not achieved at w12. For dogs that had reached target BW at w12, the energy allowance was adjusted to maintain BW. Between the measurements at w12 and w16, there were no weekly weighing by research staff, but dog owners were encouraged to weigh their dogs at home or at a veterinary clinic but these data were not included in the results.

**Table 1.** Descriptive characteristics (mean values ± SD)) of the dogs ($n$ = 16) included in a study comprising two dietary treatment groups* including a diet with high protein, low fat, and high fiber contents ($LFH_{Fibre}$), and a carbohydrate-free diet with high protein and fat contents ($H_{Fat}$)

| | $LFH_{Fibre}$ | $H_{Fat}$ |
|---|---|---|
| Body weight, kg | 31.7 ± 10.2 | 31.9 ± 12.3 |
| Body condition score (BCS; 1 to 9) | 8.2 ± 0.7 | 8.2 ± 0.9 |
| Age, years | 6.7 ± 2.0 | 6.3 ± 2.0 |
| Females/males | 2/6 | 5/3 |
| Intact/neutered | 1/7 | 2/6 |

*BWs, BCS, and age of the dogs were first prioritized to select balanced pair of dogs. The next priority was the owner preference for dietary treatment.

**Table 2.** Analyzed chemical composition, calculated metabolizable energy (ME) content, and protein–fat–carbohydrate ratios of the experimental diets

| | $LFH_{Fibre}$[1] | $H_{Fat}$[2] |
|---|---|---|
| DM, % | 92.4 | 37.3 |
| Chemical composition in % of DM | | |
| Crude protein | 33.3 | 37.94 |
| Crude fat | 9.6 | 52.0 |
| Total carbohydrate[3] | 50.2 | 1.1 |
| Crude fibre[4] | 18.0 | |
| Nitrogen-free extract, (NFE) | 32.2 | |
| Ash | 6.9 | 9.0 |
| *Energy* | | |
| ME, kJ/kg DM | 11,870[5] | 26,050[6] |
| ME, kcal/kg DM | 2,840[5] | 6,230[6] |
| Protein: fat: carbohydrate ratio, % of ME | 38:26:36[7] | 24:75:1[6] |

[1]Satiety Weight Management, Royal Canin, Aimargues, France. Ingredients: cellulose fiber, dried poultry protein, wheat gluten, tapioca, maize gluten, hydrolyzed animal protein, maize, wheat, animal fat, beet pulp, fish oil, minerals, fructooligosaccharides, soybean oil, psyllium seeds/shell, shellfish hydrolysate, hydrolysate from cartilage.
[2]Active/Salmon, Vom og Hundemad, Trøgstad, Norway. Ingredients: raw materials from chicken (meat cuttings, hide, and bones), mixed innards, beef tripe, and meat cuttings from pork and beef, salmon.
[3]Total carbohydrate = DM – (crude protein + crude fat + ash).
[4]Based on manufacturer data.
[5]Calculated based on the stepwise method (NRC, 2006).
[6]Calculated based on the Atwater factors (in kJ/g) for crude protein: 16.7 crude fat: 37.6 and NFE: 16.7 (NRC, 2006).
[7]Calculated based on the Modified Atwater factors (in kJ/g) for crude protein: 14.6 crude fat: 35.6 and NFE: 14.6 (NRC, 2006).

Throughout the study, dog owners were asked to fill in questionnaires regarding feed intake, fecal score (using a 1 to 5 scoring system (Moxham, 2001), where grade 1 represents "bullet-like," crumbles with little pressure and grade 5 represents entire liquid stool), and daily physical activity. The activity levels were divided according to durations of walks; 0 to 30, 30 to 60, 60 to 90, 90 to 120, and >120 min/d and the intensity scored as low (walk on leash), moderate (active walk, free runs), and high (sprints, high-intensity play).

## Sample collection

EE was determined before the start of energy restriction at w0, and then at w12 and w16. Additionally, body composition determinations in terms of fat mass (**FM**) and FFM were performed, and blood samples were collected for plasma hormone analyses. The day before the measurements, dogs were fasted overnight. In the morning, prior to the start of the measurements, the dogs were taken out for a short walk (~15 min) on leash.

### *Energy expenditure*

Resting EE was estimated using the $^{13}$CBT with oral isotope administration (o$^{13}$CBT). Each dog was measured on two consecutive days at w0, w12, and w16. Measurements were done indoors, at room temperature, and in the home of each dog, except for one dog where the measurement was done at another familiar place. Breath samples were collected in 1-liter breath bags (Wagner Analysen Technik GmbH, Bremen, Germany) using an anesthetic mask (Jørgen Kruuse A/S,

Langeskov, Denmark) with a two-way non-rebreathing valve system (Hans Rudolph, Inc., Kansas City, KS, USA). Before the experimental period started, the dogs had been introduced to the method (i.e., collection of expired breath by using the mask and breath bags) at a pre-start visit, where the mask was made interesting by putting liver pâté inside it and most dogs accepted the procedure immediately. After baseline samples were collected, 5 mg per kg BW of $^{13}$C-labeled sodium bicarbonate ($NaH^{13}CO_3$, 98 atom% $^{13}$C, Sigma-Aldrich, St Louis, MO, USA, or $NaH^{13}CO_3$, 99 atom% $^{13}$C, Cambridge Isotope Laboratories, Inc. Tewksbury, MA, USA) was given orally to the dogs by mixing the weighed dose with a small piece of liver pâté (MultiFit Tiernahrungs GmBH, Germany). Breath samples were then collected at 5, 10, 15, 20, 30, 40, 60, 90, 120, 180, 360, and 540 min after isotope administration (Fig. 1). To ensure that measurements were representative of resting conditions, heart rate (HR) recordings and behavioral observations were performed. A Polar H10 HR sensor (Polar Electro Oy, Kempele, Finland) with transmission gel (Echophonic, Jørgen Kruuse A/S, Langeskov, Denmark) placed on the electrodes on the strap (Polar Pro strap) before placed around the dog's thorax was used to register HR. The aim with the recordings and observations was to monitor whether or not the dogs were calm and relaxed during the measurement, but the results were not statistically analyzed. If the mean HR exceeded a threshold of 120 bpm and/or the dog exhibited an anxious behavior, had elevated respiration rate, was excessively excited or was walking around without finding rest and lie down, etc., the $RCO_2$ and EE measurements were excluded from the dataset.

### *Body composition and concentration of plasma hormones*

The body composition of each dog was estimated from total body water (**TBW**) determination by the deuterium oxide ($D_2O$) dilution technique (Son et al., 1998). To minimize invasiveness, urine was collected for measuring the baseline level of deuterium (except in one dog where it was replaced with a blood sample), and then dogs received an oral administration of 200 mg $D_2O$ solution (40% $D_2O$) per kg BW (Sigma-Aldrich/Merck Life Science A/S, Søborg, Denmark). The $D_2O$ solution was prepared by diluting 99.9% $D_2O$ with isotonic saline to a 40% solution. The deuterium solution was administered by using a syringe and slowly injecting it into the back of the oral cavity of the dogs. Syringes were weighed before and after administration of $D_2O$ to determine the exact mass of the $D_2O$ enrichment of the dog. A high concentration of $D_2O$ can reduce the amount of isotope solution; even a very small amount of the solution potentially lost during enrichment will contribute to incorrect estimates of the TBW content and consequently body composition of the dog. Thus, in case of suspicion of lost $D_2O$ solution, results were excluded. After an equilibration period of 3 h (i.e., around 5 h after $NaH^{13}CO_3$ administration when any short-term stress related to blood sampling was no longer considered to affect the EE results), blood samples were collected into heparinized tubes (2 × 5 mL Venoject tubes; Terumo Europe N.V.) by cephalic vein puncture to measure the $D_2O$ after equilibration (Fig. 1). Plasma was separated by centrifugation (Hettich centrifuge, EBA20) at 2,000 × *g* for 15 min and stored at –20 °C until analyses of $D_2O$ and measurement of concentrations of the metabolic hormones; leptin, insulin, and total ghrelin. For dogs that were not comfortable during sampling, blood samples were replaced with urine samples for determination of both the baseline level and levels of $D_2O$ after equilibration.

### Analytical procedures

The diets were analyzed for DM, ash, nitrogen (N), crude fat, and gross energy (GE). The DM was determined by evaporation at 103 °C to constant weight according to International Organization for Standardization (ISO) 5534 (2004). Ash was determined by combustion at 525 °C according to the Association of Official Analytical Chemists (AOAC), 923.03 (1990). The N content was determined by the Kjeldahl method using the VELP UDK 149 Automatic Distillation Unit (VELP Scientifica slr, Milano, Italy) and crude protein was calculated as N*6.25 (AOAC 984.13). Crude fat was determined by petroleum ether extraction using a Sotex system HT.6 after HCl hydrolysis using a Soxcap System 2047 (AOAC 963.15). The GE was determined using an adiabatic bomb calorimeter (IKA-Calorimeter system, IKA GmbH & Co. KG, Staufen, Germany) according to ISO 9831 (1998).

To assess the $^{13}$C kinetics of $CO_2$ in expired air, the concentration of $CO_2$ and the $^{13}$C:$^{12}$C ratios in each sample were analyzed using an Isotope Ratio Infrared Spectrometer (Wagner Analysentechnik, Bremen, Germany).

The atomic fraction of $D_2O$ was measured in plasma and urine samples after ultrafiltration using Centrisart-C4 microcentrifuge filters (5 kDa, Sartorius AG, Göttingen, Germany) and centrifuged at 6,000 × *g* at 20 °C for 2 h. The atomic fraction of $D_2O$ was measured as described for plasma by Theil et al. (2002).

### *Plasma hormone concentrations*

The hormone concentrations in plasma were analyzed by radioimmunoassay (RIA) at the University of Western Australia,

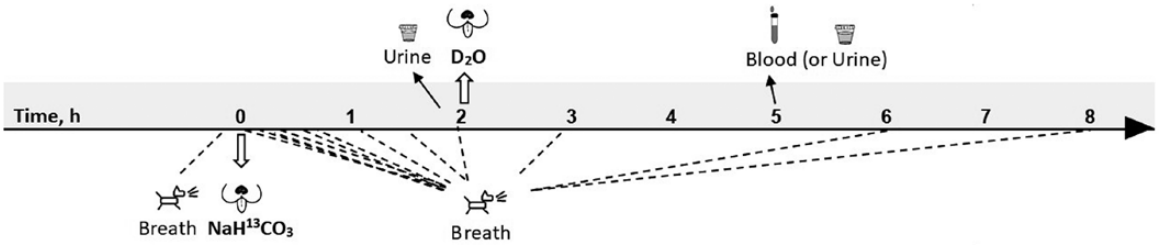


**Figure 1.** Timeline over measurements performed at the start (w0), after 12 weeks (w12), and at the end (w16) of the experimental period. energy expenditure (EE) of each dog (*n* = 16) was measured on two consecutive days in each period by using the o$^{13}$CBT ($NaH^{13}CO_3$ and breath samples). On one of these 2 d, also measurements of body composition using the deuterium dilution technique ($D_2O$, urine, and blood) and of concentration of plasma hormones were performed.

Perth. Plasma leptin was measured in duplicate using a double-antibody RIA method (Blache et al., 2000) and previously validated for dog samples (Larsson et al., 2015). All samples were processed in a single assay where the detection limit was 0.06 ng/mL and intra-assay coefficients of variation (CVs) of 2.9% (0.27 ng/mL) and 7.3% (0.54 ng/mL). Plasma insulin was assayed in duplicate by a double-antibody RIA (Tindal et al., 1978). All samples were processed in a single assay where the detection limit was 0.5 µU/mL and the intra-assay CVs of 2.9% (2.3 µU/mL) and 5.3% (6.7 µU/mL). The concentration of total ghrelin was measured by the double-antibody RIA method of Miller et al. (2009). All samples were measured in a single assay in duplicate using 50-µL aliquots of plasma. The detection limit was 0.4 ng/mL and the intra-assay CVs were of 3.1% (6.1 ng/mL) and 3.5% (21.1 ng/mL).

## Calculations

To calculate the required sample size, both a one-sided and a two-sided power tests were performed (Kaps and Lamberson, 2004), resulting in a minimum of 4 dogs per dietary treatment group.

The $^{13}C/^{12}C$ ratios in expired breath samples were reported as a relative difference from the international Pee Dee Belemnite (**PDB**) reference standard, expressed by delta (δ) values in parts per million. From the abundance of $^{13}C$ in the samples at a given time after tracer administration, the area under the $^{13}C$ enrichment–time curve (AUC) was calculated as described in Marcussen et al. (2021) and used to estimate the $RCO_2$ according to the following equation (Elia, 1991):

$$RCO_2 = \frac{D}{AUC} \cdot RF$$

where $D$ is the dose of $^{13}C$ administrated and RF is a fractional recovery factor for $^{13}C$ not recovered in the expired breath. On the basis of a previous study in overweight dogs, an estimate of 0.74 was used for the RF in this study (Larsson et al., 2014b). To estimate the EE, the modified equation of Brouwer (1965) was used:

$$EE = 5.02 \cdot RCO_2 + 16.18 \cdot \left(\frac{RCO_2}{RQ}\right)$$

where methane production and loss of urinary nitrogen are excluded from the original equation due to very low contribution in dogs (McKay and Eastwood, 1984; Junghans et al., 2007). For the respiratory quotient (**RQ**), an estimate of 0.76, based on previous studies in dogs that had been fasted overnight before measurements, was used (Larsson et al., 2014a, 2014b; McKnight et al., 2014). Results were standardized to 24 h, and the estimated EE (kJ (kcal)/d) was further calculated in relation to BW and FFM.

The TBW content in this study was calculated similar to Son et al. (1998):

$$TBW = \frac{(D_2O \text{ administrated} - ((m_1 - m_0) \cdot (D_1 - D_0/100)) \cdot 0.985 \cdot (18/20))}{(D_1 - D_0/100)}$$

where $m_1$ and $m_0$ are the BWs of the dog before $D_2O$ (g) administration and at the time of sample collection, respectively. The BW was measured only before the $D_2O$ administration, thus $m_1 = m_0$ was assumed. The $D_1$ is atom% $D_2O$ in the plasma sample obtained after equilibrium of $D_2O$, and $D_0$ is atom% $D_2O$ in the urine sample obtained before the $D_2O$ dose was administrated. The correction factor of 0.985 was used to account for incorporation of deuterium into nonexchangeable organic constituents and 18/20 as the correction factor for the difference in molecular weight between $D_2O$ and $H_2O$. Assuming that small amounts of water that may have been retained along with the fat deposit (Noblet and Etienne, 1987) can be neglected, and that FFM contains on average 73.2% moisture, the FFM was calculated as the TBW divided by 0.732, and FM was calculated as (Son et al., 1998; Wang et al., 1999)

$$FM = \text{body mass} - TBW/0.732$$

## Statistical analysis

Statistical analyses of collected data (EI, BW, BCS, WL%, WLR, FM, FFM, $RCO_2$, EE, and plasma hormone concentrations) were carried out as a repeated-measures procedure in SAS procedure MIXED (Version 9.4, SAS Institute Inc., Cary, North Carolina, USA) (Littell et al., 2006). The model comprised the fixed effects of time of measurement and dietary treatment. Interactions between fixed effects were investigated and removed from the model when nonsignificant. Dog within dietary treatment group was used as subject. The autoregressive order 1 covariance structure was used to evaluate covariance parameters. The results are presented as least squares means (**LSM**) with 95% confidence intervals (CI) or the square root of residuals (**RR**) as measures of variance. Pairwise comparisons of LSM were performed using the PDIFF option (i.e. *P*-values for DIFFerences between LSM). Differences were considered significant if $P < 0.05$.

# Results

All dogs remained healthy throughout the study based on subjective evaluation, owners' statements, and the diaries regarding feeding behavior, fecal scores, and daily activity. The dogs tolerated the diets well and the average fecal score from the reported data of both groups was a grade 2 ± 0.5, corresponding to "well-formed and does not leave a mark when picked up." The ME intake during the study was on average 72% of MEm for the estimated target BW and did not differ ($P > 0.05$) between the two dietary treatment groups (285 kJ (68 kcal) ME/kg $BW^{0.75}$/d) and (289 kJ (69 kcal) ME/kg $BW^{0.75}$/d) for dogs fed the $LFH_{Fibre}$ and the $H_{Fat}$ diet, respectively. For both groups, the energy allowance had to be reduced to maintain WL. Thus, calculated over a time period of 4 weeks, the ME intake was higher ($P < 0.001$) in the first 4 wk (w0 to w4) than the remaining period of the study (Table 3). The general activity level among the dogs was low, with most dogs having daily walks <30 min per day, such as walks on leash. However, some dogs were more frequently activated with longer daily walks and, at times, also with higher intensity, i.e., running freely during walks, play with other dogs, ball throwing games. Out of 1382 reported daily walks, 61% were in the category of <30 min/d, 13% between 30 and 60 min/d, 10% between 60 and 90 min/d, 10% between 90 and 120 min/d, and 7% >120 min/d. Even though most dogs did not reach their target BW, dog owners from both diet groups described their dogs appearing to have more energy and mobility than before the study. Some dog

**Table 3.** Body weights (BW) and body conditions scores (BCS, 1-9) measured in dogs[1] fed two different diets[2] ($LFH_{Fibre}$ or $H_{Fat}$), in a weight loss study. As well, metabolisable energy (ME) allowance, weight loss rates (WLR) in percent per week the first 4 weeks (w0-4), between w4-w8, w8-w12 and w12-w16 and, $CO_2$ production rates ($RCO_2$) and energy expenditure (EE) measured under resting conditions in fasted dogs at the start (w0), after 12 weeks (w12) and at the end of the experimental period (w16). Values are presented as the least square means along with the 95% CI in brackets.

| | Diet | | Time | | | | *P*-value, effect of: | |
|---|---|---|---|---|---|---|---|---|
| | $LFH_{Fibre}$ | $H_{Fat}$ | w0 | w12 | | w16 | Diet | Time |
| BW, kg | 28.4 (20.0-36.8) | 29.2 (20.8-37.6) | 31.8[a] (26.2-37.4) | 28.1[b] (22.5-33.7) | | 27.4[b] (21.8-33.1) | NS | <0. 001 |
| BCS | 7.1 (6.2-7.9) | 7.5 (6.6-8.3) | 8.2[a] (7.6-8.3) | 6.9[b] (6.3-7.5) | | 6.7[b] (6.1-7.3) | NS | <0.05 |
| **Energy allowance:** | | | **w0-4** | **w4-8** | **w8-12** | **w12-16** | | |
| ME, kJ/kg target $BW^{0.75}$/d | 284 (263-309) | 289 (268-309) | 309[a] (326-330) | 284[b] (268-301) | 272[b] (255-289) | 284[b] (268-301) | NS | <0.001 |
| ME, kcal/kg target $BW^{0.75}$/d | 68 (63-74) | 69 (64-74) | 74[a] (78-79) | 68[b] (64-72) | 65[b] (61-69) | 68[b] (64-72) | | |
| WLR, %/week | 1.1[a] (0.9-1.3) | 0.8[b] (0.6-1.0) | 1.4[a] (1.2-1.6) | 0.9[b] (0.7-1.2) | 0.8[b] (0.5-1.0) | 0.6[b] (0.4-0.9) | 0.04 | <0.001 |
| $RCO_2$, l/kg $BW^{0.75}$/h | 0.57 (0.49-0.65) | 0.58 (0.50-0.66) | 0.66[a] (0.59-0.73) | 0.56[b] (0.49-0.66) | | 0.52[b] (0.45-0.59) | NS | 0.005 |
| EE, kJ/kg $BW^{0.75}$/d | 361 (310-412) | 366 (316-416) | 414[a] (370-459) | 350[b] (307-393) | | 326[b] (283-370) | NS | 0.005 |
| EE, kcal/kg $BW^{0.75}$/d | 86 (74-99) | 88 (76-99) | 99[a] (88-110) | 84[b] (73-94) | | 78[b] (68-88) | | |
| EE, kJ/kg FFM/d | 345 (304-386) | 350 (315-385) | 374 (338-411) | 333 (296-369) | | 336 (299-373) | NS | NS |
| EE, kcal/kg FFM/d | 83 (73-92) | 84 (75-92) | 89 (81-98) | 80 (71-88) | | 81 (71-89) | | |
| EE, kJ/kg $FFM^{0.75}$/d | 678 (561-795) | 694 (586-801) | 758[a] (669-847) | 657[b] (568-746) | | 642[b] (553-731) | NS | 0.034 |
| EE, kcal/kg $FFM^{0.75}$/d | 162 (134-190) | 166 (140-192) | 181[a] (160-203) | 157[b] (136-178) | | 154[b] (132-175) | | |

NS: Non significant ($P > 0.05$)
[1]Number of dogs per dietary treatment: $n = 8$
[2]Diets: $LFH_{Fibre}$ = high protein, low fat and high fibre content; $H_{Fat}$ = carbohydrate-free diet with high protein and high fat content

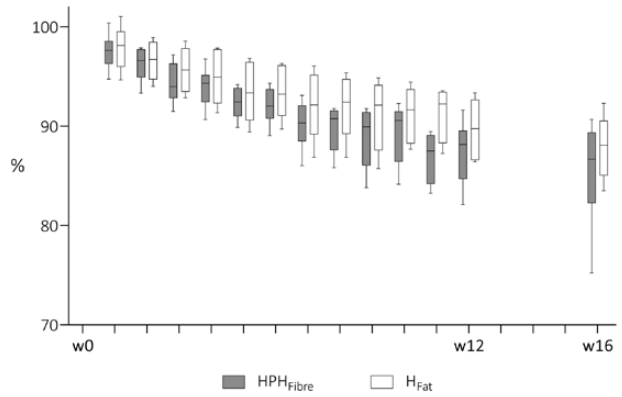


**Figure 2.** Body weights (BW) in percent of initial BW among dogs fed the LFH$_{Fibre}$ diet ($n$ = 8) and the H$_{Fat}$ diet ($n$ = 8) in a 16-wk WL study. The total BW reduction at w16 was 14.6 (CI: 12.6-16.7) % and 12.0 (CI: 10.0-14.1) % for dogs fed the LFH$_{Fibre}$ and the H$_{Fat}$ diet, respectively. The results, given as LSM with 95% CI, did not differ significantly ($P$ > 0.05) between dietary treatments.

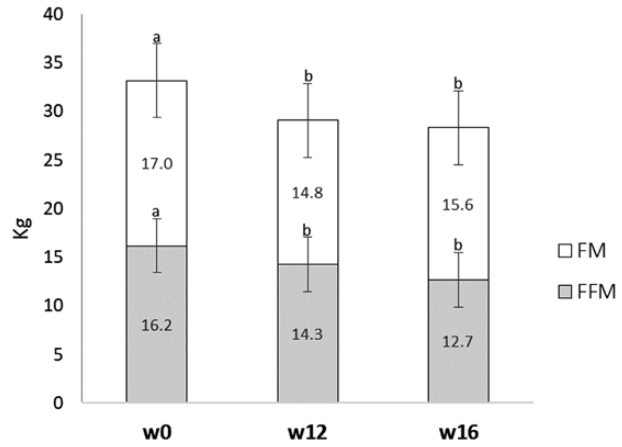


**Figure 4.** The body composition of the dogs ($n$ = 11) was determined at start (w0), after 12 wk (w12), and at the end (w16) of the experimental period. Results of fat mass (FM, kg) and fat-free mass (FFM, kg) were not significantly ($P$ > 0.05) affected by diet. Effects of time of measurements are presented as LSM along with the 95% CI, where different lower case letters (a,b) within measured parameters (FM and FFM) indicate a significant difference ($P$ < 0.05).

owners from the group fed the H$_{Fat}$, also reported better fur quality.

## BWs and body composition

There was no significant effect ($P$ > 0.05) of dietary treatment on the total BW reduction in % of initial BW (14.6 % (CI: 12.6 to 16.7) and 12.0 % (CI: 10.0 to 14.1) for dogs fed the LFH$_{Fibre}$ and the H$_{Fat}$ diet, respectively) (Fig. 2). However, the mean WLR (in % per week) was higher ($P$ < 0.05) in the dogs fed the LFH$_{Fibre}$ diet, than for dogs fed the $H_{Fat}$ diet. In both dietary groups, higher WLR ($P$ < 0.05) was observed during the first 4 wk (w0 to w4: LFH$_{Fibre}$ = 1.5 % (CI: 1.2 to 1.9) and H$_{Fat}$ = 1.3 % (CI: 0.9 to 1.6) WL/wk, respectively), than during the remaining study period. Two dogs reached target BW within the 12-wk period, one dog from each group. Three more dogs reached their target BW between w12 and w16, two from the group fed the LFH$_{Fibre}$ diet and one from

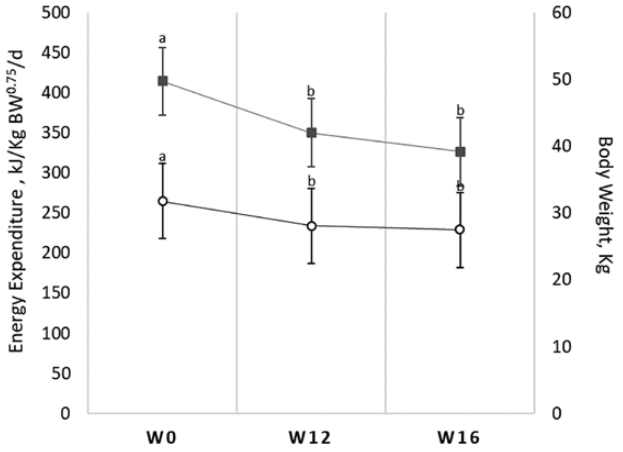


**Figure 3.** Energy expenditure (closed square) and BWs (open circle) were measured in overweight dogs ($n$ = 16) before (w0), after 12 (w12), and 16 (w16) weeks of energy restriction. Results are given as LSM with 95% CI. Different lower case letters (a,b) within measured parameters indicate a significant difference ($P$ < 0.05).

the group fed the H$_{Fat}$ diet. Accordingly, BCSs were lower ($P$ < 0.05) at w12 and w16 than at the start of the study (w0), but did not differ significantly ($P$ > 0.05) between dietary treatment groups (Table 3).

Both FM and FFM were found to be reduced ($P$ < 0.001) when measured at w12 and w16, compared with measurements at the start (w0) (Fig. 4), but results did not differ ($P$ > 0.05) between the two dietary treatment groups. However, if a dog was turning its head away during administration, or did not swallow the $D_2O$ solution immediately, results from that dog ($n$ = 5; three dogs from the LFH$_{Fibre}$ and two from the H$_{Fat}$ group, respectively) were excluded. From the measurements included ($n_{dogs}$ = 11), FM was reduced by 12.9% and FFM by 11.7% at w12. At w16, an 8.2% reduction in FM and a 21.6% reduction in FFM were measured.

## $CO_2$ production and EE

Results of $RCO_2$ and EE were not affected ($P$ > 0.05) by the day of measurement (days 1 and 2, data not shown) and values were therefore averaged, when measured at w0, w12, and w16. EE expressed per kg BW$^{0.75}$ decreased ($P$ < 0.05) from w0 to w12 and w16. However, results did not differ significantly ($P$ > 0.05) between dietary treatment groups (Fig. 3). Calculated per kg FFM, EE values were not affected ($P$ > 0.05) by the time (week) of measurement or dietary treatment, but expressed in kJ (or kcal) per kg FFM$^{0.75}$, the EE values were lower ($P$ = 0.03) at w12 and w16, than at w0 (Table 3).

## Plasma concentrations of leptin, insulin, and ghrelin

The dietary treatments did not affect ($P$ > 0.05) concentrations of leptin, insulin, and ghrelin when measured at start (w0) and at the end (w12 and w16) of the WL study. However, a diet vs. week interaction ($P$ = 0.009) for insulin concentrations was observed, and values measured at w0 were higher than values measured at w12 and w16 in the LFH$_{Fibre}$ group. Dogs fed the H$_{Fat}$ diet had higher plasma insulin concentrations at w16 than at w0 and w12 (Table 4).

**Table 4.** Fasting plasma concentrations of leptin, insulin, and total ghrelin in dogs[1] fed different diets[2] ($LFH_{Fibre}$ and $H_{Fat}$), measured at start (w0), after 12 wk of energy restriction (w12) and, at the end of the experimental period (w16)

| | Diet | | Time | | | | *P*-value, effect of | | |
|---|---|---|---|---|---|---|---|---|---|
| | $LFH_{Fibre}$ | $H_{Fat}$ | w0 | w12 | w16 | RR | Diet | Time | Diet*time[3] |
| Leptin, ng/ml | 0.132 | 0.136 | 0.133 | 0.124 | 0.144 | 0.077 | NS | NS | |
| Insulin, µU/ml | 32.0 | 30.4 | 36.5 | 26.2 | 30.9 | 22.2 | NS | NS | 0.009 |
| $LFH_{Fibre}$ | | | 46.9[a] | 30.1[b] | 18.8[b] | | | | |
| $H_{Fat}$ | | | 26.2[ab] | 22.1[b] | 42.9[a] | | | | |
| Total Ghrelin, ng/ml | 2.93 | 3.45 | 2.66 | 3.17 | 3.71 | 2.75 | NS | NS | |

[1]Number of dogs per dietary treatment: $n = 6$.
[2]Diets: $LFH_{Fibre}$ = high protein, low fat, and high fiber content; $H_{Fat}$ = carbohydrate-free diet with high protein and high-fat content.
[3]The statistical model was reduced for non-significant interaction effects.
RR: root of residuals; NS: nonsignificant ($P > 0.05$).

## Discussion

Changes in body size and body composition, i.e., BW gain or reduction, affect the daily EE and may also affect individuals differently. This study demonstrates that it is possible to obtain reliable estimates of EE in overweight dogs in a minimally invasive way. Such information may be useful to optimize the ME allowance to maintain WL and ideal BW after a successful WL program.

At the start of this experiment (w0), dogs were on average 32% overweight (mean BCS 8.2). The EE measured at this time was on average 414 kJ (99 kcal)/kg $BW^{0.75}$/d, a result that corresponds well with the MEm recommended for inactive adult pet dogs by NRC (2006) and FEDIAF (2018), i.e., 400-440 (95-105) kJ (kcal)/kg $BW^{0.75}$/d, also used for estimation of daily ME allowance. During the first 4 wk, dogs were fed on average 309 kJ (74 kcal) ME/kg $BW^{0.75}$/d, i.e., approximately 78% of MEm estimated for their target BW. This energy restriction was associated with a WL of on average 1.4% per week. However, after this period, WLRs decreased to less than 1% per week even though the ME allowance was further reduced to ~69% of the MEm estimate. WLRs between 1% and 2% per week have been achieved in research colony dogs (Laflamme & Kuhlman, 1995; Diez et al., 2002; Floerchinger et al., 2015), but based on results from previous studies (German et al., 2015; Vitger et al., 2016; Flanagan et al., 2017), WLRs <1% per week are more likely to be expected in pet dogs with naturally occurring obesity. The decrease in WLRs indicated a reduction in the EE of the dogs. This could be supported by significantly lower EE results when measured at w12 and w16. After the 16-wk WL period, the dogs had lost on average 13.8% of their initial BW. Due to the level of overweight at the start of the study, most dogs were still overweight (~18%) at the end of the study. At w16, EE was on average 326 kJ (78 kcal)/kg $BW^{0.75}$/d, similar to previously reported EE values in dogs at the same level of overweight (BCS 6.7) (Larsson et al., 2014b). However, the average ME allowance in this period was 284 kJ (68 kcal)/kg target $BW^{0.75}$/d, meaning that the ME allowance was only reduced to 87% of the measured EE value. A previous study found that the MEm requirement for obese dogs after completing a WL program was similar to this level, i.e., 285 kJ (68 kcal)/kg $BW^{0.75}$/d (German et al., 2011). Thus, the ME allowance should have been further reduced to maintain the WL of the dogs in this study. A greater WLR of more than 2% per week is, however, not recommended as it may have negative effects, such as increased loss of FFM (Prentice et al., 1991; Butterwick and Markwell, 1996). Given that FFM is more metabolically active than FM, a reduction in EE per kg $BW^{0.75}$ may indicate a WL consisting of both reduced FM and FFM, which has been observed in overweight dogs subjected to a WL program (German et al., 2011). An increased physical activity as a part of the WL program may contribute to preservation of FFM and maintain resting EE per kg $BW^{0.75}$ (Larsson et al., 2014b). Even though a loss of FFM was expected in this study, reduced FFM accounted for 46% of the WL between w0 and w12 despite the relatively low WLRs (<2% per week). It is assumed that the fraction of WL as FFM changes dynamically and is being reduced over time (Heymsfield et al., 2011). This could, however, not be supported by the results in this study where a similar reduction of FFM, as between w0 and w12, was also observed between w12 and w16, although not significant. A significantly lower proportion of FFM loss has been reported in previous WL studies in dogs (Diez et al., 2002; German et al., 2007). The use of different methods to assess body composition (i.e., dual-energy X-ray absorptiometry and the $D_2O$ dilution technique using different administration routes) might have contributed to the different results. Since only 11 dogs were included in this study, the data should also be interpreted with caution. Furthermore, excess weight at w0 differed among dogs and 6 out of these 11 dogs were between 7 and 9 years old. Factors such as baseline adiposity and age, but also sex, metabolic state or hormonal response, may affect the fractional loss of FFM during WL (Heymsfield et al., 2014).

The expression of EE raised at the power of 0.75, i.e., metabolic body size (Kleiber, 1947), is commonly used when comparing individuals within or between species. However, just as in overweight humans (Madden et al., 2016), comparing EE in overweight dogs is complex and using this allometric scaling may not be optimal when comparing EE between groups of individuals with variable FM, such as in lean and obese animals (Kaiyala et al., 2010). Therefore, EE results were correlated to the measured FFM. This calculation (i.e., EE/FFM) assumes that the contribution of fat tissue to the energy demands is zero, which is not completely true and may, therefore, also lead to confounding results (Speakman et al., 2002). It is possible that the amount of excess FM stimulates the metabolic rate of other tissues due to adipokine secretions that are positively associated with the FM (Johnstone et al., 2005; Kaiyala et al., 2010). Thus, even if the FFM is preserved during WL, EE expressed per kg $BW^{0.75}$ may be reduced due

to FFM becoming less metabolically active as a result of the reduced FM. It would have been preferable also to measure the EE after completed WL to provide more knowledge about post-WL EE, which is important in the management of weight rebound prevention. However, this was not possible within the time frame of the study.

Previous studies have investigated how different macronutrient compositions may affect preservation of FFM and satiety in dogs during WL programs (Blanchard et al., 2004; Laflamme and Hannah, 2005; Weber et al., 2007; Yamka et al., 2007; German et al., 2010). Dietary protein induces higher postprandial EE than fat and carbohydrates (Westerterp, 2004; Marcussen et al., 2021). Thus, feeding high protein diets may have beneficial effects on maintaining the daily EE and help sustaining an appropriate WLR. In this study, the percentage of ME made up by protein was higher in the $LFH_{Fibre}$ diet (38%) than in the $H_{Fat}$ diet (24%). It could therefore be expected that, with similar energy allowance, dogs fed the $LFH_{Fibre}$ would have higher postprandial EE than dogs fed the $H_{Fat}$ diet, leading to a higher WLR. This was also supported by the results where the mean WLR was higher in the $LFH_{Fibre}$ than in the $H_{Fat}$ group. However, at the end of the study, the total WL (in % of initial BW) was not significantly different between the dietary groups and the WLRs decreased in both groups. In humans, it has been suggested that after adaptation to the diet, the influence of dietary protein on the resting EE is minimal (Li et al., 2016). Although the significant difference in mean WLRs between the groups support an effect of diet-induced thermogenesis for the $LFH_{Fibre}$ diet, this could not be elucidated from the EE values, but measurements were performed on fasted dogs, which would conceal possible diet induced effects on thermogenesis.

Both high levels of fat and/or of dietary fiber are associated with slower digestion and absorption rates in the upper GI tract (Sjaastad et al., 2003; Grundy et al., 2016) which may contribute to more stable plasma glucose and insulin concentrations after feeding and, increased feeling of satiety (Kaur et al., 2016). By including dietary fiber and lower the fat content, i.e., decrease the energy density of the diet, a larger meal size can be fed, which may influence the duration of postprandial satiety. Satiety is an important factor during WL and weight management. Changed behavior, such as begging and scavenging for food, is often a reason for dog owners to give up WL programs (Porsani et al., 2020). Based on the questionnaires, it was not possible to deduce if the two diets induced different feeling of satiety. The concentrations of satiety related hormones was not different among the dietary groups but the blood samples were collected in fasted dogs, and, thus, short-term diet effects could not be evaluated. The secretion of leptin from adipose tissue increases when insulin concentration increases as a response to glucose metabolism, and contributes to satiety (Wynne et al., 2005; Trayhurn et al., 2006; Cortese et al., 2019). Plasma leptin concentration is positively correlated to body FM (Jeusette et al., 2005; Radin et al., 2009; Cortese et al., 2019). Persistent high leptin concentrations have been associated with leptin resistance, leading to lack of satiety after the meal as well as the metabolic syndrome with hyperinsulinemia and insulin resistance (Radin et al., 2009; Cortese et al., 2019). High leptin concentrations in obese dogs decrease as a result of reduced FM (Jeusette et al., 2005). This was not to be observed in this study and a likely reason is that the WL in our study (13.8%) was far below that of Jeusette et al. (2005; 31.9%). Similar to leptin, ghrelin affects energy metabolism, but high concentrations of ghrelin stimulate appetite (De Vriese & Delporte, 2008; Rhodes et al., 2018). Secretion of ghrelin from the endocrine cells in the gastrointestinal mucosa increases as a response to fasting and/or low circulating levels of glucose and insulin, and decreases after the meal (Bhatti et al., 2006; King et al., 2013; Rhodes et al., 2018). Lower fasting (24 hr) plasma ghrelin concentrations in obese than in lean dogs have been found previously and concentrations were correlated positively to BW reduction (Jeusette et al., 2005). Similar to leptin, total plasma ghrelin concentrations did not change after BW reduction in this study. The WL was probably not large enough to demonstrate a similar relationship as in the study of Jeusette et al. (2005). To get a clearer picture of the metabolic status of the dogs, it would have been preferable to also measure the concentrations of circulating glucose. It is important to note that there are other hormones not measured in this study, that are involved in the regulation of appetite and energy balance (e.g., peptide tyrosine–tyrosine [PYY], glucagon-like-peptide 1 [GLP-1], and cholecystokinin [Bosch et al., 2009; King et al., 2013]).

Limitations of this study include the number of dogs and also the short duration of the study given the variability of overweight/obesity among them. Furthermore, even though the use of private-owned dogs may better reflect the actual canine population, this also leads to a greater exposure to uncontrolled factors. When dogs are maintained at home, as in the current study, it is not possible to control, for example, food intake, allocation of treats, or physical activity, and the study relies on candid self-reported data from the dog owners. Finally, minimally invasive stable isotope methods were used to assess the EE and body composition of the dogs. Using intravenous or intramuscular, instead of oral, isotope administration may better ensure the precise doses and improve the accuracy of the results.

## Conclusions

The $o^{13}CBT$ proved to be a useful research method for determining short-term EE in field studies with overweight dogs. The results support previous observations and demonstrate reduced EE after BW reduction in overweight dogs, and also that restricted energy allowance leading to reduced FFM reduces the EE when corrected for metabolic body size.

The WLR was higher in the dogs fed the $LFH_{Fiber}$ diet, but the two diets used in this study did not differ significantly regarding the total WL, or the resting EE measured in the dogs. Furthermore, the dietary composition did not affect the fasting plasma concentrations of insulin, leptin, and ghrelin differently, and did not change as a result of WL, but the dogs did not reach target BW within the time frame of the study.

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## Conflict of Interest Statement

None declared.

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