Aanmeldingsformulier voor proeven met gewervelde dieren.

Secretariaat DEC
[redacted]

Aanvrager: [redacted]
Afdeling:

Titel dierproef: Influence of dietary macronutrients on the urinary oxalate excretion in healthy cats

Aanmeldcode / Protocol: 2010034.a
Stadia van de proef:

03-03-2010	Aangemeld	[redacted]
04-03-2010	Wijzigen	Secretaris van de DEC
04-03-2010	Gekopieerd	[redacted]

Is deze proef wetenschappelijk getoetst en goedgekeurd? Ja
Toelichting:trial (PhD research) has been approved by the supervisors, [redacted]

1.a. Met dit onderzoek te beantwoorden concrete vraag:
. Wetenschappelijke vraag m.b.t.van dieren
This DEC-request is for two related studies: a pilot study and the main study. The pilot study will be conducted to provide an indication for the adaptation period needed for the main study as to minimize the adaptation period for each diet given.

The main objective of this study:
To determine the influence of dietary macronutrients on the urinary oxalate excretion as an indicator for endogenous oxalate synthesis in healthy cats in a controlled environment.

. Sub objectives of this study
Pilot-study:
¢ To provide an indication for the time needed for urinary oxalate to reach a steady state excretion after a diet change (adaptation period).
Main study:
¢ To determine the influence of dietary protein content on the endogenous oxalate synthesis in cats fed an oxalate-free diet.
¢ To determine the influence of dietary carbohydrate content on the endogenous oxalate synthesis in cats fed an oxalate-free diet.
¢ To determine the influence of dietary fat content on the endogenous oxalate synthesis in cats fed an oxalate-free diet.
¢ To determine the time needed for urinary oxalate to reach a steady state excretion after a diet change.
¢ To determine the correlation between plasma oxalate concentration and 72h urine oxalate concentration in healthy cats.

¢ To determine the correlation between 72h urinary oxalate excretion and oxalate/creatinine ratio.

1.b. Het uiteindelijk doel (Maatschappelijke en wetenschappelijke relevantie):
Urolithiasis is the second most common cause of lower urinary tract disease (LUTD) in cats (1;2) (symptoms: difficult and painful urination, blood in the urine, partial or complete urethral obstruction) and different urolith (stone) types are known to cause urolithiasis. Over the past decades, a progressive increase in the prevalence of calcium oxalate (CaOx) uroliths (from 2% to 55-60%) has been reported in cats with LUTD in the United States of America (3) and Benelux (4). Nutrition is thought to play a major role in this increase in prevalence of CaOx urolithiasis. Today's nutritional interventions are mainly based on human medicine studies as only a few studies have been conducted in cats.

The current treatment of choice for severe CaOx urolithiasis is surgical urolith removal, followed by methods to prevent urolith recurrence. At present, the standard method for preventing CaOx urolith recurrence is to feed a CaOx urolith reducing therapeutic diet and encourage water intake. Despite these preventative measures, the prevalence and recurrence rate of CaOx uroliths remains high in cats. This means that at present we cannot prevent cats to develop CaOx urolithiasis and therefore cannot prevent them to undergo (mostly repeatedly) surgical treatment. Therefore, more knowledge regarding the etiopathogenesis of CaOx urolithiasis should be generated to improve current preventative measures and to decrease the prevalence of CaOx urolithiasis.

Urinary oxalate and calcium excretion are the two central risk factors in CaOx urolith formation, since they are the two elements to precipitate with each other in the urine. An increase in the excretion of either calcium or oxalate or both will lead to CaOx urolith formation. Since calcium metabolism is of interest for more diseases, several studies have been conducted to determine the nutrients that influence the urinary calcium excretion. This is however not the case for urinary oxalate excretion. Therefore it is essential to study the origin of oxalate excreted the urine.

In contrast with human medicine, dietary modifications to decrease endogenous oxalate synthesis have hardly been studied in carnivores. In human medicine both protein (certain amino acids) (5;6) and carbohydrates (certain sugars) (7;8) are known to act as a precursor of the endogenous oxalate synthesis. However, the metabolic pathway leading to oxalate synthesis differ between humans and carnivores ((9)(10;11)). It is therefore unclear how these dietary constituents influence endogenous oxalate synthesis in carnivores.
In cats, only one study has been published that aimed to determine the relationship between dietary protein content and urinary oxalate excretion (12). Urinary oxalate excretion was found to be inversely correlated with protein intake. However, in this experiment low protein diets did also contain a high content of carbohydrates and therefore the author could not ascribe the increased oxalate excretion to either the low protein content of the diet or to the high carbohydrate content.

Considering the enormous change in composition of diets for cats in the last decades, from small mammals (high in protein, hardly any carbohydrate) to today's pet food (containing 20-60% carbohydrates with a concomitant lower level of (animal) protein), and the increase in prevalence of CaOx uroliths at the same time, it is worthwhile to study the influences of protein en carbohydrate on the endogenous oxalate synthesis. This experiment will contribute to our understanding of the relationship between endogenous oxalate synthesis and dietary protein or carbohydrate and fat content and of the length of the needed adaptation period. Furthermore, this study may provide new insights for dietary strategies that prevent urolithiasis.

1.c. Lekensamenvatting:

2. Gepland vanaf: 01-04-2010 **tot** 31-08-2010

3. Specificatie diergroepen:

1	4	katten	Pilot study
2	12	katten	Alle dieren krijgen alle 3 de proefvoeders in 3 perioden

4.a. Nadere aanduiding gebruikte dieren:
In the pilot study n=4 cats; in the main study n=12 cats.

Intact adult Domestic Shorthair cats, all females, bodyweight 2-4 kg, age between 2 and 5 years.

4.b. Motivatie waarom is gekozen voor deze diersoort:
Cats are the target animal of this study. Since in vitro methods are not available, we have no other choice than to use cats.

4.c. Toelichting voor het aantal gebruikte dieren:
Based on a sample size calculation the conclusion was drawn that a minimum of 9 animals is needed to obtain statistical significant results in the main experiment.
(Sample size calculation: power = 90%; ? = 0,05; SD = 6,33 umol/kg/24h (12); relevant difference of 10 umol/kg/24h ' n = 8,42).

When working with animals there is a chance that some animals will deviate from the protocol (can be due to a lot of reasons) and will result in missing data. Therefore three reserve cats, one per group, will be included in the study to prevent missing values in the final data. To be able to use the reserve cats during the sample period, these cats need to have the same adaptation period as the other cats. This means that the reserve cats will follow the same procedures (adaptation and sample period) as all other cats, except for taking a blood sample in each feeding period. Of these reserve cats only a blood sample will be taken when blood sampling fails in one of three cats fed the same diet.

In addition, according the broad experienced [redacted] 9 cats is a minimum amount of animals for this type of experiment and is highly advisable to have 3 reserve cats. [redacted] can be considered to be an expert in cat experiments as he has been the head of the cat facility of [redacted]

In the pilot study 4 animals are needed to provide an indication for the length of the adaptation period of the main study.

4.d. Herkomst:

1	A. van gereg. fok/toeleveringsbedrijf in Nederland
2	A. van gereg. fok/toeleveringsbedrijf in Nederland

Toelichting:
The four cats housed in the facility are bred by [redacted]

To date (3 march 2010) the purchasing action of eight additional animals is under control of o.a. [redacted]
These animals are purchased based on the approved DEC-request of the experiment 'het testen van smakelijkheid van kattenvoerder met de 'two bowl' test en 'vloer'test' (officially approved 8-9-2009).

5.a. Accommodatie: [redacted]

The cats are housed in a group accommodation with a surface of 25 m2. During the pilot study and the adaptation period of the main study the cats will stay overnight and during feeding time in the metabolism cage (80 cm high x 100 cm length x 75 cm depth). From 11 till 17h the cats can walk freely around in the room where the metabolism cages are placed. During the 3 day during sample period of the main study the cats will be housed in the metabolism cage for the whole day.

5.b. Huisvesting & Verzorging:
The daily care during the experiments will be performed by the persons involved in the experiment (see also point 10) and will be in close contact with the
animal caretakers/staff members of the [redacted].

5.c. Voeding:
Previous to the pilot study the cats will be fed their regular dry food. At day 1-14 of the pilot study the cats will be fed a balanced and complete commercial moist food for adult cats. This food will also be used as the basis for the experimental diets. During the main study three different experimental diets will be fed. These diets will be tested on palatability before the experiment starts.
The experimental diets consist of a commercial balanced and complete moist food for adult cats with either an added protein, carbohydrate or fat source (table 1).

High protein (Hp) diet: Protein source: hydrolyzed gelatin,
High carbohydrate (Hc) diet: Carbohydrate source: gelatinized maize starch, glucose, fructose, galactose, and xylose,
High Fat (Hf) diet: Fat source: lard.

The Hf diet will serve as the control diet in this experiment. The nutrient composition of the three experimental diets is given in table 2.

Table 1: Composition of experimental diets in gram dry matter (DM).

Ingredient	Hp	Hc g DM	Hf g DM	g DM
Commercial complete moist food for adult cats		145	145	145
Palatibity enhancer (Optimizor)		4	4	4
Lard	0	0	60	
Herring oil		10	10	10
Galactose	0	25	0	
Glucose		0	25	0
Xylose	0	25	0	
Fructose		0	25	0
maize starch (gelatinized)		0	26	0
dried egg white (sterilized)		0	0	0
hydrolized gelatine (Rouselot ADF)		140	0	0
vitamin/mineral premix	?	?	?	
Total	298	285	219	

Table 2: Calculated nutrient composition of the experimental diets using the Atwater equation: (13).

		Hp	Hc	Hf	
DM	(g/100g)		36,1	35,2	28,1
MEa	(MJ/100g)	0,66	0,65	0,73	
CP	(g/MJ)	42,3	12,0	12,0	

Cfat	(g/MJ)	8,0	8,4	22,2
Cfiber	(g/MJ)	0,6	0,6	0,6
Cash	(g/MJ)	3,1	2,9	3,1
NFE	(g/MJ)	0,8	30,1	0,8

a Metabolizable energy (in KJ) per 100 gram food = 16,7 * (g/100g crude protein) + 35,5 * (g/100 g crude fat) + 16,7 * (g/100 gram NFE).
Abbreviations: DM = dry matter; ME = metabolizable energy; CP = crude protein; Cfat = crude fat; Cfiber = crude fiber; Cash = crude ash; NFE = nitrogen-free extract.

By adding the protein, carbohydrate or fat source, the diet will be diluted. Therefore a relative decrease in the content of protein, fat, essential amino acids, fatty acids, minerals and vitamins will occur. In the final formulation of this diet the nutrient(s) that will be below the minimal nutrient requirement determined by the National Research Council (NRC) for adult cats (13) will be supplemented.
For these experimental diets the protein content is set on minimum of 12 g/MJ instead of the 9,59 g/MJ recommended by the NRC to assure there will be no changes in protein metabolism due to protein deficiency in the cats. The minimal fat content is well above the recommendation of the NRC as well. At the moment the detailed nutrient composition of some ingredients is not received yet, and therefore the content of the essential amino acids (especially taurine and arginine), fatty acids, minerals and vitamins (especially vitamin B6) could not be calculated yet. Again, the essential nutrients that will be below the recommended nutrient requirements of the NRC will be supplemented in order to compose three complete experimental diets.

The amount of food per cat will be determined based on energy requirement of the individual cat by using 300 KJ/kg B.W/day (13). To assure the cats receive enough food, food intake will be monitored and the cats will be weighed weekly. Water is available ad libitum.

6.a. Proefschema / proefbehandelingen:
An overview over the training of the cats and the experiments is provided in table 3.

Table 3: Schedule for the training and the experiments with the cats plus the sampling scheme of the main study.

1, Pilot study (n=4)a

Acitvity	Time	sampling single urine	72h urine	feces	blood	weight cat
	14 days	Every day			Day 1, 7, 14	

2. Main study (n=12)

Day 0 (baseline)					yes	yes
Adaptation period Ia	. daysb	Every day				Once a week
Sample period Ic	3 days		yes	yes	yes	
Adaptation period IIa	. daysb	Every day				Once a week
Sample period IIc	3 days		yes	yes	yes	
Adaptation period IIIa	. daysb	Every day			Once a week	
Sample period IIIc	3 days		yes	yes	yes	

a The cats will be treated according the schedule in table 4.
b The amount of days will be based on the outcome of the pilot study.
c During the sample period the cats will be housed continuously in the metabolic cage for 72 hours.

Training period to metabolic cages (in approximately 2 weeks):
Previous to the pilot study and the main study the cats will be trained by feeding the cats in the metabolic cages while the door is open. When the cats are used to this procedure, the cage will be closed shortly and this will occur gradually for a longer time. The cats will also be trained to use the litter box in the cage: first by transferring feces and urine to the litter box to create the 'right' smell, and gradually offer them less choice in type and place of available litter boxes. Finally they will be trained only to use the litter boxes placed in the cages without stress.
The procedure of weighing of the cats will be trained as well.

Pilot study:
The duration of the pilot study will be 14 days. In cats and other carnivores the adaptation period has not been determined yet. In the few studies in which urinary oxalate was measured in cats, an adaptation period of at least 28 days was used, whereas in dogs a minimum period of 10 days is used (14). In an experiment with humans a minimum of 5 days is used (6). Since the main focus of the studies in dogs and humans was urinary oxalate excretion, we expect to find an adaptation period shorter than 14 days in cats as well.

Previous to the study the cats will be fed their regular dry food. At day one of the pilot study the food will be changed to the canned moist food which also will be used in the experimental diets.

During this study (and adaptation periods of the main study) the cats stay in the cage during the night and feeding time (2 times a day) (see table 3). In this way food intake per cat can be recorded. The expectation is that the cats will urinate at least once during the night. A (single) urine sample will be collected in the morning to determine the time (days) for oxalate appearance, expressed as a ratio with creatinine, to reach their steady state after a diet change. The cats that didn't urinate during the night or during feeding time will be placed back into the metabolism cage at 12h until they urinate.
At day 1, 7 and 14 the cats will be weighed.

Table 4. Daily schedule for the pilot study and adaptation period of the main study.

Time	Cat in/out of cage	Activity
19.00 - 8.30h (overnight)	Cats in cage	To collect urine sample
8.30 - 10.30h	Cats in cage	Feeding time
10.30 - 17.00h	Cats out of cage	
12.00 - till urination	Cats that didn't urinate back in cage	To collect urine sample
17.00 - 19.00h	Cats in cage	Feeding time

Based on the results of this pilot study we are able to refine the duration of the adaptation period of the main study. In addition, the pilot study can be considered as part of the adaptation process for being housed in a metabolic cage as well.

Main study:
Three diets will be tested in this experiment and therefore this experiment will consist of three feeding periods containing an adaptation period and a sample period (see table 3). The cats will receive the experimental diets according a 3x3 latin square design. This means that 3 groups of 3 cats + 1 reserve will

receive the three experimental diets in a different order (table 5). For the composition of these diets see table 1.

Table 5. 3x3 Latin square design for 12 cats.

	Feeding period I	II	III
Cat 1 + 2		B	A	C
Cat 3 + 4		A	C	B
Cat 5 + 6		C	B	A
Cat 7 + 8		B	C	A
Cat 9 + 10	A	B	C	
Cat 11 +12	C	A	B	

A = Hp diet; B = Hc diet; C= Hf diet

Each feeding period will consist of an adaption period and a sample period (see table 3). In the adaptation period the same protocol will be used as for the pilot study (table 4). This means that they will be housed in the metabolism cage overnight and during feeding time. During the sample period the cats will be housed for 24 hours in the metabolic cage in order to be able to collect 72h urine and feces (15).

Once a week the cats will receive a general clinical check-up by a veterinarian ([redacted]). In addition, the cats will be weighed once a week to assure they maintain a steady body weight. This will give an indication of the cats having a sufficient food intake. In addition food left-overs will be weighed to record the food intake.

Blood will be collected by venepuncture (V. Jugularis) at the start of the experiment to assure the cats are healthy (especially in liver and kidney function) and to obtain a baseline value. In every sample period blood will be collected to determine the plasma oxalate level to be able to correlate this to the urinary oxalate excretion. Since a shortage in vitamin B6 is known to induce endogenous oxalate synthesis and therefore will increase oxalate excretion in the urine, the liver enzymes AST and ALT will be measured in blood plasma to evaluate vitamin B6 status during this experiment.
In preparation to the blood collection, the cats will be fixated by the animal caretaker (not sedated), shaved (1x2 cm at the basis of the neck) and disinfected with ethanol. The amount of 5 ml blood sample will be taken using a blue (0,60 x 25 mm, 23 G x 1") or black needle (0,70 X 30 mm BL/LB, 22 G x 1 ¼") by a trained veterinarian ([redacted]) or another certified staff member.

During the pilot study and adaptation period of the main study urine samples will be collected. To correct for the dilution of the urine, the ratio of urine creatinine will be taken, and therefore oxalate will be expressed as oxalate/creatinine ratio. Since creatinine excretion is dependent on muscle mass, different type of diets, etc, this is a significantly less accurate method to quantify oxalate in the urine.
To answer the main research questions accurately, it is essential to quantify the oxalate by pooling the urine collected on 3 consecutive days (72h urine; umol oxalate/kg B.W./day) instead of taking the ratio with creatinine (single urine; oxalate/creatinine ratio). Since being housed in the metabolic cage during the whole main study will result in too much inconvenience, we have chosen to collect only once a 72h urine per feeding period (sample period). For the pilot study and adaptation period of each feeding period is chosen to collect a single urine sample each day.

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(9) Birdsey GM, Lewin J, Cunningham AA, Bruford MW, Danpure CJ. Differential enzyme targeting as an evolutionary adaptation to herbivory in carnivora. Mol Biol Evol 2004 Apr;21(4):632-46.
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(12) Zentek J, Schulz A. Urinary composition of cats is affected by the source of dietary protein. J Nutr 2004 Aug;134(8 Suppl):2162S-5S.
(13) National Research Council (U.S.) and Committee on Dog and Cat Nutrition. Nutrient Requirements of Dogs and Cats. Washington: The National Academies Press; 2006.
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6.b. Mate van ongerief:

1 C. Matig
2 C. Matig

6.c. Waaruit bestaat het ongerief en hoe bent u tot uw inschatting van de mate van ongerief gekomen?

The individual housing in the metabolic cage can result to inconvenience for the cat. The size of the metabolic cage (80 cm high x 100 cm length x 75 cm depth) together with having the training/adaptation period will minimize the inconvenience as much as possible.

Taking a blood sample will result in some (matig) inconvenience for the cats since they need to be fixated for this procedure and they'll experience a short sense of pain at the moment the needle will penetrate the skin.

Weighing will result in slight inconvenience for the cats since the handling of the cats for weighing will result in no to little stress for the cats.

7. Welke maatregelen heeft u getroffen om het ongerief tot een minimum te beperken?

Anesthesie:

1 A. Niet toegepast (geen aanleiding).
2 A. Niet toegepast (geen aanleiding).

Pijnbestrijding:

1 A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.
2 A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.

o The cats will be trained/adapted step-by-step to be housed in the metabolic cage for a prolonged period and for learning to urinate and defecate on the special designed litter box. In this way the stress response of cats to these new procedures will be minimized.
o During the pilot study and pre-period of the main experiment the cats are only housed in the cages during the night and feeding times. The rest of the day they can walk around freely and interact with each other.
o The metabolic cages are positioned in such a way that when the cats are housed in the cage they can make eye contact with each other, hear each other, etc.
o During daytime almost continuously researchers and/or caretakers will be present in the room were cats are housed in the metabolic cages. Also time is scheduled to socially interact with the cats.
o Additional to the set-up of the litter box described in Hendriks and co-authors (1999) (15) non-absorbable cat litter will be added. In this way the cat will be less disturbed in expressing its natural behaviour previous to urinating and defecating, i.e. scratching/digging into the 'soil'.

8. Toestand van dieren na einde van de proef:

1 Het dier is na de proef in leven gelaten.
2 Het dier is na de proef in leven gelaten.

Toelichting:

The cats will stay in the group accommodation of the cat facility of [redacted] after the experiment.

9. Welke alternatieven (vervanging, verfijning, vermindering) zijn voor de beschreven experimenten overwogen en waarom zijn deze verworpen?

Replacement is not possible since the cat is the target species for this study and no in vitro method is available.

Reduction:
o of the number of animals is not possible because a lower amount of animals will reduce the power of this experiment below an acceptable level of 80% (see 4.c.).
o of the duration of the experiment is not possible since the pre-period is already minimalised by conducting the pilot-study to determine the minimal time needed for oxalate excretion to reach its steady state/plateau phase.
In addition, based on the study conducted by Hendriks and co-authors (1999) (15) a minimum of three consecutive days (72h) collection of urine is required to obtain an accurate urine sample for measuring quantities.

All ideas for refinement of the experiment will be implicated in the protocols during the studies.

10. Namen van direct betrokkenen bij de dierproef (artikel 9- en 12-functionarissen):

[redacted]

Tabel registratiecode opties voor aanvraag 2010034.a (K14):

	1	2	3	4	5	6	7	8	9	10	11	12	13
					36	1	1	01					
1	1	Ot	1	4					01	1	1	3	3
2	1	Ot	1	12					01	1	1	3	3

Aanmeldingsformulier voor proeven met gewervelde dieren.

Secretariaat DEC
[redacted]

Aanvrager: [redacted]
Afdeling:

Titel dierproef: Influence of dietary macronutrients on the urinary oxalate excretion in healthy cats

Aanmeldcode / Protocol: 2010034.b
Stadia van de proef:

04-03-2010	Aangemeld	[redacted]
19-03-2010	Wijzigen	Secretaris van de DEC
29-03-2010	Gekopieerd	[redacted]

Is deze proef wetenschappelijk getoetst en goedgekeurd? Ja
Toelichting:trial (PhD research) has been approved by the supervisors, [redacted]

1.a. Met dit onderzoek te beantwoorden concrete vraag:
. Wetenschappelijke vraag m.b.t.van dieren
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¢ To provide an indication for the time needed for urinary oxalate to reach a steady state excretion after a diet change (adaptation period).
Main study:
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¢ To determine the time needed for urinary oxalate to reach a steady state excretion after a diet change.
¢ To determine the correlation between plasma oxalate concentration and 72h urine oxalate concentration in healthy cats.

¢ To determine the correlation between 72h urinary oxalate excretion and oxalate/creatinine ratio.

1.b. Het uiteindelijk doel (Maatschappelijke en wetenschappelijke relevantie):
Urolithiasis is the second most common cause of lower urinary tract disease (LUTD) in cats (1;2) (symptoms: difficult and painful urination, blood in the urine, partial or complete urethral obstruction) and different urolith (stone) types are known to cause urolithiasis. Over the past decades, a progressive increase in the prevalence of calcium oxalate (CaOx) uroliths (from 2% to 55-60%) has been reported in cats with LUTD in the United States of America (3) and Benelux (4). Nutrition is thought to play a major role in this increase in prevalence of CaOx urolithiasis. Today's nutritional interventions are mainly based on human medicine studies as only a few studies have been conducted in cats.

The current treatment of choice for severe CaOx urolithiasis is surgical urolith removal, followed by methods to prevent urolith recurrence. At present, the standard method for preventing CaOx urolith recurrence is to feed a CaOx urolith reducing therapeutic diet and encourage water intake. Despite these preventative measures, the prevalence and recurrence rate of CaOx uroliths remains high in cats. This means that at present we cannot prevent cats to develop CaOx urolithiasis and therefore cannot prevent them to undergo (mostly repeatedly) surgical treatment. Therefore, more knowledge regarding the etiopathogenesis of CaOx urolithiasis should be generated to improve current preventative measures and to decrease the prevalence of CaOx urolithiasis.

Urinary oxalate and calcium excretion are the two central risk factors in CaOx urolith formation, since they are the two elements to precipitate with each other in the urine. An increase in the excretion of either calcium or oxalate or both will lead to CaOx urolith formation. Since calcium metabolism is of interest for more diseases, several studies have been conducted to determine the nutrients that influence the urinary calcium excretion. This is however not the case for urinary oxalate excretion. Therefore it is essential to study the origin of oxalate excreted the urine.

In contrast with human medicine, dietary modifications to decrease endogenous oxalate synthesis have hardly been studied in carnivores. In human medicine both protein (certain amino acids) (5;6) and carbohydrates (certain sugars) (7;8) are known to act as a precursor of the endogenous oxalate synthesis. However, the metabolic pathway leading to oxalate synthesis differ between humans and carnivores ((9)(10;11)). It is therefore unclear how these dietary constituents influence endogenous oxalate synthesis in carnivores.
In cats, only one study has been published that aimed to determine the relationship between dietary protein content and urinary oxalate excretion (12). Urinary oxalate excretion was found to be inversely correlated with protein intake. However, in this experiment low protein diets did also contain a high content of carbohydrates and therefore the author could not ascribe the increased oxalate excretion to either the low protein content of the diet or to the high carbohydrate content.

Considering the enormous change in composition of diets for cats in the last decades, from small mammals (high in protein, hardly any carbohydrate) to today's pet food (containing 20-60% carbohydrates with a concomitant lower level of (animal) protein), and the increase in prevalence of CaOx uroliths at the same time, it is worthwhile to study the influences of protein en carbohydrate on the endogenous oxalate synthesis. This experiment will contribute to our understanding of the relationship between endogenous oxalate synthesis and dietary protein or carbohydrate and fat content and of the length of the needed adaptation period. Furthermore, this study may provide new insights for dietary strategies that prevent urolithiasis.

1.c. Lekensamenvatting:

2. Gepland vanaf: 01-04-2010 **tot** 31-08-2010

3. Specificatie diergroepen:

1	4	katten	Pilot study
2	12	katten	Alle dieren krijgen alle 3 de proefvoeders in 3 perioden

4.a. Nadere aanduiding gebruikte dieren:
In the pilot study n=4 cats; in the main study n=12 cats.

Intact adult Domestic Shorthair cats, all females, bodyweight 2-4 kg, age between 2 and 5 years.

4.b. Motivatie waarom is gekozen voor deze diersoort:
Cats are the target animal of this study. Since in vitro methods are not available, we have no other choice than to use cats.

4.c. Toelichting voor het aantal gebruikte dieren:
Based on a sample size calculation the conclusion was drawn that a minimum of 9 animals is needed to obtain statistical significant results in the main experiment.
(Sample size calculation: power = 90%; alpha = 0,05; SD = 6,33 umol/kg/24h (12); relevant difference of 10 umol/kg/24h ' n = 8,42).

When working with animals there is a chance that some animals will deviate from the protocol (can be due to a lot of reasons) and will result in missing values in the final data. When this occurs, this will reduce the statistical power of the study (for example the study results in trend instead of a significant difference). Therefore three extra cats will be included in the study to improve the statistical power of the study even when a cat becomes ill.

In addition, according the broad experienced [redacted], 9 cats is a minimum amount of animals for this type of experiment and it is highly advisable to have 3 extra cats in case of illness. [redacted] can be considered to be an expert in cat experiments as he has been the head of [redacted]

In the pilot study 4 animals are needed to provide an indication for the length of the adaptation period of the main study.

4.d. Herkomst:

1	A. van gereg. fok/toeleveringsbedrijf in Nederland
2	A. van gereg. fok/toeleveringsbedrijf in Nederland

Toelichting:
The four cats housed in the facility are bred by [redacted].

To date (3 march 2010) the purchasing actions of eight additional animals is under control of o.a. Mr R. Steenmans.
These animals are purchased based on the approved DEC-request of the experiment 'het testen van smakelijkheid van kattenvoerder met de 'two bowl' test en 'vloer'test' (officially approved 8-9-2009).

5.a. Accommodatie: [redacted]

The cats are housed in a group accommodation with a surface of 25 m2. During the pilot study and the adaptation period of the main study the cats will stay overnight and during feeding time in the metabolism cage (80 cm high x 100 cm length x 75 cm depth). From 11 till 17h the cats can walk freely around in the room where the metabolism cages are placed. During the 3 day during sample period of the main study the cats will be housed in the metabolism cage for the whole day.

5.b. Huisvesting & Verzorging:
The daily care during the experiments will be performed by the persons involved in the experiment (see

also point 10) and will be in close contact with the
animal caretakers/staff members of the [redacted].

5.c. Voeding:
Previous to the pilot study the cats will be fed their regular dry food. At day 1-14 of the pilot study the cats will be fed a balanced and complete commercial moist food for adult cats. This food will also be used as the basis for the experimental diets. During the main study three different experimental diets will be fed. These diets will be tested on palatability before the experiment starts.
The experimental diets consist of a commercial balanced and complete moist food for adult cats with either an added protein, carbohydrate or fat source (table 1).

High protein (Hp) diet: Protein source: hydrolyzed gelatin,
High carbohydrate (Hc) diet: Carbohydrate source: gelatinized maize starch, glucose, fructose, galactose, and xylose,
High Fat (Hf) diet: Fat source: lard.

The Hf diet will serve as the control diet in this experiment. The nutrient composition of the three experimental diets is given in table 2.

Table 1: Composition of experimental diets in gram dry matter (DM).

Ingredient	Hp	Hc g DM	Hf g DM	g DM
Commercial complete moist food for adult cats		145	145	145
Palatibity enhancer (Optimizor)		4	4	4
Lard	0	0	60	
Herring oil		10	10	10
Galactose	0	25	0	
Glucose		0	25	0
Xylose	0	25	0	
Fructose		0	25	0
maize starch (gelatinized)		0	26	0
dried egg white (sterilized)		0	0	0
hydrolized gelatine (Rouselot ADF)		140	0	0
vitamin/mineral premix	?	?	?	
Total	298	285	219	

Table 2: Calculated nutrient composition of the experimental diets using the Atwater equation: (13).

		Hp	Hc	Hf	
DM	(g/100g)		36,1	35,2	28,1
MEa	(MJ/100g)	0,66	0,65	0,73	
CP	(g/MJ)	42,3	12,0	12,0	
Cfat	(g/MJ)	8,0	8,4	22,2	
Cfiber	(g/MJ)	0,6	0,6	0,6	

Cash	(g/MJ)	3,1	2,9	3,1
NFE	(g/MJ)	0,8	30,1	0,8

a Metabolizable energy (in KJ) per 100 gram food = 16,7 * (g/100g crude protein) + 35,5 * (g/100 g crude fat) + 16,7 * (g/100 gram NFE).
Abbreviations: DM = dry matter; ME = metabolizable energy; CP = crude protein; Cfat = crude fat; Cfiber = crude fiber; Cash = crude ash; NFE = nitrogen-free extract.

By adding the protein, carbohydrate or fat source, the diet will be diluted. Therefore a relative decrease in the content of protein, fat, essential amino acids, fatty acids, minerals and vitamins will occur. In the final formulation of this diet the nutrient(s) that will be below the minimal nutrient requirement determined by the National Research Council (NRC) for adult cats (13) will be supplemented.
For these experimental diets the protein content is set on minimum of 12 g/MJ instead of the 9,59 g/MJ recommended by the NRC to assure there will be no changes in protein metabolism due to protein deficiency in the cats. The minimal fat content is well above the recommendation of the NRC as well. At the moment the detailed nutrient composition of some ingredients is not received yet, and therefore the content of the essential amino acids (especially taurine and arginine), fatty acids, minerals and vitamins (especially vitamin B6) could not be calculated yet. Again, the essential nutrients that will be below the recommended nutrient requirements of the NRC will be supplemented in order to compose three complete experimental diets.

The amount of food per cat will be determined based on energy requirement of the individual cat by using 300 KJ/kg B.W/day (13). To assure the cats receive enough food, food intake will be monitored and the cats will be weighed weekly. Water is available ad libitum.

6.a. Proefschema / proefbehandelingen:
An overview over the training of the cats and the experiments is provided in table 3.

Table 3: Schedule for the training and the experiments with the cats plus the sampling scheme of the main study.

1, Pilot study (n=4)a

Acitvity	Time	sampling				
		single urine	72h urine	feces	blood	weight cat
	14 days	Every day			Day 1, 7, 14	

2. Main study (n=12)

Day 0 (baseline)					yes	yes
Adaptation period Ia	. daysb	Every day				Once a week
Sample period Ic	3 days		yes	yes	yes	
Adaptation period IIa	. daysb	Every day				Once a week
Sample period IIc	3 days		yes	yes	yes	
Adaptation period IIIa	. daysb	Every day			Once a week	
Sample period IIIc	3 days		yes	yes	yes	

a The cats will be treated according the schedule in table 4.

b The amount of days will be based on the outcome of the pilot study.
c During the sample period the cats will be housed continuously in the metabolic cage for 72 hours.

Training period to metabolic cages (in approximately 2 weeks):
Previous to the pilot study and the main study the cats will be trained by feeding the cats in the metabolic cages while the door is open. When the cats are used to this procedure, the cage will be closed shortly and this will occur gradually for a longer time. The cats will also be trained to use the litter box in the cage: first by transferring feces and urine to the litter box to create the 'right' smell, and gradually offer them less choice in type and place of available litter boxes. Finally they will be trained only to use the litter boxes placed in the cages without stress.
The procedure of weighing of the cats will be trained as well.

Pilot study:
The duration of the pilot study will be 14 days. In cats and other carnivores the adaptation period has not been determined yet. In the few studies in which urinary oxalate was measured in cats, an adaptation period of at least 28 days was used, whereas in dogs a minimum period of 10 days is used (14). In an experiment with humans a minimum of 5 days is used (6). Since the main focus of the studies in dogs and humans was urinary oxalate excretion, we expect to find an adaptation period shorter than 14 days in cats as well.

Previous to the study the cats will be fed their regular dry food. At day one of the pilot study the food will be changed to the canned moist food which also will be used in the experimental diets.

During this study (and adaptation periods of the main study) the cats stay in the cage during the night and feeding time (2 times a day) (see table 3). In this way food intake per cat can be recorded. The expectation is that the cats will urinate at least once during the night. A (single) urine sample will be collected in the morning to determine the time (days) for oxalate appearance, expressed as a ratio with creatinine, to reach their steady state after a diet change. The cats that didn't urinate during the night or during feeding time will be placed back into the metabolism cage at 12h until they urinate.
At day 1, 7 and 14 the cats will be weighed.

Table 4. Daily schedule for the pilot study and adaptation period of the main study.

Time	Cat in/out of cage	Activity
19.00 - 8.30h (overnight)	Cats in cage	To collect urine sample
8.30 - 10.30h	Cats in cage	Feeding time
10.30 - 17.00h	Cats out of cage	
12.00 - till urination	Cats that didn't urinate back in cage	To collect urine sample
17.00 - 19.00h	Cats in cage	Feeding time

Based on the results of this pilot study we are able to refine the duration of the adaptation period of the main study. In addition, the pilot study can be considered as part of the adaptation process for being housed in a metabolic cage as well.

Main study:
Three diets will be tested in this experiment and therefore this experiment will consist of three feeding periods containing an adaptation period and a sample period (see table 3). The cats will receive the experimental diets according a 3x3 latin square design. This means that every 2 cats will receive the three experimental diets in a different order (table 5). For the composition of these diets see table 1.

Table 5. 3x3 Latin square design for 12 cats.

	Feeding period I	II	III
Cat 1 + 2		B	A	C
Cat 3 + 4		A	C	B
Cat 5 + 6		C	B	A
Cat 7 + 8		B	C	A
Cat 9 + 10	A	B	C	
Cat 11 +12	C	A	B	

A = Hp diet; B = Hc diet; C= Hf diet

Each feeding period will consist of an adaption period and a sample period (see table 3). In the adaptation period the same protocol will be used as for the pilot study (table 4). This means that they will be housed in the metabolism cage overnight and during feeding time. During the sample period the cats will be housed for 24 hours in the metabolic cage in order to be able to collect 72h urine and feces (15).

Once a week the cats will receive a general clinical check-up by a veterinarian ([redacted]). In addition, the cats will be weighed once a week to assure they maintain a steady body weight. This will give an indication of the cats having a sufficient food intake. In addition food left-overs will be weighed to record the food intake.

Blood will be collected by venepuncture (V. Jugularis) at the start of the experiment to assure the cats are healthy (especially in liver and kidney function) and to obtain a baseline value. In every sample period blood will be collected to determine the plasma oxalate level to be able to correlate this to the urinary oxalate excretion. Since a shortage in vitamin B6 is known to induce endogenous oxalate synthesis and therefore will increase oxalate excretion in the urine, the liver enzymes AST and ALT will be measured in blood plasma to evaluate vitamin B6 status during this experiment.
In preparation to the blood collection, the cats will be fixated by the animal caretaker (not sedated), shaved (1x2 cm at the basis of the neck) and disinfected with ethanol. The amount of 5 ml blood sample will be taken using a blue (0,60 x 25 mm, 23 G x 1") or black needle (0,70 X 30 mm BL/LB, 22 G x 1 ¼") by a trained veterinarian ([redacted]) or another certified staff member.

During the pilot study and adaptation period of the main study urine samples will be collected. To correct for the dilution of the urine, the ratio of urine creatinine will be taken, and therefore oxalate will be expressed as oxalate/creatinine ratio. Since creatinine excretion is dependent on muscle mass, different type of diets, etc, this is a significantly less accurate method to quantify oxalate in the urine.
To answer the main research questions accurately, it is essential to quantify the oxalate by pooling the urine collected on 3 consecutive days (72h urine; umol oxalate/kg B.W./day) instead of taking the ratio with creatinine (single urine; oxalate/creatinine ratio). Since being housed in the metabolic cage during the whole main study will result in too much inconvenience, we have chosen to collect only once a 72h urine per feeding period (sample period). For the pilot study and adaptation period of each feeding period is chosen to collect a single urine sample each day.

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6.b. Mate van ongerief:

1 B. Gering/Matig
2 C. Matig

6.c. Waaruit bestaat het ongerief en hoe bent u tot uw inschatting van de mate van ongerief gekomen?

The individual housing in the metabolic cage can result to inconvenience for the cat. The size of the metabolic cage (80 cm high x 100 cm length x 75 cm depth) together with having the training/adaptation period will minimize the inconvenience as much as possible.

Taking a blood sample will result in some (matig) inconvenience for the cats since they need to be fixated for this procedure and they'll experience a short sense of pain at the moment the needle will penetrate the skin.

Weighing will result in slight inconvenience for the cats since the handling of the cats for weighing will result in no to little stress for the cats.

7. Welke maatregelen heeft u getroffen om het ongerief tot een minimum te beperken?

Anesthesie:

1 A. Niet toegepast (geen aanleiding).

2 A. Niet toegepast (geen aanleiding).

Pijnbestrijding:

1 A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.

2 A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.

o The cats will be trained/adapted step-by-step to be housed in the metabolic cage for a prolonged period and for learning to urinate and defecate on the special designed litter box. In this way the stress response of cats to these new procedures will be minimized.

o During the pilot study and pre-period of the main experiment the cats are only housed in the cages during the night and feeding times. The rest of the day they can walk around freely and interact with each other.

o The metabolic cages are positioned in such a way that when the cats are housed in the cage they can make eye contact with each other, hear each other, etc.

o During daytime almost continuously researchers and/or caretakers will be present in the room were cats are housed in the metabolic cages. Also time is scheduled to socially interact with the cats.

o Additional to the set-up of the litter box described in Hendriks and co-authors (1999) (15) non-absorbable cat litter will be added. In this way the cat will be less disturbed in expressing its natural behaviour previous to urinating and defecating, i.e. scratching/digging into the 'soil'.

8. Toestand van dieren na einde van de proef:

1 Het dier is na de proef in leven gelaten.

2 Het dier is na de proef in leven gelaten.

Toelichting:

The cats will stay in the group accommodation of the cat facility of [redacted] after the experiment.

9. Welke alternatieven (vervanging, verfijning, vermindering) zijn voor de beschreven experimenten overwogen en waarom zijn deze verworpen?

Replacement is not possible since the cat is the target species for this study and no in vitro method is available.

Reduction:

o of the number of animals is not possible because a lower amount of animals will reduce the power of this experiment below an acceptable level of 80% (see 4.c.).

o of the duration of the experiment is not possible since the pre-period is already minimalised by conducting the pilot-study to determine the minimal time needed for oxalate excretion to reach its steady state/plateau phase.

In addition, based on the study conducted by Hendriks and co-authors (1999) (15) a minimum of three consecutive days (72h) collection of urine is required to obtain an accurate urine sample for measuring quantities.

All ideas for refinement of the experiment will be implicated in the protocols during the studies.

10. Namen van direct betrokkenen bij de dierproef (artikel 9- en 12-functionarissen):

[redacted]

Tabel registratiecode opties voor aanvraag 2010034.b (K14):

1 2 3 4 5 6 7 8 9 10 11 12 13

						36	1	1	01						
1			1	Ot	1	4					01	1	1	2	3
2			1	Ot	1	12					01	1	1	3	3

Aanmeldingsformulier voor proeven met gewervelde dieren.

Secretariaat DEC
[redacted]

Aanvrager: [redacted]
Afdeling:

Titel dierproef: Influence of dietary macronutrients on the urinary oxalate excretion in healthy cats

Aanmeldcode / Protocol: 2010034.c
Stadia van de proef:

29-03-2010	Aangemeld	[redacted]
30-03-2010	Positief advies na behandeling DEC	Secretaris van de DEC
01-07-2010	Wijzigen	[redacted]
01-07-2010	Gekopieerd	

Is deze proef wetenschappelijk getoetst en goedgekeurd? Ja
Toelichting:trial (PhD research) has been approved by the supervisors, [redacted]

1.a. Met dit onderzoek te beantwoorden concrete vraag:
. Wetenschappelijke vraag m.b.t.van dieren
This DEC-request is for two related studies: a pilot study and the main study. The pilot study will be conducted to provide an indication for the adaptation period needed for the main study as to minimize the adaptation period for each diet given.

The main objective of this study:
To determine the influence of dietary macronutrients on the urinary oxalate excretion as an indicator for endogenous oxalate synthesis in healthy cats in a controlled environment.

Sub objectives of this study
Pilot-study:
¢ To provide an indication for the time needed for urinary oxalate to reach a steady state excretion after a diet change (adaptation period).
Main study:
¢ To determine the influence of dietary protein content on the endogenous oxalate synthesis in cats fed an oxalate-free diet.
¢ To determine the influence of dietary carbohydrate content on the endogenous oxalate synthesis in cats fed an oxalate-free diet.
¢ To determine the influence of dietary fat content on the endogenous oxalate synthesis in cats fed an oxalate-free diet.
¢ To determine the time needed for urinary oxalate to reach a steady state excretion after a diet change.
¢ To determine the correlation between plasma oxalate concentration and 72h urine oxalate

concentration in healthy cats.
¢ To determine the correlation between 72h urinary oxalate excretion and oxalate/creatinine ratio.

1.b. Het uiteindelijk doel (Maatschappelijke en wetenschappelijke relevantie):
Urolithiasis is the second most common cause of lower urinary tract disease (LUTD) in cats (1;2) (symptoms: difficult and painful urination, blood in the urine, partial or complete urethral obstruction) and different urolith (stone) types are known to cause urolithiasis. Over the past decades, a progressive increase in the prevalence of calcium oxalate (CaOx) uroliths (from 2% to 55-60%) has been reported in cats with LUTD in the United States of America (3) and Benelux (4). Nutrition is thought to play a major role in this increase in prevalence of CaOx urolithiasis. Today's nutritional interventions are mainly based on human medicine studies as only a few studies have been conducted in cats.

The current treatment of choice for severe CaOx urolithiasis is surgical urolith removal, followed by methods to prevent urolith recurrence. At present, the standard method for preventing CaOx urolith recurrence is to feed a CaOx urolith reducing therapeutic diet and encourage water intake. Despite these preventative measures, the prevalence and recurrence rate of CaOx uroliths remains high in cats. This means that at present we cannot prevent cats to develop CaOx urolithiasis and therefore cannot prevent them to undergo (mostly repeatedly) surgical treatment. Therefore, more knowledge regarding the etiopathogenesis of CaOx urolithiasis should be generated to improve current preventative measures and to decrease the prevalence of CaOx urolithiasis.

Urinary oxalate and calcium excretion are the two central risk factors in CaOx urolith formation, since they are the two elements to precipitate with each other in the urine. An increase in the excretion of either calcium or oxalate or both will lead to CaOx urolith formation. Since calcium metabolism is of interest for more diseases, several studies have been conducted to determine the nutrients that influence the urinary calcium excretion. This is however not the case for urinary oxalate excretion. Therefore it is essential to study the origin of oxalate excreted the urine.

In contrast with human medicine, dietary modifications to decrease endogenous oxalate synthesis have hardly been studied in carnivores. In human medicine both protein (certain amino acids) (5;6) and carbohydrates (certain sugars) (7;8) are known to act as a precursor of the endogenous oxalate synthesis. However, the metabolic pathway leading to oxalate synthesis differ between humans and carnivores ((9)(10;11)). It is therefore unclear how these dietary constituents influence endogenous oxalate synthesis in carnivores.
In cats, only one study has been published that aimed to determine the relationship between dietary protein content and urinary oxalate excretion (12). Urinary oxalate excretion was found to be inversely correlated with protein intake. However, in this experiment low protein diets did also contain a high content of carbohydrates and therefore the author could not ascribe the increased oxalate excretion to either the low protein content of the diet or to the high carbohydrate content.

Considering the enormous change in composition of diets for cats in the last decades, from small mammals (high in protein, hardly any carbohydrate) to today's pet food (containing 20-60% carbohydrates with a concomitant lower level of (animal) protein), and the increase in prevalence of CaOx uroliths at the same time, it is worthwhile to study the influences of protein en carbohydrate on the endogenous oxalate synthesis. This experiment will contribute to our understanding of the relationship between endogenous oxalate synthesis and dietary protein or carbohydrate and fat content and of the length of the needed adaptation period. Furthermore, this study may provide new insights for dietary strategies that prevent urolithiasis.

1.c. Lekensamenvatting:

2. Gepland vanaf: 01-04-2010 **tot** 31-08-2010

3. Specificatie diergroepen:

1	4	katten	Pilot study
2	12	katten	Alle dieren krijgen alle 3 de proefvoeders in 3 perioden

4.a. Nadere aanduiding gebruikte dieren:
In the pilot study n=4 cats; in the main study n=12 cats.

Intact adult Domestic Shorthair cats, all females, bodyweight 2-4 kg, age between 2 and 5 years.

4.b. Motivatie waarom is gekozen voor deze diersoort:
Cats are the target animal of this study. Since in vitro methods are not available, we have no other choice than to use cats.

4.c. Toelichting voor het aantal gebruikte dieren:
Based on a sample size calculation the conclusion was drawn that a minimum of 9 animals is needed to obtain statistical significant results in the main experiment.
(Sample size calculation: power = 90%; alpha = 0,05; SD = 6,33 umol/kg/24h (12); relevant difference of 10 umol/kg/24h ' n = 8,42).

When working with animals there is a chance that some animals will deviate from the protocol (can be due to a lot of reasons) and will result in missing values in the final data. When this occurs, this will reduce the statistical power of the study (for example the study results in trend instead of a significant difference). Therefore three extra cats will be included in the study to improve the statistical power of the study even when a cat becomes ill.

In addition, according the broad experienced [redacted], 9 cats is a minimum amount of animals for this type of experiment and it is highly advisable to have 3 extra cats in case of illness. [redacted] can be considered to be an expert in cat experiments as he has been the head of [redacted] for 12 years.

In the pilot study 4 animals are needed to provide an indication for the length of the adaptation period of the main study.

Toelichting:

Er is gekozen om de katten te voeren volgens een 'repeated measure latin square design'.. Het grote voordeel van een latijns vierkant is dat alle dieren hun eigen controle zijn en dat er door het voeren van alle proefdieten in alle voerperiodes geen correctie nodig is voor de volgorde van de gegeven voeders. Dit vergroot de statistische power, waardoor er met relatief weinig proefdieren gewerkt kan worden. Nadeel van het gebruik van het latijnse vierkant is dat als er een dier uitvalt, om welke reden dan ook, het latijns vierkant verbroken wordt.
Er zal dan andere statistiek toegepast moeten worden met groot verlies van 'power' van de proef. Door een 'repeated' variant van het latijns vierkant uit te voeren, wordt er meer zekerheid bereikt dat de het latijnse vierkant niet verbroken gaat worden doordat er een dier uitvalt. Deze 'repeated' variant kan alleen uitgevoerd worden als er 3 dieren extra worden toegevoegd aan de groep.

	Feeding period			
	I	II	III	
Cat 1 + 2		B	A	C
Cat 3 + 4		A	C	B
Cat 5 + 6		C	B	A
Cat 7 + 8		B	C	A
Cat 9 + 10	A	B	C	

Cat 11 +12 C A B

4.d. Herkomst:

1 A. van gereg. fok/toeleveringsbedrijf in Nederland
2 A. van gereg. fok/toeleveringsbedrijf in Nederland

Toelichting:

The four cats housed in the facility are bred by [redacted].

To date (3 march 2010) there are purchasing actions for additional animals, which come from another origin (andere herkomst).

These animals are purchased based on the approved DEC-request of the experiment 'het testen van smakelijkheid van kattenvoerder met de 'two bowl' test en 'vloer'test' (officially approved 8-9-2009).

5.a. Accommodatie: [redacted]

The cats are housed in a group accommodation with a surface of 25 m2. During the pilot study and the adaptation period of the main study the cats will stay overnight and during feeding time in the metabolism cage (80 cm high x 100 cm length x 75 cm depth). From 11 till 17h the cats can walk freely around in the room where the metabolism cages are placed. During the 3 day during sample period of the main study the cats will be housed in the metabolism cage for the whole day.

5.b. Huisvesting & Verzorging:

The daily care during the experiments will be performed by the persons involved in the experiment (see also point 10) and will be in close contact with the animal caretakers/staff members of the [redacted].

5.c. Voeding:

Previous to the pilot study the cats will be fed their regular dry food. At day 1-14 of the pilot study the cats will be fed a balanced and complete commercial moist food for adult cats. This food will also be used as the basis for the experimental diets. During the main study three different experimental diets will be fed. These diets will be tested on palatability before the experiment starts.
The experimental diets consist of a commercial balanced and complete moist food for adult cats with either an added protein, carbohydrate or fat source (table 1).

High protein (Hp) diet: Protein source: hydrolyzed gelatin,
High carbohydrate (Hc) diet: Carbohydrate source: gelatinized maize starch, glucose, fructose, galactose, and xylose,
High Fat (Hf) diet: Fat source: lard.

The Hf diet will serve as the control diet in this experiment. The nutrient composition of the three experimental diets is given in table 2.

Table 1: Composition of experimental diets in gram dry matter (DM).

Ingredient	Hp	Hc g DM	Hf g DM	g DM

Commercial complete moist food for adult cats		145	145	145.
Palatibity enhancer (Optimizor)		4	4	4
Lard	0	0	60	
Herring oil		10	10	10
Galactose	0	25	0	
Glucose		0	25	0
Xylose	0	25	0	
Fructose		0	25	0
maize starch (gelatinized)		0	26	0
dried egg white (sterilized)		0	0	0
hydrolized gelatine (Rouselot ADF)		140	0	0
vitamin/mineral premix	?	?	?	
Total	298	285	219	

Table 2: Calculated nutrient composition of the experimental diets using the Atwater equation: (13).

		Hp	Hc	Hf	
DM	(g/100g)		36,1	35,2	28,1
MEa	(MJ/100g)	0,66	0,65	0,73	
CP	(g/MJ)	42,3	12,0	12,0	
Cfat	(g/MJ)	8,0	8,4	22,2	
Cfiber	(g/MJ)	0,6	0,6	0,6	
Cash	(g/MJ)	3,1	2,9	3,1	
NFE	(g/MJ)	0,8	30,1	0,8	

a Metabolizable energy (in KJ) per 100 gram food = 16,7 * (g/100g crude protein) + 35,5 * (g/100 g crude fat) + 16,7 * (g/100 gram NFE).
Abbreviations: DM = dry matter; ME = metabolizable energy; CP = crude protein; Cfat = crude fat; Cfiber = crude fiber; Cash = crude ash; NFE = nitrogen-free extract.

By adding the protein, carbohydrate or fat source, the diet will be diluted. Therefore a relative decrease in the content of protein, fat, essential amino acids, fatty acids, minerals and vitamins will occur. In the final formulation of this diet the nutrient(s) that will be below the minimal nutrient requirement determined by the National Research Council (NRC) for adult cats (13) will be supplemented.
For these experimental diets the protein content is set on minimum of 12 g/MJ instead of the 9,59 g/MJ recommended by the NRC to assure there will be no changes in protein metabolism due to protein deficiency in the cats. The minimal fat content is well above the recommendation of the NRC as well. At the moment the detailed nutrient composition of some ingredients is not received yet, and therefore the content of the essential amino acids (especially taurine and arginine), fatty acids, minerals and vitamins (especially vitamin B6) could not be calculated yet. Again, the essential nutrients that will be below the recommended nutrient requirements of the NRC will be supplemented in order to compose three complete experimental diets.

The amount of food per cat will be determined based on energy requirement of the individual cat by using 300 KJ/kg B.W/day (13). To assure the cats receive enough food, food intake will be monitored and the cats will be weighed weekly. Water is available ad libitum.

6.a. Proefschema / proefbehandelingen:

An overview over the training of the cats and the experiments is provided in table 3.

Table 3: Schedule for the training and the experiments with the cats plus the sampling scheme of the main study.

1, Pilot study (n=4)a

Acitvity	Time	sampling single urine	72h urine	feces	blood	weight cat
	14 days	Every day			Day 1, 7, 14	

2. Main study (n=12)

Day 0 (baseline)					yes	yes
Adaptation period Ia	. daysb	Every day				Once a week
Sample period Ic	3 days		yes	yes	yes	
Adaptation period IIa	. daysb	Every day				Once a week
Sample period IIc	3 days		yes	yes	yes	
Adaptation period IIIa	. daysb	Every day			Once a week	
Sample period IIIc	3 days		yes	yes	yes	

a The cats will be treated according the schedule in table 4.
b The amount of days will be based on the outcome of the pilot study.
c During the sample period the cats will be housed continuously in the metabolic cage for 72 hours.

Training period to metabolic cages (in approximately 2 weeks):
Previous to the pilot study and the main study the cats will be trained by feeding the cats in the metabolic cages while the door is open. When the cats are used to this procedure, the cage will be closed shortly and this will occur gradually for a longer time. The cats will also be trained to use the litter box in the cage: first by transferring feces and urine to the litter box to create the 'right' smell, and gradually offer them less choice in type and place of available litter boxes. Finally they will be trained only to use the litter boxes placed in the cages without stress.
The procedure of weighing of the cats will be trained as well.

Pilot study:
The duration of the pilot study will be 14 days. In cats and other carnivores the adaptation period has not been determined yet. In the few studies in which urinary oxalate was measured in cats, an adaptation period of at least 28 days was used, whereas in dogs a minimum period of 10 days is used (14). In an experiment with humans a minimum of 5 days is used (6). Since the main focus of the studies in dogs and humans was urinary oxalate excretion, we expect to find an adaptation period shorter than 14 days in cats as well.

Previous to the study the cats will be fed their regular dry food. At day one of the pilot study the food will be changed to the canned moist food which also will be used in the experimental diets.

During this study (and adaptation periods of the main study) the cats stay in the cage during the night and feeding time (2 times a day) (see table 3). In this way food intake per cat can be recorded. The expectation is that the cats will urinate at least once during the night. A (single) urine sample will be collected in the morning to determine the time (days) for oxalate appearance, expressed as a ratio with

creatinine, to reach their steady state after a diet change. The cats that didn't urinate during the night or during feeding time will be placed back into the metabolism cage at 12h until they urinate.
At day 1, 7 and 14 the cats will be weighed.

Table 4. Daily schedule for the pilot study and adaptation period of the main study.

Time	Cat in/out of cage	Activity
19.00 - 8.30h (overnight)	Cats in cage	To collect urine sample
8.30 - 10.30h	Cats in cage	Feeding time
10.30 - 17.00h	Cats out of cage	
12.00 - till urination	Cats that didn't urinate back in cage	To collect urine sample
17.00 - 19.00h	Cats in cage	Feeding time

Based on the results of this pilot study we are able to refine the duration of the adaptation period of the main study. In addition, the pilot study can be considered as part of the adaptation process for being housed in a metabolic cage as well.

Main study:
Three diets will be tested in this experiment and therefore this experiment will consist of three feeding periods containing an adaptation period and a sample period (see table 3). The cats will receive the experimental diets according a 3x3 latin square design. This means that every 2 cats will receive the three experimental diets in a different order (table 5). For the composition of these diets see table 1.

Table 5. 3x3 Latin square design for 12 cats.

	Feeding period I	II	III
Cat 1 + 2	B	A	C
Cat 3 + 4	A	C	B
Cat 5 + 6	C	B	A
Cat 7 + 8	B	C	A
Cat 9 + 10	A	B	C
Cat 11 +12	C	A	B

A = Hp diet; B = Hc diet; C= Hf diet

Each feeding period will consist of an adaption period and a sample period (see table 3). In the adaptation period the same protocol will be used as for the pilot study (table 4). This means that they will be housed in the metabolism cage overnight and during feeding time. During the sample period the cats will be housed for 24 hours in the metabolic cage in order to be able to collect 72h urine and feces (15).

Once a week the cats will receive a general clinical check-up by a veterinarian ([redacted]).
In addition, the cats will be weighed once a week to assure they maintain a steady body weight. This will give an indication of the cats having a sufficient food intake. In addition food left-overs will be weighed to record the food intake.

Blood will be collected by venepuncture (V. Jugularis) at the start of the experiment to assure the cats are healthy (especially in liver and kidney function) and to obtain a baseline value. In every sample period blood will be collected to determine the plasma oxalate level to be able to correlate this to the urinary oxalate excretion. Since a shortage in vitamin B6 is known to induce endogenous oxalate synthesis and therefore will increase oxalate excretion in the urine, the liver enzymes AST and ALT will be measured in blood plasma to evaluate vitamin B6 status during this experiment.
In preparation to the blood collection, the cats will be fixated by the animal caretaker (not sedated), shaved (1x2 cm at the basis of the neck) and disinfected with ethanol. The amount of 5 ml blood sample will be taken using a blue (0,60 x 25 mm, 23 G x 1") or black needle (0,70 X 30 mm BL/LB, 22 G x 1 ¼") by a trained veterinarian ([redacted]) or another certified staff member.

During the pilot study and adaptation period of the main study urine samples will be collected. To correct for the dilution of the urine, the ratio of urine creatinine will be taken, and therefore oxalate will be expressed as oxalate/creatinine ratio. Since creatinine excretion is dependent on muscle mass, different type of diets, etc, this is a significantly less accurate method to quantify oxalate in the urine.
To answer the main research questions accurately, it is essential to quantify the oxalate by pooling the urine collected on 3 consecutive days (72h urine; umol oxalate/kg B.W./day) instead of taking the ratio with creatinine (single urine; oxalate/creatinine ratio). Since being housed in the metabolic cage during the whole main study will result in too much inconvenience, we have chosen to collect only once a 72h urine per feeding period (sample period). For the pilot study and adaptation period of each feeding period is chosen to collect a single urine sample each day.

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calcium and oxalate on urine composition and calcium oxalate relative supersaturation in healthy adult dogs. Res Vet Sci 2003 Aug;75(1):33-41.

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6.b. Mate van ongerief:

1 B. Gering/Matig
2 C. Matig

6.c. Waaruit bestaat het ongerief en hoe bent u tot uw inschatting van de mate van ongerief gekomen?

The individual housing in the metabolic cage can result to inconvenience for the cat. The size of the metabolic cage (80 cm high x 100 cm length x 75 cm depth) together with having the training/adaptation period will minimize the inconvenience as much as possible.

Taking a blood sample will result in some (matig) inconvenience for the cats since they need to be fixated for this procedure and they'll experience a short sense of pain at the moment the needle will penetrate the skin.

Weighing will result in slight inconvenience for the cats since the handling of the cats for weighing will result in no to little stress for the cats.

7. Welke maatregelen heeft u getroffen om het ongerief tot een minimum te beperken?

Anesthesie:

1 A. Niet toegepast (geen aanleiding).
2 A. Niet toegepast (geen aanleiding).

Pijnbestrijding:

1 A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.
2 A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.

o The cats will be trained/adapted step-by-step to be housed in the metabolic cage for a prolonged period and for learning to urinate and defecate on the special designed litter box. In this way the stress response of cats to these new procedures will be minimized.
o During the pilot study and pre-period of the main experiment the cats are only housed in the cages during the night and feeding times. The rest of the day they can walk around freely and interact with each other.
o The metabolic cages are positioned in such a way that when the cats are housed in the cage they can make eye contact with each other, hear each other, etc.
o During daytime almost continuously researchers and/or caretakers will be present in the room were cats are housed in the metabolic cages. Also time is scheduled to socially interact with the cats.
o Additional to the set-up of the litter box described in Hendriks and co-authors (1999) (15) non-absorbable cat litter will be added. In this way the cat will be less disturbed in expressing its natural behaviour previous to urinating and defecating, i.e. scratching/digging into the 'soil'.

8. Toestand van dieren na einde van de proef:

1 Het dier is na de proef in leven gelaten.
2 Het dier is na de proef in leven gelaten.

Toelichting:

The cats will stay in the group accommodation of the cat facility of [redacted] after the experiment.

9. Welke alternatieven (vervanging, verfijning, vermindering) zijn voor de beschreven

experimenten overwogen en waarom zijn deze verworpen?
Replacement is not possible since the cat is the target species for this study and no in vitro method is available.

Reduction:
o of the number of animals is not possible because a lower amount of animals will reduce the power of this experiment below an acceptable level of 80% (see 4.c.).
o of the duration of the experiment is not possible since the pre-period is already minimalised by conducting the pilot-study to determine the minimal time needed for oxalate excretion to reach its steady state/plateau phase.
In addition, based on the study conducted by Hendriks and co-authors (1999) (15) a minimum of three consecutive days (72h) collection of urine is required to obtain an accurate urine sample for measuring quantities.

All ideas for refinement of the experiment will be implicated in the protocols during the studies.

10. Namen van direct betrokkenen bij de dierproef (artikel 9- en 12-functionarissen):

[redacted]

Tabel registratiecode opties voor aanvraag 2010034.c (K14):

	1	2	3	4	5	6	7	8	9	10	11	12	13
					36	1	1	01					
1	1	Ot	1	4					01	1	1	2	3
2	1	Ot	1	12					01	1	1	3	3

Aanmeldingsformulier voor proeven met gewervelde dieren.

Secretariaat DEC
[redacted]

Aanvrager: [redacted]
Afdeling:

Titel dierproef: Influence of dietary macronutrients on the urinary oxalate excretion in healthy cats

Aanmeldcode / Protocol: 2010034.d
Stadia van de proef:

01-07-2010	Aangemeld	[redacted]
01-07-2010	Wijzigen	
06-07-2010	Gekopieerd	

Is deze proef wetenschappelijk getoetst en goedgekeurd? Ja
Toelichting:trial (PhD research) has been approved by the supervisors [redacted]

1.a. Met dit onderzoek te beantwoorden concrete vraag:
. Wetenschappelijke vraag m.b.t.van dieren
This DEC-request is for two related studies: a pilot study and the main study. The pilot study will be conducted to provide an indication for the adaptation period needed for the main study as to minimize the adaptation period for each diet given.

The main objective of this study:
To determine the influence of dietary macronutrients on the urinary oxalate excretion as an indicator for endogenous oxalate synthesis in healthy cats in a controlled environment.

Sub objectives of this study
Pilot-study:
¢ To provide an indication for the time needed for urinary oxalate to reach a steady state excretion after a diet change (adaptation period).
Main study:
¢ To determine the influence of dietary protein content on the endogenous oxalate synthesis in cats fed an oxalate-free diet.
¢ To determine the influence of dietary carbohydrate content on the endogenous oxalate synthesis in cats fed an oxalate-free diet.
¢ To determine the influence of dietary fat content on the endogenous oxalate synthesis in cats fed an oxalate-free diet.
¢ To determine the time needed for urinary oxalate to reach a steady state excretion after a diet change.
¢ To determine the correlation between plasma oxalate concentration and 72h urine oxalate concentration in healthy cats.

¢ To determine the correlation between 72h urinary oxalate excretion and oxalate/creatinine ratio.

1.b. Het uiteindelijk doel (Maatschappelijke en wetenschappelijke relevantie):
Urolithiasis is the second most common cause of lower urinary tract disease (LUTD) in cats (1;2) (symptoms: difficult and painful urination, blood in the urine, partial or complete urethral obstruction) and different urolith (stone) types are known to cause urolithiasis. Over the past decades, a progressive increase in the prevalence of calcium oxalate (CaOx) uroliths (from 2% to 55-60%) has been reported in cats with LUTD in the United States of America (3) and Benelux (4). Nutrition is thought to play a major role in this increase in prevalence of CaOx urolithiasis. Today's nutritional interventions are mainly based on human medicine studies as only a few studies have been conducted in cats.

The current treatment of choice for severe CaOx urolithiasis is surgical urolith removal, followed by methods to prevent urolith recurrence. At present, the standard method for preventing CaOx urolith recurrence is to feed a CaOx urolith reducing therapeutic diet and encourage water intake. Despite these preventative measures, the prevalence and recurrence rate of CaOx uroliths remains high in cats. This means that at present we cannot prevent cats to develop CaOx urolithiasis and therefore cannot prevent them to undergo (mostly repeatedly) surgical treatment. Therefore, more knowledge regarding the etiopathogenesis of CaOx urolithiasis should be generated to improve current preventative measures and to decrease the prevalence of CaOx urolithiasis.

Urinary oxalate and calcium excretion are the two central risk factors in CaOx urolith formation, since they are the two elements to precipitate with each other in the urine. An increase in the excretion of either calcium or oxalate or both will lead to CaOx urolith formation. Since calcium metabolism is of interest for more diseases, several studies have been conducted to determine the nutrients that influence the urinary calcium excretion. This is however not the case for urinary oxalate excretion. Therefore it is essential to study the origin of oxalate excreted the urine.

In contrast with human medicine, dietary modifications to decrease endogenous oxalate synthesis have hardly been studied in carnivores. In human medicine both protein (certain amino acids) (5;6) and carbohydrates (certain sugars) (7;8) are known to act as a precursor of the endogenous oxalate synthesis. However, the metabolic pathway leading to oxalate synthesis differ between humans and carnivores ((9)(10;11)). It is therefore unclear how these dietary constituents influence endogenous oxalate synthesis in carnivores.
In cats, only one study has been published that aimed to determine the relationship between dietary protein content and urinary oxalate excretion (12). Urinary oxalate excretion was found to be inversely correlated with protein intake. However, in this experiment low protein diets did also contain a high content of carbohydrates and therefore the author could not ascribe the increased oxalate excretion to either the low protein content of the diet or to the high carbohydrate content.

Considering the enormous change in composition of diets for cats in the last decades, from small mammals (high in protein, hardly any carbohydrate) to today's pet food (containing 20-60% carbohydrates with a concomitant lower level of (animal) protein), and the increase in prevalence of CaOx uroliths at the same time, it is worthwhile to study the influences of protein en carbohydrate on the endogenous oxalate synthesis. This experiment will contribute to our understanding of the relationship between endogenous oxalate synthesis and dietary protein or carbohydrate and fat content and of the length of the needed adaptation period. Furthermore, this study may provide new insights for dietary strategies that prevent urolithiasis.

1.c. Lekensamenvatting:

2. Gepland vanaf: 01-04-2010 **tot** 31-08-2010

3. Specificatie diergroepen:

1	4	katten	Pilot study
2	12	katten	Alle dieren krijgen alle 3 de proefvoeders in 3 perioden

4.a. Nadere aanduiding gebruikte dieren:
In the pilot study n=4 cats; in the main study n=12 cats.
Due to social reasons the 13th cat will follow the same treatment as the cats included in the experiment.

Intact adult Domestic Shorthair cats, all females, bodyweight 2-4 kg, age between 1 and 5 years.

4.b. Motivatie waarom is gekozen voor deze diersoort:
Cats are the target animal of this study. Since in vitro methods are not available, we have no other choice than to use cats.

4.c. Toelichting voor het aantal gebruikte dieren:
Based on a sample size calculation the conclusion was drawn that a minimum of 9 animals is needed to obtain statistical significant results in the main experiment.
(Sample size calculation: power = 90%; alpha = 0,05; SD = 6,33 umol/kg/24h (12); relevant difference of 10 umol/kg/24h ' n = 8,42).

When working with animals there is a chance that some animals will deviate from the protocol (can be due to a lot of reasons) and will result in missing values in the final data. When this occurs, this will reduce the statistical power of the study (for example the study results in trend instead of a significant difference). Therefore three extra cats will be included in the study to improve the statistical power of the study even when a cat becomes ill.

In addition, according the broad experienced [redacted], 9 cats is a minimum amount of animals for this type of experiment and it is highly advisable to have 3 extra cats in case of illness. [redacted] can be considered to be an expert in cat experiments as he has been the head of [redacted]

In the pilot study 4 animals are needed to provide an indication for the length of the adaptation period of the main study.

Toelichting:

Er is gekozen om de katten te voeren volgens een 'repeated measure latin square design'.. Het grote voordeel van een latijns vierkant is dat alle dieren hun eigen controle zijn en dat er door het voeren van alle proefdieten in alle voerperiodes geen correctie nodig is voor de volgorde van de gegeven voeders. Dit vergroot de statistische power, waardoor er met relatief weinig proefdieren gewerkt kan worden. Nadeel van het gebruik van het latijnse vierkant is dat als er een dier uitvalt, om welke reden dan ook, het latijns vierkant verbroken wordt.
Er zal dan andere statistiek toegepast moeten worden met groot verlies van 'power' van de proef. Door een 'repeated' variant van het latijns vierkant uit te voeren, wordt er meer zekerheid bereikt dat de het latijnse vierkant niet verbroken gaat worden doordat er een dier uitvalt. Deze 'repeated' variant kan alleen uitgevoerd worden als er 3 dieren extra worden toegevoegd aan de groep.

	Feeding period			
	I	II	III	
Cat 1 + 2		B	A	C
Cat 3 + 4		A	C	B
Cat 5 + 6		C	B	A
Cat 7 + 8		B	C	A
Cat 9 + 10	A	B	C	

Cat 11 +12 C A B

4.d. Herkomst:

1 A. van gereg. fok/toeleveringsbedrijf in Nederland
2 A. van gereg. fok/toeleveringsbedrijf in Nederland

Toelichting:

The four cats housed in the facility are bred by [redacted].

To date (3 march 2010) there are purchasing actions for additional animals, which come from another origin (andere herkomst).

These animals are purchased based on the approved DEC-request of the experiment 'het testen van smakelijkheid van kattenvoerder met de 'two bowl' test en 'vloer'test' (officially approved 8-9-2009).

5.a. Accommodatie: [redacted]

The cats are housed in a group accommodation with a surface of 25 m2. During the pilot study and the adaptation period of the main study the cats will stay overnight and during feeding time in the metabolism cage (80 cm high x 100 cm length x 75 cm depth). From 10.30 till 13.00h and 14.00 till 16.30h the cats can walk freely around in the group accomodation. During the 3 day during sample period of the main study the cats will be housed in the metabolism cage for the whole day.

5.b. Huisvesting & Verzorging:

The daily care during the experiments will be performed by the persons involved in the experiment (see also point 10) and will be in close contact with the animal caretakers/staff members of the [redacted]

5.c. Voeding:

Previous to the pilot study the cats will be fed their regular dry food. At day 1-14 of the pilot study the cats will be fed a balanced and complete commercial moist food for adult cats. This food will also be used as the basis for the experimental diets. During the main study three different experimental diets will be fed. These diets will be tested on palatability before the experiment starts.
The experimental diets consist of a commercial balanced and complete moist food for adult cats with either an added protein, carbohydrate or fat source (table 1).

High protein (Hp) diet: Protein source: hydrolyzed gelatin,
High carbohydrate (Hc) diet: Carbohydrate source: gelatinized maize starch, sucrose and xylose,
High Fat (Hf) diet: Fat source: lard.

The Hf diet will serve as the control diet in this experiment. The nutrient composition of the three experimental diets is given in table 2.

Table 1: Composition of experimental diets in gram dry matter (DM).

Ingredient	Hp g DM	Hc g DM	Hf g DM

Commercial complete moist food for adult cats	145	145	145
Palatibity enhancer (Optimizor)	4	4	4
Lard	0	0	60
Salmon oil	10	10	10
Sucose	0	25	0
Xylose	0	25	0
maize starch (gelatinized)	0	26	0
Acid casein	140	0	0
vitamin B6	0.3	0.3	0.3
Total	298	285	219

Table 2: Calculated nutrient composition of the experimental diets using the Atwater equation: (13).

		Hp	Hc	Hf	
DM	(g/100g)		36,1	35,2	28,1
MEa	(MJ/100g)	0,66	0,65	0,73	
CP	(g/MJ)	42,3	12,0	12,0	
Cfat	(g/MJ)	8,0	8,4	22,2	
Cfiber	(g/MJ)	0,6	0,6	0,6	
Cash	(g/MJ)	3,1	2,9	3,1	
NFE	(g/MJ)	0,8	30,1	0,8	

a Metabolizable energy (in KJ) per 100 gram food = 16,7 * (g/100g crude protein) + 35,5 * (g/100 g crude fat) + 16,7 * (g/100 gram NFE).
Abbreviations: DM = dry matter; ME = metabolizable energy; CP = crude protein; Cfat = crude fat; Cfiber = crude fiber; Cash = crude ash; NFE = nitrogen-free extract.

By adding the protein, carbohydrate or fat source, the diet will be diluted. Therefore a relative decrease in the content of protein, fat, essential amino acids, fatty acids, minerals and vitamins will occur. In the final formulation of this diet the nutrient(s) that will be below the minimal nutrient requirement determined by the National Research Council (NRC) for adult cats (13) will be supplemented.
For these experimental diets the protein content is set on minimum of 12 g/MJ instead of the 9,59 g/MJ recommended by the NRC to assure there will be no changes in protein metabolism due to protein deficiency in the cats. The minimal fat content is well above the recommendation of the NRC as well. At the moment the detailed nutrient composition of some ingredients is not received yet, and therefore the content of the essential amino acids (especially taurine and arginine), fatty acids, minerals and vitamins (especially vitamin B6) could not be calculated yet. Again, the essential nutrients that will be below the recommended nutrient requirements of the NRC will be supplemented in order to compose three complete experimental diets.

The amount of food per cat will be determined based on energy requirement of the individual cat by using 300 KJ/kg B.W/day (13). To assure the cats receive enough food, food intake will be monitored and the cats will be weighed weekly. Water is available ad libitum.

6.a. Proefschema / proefbehandelingen:
An overview over the training of the cats and the experiments is provided in table 3.

Table 3: Schedule for the training and the experiments with the cats plus the sampling scheme of the main

study.

1, Pilot study (n=4)a

Acitvity	Time	sampling single urine	72h urine		feces	blood	weight cat
	14 days	Every day				Day 1, 7, 14	

2. Main study (n=12)

Day 0 (baseline)						yes
Adaptation period Ia	7 daysb					Once a week
urine sample period Ic	3 days		yes	yes		
Blood sampling	1 day				yes	
Adaptation period IIa	7 daysb					Once a week
Urine sample period IIc	4 days			yes	yes	
Blood sampling	1 day				yes	
Adaptation period IIIa	7 daysb					Once a week
urine sample period IIIc	4 days			yes	yes	
Blood sampling	1 day				yes	

a The cats will be treated according the schedule in table 4.
b The amount of days will be based on the outcome of the pilot study.
c During the sample period the cats will be housed continuously in the metabolic cage for 72 hours.

Training period to metabolic cages (in approximately 2 weeks):
Previous to the pilot study and the main study the cats will be trained by feeding the cats in the metabolic cages while the door is open. When the cats are used to this procedure, the cage will be closed shortly and this will occur gradually for a longer time. The cats will also be trained to use the litter box in the cage: first by transferring feces and urine to the litter box to create the 'right' smell, and gradually offer them less choice in type and place of available litter boxes. Finally they will be trained only to use the litter boxes placed in the cages without stress.
The procedure of weighing of the cats will be trained as well.

Pilot study:
The duration of the pilot study will be 14 days. In cats and other carnivores the adaptation period has not been determined yet. In the few studies in which urinary oxalate was measured in cats, an adaptation period of at least 28 days was used, whereas in dogs a minimum period of 10 days is used (14). In an experiment with humans a minimum of 5 days is used (6). Since the main focus of the studies in dogs and humans was urinary oxalate excretion, we expect to find an adaptation period shorter than 14 days in cats as well.

Previous to the study the cats will be fed their regular dry food. At day one of the pilot study the food will be changed to the canned moist food which also will be used in the experimental diets.

During this study (and adaptation periods of the main study) the cats stay in the cage during the night and feeding time (2 times a day) (see table 3). In the afternoon the cats will be placed back in the metabolism cage from 13.00-14.00h to eat and use the litterbox. In this way food intake per cat can be recorded. The expectation is that the cats will urinate at least once during the night. A (single) urine sample will be

collected in the morning to determine the time (days) for oxalate appearance, expressed as a ratio with creatinine, to reach their steady state after a diet change. The cats that didn't urinate during the night or during feeding time will be placed back into the metabolism cage at 12h until they urinate.
At day 1, 7 and 14 the cats will be weighed.

Table 4. Daily schedule for the pilot study and adaptation period of the main study.

Time	Cat in/out of cage	Activity
16.30 - 10.30h	Cats in cage	To feed and use of litterbox
10.30 - 13.00h	Cats out of cage	
13.00 - 14.00	back in cage	To feed and use of litterbox
14.00 - 16.30	Cats out of cage	

Based on the results of this pilot study we are able to refine the duration of the adaptation period of the main study. In addition, the pilot study can be considered as part of the adaptation process for being housed in a metabolic cage as well.

Main study:
Three diets will be tested in this experiment and therefore this experiment will consist of three feeding periods containing an adaptation period and a sample period (see table 3). The cats will receive the experimental diets according a 3x3 latin square design. This means that every 2 cats will receive the three experimental diets in a different order (table 5). For the composition of these diets see table 1.

Table 5. 3x3 Latin square design for 12 cats.

	Feeding period I	II	III
Cat 1 + 2		B	A	C
Cat 3 + 4		A	C	B
Cat 5 + 6		C	B	A
Cat 7 + 8		B	C	A
Cat 9 + 10	A	B	C	
Cat 11 +12	C	A	B	

A = Hp diet; B = Hc diet; C= Hf diet

Each feeding period will consist of an adaption period and a sample period (see table 3). In the adaptation period the same protocol will be used as for the pilot study (table 4), except for that in the first 5 days of the adaptation period normal cat grit will be used, on day 6 mixed with polyethylene grit and at day 7 only with polyethylene grit. This is to adapt the cats gradually to the new cat litter for the urine collection. Consequence is that no urine will be collected during the adaptation period (in the pilot study sufficient data was collected for determination of the adaptation period).This means that they will be housed in the metabolism cage overnight and during feeding time, and additionally from 13.00-14.00 they will be placed in the cage again to be able to eat and use the litterbox. During the sample period the cats will be housed for 24 hours in the metabolic cage in order to be able to collect 72h urine and feces (15).

Once a week the cats will receive a general clinical check-up by a veterinarian ([redacted]). In addition, the cats will be weighed once a week to assure they maintain a steady body weight. This will give an indication of the cats having a sufficient food intake. In addition food left-overs will be weighed to record the food intake.

Blood will be collected by venepuncture (V. Jugularis) at the start of the experiment to assure the cats are healthy (especially in liver and kidney function) and to obtain a baseline value. Blood will also be collected of cat nr 13 to check the health status. In every sample period blood will be collected to determine the plasma oxalate level to be able to correlate this to the urinary oxalate excretion. Since a shortage in vitamin B6 is known to induce endogenous oxalate synthesis and therefore will increase oxalate excretion in the urine, the liver enzymes AST and ALT will be measured in blood plasma to evaluate vitamin B6 status during this experiment.
In preparation to the blood collection, Xylocaine-gel will be applied on the spot of venepuncture for sedation. The cats will be fixated by the animal caretaker (not sedated), shaved (1x2 cm at the basis of the neck) and disinfected with ethanol. The amount of 5 ml blood sample will be taken using a blue (0,60 x 25 mm, 23 G x 1") or black needle (0,70 X 30 mm BL/LB, 22 G x 1 ¼") by a trained veterinarian ([redacted]) or another certified staff member.

During the pilot study and adaptation period of the main study urine samples will be collected. To correct for the dilution of the urine, the ratio of urine creatinine will be taken, and therefore oxalate will be expressed as oxalate/creatinine ratio. Since creatinine excretion is dependent on muscle mass, different type of diets, etc, this is a significantly less accurate method to quantify oxalate in the urine.
To answer the main research questions accurately, it is essential to quantify the oxalate by pooling the urine collected on 3 consecutive days (72h urine; umol oxalate/kg B.W./day) instead of taking the ratio with creatinine (single urine; oxalate/creatinine ratio). Since being housed in the metabolic cage during the whole main study will result in too much inconvenience, we have chosen to collect only once a 72h urine per feeding period (sample period). For the pilot study and adaptation period of each feeding period is chosen to collect a single urine sample each day.

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6.b. Mate van ongerief:

1 B. Gering/Matig
2 C. Matig

6.c. Waaruit bestaat het ongerief en hoe bent u tot uw inschatting van de mate van ongerief gekomen?

The individual housing in the metabolic cage can result to inconvenience for the cat. The size of the metabolic cage (80 cm high x 100 cm length x 75 cm depth) together with having the training/adaptation period will minimize the inconvenience as much as possible.

Taking a blood sample will result in some (matig) inconvenience for the cats since they need to be fixated for this procedure and they'll experience a short sense of pain at the moment the needle will penetrate the skin.

Weighing will result in slight inconvenience for the cats since the handling of the cats for weighing will result in no to little stress for the cats.

7. Welke maatregelen heeft u getroffen om het ongerief tot een minimum te beperken?

Anesthesie:

1 A. Niet toegepast (geen aanleiding).
2 A. Niet toegepast (geen aanleiding).

Pijnbestrijding:

1 A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.
2 A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.

o The cats will be trained/adapted step-by-step to be housed in the metabolic cage for a prolonged period and for learning to urinate and defecate on the special designed litter box. In this way the stress response of cats to these new procedures will be minimized.
o During the pilot study and pre-period of the main experiment the cats are only housed in the cages during the night and feeding times. The rest of the day they can walk around freely and interact with each other.
o The metabolic cages are positioned in such a way that when the cats are housed in the cage they can make eye contact with each other, hear each other, etc.
o During daytime almost continuously researchers and/or caretakers will be present in the room were cats are housed in the metabolic cages. Also time is scheduled to socially interact with the cats.
o Additional to the set-up of the litter box described in Hendriks and co-authors (1999) (15) non-absorbable cat litter will be added. In this way the cat will be less disturbed in expressing its natural behaviour previous to urinating and defecating, i.e. scratching/digging into the 'soil'.

8. Toestand van dieren na einde van de proef:

1 Het dier is na de proef in leven gelaten.
2 Het dier is na de proef in leven gelaten.

Toelichting:
The cats will stay in the group accommodation of [redacted] after the experiment.

9. Welke alternatieven (vervanging, verfijning, vermindering) zijn voor de beschreven experimenten overwogen en waarom zijn deze verworpen?
Replacement is not possible since the cat is the target species for this study and no in vitro method is available.

Reduction:
o of the number of animals is not possible because a lower amount of animals will reduce the power of this experiment below an acceptable level of 80% (see 4.c.).
o of the duration of the experiment is not possible since the pre-period is already minimalised by conducting the pilot-study to determine the minimal time needed for oxalate excretion to reach its steady state/plateau phase.
In addition, based on the study conducted by Hendriks and co-authors (1999) (15) a minimum of three consecutive days (72h) collection of urine is required to obtain an accurate urine sample for measuring quantities.

All ideas for refinement of the experiment will be implicated in the protocols during the studies.

10. Namen van direct betrokkenen bij de dierproef (artikel 9- en 12-functionarissen):
[redacted]

Tabel registratiecode opties voor aanvraag 2010034.d (K14):

	1	2	3	4	5	6	7	8	9	10	11	12	13
					36	1	1	01					
1	1	Ot	1	4					01	1	1	2	3
2	1	Ot	1	12					01	1	1	3	3

Aanmeldingsformulier voor proeven met gewervelde dieren.

Secretariaat DEC
[redacted]

Aanvrager: [redacted]
Afdeling:

Titel dierproef: Influence of dietary macronutrients on the urinary oxalate excretion in healthy cats

Aanmeldcode / Protocol: 2010034.e
Stadia van de proef:

06-07-2010	Aangemeld	[redacted]
06-07-2010	Positief advies	
18-01-2011	Opmerkingen	
21-02-2011	Welzijnsevaluatie aangemaakt	[redacted]

Is deze proef wetenschappelijk getoetst en goedgekeurd? Ja
Toelichting:trial (PhD research) has been approved by the supervisors, [redacted]

1.a. Met dit onderzoek te beantwoorden concrete vraag:
. Wetenschappelijke vraag m.b.t.van dieren
This DEC-request is for two related studies: a pilot study and the main study. The pilot study will be conducted to provide an indication for the adaptation period needed for the main study as to minimize the adaptation period for each diet given.

The main objective of this study:
To determine the influence of dietary macronutrients on the urinary oxalate excretion as an indicator for endogenous oxalate synthesis in healthy cats in a controlled environment.

Sub objectives of this study
Pilot-study:
¢ To provide an indication for the time needed for urinary oxalate to reach a steady state excretion after a diet change (adaptation period).
Main study:
¢ To determine the influence of dietary protein content on the endogenous oxalate synthesis in cats fed an oxalate-free diet.
¢ To determine the influence of dietary carbohydrate content on the endogenous oxalate synthesis in cats fed an oxalate-free diet.
¢ To determine the influence of dietary fat content on the endogenous oxalate synthesis in cats fed an oxalate-free diet.
¢ To determine the time needed for urinary oxalate to reach a steady state excretion after a diet change.
¢ To determine the correlation between plasma oxalate concentration and 72h urine oxalate

concentration in healthy cats.
¢ To determine the correlation between 72h urinary oxalate excretion and oxalate/creatinine ratio.

1.b. Het uiteindelijk doel (Maatschappelijke en wetenschappelijke relevantie):
Urolithiasis is the second most common cause of lower urinary tract disease (LUTD) in cats (1;2) (symptoms: difficult and painful urination, blood in the urine, partial or complete urethral obstruction) and different urolith (stone) types are known to cause urolithiasis. Over the past decades, a progressive increase in the prevalence of calcium oxalate (CaOx) uroliths (from 2% to 55-60%) has been reported in cats with LUTD in the United States of America (3) and Benelux (4). Nutrition is thought to play a major role in this increase in prevalence of CaOx urolithiasis. Today's nutritional interventions are mainly based on human medicine studies as only a few studies have been conducted in cats.

The current treatment of choice for severe CaOx urolithiasis is surgical urolith removal, followed by methods to prevent urolith recurrence. At present, the standard method for preventing CaOx urolith recurrence is to feed a CaOx urolith reducing therapeutic diet and encourage water intake. Despite these preventative measures, the prevalence and recurrence rate of CaOx uroliths remains high in cats. This means that at present we cannot prevent cats to develop CaOx urolithiasis and therefore cannot prevent them to undergo (mostly repeatedly) surgical treatment. Therefore, more knowledge regarding the etiopathogenesis of CaOx urolithiasis should be generated to improve current preventative measures and to decrease the prevalence of CaOx urolithiasis.

Urinary oxalate and calcium excretion are the two central risk factors in CaOx urolith formation, since they are the two elements to precipitate with each other in the urine. An increase in the excretion of either calcium or oxalate or both will lead to CaOx urolith formation. Since calcium metabolism is of interest for more diseases, several studies have been conducted to determine the nutrients that influence the urinary calcium excretion. This is however not the case for urinary oxalate excretion. Therefore it is essential to study the origin of oxalate excreted the urine.

In contrast with human medicine, dietary modifications to decrease endogenous oxalate synthesis have hardly been studied in carnivores. In human medicine both protein (certain amino acids) (5;6) and carbohydrates (certain sugars) (7;8) are known to act as a precursor of the endogenous oxalate synthesis. However, the metabolic pathway leading to oxalate synthesis differ between humans and carnivores ((9)(10;11)). It is therefore unclear how these dietary constituents influence endogenous oxalate synthesis in carnivores.
In cats, only one study has been published that aimed to determine the relationship between dietary protein content and urinary oxalate excretion (12). Urinary oxalate excretion was found to be inversely correlated with protein intake. However, in this experiment low protein diets did also contain a high content of carbohydrates and therefore the author could not ascribe the increased oxalate excretion to either the low protein content of the diet or to the high carbohydrate content.

Considering the enormous change in composition of diets for cats in the last decades, from small mammals (high in protein, hardly any carbohydrate) to today's pet food (containing 20-60% carbohydrates with a concomitant lower level of (animal) protein), and the increase in prevalence of CaOx uroliths at the same time, it is worthwhile to study the influences of protein en carbohydrate on the endogenous oxalate synthesis. This experiment will contribute to our understanding of the relationship between endogenous oxalate synthesis and dietary protein or carbohydrate and fat content and of the length of the needed adaptation period. Furthermore, this study may provide new insights for dietary strategies that prevent urolithiasis.

1.c. Lekensamenvatting:

2. Gepland vanaf: 01-04-2010 **tot** 31-08-2010

3. Specificatie diergroepen:

1	4	katten	Pilot study + main study: 3 proefvoeders in 3 perioden
2	8	katten	Main study: 3 proefvoeders in 3 perioden
3	1	katten	reserve main study: 3 proefvoeders in 3 perioden

4.a. Nadere aanduiding gebruikte dieren:
In the pilot study n=4 cats; in the main study n=12 cats.
Due to social reasons the 13th cat will follow the same treatment as the cats included in the experiment.

Intact adult Domestic Shorthair cats, all females, bodyweight 2-4 kg, age between 1 and 5 years.

4.b. Motivatie waarom is gekozen voor deze diersoort:
Cats are the target animal of this study. Since in vitro methods are not available, we have no other choice than to use cats.

4.c. Toelichting voor het aantal gebruikte dieren:
Based on a sample size calculation the conclusion was drawn that a minimum of 9 animals is needed to obtain statistical significant results in the main experiment.
(Sample size calculation: power = 90%; alpha = 0,05; SD = 6,33 umol/kg/24h (12); relevant difference of 10 umol/kg/24h ' n = 8,42).

When working with animals there is a chance that some animals will deviate from the protocol (can be due to a lot of reasons) and will result in missing values in the final data. When this occurs, this will reduce the statistical power of the study (for example the study results in trend instead of a significant difference). Therefore three extra cats will be included in the study to improve the statistical power of the study even when a cat becomes ill.

For social reasons the 13th (reserve) cat will follow the same procedure as the other cats, except for drawing blood. The 13th cat (reserve cat) will receive the same diet as the cats known to be difficult eaters. When one of these cats refuses to eat the diet for more than 2 days, the reserve cat will replace this cat. Of this reserve cat also urine will be collected.

In addition, according the broad experienced [redacted], 9 cats is a minimum amount of animals for this type of experiment and it is highly advisable to have 3 extra cats in case of illness. [redacted] can be considered to be an expert in cat experiments as he has been the head of [redacted]

In the pilot study 4 animals are needed to provide an indication for the length of the adaptation period of the main study.

Toelichting:

Er is gekozen om de katten te voeren volgens een 'repeated measure latin square design'.. Het grote voordeel van een latijns vierkant is dat alle dieren hun eigen controle zijn en dat er door het voeren van alle proefdieten in alle voerperiodes geen correctie nodig is voor de volgorde van de gegeven voeders. Dit vergroot de statistische power, waardoor er met relatief weinig proefdieren gewerkt kan worden. Nadeel van het gebruik van het latijnse vierkant is dat als er een dier uitvalt, om welke reden dan ook, het latijns vierkant verbroken wordt.
Er zal dan andere statistiek toegepast moeten worden met groot verlies van 'power' van de proef. Door een 'repeated' variant van het latijns vierkant uit te voeren, wordt er meer zekerheid bereikt dat de het latijnse vierkant niet verbroken gaat worden doordat er een dier uitvalt. Deze 'repeated' variant kan alleen uitgevoerd worden als er 3 dieren extra worden toegevoegd aan de groep.

	Feeding period			
	I	II	III	
Cat 1 + 2		B	A	C
Cat 3 + 4		A	C	B
Cat 5 + 6		C	B	A
Cat 7 + 8		B	C	A
Cat 9 + 10	A	B	C	
Cat 11 +12	C	A	B	

4.d. Herkomst:

1 A. van gereg. fok/toeleveringsbedrijf in Nederland
2 A. van gereg. fok/toeleveringsbedrijf in Nederland
3 A. van gereg. fok/toeleveringsbedrijf in Nederland

Toelichting:

The four cats housed in the facility are bred by [redacted].

To date (3 march 2010) there are purchasing actions for additional animals, which come from another origin (andere herkomst).

These animals are purchased based on the approved DEC-request of the experiment 'het testen van smakelijkheid van kattenvoerder met de 'two bowl' test en 'vloer'test' (officially approved 8-9-2009).

5.a. Accommodatie: [redacted]

The cats are housed in a group accommodation with a surface of 25 m2. During the pilot study and the adaptation period of the main study the cats will stay overnight and during feeding time in the metabolism cage (80 cm high x 100 cm length x 75 cm depth). From 10.30 till 13.00h and 14.00 till 16.30h the cats can walk freely around in the group accomodation. During the 3 day during sample period of the main study the cats will be housed in the metabolism cage for the whole day.

5.b. Huisvesting & Verzorging:

The daily care during the experiments will be performed by the persons involved in the experiment (see also point 10) and will be in close contact with the animal caretakers/staff members of the [redacted]

5.c. Voeding:

Previous to the pilot study the cats will be fed their regular dry food. At day 1-14 of the pilot study the cats will be fed a balanced and complete commercial moist food for adult cats. This food will also be used as the basis for the experimental diets. During the main study three different experimental diets will be fed. These diets will be tested on palatability before the experiment starts.
The experimental diets consist of a commercial balanced and complete moist food for adult cats with either an added protein, carbohydrate or fat source (table 1).

High protein (Hp) diet: Protein source: hydrolyzed gelatin,
High carbohydrate (Hc) diet: Carbohydrate source: gelatinized maize starch, sucrose and xylose,
High Fat (Hf) diet: Fat source: lard.

The Hf diet will serve as the control diet in this experiment. The nutrient composition of the three experimental diets is given in table 2.

Table 1: Composition of experimental diets in gram dry matter (DM).

Ingredient	Hp	Hc g DM	Hf g DM	g DM
Commercial complete moist food for adult cats		145	145	145
Palatibity enhancer (Optimizor)		4	4	4
Lard	0	0	60	
Salmon oil	10	10	10	
Sucose	0	25	0	
Xylose	0	25	0	
maize starch (gelatinized)		0	26	0
Acid casein	140	0	0	
vitamin B6	0.3	0.3	0.3	
Total	298	285	219	

Table 2: Calculated nutrient composition of the experimental diets using the Atwater equation: (13).

		Hp	Hc	Hf	
DM	(g/100g)		36,1	35,2	28,1
MEa	(MJ/100g)	0,66	0,65	0,73	
CP	(g/MJ)	42,3	12,0	12,0	
Cfat	(g/MJ)	8,0	8,4	22,2	
Cfiber	(g/MJ)	0,6	0,6	0,6	
Cash	(g/MJ)	3,1	2,9	3,1	
NFE	(g/MJ)	0,8	30,1	0,8	

a Metabolizable energy (in KJ) per 100 gram food = 16,7 * (g/100g crude protein) + 35,5 * (g/100 g crude fat) + 16,7 * (g/100 gram NFE).
Abbreviations: DM = dry matter; ME = metabolizable energy; CP = crude protein; Cfat = crude fat; Cfiber = crude fiber; Cash = crude ash; NFE = nitrogen-free extract.

By adding the protein, carbohydrate or fat source, the diet will be diluted. Therefore a relative decrease in the content of protein, fat, essential amino acids, fatty acids, minerals and vitamins will occur. In the final formulation of this diet the nutrient(s) that will be below the minimal nutrient requirement determined by the National Research Council (NRC) for adult cats (13) will be supplemented.
For these experimental diets the protein content is set on minimum of 12 g/MJ instead of the 9,59 g/MJ recommended by the NRC to assure there will be no changes in protein metabolism due to protein deficiency in the cats. The minimal fat content is well above the recommendation of the NRC as well. At the moment the detailed nutrient composition of some ingredients is not received yet, and therefore the content of the essential amino acids (especially taurine and arginine), fatty acids, minerals and vitamins (especially vitamin B6) could not be calculated yet. Again, the essential nutrients that will be below the recommended nutrient requirements of the NRC will be supplemented in order to compose three complete experimental diets.

The amount of food per cat will be determined based on energy requirement of the individual cat by using

300 KJ/kg B.W/day (13). To assure the cats receive enough food, food intake will be monitored and the cats will be weighed weekly. Water is available ad libitum.

6.a. Proefschema / proefbehandelingen:
An overview over the training of the cats and the experiments is provided in table 3.

Table 3: Schedule for the training and the experiments with the cats plus the sampling scheme of the main study.

1, Pilot study (n=4)a

Acitvity	Time	sampling single urine	72h urine		feces	blood	weight cat
	14 days	Every day				Day 1, 7, 14	

2. Main study (n=12)

Day 0 (baseline)						yes
Adaptation period Ia	7 daysb					Once a week
urine sample period Ic	3 days		yes	yes		
Blood sampling	1 day				yes	
Adaptation period IIa	7 daysb					Once a week
Urine sample period IIc	4 days			yes	yes	
Blood sampling	1 day				yes	
Adaptation period IIIa	7 daysb					Once a week
urine sample period IIIc	4 days			yes	yes	
Blood sampling	1 day				yes	

a The cats will be treated according the schedule in table 4.
b The amount of days will be based on the outcome of the pilot study.
c During the sample period the cats will be housed continuously in the metabolic cage for 72 hours.

Training period to metabolic cages (in approximately 2 weeks):
Previous to the pilot study and the main study the cats will be trained by feeding the cats in the metabolic cages while the door is open. When the cats are used to this procedure, the cage will be closed shortly and this will occur gradually for a longer time. The cats will also be trained to use the litter box in the cage: first by transferring feces and urine to the litter box to create the 'right' smell, and gradually offer them less choice in type and place of available litter boxes. Finally they will be trained only to use the litter boxes placed in the cages without stress.
The procedure of weighing of the cats will be trained as well.

Pilot study:
The duration of the pilot study will be 14 days. In cats and other carnivores the adaptation period has not been determined yet. In the few studies in which urinary oxalate was measured in cats, an adaptation period of at least 28 days was used, whereas in dogs a minimum period of 10 days is used (14). In an experiment with humans a minimum of 5 days is used (6). Since the main focus of the studies in dogs and humans was urinary oxalate excretion, we expect to find an adaptation period shorter than 14 days in cats as well.

Previous to the study the cats will be fed their regular dry food. At day one of the pilot study the food will be changed to the canned moist food which also will be used in the experimental diets.

During this study (and adaptation periods of the main study) the cats stay in the cage during the night and feeding time (2 times a day) (see table 3). In the afternoon the cats will be placed back in the metabolism cage from 13.00-14.00h to eat and use the litterbox. In this way food intake per cat can be recorded. The expectation is that the cats will urinate at least once during the night. A (single) urine sample will be collected in the morning to determine the time (days) for oxalate appearance, expressed as a ratio with creatinine, to reach their steady state after a diet change. The cats that didn't urinate during the night or during feeding time will be placed back into the metabolism cage at 12h until they urinate.
At day 1, 7 and 14 the cats will be weighed.

Table 4. Daily schedule for the pilot study and adaptation period of the main study.

Time	Cat in/out of cage	Activity
16.30 - 10.30h	Cats in cage	To feed and use of litterbox
10.30 - 13.00h	Cats out of cage	
13.00 - 14.00	back in cage	To feed and use of litterbox
14.00 - 16.30	Cats out of cage	

Based on the results of this pilot study we are able to refine the duration of the adaptation period of the main study. In addition, the pilot study can be considered as part of the adaptation process for being housed in a metabolic cage as well.

Main study:
Three diets will be tested in this experiment and therefore this experiment will consist of three feeding periods containing an adaptation period and a sample period (see table 3). The cats will receive the experimental diets according a 3x3 latin square design. This means that every 2 cats will receive the three experimental diets in a different order (table 5). For the composition of these diets see table 1.

Table 5. 3x3 Latin square design for 12 cats.

	Feeding period I	II	III	
Cat 1 + 2		B	A	C
Cat 3 + 4		A	C	B
Cat 5 + 6		C	B	A
Cat 7 + 8		B	C	A
Cat 9 + 10	A	B	C	
Cat 11 +12	C	A	B	

A = Hp diet; B = Hc diet; C= Hf diet

Each feeding period will consist of an adaption period and a sample period (see table 3). In the adaptation period the same protocol will be used as for the pilot study (table 4), except for that in the first 5 days of

the adaptation period normal cat grit will be used, on day 6 mixed with polyethylene grit and at day 7 only with polyethylene grit. This is to adapt the cats gradually to the new cat litter for the urine collection. Consequence is that no urine will be collected during the adaptation period (in the pilot study sufficient data was collected for determination of the adaptation period).This means that they will be housed in the metabolism cage overnight and during feeding time, and additionally from 13.00-14.00 they will be placed in the cage again to be able to eat and use the litterbox. During the sample period the cats will be housed for 24 hours in the metabolic cage in order to be able to collect 72h urine and feces (15).

Once a week the cats will receive a general clinical check-up by a veterinarian ([redacted]). In addition, the cats will be weighed once a week to assure they maintain a steady body weight. This will give an indication of the cats having a sufficient food intake. In addition food left-overs will be weighed to record the food intake.

Blood will be collected by venepuncture (V. Jugularis) at the start of the experiment to assure the cats are healthy (especially in liver and kidney function) and to obtain a baseline value. Blood will also be collected of cat nr 13 to check the health status. In every sample period blood will be collected to determine the plasma oxalate level to be able to correlate this to the urinary oxalate excretion. Since a shortage in vitamin B6 is known to induce endogenous oxalate synthesis and therefore will increase oxalate excretion in the urine, the liver enzymes AST and ALT will be measured in blood plasma to evaluate vitamin B6 status during this experiment.
In preparation to the blood collection, Xylocaine-gel will be applied on the spot of venepuncture for sedation. The cats will be fixated by the animal caretaker (not sedated), shaved (1x2 cm at the basis of the neck) and disinfected with ethanol. The amount of 5 ml blood sample will be taken using a blue (0,60 x 25 mm, 23 G x 1") or black needle (0,70 X 30 mm BL/LB, 22 G x 1 ¼") by a trained veterinarian ([redacted]) or another certified staff member.

During the pilot study and adaptation period of the main study urine samples will be collected. To correct for the dilution of the urine, the ratio of urine creatinine will be taken, and therefore oxalate will be expressed as oxalate/creatinine ratio. Since creatinine excretion is dependent on muscle mass, different type of diets, etc, this is a significantly less accurate method to quantify oxalate in the urine.
To answer the main research questions accurately, it is essential to quantify the oxalate by pooling the urine collected on 3 consecutive days (72h urine; umol oxalate/kg B.W./day) instead of taking the ratio with creatinine (single urine; oxalate/creatinine ratio). Since being housed in the metabolic cage during the whole main study will result in too much inconvenience, we have chosen to collect only once a 72h urine per feeding period (sample period). For the pilot study and adaptation period of each feeding period is chosen to collect a single urine sample each day.

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6.b. Mate van ongerief:

1 B. Gering/Matig
2 C. Matig
3 A. Gering

6.c. Waaruit bestaat het ongerief en hoe bent u tot uw inschatting van de mate van ongerief gekomen?

The individual housing in the metabolic cage can result to inconvenience for the cat. The size of the metabolic cage (80 cm high x 100 cm length x 75 cm depth) together with having the training/adaptation period will minimize the inconvenience as much as possible.

Taking a blood sample will result in some (matig) inconvenience for the cats since they need to be fixated for this procedure and they'll experience a short sense of pain at the moment the needle will penetrate the skin.

Weighing will result in slight inconvenience for the cats since the handling of the cats for weighing will result in no to little stress for the cats.

7. Welke maatregelen heeft u getroffen om het ongerief tot een minimum te beperken?

Anesthesie:

1 A. Niet toegepast (geen aanleiding).
2 A. Niet toegepast (geen aanleiding).
3 A. Niet toegepast (geen aanleiding).

Pijnbestrijding:

1 A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.
2 A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.
3 A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.

o The cats will be trained/adapted step-by-step to be housed in the metabolic cage for a prolonged period and for learning to urinate and defecate on the special designed litter box. In this way the stress response of cats to these new procedures will be minimized.
o During the pilot study and pre-period of the main experiment the cats are only housed in the cages during the night and feeding times. The rest of the day they can walk around freely and interact with

each other.
o The metabolic cages are positioned in such a way that when the cats are housed in the cage they can make eye contact with each other, hear each other, etc.
o During daytime almost continuously researchers and/or caretakers will be present in the room were cats are housed in the metabolic cages. Also time is scheduled to socially interact with the cats.
o Additional to the set-up of the litter box described in Hendriks and co-authors (1999) (15) non-absorbable cat litter will be added. In this way the cat will be less disturbed in expressing its natural behaviour previous to urinating and defecating, i.e. scratching/digging into the 'soil'.

8. Toestand van dieren na einde van de proef:

1 Het dier is na de proef in leven gelaten.
2 Het dier is na de proef in leven gelaten.
3 Het dier is na de proef in leven gelaten.

Toelichting:

The cats will stay in the group accommodation of the cat facility of [redacted] after the experiment.

9. Welke alternatieven (vervanging, verfijning, vermindering) zijn voor de beschreven experimenten overwogen en waarom zijn deze verworpen?

Replacement is not possible since the cat is the target species for this study and no in vitro method is available.

Reduction:
o of the number of animals is not possible because a lower amount of animals will reduce the power of this experiment below an acceptable level of 80% (see 4.c.).
o of the duration of the experiment is not possible since the pre-period is already minimalised by conducting the pilot-study to determine the minimal time needed for oxalate excretion to reach its steady state/plateau phase.
In addition, based on the study conducted by Hendriks and co-authors (1999) (15) a minimum of three consecutive days (72h) collection of urine is required to obtain an accurate urine sample for measuring quantities.

All ideas for refinement of the experiment will be implicated in the protocols during the studies.

10. Namen van direct betrokkenen bij de dierproef (artikel 9- en 12-functionarissen):

[redacted]

Tabel registratiecode opties voor aanvraag 2010034.e (K14):

	1	2	3	4	5	6	7	8	9	10	11	12	13
					36	1	1	01					
1	1	Ot	1	4					01	1	1	2	3
2	1	Ot	1	8					01	1	1	3	3
3	1	Ot	1	1					01	1	1	1	3

Uw aanvraag 2010034.a, door u aangemeld vanuit DRS heeft van de Secretaris DEC de status: 'Wijzigen' gekregen.

Indien de status op 'wijzigen' is gezet en u wilt deze aanvraag gaan wijzigen, dan selecteert u deze aanvraag en kiest u vanuit het menu 'bewerken aanvraag', en dan de optie 'wijzigen'. Er wordt dan een kopie van de originele aanvraag gemaakt. Deze kopie kunt u vervolgens wijzigen, en opnieuw aanmelden.

Met vriendelijke groet,

[redacted]
Secretaris DEC

Uw aanvraag 2010034.b, door u aangemeld vanuit DRS heeft van de DEC de status: 'Wijzigen' gekregen.

De DEC is van mening dat het doel van de proef opweegt tegen het te verwachten matige ongerief dat de dieren ondergaan. Voorafgaand aan een definitief advies heeft de DEC de volgende vragen en opmerkingen:
De DEC verzoekt u bij 4.c. (toelichting aantal dieren) het aantal reservedieren (3 op een groep van 9) uitgebreider te onderbouwen, aangezien haar dit erg veel lijkt.
Daarnaast verzoekt de DEC u bij 4.d. de herkomst van de dieren correct aan te geven (andere herkomst). Bovendien verzoekt de DEC u de passage, dat de aanvoer van de dieren plaatsvindt onder verantwoordelijkheid van [redacted] te verwijderen, aangezien dit niet juist is.

Na aanpassing zal de proef door de secretaris van de DEC worden afgehandeld.

Uw aanvraag 2010034.c, door u aangemeld vanuit DRS heeft van de Secretaris DEC de status: 'Positief advies na behandeling DEC' gekregen.

De DEC is van mening dat het doel van de proef opweegt tegen het te verwachten maximaal matige ongerief dat de dieren ondergaan en dat de vraag m.b.t. alternatieven voldoende is beantwoord.

Met vriendelijke groet,

[redacted]
Secretaris DEC

Uw aanvraag 2010034.c, door u aangemeld vanuit DRS heeft van de PD de status: 'Wijzigen' gekregen.

Indien de status op 'wijzigen' is gezet en u wilt deze aanvraag gaan wijzigen, dan selecteert u deze aanvraag en kiest u vanuit het menu 'bewerken aanvraag', en dan de optie 'wijzigen'. Er wordt dan een kopie van de originele aanvraag gemaakt. Deze kopie kunt u vervolgens wijzigen, en opnieuw aanmelden.

Na aanmelding wordt de wijzigingsaanvraag in eerste instantie door de proefdierdeskundige afgehandeld, eventueel beslist de proefdierdeskundige de proef door te sturen naar de Kleine Commissie.

Met vriendelijke groet,

[redacted]
Proefdierdeskundige

Uw aanvraag 2010034.d, door u aangemeld vanuit DRS heeft van de PD de status: 'Wijzigen' gekregen.

U wordt verzocht kat nummer dertien bij het totaal aantal proefdieren te tellen, en de groepen bij "3. specificatie diergroepen" zo op te schrijven dat u op een totaal van 13 uitkomt. Een suggestie is 4 katten: pilot+3 proefvoeders; 8 katten: 3 proefvoeders; 1 kat: reserve.
Onder 4c dient het meenemen van een reserve gemotiveerd te worden, en moet aangegeven worden welk deel van de proef de reserve zal meelopen.
Onder 6b kunt u het ongerief van de reserve clasificeren als gering.

Indien de status op 'wijzigen' is gezet en u wilt deze aanvraag gaan wijzigen, dan selecteert u deze aanvraag en kiest u vanuit het menu 'bewerken aanvraag', en dan de optie 'wijzigen'. Er wordt dan een kopie van de originele aanvraag gemaakt. Deze kopie kunt u vervolgens wijzigen, en opnieuw aanmelden.

Na aanmedling zal de aanvrag worden behandeld door de proefdierdeskundige.

Met vriendelijke groet,

[redacted]
Proefdierdeskundige

Uw aanvraag 2010034.e, een wijziging op de c-versie die eerder van positief advies was voorzien, door u aangemeld vanuit DRS heeft van de PD de status: 'Positief advies' gekregen.

Met vriendelijke groet,

[redacted]
proefdierdeskundige