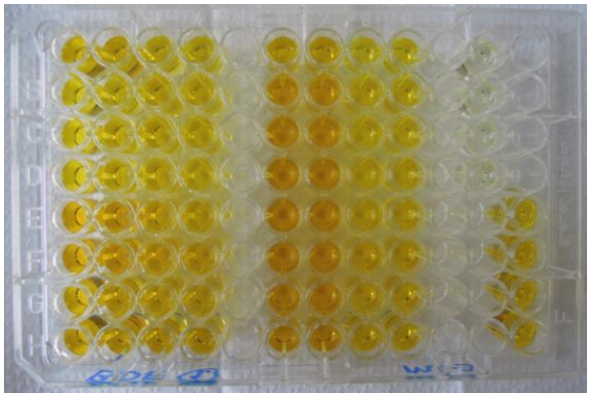
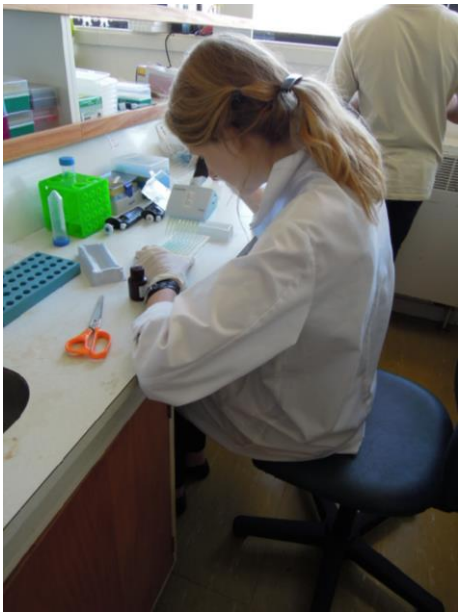
Immunological response to dietary proteins in cats

Veterinary Medicine, Research Project at Massey University, Palmerston North





Research Project, Veterinary Medicine

Student:	Manon de Wit, 3260313
Supervisors:	
Massey University, New Zealand	Nick Cave, BVSc. MVSc. PhD. MANZCVS. DipAC.
Utrecht University, The Netherlands	Esther Hagen-Plantinga, PhD. DVM. MSc.
Date:	
Research internship	Nov 2012- Jan 2013
Essay and Presentation	August 2013

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Universiteit Utrecht

Contents

<u>Abbreviations:</u> HSF, Hemp Seed Flour; HSFP, Hemp Seed Flour Protein; ELISA, Enzyme-Linked Immunosorbent Assay; MER, Maintenance Energy Requirements; PBS, Phosphate buffered saline; PBST, Phospate buffered saline with Tween; BDE, Basal Diet Extract; HSFE, Hemp Seed Flour Extract; HHDE, Heated Hemp Diet Extract; CBD, Cannabidiol.

- *Diet 1 = (Basal diet → heated) + HSF*
- *Diet 2 = (Basal diet + HSF) → heated*

Abstract

This study was conducted to gain more information about antibody production against novel and commonly exposed proteins, using hemp seed flour protein (from Cannabis Sativa plant) as a novel protein source. In this trial, two groups of eight domestic shorthair cats were fed with different diets, a basal canned diet mixed with raw hemp seed flour protein (diet 1) or diet 2 in which the canned diet was first mixed with hemp seed flour and heated afterwards. Antibody production was measured via indirect ELISA, using tree antigens (basal diet extract, raw hemps seed flour extract and heated hemp diet extract) and cats sera of 3 time moments (day 0, 11 and 25 after starting diets). Using raw or heated hemp protein in the diets, the effects of canning (heating) the hemp protein could also be reviewed. Based on results of indirect ELISA robust IgG antibodies were found against all the antigens in both group of cats. This is not in line with oral tolerance theory, in which cats should have lower antibody levels against commonly exposed proteins like the basal diet extract. Some differences were found between IgG levels against hemp protein between the two groups, with a possible explanation that cannabidiol from the hemp seed flour extract induced immune stimulation in the group receiving non-processed, raw hemp diet (diet 1).
For more confirmation and clarification about antibody production against dietary proteins in cats, further research is necessary consisting of optimizing ELISA experiments and results.

1. Introduction

Adverse reactions to food are more common these days, they are found in approximately 29% of all cats (n=55) with chronic gastrointestinal disease[1]. A possible explanation for this increase in adverse feline food reactions may be the manufacturing process of commercial pet food. Commercial pet foods are fabricated during a process of heating which could change the 3-dimensional conformation of food proteins[2]. These conformational changes may lead to the creation of new antigenic proteins, for example melanoidins.
A previous experiment by Cave et al.[2] showed that cats (without showing any clinical signs) develop robust serum IgG and IgA responses to novel dietary proteins (soy and casein), either unprocessed or as part of a canned diet. An explanation could be that the production of these antibodies is a method of removing opsonized dietary antigens out of the circulation by phagocytosis. However, this could induce the formation of antigen-antibody complexes which can cause obstruction and trigger antibody-mediated diseases like polyarthritis or glomerulonephritis[2]. Cave et al.[2] did not investigate whether the robust serum immunoglobulin production decreased after a period of time, both casein and soy were novel proteins presented to the cats over a relatively short period of time.
Based on these results of Cave et al. it is worth looking at the immunoglobulin production of novel dietary proteins compared to proteins the cats have previously been exposed to (a "known protein"). This could characterize if they develop a peripheral self-tolerance causing an overtime decrease in food-specific antibody responses against known proteins or on a contrary, equal immunoglobulin levels against novel or continuously presented proteins.
The goal of this research is to retest antibody formation against dietary proteins in healthy cats and to compare if heating (canning process) causes differences in immune response. Another point of interest is to see if there is a difference in antigen specific immune response between novel proteins and known proteins, to possibly confirm the principle of oral tolerance.

2. Background information

2.1 Immunologic reactions to food

Adverse reactions to food includes food intoxication, food allergy and food intolerance. They can be divided in immunological responses (food allergy/food hypersensitivity) and those with no immunological basis like *food intolerance*, a digestive enzyme deficiencies e.g. lactose intolerance[3]. *Food intoxication* is caused by bacteria/toxins[4] in food that trigger immune system and are immediately bound by secretory IgA (block adherence to mucosal surface) or taken up by M cells which main target is to screen intestinal content for foreign, pathogenic substances. In *food hypersensitivity*[3, 5] these M-cells are also active, sampling undigested particles/proteins from the intestinal content. Normally individuals create oral-tolerance to these antigens, induced by several factors participating different cells of the immune system with regulatory T cells being the most important[5]. Food hypersensitivity can develop through disturbances at different steps of generating oral-tolerance, resulting in an allergic immune response to little, otherwise harmless, dietary components (allergens)[3]. Normally after been taken up by M-cells, antigens are processed by antigen presenting cells (macrophages and dendritic cells), then presented to local, inactive T lymphocytes. After finding the matching antigen presented on the MHC-II receptor on the APC (Antigen Presenting Cell) surface (binding to the T-Cell Receptor, TCR), the T-cell becomes activated and will produce cytokines and also stimulate B lymphocytes, followed by antibody production[6]. This reaction can be suppressed by T-regulatory cells, which happens in case of oral tolerance.

2.2 Manufacturing of pet food

Melanoidins are less soluble and less digestible compounds created through heating of food proteins which causes a nonenzymatic protein glycosylation called the Maillard reaction[7, 8]. Melanoidins give a characteristic brown color to food[2, 7, 8] and are suspected to increase antigenicity based on an increase in the amount of undigested protein complexes that are being absorbed across the intestinal mucosa. Especially in cats the decreased digestibility by commercial canning could be a problem, cats have a relatively short intestinal tract, so less ability to digest their food properly[2]. It is conceivable that atopic animals may be more likely to produce diet-specific IgE reactions when they eat certain poorly digestible, canned diets. Also, in case of eating poorly digestible diets in periods of enteritis, this could increase the level of immune responses to those protein complexes, possible of causing clinical signs like food hypersensitivity[2].

2.3 Other research about immunologic reactions against food proteins

So far only two articles have been published about antibody responses to food proteins in cats, the previously discussed article about immune responses against heated or unheated food proteins in cats (N. Cave) and another report by Halliwell et al[9].

Cave et al.[2] hypothesized about the reason why cats produce robust antibody responses against harmless dietary proteins. Besides removing opsonized dietary antigens out of the circulation, it could be that the cats in this experiment were fed with an overdose of those novel dietary proteins, which triggers a higher antibody response based on uptake of food proteins.

Halliwell et al[9] investigated the antibody responses to specific antigen (HSA, Human Serum Albumin) administered with food to two groups of kittens, one group was Toxocara cati

positive and the other group was parasite-free. Halliwell reported that the parasitized cats had a significantly higher antigen-specific IgE response in week 2 and 4 of feeding compared to the parasite-free kittens, they also had a significant higher antigen-specific IgG and IgA response during week 4 and 8[9].
At least these articles of Halliwell et al. and Cave et al. confirm the hypothesis that non-symptomatic/healthy cats produce antibodies against food proteins. It is important to know whether or not cats produce food specific antibodies and to find out when and why cats are producing them. This because there could be a relationship between the circulating immune complexes and common diseases, for example chronic kidney disease in cats (possible trapping of the immune complexes in renal glomeruli). Also the reliability of serological tests in patients with food allergy needs to be reconsidered, in which they test for food specific IgE and IgG in blood of humans and animals with food allergy, to find the causative allergens/antigens. If also clinically healthy cats produce IgG against food proteins, the usefulness and reliability of these test must be strongly reconsidered.

2.4 Cannabis sativa L. seeds

As a novel protein in this research project, hemp seed flour was used. Hemp seed flour was derived from the seeds of a Cannabis sativa L. plant. This plant is widely used for different purposes, especially as source of industrial fiber, seed oil, food, medicine and psychoactive drugs. Each part of the plant is harvested differently, depending on the purpose of its use. A Cannabis sativa plant contains more than 60 different kinds of phytocannabinoids, the best-studied phytocannabinoid is THC (δ-9-tetrahydrocannabinol), the psychoactive constituent of C. sativa[10]. Cannabis plants are divided into fiber-type and drug-type plants, based on their THC content, when the ratio [THC+CBN]/CBD (in which CBN= cannabinol and CBD= cannabidiol) is less than 1, it is a *fiber-type plant*. When the ratio is greater than 1, the cannabis plant is classified as a *drug-type plant*[11, 12]. THC has especially been found in hemp oil and in the leaves which are commonly used for tea[11]. Hemp, a term for seed and fiber products of different Cannabis plants, is usually bred with low THC content and high CBD content. THC and CBD have a common precursor, so this explains why CBD is mostly present in low-THC plants.
Hemp seeds are an interesting byproduct of commercial utilization of hemp fiber, containing mean(±SD): 30,4$_{(2,7)}$% oil and 24,0$_{(2,1)}$% protein before dehulling and 46,7$_{(5,0)}$% oil and 35,9$_{(3,6)}$ % protein afterwards[13]. Hemp seed protein consists of two main proteins, the storage protein edestin (60-80%) and albumin accounting for the rest[12]. Edestin is an easily digested protein and contains significant amounts of all essential amino acids[12]. Albumin is also highly digestable and functions, like edestin, as a high-quality storage protein.
Because of the high protein content, hemp seed powder (obtained after defatting) is very suitable as a novel protein source for this dietary trial. Hemp seed proteins are also known to be excellent digestible proteins (improved digestibility after dehulling[13]), this because hemp seeds lack the anti-nutritional trypsin-inhibiting factors (factors that bind to trypsin, preventing this enzyme to degradate proteins), that are present in most other vegetable products like soy[12]. Based on this, a greater proportion of proteins can be digested and absorbed through the intestinal tract, only processes like heating could change digestibility, increasing the uptake of the proteins by M-cells possible resulting in higher antibody production against undigestable food proteins.
So hemp seed protein is an interesting protein to use in this research, it is a novel, excellent digestible protein, which could be changed by canning the food.

3. Material and Methods

3.1 Study design

Cats were randomized into two groups of eight cats; each group contained five males and three females (see Appendix 1A). Three weeks before the start of the feeding trial the cats were placed in these groups, to adapt to their environment. They were weighed every week and food intake was measured daily. Cats received ad libitum food, every day the leftovers were weighed and new canned food was given. One group was fed the basal diet mixed with raw hemp seed flour (Diet 1), the other group was fed with the basal diet mixed with the same amount of hemp seed flour and heated/canned after mixing (Diet 2). Blood was collected for serum at day 0, 11 and 25 after the start of the feeding trial. It was taken by jugular vein puncture, permitted to clot around 20˚C, followed by centrifugating with aspiration of the serum. Collected serum was frozen in a -80˚C freezer until analyses.

3.2 Animals and husbandry

The Domestic Shorthair cats in this experiment were part of the Feline Unit from the Massey University, Palmerston North. Age of the cats was between three and ten years at the time of the study. They were exposed to a basal diet of one brand (Chef®) through their entire life's. Through time, different protein sources have been used to manufacture this diet, with flavours as chicken, lamb, beef, jellymeat and fish. The cats used in this study have never shown signs of pruritic skin disease, food allergy or food intolerance and it was also confirmed that the cats have never been in contact with the novel protein (hemp seed flour protein) before. The cats were housed in two separate colony cages (4,5x1,4x2,5m) in an outside courtyard of the Feline Unit. The pens were partially outside and partially covered by a roof. Temperature and light-and-dark cycle were equal to the temperature and day lengths outside (December 2012, summer), wind was blocked by the walls of the Feline Unit house. Water and food were available ad libitum, only food intake was measured daily. Exclusion criteria before and during the study included any sign of systemic illness determined by results of physical examination, complete blood count and serum biochemical analyses.

3.3 Diets

Two canned diets were prepared. One contained the basal diet (Tasty Lamb Classic) mixed with raw hemp seed flour (Diet 1). The other basal diet (Tasty Lamb Classic) was heated to 25-30˚C, mixed with the same amount of raw hemp seed flour and then sterilised to 120.5˚C for 90 minutes (Diet 2).
Preparation of the diets:

- Diet 1: In total 2,5 kg of HSF was mixed in 150 kg of Tasty Lamb Classic canned diet. This mixture was prepared every day in which 69 grams of HSF was added to 4140 gram (6 cans) of Tasty Lamb Classic canned diet, resulting in a HSF/basal canned diet content of 1,64% (69/4209).
- Diet 2: 2,5 kg of HSF was mixed in 150 kg of Tasty Lamb Classic canned diet, followed by heating and sterilisation of the mixture. Each day the cats in group 2 received 4140 grams of this heated diet.

The amount of energy the cats required for their maintenance (MER) was calculated with the following formula:

$$MER = 1{,}4 \times [(\text{body weight in kg})^{0{,}67} \times 293]$$

Body weight of all the cats have been measured every week with a mean body weight of 3,83 kg. MER calculations were performed in advance of the study to assure adequate energy intake. Based on this energy requirements (MER= 1009 kJ/cat; Energy content diet= 2388 kJ/kg), one pen of eight cats should at least be fed with 3,38 kg (4,90 cans) of the diet, to confirm ad lib feeding six cans (4140 gram) were fed to each pen on daily basis.

The canned diets were formulated according to AAFCO standards, containing as-fed 85.8% moisture (14,2% dry matter), 8.0% crude protein (56% on a DM basis), 3.0% crude fat (21% on a DM basis) and 1.0% carbohydrates (12,5% on a DM basis).

Based on rough conclusions of two experiments[2, 9] an antigen level around 500 mg/kg was used in this trial. Ending up with adding 2,5 kg of hemp seed flour to 150 kg of basal diet, giving a hemp seed flour content of 1,64% in both diets. Assuming all the content of six food cans was eaten every day, equally divided over 8 cat per pen, each cat will eat around 450 grams of food per day. Resulting in 7,38 gram hemp seed flour/cat/day and (with a protein content of 30,1% for the hemp seed flour (see Appendix 2A)) 2,22 grams of hemp protein/cat/day. Assumption has been made that around 25% of the present proteins in hemp seed flour is antigenic (based on the two main proteins in hemp), giving 555 mg antigen/cat/day besides the proteins already present in the basal diet (8.0% crude protein).

3.4 Materials

Organic Hemp Protein Powder (defatted hemp seed flour) was ordered at Eternal Delight, New Zealand. Hemp seed flour is a by-product of the isolation process of hemp seed oil. This process of dehulling, disintegrating and defatting was carried out at a low temperature (less than 28˚C), to decrease denaturation of proteins in the powder. The Organic Hemp Protein Powder contains (cited by Eternal Delight): 37% Edestin and Albumin protein, 43% fiber, 0% "net" carbs, 9% beneficial fats and vitamin E and iron[14].

Diet 1, the Tasty Lamb Classic canned diet was obtained from the Heinz Wattie's factory, (Hastings, New Zealand), this was mixed with HSF at the Feline Unit (Massey University Palmerston North). Diet 2 was prepared and heated at the Heinz Wattie's factory, Hastings, New Zealand.

3.5 Test antigens

There are 3 antigens used in the indirect ELISA:

- BDE, a protein extract from the basal diet (Basal Diet Extract)
- HSFE, a protein extract from the hemp seed flour (Hemp Seed Flour Extract)
- HHDE, a protein extract from the second diet in which the diet was heated after mixing with the hemp seed flour. (Heated Hemp Diet Extract)

These proteins were extracted by an adaptation of the method of Pastorello et al. and Calabozo et al. (See Appendix 2B), previously used by N. Cave[2].

3.6 Immunoglobulin determination by indirect ELISA

Indirect ELISA was used to determine the serum IgG antibody titer against three antigens, an extract of the basal diet, called BDE; HSF protein extract (HSFE) and against an extract of Diet 2 (HHDE). First an ELISA titration experiment was performed, followed by ELISA trial and final ELISA experiment.

3.6.1 ELISA Titration experiment with BDE and pooled serum sample:

To limit the amount of ELISA tests with the experimental blood samples from day 0, 11 and 25, a pre-ELISA was performed to determine the optimal primary and secondary antibody concentration. For protocol and ELISA preparations see Appendix 3A and B. Optimal dilution is the concentration which gives the best staining with a minimum of background staining (non-specific binding). A pooled serum sample of the cats was used (taken before start of trial) to determine these optimal concentrations. The best way of defining these dilutions will be using single serum samples of all the cats, to determine for each cat their specific optimal primary and secondary antibody concentration, and also identify the optimal concentration for the different antigens (BDE, HSFE, HHDE). In case of time and money, there was chosen for a pooled serum sample titration experiment with one antigen (basal diet extract). See Appendix 4A for format of the ELISA titration experiment plate.

Based on results of ELISA titration experiment(Appendix 4B), first mean absorbance was calculated of duplicated values of primary and secondary antibody concentration and for the negative control sample (Appendix 4C). To find the right primary antibody concentration the signal:noise ratio was calculated (Appendix 4E) to show the relation between the absorbance of the different sera ("signal") and the mean absorbance of the corresponding negative control ("noise"). The signal:noice ratio shows the compartment of noise in the signal value in which 'noice' is the amount of staining not due to the presence of primary antibodies.

3.6.2 ELISA trial with BDE, HSFE and serum day 0 and 11:

Main goal of this ELISA trial was practice and to optimize results for the final ELISA. Basal diet protein extract (BDE) and hemp seed flour extract (HSFE) have been used as antigens to coat the ELISA wells, individual cat sera of day 0 and 11 were used as (possible) primary antibody source. Appendix 5A for ELISA trial protocol; Appendix 5B,C for formats of the ELISA trial plates.

3.6.3 Final ELISA experiment:

With four ELISA plates left, for the final ELISA HSFE and BDE day 11 serum samples were repeated under new, adapted circumstances (see Discussion) and new serum sample day 25 was used and tested for both antigens. The last antigen, HHDE, was coated on two ELISA plates to investigate individual cat serum antibody level on day 0, 11 and 25. See Appendix 6 A,B for final ELISA protocol and preparations; Appendix 7A-D for formats of the final ELISA plates.

3.7 Statistical analyses

Results of the ELISA trial and final ELISA were corrected for the mean negative control value and then average was taken of the duplicate values. Based on ELISA trial results, adjustments were made for the final ELISA experiment protocol.

For ELISA trial and Final ELISA statistics were performed. Differences between the pens and days of sampling were assessed by use of a paired sample T-test. Paired sample T-test requires a normal distribution of the results, this was confirmed before starting T-test. T-test was performed for both antigens in ELISA trial (BDE and HSFE) to compare OD of Pen 14 day 0 vs day 11, the same was done for pen 15. Finally a comparison was made between the results of Pen 14 and 15 for the two different time samples and antigens. The same was

performed on Final ELISA results, day 11 and 25 for BDE and HSFE were compared for pen 14 and 15 and results of both groups were tested for significant differences between the pens. For HHDE antigen an repeated measures ANOVA was performed to compare the three blood samples (day 0, 11 and 25) for the different pens. ANOVA statistics was assessed to test if there is a difference in the results (absorbance) between the different days of blood sampling ("time effect") and/or between the pens ("pen effect").
The null hypothesis for both paired sample T-test and repeated measures ANOVA was formed and stated that there is no difference between the results of Pen 14 and Pen 15 and also no difference between the different time samples within the pens.
With a confidence interval of 95%, H0 will be rejected if $P < 0,05$.

3.8 SDS Page and Western Blot

Besides an indirect ELISA test, a Western Blot has been performed to look at the effect of heating on different proteins present in the diet. All different protein extracts (BDE, HSFE and HHDE) were diluted in carbonate buffer and mixed with a reducing agent, sample buffer and boiled at 100 degrees for 10 minutes. The different protein extracts were injected in separate lanes of a NuPAGE® 4-12% in a gel tank filled with running buffer. After electrophoresis the proteins run through the gel based on their molecular weight. The gel was stained with Coomassie Brilliant Blue until protein bands were clearly visible. The goal of SDS Page was to see if there are different proteins present in the basal diet extract compared to the heated hemp diet extract, and if the hemp proteins in the HHDE have the same molecular weight as the non-heated hemp proteins (HSFE). This could provide information on a possible heat-related reaction (for example Maillard reaction), which may have caused conformational changes of the food proteins.
After this stained gel another SDS Page was conducted, continued as a Western Blot, to see if the cats produced antibodies against the different proteins in the diets and, in case, which proteins (antigens) triggered the immune system to produce antibodies.
Results of the SDS Page and Western Blot are discussed in the thesis of Jet Kuijers, Utrecht University[15].

4. Results

4.1 Food intake and weight of the cats

All the cats (Appendix 1) completed the study without developing any clinical signs. The food intake was measured per pen on a daily basis, as shown in the graph of food intake/kg cat (*Figure 1*), at 17th of December 2012 (green arrow) the trial started of feeding the two diets to the corresponding pens. At the 16th of January 2013 the trial stopped (red arrow), cats were receiving their previous diets again consisting of basal Tasty Lamb canned diet. As can be concluded from Figure 1 food intake before, during and after the feeding trial showed a large variation. Before the trial the food intake for each pen is almost equal, during the trail pen 15 (heated hemp diet) consistently ate less than pen 14 (basal diet with raw hemp). From 17th of January the food intake of especially pen 15 increased, which may have been caused by the fact that from this day on the cats were fed their previous basal diet again.
All the cats were individually weighed every week (Figure 2 and 3), the weight showed no significant difference during the feeding trial[15].

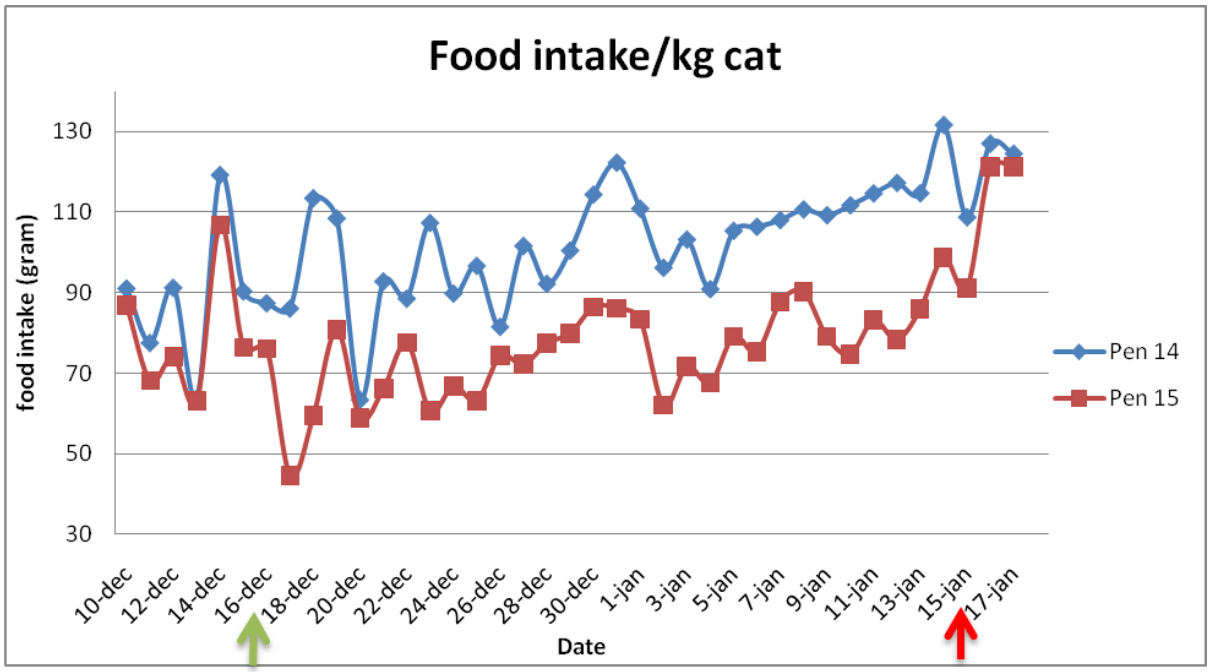


Figure 1 - *Food intake per pen. Green arrow = starting feeding trial; red arrow = stop feeding trial*

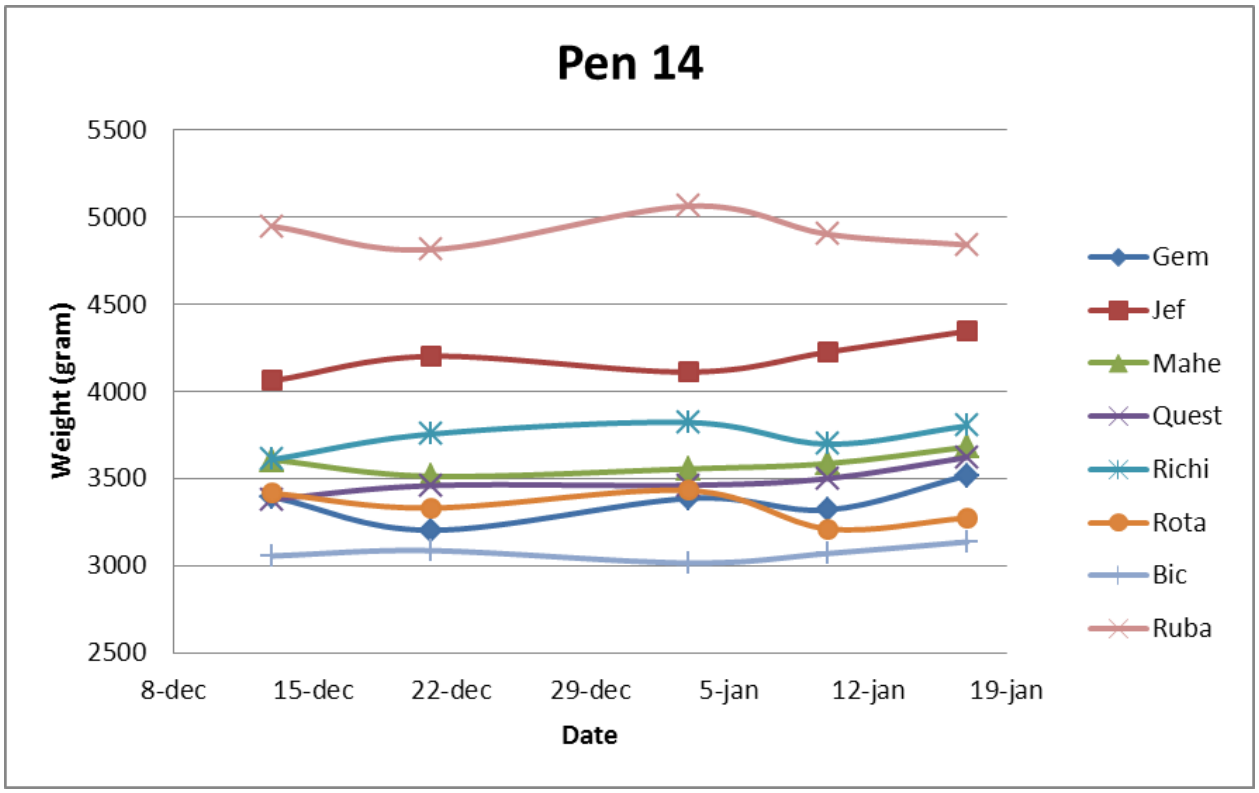


Figure 2 - *Weights of the individuals cats in pen 14 (basal diet mixed with raw hemp seed flour). Start feedings trial on 17th of December 2013. Stop feeding trial on 16th of January 2013*

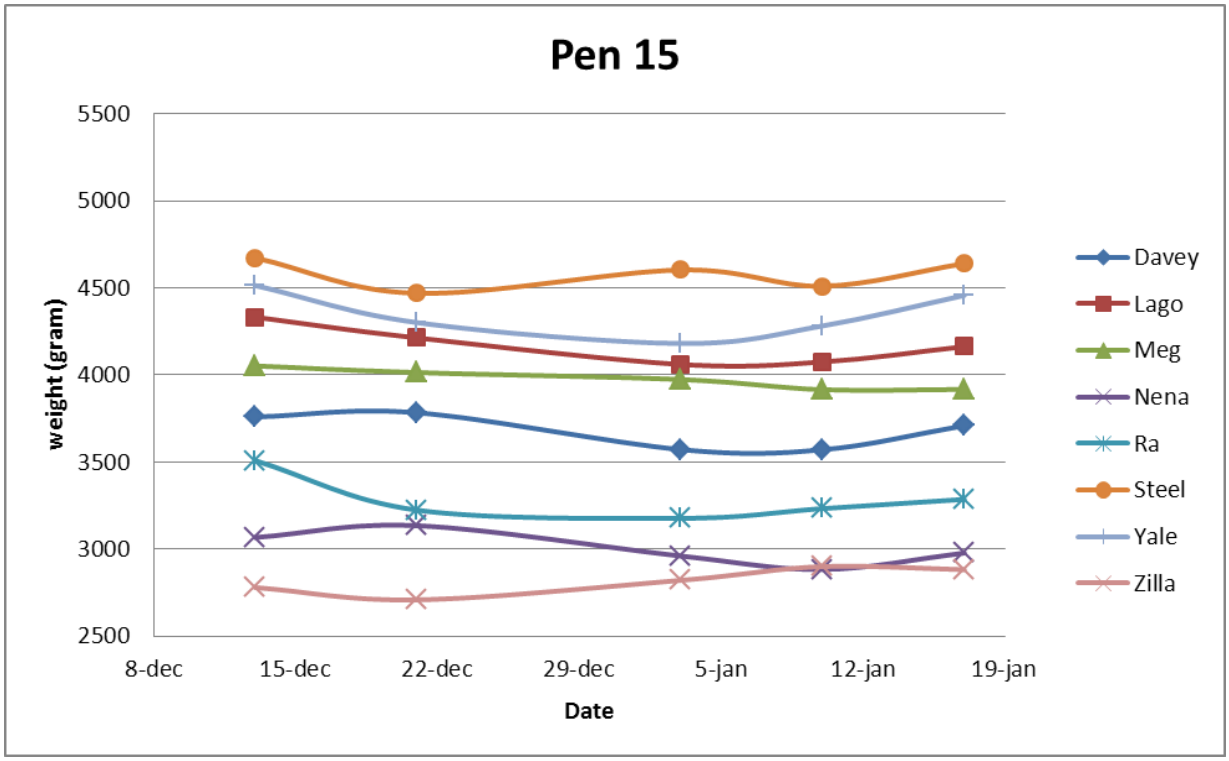


Figure 3 - *Weights of the individuals cats in pen 15 (heated hemp diet). Start feedings trial on 17th of December 2013. Stop feeding trial on 16th of January 2013.*

4.2 ELISA Titration experiment

Secondary antibody concentration:
To make assumptions about the right secondary antibody concentration, a graph was made of the mean absorbance of negative control sample for the different secondary antibody concentrations (Appendix 4D). Steep decrease in absorbance was seen from 1/10.000 to 1/20.000 secondary antibody concentration. At the 1/10.000 concentration the absorbance of the negative control sample is relatively high, so the "noise" for this concentration is high. The noise lowers while diluting the secondary antibody concentration and begins to be at a lower level from 1/20.000 dilution. Lower secondary antibody concentrations than 1/20.000 are not preferred because lower concentrations could increase risk of false negative results, in which there are not enough secondary antibodies present to bind to the primary antibodies in the serum. Because at 1/20.000 secondary antibody concentration the signal is highest compared to the noise, the dilution of 1/20.000 will be used for the indirect ELISA experiments.

Primary antibody concentration:
To choose the optimal primary antibody concentration, the steepest point at 1/20.000 secondary antibody concentration line was used (because this [Sec Ab] will be used in next ELISA experiments) (Appendix 4F). At this (steepest) point a little difference in primary antibody concentration in the serum will give a bigger and clearer difference in absorbance of the graph, so a bigger range between the results. It will be easier to detect differences in absorbance in the upcoming ELISAs when there is a bigger range for the different primary antibody levels of the individual cats.
The steepest point of the 1/20.000 line is around 1:100 primary antibody concentration. More dilute serum samples give a flattened line, primary antibody concentration of 1:100 will be used for next ELISAs to compare the different absorbances of the individual cat sera.

Negative control:
The values of other negative control lanes with no antigen have not been evaluated because not enough blocking buffer was used to prevent attachment of primary antibodies to the well. When no antigen is coated on the wells, more blocking buffer is needed to block the wells otherwise primary and secondary antibodies will attach to the wells, causing false positive, high absorbance values.

Other antigens:
This titration experiment was performed with only basal diet protein extract (BDE) as antigen. In the upcoming ELISAs other antigens (HSFE, HHDE) will be added to have a total of three antigens that will be tested via indirect ELISA. Only BDE was used for the titration experiment because this was the antigen expected to give the lowest antibody concentration in the cats due to oral tolerance. The hemp protein is a novel protein expected to induce higher antibody levels, this will give a higher optical density (OD) in the upcoming ELISAs. With the 1:100 primary antibody dilution in combination with the 1:20.000 secondary antibody concentration, there is enough range left for an increase in OD for the HSFE and HHDE antigens.

4.3 ELISA trial

Results of the paired sample T-test (after confirming a normal distribution for the differences within and between the pens):

BDE		**HSFE**	
Pen 14:		Pen 14:	
Day 11 vs day 0	P = 0,330	Day 11 vs day 0	*P = 0,041*
Pen 15:		Pen 15:	
Day 11 vs day 0	*P = 0,037*	Day 11 vs day 0	P = 0,914
Pen 14 vs Pen 15:		Pen 14 vs Pen 15:	
Day 0	P = 0,599	Day 0	P = 0,916
Day 11	P = 0,489	Day 11	P = 0,090

Based on the confidence interval, H0 was rejected for BDE pen 15 day 11 vs day 0, this implies that there is a difference between the OD of pen 15 for BDE between day 11 and day 0. H0 has also been rejected for HSFE pen 14, day 11 vs. day 0, giving a significant difference between OD at day 11 and day 0.
Graphs were made for BDE and HSFE, with separate curves per pen for absorbance from day 0 to day 11 (Appendix 5F,G). These graphs show, for each antigen, the different absorbances per pen at day 0 and day 11, with SEM (Standard Error of the Mean).
In graphic of BDE (Appendix 5F) is shown that for Pen 15 at day 11 the OD is slightly lower than at day 0, suggesting an (significant) antibody level decrease during this period. For Pen 14 the curve shows a non-significant decrease between day 0 and 11.
Based on calculations there is no significant difference between pen 14 and 15.
The HSFE graph (Appendix 5G) indicates an (significant) increase in OD for pen 14 between day 0 and 11, which points out an increase in antibody level between these days. For pen 15 the absorbance stays at approximately the same level, with no significant difference between day 0 and 11. At day 0 there was no difference visible in the graph between pen 14 and 15, at sample day 11 there is a noticeable difference in absorbance, but not of significant value based on statistics.

4.4 Final ELISA experiment:

With some adaptations of the protocol based on the ELISA trial, the final ELISA was performed with three antigens, BDE, HSFE and HHDE.
After correcting for negative control and calculating the mean of the duplicate value for all the antigens (Appendix 8A-C), graphics were conducted for day 11 and 25 for BDE and HSFE and one graph for the HHDE results of day 0, 11 and 25 (Appendix 8D-F). The graph of BDE shows an increase in absorbance for pen 14 between day 11 and 25. This was confirmed by statistics, giving a significant difference in absorbance for pen 14 between day 11 and 25. Statistics also provoke a rejection of H0 (no difference between data of pen 14 and 15) for BDE based on the P-value day 25 comparing pen 14 and 15.
No significant difference is found for BDE pen 15 between day 11 and 25 serum samples, which is also visible as a relatively flat OD curve.

Statistics (paired sample T-test):

BDE		**HSFE**	
Pen 14:		Pen 14:	
Day 25 vs day 11	*P = 0,018*	Day 25 vs day 11	P = 0,835
Pen 15:		Pen 15:	
Day 25 vs day 11	P = 0,155	Day 25 vs day 11	P = 0,300
Pen 14 vs Pen 15:		Pen 14 vs Pen 15:	
Day 11	P = 0,132	Day 11	P = 0,508
Day 25	*P = 0,001*	Day 25	P = 0,442

For HSFE curves for both pen 14 and 15 stay at approximately the same level between day 11 and 25 serum sample, equal results were found with statistics. Concluding that for HSFE no significant difference is found between day 11 and 25 serum samples for both pen 14 and 15, as well as no difference is found between pen 14 en 15 for the different time serum samples.

HHDE

Results of the repeated measure ANOVA for HHDE were as following:

Time effect = 0,46
Pen effect = 0,387
Interaction Time*Pen = 0,922

Based on the above, no significant difference was found between the different time samples nor between the different pens.
In graph (Appendix 8F) there is a slight increase visible for both pen 14 and 15 between day 0 and 11 and a slight decrease between day 11 and 25, but not of significant value based on statistics.

5. Discussion

5.1 Finding the right protein and concentration of exposure

For this research it was of interest to measure the immune response cats against known and new proteins, and to see if there is a difference in antibody response based on the processing of the diets. As a novel protein there must be chosen for a protein the cats have never been exposed to during their lives, for this Heinz Watties pet food manufacturer was contacted, preparing food for all the colony cats. First, the use of goat milk casein was preferred as a novel food protein, but after some literature research it was found that goat milk casein cross reacts with cow milk casein, so having the same antigens capable of causing antibody responses. Colony cats have been exposed to cow milk casein via their food from Heinz Watties, so there is a change of cross reactivity when using goat milk casein[16].

Second, peanut protein was chosen as novel protein, with Ara h 1 and Ara h 2 as major allergens also known to be more allergenic after heating (roasting) because of Maillard reactions decreasing digestion of the allergens[17]. Unfortunately, Heinz Watties factory did not want to use peanut proteins because of possible cross contamination with human food, also manufactured at the same factory.

Third, using fish extracts like shrimp or mollusc was an interesting choice as novel protein, mainly because of the high protein content in fish. Fish allergy is quite common in which parvalbumins are the major codfish allergens and tropomyosin in crustaceans like shrimp, crabs and molluscs. After some research it was found that both parvalbumins and tropomyosins induce cross reactivity between different fish species[18, 19] and Heinz Watties could not rule out that cats did receive some fish in their diets. Besides, some fish like green lip mussels have immune-modulating effects which is not desirable in this study, looking at immune responses to dietary proteins[20].

In the end, hemp seed protein was used as a novel protein. Important in this protein source is the low THC content, because THC is a phytocannabinoid, which can bind to cannabinoid receptors in both human and animals, having an effect on function of various immune cells and their cytokine production, for example reducing antibody production and B cell proliferation[21]. Besides this well-known immune-modulatory effect of the cannabinoid, cannabidiol (the major nonpsychoactive component of cannabis) has also been found to induce both immunosuppressive and anti-inflammatory effects tested in mice with collagen-induced arthritis[22]. Our hemp seed flour will probably contain mostly cannabidiol because it is made from the seeds of a fibre-type plant, containing low THC. It was thought that hemp seed flour was a good novel protein, because of high protein content and low THC levels, so no immune-modulatory effect. After reading the article of Malfait et al.[22] this idea must be reconsidered, it could be possible that the used hemp seed flour may have immune-modulatory effects like immune suppression or immune-stimulation.

To determine antigen concentration in the diet it is important that it is not to high (possible massive immune responses) nor to low (less likely to cause an immunologic response). Halliwell et al.[9] (as discussed in Introduction) gave 100 mg of HSA (antigen) to each cat on a daily basis, inducing antigen-specific serum IgE, IgG and IgA responses. Cave et al.[2] gave in their research a diet containing 23% of protein added with 2 grams of unprocessed protein/kg body weight/day. All together N. Cave fed significantly more protein to the cats per day compared to Halliwell et al., attended with higher uptake of antigens. Halliwell discussed that their 100 mg HSA might not be enough to develop clinical signs of food allergy in cats[9]. On a contrary, Cave et al. discussed that cats are not used to consuming large amounts of a single protein, which could cause high IgG responses as happened in their research[2]. Based on the

rough conclusions of those articles, an antigen level of around 500 mg/kg cat was used in this trial to feed the cats on a daily basis.

5.2 Allergens vs antigens

Often both terms, allergens and antigens, are used to indicate a substance that is triggering an immune response. However, there is an important difference: an antigen is usually (but not always) a protein and capable of eliciting antibody production. An allergen is more specific, capable of binding to IgE receptor, inducing mast cell degranulation[23]. In this articles hemp seed flour has been used as novel protein source and basal diet extract as not-novel protein source. Serum of the cats were tested for IgG antibodies against three protein extracts and possible antigens, BDE, HSFE and HHDE. Because IgG antibodies are found for all three protein extracts, BDE, HSFE and HHDE can be called 'antigens' but not 'allergens', because cat sera were not tested for IgE antibodies.

5.3 Immunologic reactions

The reason why some mammals will develop an allergic, IgE-mediated, reaction to specific proteins and others will not, is based on the epitopes the IgE of that mammal can bind to. Mast cells are present in different tissues throughout the body, especially in association with structures such as blood vessels, nerves and surfaces in contact with the external environment[24]. They have IgE receptors on their surface which can bind to a specific epitope. If this epitope is, for example, part of a peanut protein, the mammal will develop an immediate allergic reaction after contact with this specific peanut protein (after eating or contact with external surfaces). After activation of the IgE receptors the mast cells will degranulate, which will trigger the release of different cytokines like histamine. So it depends on the matching IgE to which epitope the organism will develop an allergic response.

This is almost the same for the development of an IgG response against certain antigens in an organism, except in this case no mast cells are involved. To induce a humoral immune response against a certain peptide, there has to be a MHC-II receptor that can specifically bind to this peptide. Genetic material of an organism determines to which peptide a MHC-II receptor can bind, although there is a rich polymorphism of the MHC region to maximize protection of species against diverse micro-organisms.
Antigen Presenting Cells (APC) take up antigens out of the blood stream or lymph circulation, they process the antigen into small particles, called peptides. If the MHC-II receptors of the APC recognize one of these peptides, the MHC-II receptor will bind this peptide and will present it on the surface of the APC. After this, the APC will go to a regional lymph node where it will get in contact with a matching mature, naïve T-lymphocyte. When one of those T-lymphocytes recognizes the antigen presented by the APC, the T-lymphocyte will become activated and will cause cell death of the APC (in case of CD8 T-cell) or will produce cytokines and cause activation of B-cells (in case of a CD4 T-cell). What type of cytokines will be produced is dependent of the antigen causing this immune reaction, different cytokines stimulate the production of different immunoglobulins[25].
In this ELISA experiment goat anti-cat IgG has been used as secondary antibody to detect primarily or exclusively IgG antibodies in the cats serum. IgG, as well as IgA, are not necessarily associated with adverse food reactions, presence of those food specific antibodies are thought to reflect previous food exposure[9].

5.4 M-cells and undigestable proteins

M-cells are special epithelial cells in the ileum, through which antigens can pass into the gut-associated lymphoid tissues. M-cells control the feces for proteins that are still present in the intestinal content in ileum. Normally, most proteins are already absorbed before feces reaches the ileum, to be used as a nutrient. Proteins, that are still present in the feces when they enter the ileum, are mostly micro-organisms. M-cells take up these micro-organisms and scan what kind of micro-organisms they are and if they are harmful. The micro-organisms will be digested by APC's and matching T-cells will be found, this will promote the production of T-helper cells. Those T-helper cells will be activated based on repeatable exposure to those antigens/micro-organisms.

When a diet becomes less digestible (for example because of Maillard reaction), those less digestible compounds will enter the ileum. M-cells absorb those less digestible proteins and matching T-cells will be found. T-cells are not activated yet, so not causing any cytotoxic reaction nor producing antibodies via stimulation of B-cells. But when there will be some antigen triggering a reaction in the gut, the cytokines that are produced can also trigger the inactive diet-protein matching T-cells, this T-cells will trigger B-cells and cause production of antibodies against food proteins. In this way the production of IgG against food proteins is more likely to occur for less digestible proteins, because highly digestible proteins will be absorbed before entering last part of the intestinal tract where M-cells are located.

With this information, processing food could trigger Maillard reaction, giving rise to melanoidins (discussed in Introduction)[2, 7, 8]. These melanoidins could be involved in increasing immunologic food reactions in cats, because they lower digestibility, possible of triggering antibody production. Especially cats are susceptible for low digestible diets because they have a relatively short intestinal tract, suitable for highly digestible dietary proteins[2].

Increasing immunologic food reactions based on heating/processing of diets results in the hypothesis that antibody production against BDE and HHDE will be higher compared to HSFE. Unfortunately, based on this results no conclusions can be made about the amount of antibodies present against different antigens because different primary and secondary antibody concentrations have been used (will be discussed in 5.6). Conclusions can only be drawn about the course of antibody concentration during time.

5.5 Oral tolerance

The results of this study are in contrast with the hypothesis of oral tolerance because food-specific antibodies were found in clinically normal cats. A possible reason for the presence of (high levels of) antibodies against normal, previously exposed dietary proteins is a decrease in the development of oral tolerance in the cats.

The lack of oral tolerance may be due to age of the host, genetics, normal flora (neonates have stronger immunologic reactions), incorrect antigen concentration (as discussed above) or wrong form of ingested antigen[26]. Research in others species revealed that in chickens oral tolerance development is dependent of the physical form of the proteins at the moment of ingestion, when proteins are solid (non-liquid) they develop lower food-specific antibody levels, so inducing oral-tolerance. Otherwise when soluble proteins have a quicker passage through the intestines leading to less enzymatic digestion, giving higher molecular weight peptides in ileum and large intestine. These larger peptides would stimulate antibody production under inflammatory conditions, in which development of oral-tolerance was prevented[26].

Another possibility of preventing oral tolerance is when antigens are encapsulated which protects them from acid and enzymatic breakdown keeping the antigen intact until last part of the intestines, capable of inducing an immune response[26].

Concerning antigen concentration there are two forms of inducing oral tolerance based on the antigen dose, called high-dose and low-dose tolerance. The low-dose tolerance is mediated by suppressor CD8+ T-cells and regulatory CD4+ T-cells[26]. High-dose tolerance is induced by lymphocyte anergy, which can occur when T-cell receptors do bind but no co-stimulatory signals are present (normally produced by cytokines or interaction between T-cell receptors and APC)[26].
Another reason for not developing oral tolerance may be because of parasite infection like Toxocara cati as suggested in research by Halliwell et al[9]. As discussed in the introduction, Halliwell investigated the antibody responses to HSA administered with food to two groups of kittens, one T. cati positive and the other group was parasite-free. Halliwell et al. suggested that the increased IgE response in T. cati positive cats could be T. cati preventing the development of oral tolerance. Instead T. cati could cause activation of CD4+ cells leading to induction of helper and memory cells. Another hypothesis Halliwell proposed is that an increase of HSA-specific IgG in parasitized cats reflect the increased permeability of the intestinal mucosa because of the helminth infection, leading to an increased antigen exposure. The cats used for the hemp feeding trial were not infected with T. cati, based on fecal parasitological examination, also there is no reason to suggest that the intestines of the cats were more permeable for substances like proteins, because no clinical signs like diarrhea were present before, during or after the study. The antigens were already present (BDE) or have been mixed (HSFE, HHDE) with the diets of the cats. Antigens were given as particles mixed in wet diet, which is thought to pass the intestines more quickly because of soluble basis. This could lower the induction of oral tolerance, corresponding to high antibody responses that have been found in this research.

5.6 Analysing the results

5.6.1 Food intake and weight of the cats

Based on food intake graph (Results Figure 1), food intake of pen 14 is higher than intake of pen 15. This could be caused by the taste of the diets, heating of the hemp seed flour possibly changed and negatively influenced the taste of basal canned diet. Although before starting this feeding trial there was already a difference in food intake between the pens, consisting of a higher intake for pen 14. So whether the higher food intake of pen 14 during the trial is due to palatability or based on a consistent difference between the pens (possibly due to the individual cats in the pen) is not certain. An argument for the change in palatability is the rapid increase in food intake, of especially pen 15, after stopping the dietary trial (red arrow) and changing the food back to basal Tasty Lamb canned diet again. Assuming that they like the basal diet far more that heated hemp diet, so heating the mixture of hemp and basal diet is supposed to decrease palatability of the diet.
For the weight of the cats no significant difference was found, also visible in the graphs (Results Figure 2,3) as relatively flat weight lines during the period of the 13th of December 2012 until 17th of January 2013.

5.6.2 ELISA trial:

Adaptations for Final ELISA:

Primary antibody concentration

Compared to the pooled serum sample for the ELISA titration experiments, the cats used for this ELISA trial had a higher OD, a higher antibody titer at day 0. This may be due to the high background noise, but also because of the primary antibody dilution. Primary antibody

dilution of 1:100 has been used based on the results of the ELISA titration experiment. This ELISA trial showed that the OD is much higher for the basal diet extract (OD=1,5) and HSFE (OD=2,0). To create a bigger range for a possible increase in OD in the final ELISA trial, primary antibody concentration was decreased from 1:100 to 1:200, to decrease the antibody attachment, lowering the OD. Hopefully more difference can be seen with comparing the ODs of the different cat serum samples and also specificity increases.

Negative control (no serum)
The OD of the negative control is relatively high, there is a lot of background noise which decreases the specificity of this trial, increasing the risk of false positive results. This may be due to the serum incubation time, if incubation time is too long the primary antibodies can cause detachment of the antigen from the ELISA wells, the primary antibodies from the serum may attach to the empty places in the wells and will cause false positive results. The high level of background noise may also be due to the blocking of the ELISA wells, when the amount of blockings solution or the blocking time is not enough, the primary or secondary antibodies are able to attach to the ELISA wells, causing false positive results.
Based on these findings the ELISA protocol was adjusted, decreasing the serum incubation time from 2 hours to 1 hour and increasing the blocking time from 1 hour to 2 hours. The volume of blocking solution did not alter, 150 µl was estimated to be enough to block the wells because 100 µl is the biggest volume added to the wells.

5.6.3 Overall ELISA experiments:
Based on overall ELISA results, first the possibilities of false positive results must be discussed. All ELISA experiments gave relatively high negative control samples and also positive OD results for HSFE on day 0 were not expected, because this must be a novel protein.
The results the ELISA trial suggest that the cats, of both pen 14 and 15 did have antibodies against HSFE, on day 0 as well as day 11 and 25. A theoretical possibility is that the cats have been in contact with hemp before, but this is very unlikely because all the cats were born in the Feline Unit and food intake have always been controlled. The high OD at day 0 can also be caused by cross-reactivity of the cat antibodies to parts of the hemp protein that are conformational the same as other previously exposed antigens for which the cats have matching MHC-complexes and T-cel Receptors. Implying that there is a cross-reactivity of hemp seed flour extract and another not-novel antigen.
The high HSFE OD at day 0, but also relatively high negative control values, could also be explained with a fault in protein extraction method giving a non-representative antigen extract to which cat antibodies bind giving a possible false positive result.
Other possibilities for false positive results are that the antigens (BDE, HSFE and/or HHFDE) contained particles that were too big to coat the ELISA plate. Those particles can cluster and block the ELISA wells. Because of the size of these clusters they detach easily, when this happens the ELISA plate contains empty places (uncoated places) to which primary and secondary antibodies can bind. To prevent the clustering, the final ELISA protocol was adjusted by centrifuging the antigen solution before adding to the wells. This to remove the big clusters from the solution, to provide nicely coated ELISA wells. Although, no difference is seen in negative control value between ELISA trial en Final ELISA, probably only centrifuging is not enough to remove the bigger particles prone to clustering.
Another reason for the high background noise, which could also explain high HSFE OD at day 0, is the possibility of primary antibodies binding to blocking buffer instead of antigens; secondary antibodies binding to antigen, blocking buffer and/or binding to the wall of ELISA

wells. However, in ELISA Titration Experiment (Appendix 4C) there can be seen that (for a certain secondary antibody concentration) absorbance decreases when primary antibody concentration declines, meaning that the secondary antibody binding is (also) dependent of the amount of primary antibodies present. If the secondary antibodies were not binding to the primary antibodies at all, absorbances are expected to stay at approximately the same level, independent of changing primary antibody concentration.
Taken this into account, it could be possible that part of the absorbance value is due to false positive results, based on high negative control values and positive results at especially day 0 for HSFE, but positive OD values are also due to the desired binding of secondary to primary antibodies present in cats serum.

Negative antigen samples:
In Final ELISA negative antigen samples have been performed, giving very variable absorbances. These results have not been used because high absorbances can be due to lack of blocking buffer. Normally blocking buffer blocks the spare places in the wells that have not been coated with antigen. When no antigen is present, more blocking buffer should be added otherwise there are not enough blocking proteins to block the ELISA wells. This gives primary and secondary antibodies space to bind to ELISA well giving false positive results. These negative antigen samples were performed with the idea to conclude something about false positive results due to binding of primary or secondary antibodies to blocking buffer or wells instead of binding to antigen. Based on above information about not using enough blocking buffer for antigen negative wells, negative antigen results were ignored.

No control group:
No separate control group was used, receiving only basal diet and testing for all tree antigens. At the moment a separate control group was not considered as necessary, as the bleeding prior to oral sensitisation served as a negative control.

5.6.4 ELISA trial and Final ELISA discussion per antigen:
The ELISA trial (day 0 and 11) and Final ELISA (day 11 and 25) graphs of BDE and HSFE could not be combined because ELISA trial and Final ELISA were carried out under different circumstances. For example ELISA trial serum incubation time was 2h, blocking time 1h and primary Ig concentration was 1:100. In Final ELISA these values were changed based on the results of ELISA trial: serum incubation time is 1h, blocking time increased to 2h and primary Ig concentration of 1:200. Besides these adaptation the ELISA trial was performed on a different day as the final ELISA, so environmental factors could also influence results. Also practical skills of performing ELISAs was better during final ELISA. All these reasons combined led to the decision of making separate graphs per antigen for day 0 and 11 (Appendix 5F,G) and a graph for BDE and HSFE for day 11 and 25 under Final ELISA circumstances (Appendix 8D,E).

5.6.4.1 Basal Diet Extract:
Before starting the diet, both pens already had high antibody titers against the BDE. This could suggest that no oral tolerance is present which would reduce antibody production against commonly exposed dietary proteins like BDE. Although, these results could also be explained by false positive results based on errors in ELISA experiment as discussed above.
For pen 14, receiving this basal diet mixed with raw HSF, no significant difference was seen between day 0 and 11 suggesting that raw HSF did not influence antibody production against BDE causing significant difference during that period. Between day 11 and 25 a significant

increase was found in BDE antibody titer for pen 14, this will be discussed underneath. For pen 15 antibody titer against BDE decreased between day 0 and 11 in ELISA trial, in Final ELISA the BDE IgG titer between day 11 and 25 did not differ.

ELISA trial - Significant antibody level decrease for BDE pen 15 between day 0 and 11:
Reliability of these results are questionable because of high noise value on ELISA plates, as discussed above. But in case results are (partly) reliable, the following explanations can be given based on statistical significant differences (corrected for negative control).

Pen 15 was given the Heated Hemp Diet (HHD), so the HSF was first mixed with the raw basal diet and then heated together. An hypothesis for the significant decrease in antibody level of pen 15 for BDE is that heating HSF has an influence on the BDE component of the basal diet. So during heating the HSF causes, for example, an conformational change of the BDE antigen, in time decreasing the antibody production against the original BDE antigen. Another possibility is that the heated hemp seed flour component contains substances that lower antibody responses against proteins, this could explain the significant decrease in antibody production of pen 15 against BDE and also for other antigens that will be discussed next.
Suggesting that heated hemp seed flour has an immune-modulating effect which is expected to come from the higher CBD/THC ratio of our Organic Hemp Protein Powder (stated by Eternal Delight, no THC is present in the Hemp Powder).
Research of Eichler et al.[27] consisted of measuring plasma concentration after oral administration of heated (140°C for 12 min) or unheated Cannabis extracts. Both heated and unheated extracts contained approximately the same amount of total THC and CBD, but median CBD plasma concentration was almost 2-fold higher after oral administration of unheated compared to the heated extract[27]. This implies that the unheated version of Cannabis extracts provide a higher plasma CBD concentration (so possible higher immune modulating effect) and inhibits the uptake of THC through changes in absorption, metabolic activity or elimination processes.
Suggesting these results would apply for our research, one would expect to have higher CBD levels in pen 14, receiving the raw/unheated hemp seed flour, and lower CBD levels in pen 15 receiving the heated hemp diet.
In a review article of Croxford et al.[21], most articles found an immune suppressive effect of cannabinoids, resulting in a decrease in antibody production and B cell proliferation. However there are some articles published about immune stimulating effects of cannabidiol, like enhancing cytokine production under some circumstances depending on age, cel type or type and/or magnitude of cellular activation[28]. This matches with our results, in this case: pen 15 receiving the heated hemp, therefore having a lower CBD plasma concentration[27], less influenced by the immune stimulating effect of CBD, resulting in lower IgG serum concentrations against the food antigens (BDE in this case).

Final ELISA - Significant antibody level increase for BDE pen 14 between day 11 and 25:

Interpreting the results (in case they are valid), they are corresponding with the theory discussed above. Pen 14 was receiving the basal diet mixed with unheated hemp protein, giving possible higher CBD plasma levels. If CBD has an immune stimulating effect this could explain the increase in IgG in pen 14 between day 11 and 25 in which CBD is promoting antibody production against food antigens like BDE. Why CBD could have immune suppressive and immune stimulating effects is not completely clear, but it is thought that the amount of antigen, antigen concentration and way of exposure influences the immune modulating effect[27].

Final ELISA - Significant difference in BDE antibody level between pen 14 and 15 at day 25:

Based on graph (Appendix 8D) and calculations there is a significant difference for BDE between pen 14 and 15 at day 25. This could be the result of the diets pens were receiving, pen 14 received the plain BDE, without processing, but with substitution of HSFE (unprocessed). But reliability of results is uncertain because of high background noise. If an explanation must be given, in this case it could also be the cannabidiol in the HSF that stimulated immune reaction against BDE, an antigen/protein extract present in this form in the diet of pen 14. Pen 15 received the heated version of BDE mixed with HSFE, it could be possible that heated HSFE does not give a plasma CBD concentration high enough to trigger this immune stimulating effect, so antibodies against BDE stay at the same level in pen 15, creating a significant difference between pen 14 (expressing the immune stimulating effect) and pen 15 at day 25 of feeding.

5.6.4.2 Hemp Seed Flour Extract

For HSFE no significant difference was found for pen 15 between day 0 and 11 in ELISA trial, nor between day 11 and 25 in Final ELISA. For pen 14 an increase in IgG was seen between day 0 and 11 (discussed underneath), but no difference between day 11 and 25 in Final ELISA. Following assumptions can be made in case ELISA results are valid.

ELISA trial - Significant increase in antibody level for HSFE pen 14 between day 0 and 11:

Pen 14 was given the basal diet mixed with the raw hemp seed flour, so this is the only pen given the raw hemp seed flour. The fact that pen 14 has a significant difference between day 0 and 11, and pen 15 not, indicating that there would be a conformational change of the hemp seed flour proteins during heating because otherwise you would expect an increase for pen 15 on the HSFE as well. So this is in accordance with the hypotheses that heating could change the conformation of the proteins in a way this could change antigenity as well. It could also be that CBD stimulates IgG production against HSFE, like what is suspected to have happened for BDE.

Final ELISA - No significant difference between pen 14 and 15 at day 11 and 25:

No differences were found for HSFE between day 11 and 25 in both pen 14 and 15. The possible immune stimulating effect of CBD in pen 14 is not visible in Final ELISA results for HSFE.

5.6.4.3 Heated Hemp Diet Extract

If no conformational changes take place during the heating of basal diet with raw hemp mixture, you would expect that the same amount of antibodies that did bind to BDE would also bind to this HHDE plus extra IgG binding to the heated hemp component of the HHDE antigen. Antibody levels of both pen 14 and 15 (at day 11 and 25 of Final ELISA) are lower for HHDE than for BDE, suggesting that BDE binding antibodies do not bind to the HHDE. This could be explained by the change in conformation of the antigenic part of the basal diet antigenic proteins when it is heated together with hemp. So in this case it is likely to assume that heating causes conformational changes which lowers antibody production, reducing antigenic component of the food proteins or inducing oral tolerance.

Because statistics result in no difference between pen 14 and 15 at all different serum samples, suggest that the HHDE antigenic component the antibodies are binding to is irrelevant to the diet the cats are receiving. HHDE antigen is both present in the diet of pen 14 and 15 and is not influenced by raw or heated hemp. The HHDE antibody levels do not match

with the BDE antibody levels (as discussed above) neither HHDE antibody levels matches with antibody level against HSFE (day 11 and 25, Final ELISA), so HHDE is a new antigenic protein independent of the processing of hemp seed flour.

5.7 Why antibody production against food proteins?

If the responses found in our research are normal, the next question raises: Why would cats develop such an antibody response to daily presented food proteins? To answer to this question is mostly unclear, but there are some speculations. As discussed in Introduction, antibodies could be useful for removing of circulation dietary antigens, by phagocytosis or opsonization. However, due to common antigen exposure through the diet, cats will produce antibody-antigen complexes based on repeated challenge. These antibody-antigen complexes could also cause problems like antibody-mediated diseases, when they got stuck in the kidney (glomerulonephritis), joints (polyarthritis) or causing thrombocytopenia[2]. Nevertheless, these diseases are not very common in general cat population, indicating that or antigen-antibody complex forming will not occur in excessive amounts or formed complexes will not get stuck in the organs because of quick break down of complexes. However chronic interstitial nephritis is a common disease in cats, in which antibody-antigen complexes could play a role in damaging renal tubuli[2]. Further research needs to be done to see if there really is a relation between prevalence of chronic interstitial nephritis and antibody production against harmless substances like food proteins. What we did prove in this research is that cats do produce antibodies against food protein, despite the fact that we could not see a difference in antibody production between novel proteins or previous exposed ones. Based on these results it is worth reflecting the performance of some clinical tests like measuring antibody levels in cats with food allergies. Results of these tests are not reliable because healthy, non-symptomatic cats also produce antibodies against commonly and new dietary proteins, confirmed by Halliwell et al[9], previous[2] and current research of N. Cave. More research needs to be done about production of immunologic reaction to dietary proteins in cats, but also dietary immunologic reaction in other animals, like dogs, should be investigated.

6. Conclusion

It is difficult to make conclusions about the study presented in this paper. High antibody titers have been found for all the antigens, but negative control values were also relatively high. The high OD of HSFE at day 0 is contradicting, because the cats have never been exposed to hemp proteins before. Possible explanations for the false positive results are mentioned, like inconsistencies in performing indirect ELISAs or in protein extraction method, giving possible cluster forming of the antigens resulting in false binding of primary and secondary antibodies to the ELISA well. To prevent attachment of antibodies to the wells ELISA protocol must be improved, changing the antigen solution and coating and blocking solution, volume and/or blocking time. Another possibility is cross-reactivity of HSF with another, known antigen which would explain high OD for HSFE day 0 (both pens).
Although, in ELISA Titration Experiment an decrease in absorbance can be seen when primary antibody concentration declines, suggesting that there is also some desired binding of secondary antibodies to primary serum IgG.
With a cautious conclusion that the results are partly based on the desired indirect ELISA binding and partly based on false positive results, some suggestions were made based on the significant differences in absorbance. Results indicate that cats do produce robust serum IgG responses against dietary presented proteins, whether novel or previously exposed, heated or unheated. This in contrast with the expected oral tolerance for the BDE antigen. Also seen in results is the consistent higher OD of pen 14 compared to pen 15, although only at one point significant (BDE day 25). A theoretical possibility for this increase is that unheated hemp could provide higher CBD (cannabidiol) plasma concentrations compared to heated hemp. In some literature CBD has immunosuppressive effects and others found immune stimulating effects. Last one would be likely in this study in which pen 14, exposed to the unheated hemp diet, gives higher OD results than pen 15.
Coming to the conclusion that further research needs to be done, to optimize ELISA results and look for more confirmation and clarification for the antibody production against dietary proteins in cats and the possible effects of CBD on immune system.

7. Acknowledgements

I would like to thank some people who made it possible for me to perform and finish this research project, an amazing experience I will never forget.
First of all I would like to thank Jet Kuijers, a co-student and friend who wanted to travel with me to the other side of the world, going away from home for four month, diving into a new experience. She supported me in difficult times and always took time to explain things when needed. A new friendship which will be continued back home, crowned with a tandem bungee-jump from a 47 meter high Kawarau bridge in Queenstown!
Second I want to thank Nick Cave who invited me and Jet to come to New Zealand and providing us all the information, knowledge and enthusiasm needed to perform this project. Karin Weidgraaf and Margreet Hekman, two Dutch vet technicians working at the Feline Unit (Massey University, Palmerston North), were always there for us. They helped us a lot with the practical parts of the research like taking blood from the cats, preparing the diets, collecting all the instruments needed for ELISA and Western Blot and they were always in for a chat, gave us a lot of travel information and let us experience and taste some typical New Zealand traditions like celebrating Christmas and barbecuing.
Last but not least, I would like to thank my Dutch supervisor, Esther Plantinga, who gave me a lot of positive feedback and motivation to write this paper and helped a lot with performing statistics on the results of this experiment. She supported me and gave me some extra time needed to finish this project. A project I will never forget, I learned a lot and made it easier for me to read and understand articles. I even start to like doing research, learning more about a typical subject, although this is just a little taste of doing research.
For me, an amazing experience on the beautiful islands of New Zealand!

8. References

1. Guilford WG, Jones BR, Markwell PJ, et al. Food Sensitivity in Cats with Chronic Idiopathic Gastrointestinal Problems. J Vet Intern Med 2001;15:7-13.

2. Cave NJ, Marks SL. Evaluation of the Immunogenicity of Dietary Proteins in Cats and the Influence of the Canning Process. Am J Vet Res 2004;65:1427-1433.

3. Veenhof E. Immune Responses in Dogs with Cutaneous Adverse Food Reactions. 2012.

4. Gaschen FP, Merchant SR. Adverse Food Reactions in Dogs and Cats. Vet Clin North Am Small Anim Pract 2011;41:361-379.

5. Chehade M, Mayer L. Oral Tolerance and its Relation to Food Hypersensitivities. J Allergy Clin Immunol 2005;115:3-12; quiz 13.

6. Furness JB, Kunze WA, Clerc N. Nutrient Tasting and Signaling Mechanisms in the Gut. II. the Intestine as a Sensory Organ: Neural, Endocrine, and Immune Responses. Am J Physiol 1999;277:G922-8.

7. Hayase F, Usui T, Watanabe H. Chemistry and some Biological Effects of Model Melanoidins and Pigments as Maillard Intermediates. Mol Nutr Food Res 2006;50:1171-1179.

8. Kim SW, Rogers QR, Morris JG. Maillard Reaction Products in Purified Diets Induce Taurine Depletion in Cats which is Reversed by Antibiotics. J Nutr 1996;126:195-201.

9. Gilbert S, Halliwell RE. The Effects of Endoparasitism on the Immune Response to Orally Administered Antigen in Cats. Vet Immunol Immunopathol 2005;106:113-120.

10. Capasso R, Borrelli F, Aviello G, et al. Cannabidiol, Extracted from Cannabis Sativa, Selectively Inhibits Inflammatory Hypermotility in Mice. Br J Pharmacol 2008;154:1001-1008.

11. Lachenmeier DW, Kroener L, Musshoff F, Madea B. Determination of Cannabinoids in Hemp Food Products by use of Headspace Solid-Phase Microextraction and Gas Chromatography-Mass Spectrometry. Anal Bioanal Chem 2004;378:183-189.

12. Park SK, Seo JB, Lee MY. Proteomic Profiling of Hempseed Proteins from Cheungsam. Biochim Biophys Acta 2012;1824:374-382.

13. House JD, Neufeld J, Leson G. Evaluating the Quality of Protein from Hemp Seed (Cannabis Sativa L.) Products through the use of the Protein Digestibility-Corrected Amino Acid Score Method. J Agric Food Chem 2010;58:11801-11807.

14. **Eternal Delight New Zealand**
Organic Hemp Protein Powder
Http://www.Eternaldelight.Co.nz/2012/01/hemp/ .

15. Kuijers J. **Immunological Respons to Dietary Proteins in Cats.**
SDS Page and Western Blot. *Thesis of Utrecht University*. 2013.

16. Sanz Ceballos L, Sanz Sampelayo MR, Gil Extremera F, Rodriguez Osorio M. Evaluation of the Allergenicity of Goat Milk, Cow Milk, and their Lactosera in a Guinea Pig Model. J Dairy Sci 2009;92:837-846.

17. Maleki SJ, Chung SY, Champagne ET, Raufman JP. The Effects of Roasting on the Allergenic Properties of Peanut Proteins. J Allergy Clin Immunol 2000;106:763-768.

18. Taylor SL. Molluscan Shellfish Allergy. Adv Food Nutr Res 2008;54:139-177.

19. Kuehn A, Scheuermann T, Hilger C, Hentges F. Important Variations in Parvalbumin Content in Common Fish Species: A Factor Possibly Contributing to Variable Allergenicity. Int Arch Allergy Immunol 2010;153:359-366.

20. Brien S, Prescott P, Coghlan B, Bashir N, Lewith G. Systematic Review of the Nutritional Supplement Perna Canaliculus (Green-Lipped Mussel) in the Treatment of Osteoarthritis. QJM 2008;101:167-179.

21. Croxford JL, Yamamura T. Cannabinoids and the Immune System: Potential for the Treatment of Inflammatory Diseases? J Neuroimmunol 2005;166:3-18.

22. Malfait AM, Gallily R, Sumariwalla PF, et al. The Nonpsychoactive Cannabis Constituent Cannabidiol is an Oral Anti-Arthritic Therapeutic in Murine Collagen-Induced Arthritis. Proc Natl Acad Sci U S A 2000;97:9561-9566.

23. Cave NJ. Hydrolyzed Protein Diets for Dogs and Cats. Vet Clin North Am Small Anim Pract 2006;36:1251-68, vi.

24. Metcalfe DD, Baram D, Mekori YA. Mast Cells. Physiol Rev 1997;77:1033-1079.

25. Janeway CA, Travers P, Walport M, Shlomchik MJ. Humoral Immune Response. In: Immunobiology, the immune system in health and disease. 6th ed. ; 2005:367.

26. Klipper E, Sklan D, Friedman A. Response, Tolerance and Ignorance Following Oral Exposure to a Single Dietary Protein Antigen in Gallus Domesticus. Vaccine 2001;19:2890-2897.

27. Eichler M, Spinedi L, Unfer-Grauwiler S, et al. Heat Exposure of Cannabis Sativa Extracts Affects the Pharmacokinetic and Metabolic Profile in Healthy Male Subjects. Planta Med 2012;78:686-691.

28. Karmaus PW, Wagner JG, Harkema JR, Kaminski NE, Kaplan BL. Cannabidiol (CBD) Enhances Lipopolysaccharide (LPS)-Induced Pulmonary Inflammation in C57BL/6 Mice. J Immunotoxicol 2012.

Appendix

Page

Abbreviations: ***HSF****, Hemp Seed Flour;* ***HSFP****, Hemp Seed Flour Protein;* ***BDE****, Basal Diet Extract;* ***HSFE****, Hemp Seed Flour Extract;* ***HHDE****, Heated Hemp Diet Extract;* ***2-ME****, 2-mercaptoethanol;* ***PBS****, Phosphate buffered saline;* ***PBST****, Phosphate buffered saline with Tween 20;* ***ELISA****, Enzyme-Linked Immunosorbent Assay;* ***HSA****, Human Serum Albumin;* ***[Prim Ab]****, Primary Antibody Concentration;* ***[Sec Ab]****, Secondary Antibody Concentration..*

Appendix 1

Format of the pens: number, name, gender and age of the cats.

Pen 14			
Number	**Name**	**Gender**	**Age**
1	Bic	Female	7 years
2	Gem	Female	7 years
3	Jef	Male	8 years
4	Mahe	Male	3 years
5	Quest	Male	8 years
6	Richi	Male	6 years
7	Rota	Female	8 years
8	Ruba	Male	11 years

Pen 15			
Number	**Name**	**Gender**	**birth date**
9	Davey	Male	3 years
10	Lago	Male	7 years
11	Meg	Female	8 years
12	Nena	Female	8 years
13	Ra	Male	8 years
14	Steel	Male	11 years
15	Yale	Male	6 years
16	Zilla	female	6 years

Appendix 2

A) *Eternal Delight product information about Hemp protein powder (hemp seed flour)*

Hemp protein powder

Product Name: Hemp protein powder
Botanical Name: Cannabis Sativa
Extraction Method: Cold Pressed and Cold Processed (Milled)
Appearance: A dark green to brown ground flour with characteristic taste and odor
Shelf life: If stored in its original packaging at less than 20°C, this product can expect to have a shelf life of 12 months.
Common Dosage: Functional food ingredient
Product Variety: Hemp Seed Flour cold pressed organic Hemp protein powder is a byproduct of THC free Hemp Seed (Cannabis Sativa) after the oil has been extracted. This fibre originates in a pellet form which is commonly referred to as Hemp Cake and is then further milled (Cold Processed) to produce the final fibre product (Flour). Hemp Seed Flour contains protein and high levels of dietary fibre both soluble and insoluble. In addition, the oil component provides one of the richest sources of Essential Fatty Acids (EFA's) Linoleic Acid (Omega 6, LA) and Alpha Linolenic Acid (Omega 3, ALA) as well as smaller amounts of Gamma Linolenic Acid (GLA), and Stearidonic Acid (SDA).
Hemp Seed Fibre provides nutrients that will ensure optimum health and wellbeing for the whole family. It is an excellent source of protein, fibre, Vitamin C and E as well as important amino acids. It can easily be incorporated into your food and beverages, which makes it a get way to get extra goodness into your diet. Hemp flour is truly a super food because it is so nutritionally dense and has a pleasant nutty flavour that your family will love.
Five star rating: nutrional & culinary

Typical hemp seed flour profile
Oil Content (Fat % m/m) 13.7%
Moisture (% m/m) 9.1%
Fibre (dietary) (% m/m) 37.7%
Protein (% m/m) 30.1%

Typical hemp seed flour fatty acid profile (Of oil component)
Oleic Acid C 18:1 (Omega 9) 8.4%
Linoleic Acid C 18:2 (Omega 6) 53.9%
Alpha Linolenic Acid C 18:3 (Omega 3) 20.4%

Disclaimer: These profiles prepared are intended to describe the product with regard to the necessary nutritional requirements. They do not guarantee special characteristics and are made to the best of our current knowledge.

Eternal Delight since 2009
Eternal Delight Ltd,
PO Box 588, Christchurch, New Zealand
www.eternaldelight.co.nz
info@eternaldelight.co.nz

Appendix 2

A) *Eternal Delight product information about Hemp protein powder (hemp seed flour)*

Certificate of Analysis

Product Name: Hemp protein powder
Botanical Name: Cannabis Sativa
Batch Number: GPFAS1103HMP-HF
Quantity: Sample
DOM: 20th May 2012
Expiry Date: 20th May 2013
Description:
This Hemp Seed Fibre is produced from the residue or byproduct of the production of Cold Pressed Hemp Seed oil extracted from the seeds of the Hemp plant (Cannabis sativa). Hemp Seed Fibre is suitable for both dietary supplements and functional food applications. This product contains no solvent residues, and has been produced in a GMO/GE free production process, and contains no THC.

Test	**Result**	**Limit**
Appearance	Pass	
Fibre (dietary)		
Carbohydrates		Dark green, ground powder with
Oil Content	37,00% m/m	characteristic taste and odour
Ash	0,60% m/m	
Moisture	14,90% m/m	
Gluten	6,00% m/m	
Heavy Metals	5.10% m/m	
THC Content	<3	<3
Fatty Acid Composition	Pass	NMT 10 ppm
C16:0 Palmitic	Not detected	Not detected
C18:0 Stearic		
C18:1 Oleic	5,80%	4,0-10,0%
C18:2 Linoleic	2,70%	1,0-4,0%
C18:3 a-Linolenic	8,30%	6,0-20,0%
C18:3 y-Linolenic	53,80%	45,0-65,0%
Microbiological	21,50%	14,0-28,0%
TPC (cfu/g)	3,40%	1,0-5,0%
Confirmed Coliform Count (per g)	<50	<100.000
E. coli Count (per g)	Not detected	Not detected
Yeast & Mould Count (cfu/g)	Not detected	Not detected
Salmonella Isolation (per 25g)	<50	<500

Eternal Delight since 2009
Eternal Delight Ltd,
PO Box 588, Christchurch, New Zealand
www.eternaldelight.co.nz
info@eternaldelight.co.nz

Appendix 2

B) *Protein purification protocol.*

Protein purification protocol adaptation of methods of Calabozo et al. and Pastorello[1]*:*

1.
    - 100 gram of canned Tasty Lamb diet will be mixed using a blender, the resulting material will be mixed with 1:4 wt/vol of 0,1 mol/l PBS, pH 7.4 and stirred overnight at room temperature (app. 20˚C).
    - For the HSFP extract we mixed 50 gram in 1:10 wt/vol 0,1 mol/l PBS solution, stirring overnight at room temperature (app. 20˚C).
    - For the canned hemp diet, 100 gram was mixed in 1:4 wt/vol 0,1 mol/l PBS solution and stirred overnight at room temperature (app. 20˚C).

2. Solid ammonium sulphate will be gradually added to saturation (0.65 g/ml PBS → 400 mL PBS → 260 grams ammonium sulphate). The mixture will be stirred for 2 hours at room temperature (app. 20˚C) and then centrifuged (in 3 S-R Heraeus centrifuge) at 3450 g for 10 min (after 5 min removal of the floating fat layer during canned diet extraction).

3. The supernatant will be discarded and the precipitate will be re-suspended with mixture 1* until 50 ml of sample was obtained. This mixture will be sieved and then dialysed for 24 hours against 1 mM 2-ME in distilled water using a 3500 Dalton cut-off dialysis membrane in a sample:dialysate ratio of 1:100, changed for 3 times creating a 1:300 ratio (changed every 8 hours). Equilibrium was reached after 4-8 hours of dialysating.
   (* mixture 1 = 0,2797 ml 2-ME in 4L dH_20)

4. The resulting suspension will be paper filtered through 5-10 µm Whatman filter paper under low vacuum.

5. This will yield the protein suspension, which will be frozen in 20 mL volumes in 50 mL falcon tubes to create a frozen sample layer over the inside surface (increase surface area for lyophilization). After freezing, air-holes will be fitted in the caps of the falcon tubes (8 holes with 20G needle, airholes are above fluid level) and the samples will be lyophilized in the freeze drier for approximately 2 days.

6. Prior to use in ELISA, the purified protein powder will be reconstituted in a solution in 0,2M carbonate buffer. The suspension will be vortexed before coating the ELISA plates. Prior to use for Western Blotting, the purified protein suspension will be centrifuged for 30 minutes at 3.300xg, only the supernatant will be used as an antigen to be added to the SDS page gel.

Appendix 3

ELISA titration experiment:

A) *Protocol.*

The protein extract of the basal lamb diet (BDE) has been used as an antigen by dissolving the protein extract in 0,2 carbonate buffer to finally acquire a 0,01% dilution, adding 100 µL (containing 10 µg of antigen) per ELISA well. After overnight incubation at 4˚C, the plates were washed three times with PBST. After this the wells were blocked with 150 µL of 1% human serum albumin in PBST, followed by 1 hour incubation at 37˚C. After one hour, the plates were washed and 100 µL pooled feline serum sample was added to each well. The pooled serum sample has been diluted in PBST in the following concentrations: 1/50, 1/100, 1/200, 1/400, 1/800 added to different wells *(Appendix 4A)*. The plate was incubated for 1 h at 37˚C. Prior to adding 100 µL of secondary goat anti-cat IgG, the wells have been washed three times with PBST. Secondary antibodies have been diluted with PBST to the following concentrations: 1/10.000, 1/20.000 1:40.000 and 1/80.000 and added to the wells *(Appendix 4A)*, followed by 1 h incubation at 37˚C. Again three times washing with PBST and adding 100 µL of TMB (tetramethylbenzidin) to all the wells. After 15 minutes the reaction was stopped with 100 µL of 2M sulphuric acid. Optical density (OD) was measured at 450 nm in an ELISA microplate reader. All ELISA determinations were carried out in duplicate.

B) *Preparations.*

Antigen:

Antigen = basal diet protein extract

- Per well = 100 µL antigen/carbonate buffer mixture, this contains 10 µg of antigen.
- **Requirements** = 48 antigen coated wells, with 100 µL per well → 4800 µL antigen/carbonate buffer mixture needed → approximately 5000 µL.
- **Making stock solution** = 1% dilution (10 mg/ml), 100x diluting before usage.
  → 1% dilution = 10 mg of antigen + 1000 µL 0,2M carbonate buffer
  (100 µL = 0,1 mL = [1000 µg x 1 mL] / 10.000 µg).
- **Usage** = 0,01% = 100 µg/ml.
  → 0,01% dilution = 100 µL of the 1% mixture + 9900 µL 0,2M carbonate buffer
  (100x diluting the 1% mixture, factor 100 difference [50/5000])

Blocking buffer:

Mix all the Human Serum Albumin (HSA, 1000mg) with 1% PBST (1L PBS 1% + 500 µL T20), store this in falcon tubes.

- Per well = 150 µL blocking buffer. 56 wells in total.
- **Requirements** = 150 µL blocking buffer per well x 56 wells → 8400 µL of blocking solution needed. Best to make approximately 10 mL of 1% HSA/PBST blocking solution.

- **Making** = 10% dilution = 100 mg/mL.
  → We have got 1000 mg HSA, we will use this all to make the blocking buffer. Then we would have to dissolve 1000 mg HSA in 10 mL PBST. Store in fridge.
- **Usage** = 1% dilution = 10 mg/ml.
  → 1 mL of the 10% dilution + 9 mL of PBST.

Pooled serum (primary antibodies):

Serum dilutions 1:50 1:100 1:200 1:400 1:800

- 8 wells per dilution, 100 µL serum in every well → 800 µL serum per dilution. Make some extra (double amount) of all the solutions to make it easier to use the Multi-pipettes for the ELISA wells.
- Dilution 1:100 is used for the negative control samples (without antigen), so of this dilution we will need 16 x 100 µL = 1600 µL (double this amount in case of Multi-pipettes use).

Dilutions that we are going to make	Amount of previous dilution	Amount of PBST	Needed amount for wells	Needed amount for next dilutions	Total Volume	Volume Left
1/50	100 µl	4900 µl	800 µl	3000 µl	5000 µl	1200 µl
1/100	3000 µl	3000 µl	1600 µl	2000 µl	6000 µl	2400 µl
1/200	2000 µl	2000 µl	800 µl	2000 µl	4000 µl	1200 µl
1/400	2000 µl	2000 µl	800 µl	2000 µl	4000 µl	1200 µl
1/800	2000 µl	2000 µl	800 µl	-	4000 µl	3200 µl

Total amount of PBST needed: 13.900 µl = 13,9 mL.

Secondary antibodies:

Dilutions: 1:10.000 1:20.000 1:40.000 1:80.000

- We have 2 mL of 0,8 mg/ml goat anti-cat IgG. Mix it with PBST.
- **Requirements** = 100 µL per well x 14 wells per dilution → 1400 µL needed.

1:10.000 → 1 µL secondary antibodies + 9.999 µL PBST = 10 mL solution
1:20.000 → 2 mL of 1:10.000 solution + 2 mL PBST = 4 mL solution
1:40.000 → 2 mL of 1:20.000 solution + 2 mL PBST = 4 mL solution
1:80.000 → 2 mL of 1:40.000 solution + 2 mL PBST = 4 mL solution

Only store the stock solution of 2 mL (0,8 mg/ml), store in the fridge.

Negative control:

- No serum = antigen + blocking buffer + **no serum** + secondary antibodies
- No antigen = **no antigen** + blocking buffer + serum + secondary antibodies

Appendix 4

ELISA, Titration experiment with pooled serum sample.

A) *Format of ELISA plate.*

ELISA wells	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A 1/10.000		1/50	1/100	1/200	1/400	1/800			No serum		No antigen	
B 1/10.000		1/50	1/100	1/200	1/400	1/800			No serum		No antigen	
C 1/20.000		1/50	1/100	1/200	1/400	1/800			No serum		No antigen	
D 1/20.000		1/50	1/100	1/200	1/400	1/800			No serum		No antigen	
E 1/40.000		1/50	1/100	1/200	1/400	1/800			No serum		No antigen	
F 1/40.000		1/50	1/100	1/200	1/400	1/800			No serum		No antigen	
G 1/80.000		1/50	1/100	1/200	1/400	1/800			No serum		No antigen	
H 1/80.000		1/50	1/100	1/200	1/400	1/800			No serum		No antigen	

B) *Results ELISA Titration experiment.*

ELISA Wells	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
		1/50	*1/100*	*1/200*	*1/400*	*1/800*			*No serum*		*No antigen*	
A 1/10.000		1,168	0,859	0,670	0,490	0,420			0,329		0,788	
B 1/10.000		1,099	0,827	0,597	0,549	0,485			0,335		0,821	
C 1/20.000		0,530	0,386	0,261	0,253	0,226			0,151		0,368	
D 1/20.000		0,492	0,359	0,316	0,201	0,193			0,143		0,352	
E 1/40.000		0,250	0,188	0,119	0,150	0,119			0,097		0,198	
F 1/40.000		0,218	0,190	0,122	0,131	0,131			0,099		0,193	
G 1/80.000		0,118	0,112	0,097	0,089	0,080			0,078		0,112	
H 1/80.000		0,154	0,120	0,102	0,099	0,098			0,083		0,124	

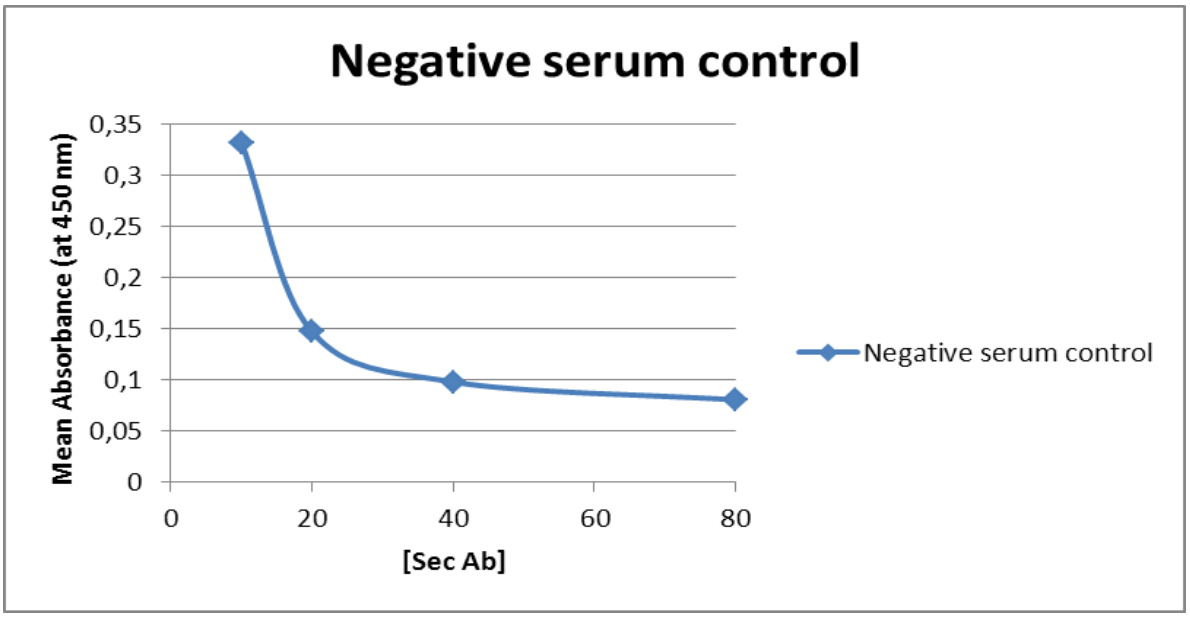
Appendix 4

ELISA, Titration experiment with pooled serum sample.

C) *Mean of the duplicate trial.*

Mean serum dilutions		**[Prim Ab]**					
		1/50	*1/100*	*1/200*	*1/400*	*1/800*	*No serum*
[Sec Ab]	*1/10.000*	1,1335	0,8430	0,6335	0,5195	0,4525	0,3320
	1/20.000	0,5110	0,3725	0,2885	0,2270	0,2095	0,1470
	1/40.000	0,2340	0,1890	0,1205	0,1405	0,1250	0,0980
	1/80.000	0,1360	0,1160	0,0995	0,0940	0,0890	0,0805

D) *Figure of the mean absorbance of the negative control sample (no serum) for different secondary antibody concentrations. (20 = 1/20.000 [Sec Ab] etc.)*



E) *Signal:Noise table. Serum absorbance / negative control absorbance (no serum).*

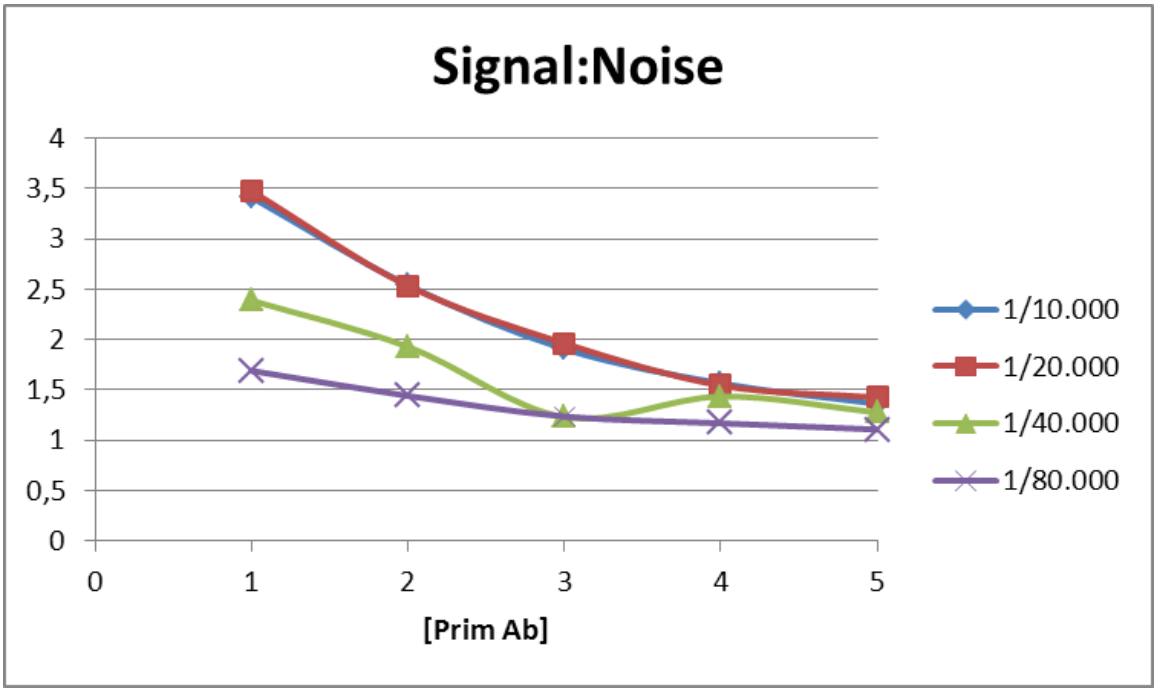
Signal:Noise	**[Prim Ab]**				
[Sec Ab]	*1/50*	*1/100*	*1/200*	*1/400*	*1/800*
1/10.000	3,4142	2,5392	1,9081	1,5648	1,3630
1/20.000	3,4762	2,5340	1,9626	1,5442	1,4252
1/40.000	2,3878	1,9286	1,2296	1,4337	1,2755
1/80.000	1,6894	1,4410	1,2360	1,1677	1,1056

Appendix 4

ELISA, Titration experiment with pooled serum sample.

F) *Figure of the Signal:Noice ratio calculated for different primary and secondary antibody concentrations.*

- *Legend = Secondary Antibody Concentration*
- *Horizontal axis ([Prim Ab]): 1= 1:50 2= 1:100 3= 1:200 4= 1:400 5= 1:800*



Appendix 5

ELISA trial:

A) *Protocol.*

10 mg of powdered protein extract (from basal diet, HSF or heated diet) was dissolved in 0,2M carbonate buffer (pH 9,6) to acquire a 0,01% solution, adding 100 µL (containing 10 µg of antigen) per ELISA well for coating. ELISA plate was incubated overnight at 4˚C (to prevent bacterial growth). The plates were washed three times with PBST (1% PBS plus 0,05% Tween 20). Wells were blocked with 150 µL of 1% human serum albumin in PBST and incubated for 1 h at 37˚C, followed by a washing cycle (ELISA washing machine, 3 times washing with PBST). 100 µL of feline serum was optimally diluted (1:100) in PBST and added to the wells, followed by incubation for 2 h at 37˚C. Plates were washed three times with PBST followed by adding 100 µL of secondary goat anti-cat IgG diluted in PBST (optimal concentration of 1:20.000), followed by 1h incubation. After three times washing with PBST, 100 µL of tetramethylbenzidine (TMB) and hydrogen peroxide was added. 15 minutes later the reaction was stopped with 100 µL of 2M sulphuric acid. Optical absorbance was read at 450 nm in an ELISA microplate reader. All ELISA determinations were carried out in duplicate.

Appendix 5

ELISA trial: BDE day 0 and 11

B) *Format of ELISA plate and Results, Basal Diet Extract (BDE) at day 0 and 11.*

Lane 5 and 9 have been coated with antigens, blocked and washed in case spare lanes were needed.

Format per well: ANTIGEN

Cat # (See Appendix 1A for corresponding information about the cats)

Result ELISA

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
1 ***Day 0***	BDE *Cat 1 –* **1,727**	BDE *Cat 2 –* **1,100**	BDE *Cat 3 –* **1,196**	BDE *Cat 4 –* **1,441**	BDE *Cat 5 –* **2,993**	BDE *Cat 6 –* **1,435**	BDE *Cat 7 –* **1,800**	BDE *Cat 8 –* **1,982**
2 ***Day 0***	BDE *Cat 1 –* **1,844**	BDE *Cat 2 –* **0,941**	BDE *Cat 3 –* **0,862**	BDE *Cat 4 –* **1,296**	BDE *Cat 5 –* **3,042**	BDE *Cat 6 –* **1,521**	BDE *Cat 7 –* **1,176**	BDE *Cat 8 –* **2,165**
3 ***Day 0***	BDE *Cat 9 –* **1,132**	BDE *Cat 10 –* **1,081**	BDE *Cat 11 –* **1,709**	BDE *Cat 12 –* **1,200**	BDE *Cat 13 –* **1,884**	BDE *Cat 14 –* **1,969**	BDE *Cat 15 –* **2,025**	BDE *Cat 16 –* **2,112**
4 ***Day 0***	BDE *Cat 9 –* **1,070**	BDE *Cat 10 –* **1,167**	BDE *Cat 11–* **1,107**	BDE *Cat 12 –* **1,218**	BDE *Cat 13 –* **1,669**	BDE *Cat 14 –* **1,186**	BDE *Cat 15 –* **1,935**	BDE *Cat 16 –* **1,789**
5								
6 ***Day 11***	BDE *Cat 1 –* **2,563**	BDE *Cat 2 –* **1,140**	BDE *Cat 3 –* **0,617**	BDE *Cat 4 –* **1,308**	BDE *Cat 5 –* **2,776**	BDE *Cat 6 –* **1,519**	BDE *Cat 7 –* **1,623**	BDE *Cat 8 –* **0,968**
7 ***Day 11***	BDE *Cat 1 –* **1,618**	BDE *Cat 2 –* **0,726**	BDE *Cat 3 –* **0,531**	BDE *Cat 4 –* **1,307**	BDE *Cat 5 –* **2,524**	BDE *Cat 6 –* **1,493**	BDE *Cat 7 –* **2,007**	BDE *Cat 8 –* **1,337**
8 ***Day 11***	BDE *Cat 9 –* **0,720**	BDE *Cat 10 –* **0,663**	BDE *Cat 11 –* **1,367**	BDE *Cat 12 –* **1,207**	BDE *Cat 13 –* **1,816**	BDE *Cat 14 –* **1,242**	BDE *Cat 15 –* **2,368**	BDE *Cat 16 –* **1,544**
9 ***Day 11***	BDE *Cat 9 –* **0,566**	BDE *Cat 10 –* **0,532**	BDE *Cat 11–* **1,275**	BDE *Cat 12 –* **1,177**	BDE *Cat 13 –* **0,682**	BDE *Cat 14 –* **2,204**	BDE *Cat 15 –* **1,856**	BDE *Cat 16 –* **1,462**
10								
11 NEG Control	No serum **0,158**	No serum **0,158**	No serum **0,167**	No serum **0,167**				
12								

Appendix 5

ELISA trial: HSFE day 0 and 11

C) *Format of ELISA plate and Results, HSFE at day 0 and 11.*

Lane 5 and 9 have been coated with antigens, blocked and washed in case spare lanes were needed.

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
1 ***Day 0***	HSFE *Cat 1 –* **2,325**	HSFE *Cat 2–* **1,756**	HSFE *Cat 3–* **1,432**	HSFE *Cat 4–* **1,930**	HSFE *Cat 5–* **3,188**	HSFE *Cat 6–* **1,997**	HSFE *Cat 7–* **2,420**	HSFE *Cat 8–* **3,373**
2 ***Day 0***	HSFE *Cat 1 –* **3,114**	HSFE *Cat 2–* **1,922**	HSFE *Cat 3–* **1,557**	HSFE *Cat 4–* **1,970**	HSFE *Cat 5–* **3,375**	HSFE *Cat 6–* **2,379**	HSFE *Cat 7–* **2,533**	HSFE *Cat 8–* **3,320**
3 ***Day 0***	HSFE *Cat 9–* **1,863**	HSFE *Cat 10–* **2,241**	HSFE *Cat 11–* **2,424**	HSFE *Cat 12–* **1,888**	HSFE *Cat 13–* **2,454**	HSFE *Cat 14–* **3,043**	HSFE *Cat 15–* **2,816**	HSFE *Cat 16–* **3,203**
4 ***Day 0***	HSFE *Cat 9–* **1,809**	HSFE *Cat 10–* **1,465**	HSFE *Cat 11–* **2,006**	HSFE *Cat 12–* **1,869**	HSFE *Cat 13–* **2,295**	HSFE *Cat 14–* **2,997**	HSFE *Cat 15–* **2,635**	HSFE *Cat 16–* **3,061**
5								
6 ***Day 11***	HSFE *Cat 1 –* **2,921**	HSFE *Cat 2–* **0,924**	HSFE *Cat 3–* **2,513**	HSFE *Cat 4–* **1,841**	HSFE *Cat 5–* **4,155**	HSFE *Cat 6–* **4,434**	HSFE *Cat 7–* **3,589**	HSFE *Cat 8–* **3,749**
7 ***Day 11***	HSFE *Cat 1 –* **3,724**	HSFE *Cat 2–* **1,762**	HSFE *Cat 3–* **2,690**	HSFE *Cat 4–* **1,890**	HSFE *Cat 5–* **4,066**	HSFE *Cat 6–* **4,431**	HSFE *Cat 7–* **3,613**	HSFE *Cat 8–* **4,030**
8 ***Day 11***	HSFE *Cat 9–* **0,912**	HSFE *Cat 10–* **1,981**	HSFE *Cat 11–* **1,852**	HSFE *Cat 12–* **1,741**	HSFE *Cat 13–* **2,172**	HSFE *Cat 14–* **3,066**	HSFE *Cat 15–* **2,930**	HSFE *Cat 16–* **3,247**
9 ***Day 11***	HSFE *Cat 9–* **1,390**	HSFE *Cat 10–* **2,379**	HSFE *Cat 11–* **2,307**	HSFE *Cat 12–* **1,658**	HSFE *Cat 13–* **1,969**	HSFE *Cat 14–* **3,054**	HSFE *Cat 15–* **2,883**	HSFE *Cat 16–* **3,201**
10								
11 ***NEG control***	No serum **0,333**	No serum **0,315**	No serum **0,306**	No serum **0,315**				
12								

Appendix 5

ELISA trial: BDE and HSFE day 0 and 11

D) *Mean corrected values for BDE at day 0 and 11.*

Each result was corrected for the mean negative control value (no serum) and then an average was taken of the duplicate results per cat.

Pen 14 = basal diet mixed with hemp seed flour. Pen 15 = heated hemp diet.

Mean corrected values BDE		***Cats***								
		Pen 14								
		1	2	3	4	5	6	7	8	**Mean**
OD	*Day 0*	1,623	0,858	0,867	1,206	2,855	1,316	1,326	1,911	1,495
	Day 11	1,928	0,771	0,412	1,145	2,488	1,344	1,653	0,990	1,341
		Pen 15								
		9	10	11	12	13	14	15	16	**Mean**
	Day 0	0,939	0,962	1,246	1,047	1,614	1,415	1,818	1,788	1,353
	Day 11	0,481	0,435	1,159	1,030	1,087	1,561	1,950	1,341	1,130

E) *Mean corrected values for HSFE at day 0 and 11.*

Each result was corrected for the mean negative control value (no serum) and then an average was taken of the duplicate results per cat.

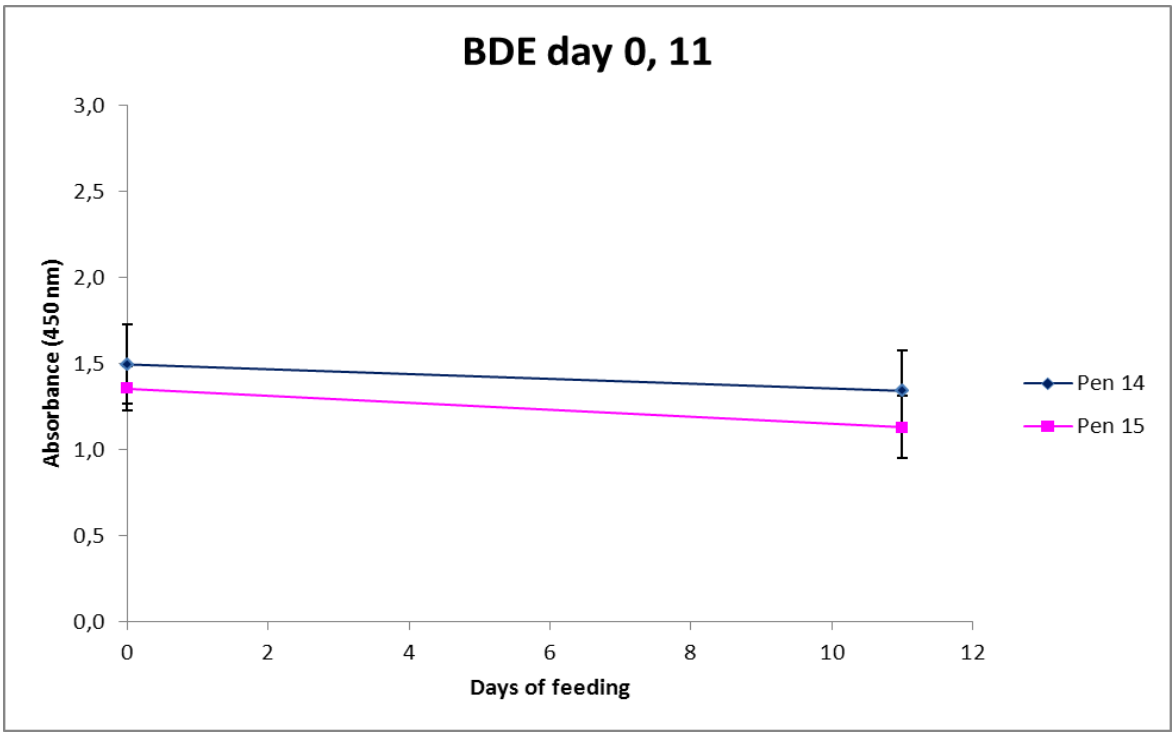
Pen 14 = basal diet mixed with hemp seed flour. Pen 15 = heated hemp diet.

Mean corrected values HSFE		***Cats***								
		Pen 14								
		1	2	3	4	5	6	7	8	**Mean**
OD	*Day 0*	2,402	1,522	1,177	1,633	2,964	1,871	2,159	3,029	2,095
	Day 11	3,005	1,026	2,284	1,548	3,793	4,115	3,284	3,572	2,829
		Pen 15								
		9	10	11	12	13	14	15	16	**Mean**
	Day 0	1,519	1,536	1,898	1,561	2,057	2,703	2,408	2,815	2,062
	Day 11	0,834	1,863	1,762	1,382	1,753	2,743	2,589	2,907	1,979

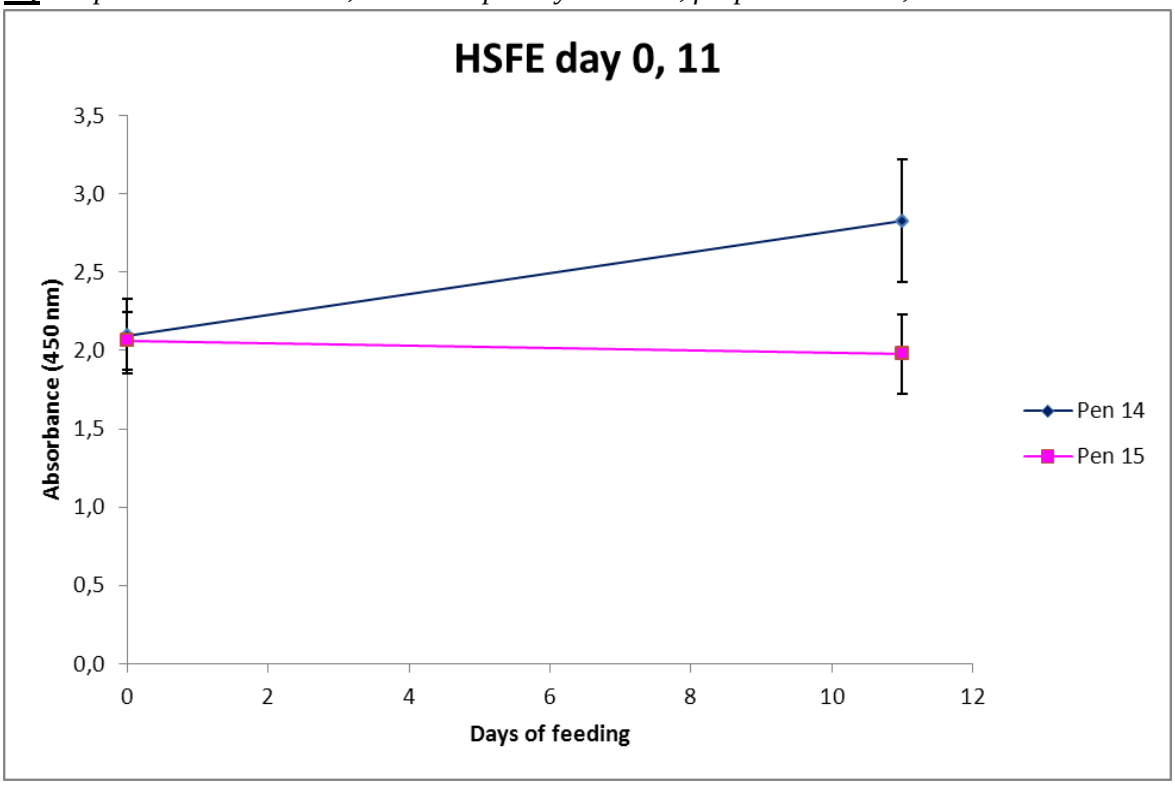
Appendix 5

ELISA trial: BDE and HSFE day 0 and 11

F) *Graph absorbance BDE, blood sample day 0 and 11, for pen 14 and 15, with SEM.*



G) *Graph absorbance HSFE, blood sample day 0 and 11, for pen 14 and 15, with SEM.*



Appendix 6

Final ELISA

A) *Protocol.*

20 mg of powdered protein extract (from basal diet, HSF or heated diet) was dissolved in 0,2M carbonate buffer (pH 9,6) to acquire a 0,02% (200 µg/ml) solution. This solution was centrifugated for 5 minutes at 3000g. Add 100 µL (containing 20 µg of antigen) per ELISA well for coating. ELISA plate was incubated overnight at 4˚C (to prevent bacterial growth). The plates were washed three times with PBST (1% PBS plus 0,05% Tween 20). Wells were blocked with 150 µL of 1% human serum albumin in PBST and incubated for 2 h at 37˚C, followed by a washing cycle (ELISA washing machine, 3 times washing with PBST). 100 µL of feline serum was optimally diluted (1:200) in PBST and added to the wells, followed by incubation for 1 h at 37˚C. Plates were washed three times with PBST followed by adding 100 µL of goat anti-cat IgG diluted in PBST (secondary antibody solution, in optimal dilution of 1:20.000). 1h incubation at 37˚C. After three times washing with PBST, 100 µL of tetramethylbenzidine (TMB) and hydrogen peroxide was added. 15 minutes later the reaction was stopped with 100 µL of 2M sulphuric acid. Optical absorbance was read at 450 nm in an ELISA microplate reader. All ELISA values were carried out in duplicate.

Appendix 6

Final ELISA

B) *Preparations.*

Antigen:

Antigen = BDE or HSFE

- **Requirements** = 88 antigen coated wells, with 100 uL per well → 8800 uL antigen/carbonate buffer mixture needed → approximately 10 mL.
- **Making stock solution** = 1% dilution (10 mg/ml), 100x diluting before usage.
  → 1% dilution = 10 mg of antigen + 1000 uL 0,2M carbonate buffer
  Total amount = 1 mL.
- **Usage centrifugation** = 0,02% = 200 ug/ml
  → 0,02% dilution = 200 uL of the 1% mixture + 9800 uL 0,2M carbonate buffer
  Total amount = 10 mL.
  Per well = 100 uL antigen/carbonate buffer mixture, this contains 20 ug of antigen.

Antigen = HHDE

- Per well = 100 uL antigen/carbonate buffer mixture, this contains 10 ug of antigen.
- **Requirements** = 144 antigen coated wells, with 100 uL per well → 14.400 uL antigen/carbonate buffer mixture needed → approximately 20 mL.
- **Making stock solution** = 1% dilution (10 mg/ml), 100x diluting before usage.
  → 1% dilution = 10 mg of antigen + 1000 uL 0,2M carbonate buffer
  Total amount = 1 mL
- **Usage centrifugation** = 0,02% = 200 ug/ml
  → 0,02% dilution = 400 uL of the 1% mixture + 19.600 uL 0,2M carbonate buffer. Total amount = 20 mL.

Blocking buffer:

Mix all the HSA(1000mg) with 1% PBST (1L PBS 1% + 500 uL T20), store this in falcon tubes.

- Per well = 150 ul blocking buffer. 288 wells in total.
- **Requirements** =
  - 150 uL blocking buffer per well x 352 wells → 43.200 uL of blocking solution needed. Make = 50 mL.
- **Making** = 10% dilution = 100 mg/ml.
  → We have got 1000 mg HSA, we will use this all to make the blocking buffer. Then we would have to dissolve 1000 mg HSA in 10 mL PBST. Store in fridge.
- **Usage** = 1% dilution = 10 mg/ml.
  →5 mL of the 10% dilution + 45 mL of PBST → Total = 50 mL

Serum cats: 1:200 dilution 100 uL per well

Example described for the serum of Cat 1, this counts for cat 1-16:

Cat 1, day 0: T1

- 2 wells → HHDE → 200 uL needed
- Make 400 uL solution per cat = 2 uL serum in 398 uL PBST (2/400 = 1/200)
- Total PBST needed = 16 cats x 398 uL = 6.368 uL

Cat 1, day 11: T2

- 6 wells → BDE, HSFE, HHDE → 600 uL needed
- Make 800 uL solution per cat = 4 uL serum in 796 uL op PBST (4/800 = 1/200)
- Total PBST needed = 16 cats x 796 uL = 12.736 uL

Cat 1, day 25: T3

- 6 wells → BDE, HSFE, HHDE → 600 uL needed
- Make 800 uL solution per cat = 4 uL serum in 796 uL op PBST (4/800 = 1/200)
- Total PBST needed = 16 cats x 796 uL = 12.736 uL

Total needed:

- We need 16 eppendurf cups per time serum sample, we have 3 time samples, so that will be 16 cats x 3 time samples = 48 eppendurf cups
- PBST = 6368 + 12736 +12736 = 31.840 uL = around 35 mL of PBST
- Best = Make plate 1&2 at the same time. After this, plate 3&4 togheter. Otherwise the plates will be dry.

Secondary antibodies: 1:20.000 100 uL per well

We have 2 mL of 0,8 mg/ml goat anti-cat IgG. Mix it with PBST.

- **Requirements** = 100 uL per well x 256 wells per dilution → 25.600 uL needed.
- Make 30 mL of secondary antibody solution → 1,5 uL sec Ig in 29.998,5 uL PBST.

TMB = (72x3)+40 = 256 wells 100 uL per well → 25.600 uL = 25,6 mL

Stop sol. = Sulphuric acid = 100 uL per well, 256 wells → 25.600 uL = 25,6 mL

Negative control:

- No serum = antigen + blocking buffer + **no serum (add 100 uL of PBST)** + sec Ig
- No antigen = **no antigen** + blocking buffer + serum + sec Ig

Appendix 7

Final ELISA: BDE day 11 and 25

A) *Format of ELISA plate and Results, BDE day 11 and 25:*

	A	B	C	D	E	F	G	H
1 *Day 11*	BDE *Cat 1 –* **3,560**	BDE *Cat 2–* **2,081**	BDE *Cat 3–* **2,325**	BDE *Cat 4–* **2,398**	BDE *Cat 5–* **4,319**	BDE *Cat 6–* **4,426**	BDE *Cat 7–* **3,599**	BDE *Cat 8–* **3,965**
2 *Day 11*	BDE *Cat 1 –* **3,548**	BDE *Cat 2–* **2,005**	BDE *Cat 3–* **2,382**	BDE *Cat 4–* **2,296**	BDE *Cat 5–* **4,343**	BDE *Cat 6–* **4,345**	BDE *Cat 7–* **3,591**	BDE *Cat 8–* **3,838**
3 *Day 11*	BDE *Cat 9 –* **1,916**	BDE *Cat 10–* **2,572**	BDE *Cat 11–* **2,365**	BDE *Cat 12–* **2,233**	BDE *Cat 13–* **2,624**	BDE *Cat 14–* **3,501**	BDE *Cat 15–* **2,994**	BDE *Cat 16–* **3,469**
4 *Day 11*	BDE *Cat 9 –* **1,900**	BDE *Cat 10–* **2,481**	BDE *Cat 11–* **2,256**	BDE *Cat 12–* **1,792**	BDE *Cat 13–* **2,604**	BDE *Cat 14–* **3,465**	BDE *Cat 15–* **3,091**	BDE *Cat 16–* **3,589**
5	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*
6 *Day 25*	BDE *Cat 1 –* **3,524**	BDE *Cat 2–* **4,529**	BDE *Cat 3–* **OVRFL**	BDE *Cat 4–* **4,532**	BDE *Cat 5–* **OVRFL**	BDE *Cat 6–* **4,574**	BDE *Cat 7–* **4,529**	BDE *Cat 8–* **4,562**
7 *Day 25*	BDE *Cat 1 –* **3,558**	BDE *Cat 2–* **4,532**	BDE *Cat 3–* **4,485**	BDE *Cat 4–* **4,563**	BDE *Cat 5–* **4,579**	BDE *Cat 6–* **4,607**	BDE *Cat 7–* **4,534**	BDE *Cat 8–* **4,547**
8 *Day 25*	BDE *Cat 9 –* **1,281**	BDE *Cat 10–* **2,284**	BDE *Cat 11–* **1,864**	BDE *Cat 12–* **1,965**	BDE *Cat 13–* **2,220**	BDE *Cat 14–* **3,194**	BDE *Cat 15–* **2,811**	BDE *Cat 16–* **2,952**
9 *Day 25*	BDE *Cat 9 –* **1,449**	BDE *Cat 10–* **2,220**	BDE *Cat 11–* **2,064**	BDE *Cat 12–* **2,253**	BDE *Cat 13–* **2,361**	BDE *Cat 14–* **3,291**	BDE *Cat 15–* **2,839**	BDE *Cat 16–* **3,002**
10								
11 *No serum*	**0,210**	**0,204**	**0,195**	**0,206**				
12 *No antigen*					*Cat 13T1* **2,136**	*Cat 14T1* **3,155**	*Cat 15T1* **2,730**	*Cat 16T1* **2,885**

'No serum' = antigen + blocking + PBST (without serum) + sec Ig

'No antigen' = blocking + serum of 4 different cats + sec Ig (no antigen coating in wells)

Lane 5 = spare lane filled with antigen + blocking buffer.

13T3 = serum of cat 13 from time sample 3 = day 25

OVRFL = overflow of solution, no data

Appendix 7

Final ELISA: HSFE day 11 and 25

B) *Format of ELISA plate and Results, HSFE day 11 and 25:*

	A	B	C	D	E	F	G	H
1 *Day 11*	HSFE *Cat 1 –* **2,414**	HSFE *Cat 2–* **1,640**	HSFE *Cat 3–* **1,683**	HSFE *Cat 4–* **1,871**	HSFE *Cat 5–* **4,089**	HSFE *Cat 6–* **2,164**	HSFE *Cat 7–* **2,322**	HSFE *Cat 8–* **3,073**
2 *Day 11*	HSFE *Cat 1 –* **2,446**	HSFE *Cat 2–* **1,744**	HSFE *Cat 3–* **1,683**	HSFE *Cat 4–* **1,803**	HSFE *Cat 5–* **4,055**	HSFE *Cat 6–* **2,453**	HSFE *Cat 7–* **2,544**	HSFE *Cat 8–* **2,330**
3 *Day 11*	HSFE *Cat 9 –* **1,682**	HSFE *Cat 10–* **1,658**	HSFE *Cat 11–* **1,292**	HSFE *Cat 12–* **1,856**	HSFE *Cat 13–* **2,346**	HSFE *Cat 14–* **2,669**	HSFE *Cat 15–* **2,581**	HSFE *Cat 16–* **3,325**
4 *Day 11*	HSFE *Cat 9 –* **1,594**	HSFE *Cat 10–* **1,695**	HSFE *Cat 11–* **2,037**	HSFE *Cat 12–* **1,871**	HSFE *Cat 13–* **2,317**	HSFE *Cat 14–* **3,214**	HSFE *Cat 15–* **2,698**	HSFE *Cat 16–* **3,185**
5	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*
6 *Day 25*	HSFE *Cat 1 –* **1,605**	HSFE *Cat 2–* **1,518**	HSFE *Cat 3–* **1,257**	HSFE *Cat 4–* **2,451**	HSFE *Cat 5–* **4,282**	HSFE *Cat 6–* **1,755**	HSFE *Cat 7–* **2,503**	HSFE *Cat 8–* **3,049**
7 *Day 25*	HSFE *Cat 1 –* **1,715**	HSFE *Cat 2–* **1,606**	HSFE *Cat 3–* **1,360**	HSFE *Cat 4–* **2,431**	HSFE *Cat 5–* **4,223**	HSFE *Cat 6–* **2,277**	HSFE *Cat 7–* **2,623**	HSFE *Cat 8–* **3,114**
8 *Day 25*	HSFE *Cat 9 –* **1,275**	HSFE *Cat 10–* **1,919**	HSFE *Cat 11–* **1,731**	HSFE *Cat 12–* **1,778**	HSFE *Cat 13–* **2,091**	HSFE *Cat 14–* **2,993**	HSFE *Cat 15–* **2,617**	HSFE *Cat 16–* **2,735**
9 *Day 25*	HSFE *Cat 9 –* **1,116**	HSFE *Cat 10–* **1,365**	HSFE *Cat 11–* **1,725**	HSFE *Cat 12–* **1,786**	HSFE *Cat 13–* **2,158**	HSFE *Cat 14–* **2,926**	HSFE *Cat 15–* **2,550**	HSFE *Cat 16–* **2,628**
10								
11 *No serum*	**0,164**	**0,166**	**0,158**	**0,169**				
12 *No antigen*					*Cat 13T1* **4,442**	*Cat 14T1* **2,120**	*Cat 15T1* **2,438**	*Cat 16T1* **2,902**

'No serum' = antigen + blocking + PBST (without serum) + sec Ig
'No antigen' = blocking + serum of 4 different cats + sec Ig (no antigen coating in wells)
Lane 5 = spare lane filled with antigen + blocking buffer.
5T3 = serum of cat 5 from time sample 3 = day 25

Appendix 7

Final ELISA: HHDE day 0 and 11

C) *Format of ELISA plate and Results, HHDE day 0 and 11:*

	A	B	C	D	E	F	G	H
1 *Day 0*	HHDE *Cat 1 –* **2,082**	HHDE *Cat 2–* **1,116**	HHDE *Cat 3–* **1,172**	HHDE *Cat 4–* **1,553**	HHDE *Cat 5–* **3,637**	HHDE *Cat 6–* **1,933**	HHDE *Cat 7–* **2,291**	HHDE *Cat 8–* **2,223**
2 *Day 0*	HHDE *Cat 1 –* **2,412**	HHDE *Cat 2–* **1,299**	HHDE *Cat 3–* **1,468**	HHDE *Cat 4–* **1,546**	HHDE *Cat 5–* **3,665**	HHDE *Cat 6–* **1,955**	HHDE *Cat 7–* **2,264**	HHDE *Cat 8–* **1,589**
3 *Day 0*	HHDE *Cat 9 –* **1,318**	HHDE *Cat 10–* **1,692**	HHDE *Cat 11–* **1,307**	HHDE *Cat 12–* **1,404**	HHDE *Cat 13–* **1,669**	HHDE *Cat 14–* **2,787**	HHDE *Cat 15–* **0,632**	HHDE *Cat 16–* **2,946**
4 *Day 0*	HHDE *Cat 9 –* **1,239**	HHDE *Cat 10–* **1,675**	HHDE *Cat 11–* **1,346**	HHDE *Cat 12–* **1,286**	HHDE *Cat 13–* **1,738**	HHDE *Cat 14–* **2,598**	HHDE *Cat 15–* **0,647**	HHDE *Cat 16–* **3,024**
5	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*
6 *Day 11*	HHDE *Cat 1 –* **2,286**	HHDE *Cat 2–* **1,308**	HHDE *Cat 3–* **1,411**	HHDE *Cat 4–* **1,596**	HHDE *Cat 5–* **4,227**	HHDE *Cat 6–* **2,112**	HHDE *Cat 7–* **2,252**	HHDE *Cat 8–* **1,935**
7 *Day 11*	HHDE *Cat 1 –* **2,228**	HHDE *Cat 2–* **1,219**	HHDE *Cat 3–* **1,375**	HHDE *Cat 4–* **1,593**	HHDE *Cat 5–* **4,135**	HHDE *Cat 6–* **2,071**	HHDE *Cat 7–* **2,123**	HHDE *Cat 8–* **1,941**
8 *Day 11*	HHDE *Cat 9 –* **1,177**	HHDE *Cat 10–* **1,308**	HHDE *Cat 11–* **1,414**	HHDE *Cat 12–* **1,379**	HHDE *Cat 13–* **1,673**	HHDE *Cat 14–* **2,928**	HHDE *Cat 15–* **2,254**	HHDE *Cat 16–* **2,546**
9 *Day 11*	HHDE *Cat 9 –* **1,174**	HHDE *Cat 10–* **1,330**	HHDE *Cat 11–* **1,358**	HHDE *Cat 12–* **1,393**	HHDE *Cat 13–* **1,605**	HHDE *Cat 14–* **2,903**	HHDE *Cat 15–* **2,140**	HHDE *Cat 16–* **2,538**
10								
11 *No serum*	**0,152**	**0,144**	**0,164**	**0,147**				
12 *No antigen*					*Cat 13T1* **1,908**	*Cat 14T1* **3,129**	*Cat 15T1* **0,689**	*Cat 16T1* **2,655**

'No serum' = antigen + blocking + PBST (without serum) + sec Ig
'No antigen' = blocking + serum of 4 different cats + sec Ig (no antigen coating in wells)
Lane 5 = spare lane filled with antigen + blocking buffer.
13T1 = serum of cat 13 from time sample 1 = day 0

Appendix 7

Final ELISA: HHDE day 25

D) *Format of ELISA plate and Results, HHDE day 25:*

	A	B	C	D	E	F	G	H
1 *Day 25*	HHDE *Cat 1 –* **1,528**	HHDE *Cat 2–* **1,464**	HHDE *Cat 3–* **1,228**	HHDE *Cat 4–* **2,114**	HHDE *Cat 5–* **4,227**	HHDE *Cat 6–* **1,843**	HHDE *Cat 7–* **2,064**	HHDE *Cat 8–* **2,035**
2 *Day 25*	HHDE *Cat 1 –* **1,520**	HHDE *Cat 2–* **1,481**	HHDE *Cat 3–* **1,181**	HHDE *Cat 4–* **2,083**	HHDE *Cat 5–* **4,170**	HHDE *Cat 6–* **1,864**	HHDE *Cat 7–* **2,107**	HHDE *Cat 8–* **1,529**
3 *Day 25*	HHDE *Cat 9 –* **0,968**	HHDE *Cat 10–* **1,429**	HHDE *Cat 11–* **1,359**	HHDE *Cat 12–* **1,531**	HHDE *Cat 13–* **1,686**	HHDE *Cat 14–* **2,617**	HHDE *Cat 15–* **2,105**	HHDE *Cat 16–* **1,939**
4 *Day 25*	HHDE *Cat 9 –* **0,959**	HHDE *Cat 10–* **1,374**	HHDE *Cat 11–* **1,245**	HHDE *Cat 12–* **1,471**	HHDE *Cat 13–* **1,394**	HHDE *Cat 14–* **2,664**	HHDE *Cat 15–* **1,685**	HHDE *Cat 16–* **1,987**
5	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*	*Antigen+ blocking*
6								
7								
8								
9								
10								
11 *No serum*	**0,144**	**0,174**	**0,157**	**0,153**				
12 *No antigen*					*Cat 1T3* **1,470**	*Cat 2T3* **1,351**	*Cat 3T3* **1,142**	*Cat 4T3* **1,875**

‘‘No serum’ = antigen + blocking + PBST (without serum) + sec Ig
‘No antigen’ = blocking + serum of 4 different cats + sec Ig (no antigen coating in wells)
Lane 5 = spare lane filled with antigen + blocking buffer.
1T3 = serum of cat 1 from time sample 3 = day 25

Appendix 8

Final ELISA Results

A) *Mean values of BDE, after correcting for negative control (no serum), day 11 and 25:*

Mean corrected values BDE		***Cats***								
		Pen 14								
		1	2	3	4	5	6	7	8	**Mean**
OD	*Day 11*	3,350	1,839	2,150	2,143	4,127	4,182	3,391	3,698	3,110
	Day 25	3,337	4,327	4,281	4,344	4,375	4,387	4,328	4,351	4,216
		9	10	11	12	13	14	15	16	**Mean**
	Day 11	1,704	2,323	2,107	1,809	2,410	3,279	2,839	3,325	2,475
	Day 25	1,161	2,048	1,760	1,905	2,087	3,039	2,621	2,773	2,174

B) *Mean values of HSFE, after correcting for negative control (no serum), day 11 and 25:*

Mean corrected values HSFE		***Cats***								
		Pen 14								
		1	2	3	4	5	6	7	8	**Mean**
OD	*Day 11*	2,266	1,528	1,519	1,673	3,908	2,144	2,269	2,537	2,231
	Day 25	1,496	1,398	1,144	2,277	4,088	1,852	2,399	2,917	2,196
		Pen 15								
		9	10	11	12	13	14	15	16	**Mean**
	Day 11	1,474	1,512	1,500	1,699	2,167	2,777	2,475	3,091	2,087
	Day 25	1,031	1,478	1,564	1,618	1,960	2,795	2,419	2,517	1,923

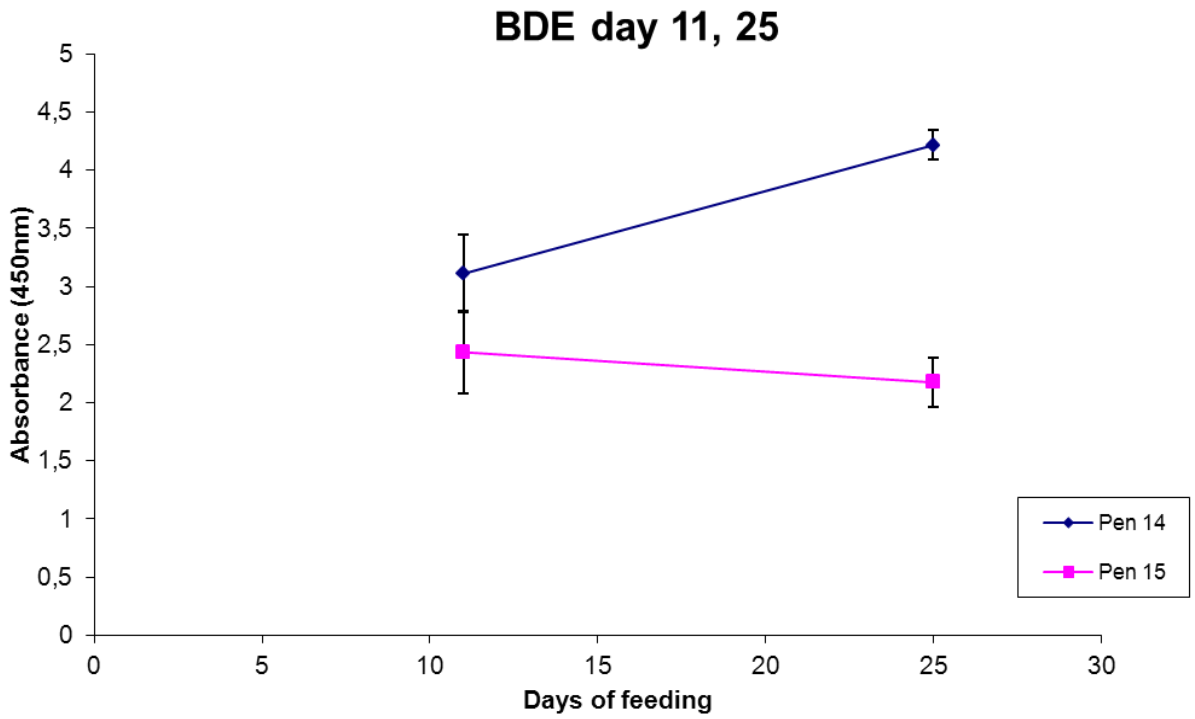
C) *Mean values HHDE, after correcting for negative control (no serum), day 0, 11 and 25:*

Mean corrected values HHDE		***Cats***								
		Pen 14								
		1	2	3	4	5	6	7	8	**Mean**
OD	*Day 0*	2,095	1,056	1,168	1,398	3,499	1,792	2,126	1,754	1,861
	Day 11	2,105	1,112	1,241	1,443	4,029	1,940	2,036	1,786	1,962
	Day 25	1,367	1,316	1,048	1,942	4,042	1,697	1,929	1,625	1,870
		Pen 15								
		9	10	11	12	13	14	15	16	**Mean**
	Day 0	1,127	1,532	1,175	1,193	1,552	2,541	0,488	2,833	1,555
	Day 11	1,024	1,167	1,234	1,234	1,487	2,764	2,045	2,390	1,668
	Day 25	0,807	1,245	1,145	1,344	1,383	2,484	1,738	1,806	1,494

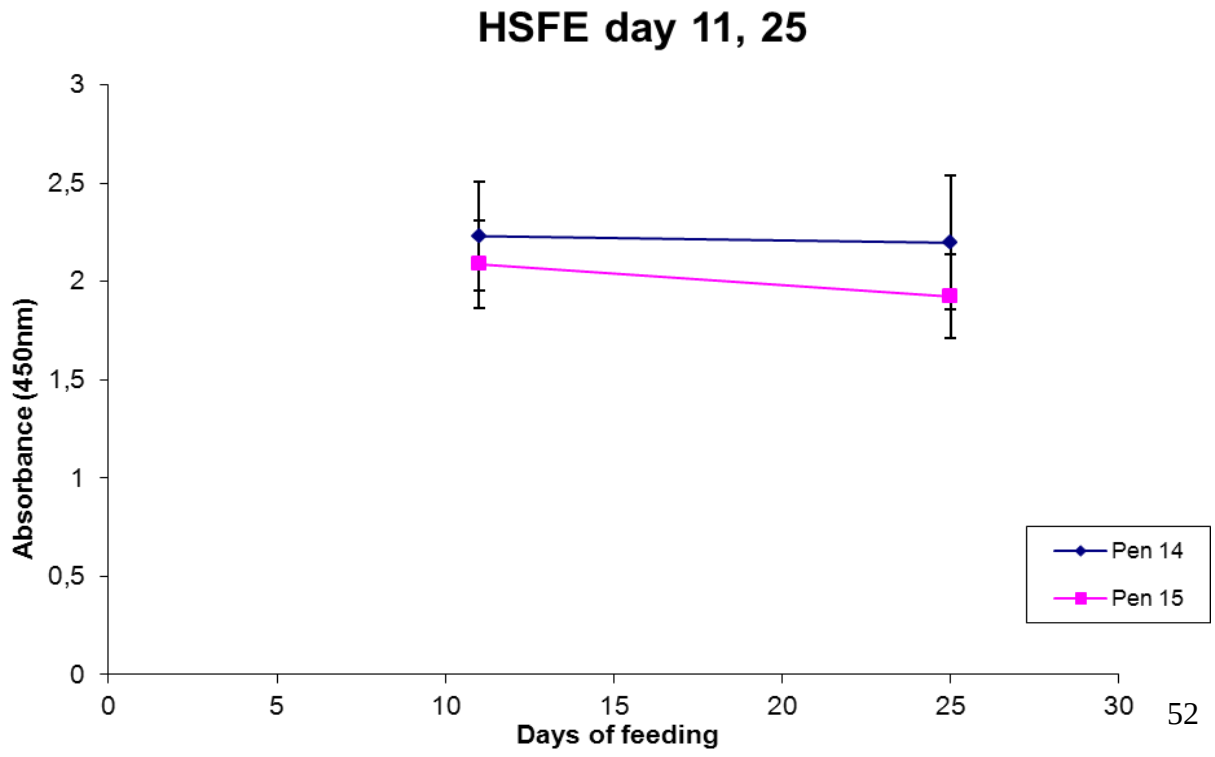
Appendix 8

Final ELISA Results

D) *Graph absorbance BDE, blood sample day 11 and 25, for pen 14 and 15, with SEM.*



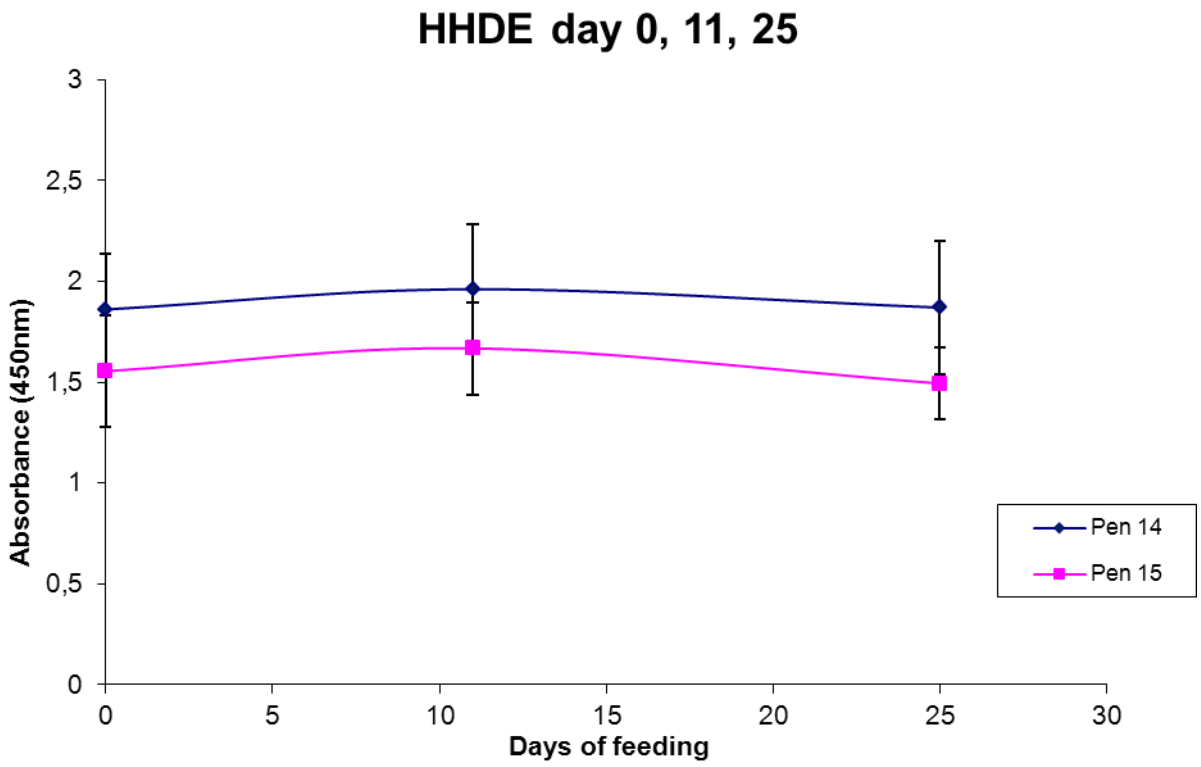
E) *Graph absorbance HSFE, blood sample day 11 and 25, for pen 14 and 15, with SEM.*



Appendix 8

Final ELISA Results

F) *Graph absorbance HHDE, blood sample day 0, 11 and 25, for pen 14 and 15, with SEM.*



References

1. Pastorello EA, Trambaioli C. Isolation of Food Allergens. J Chromatogr B Biomed Sci Appl 2001;756:71-84.