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Department of Animal and Aquacultural Sciences
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Main Supervisor: Øystein Ahlstrøm

The effect of tetradecylthioacetic acid (TTA) on body weight management in growing silver foxes (*Vulpes vulpes*) as a model for dogs (*Canis familiaris*)

Xue Chen
Master in Feed Manufacturing Technology
Faculty of Biosciences

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Abbreviations

TTA	Tetradecylthioacetic acid
MER	Metabolizable energy requirement
IBW	Ideal body weight
TAG	Triacylglycerol
LDL-cholesterol	Low density lipoprotein- cholesterol
HDL-cholesterol	How density lipoprotein- cholesterol
PPARs	Peroxisome proliferator-activated receptors
FFA	Free fatty acids
NRC	National Research Council
AAFCO	The Association of American Feed Control Officials
FEDIAF	The European Pet Food Industry Federation
APOP	Association for Pet Obesity Prevention

Summary

This thesis consists of two parts, one general part concerning obesity and obesity related health problems in dogs. In addition, the general part gives an overview of methods to prevent obesity with restricted energy intake, low energy diets or dietary supplements. Also, a literature review over tetradecylthioacetic acid (TTA) background is given. In the second part, the experimental study was performed on growing silver foxes (*Vulpes vulpes*) to reveal if TTA supplement can influence animal body growth, feed intake and plasma lipids profile. Foxes were chosen as a model for dogs as they have similar food preference, and both belong to the *Canidae family*. The experiment was performed during growing- furring period, staring from September with finishing on December.

During this 85 days study, we examined the effect of different doses of TTA on growth and fat deposition in foxes as a model for dogs. Three groups of 7 animals were applied to these treatments: control, 1.2 g TTA and 4.8 g TTA/kg feed. High TTA dose (4.8 g/kg feed) treatment had a significant effect in reducing feed consumption, with a subsequent lower body weight gain ($P < 0.05$). A significant correlation between body weight and kidney fat indicated that, kidney fat was influenced by ME intake rather than TTA supplementation, considering there was almost no difference in the kidney fat between control and the low TTA group (1.2 g/kg feed). The effect of TTA on plasma lipids level were mainly in the changes in TG, LDL-cholesterol and FFA levels. The effects were dose dependent and more pronounced with the lowest TTA supplement. It is possible that the lowered plasma lipids due to upregulated lipid metabolism , followed by a removal of lipids from plasma and liver. Significant higher liver mass ($P < 0.05$) were found in both TTA treated groups.

The combined effects of TTA on reducing body weight gain as an effect of lower energy intake and improving plasma lipids profile suggested that, TTA might play a role in treating obesity problems in dogs.

Sammendrag

Denne oppgaven består av to deler. Den første delen omhandler helseproblemer med overvekt og fedme hos hunder, og metoder for å redusere problemet ved å redusere energitildeling, med spesielle fôrsammensetninger og ved hjelp av tilsetninger. Også effekt av tetradecylthioacetic syre (TTA) på fettomsetning blir gjennomgått i denne delen. I den andre delen, blir resultater fra et forsøk med sølvrevvalper rapportert hvor effekter av TTA på tilvekst, fôropptak og plasma lipider ble undersøkt. Rev ble brukt som model for hund i forsøket siden de har samme fôrpreferanse og begge tilhører canidene. Forsøket ble gjennomført i valpenes vekstperiode fra september til desember.

Under dette 85-dagers forsøket ble effekten av TTA undersøkt på kroppsvektutvikling og fettavleiring i forhold til energiopptak hos sølvrevvalper som modell for hund. Det ble brukt 7 sølvrevhanner i tre grupper: kontroll, 1.2 g TTA og 4.8 g TTA pr kg fôr. Behandlingen med høy TTA dose (4.8 g / kg) hadde en signifikant effekt i å redusere fôrforbruket, og dermed lavere kroppsvektsøkning. En signifikant korrelasjon mellom kroppsvekt og nyrefett indikerte at nyrefett var påvirket av e energiinntaket i stedet for TTA-tilskuddet.. Rever i begge TTA-behandlede grupper hadde redusert lipidnivå, spesielt signifikant reduksjon i TTA-gruppen på laveste nivå. Det er mulig at effekten på plasmalipidnivået skyldtes oppregulering av lipidmetabolismen,, etterfulgt av fjerning av lipider fra plasma og lever. Hos begge TTA gruppene ble det funnet en signifikant høyere levervekt i frohold til kontroll ($P < 0.05$).

e Effektene av TTA som gi reduksjon i kroppsvektsøkning som følge av redusert energiinntak og forbedring av plasma lipidprofilen tyder på at TTA kan spille en rolle i behandlingen av overvekt hos hund.

1. General introduction

Obesity is considered as a serious and widespread health related disease, not only in human, but also in companion animals. Obesity is widely considered to be related to various chronic health problems, such as glucose intolerance, insulin resistance/diabetes type II, hypertension, hyperlipidemia and coronary heart disease (Cefalo et al., 2018; Furukawa et al., 2017; Lustig et al., 2016). The main reason for being obese is the imbalance between energy expenditure and energy intake (Cahill et al., 2018), thus leading to excessive fat deposited all over body, especially in the adipose tissue. Obesity comes with an elevated body weight gain, it is thought that more than 15% to 20% above normal weight is an indication for obesity.

Lack of exercise is a direct cause leading to body fat accumulation. In addition, companion animals are always fed with energy-rich and palatable food, which makes them to consume more food than they need. In order to reduce the fat stored in the body, the inclusion of regular exercise and diet with less energy can be positive treatments affecting body weight in some ways. In addition to the important role of controlled fat consumption in body fat storage, some types of fatty acids are involved in enhancing fat metabolism, lowering triacylglycerol and cholesterol levels in the blood, thus used as feed additives, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Calder, 2004). In recent years, TTA and L-carnitine are also becoming the focus of attention in treating obesity (Center et al., 2000). There have been several studies documenting the effects of TTA on fat metabolism in humans and animals (Lovas et al., 2009). TTA is considered as a health factor in terms of reducing body fat storage, increasing fatty acid catabolism, improving insulin response, anti-inflammatory, improving plasma lipids level and promoting health (Arge et al., 2018b; Gudbrandsen et al., 2005; Morken et al., 2011). In the experiment part of this thesis, the effect of body weight gain regarding to TTA in foxes as a model for dogs will be studied in detail.

The thesis consists of a background literature review and a practical experiment. The purpose of this literature review is to remind readers the serious obesity problem dogs are facing with, and some successful experiments which helped dogs to lose weight would be illustrated. In addition, an overview over TTA chemical properties, and metabolism is presented. Since silver foxes (*Vulpes vulpes*) were used in animal study, a short description of the fox in the wild and as a farmed fur animal is included.

The experimental study contains two parts: a feeding experiment and subsequent sampling of blood and tissue from the animals. Feed consumption and foxes weight were checked and recorded periodically by employed staff in the fox farm to measure body weight growth and feed efficiency. After foxes being euthanized, blood sample, liver and kidney fat were collected with the assistance from professional technicians. Following analyses of plasma lipids were conducted. The two parts of the study are reported in Part II of the thesis.

The experimental study is a pilot study, foxes are chosen to be the animal models for dogs. If TTA shows to have body fat reducing and reduced body weight gain effects in this study, then can support the application of TTA to be further used on the treatment with obese dogs.

Part I. Literature review

2. Background –obesity problems in dogs

In the ancient time, our ancestors struggle to get some food so they could survive. Their bodies are used to energy saving status. However, modern people are now more tangled in the easy accessibility to the food, and the difficulty to refuse the temptation of a delicious meal. With the prevalence of sedentary habit and changes in life style, people tend to get overweight (Skinner et al., 2016). Therefore, obesity is becoming a serious health concern due to the over intake of food and energy. People who have their body mass index (BMI) $\geqslant$25 are considered to be overweight, and with a value over 30 are considered to be obese (Finucane et al., 2011).

In addition to causing immobility and a bad body-shape looking, excessive body weight is also a major risk factor for many kinds of health disease, such as diabetes and cardiovascular disease.

Not only in the human population, but also in companion animal, obesity has become a considerable health concern. The tendency towards obesity has been shown an increasing direction in the last decades. In the 1978, nearly one third of the dogs were found in the obese state. The problems with obesity in dogs increase with age, and were more common in females than males, in neutered than non-neutered (Sibley., 1984). At that time, when commercial foods were not as commonly applied as today, dogs' diets contained more household waste such as trimmed fat. This high energy density and unbalanced food regime with little focus on the content of essential nutrients could put dogs in an unfavorable health situation. With the development of economy, more people can afford to have a dog or a cat for companion. Because of the increased popularity for keeping pets, the commercial pet food industry has grown large and apply research to obtain knowledge that will improve and develop their products in accordance with scientific based recommendations. Modern feed manufacturing technology have made the production effective and safe, with a nutrition-balanced product.

Today, the commercial foods are nutritionally balanced for all the essential nutrients and the daily food allowance for the dog is given on the food declaration. However, it is surprising to find out obesity is still a major health concern in companion animals nowadays, especially in well-developed countries. It is reported that nearly 59% of cats and 54% of dogs are affected by obesity, also related health issues in 2016 in the U.S (APOP, 2016). The obesity problems in companion animals may due to various reasons. On the one hand, overeating is a problem in dogs because of the general greedy feeding behavior and their ability to consume large meals rapidly. The lack of a correct feeding program and resistance from the owner to give in when the dog begs for more food is another important factor that produce overweight (Chandler et al., 2017). This extra energy intake will exceed the energy requirement and make the dog deposit body fat and increased body weight. For dog owners, it is very important to observe dog behavior and stop to giving them food when it exceeds the requirement. A clear attitude to this begging habit is especially required to prevent obesity problems to develop. In addition, the recommended food intake given on the declaration on the package are general suggestions, thus, it is not a good way to feed different dogs with the same food amount, as the physical activity among dog breeds and individual dogs can be very different. It is therefore crucial that the dog owners are aware of what body weight and body conditions score the dog should have and adjust the food allowance accordingly.

Another factor, which puts dog in the unbeneficial situation for obtaining low body condition score is that many dog owners do not spend enough time to exercise their dogs. Dogs have high capacity for physical activity and it is the dog owners' responsibility to give their dogs the opportunity exercise such as walk their dogs on a leash or do some other energy consuming physical activity that will prevent obesity problems to develop. Thus, the correct balance between dietary energy intake and expenditure is the key factor to prevent obesity in the long run (Case et al., 2011).

2.1. Diagnosis of obesity in dogs and related health problems

The easiest way to assess the body fat level of the dog is by using body condition observation(Case et al., 2011). The determination of body condition score (BCS) is through visual and tactile assessment of body fat distribution, with the main places being abdominal and subcutaneous fat (Chandler et al., 2017). If the ribs, lumbar vertebrae and pelvic bones of the dog are easily visible, without obvious fat, then the dog is too thin. If ribs are easily apparent, together with some fat covering but not enough, then this dog is underweight. For ideal health condition, there is slightly more fat covering the ribs and dog bodies. This body score is preferable. If ribs are covered by a slight excessive fat, with waist and abdomen can be seen but not very prominent, then we can say the dog is overweight. In a worse situation when the dog is obese, a heavy fat will cover the whole dog body. In this situation, dog owners must do something to help dogs lose weight, because obese dogs are in a bad health condition, and there may come up with some other disease; Lethargy, a preference for warm place and reduced mobility, a slow heart rate can be easily observed in cases of obese dogs. Obese dogs do not like going out and walking around, which furthermore speeds up the degree of getting fatter. There are also some other health problems that are common in obese dogs. As like human, obesity is often accompanied by diabetes and cardiovascular disease (Andre et al., 2017) . Obesity is also related to cause disorder in organ function in the body, like liver and renal, leading to an interference in metabolism. Obese dogs are also found in the increased risk of developing osteoarthritis. Dogs with osteoarthritis can feel pain when they walk, resulting in a poorer quality of life. In many instances, one condition leads to another and the only outcome for obese dogs is a short life span (Andre et al., 2017; German, 2016; Kealy et al., 2002; Sibley., 1984)

In addition to the mentioned body disorders, there is interesting study indicating the relation between gut microbiota (GM) composition and dog longevity. Under the limitation of the same diet fed to the dogs, the obvious changing amount of various microbiota during different weight loss rate can be observed. Thus, authors concluded GM composition and fecal short chain fatty acid levels are strongly correlated to weight loss rate (Kieler et al., 2017). Then it is no wonder microbiota plays an essential role in balancing intestine health, thus may can also regulate the body weight change and affect longevity in the long run (Lawler et al., 2007).

2.2. Energy content in dog foods

Animals need to eat food or use body reserves to acquire energy for living. The basic required amount of energy is needed to maintain body's normal metabolism, also for physical activity and body temperature maintenance. Maintenance energy requirement (MER) is the term used to describe the required amount of energy for a moderately active adult animal. Values can vary widely due to the variation in dog size, breeds, age, physical activity, temperature, insulation characteristics of skin and hair and health condition.

Energy evaluation of dog food is based on the content of the energy contributing nutrients; protein, fat and carbohydrates. The gross energy (GE) content of main nutrients or a food sample can be determined by bomb calorimetry of which the heat energy generated from complete combustion of the sample is measured (NRC, 2006). For fat the GE is in the range of 8.7-9.5 kcal/g dependent on the fatty acid chain length and saturation level, crude protein GE content of 5.3-5.8 kcal/g have been reported, while carbohydrates GE have been determined from 3.3 – to 4.3 kcal/ g(NRC, 2006). Typical GE values applied in pet food is 9.4 kcal /g fat, 5.7 kcal/g protein and 4.1 kcal/g carbohydrates.

Gross energy can be easily determined, but in the real situation considering the digestibility of foods, digestible energy (DE), metabolizable energy (ME) and net energy (NE) are more commonly applied and these provides more accurate energy information animals can get through consuming. GE stands for the total energy that foods contain, after subtracting the energy losses in feces, DE is defined. When take the energy loss from urine and fermentation gas into account, then that is ME. The last transformation from ME into NE is due to the generated heat loss mainly during food digestion.

In the case of dogs, the energy evaluation of system of ME is often used. Thus, ME value can be calculated by the subtraction for the energy content of undigestible nutrients in feces together with the energy of digestible protein in the urine from food GE. The digestibility of different prepared pet foods in the market may differ widely, ranging from 70% to more than 90%. There are some different ways in counting ME value in the dog foods. The most well-known one is using Atwater method. Atwater values for the energy contributing nutrients are 4, 4 and 9 kcal/g (1cal =4,184J) for carbohydrate, protein and fat.

Originally, ME value is described as:

$$ME \text{ (kcal)} = (4 * g \text{ carbohydrate}) + (4 * g \text{ protein}) + (9 * g \text{ fat})$$

The modified Atwater factors applied for dogs in practice are 3.5 kcal/14.64 kJ per g of protein and carbohydrates, and 8.5kcal/35.56 kJ/g of fat. These factors assume digestibility values of 80, 85 and 90% for protein, carbohydrates and fat, respectively (NRC, 2006).

Fat as the main energy contributing nutrient provides a big part of energy required in animals. Partly because it is the most energy main nutrient and partly because of high digestibility (McDonald et al., 2011). Furthermore, the metabolic efficiency from absorbed fat to storage body fat is high compared to that of protein and carbohydrates. Protein and carbohydrates need to be converted to body fat through energy consuming metabolic processes which reduce the efficiency (Donato & Hegstad, 1985). Since fat is the most efficient, a low dietary fat content is a critical factor to obtain body weight reduction in obese animals. Even when total energy intake is under restriction, diets high in fat tend to preserve body fat more efficiently (Donato & Hegsted, 1985).This means the high dietary fat levels will be negative on body fat oxidation rates, thus not helpful for losing body weight. Low fat diets, with high protein and fiber content is therefore recommended in diets for body weight reduction programs.

2.2.1. Energy and main nutrient requirement for the dog

According to the research made by The European Pet Food Industry Federation, energy allowance sometimes range from less than 90 kcal ME/ $kg^{0.75}$ to 200 kcal ME/ $kg^{0.75}$ per day in the cases of dog in different age (FEDIAF, 2017). Table 1: Daily metabolizable energy (ME) requirement for adult dogs at different activity levelsTable 1 shows the energy requirement of adult dogs with consideration for the effect of physical activity. As shown in the Table 1, a recommendation for 523 kJ/$kg^{0.75}$ /day is required for an adult dog with the accessibility to moderate activity per day. This can be a guideline for most dogs, since moderate level of physical activity is easy to achieve. Besides, dogs in different life stages have different energy requirements. There is an increasing requirement for ME when dogs are in one of the following conditions: post weaned, growth, gestation, lactation, prolonged physical work, or exposed to extreme peripheral temperature.

Table 1: Daily metabolizable energy (ME) requirement for adult dogs at different activity levels (FEDIAF, 2017)

Activity level	kcal ME / $kg^{0.75}$	kJ ME / $kg^{0.75}$
Low activity (<1h/day)	95	398
Moderate activity (1-3h/day)	125	523
High activity (3-6h/day)	150-175	628-732
Obese prone dogs	≤90	≤377

Three common macronutrients contribute to the provision of energy - fat, protein and carbohydrates. Since dog has a special need for protein and fat in their daily diet, the role of these two macronutrients in energy and nutrition supply are mostly studied. Table 2 shows the recommended food nutrient profile in accordance to the dog requirement. As can be seen from the table, a minimum requirement for 21% protein and 5.5% fat in 100g foods is essential for adult dog (FEDIAF, 2017). The Association of American Feed Control Officials (AAFCO) recommends the same amount of fat and has a slightly reduction in protein ratio (18.0%) (AAFCO, 2014).

Table 2: Recommended dog main nutrient profile based on food dry matter (AAFCO, 2014; FEDIAF, 2017)

	Nutrients	Growth & reproduction	Adult maintenance
AAFCO	Crude protein	22.5%	18.0%
	Crude fat	8.5%	5.5%
FEDIAF	Crude protein	25%	21.0%
	Crude fat	8.5%	5.5%

2.3. Obese dogs and suggestions for treatment

The fundamental underlying reason for obesity is due to excessive food intake accompanied with inadequate energy expenditure. The consumed excessive energy will be stored in the body as fat, resulting in weight gain and a change in body composition resulting in reduced mobility. For obese dogs, a combination of reduced energy intake and moderately increased physical activity will be a good way to reduce body weight. Table 3 lists some weight loss program conducted on obese dogs. During dogs receiving programming treatment, energy allocation and diet composition are the two aspects should be noted concerning energy intake. In the cases, a reduced energy intake usually comes with a high protein combined with high fiber diet.

Table 3: Overview of weight loss program in obese dogs (Diez et al., 2002; Flanagan et al., 2017; German et al., 2007)

N	BCS	Duration /weeks	Energy allocation	Weight loss % of BW	diet
926	8/9	12	60-80kcal/TBW $kg^{0.75}$	11	High protein high fiber
19		20.5	60% MER for IBW	18	High protein moderate fiber
9	4.6/5	18.3	50-75% MER for IBW	30%	High protein Low starch

N: number of animals; BCS: Body Condition Score; MER: Maintenance Energy Requirement;
IBW: Ideal body weight.

- Energy intake restriction

For overweight dogs or dogs that just need a moderate weight reduction for health consideration, a slight energy intake restriction will be sufficient to obtain the required BW loss. Regular BW registrations are important to monitor the BW development. In more severe cases, when pets are more than 15% over their ideal body weight, a long term BW loss program should be applied. It is approved that a goal of 1% to 2% of body weight loss per week is optimal for dogs (German, 2016). When the weight loss outcome less than 1%, veterinary professionals would suggest somehow a degree of reduction in food intake on the circumstances of the case (Flanagan et al., 2017). In most cases, a level of 60% to 70% of MER for ideal body weight provided are most applied in weight loss program, which is also approved to be effective. Severe energy restriction may lead to a significant feeling of hunger in the animal, and a reduction in physical activity, which furthermore result in muscle mass loss.

To make it clear, take a dog weighing 30kg for an example. The dog's ideal weight is 25kg, then target weight loss per week is 0.6kg. After being put on a weight loss program, the dog's actual weight can be observed every week to see if the goal has been achieved. If dog is quite active and received regular physical activities, ME requirement $=0.60*132*30^{0.75} = 1015$kcal/day.

- A proper target weight loss goal

As stated above, a proper target BW loss goal set is important. It is concluded from some weight management programs that their owners have prematurely stopped a considerable number of obese dogs during program, because they think the weight loss progress is too slow or have not achieve the set goal. A quick weight loss setting does not only destroy owners' confidence, but also harmful for dog health. Furthermore, a rapid BW loss will often result in a weight regain shortly after the end of the program (Case et al., 2000). Experiences suggest that a body weight loss goal of 1-2% per week is proper and achievable (Flanagan et al., 2017).

- Portion-controlled feeding

After the calculation of daily energy intake has been made, it is easy to determine how much food should be fed every day. Portion-controlled feeding is widely believed to be a healthy feeding regime (Fascetti, 2010). After the food amount has been determined, this big meal can be separated into many small meals. It is not as strict as time-controlled feeding, owners can take away the feeding bowls when he thinks the dog has consumed the targeted amount of food. And the small meal can be served any time in one day when the dog gets hungry on the basis of the total amount of food have been determined. Owners can also choose to offer their dogs with less tasty food or allow less time to eat, in such a way to prevent dogs from eating too much food. Portion-controlled feeding can allow owners to understand dog's eating habit better, by observing food intake and hunger time.

- Diet choice- a high protein and high fiber diet

An effective weight loss diet should have high protein and fiber to support a low energy density and sufficient protein supply for maintenance. The problem of obesity is usually due to the high fat and energy dense food in the diet. Dogs' requirement for fat content from daily diet goes to 5.5%, while most of commercial dog food in the market has far more fat, ranging from 14-20%. A combination of high protein and fiber in the diet has been approved to have greater effect than high protein or high fiber diet alone, in reducing dog's voluntary energy intake, which indicates a greater satiating effect (Weber et al., 2007). In this way, dogs would be easy to feel full without consuming too much food for effective weight control. A consistent supplement of protein also can make sure dogs can get enough protein supplement when losing weight may cause lean mass loss (German et al., 2007). The quality and digestibility of protein should also be taken into consideration, especially when feed manufacturing technologist process the food product. A good protein quality gives a higher nitrogen retention in the body, and promotes the growth more. Adult dogs' requirement for protein content from daily diet goes to 18% on a dry matter basis (AAFCO, 2014) For dogs which need to lose some weight, protein becomes more important and should increase the percentage in the daily diet. Because when animals start to lose weight, the energy requirement still remain similar in the first place, but at the same time, energy allowance is decreased, which may promote animal to consume their own muscle to cover the energy requirement, hence cause the lean mass loss if keeping consuming the low protein diet. In addition, fiber is a health factor which is good for gastrointestinal health by helping to forming a favorable environment for the bacteria in the large intestine. A typical diet for normal adult dogs often contains between 2.5% and 4.5% fiber.

In some successful weight loss program, the diet is offered containing 33g/100gDM protein and 18g/100g DM fiber. At the end of 3 months weight loss program, it shows 896 of 926 dogs lose weight. The result indicates a diet that consists of high protein and high fiber is an important factor contributing to the weight loss success to a certain degree (Flanagan et al., 2017).

- Usage of drugs

Since the rate of weight loss in dogs is sometimes disappointing, clinicians and dog owners start to focus on the tailoring program introducing drugs. Dirlotapide is a great example. It is the first FDA-approved product used to treat obese dogs, and the function of this drug in weight loss can be explained in biology. The whole name of dirlotapide is called selective microsomal triglyceride transfer protein (MTP) inhibitors. MTP is essential in the synthesis of both chylomicron and very low-density lipoprotein (LDL). When the role of transferring protein is inhibited, the tendency of lipoprotein releasing into bloodstream will be blocked (Klonoff, 2007). Thus, weight loss task can be achieved by reducing fat absorption and increasing fat excretion. However, with frequent news about the side effects in dogs are exposed later, including vomiting, lethargy, diarrhea and soften feces during treatment. The usage of dirlotapide is stopped and the product has been withdrawn from the market in EU. Abandon using dirlotapide reminds us more the positive effect of one drug is not the only thing we should pay attention to, sometimes a mistake or side effects can turn all the effort into useless, but only trials and experiments can prove that.

3. Tetradecylthioacetic acid (TTA)

3.1. TTA – structure and production

TTA, tetradecylthioacetic acid, is a saturated fatty acid with 16 carbon atoms, in which a sulfur atom is placed in the third position in the carbon backbone counting from carboxyl end (Figure 1). Due to the existence of sulfur atom, TTA belongs to thia fatty acids. There has been a long history for scientists to synthesize this kind of fatty acids with sulfur atom, because it is related to stimulate mitochondrial fatty acid oxidation and have effect in hypolipidemic (Bremer, 2001).

$$\mathbf{CH_3{-}(CH_2)_{13}{-}S{-}CH_2{-}COOH}$$

Figure 1: TTA structure (Bremer, 2001)

TTA is not a natural occurring fatty acid, but is produced chemically from a sulphur containing acid and potassium hydroxide dissolved in methanol. Tetraadcylbromide, which is the molecule basis for the TTA is added to the solution and through heating and pH regulation, the necessary reactions will produce the TTA (Madsen et al., 1997). TTA is a dry, white crystalline product which is offered commercially.

3.2. TTA metabolism – 3 thia fatty acid

The dietary fat will be transported to adipose tissue for storage after consumption. When the body requires energy for maintenance or physical activities purposes, fat can perform it's function and provide unexpected amount of energy through oxidation, much higher than carbohydrate can do.

The β-oxidation pathway

Fat oxidation is a step-by-step work. For most of fat, the main way to release energy is through β-oxidation. Mitochondria is the place where the action happens. Fatty acids are originally located in the cytosol inside the cells, and can't permeate through mitochondrial membrane. With the help of a series of fatty acyl-CoA ligases, fatty acids can be converted into fatty acyl-CoA firstly. Then another substance carnitine comes into play, to move Acetyl CoAs into the mitochondria matrix, where oxidation occurs.

Once fatty acyl-CoAs enters the mitochondrial matrix, they are then further oxidized with the initial oxidation in the β-carbon. That's why the whole oxidation pathway is called β-oxidation pathway. Take a look into how a saturated fatty acyl-CoA oxidized.

A fatty acyl-CoA oxidation involves the following steps: 1) dehydrogenation to give an enoyl derivative; 2) hydration of the resultant double bond, with the β -carbon undergoing hydroxylation; 3) dehydrogenation of the hydroxyl group; 4) cleavage by attack of a second molecule of coenzyme A on the β-carbon, to release acetyl-CoA and a fatty acyl-CoA two carbons shorter than the original substrate. The pathway is cyclic, so the product acyl-CoA then goes into the same steps as shown above.

Acyl-CoA from β-oxidation enters the citric acid cycle, where it is oxidized to carbon dioxide, and generates energy in the form of ATP to meet the body requirement.

TTA oxidation- ω-oxidation pathway

The unique in the structure of TTA makes it differ from other normal fatty acids in metabolism. The sulfur atom in the β position inhibits the first dehydration step. So TTA cannot be β-oxidized directly. They are metabolized through ω-oxidation. As the name indicates, ω end of the fatty acid chain firstly being processed. To convert methyl group in ω end to carboxylic group for the purpose of following catabolism, TTA is firstly ω-hydroxylated in the endoplasmatic reticulum (Figure 2). This is also accompanied by sulphur being oxidized. Secondly, the formation of carboxylic group from hydroxyl group is produced in the cytosol. The production: dicarboxylic acids are finally formed, and thereafter undergo β-oxidation from the new formed carboxylic end. This process takes place in the peroxisome. Chain shortened dicarboxylic acids as one of the end products during peroxisome β-oxidation are then excreted by urine (Skrede et al., 1996).

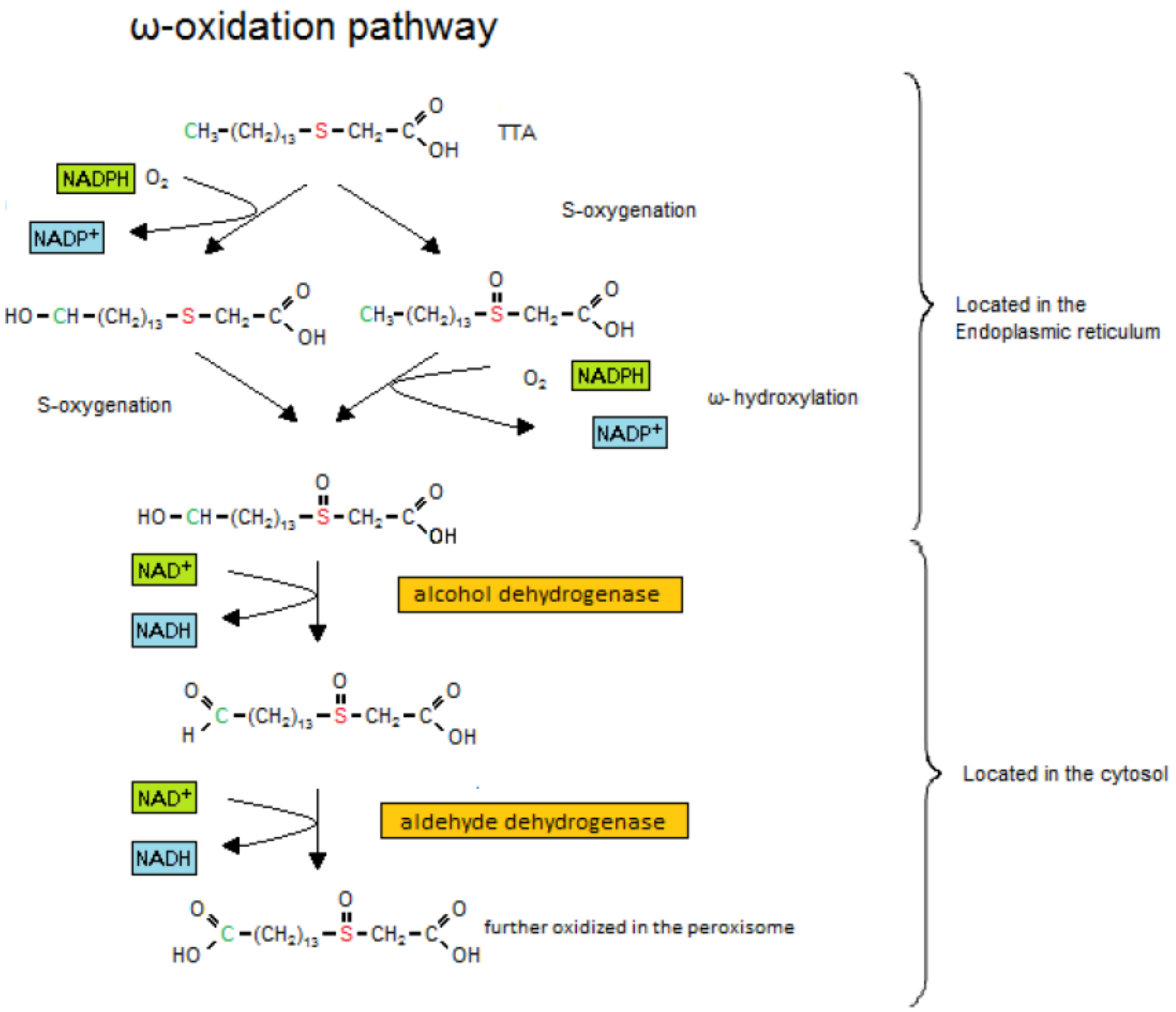

Figure 2: TTA ω oxidation pathway: the first carbon from ω end must be hydroxylated and then oxidized for the forthcoming β-oxidation in the peroxisome (Norbeck & Wedin, 2011b).

3.3. L-carnitine supplementation to increase fat oxidation

Carnitine is essential in the way of fat oxidation as an important transporter. So there are some hypothesis and researches about carnitine in the field of obesity control regarding it's metabolism function (Amin & Nagy, 2009; Arslan, 2006; Bremer, 1962). The effect of L-carnitine on the obese rats that are caused by high fat diet have been reported (Amin & Nagy, 2009). The results revealed that dietary L-carnitine supplementation reduced feed intake and therefore probably reduced the appetite of the animals. So the effect was lower body weight and adipose tissue accumulation. Rats in the carnitine treatment group has an improved body plasma lipid profile. Results indicated a better balance between different kinds of lipoprotein, a significant reduce in VLDL and LDL, accompanied by an increase in HDL amount. Some other studies also reported a greater and faster weight loss induced by supplemental carnitine (Center et al., 2000), but author also honestly claimed without the body composition studies, decision that carnitine facilitated body fat loss can be easily made. Although the essential effect of carnitine on transporting long-chain fatty acids across the inner mitochondrial membrane has been approved and widely accepted, we should remain critical attitude towards carnitine when it comes to treating obesity issue. Because L-carnitine can be produced endogenously in the animals, supplemental carnitine may cause some side effects in the body. A largely increased fatty acid oxidation indicates that person who take the extra carnitine may have the symptoms of fast heart beat rates, increase blood pressure, restlessness, sleeping difficulty etc.

4. The silver fox (*Vulpes vulpes*)

Foxes, the well-known smart and cunning animals, are the typical wild animals that can be found in many places all over the world. Foxes have a flattened skull, upright ears with a triangular shape, a pointed and slightly upturned snout, and a long bushy tail. They originally live in the wild, depending on praying some small animals for a living. Today, silver fox is an important species in fur farming and is occasionally used as model animal in research.

4.1. Feeding habit of wild foxes

Both fox and dogs belong to the same family (*Canidae*) and diverged from a common ancestor 10-12 million years ago. So foxes share some similarities with dogs, such as the body skeletal structure and meat lover habit, but have a relatively smaller weight and size than most dog breeds. Foxes have sharp teeth, which makes them excellent in gripping prey and shearing tough material like flesh (see Figure 3). The diet of foxes is largely made up of invertebrates like insect, and small vertebrates such as rabbits, hens, reptiles and birds. There is a large chance for foxes to attack poultry or other livestock if they are nearby, that is also the reason why people's opinion on foxes as nuisance creatures. Sometimes plants and berries are also their favorites.

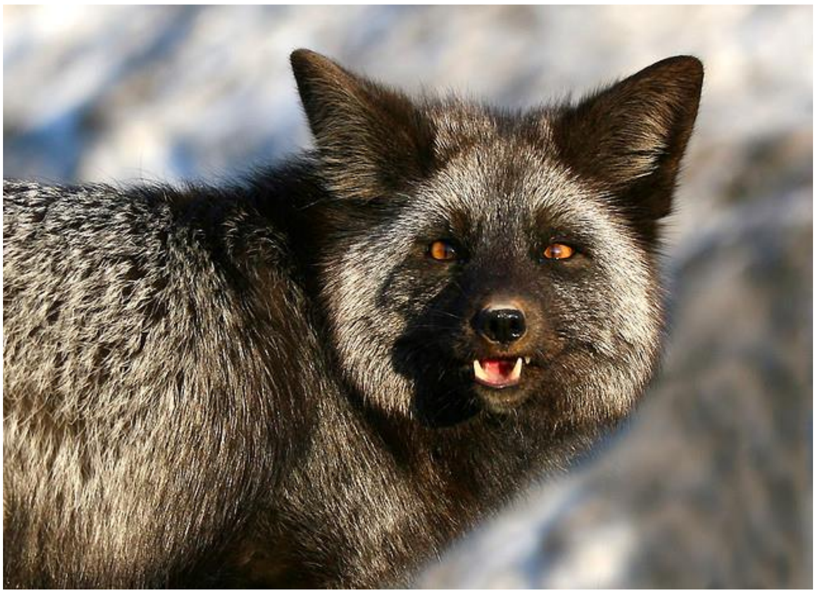

Figure 3: The silver fox

For fox, the life conditions vary largely in different parts of the world. In the remote cold places like North America and Scandinavia, life for the fox is quite challenging. The weather, snow, wind itself in winter pose a threat to the creatures living there. While in the Mediterranean coastal area, there is a richer variety of potential food resources for the fox all year round, and this is a satisfying condition for them because they have broad food preference. It can be expected that there exists a great diet difference due to the distinct environmental characteristics. Even in the same area, food can be different because of climate changing and food availability. In winter and spring when fruits are still scarce, fox can make a living out mainly by catching small mammals (mostly rodents,38-40%), caterpillars (12.2-40%) and earthworms (20-26%)

(Ciampalini & Lovari, 1985). Whereas the number of grasshoppers, beetles, also berries start to build up greatly in the summer. Winter period is a factor that makes foxes' lives harder because of food scarcity. Snow covers almost everything can be eaten, so it takes fox sometimes a lot of time and energy to find edible food. So climate and regional differences appear to be important in regulating foxes eating habit. What's makes it interesting is that breed variety is also a main factor which influencing eating habit of the foxes. Hockman studied the feeding habit of red foxes and gray foxes by analyzing fox stomach, and concluded the red fox is naturally small mammals predators. In contrast, Gray foxes show a preference for plants and fruits, and their diet is more balanced in the resources of foods, so they are more omnivorous in the feeding habit than the red fox (Hockman & Chapman, 1983).

4.2 Farmed foxes

Fur animal farming started in Canada in 1895 with silver foxes (*Vulpes vulpes*), which was caught in the wild and kept for breeding because of the very valuable fur at that time. The silver fox is a natural color mutant of the red fox (*Vulpes vulpes*). The pioneer breeding attempts was successful, and this was the start of todays' commercial fur farming. Breeding animals were imported from Canada to Norway and other countries in the 1920s, and they were the start of silver fox fur production(Nes et al., 1987). Nordic area was the biggest fur producer back then, with 2.5 million fox pelts produced in Finland (Farstad, 1998). In recent years, fox farming in Norway has been decreasing considering animal welfare, with a big fox fur market dominated by North America, China and Finland. Several other species is also used for fur animal production, the most common are the American mink (*Neovison vison*) and blue foxes (*Vulpes lagopus*)(Nes et al., 1987).

4.3. Fox farming production and management

Silver foxes have yearly reproduction and fur growth cycle that is governed by the annual day light cycle. Farmed foxes have the same cycle as wild foxes, and both males and females have breeding season from January to March. The litter size is average 3-4kg and the cubs are born in April and May (gestation 52 d). Silver fox cubs are weaned at the age of 7-8 weeks. In December, the cubs are 6-7 months old and they have reached adult body size and have grown a mature winter pelt. In fur farming, the winter pelt is the product, which is sold at world auctions. The feed applied for foxes is a commercial wet feed or a dry extruded food. The ingredients applied in fox feed are to a large degree the same as those applied in dog food, and the food preference and nutrient requirement in foxes are similar to that in dogs (Lassen et al., 2012; NRC, 2006).

4.4. Energy and nutrient recommendations for foxes

Fox requires energy every day for body maintenance, for body biochemical reactions, for physical activity and for generating body heat. Additional daily energy is needed when fox is in one or more of the following life-stage: growth, fur production, reproduction, and lactation. Since the experimental fox is under post-growing period, the recommendation for energy intake as stated below is suitable for maintenance demanding.

The first pioneer studies estimated the daily gross energy (GE) requirements of mature foxes to average 121 kcal per kilogram of body weight (Hodson & Smith, 1942). Based on this figure and animal weight, the suggestion for daily feed intake can be calculated. National Research Council suggests a maintenance diet for foxes should contain 3227 kcal of energy per kilogram of dry matter (NRC, 1968). Considering the various growth period, there are some detailed articles in nutrition field published later. Metabolized energy requirement achieves 545-590 kcal per day for each adult female during March to September. When it comes to furring period (September to December), Perel’dik recommended a range from 470 to 600 kcal is optimal (Perel'dik et al., 1972). Inadequate energy intake could cause poor fur quality, growth retardation and less milk yield.

Energy supplement comes from three main macronutrients: fat, protein and carbohydrates. Fat content plays an important role in diet energy density and resulting in various pelt quality. Perel’dik recommends 23-49 percent of energy from fat (Perel'dik et al., 1972). Protein is also reported to make a difference in skin length and pelt quality. A minimum of 22 % of ME from protein is required for maintenance (Rimeslåtten, 1976a). Studies on carbohydrates is limited and it is recommended not to excess 35% of DE(NRC, 1982).

More recent recommendations from Lassen (Lassen et al., 2012) is given in Table 4. Fur animals go through a yearly cycle divided into four production periods, of which main nutrient content and dietary energy content vary. Protein levels are minimum, fat levels are given as intervals and carbohydrates as maximum levels. In the growing-furring period from September to pelting, the fat level and energy density of the feed are increasing to facilitate body fat deposition that will produce high body weights and thereby give a long skin. Skin length is the most decisive factor for the market price of fur.

Table 4: Recommendations for silver foxes (% of metabolizable energy). Protein is given as a minimum, fat as an interval and carbohydrates as a maximum(Lassen et al., 2012).

Production period	Protein	Fat	Carbohydrates
December – parturition	34	20-50	35
Parturition – 8 weeks	36	40-50	25
9 – 18 weeks/Aug 31	27	35-55	30
September 1 – pelting	25	30-55	35

Part II Animal study

5. Introduction

The reason for obesity is not only related to the consumed fat amount, but also the different types of fat (Hu et al., 2001). A large accumulation of saturated and trans fatty acid in the body is said be bad for the health, which tend to increase hepatic LDL cholesterol level and pose a risk for fat deposition. On the contrary, some polyunsaturated fatty acids, such as EPA and DHA, thought to be beneficial, since their published lowering triacylglycerol level and chylomicron clearance function (Harris et al., 2008). However, there is an oxidative stress concern for EPA and DHA because of existed double bonds. TTA, a chemically synthesized saturated fatty acid, has gained attention for these decades, due to the enhanced fatty acid oxidation and obesity prevention potential. Tetradecylthioacetic acid **(**TTA) is an artificially produced fatty acid with a sulphur atom replacing the third methyl group from the carboxyl end. In animal studies like rats and mink, TTA exerted increased mitochondrial β-oxidation (Asiedu et al., 1993) and reduced body fat deposition and body weight gain (Norbeck & Wedin, 2011a; Wensaas et al., 2009). Mechanism behind this is widely explained by TTA as an activation factor for peroxisome proliferator activated receptor (PPARα), which have effect on regulating growth and differentiation of the body cells, and upregulating fatty acids metabolism (Westergaard et al., 2001). Besides that, several enzymes involved in lipid metabolism are induced after TTA treatment, including carnitine acetyltransferase and palmitoyl-CoA hydrolase, palmitoyl-CoA synthetase, acyl- CoA hydrolase etc (Skrede et al., 1997; Westergaard et al., 2001). TTA can therefore be classified as functional ingredient with a potential to affect fat metabolism, also in other species. In dogs, obesity is a large health concern in developed countries and dietary TTA supplementation may therefore be a contribution in reducing body fat deposition.

In this study, silver foxes will be models for dogs as they are closely related carnivores in *Canidae family*, with similar nutrient requirement, digestive tract and digestive capacity. Furthermore, it is reported that the digestibility for three main nutrients is quite similar (Ahlstrøm & Skrede, 1998). Responds of TTA on fat metabolism in foxes and dogs will therefore most likely be the same. If the responds of TTA on fat deposition in foxes are effective, a follow-up study in dogs can be carried out for further and more solid confirmation on the target species.

The hypothesis for the study was that TTA will reduce body weight in growing silver foxes with similar dietary energy consumption. It is expected that foxes fed the TTA diet will have a slower growth rate due to reduced fat deposition compared to foxes fed the control diet. Besides, there is expectation for less fat deposition observed when foxes fed with higher dietary TTA, compared to lower level, which may indicate high TTA dose is more responsive in reducing body growth. Furthermore, it is expected that TTA can also play a role in regulating fat deposition on the organs, such as kidney. In addition, an improved plasma lipids profile of the foxes in TTA treated groups is expected to see. The objective of this study was to elucidate the effect of TTA on body fat deposition and body weight gain in male fox during growing-furring period. The difference due to different TTA dose levels can make to the result will be investigated.

6. Material and methods

6.1. Animals and diets

The study started September 14th and finished December 8th in 2017 (85 days). The silver foxes cubs were 21 males (seven in each group) raised at the same farm. During the experiment, the animals were kept under conventional farm conditions in semi-outdoor houses. One exception was that the animals were housed individually in each cage. Normally, two cubs, one male and one female, are kept in each cage. The size of the cage was 1.5 x 1.0 x 1.0 m equipped with a top nest box. Each cage had a metal feeding board, which allowed for exact measurement of feed consumption. Two rows of cages in the house were applied for the experiment. Each group of seven animals were placed three on one row and four on the opposite row. The males were mainly two or three siblings that were evenly distributed to the three groups; Farmed foxes see in Figure 4.

Figure 4: Experimental foxes in the farm

1. Control group (no TTA)

2. TTA-1 group, 1.2 g TTA/kg feed

3. TTA-2 group 4.8 g TTA/kg feed

The TTA levels were based on a feed intake of approximately 250 g dry matter/animal/day, corresponding to 50 mg and 200 mg/ kg body weight per day with a six kg animal for the 1.2g and 4.8 g TTA group, respectively.

The feed was an extruded dry dog food produced at Center for Feed Technology. The food was prepared from a dog food premix, poultry fat and added the respective TTA levels. The dog food premix was made at Felleskjøpet, Vaksdal, Norway. The ingredients were wheat, corn, rice flour, poultry meal, fishmeal, beet pulp, lime stone meal, vitamins, and mineral mixture.

Diet composition was adjusted to cover the nutrient requirement for silver foxes during the growing-furring period(Lassen et al., 2012). The planned diet composition of the diet should provide metabolizable energy (ME) content of 4800 kcal/kg or 20 MJ/kg, and ME distribution from protein, fat and carbohydrates of 23, 58 and 19 %, respectively (Lassen et al., 2012). This corresponds to approximately 30 % protein, 33 % fat and 23 % carbohydrates per kg food. Each batch of food was approximately 250 kg. After drying, the extruded food was stored in 20 kg airtight bags at -20°C until use.

6.2. Feeding regime and body weights

A moderate restricted feeding regime was adopted to have best possibility to have similar daily feed intake in all animals. Similar daily feed intake through the experimental period would give the similar energy consumption. Differences in body growth and body fat deposition between groups would then be due to the TTA supplementation only in addition to random variation. The body weight of the animals were 6-6.5 kg at the start of the experiment. Maximum feed intake was estimated to be 250 g extruded food per day (5 000 kJ) corresponding to the requirement for daily energy consumption for approximately 1300 kJ/kg BW $^{0.75}$.

Water was added to the feed in the ratio 1part feed: 1.5 part water to make a wet feed mixture. The water was added to the feed the day before. Feed was given out to the foxes once daily in the morning, shortly after consumption of a meal, the farm technicians collected leftovers on the feeding board. Both offered feed and leftovers were recorded individually every day in the experimental period.

Body weight of the animals were registered at the start of the experiment, and after that every third week, and at the end. Procedure for body weight registration was that one person holding the animal stepped on weight plate. On beforehand the weight been adjusted for the body weight of the person and the body weight of the animal could be read directly from the display to the nearest 50 g.

6.3. Chemical analyses

Samples of the feed were analyzed for dry matter (DM), ash, crude protein (CP), crude fat (CF) and fatty acid composition at Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, Ås, Norway. Dry matter was determined by drying of the samples to constant weight at 103°C, whereas samples were combusted at 550°C for 10 hours for determination of ash. Nitrogen was analyzed by use of a Kjeltec 1015 Digester at 420°C and a Kjeltec Auto 2400/2600 (Foss Tecator AB, Höganäs, Sweden), and CP was determined as Kjeldahl-N × 6.25. Crude fat was determined by extraction with petroleum ether and acetone in an Accelerated Solvent Extractor (ASE 200) from Dionex (Sunnyvale, CA, USA). Fatty acids were determined by treating the feed samples with hydrochloric acid prior to extraction of lipids by chloroform:methanol 2:1 (Folch et al., 1956).Fatty acids were methylated by methanol in sulphuric acid (Welch, 1976), and the methylesters were separated and quantified by gas liquid chromatography. Gross energy of the diet was determined using bomb calorimeter. Carbohydrates was calculated by difference: carbohydrates = DM – (CP + crude fat + ash).

6.4. Blood sampling and serum analyses

At the end of the study, the foxes were put to death by electrocution with special purpose apparatus according to Norwegian regulations. Before euthanization, the animals had been fasted for 24 h. Immediately after death the animals were blood sampled by hearth puncture. After that, liver, kidney fat and gut samples were collected for histology examination. Only liver weight and kidney fat weight will be reported here.

Blood was collected in 15 ml vacutainer tubes from heart puncture and blood was allowed to coagulate for 30 minutes before centrifuged in 15 minutes. Serum was collected and kept frozen at -80°C pending analyses of serum lipids. Analyses were carried out at Laboratory for Clinical Biochemistry, Haukeland University Hospital, Bergen, Norway. Serum lipids were enzymatically determined on a Hitachi 917 system (Roche Diagnostics GmbH, Mannheim, Germany) using the total cholesterol (CHOL), LDL-cholesterol (LDL-C plus), HDL-cholesterol (HDL-C plus), and TAG (triacylglycerol GPO-PAP) kit from Roche Diagnostics, and the free cholesterol (Free Cholesterol FS), non-esterified fatty acid or free fatty acids (FFA, NEFA FS) and phospholipid kit (Phospholipids FS) from DiaSys Diagnostic Systems GmbH (Holzheim, Germany).

6.5. Ethical approval

The experimental procedures were approved by the Norwegian Animal Research Authority and followed institutional and national guidelines for the care and use of animals (the Norwegian Animal Welfare Act, and the Norwegian Regulation on Animal Experimentation).

6.6. Statistical analyses

The SAS 9.4 computer software (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses. Data were analyzed by use of the GLM procedure. The effect of diet (TTA level) was tested by one-way ANOVA according to the following model: $Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, where μ = general mean, α_i = fixed effect diet and ε_{ij} = random error component. The results are expressed as least-square means, with the variance given as pooled standard error of the means (SEM). Significant differences between means ($p \leqslant 0.05$) were determined and ranked by use of the PDIFF option. The CORR procedure of SAS was applied to test correlations.

7. Results

7.1. Diet data

7.1.1. Diet analysis

The proximate chemical analyses of the experimental diets showed that they were as expected, very similar (Table 5). The slight increase in fat content with inclusion level of TTA was likely because of TTA itself. Gross energy (GE) and metabolizable energy (ME) levels were also reflected by the slight increase in fat content.

The planned diet composition of the diet should provide ME content of 4800 kcal/kg or 20 MJ/kg. Compared to the planned content of main nutrients and energy, the chemical analyses showed that the protein and fat content was slightly lower, and the carbohydrate content was higher. The resulted in a lower ME content, about 19 MJ/kg compared with 20 MJ/kg in the planned formulation.

Table 5: Nutrient composition (g/kg) and energy data (MJ/kg) in the experimental diets.

Diet	Control	TTA-1	TTA-2
Dry matter	972	971	971
Ash	84	85	84
Crude protein	260	260	251
Crude fat	276	283	287
Carbohydrate	352	343	349
Gross energy	23.15	23.55	23.72
Metabolizable *	18.78	18.89	18.99

*Modified Atwater factors applied in calculations for metabolizable energy; 14.64 kJ/g protein and carbohydrates, 35.56 kJ/g fat (NRC, 2006)

7.1.2. Fatty acid composition in the diet

The fatty acid composition of the diets is given in Table 6. Total fatty acids are divided into three main groups: saturated, monounsaturated, and polyunsaturated fatty acids. Monounsaturated fatty acids account for a largest part amount, 41.7, 41.5 and 40.7% for control, TTA-1 and TTA-2 group, respectively. The second largest was the saturated fatty acids (mainly from poultry fat), 32.1, 32.8 and 33.9%, respectively. Finally, the polyunsaturated fatty acids (most likely from fish meal) accounted for, 26.2, 25.7 and 25.3%. As expected, there was not a big variation in the percentage of different fatty acids in three groups.

The total fatty acids amount in control, TTA-1 and TTA-2 group are 241.94, 247.83 and 250.41 g/kg fat, which corresponds well with crude fat analysis (Table 5). TTA was not analyzed for, but it seemed that since C21:0 increased with the TTA inclusion level, that it might represent the TTA in the analysis (Table 6).

Table 6: Fatty acids composition in the diets (g/kg fat).

Fatty acids	Control	1.2g/kg TTA	4.8 g/kg TTA
C12:0	1.50	1.49	1.46
C14:0	3.57	3.64	3.62
C15:0	0.36	0.36	0.38
C16:0(PA)	57.55	58.96	60.05
C17:0	0.37	0.39	0.40
C18:0(SA)	13.64	14.42	15.16
C19:0	0.18	0.18	0.18
C20:0	0.32	0.40	0.51
C21:0	0.09	1.23	2.97
C22:0	0.10	0.15	0.19
C24:0	0.05	0.07	0.08
Total saturated	77.73	81.3	85.00
C14:1n7	0.43	0.43	0.43
C16:1n7	12.24	12.37	12.20
C18:1n9	0.76	0.76	0.76
C18:1n9(OA)	87.29	89.07	88.42
C24:1	0.17	0.18	0.19
Total monounsaturated	100.89	102.81	102.00
C18:2n6(LA)	53.96	54.16	53.79
C18:3n6	0.66	0.66	0.65
C18:3n3(ALA)	4.18	4.16	4.12
C20:3n6	0.23	0.24	0.25
C20:3n3	0.08	0.08	0.08
C20:4n6	0.92	0.93	0.89
C22:2	0.15	0.17	0.17
C20:5n3(EPA)	1.20	1.32	1.36
C22:5n3	0.26	0.29	0.27
C22:6n3(DHA)	1.68	1.71	1.83
Total polyunsaturated	63.32	63.72	63.41
Total fatty acids	241.94	247.83	250.41

PA, palmitic acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid; ALA, linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

7.2. Feeding period data

7.2.1. Feed intake recording

The course of the daily feed consumption for the foxes are shown in Figure 5 and Figure 6.

From Figure 5, we can see that feed intakes were generally quite low in the first eight days either in control or TTA treatment groups. Feed consumption started to have a significant increase afterwards, we assume that it was because the diet was not very tasty as they were used to eat a commercial wet feed before the experiment started. But the feed intake increased after approximately one week. However, foxes in TTA-2 group (4.8 g/kg feed TTA included in the diet) appeared to consume much less feed than the control group (no TTA) or TTA-1 group (1.2mg/kg feed TTA). There were some overlap in the amount of feed intake between control and TTA-1 group, overall there existed a slightly higher feed intake in TTA-1 group.

There was not a significant change in feed consumption as the time went to the half the experiment, although fluctuation in feed intake between days could be seen.

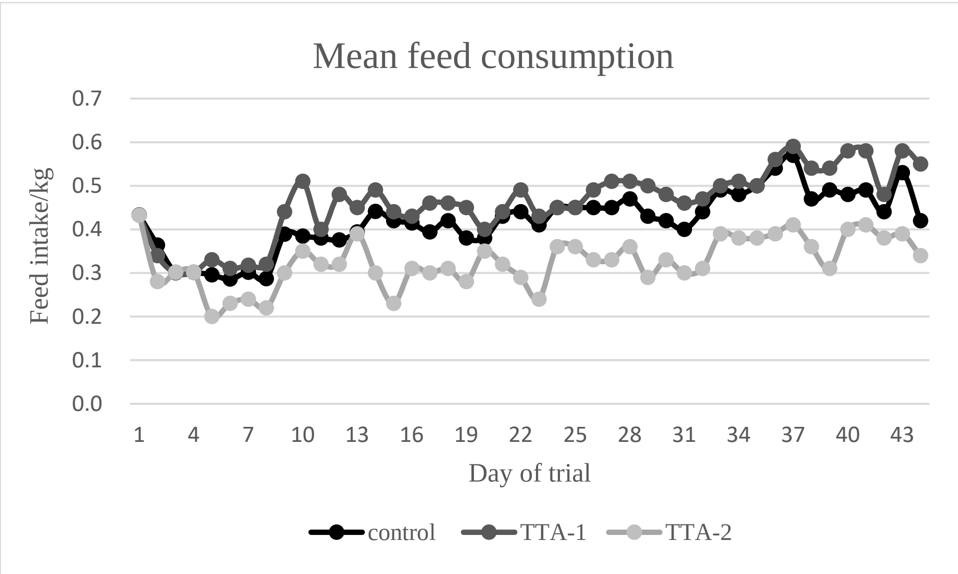

Figure 5: Diagram regarding mean wet feed consumption in the control, TTA-1 and TTA-2 groups, from day 1 to 44 (September-October).

In the following days, foxes consumed more feed in accordance to the increasing energy requirement in the fur-growing period. Wet feed consumption in TTA-2 group fluctuated around 0.3 -0.4kg/d, while the control and TTA-1 groups consumed more, 0.5-0.6 kg/d (Figure 6). At the end of the experiment, the feed intakes started to even among the groups (Figure 6), but the TTA 2 group did not compensate for the low feed intake at the start of the experiment. Daily variation on feed intake can be seen in all the groups.

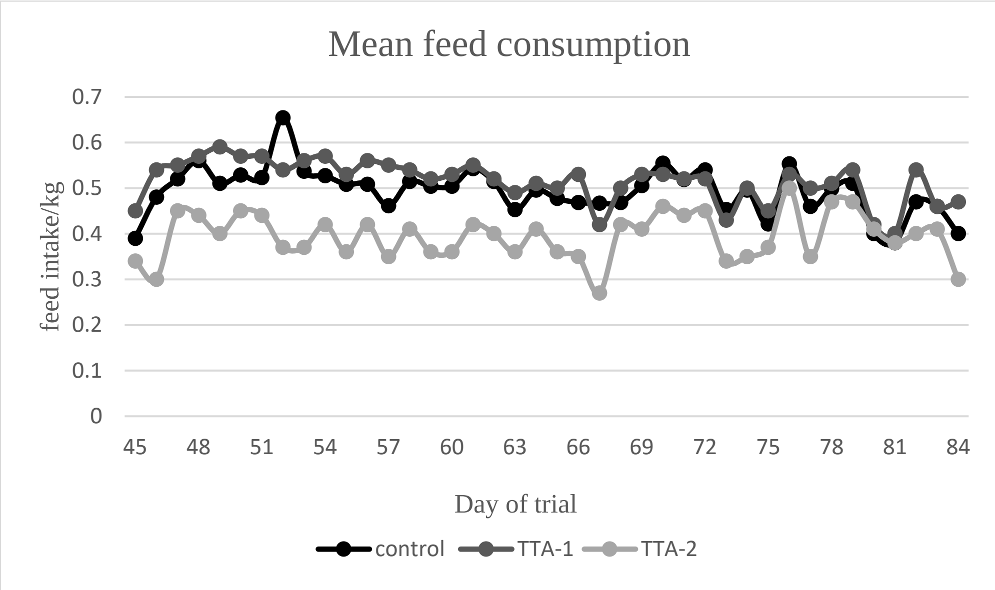

Figure 6: Diagram regarding mean wet feed consumption in the control, TTA-1 and TTA-2 groups, from day 45 to 85 (October-December).

Feed taken by foxes were prepared mixing with 60% water. The above figures showed wet feed consumption during experiment, and the following Figure 7 exhibited dry feed intake per day, recorded in each month.

The TTA-2 group revealed the lowest feed intake as shown with the wet feed intakes in Figure 5 and 6. The difference between the TTA-2 and the other groups was highest in October and November. The feed intake in the TTA-2 group was only around 2/3 of the feed intake of control and TTA-1 groups. The feed consumption between control and TTA-1 groups were quite similar, for the entire period, mean feed intake was highest with TTA-1 diet, but not significantly (Figure 7, Table 7).

Overall there was an increasing trend towards feed consumption in the first 2-3 months, and feed intake has been shown to decrease when time moved to December. This indicated an increasing energy consumption followed by a reduction, which is in accordance to the energy consumption changes through the year for silver foxes (Lassen et al., 2012).

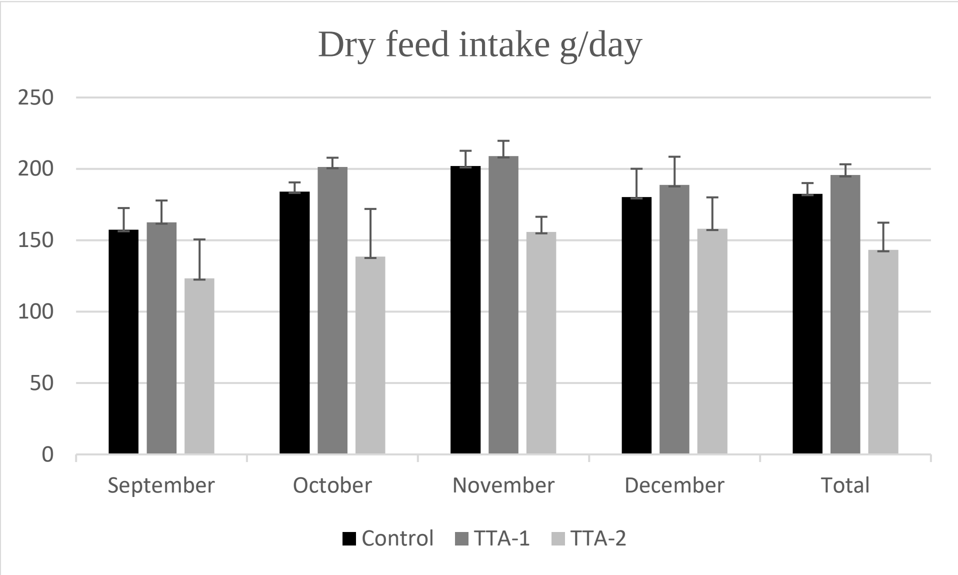

Figure 7: Diagram over average daily day matter intake on a monthly basis.

7.2.2. Body weights

At start of the experiment (8th of September), there were no significant differences in mean body weight (BW) in all the three groups, although the TTA-2 group was slightly heavier than the others.

At the first four weeks of the experiment there was body growth in the control and TTA-1 animals, while the TTA-2 animals lost body weight, thus reflecting the poor feed intake. From October, body weights started to increase in the control and TTA-1 group, while this did occur with the TTA-2 group until late October (Figure 8, Table 7). From October 25 the body weights were significantly lower with the TTA2 diet (Table 7), which lasted until the end of the experiment. The body growth was significantly lower with the TTA-2 diet, less than 0.8 kg during the experimental period, compared to control and TTA1, with 2-2.5 kg growth (Table 7). This body growth differences partly due to different feed intake, thus resulting in significant lower feed efficiency (kg body growth/ kg feed) (Table 7), and close to reduced body length, but not significant ($P<0.08$) (Table 7).

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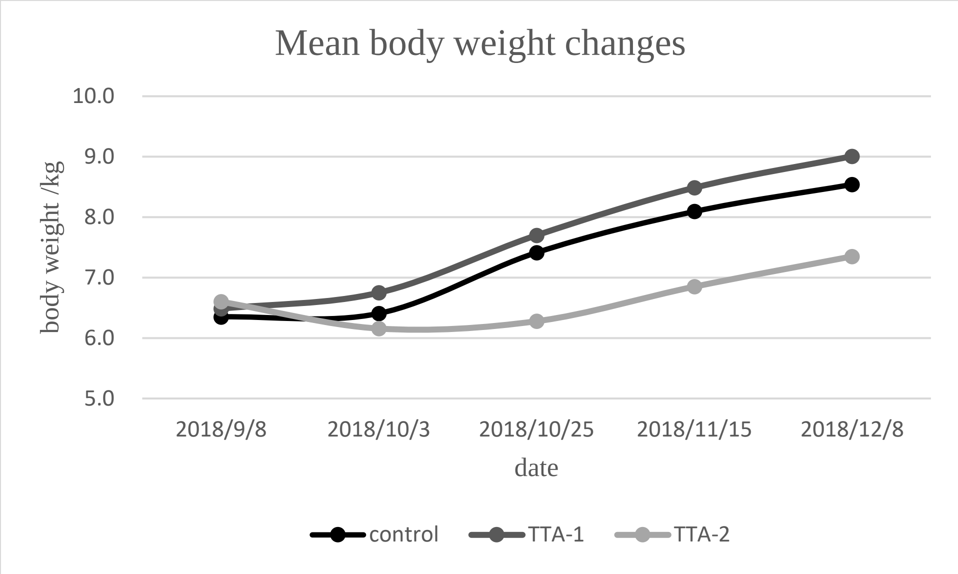


Figure 8: Diagram over mean body weight changes in control and TTA groups, from September to December.

Table 7: Body weights (kg), body length (cm), dry matter feed intake (kg) and growth parameters.

	Control	TTA-1	TTA-2	SEM	P-value
Body weights, kg					
September 14	6.35	6.49	6.60	0.18	0.63
October 3	6.41	6.75	6.15	0.20	0.15
October 25	7.41a	7.70a	6.28b	0.29	0.008
November 15	8.09a	8.49a	6.85b	0.31	0.004
December 8	8.54a	9.00a	7.35b	0.38	0.02
Growth, kg	2.19a	2.52a	0.75b	0.29	0.001
Body length, cm	67.4	67.4	65.7	0.58	0.08
Feed intake, DM, kg	15.2a	16.2a	11.9b	0.57	0.001
Growth kg /kg feed DM	0.14a	0.15a	0.06b	0.02	0.002

Results were analyzed by SAS, and the same letter means results are not significantly different from each other. ($P < 0.05$).

7.3. Organ sampling data

7.3.1. Liver and kidney fat

In Figure 9, foxes fed with supplemental TTA diet had a significant higher liver mass compared to control group, not only in the liver weight itself, but also percentage of BW. The liver weight in TTA-1 and TTA-2 groups were quite similar, but the significant higher percentage in liver weight of BW in TTA-2 group showed the effect of TTA or that the low feed intake had produced this effect.

For the kidney, there was a tendency towards lower kidney fat in the TTA group, and there was a significant fat reduction in TTA-2 group ($p < 0.05$) compared to the others (Table 8). The description also applied well when presented as kidney fat of BW.

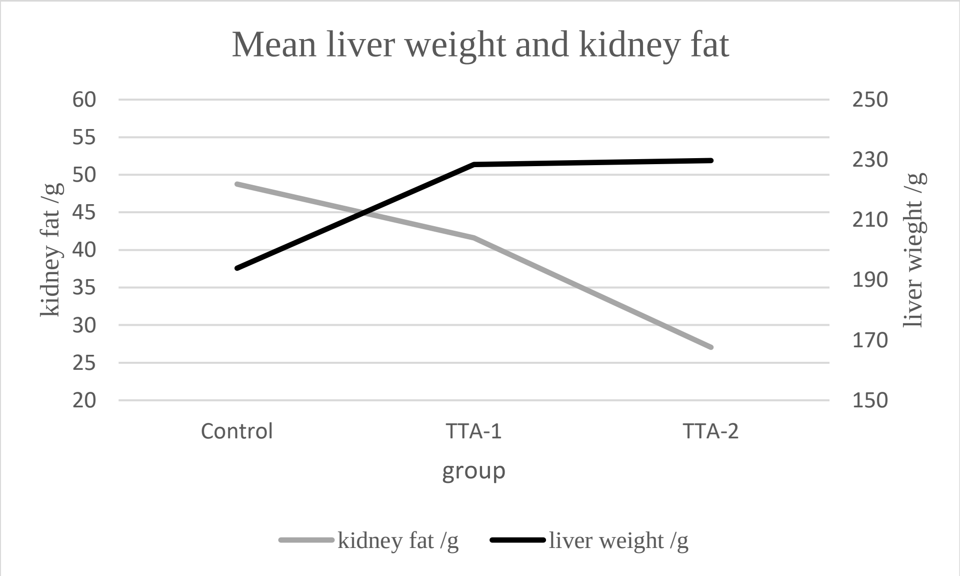
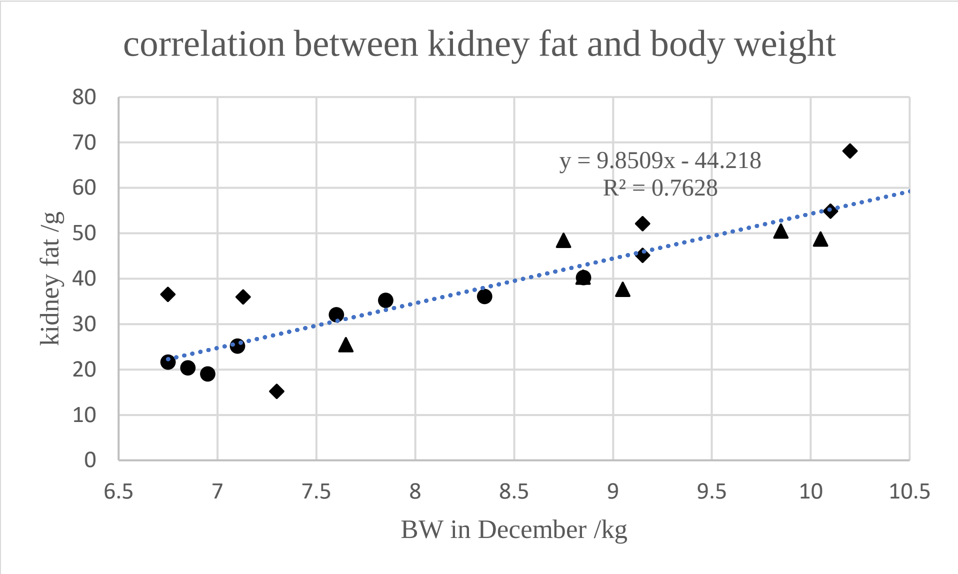


Figure 9: Diagram over mean liver weight and kidney fat of the foxes in three groups.

Table 8: Liver weights, kidney fat.

	Control	TTA-1	TTA-2	SEM	P-value
Liver weight (g)	193.9b	228.5a	229.7a	9.2	0.02
Liver weight % of BW	2.30a	2.55a	3.13b	0.1	0.0001
Kidney fat (g)	44.0a	42.0a	27.1b	4.4	0.03
Kidney fat % of BW	0.50a	0.46ab	0.36b	0.04	0.05

Figure 10 showed the relation between body weight and kidney fat. An increasing kidney fat always came with increasing body weight (R^2 =0.77). Correlation between body weight and kidney fat was highly significant ($P<0.001$) (data not shown).This result indicated a low fat deposition in the kidney is positively related to the slow growth rate of animal.



Square: control triangle: TTA-1 circular: TTA-2

Figure 10: Diagram over correlation between kidney fat and body weight

7.3.2. Serum lipids and glucose

We studied the impact of different dose of TTA on key parameters related to lipid metabolism. The blood sample were taken at the end of the experiment, and results demonstrated TTA had a lipid-lowering potential in the foxes through 3 months treatment.

Triacylglycerol (TAG) level was significantly reduced for foxes fed with 1.2g/kg feed TTA compared to control group with a reduction of 34%. There was almost no difference regarding to average TAG level between control and TTA-2 groups, though there existed variation among individuals inside TTA-2 group. TTA treatment was effective in lowering FFA level in the plasma, with a reduction of 17% in TTA-1 group, and 47% in TTA-2 group, compared to control diet.

There was a significant lower level of low density lipoprotein-cholesterol (LDL-cholesterol) in TTA-1 group, compared to control group with a reduction of 67%, indicating a substantial LDL-cholesterol lowering effect. 4.8g/kg supplemental TTA included diet tended to decrease LDL-cholesterol level by a reduction of 24%. In contrast, the supplement of TTA seemed had little impact on HDL-cholesterol level.

Low dose of TTA decreased cholesterol level by 16% compared to control diet, while high dose supplemental TTA had no effect on lowering cholesterol level. This also happened in free cholesterol level, while low TTA dose decreased free cholesterol level by a small amount and high TTA dose almost had no impact. TTA treatment showed almost no impact on glucose and phospholipids level, except there was a small increase in serum glucose level in TTA-2 group.

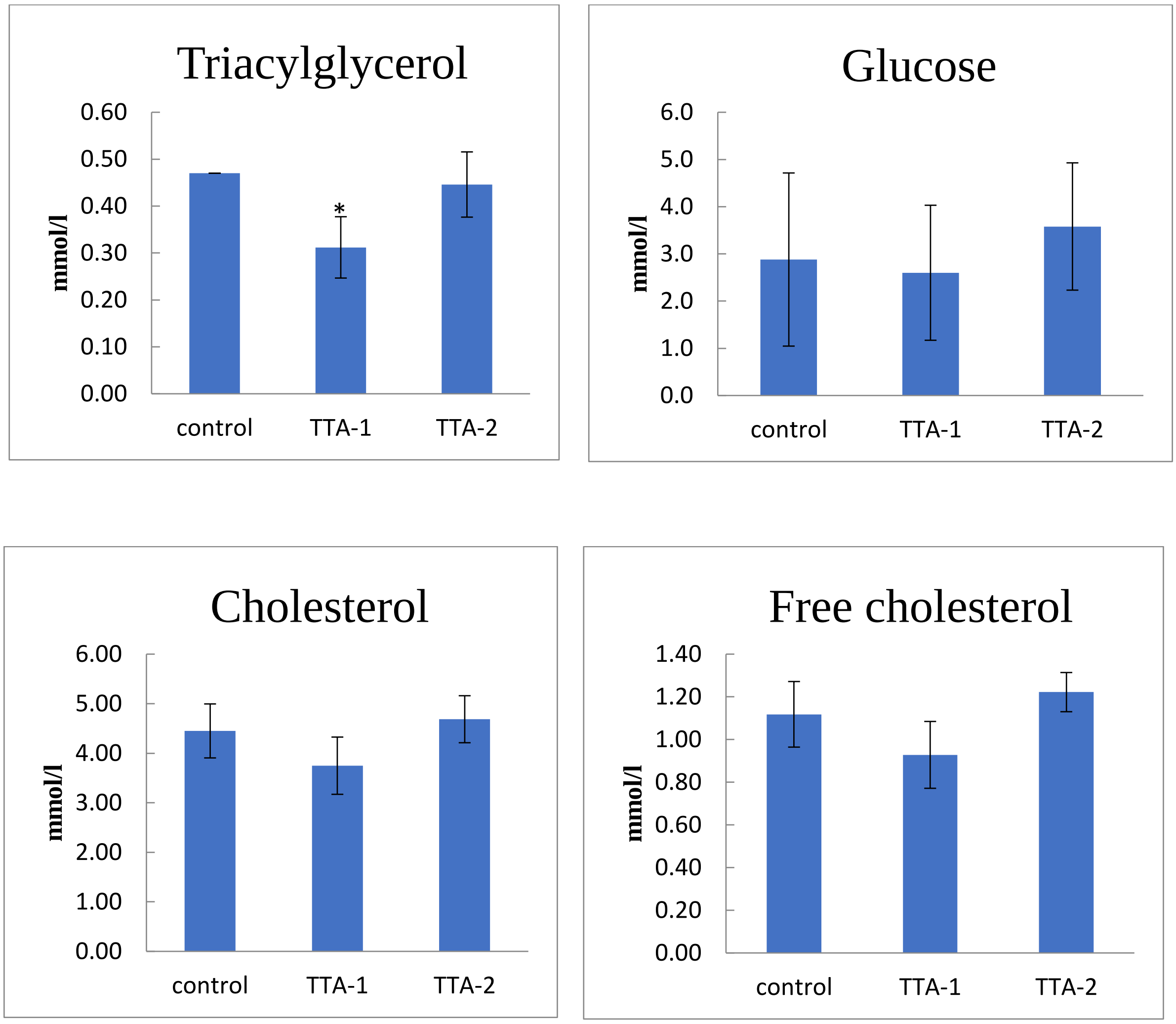


Figure 11: Serum triacylglycerol, glucose, cholesterol and free cholesterol at the end of the experiment (mmol/L)

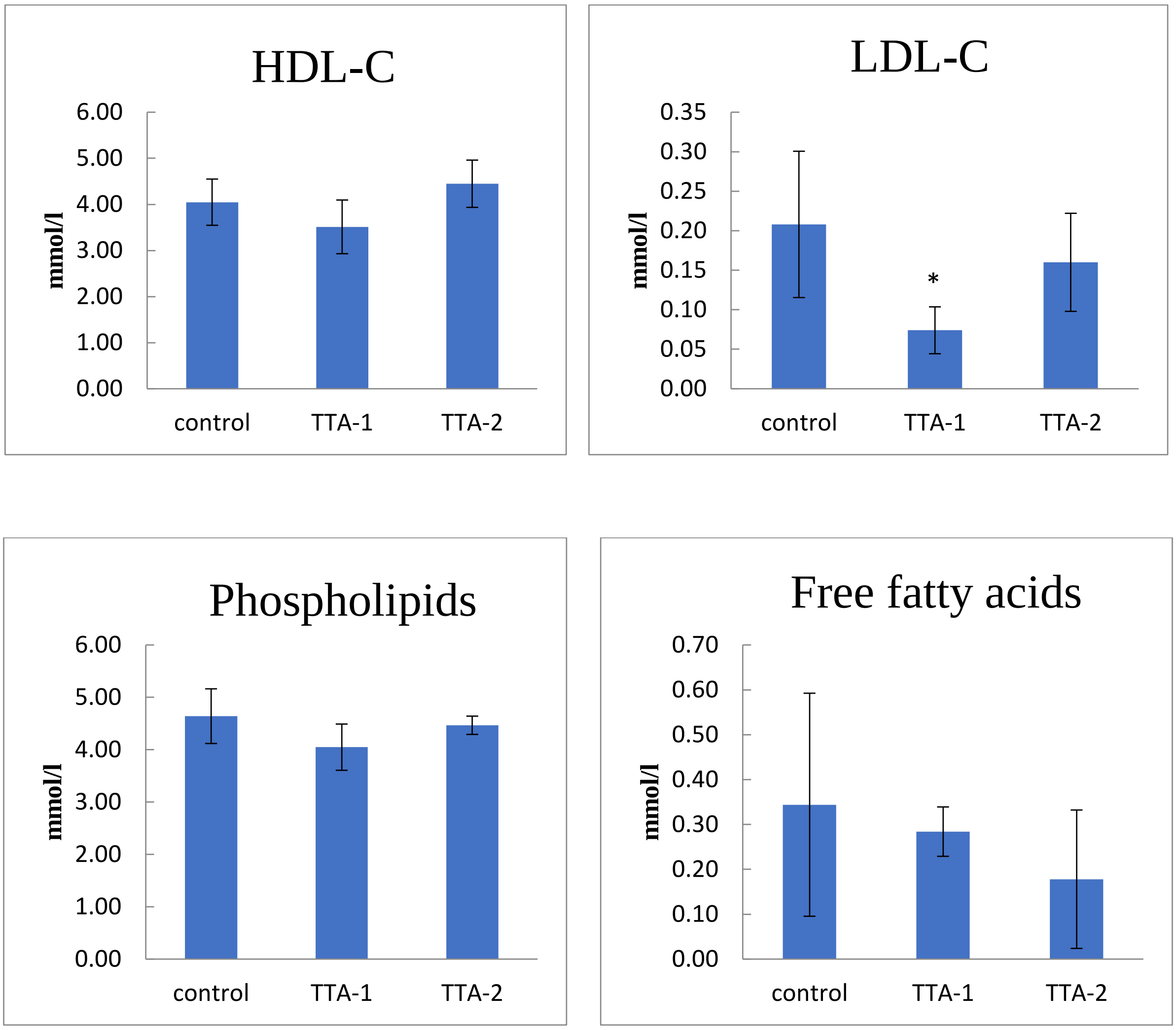


Figure 12: Serum high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), phospholipids and free fatty acids (mmol/L) at the end of the experiment.

8. Discussion

TTA is reported to play a role in regulating feed intake, promoting hepatic mitochondria and peroxisome proliferation, increasing fatty acid oxidation through its action as an agonist for peroxisome proliferator-activated receptors (PPARs), thus having effect on regulating body growth (Arge et al., 2018a). Moreover, it is considered as a beneficial fatty acid in improving plasma lipids composition to a healthier direction with less LDL-cholesterol. The results in this experiment showed TTA, irrespective of TTA dose, possessed the ability of reducing weight growth, and decreasing TG, LDL-cholesterol, which were in the agreement of the hypothesis. However, the picture was not consistent when comparing the effect of TTA dose. TTA dose seemed to have a limited capacity in improving plasma lipids profile, since foxes in TTA-2 group (4.8g TTA/kg feed) did not show decrease in TAG and cholesterol compared with control. The significant reduced weight growth when fed with 4.8g TTA/kg feed was not seen in the dose of 1.2g TTA/kg feed.

Besides, a higher liver mass and lower kidney fat were also discovered, which may can be explained by hepatic mitochondria proliferation and enhanced fatty acid oxidation. Generally, the effect of TTA on feed consumption with the highest TTA level was not in agreement with other animal studies.

8.1. Feed intake

It is well established that too high energy intake in relation to the energy requirement is responsible for the development of obesity. Supplemental TTA showed the ability to decrease feed consumption when given in a high dose in this experiment, in that respect TTA prevented excessive fat accumulation in the body. The effect of TTA on reduced feed intake is ambiguous, since the low TTA dose did not produce significant difference in feed intake while high TTA dose induced a reduction in feed consumption. In a previous study in rats, there was an increased feed intake in rats fed with the TTA diet (Wensaas et al., 2009), similar result was also reported by Kærulf (Kjærulf, 2017), but still the increased energy intake was not reflected in increased body weight gain, thus indicating either higher metabolic rate or lower digestibility.

The overall low feed intake for the animals at the start of the experiment may be due to several factors: palatability, energy density, or processing conditions. Before the study, the animals had received another type of wet feed that were partly made from other ingredients. Physiological factors may also have caused the poor appetite as the environment had changed when moving to the new farm. This may be the most likely reason since feed intake increased during the course of the experiment for the control and TTA-1 animals.

In TTA-2 group, the average dry feed intake was 143g per day (Figure 7) for the whole experimental period, corresponding to 2717kJ metabolizable energy (ME), which is not in accordance with the recommended ME allowance for this period which is 5000kJ (Lassen et al., 2012).

As far as the result indicated, a small dose of TTA (1.2g TTA/kg feed) almost made no difference in feed intake, compared to control group. However, a high dose of TTA(4.8g TTA /kg feed) induced a significant reduction in feed intake. It may explained by the feed was less tasty because of the high TTA inclusion level.

There is concern that the limited number of foxes may influence experimental outcome, though siblings of the foxes have been distributed to the three groups, and this may reduce the individual effect for feed consumption to some extent. It would have been more convincing to trace the feed intake before the study, to decrease individual difference effect.

8.2. Body weight

In all the groups, body growth exhibited an increasing trend during the growing-furring period, though foxes in TTA-2 group had the main growth from the end of October. The average final body weight achieved 8.54kg in control group, which is slightly lower than the typical BW of 9.3kg (Lassen et al., 2012). There were in particular two animals in the TTA-2 group that showed very low feed intake, which reduced the mean feed intake and body growth for this group. One animal revealed similar feed intake and body weight development as in the control and TTA-1 groups.

Foxes in TTA-2 group had a much slower body growth, an average level of 0.75kg, compared to 2.19kg in control group through the experiment period. The attempt to identify the effect of TTA on reduced body weight of foxes gave no clear answer, since there was a significant reduction in feed consumption associated with the highest dose of TTA. When feed consumption was that low, the maintenance energy requirement (MER) was probably only just covered, and there was little energy left for body fat deposition. The MER in silver foxes has been estimated to be around 600 kJ / kg body weight $^{0.75}$ (Lassen et al., 2012), which would be approximately 2440 kJ/d for 6.5 kg fox. Compared to the mean ME intake of 2717kJ/d recorded for the TTA2 group, reason for the low body growth in our study may mainly due to low feed intake, but not an effect of TTA increasing fat oxidation.

An additional effect of TTA over decreased fat digestibility in diets including TTA was observed (Kjærulf, 2017). The latter study showed that TTA fat digestibility in a vegetable oil-based diet was reduced from 95% to 41 % for mink. We did not carry out a digestibility determination in our study, but if fat digestibility was lowered by TTA, this could also contribute to the low body growth in addition to the low feed intake.

8.3. Liver weight

A significant increased liver weight was found in both TTA group, and it is in accordance with the result shown in other studies (Madsen et al., 2002; Norbeck & Wedin, 2011a). The explanation could be due to the mitochondria proliferation in the liver. With a higher amount of mitochondria, the liver weight will increase. The cellular metabolic activity is highly correlated to mitochondrial content, evidences showed an increased hepatic mitochondrial β-oxidation when treated with TTA diet (Asiedu et al., 1996), which excluded the possibility over the higher liver weight due to fat accumulation. With more hepatic fat being oxidized and working as fuel (Wensaas et al., 2009), a reduced fat content in the liver was reasonable.

8.4. Kidney fat

It was not surprising to find out a significant lower kidney fat level in TTA-2 group ($p < 0.05$), which showed a much lower fat accumulation in the kidney (Table 8). This coincided with lower body growth in the TTA-2 group, and therefore less excessive energy for fat deposition. But the kidney fat when treated with the low dose of TTA was not significantly different from control, so we assume the effect of kidney fat was an effect of energy intake and subsequently body weight as presented in Figure 10. Total body scanning was planned to get a better impression of the muscle and fat ratio of the animals, but that was stopped as the energy intakes came so different.

Apart from reduced feed intake inducing less fat accumulation in kidney, some researches reported that TTA's pronounced effect on increased peroxisomes proliferation contributing to the significant decreased kidney fat. TTA is an agonist for PPAR, known as working on upregulating fatty acid metabolism (Raspe et al., 1999). Because of this, an enhanced energy expenditure can be expected. The result of reduced kidney fat was in agreement with others' studies (Asiedu et al., 1996; Norbeck & Wedin, 2011b). Asiedu also discovered the activity of the key enzyme involved in peroxisomal β-oxidation – fatty acyl-CoA oxidase in the kidney had a significant increase, which furthermore proved the effect of TTA on increased fatty acid oxidation(Asiedu et al., 1996). But in our study, the increased fat oxidation theory did not show effect on the kidney fat of foxes in TTA-1 group, on the contrary, there was significant correlation between kidney fat and body weight, indicating feed consumption and body weight were the main reasons for reducing kidney fat.

8.5. Plasma lipids

A mammal contains 5-25%, or more, of its body weight as lipid. Body lipids mainly exist in the form of triacylglycerol(TAG), but also as cholesterol, phospholipids, high density lipoprotein (HDL) and low density lipoprotein (LDL) and free fatty acids (FFA). They constitute to the primary energy reserve, but their function is more than that. Some lipids (phospholipids) attributes to the construction of cell membrane. Fat also serves as cushion for the organs against shock, and provides efficient thermal insulator. Body lipids contents can be partly synthesized and regulated by body itself, and also influenced by dietary fats. Dietary fats are digested into FFA and glycerol with the participation of lipase and colipase in the small intestine. Some small lipids particles, combined with digestion products are then surrounded by bile salt, thus micelles formed. Re-esterified TAG, cholesterol and small fat droplets with little protein would be restricted into chylomicrons in the enterocytes, and be transported to lymphatics, further going into blood, liver or fat tissues to be stored, processed or oxidized (Mathews & Van Holde, 1990a).

Plasma lipids, as a health index, stands for a human or animal health condition, thus usually be tested. It's important to keep TAG, FFA, cholesterol levels in the plasma under the control, because any abnormal elevated levels may indicate health problems. Problem in liver malfunction, and adipose tissue inflammation can cause fat metabolism regulated hormones unbalance, thus may induce an abnormal elevated plasma lipids levels (Mathews & Van Holde, 1990b).

8.5.1. Cholesterol, FFA, and TAG

TAG accounts for as much as 90% of the lipids in the body. The degradation of TG to produce glycerol and FFA makes TAG an effective energy source. TAG can be transported out of the hepatocytes in the form of very low-density lipoprotein (VLDL) when an accumulation in the liver happen. Cholesterol is a member of a large group of substances called steroids, which include a number of important hormones. Cholesterol is essential for the synthesis of some important substances in the body, such as hormones, bile and vitamin D. cholesterol is a fat like substance made by liver, and also comes from the dietary food intake. Liver can package cholesterol with a certain amount of polypeptides to form lipoproteins. However, an abnormally elevated level of cholesterol in the blood is associated with risk of heart disease, since the difficulty in moving highly insoluble cholesterol may lead to deposition on the inside walls of blood vessel, thus eventually harden to become plaque and cause heart disease (Mathews & Van Holde, 1990a). HDL and LDL are the most common lipoproteins we talk when referring to health issues. They are various in the protein and cholesterol content distribution. LDL travels through blood stream, delivering cholesterol to the cells that need it. However, a high amount of LDL in the body may build up in the walls of the arteries, combined with other substances to form fatty deposit called plaque (Esterbauer et al., 1992). Plaque is a risky factor posing risk over narrowing the artery and reducing blood flow, with the main target plaque forming being coronary arteries. On the contrary, HDL does the opposite work of LDL. HDL has a relatively higher content of protein and lower cholesterol content, thus can remove excess cholesterol from peripheral tissues, arteries and plaque, then transport it to the liver to be processed. In this way HDL is considered as good lipoprotein while LDL is bad. So it's of great importance to keep LDL level under control (Rohatgi et al., 2014).

After 24h starvation-gradual depletion of glucose and glycogen, body starts to use fat stored in the adipose tissue to provide energy, the produced FFA would be released to the blood thus causing FFA level in the plasma to increase. FFA are then β-oxidized in the mitochondria or peroxisome to form acetyl-CoA, entering the Citric Acid Cycle and provide energy.

Our study showed there was a reduction in FFA level in TTA treatment groups, but not a significant change. Despite one fox in the TTA-2 group exceeded average FFA level compared to control group, others' FFA level were all below 0.14 mmol/l-the lowest level counting in control and TTA-1 groups (data not shown). One explanation to this may be the speed-up fatty acids oxidation induced by high level of TTA in the body, promoting free fatty acids to be transported from blood to the cells, which was mainly driven by concentration. As a result, a lower FFA level would be observed.

Besides, the low TTA dose treatment in this experiment has been shown to significantly decrease the TG and LDL-cholesterol levels, which indicated an improved plasma lipids profile in the fox after TTA treatment. Since most of TTA is incorporated into phospholipids of cell membrane and may exert effects on the physico-chemical characteristics of receptors and enzymes (Skrede et al., 1997). Explanation for reduced lipids level may be TTA activating PPARα and gene expression of enzymes involved in fat metabolism, such as lipoprotein lipase. Afterwards, TAG and LDL in the blood are more ready to be degraded to various FFA, and crossing the cells to be utilized. Evidence showed PPARα worked as controller over target genes involved in fatty acid metabolism, and they could be triggered or activated by fatty acids metabolites or functional supplemental fatty acids, such as TTA (Vigerust et al., 2012). Furthermore, a upregulated LDL-receptor gene expression was also be found to be induced by TTA treatment (Asiedu et al., 1996). The function of lowering plasma lipids has been widely reported. Løvås recruited 16 patients with type 2 diabetes mellitus and treated them with a daily dose of 1g TTA for 28 days(Lovas et al., 2009). Interesting results in plasma lipids were found afterwards, with a significant decrease in cholesterol level (LDL-cholesterol in particular), total fatty acids, and a reduction in serum triacylglycerol. Similar results were often found in animals after TTA treatment(Asiedu et al., 1996; Madsen et al., 2002; Wensaas et al., 2009).

Relatively, fox treated with high dose of TTA also decreased the LDL-cholesterol content and TG level, but to a less extent. Experiment in our study did not show the reason, but we suggest it may due to the increased activities of some hepatic lipogenic enzymes involved in triacylglycerol, cholesterol, and lipogenic synthesizing in the mitochondria(Bjorndal et al., 2011), promoting the synthesis of TAG and cholesterol, to prevent an excessive reduction in lipids level resulting in an unbalance between lipolysis and lipogenesis, in a condition of the lowest feed intake. The increased lipogenic enzymes activities in prolonged 3% TTA treatment in rats have been observed in Asiedu' report(Asiedu et al., 1996).

As for the changes in total cholesterol, HDL-cholesterol, and free cholesterol levels, the effect of TTA seemed to be limited in such a short-term experiment. Although lower dose of TTA led to a relative reduction compared to control group, higher dose always displayed the opposite effect.

8.5.2. Phospholipids and glucose

Phospholipids constitutes to the cell membrane construction in the body, thus a stable amount of phospholipids is required for a well functioned body. TTA could be incorporated into phospholipids after oral supplement, but did not seem to make a significant change in the phospholipids amount.

Glucose in the plasma can be from dietary glucose, glycogen degradation (glycogenolysis) and gluconeogenesis, and glucose level is mainly balanced by liver and hormones (Mathews & Van Holde, 1990b). Earlier study reported a 7mmol/l level of serum glucose content in silver fox (Berestov et al., 1989) , while our study showed a relatively lower level either in control or TTA treatment groups. It is difficult to distinguish the effect on glucose level, since many factors may make a difference. For example, foxes in our study were fasted for 24 h before euthanasia, blood glucose would have a decrease without supplemental feed supply. Different levels of stress also made a difference in the speed of glucose depletion. A small increase in blood glucose level in TTA-2 group was observed in our study, the upregulated fat metabolism increasing the availability of fatty acids to be transformed to glucose, may be part of the reason.

9. Conclusion

The experiment study has shown that tetradecylthioacetic acid (TTA) exerted effects on the foxes when given as a supplemental feed additive. The main effects reflected in the changes over feed consumption, body growth and body plasma lipids after TTA treatment.

The significant reduced body weight gain, accompanied by decreased feed intake in TTA-2 group indicated that, differences in body weight gain was mainly affected by ME intake. Furthermore, there was no significant difference in kidney fat in TTA-1 group, compared to control group, but the highly significant correlation between body weight and kidney fat suggested that, reduced ME intake was the main factor causing reduced kidney fat, irrespective of TTA supplementation.

Regarding to plasma lipids profile, low TTA dose (1.2g TTA/kg feed) can significantly reduce

TAG and LDL-cholesterol, while the effect of high TTA dose (4.8 g TTA/kg feed) was not considerable.

To conclude, different dietary TTA concentration may have a different impact on the outcome.

TTA effects verified by the experiment study were:

1) High dose 4.8 g TTA/kg feed can significantly reduce ME intake and subsequently BW gain.
2) TTA supplementation increased liver weight.
3) Low dose 1.2 g TTA/kg feed can significantly reduce serum triacylglycerol and LDL-cholesterol.

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Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway