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Diet during pregnancy and weaning and adult disease in the offspring in the rat

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Abstract

Several epidemiological as well as experimental studies suggest that the composition of the maternal diet during pregnancy may have a programming effect on the fetus leading to epigenetic predisposition of chronic diseases in adulthood.
Here, effects of various diets given during pregnancy and weaning on parameters related to chronic diseases during adulthood were studied in the rat.
Seven groups of sixteen animals were put on an isocaloric semi-synthetic high-fat diet two weeks before pregnancy, and continuing throughout pregnancy and weaning. Diets differed in macronutrient – or fatty acid composition. Fetal development was studied on gestational day 21. After birth, litter size in parallel groups was reduced to eight pups. A series of physiological parameters were studied in the pups at age 12 weeks.

The offspring of dams put on a low protein diet or a fish oil diet *in utero* and during weaning had a significantly reduced body weight gain from birth up to age 12 weeks in comparison to the control group.
Male offspring aged 12 weeks fed the coconut oil diet *in utero* and during weaning appeared slightly insulin-resistant. No difference in the volume of the islets of Langerhans could be demonstrated in this group.
Despite significantly smaller islets of Langerhans and a reduced number of large islets in the pancreas of 12-week-old male rats given the low protein diet *in utero* and during weaning, no differences in glucose or insulin concentration were found in comparison to the control group.
Maternal diet during pregnancy and weaning did not influence immune function or blood pressure in the offspring at the age of 12 weeks.
In conclusion, prenatal diet influences postnatal development, as determined by anatomical and physiological parameters.

Samenvatting

Uit zowel epidemiologische als dierexperimentele studies komen steeds meer aanwijzingen dat de samenstelling van de voeding tijdens de zwangerschap een belangrijke invloed heeft op de vatbaarheid van het nageslacht voor chronische ziekten op latere leeftijd.
In deze dierexperimentele studie zijn de effecten van diervoeders met een verschillende samenstelling onderzocht op parameters voor chronische ziekten op volwassen leeftijd. Aan zeven groepen van elk zestien dieren werd een isocalorisch hoog-vet voer gegeven met een verschillende macronutriënt- en vetzuursamenstelling vanaf twee weken voor de dracht tot aan het einde van de speenperiode. Op dag 21 van de dracht werd de foetale ontwikkeling onderzocht. Op de leeftijd van 12 weken is een aantal fysiologische en morfologische parameters bepaald.
Nageslacht dat *in utero* en tijdens de speenperiode was blootgesteld aan een laag eiwit voer of een voer rijk in visvetzuren had een significant lager lichaamsgewicht vanaf de geboorte tot aan de leeftijd van 12 weken, ten opzichte van de controlegroep.
Het mannelijk nageslacht van dieren van de cocosnootgroep (rijk aan verzadigde vetten) lijkt licht insuline resistent te zijn. Er was echter geen verschil in volume van de eilandjes van Langerhans ten opzichte van de controlegroep.
Ondanks significant kleinere eilandjes van Langerhans en de aanwezigheid van minder grotere eilandjes in de pancreas bij 12-weken oude dieren uit de laag eiwit groep verschilde deze groep niet van de controlegroep in glucose- of insuline respons na een orale glucose load.
De immuunfunctie en systolische en diastolische bloeddruk in het nageslacht op volwassen leeftijd leek niet te worden beïnvloed door de samenstelling van de maternale voeding *in utero* en tijdens spenen
Concluderend kan worden gezegd dat de samenstelling van de voeding tijdens de dracht en de vroege postnatale periode de postnatale ontwikkeling van het nageslacht beïnvloedde, hetgeen bleek uit veranderingen in zowel morfologische als fysiologische parameters.

CONTENTS

1. Introduction

Several decades of research have demonstrated that significant contributions to the risk of chronic diseases are made by adult lifestyle factors such as smoking, inactivity and a high fat diet. More recent research findings suggest that the risk of chronic diseases may be determined partly also by the environment before birth (1-4). The fetus appears to respond to the nutritional environment *in utero* in terms of anatomic and physiologic adaptations, with the aim to be adequately prepared for the nutritional conditions that will be encountered after birth. Consequently, when the postnatal environment is different than anticipated by the fetus, the adaptations may be detrimental to health and may in this way increase the susceptibility to cancer, cardiovascular disease, obesity, diabetes and infectious diseases (4,11). In view of the increasing incidence of the major chronic diseases, more insight into the role of prenatal nutrition on determining disease risk in adulthood may contribute to the prevention of future cases of chronic diseases.
Evidence for prenatal programming comes from a large number of retrospective epidemiological studies throughout the world, as well as from animal studies (29). Whereas human studies may be confounded by uncontrollable social and genetic factors, animal studies permit the investigation of the fetal origins of adult disease hypothesis in a more standardized and mechanistic manner.

1.1 Aim and relevance of the study

Food consumption data (30) show that in the Netherlands, the intake of polyunsaturated fatty acids is increasing and that the consumption of saturated fatty acids is still higher than desirable. In addition, the intake of dietary fiber is below the recommended dietary intake. In this investigation, we mimicked these current dietary trends in the rat. The effects of a diet with different fatty acid compositions, protein content and fiber content during pregnancy and lactation on parameters for chronic diseases in adulthood were studied. Oral glucose tolerance was determined as a marker for the development of diabetes, and blood pressure was measured as a parameter for cardiovascular disease risk. Immune response to an infective challenge was determined. The degree of responsivity of the hypothalamus-pituitary-adrenal axis, which seems to play an important role in the proces of prenatal programming, was studied *in vitro*. A variety of organs involved in the hypothesis of fetal programming were examined histologically.

2. Materials and methods

2.1 Experimental design

The experimental design is fully described elsewhere (42; 43). In figure 1, a schematic overview of the study is given.
Briefly, the animals were assigned randomly to one of the seven experimental diets. Diets differed in fatty acid composition (e.g. diets based on coconut oil, flaxseed oil, menhaden oil, safflower oil,) or in macronutrient composition (e.g. low fiber content, low protein content). A diet based on soy oil was used as a control. For more details on dietary composition, see section 2.3.
After the 2-week acclimatisation period and the 2-week premating period (in which the experimental diet was given to the animals) animals were mated. Because of practical reasons mating period was spanned over 4 weeks. Animals were fed the experimental diet until weaning was completed. Consequently, the duration of exposure to the experimental diet differed for animals from 9-13 weeks. Because of the randomisation no difference in duration of exposure between groups was present.
Half of the females were sacrificed at day 21 of pregnancy, the other half carried out their pregnancy. At birth, litter size was culled to eight pups. The offspring was weaned at the age of 3 weeks. At this timepoint, the dams were sacrificed and the control diet was supplied to all offspring. The pups of each nest were grouped according to sex. At 12 weeks of age, oral glucose tolerance as well as blood pressure and susceptibility to infection were measured. Histology of organs relevant to the hypothesis of fetal programming, was also included. The sensitivity of the hypothalamus-pituitary-adrenal-axis (HPA-axis) was determined *in vitro*.
To exclude dependence within nests, one (male) pup of each nest (leading to a total of 8 pups per diet group) was included in the measurements.

2.2 Test animals

In this study we used rats of the Harlan Cpb-WU strain because most experience and knowledge with this rat strain is available at our institute. Upon arrival, the females were about 9 weeks of age, males were about 11 weeks old. The rats were acclimatized for 2 weeks. Animals were housed by sex in groups in macrolon type 2 cages. After mating, females were housed individually. During the acclimatization period, they were fed RMH-GS chow (irradiated breeding and maintenance diet for rats, mice and hamster) in pellets (Hope Farms, Woerden) and tap water ad libitum.
Throughout the study, a constant day/night regime with the light period between 12.00 a.m. and 12.00 p.m was applied. Temperature was 20-24° C and relative humidity 50-70%.

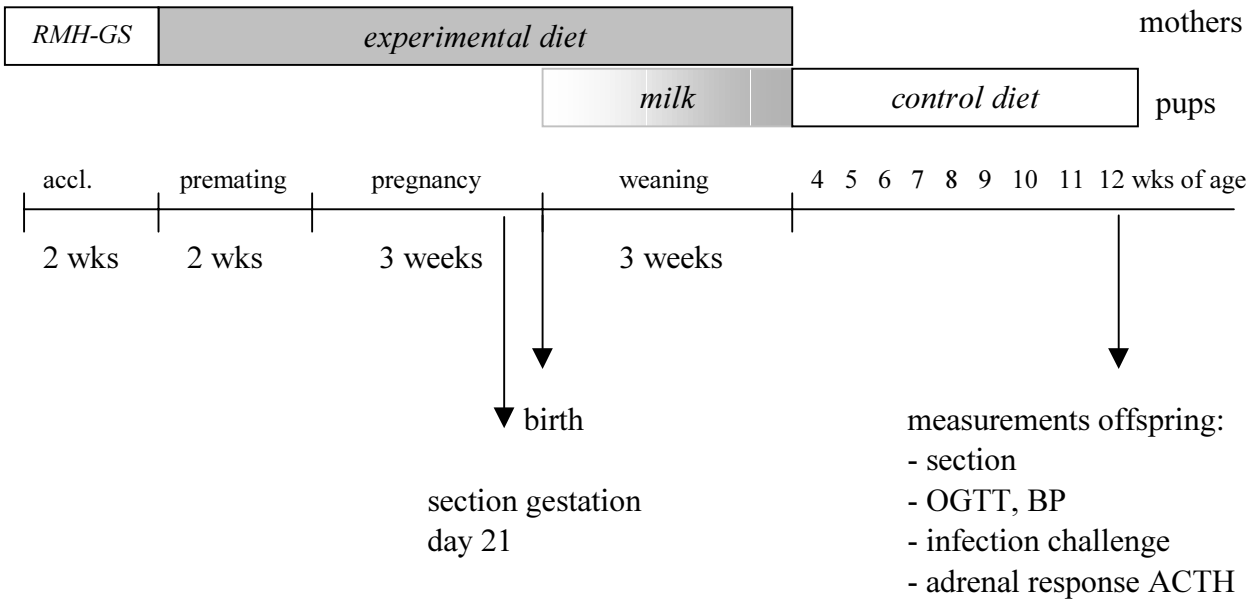

Figure 1: Study design. (OGTT: oral glucose tolerance test. BP: blood pressure)

2.3 Experimental diets

In the premating period, the experimental diets were given to the animals. All diets were semi-synthetic, contained 20% fat, were isocaloric and were pelleted. The fat percentage of 20% was chosen to be able to apply large differences in fat composition between the diets and, secondly, because this percentage is comparable to the human dietary intake of about 40% of energy derived from fat. No additional vitamin E was added to the diets, the natural anti-oxidants in the oil were expected to be sufficient to prevent oxidation of the fatty acids. Diets were manufactured by TNO Nutrition, Zeist. Oil was purchased from ICN Biochemicals, Zoetermeer (coconut oil, safflower oil, soy oil, menhaden fish oil), from Remia, Woerden (soy oil) and from Linora, Rotterdam (flaxseed oil). Due to logistic reasons, in the first batch of experimental diets (420 kg, till weaning age), soy oil from ICN was included in the control diet whereas soy oil from Remia was present in the low fiber diet and in the low protein diet. The different soy oils differed in content of the fatty acid C14:0 and C16:0, which we did not expect to influence our parameters. The second batch of experimental diet (570 kg) existed of only control diet and contained soy oil from Remia. Diets were packed in plastic bags in portions of 100 and 500 grams. Diets were stored at -20º C and each day a freshly thawed portion was offered to the rats. A detailed composition of the diets is given in appendix 1.1 and 1.2.
Peroxide levels of the experimental diets after 3 months of storage and after 24 hours at room temperature were analysed. All dietary analyses were performed by TNO Nutrition, Zeist.

2.4. Measurements from mating until weaning

2.4.1 Body weight and food intake

*** Dams**

During the pre-mating period, the body weight of the dams was measured once per week. Food intake was measured twice weekly in this period.
During pregnancy, body weight was measured on day 0, day 6, day 11, day 16 and day 21. Food intake was monitored daily during pregnancy.
In the weaning period, food intake was measured on postnatal day 1, day 7, day 14 and day 21.

*** Pups**

Body weight of the pups was determined on postnatal day 1, day 7, day 14 and day 21. The average body weight per sex per litter was measured during the weaning period. After weaning at age 3 weeks, the pups were individually marked and individual body weights were monitored weekly.

2.4.2 Autopsy gestation day 21

*** Dams**

Eight dams of each experimental diet group were sacrificed on gestation day 21 to study maternal and fetal parameters. Maternal parameters determined were: number of resorptions, number of corpora lutea, uterus weight and the ratio uterus weight / number of fetuses. About 2 ml. of abdominal blood was collected in EDTA containing tubes and 2 ml. of blood was allowed to coagulate in glass tubes. Within 2 hours after blood collection, plasma was separated from the blood cells by centrifugation (rpm=4200, 503g, t=10 min., 4˚ C) and collected in Eppendorf tubes which were tightly closed under a stream of nitrogen and stored at -80˚C . The remaining RBC were washed two times with isotonic saline, centrifuged (rpm=4200, g=503, t=10 min., 4˚ C) and collected in Eppendorf tubes, in which 2 µl of BHT (butylated hydroxytoluene, antioxidant) was present, and were closed under nitrogen. Serum was collected within 18 hours and stored at -80˚C until further analysis. These materials were stored to be able to determine fatty acid composition if desirable.

*** Fetuses**

Fetal parameters determined were: sex, fetal weight and head - and rump length. Of each litter, even numbered fetuses were fixed in Bouin for pathology and odd fetuses were stained for examination of cartilage and bone. For this purpose, the skin as well as the fetal internal organs were removed from the abdominal cavity. Fetuses were stained during 2-3 days in an Alcian Blue solution. After this period, dehydration of the fetus took place during 5 days in ethanol 100%, after which the fetuses were immersed during 3-4 days in KOH-solution 1%. The next step was staining of the fetuses during 2 days with 0.001% alizarin red S solution in 1% KOH. Subsequently, the fetuses were immersed for 3 consecutive days in destilled aqua with glycerin with increasing concentrations (25%, 50%, 80%). The double stained fetuses were stored in 100% glycerin until analysis (5).

2.4.3 Birth

At postnatal day 1, litter size, sex ratio and birth weight of the pups were recorded, within 24 hours after birth. Litter size was culled to 8 pups (6 m, 2 f).

2.4.4 Weaning at age 3 weeks

At the age of 3 weeks, males and females were housed separately and marked individually. At this time, all groups were switched to the control diet. Dams were sacrificed and an abdominal blood sample was drawn from them; the blood was processed according to the protocol described above (section 2.4.2)

2.5. Measurements in the offspring at the age of 12 weeks.

2.5.1 Oral glucose tolerance test

Male offspring underwent an oral glucose tolerance test at age 12 weeks. The rats were fasted overnight, and glucose (2 g/kg body weight) was administered by oral gavage between 8.30 a.m. and 10 a.m. Blood was collected in heparinized cups (Microvette CB300, Sarstedt Germany) by tail snipping at 0, 5, 10, 20, 30 and 50 minutes after glucose administration. Blood samples were immediately centrifuged and plasma was stored at -80° C. Glucose concentration was measured by a glucose oxidase system (Unimate 5 GLUC HK, Roche), insulin concentration was measured by a RAT insulin ELISA-kit (Mercodia), both within two months after storage.

2.5.2 Blood pressure and heart rate

After 3 days of recovery from the oral glucose tolerance test, blood pressure and heart rate were measured in the same male offspring. The rats were anaesthetized with O_2 , N_2O (1 : 2) mixed with 3% isoflurane (Isoflurane Vaporiser IMS England)
During and after surgery the isoflurane was reduced to 2 % and 1.75%, respectively.
All rats were tracheotomized and an intratracheal cannula was inserted. The intratracheal cannula was connected to a constant volume ventilator (Harvard ventilator Model 683, small animal respirator). Rats were ventilated with a tidal volume of 1 ml.100 g^{-1} at a pump with a frequency of 55/min. The left jugular vein was cannulated and saline, at a rate of 1.5 ml/hr (Harvard apparatus 22 perfusor) was infused to prevent dehydration.
The right femoral artery was cannulated for measuring the arterial blood gas values.
The right artery carotis was cannulated for the measurement of arterial blood pressure and heart rate.
The pressure signal was transduced to Hugo Sachs Electronic (HSE 7806) equipment by means of a Viggo Spectramed disposable pressure transducer and recorded continuously.
The heart rate (HR) was automatically derived from the arterial blood pressure.
Arterial blood gas values (pH, PaO_2 and $PaCO_2$) were determined by using a Ciba-Corning 288 Blood Gas System (Ciba Corning Limited, Houten, The Netherlands).
Throughout the experiments rectal temperature (TMM module, Hugo Sachs Electronic) was monitored and the body temperature was kept between 37 – 39 ^{o}C by placing the animals on heated pads and by radiant heat.

Thirty minutes after registration of basal cardiovascular values N(omega)-L-arginine methyl ester (L-NAME) was cumulatively dosed (0.3, 1, 3, 10, 30 mg/kg) via the left jugular vein. Each dose was infused (1.5 ml/hr) over 10 minutes. L-NAME is a competetive NO-synthase inhibitor. As a result, less NO, which is a vasodilator, is synthesized and, as a consequence, blood pressure rises. The magnitude of the rise in blood pressure is a measure for the quality of the endothelium of the wall in the rat, with the animal having the largest increase having the best endothelium since there was the highest natural NO synthesis in the vessel available for inhibition. Overall, a decrease in endothelial function is associated with a decrease of the quality of the vessel wall.

2.5.3 Infection with *Listeria monocytogenes*

Rats were intravenously injected with 0.5 ml. of a *Listeria monocytogenes* (strain L242/73 type 4b) solution in the tail vein for assessing bacteria clearance. Four days after the infection with activated *Listeria,* spleen was excised, weighed and stored in 10% w/v suspensions of PBS. A period of four days was chosen; that both innate effects on phagocytosis and effects on acquired immunity, if any, would be revealed.
After homogenization, serial dilutions were plated to determine the viable counts of *L. monocytogenes*. *L. monocytogenes* were cultured as described by Ruitenberg and Van Noorle Jansen (6). The number of colony forming units (CFUs) of Listeria bacteria in the spleen was determined, which gives an estimate of the severity of infection and the effectiveness of the clearance.
For practical and technical reasons, the experiment was performed on two different days, As a consequence, infection dose differed between these two days (5×10^5 bacteria/ml and 1.2×10^6 bacteria/ml). Data of the experimental diet groups is expressed as a percentage of the data of the control group to be able to compare data from the two experimental days. When the data from the different days are compared, no major differences could be shown, which is a justification for combining the data from the two infection days. Combining the data results in sufficient number of animals for statistics.
In this experiment a relatively low, far from lethal bacterial dose was used.
The protocol was performed according to SOP PAT-090 and PAT-091 with the exception of the egg passage. Animals with injection error were excluded in the analysis.

2.5.4 Autopsy

After determination of body weight, rats were anesthesized (CO_2:O_2 = 2:1). A blood sample was drawn from the abdominal aorta. Serum and plasma was obtained and stored at -80°C until analysis. The following organs were excised, weighed and fixed with phosphate buffered 4% formaldehyde: pancreas, liver, kidneys, adrenal glands, spleen, brains, thymus, heart, pituitary. Moreover, a small sample of abdominal fat and muscle was taken and stored at -80ºC. A liver lobe and the brains were quickly snap-frozen in liquid N_2 after weighing.

2.5.5 Pathology

For histological examination 5 µm paraplast sections of the adrenals, heart, kidney, liver, pancreas, pituitary, spleen and thymus were cut and stained with hematoxylin and eosin (HE).

For morphometry of the pancreas the number and surface area of the islets of the pancreas were measured with the analySIS soft imaging system (SIS, Münster, Germany).

2.5.6 Corticosterone response after ACTH stimulation *in vitro*

In this test, ACTH-responsiveness of an adrenocortical cell culture was measured according to Goverde et al. (7;8). The procedure consists of three parts: 1) the isolation of adrenal cells and the preparation of the purified cells, 2) incubation of the suspensions with ACTH, 3) measurement of corticosterone concentration.
On a test day, one animal at a time was taken to the section room and decapitated as soon as possible. After decapitating, the adrenals were excised and were collected in ice-cold Krebs-Ringer buffer (KRB). After each decapitation, all equipment was rinsed and cleaned thoroughly to avoid stress in the next rat and to avoid contamination. Four male Wistar rats of each group were decapitated. The adrenals were transferred on ice to the lab. Adrenal cells were dissociated by means of collagenase, after which the tissue was disrupted and washed two times to obtain a crude cell suspension. This crude suspension was purified by layering 1 ml. portions upon 8 ml. 5% BSA in KRBCa. After 30 minutes, the upper layer was removed and the number of cells per ml. 5% BSA, which by then contained the purified cells, was calculated. When necessary, the cell suspension was diluted to obtain 4 x 10^4 cells/ml. The cells were pre-incubated at 37º C for 60 minutes, prior to the addition of different concentrations of ACTH to the cell suspension. The tubes were incubated for two hours in a shaker. All observations were made in duplicate, unless stated otherwise. Samples were frozen at -20º C until the measurement of corticosterone. For this purpose, dichloormethane was added to the tubes. After extraction by shaking for 2 minutes, the tubes were centrifuged and the aqueous phase was removed and a sulphuric acid – ethanol reagent was added. After 1 minute of shaking, the upper organic phase was aspirated and the fluorescence was measured after 50 minutes using a spectrophotofluorometer at 470 nm and 530 nm. Process corticosterone was used as the standard.
In order to obtain sufficient cell numbers, we pooled cells from four animals to make one cell suspension; one curve therefore reflects a cell suspension from 4 animals (8 adrenals). We had 8 animals per group, except in the control group (n= 5-7). The experimental procedure is described in appendix 14.

2.5.7. UDP-hexosamine metabolites content in rat muscle.

Muscle tissue was obtained from the m.adductor magnus to determine UDP-hexosamine metabolites content. It has been suggested that the hexosamine pathway is involved in the pathogenesis of insulin resistance (41).
Tissues were homogenized with homogenization buffer in a dismembrator using liquid nitrogen. After centrifugation at 60 000g for 15 min at 4º C, 1600 pmol of UDP-mannose and 25 pmol of UDP-xylose were added to the supernatant as internal standards. The samples were deproteinized and diluted with KH_2PO_4 and applied to solid-phase extraction columns. After the columns were washed (with KH_2PO_4 solutions differing in molarity and pH), the UDP-sugars were eluted with KH_2PO_4. UDP-sugars were separated and quantified by HPLC and ultraviolet detection at 262 nm. For more details, one is referred to the article by Span et al. (41).

2.6 Statistical analysis

Data of all groups were compared to the control group. All data were reported as means ± SEM. The data were analyzed using a one-way analysis of variance (ANOVA) when appropriate. When significant interactions were noted, Dunnett's test was used for determination of difference with the control group. A p-value of <0.05 was considered to imply a significant difference.

3. Results

3.1 Dietary composition

In appendix 1.1, the composition of the experimental diets is given; in appendix 1.2 the macronutrient composition is shown. The high protein diets consisted of ± 15.5% protein; the low protein diet contained 8.3% protein. Fat content was ± 18.4%. Carbohydrate content and energy content was comparable between diet groups.
In appendix 1.3 the fatty acid composition of the fat in the experimental diets is given. The flaxseed oil diet was high in linolenic acid (53.6% of total fatty acids), the menhaden fish oil diet contained a considerable amount of eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA). The safflower oil diet was high in the unsaturated linoleic acid (71.6 % of total fatty acids) whereas the coconut oil diet contained a substantial proportion of the saturated fatty acids (42.3% lauric acid). Fatty acid composition of the low fiber diet and low protein diet, both based on soy oil, was nearly identical to the fatty acid composition of the control diet (also based on soy oil but from a different supplier) except for the proportion of myristic acid (14:0) and palmitic acid (16:0). The control diet did not contain palmitic acid, the low fiber and low protein diet did not contain myristic acid.
Peroxide level after 3 months of storage was highest in the safflower oil diet (appendix 1.4): 35.9 mmol O/kg diet compared to 2.4 mmol O/kg diet in the control diet.

3.2 Maternal parameters

3.2.1 Acclimatisation period after arrival

After randomisation, at the end of the acclimatisation period, there was no difference in average body weight of the females between diet groups (data not shown).

3.2.2 Premating period

In the first week on the experimental diet, the increase in body weight of the fish oil group was significantly lower than the control group (resp. 2.8 g vs 9.3 g; $p<0.001$, n=24, appendix 2.1); in the second week no differences were found. Over the 2-wk period as a whole, no significant differences in body weight increase were found. During the premating period, daily food intake was monitored four times (day 1, 3, 8, 10). On day 1 and day 3, the fish oil group consumed significantly less food (12.4 g vs 15.7 g on day 1; 11.1 g vs 13.5 gram in the control group on day3; $p <0.001$, appendix 2.1). On day 8, the coconut oil group consumed significantly less food compared to the control group (12.9 g vs 14.7 g; $p>0.01$). Total food consumption during the premating period (cumulative of the 4 measurements) was significantly lower in the fish oil group (49.5 g vs 57.5 g; $p<0.0001$).
Body weight was ± 205 g in all groups at the end of the premating period.

3.2.3 Food intake and body weight gain during pregnancy

Total food consumption during pregnancy was significantly lower in the fish oil group compared to the control group (339.4 g vs 366.4 g; n=16, $p<0.05$). This lower food intake was clearly seen from gestation day 7-11 (figure 2).

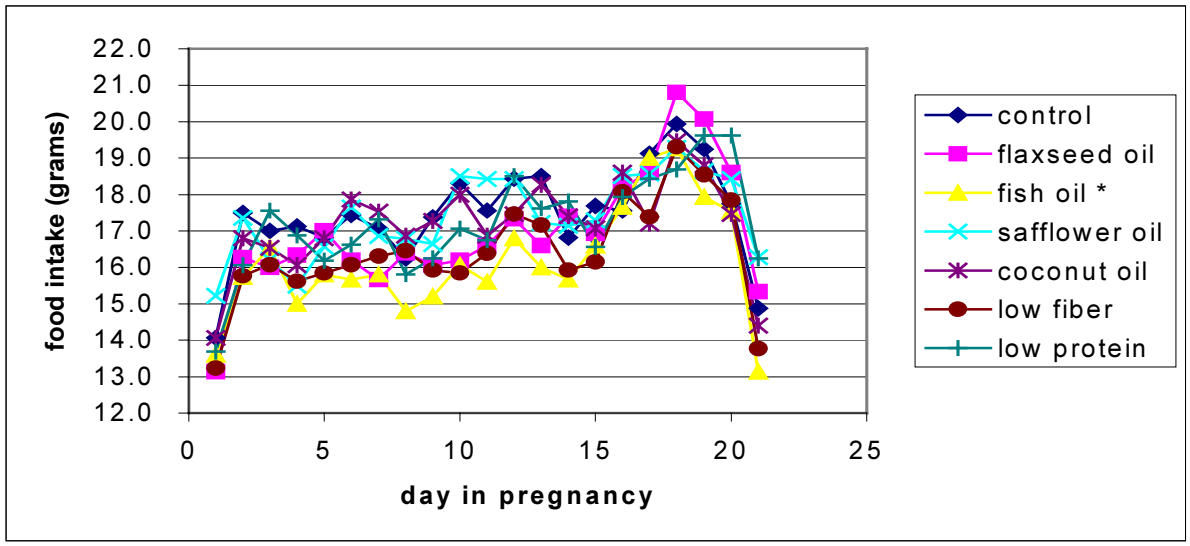

*Figure 2: Food intake during pregnancy of mothers fed different diets; n=16 per diet group. Data are expressed as mean and compared to the control group. *: p<0.05*

Total body weight gain during pregnancy was significantly lower in the fish oil group (116.7 g compared to 135.2 in the control group); this was primarily caused by a significantly lower body weight gain during day 17-21 of pregnancy (figure 3; appendix 2.2).

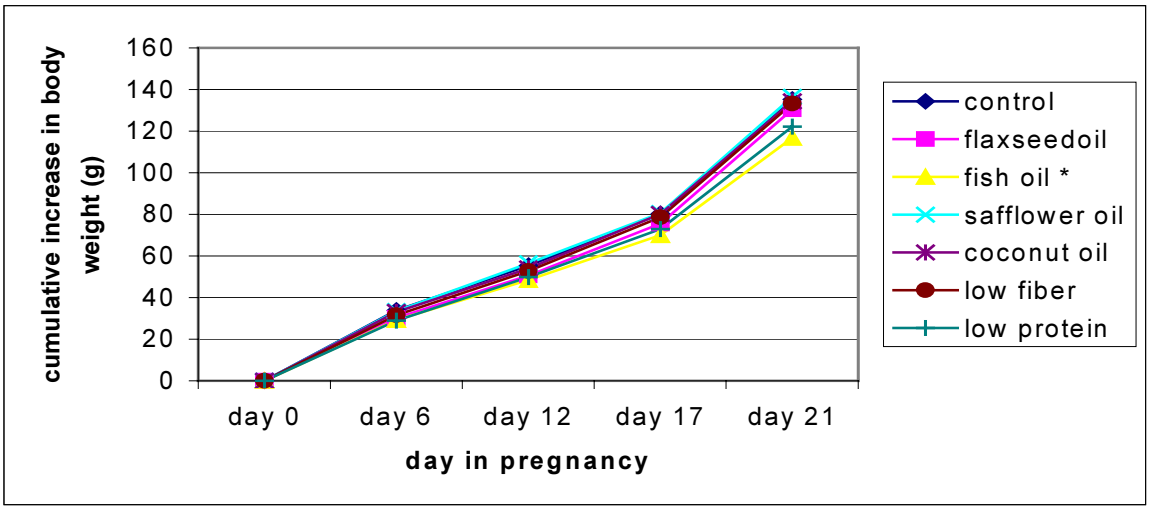

*Figure 3: Cumulative body weight gain of dams fed different diets during pregnancy; n=16. Data are expressed as mean and compared to the control group. *: p < 0.05*

3.2.4 Gestation duration

No signicant effect of the diet on gestation duration was found (table 1).

Table 1: Numbers of animals per group according to pregnancy duration

pregnancy duration:	control	flaxseed oil	fish oil	safflower oil	coconut oil	low fiber	low protein
22 days	1					1	1
23 days	8	8	6	8	10	7	9
24 days			3				

3.2.5 Food intake during lactation

With respect to food intake, on day 8, 15 and 22 postnatally, mothers on the low protein diet consumed significantly less food compared to the control group. On day 15, food intake of the menhaden fish oil group was significantly lower than that of the control group (41 ± 3 compared to 55 ± 2 in the control group, appendix 2.3), see figure 4.

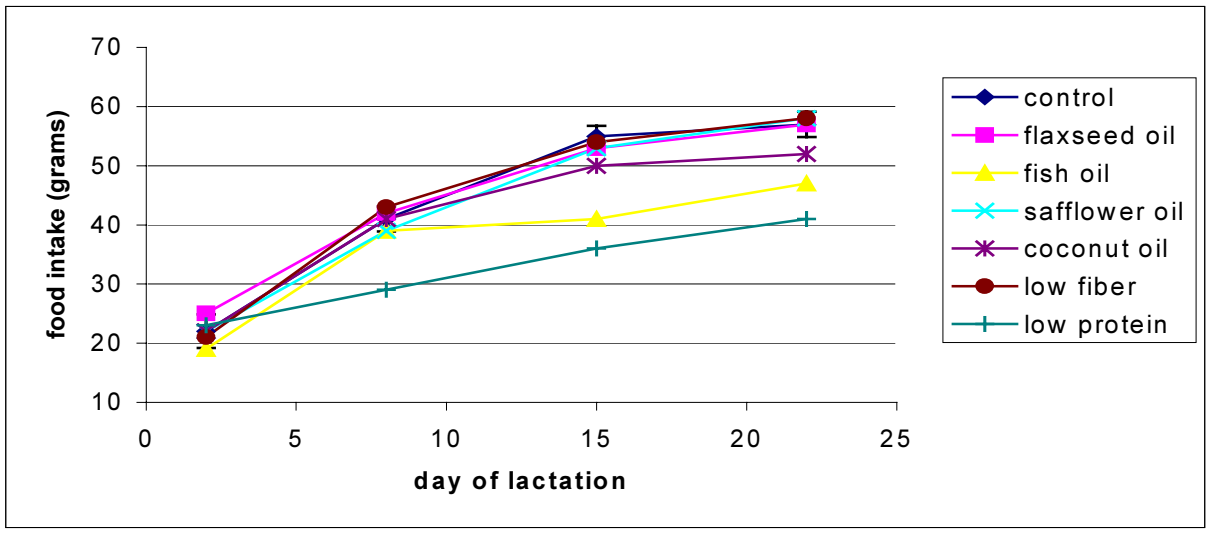

*Figure 4: Food intake during lactation of mothers fed different diets; n=8. Data are expressed as mean ± SEM (control group) and compared to the control group. *: p<0.05*

3.2.6 Number of fetuses, litter size and survival

The number of fetuses was lowest in dams fed the fish oil diet (appendix 3.1). This same group also had a significantly smaller litter size compared to the control group (7.8 pups vs. 12.1 pups, appendix 3.7). Strikingly, litter size was smaller than the average number of fetuses in this group at gestation day 21 (7.8 pups vs. 10.5 fetuses).
Survival was lowest in the fish oil group: 70% of the pups reached the 3-week old age. Survival of pups fed the coconut oil diet *in utero* was 85%; of the pups fed a low protein diet *in utero*, survival was 79% up to age 3 weeks.

3.3 Fetal parameters

3.3.1 Fetal weight

Fetal weight was significantly higher in the low fiber fed group (4.47 g vs 4.14 g, n=64 vs n-96, p<0.001) for males and females (male fetuses: see figure 5). Females of the safflower oil fed group were significantly heavier than the control group (4.2 g vs 3.95, n= 33, p<0.05, appendix 3.3).

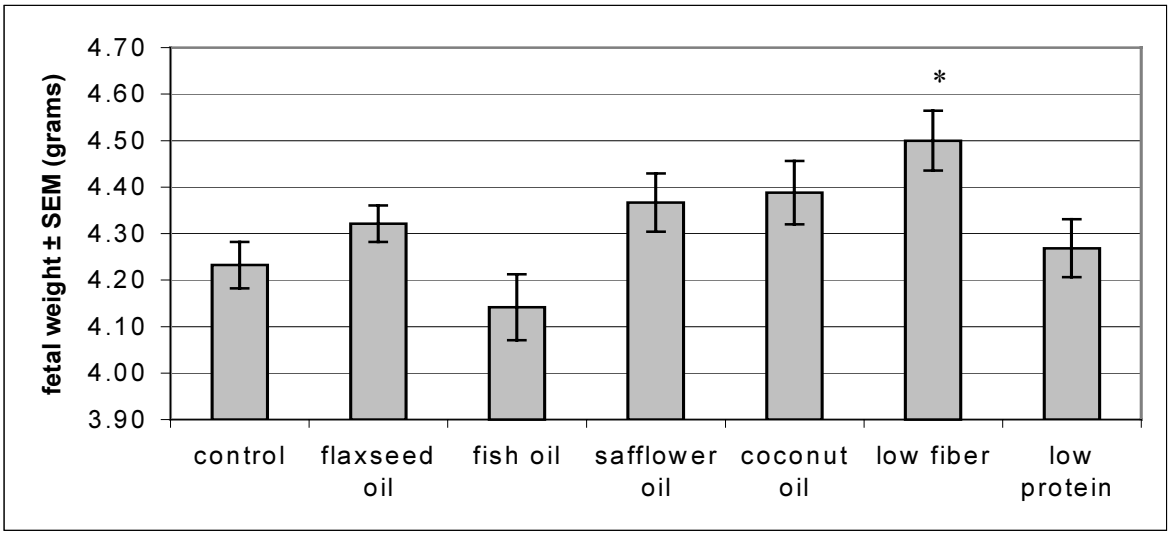

*Figure 5: Fetal weight of male fetuses on gestation day 21 of dams fed different diets during pregnancy (control:n=63; flaxseed:n=48; fish oil:n=42; safflower oil: n=35, coconut oil:n=47; low fiber:n=35, low protein: n=53). Data are expressed as mean ± SEM and are compared to the control group. *: p<0.05*

3.3.2 Placental weight

Placental weight of male and female fetuses of the low protein group was significantly lower compared to the control group (0.49 g vs 0.56 g, n=96, p<0.001, appendix 3.4). In figure 6, mean placental weight of male fetuses is shown. In appendix 3.5, the ratio fetal / placental weight is given.

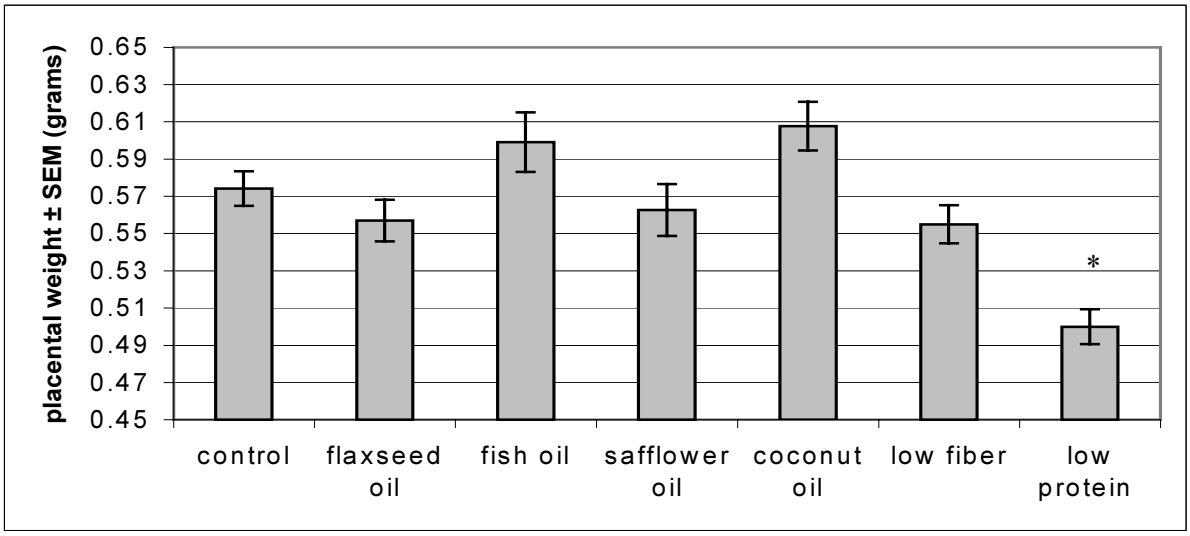

*Figure 6: Placental weight of male fetuses on gestation day 21 of dams fed different diets during pregnancy (control:n=63; flaxseed:n=48; fish oil:n=42; safflower oil: n=35, coconut oil:n=47; low fiber:n=35, low protein: n=53). Data are expressed as mean ± SEM and are compared to the control group. *: p<0.05*

3.3.3 Growth parameters

The ratio body length/head length was significantly higher in fetuses of mothers fed the fish oil diet (2.2 vs 2.1, n=82 vs n=96, appendix 3.6)

3.3.4 Live fetuses, resorptions, corpora lutea, sex ratio, uterus weight

No differences in number of fetuses per dam, corpora lutea or resorptions were found between dams. Uterus weight and ratio uterus weight/number of fetuses were not different between the dams (appendix 3.7).

3.3.5 Examination of the fetuses gestation day 21

- Pathological examination

In appendix 4 results of the gross pathological examination of the fetuses on gestation day 21 are given. A remarkable observation was the high incidence of thymus abnormalities in all groups in which thymus tissue was present under the parathyroid gland.

- Skeletal analysis

Appendix 5 shows the results of the skeletal analysis. In all groups except the flaxseed oil group and the low fiber group an unexpectedly high number of fetuses was found in which one or two ribs were not fused with the sternum. Since the fatty acid composition of the low fiber diet was similar to the control diet, the difference in incidence cannot be attributed to the type of fatty acids. The low fiber group also had the highest incidence of ossified front proximal bone (appendix 5.3) which would point to a higher degree of ossification. In accordance with this is a significantly higher fetal weight in this group compared to the control group (appendix 3.1).

3.4 The offspring

3.4.1 Visual inspection of the pups

Pups of the mothers fed the coconut oil diet showed less and diffuse hairgrowth up to about 8 weeks.

3.4.2 Body weight increase in the offspring

3.4.2.1 Males

Male offspring of mothers fed the fish oil diet were significantly lighter from birth until age 12 weeks, compared to pups of the control group. Male pups of mothers fed coconut oil were significantly smaller at 1 and 2 weeks of age. A maternal low fiber diet resulted in heavier pups from age 5 weeks until age 9 weeks (appendix 6.1). Finally, the low protein diet *in utero* and throughout weaning was found to reduce body weight from week 2 up to age 12 weeks (figure 7).

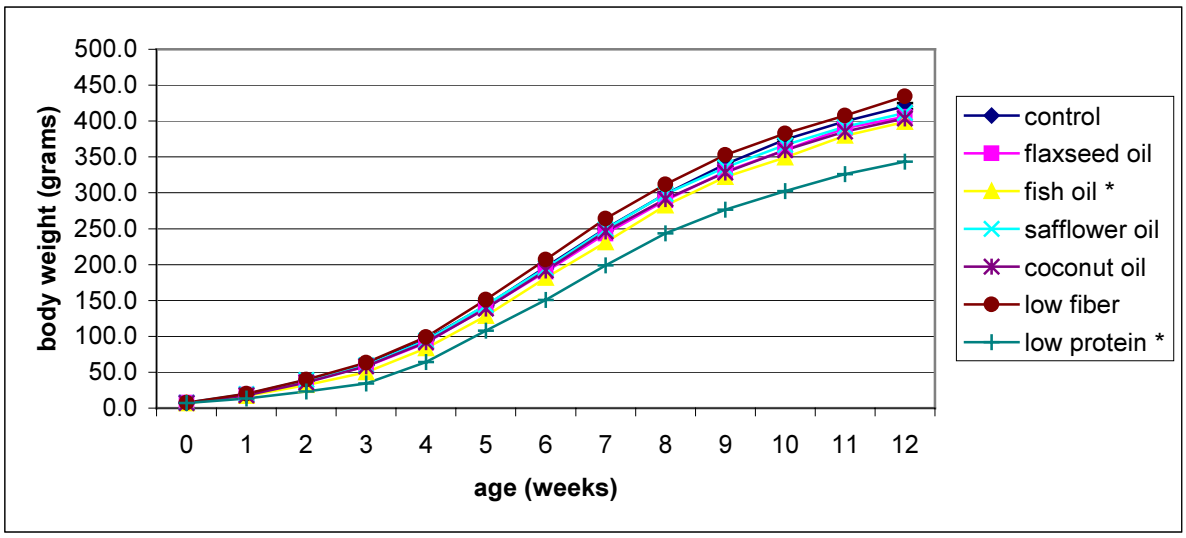

Figure 7: Increase in body weight in male offspring fed different diets in utero. Pups of the fish oil and low protein group gained significantly less body weight up to 12 weeks compared to the control group (control: n=45; flaxseed: n=44, fish oil: n=32; safflower oil: n=36, coconut oil: n=34; low fiber: n=39; low protein: n=36). Data are expressed as mean ± SEM (control group) and are compared to the control group.: p<0.05*

3.4.2.2. Females

A maternal fish oil diet resulted in significantly lighter pups from age 2 weeks till age 7 weeks, compared to the control group (appendix 6.2). The female offspring of mothers who were protein restricted during pregnancy and weaning is also significantly smaller from 7 to 14 weeks of age.

3.4.3 Oral glucose tolerance test

At 12 weeks of age, no differences in fasting insulin or glucose concentrations were found between male offspring of different groups during an oral glucose tolerance test compared to the control group. After an oral glucose load, insulin level rose to statistically significantly higher levels in the coconut oil group compared to the control group up to 10 minutes after the load (760.2 pmol/l compared to 409.8 pmol/l in the control group, figure 8, appendix 7.1).

Glucose level was significant lower at 30 minutes after the load in the coconut oil group compared to the control group (figure 9, appendix 7.2). This clearly shows the time-lag effect of insulin on decreasing glucose levels.

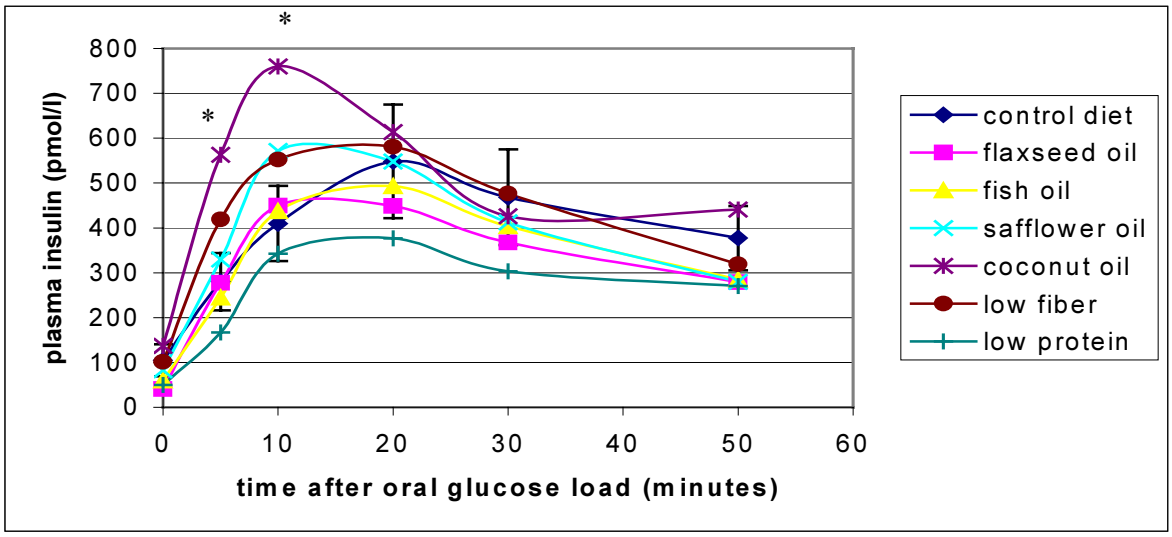

Figure 8: Plasma insulin response after oral glucose load in male offspring of 12 weeks old fed different diets in utero and during weaning ; n=8. Data are expressed as mean (± SEM for control group) and are compared to the control group.
**: p<0.05*

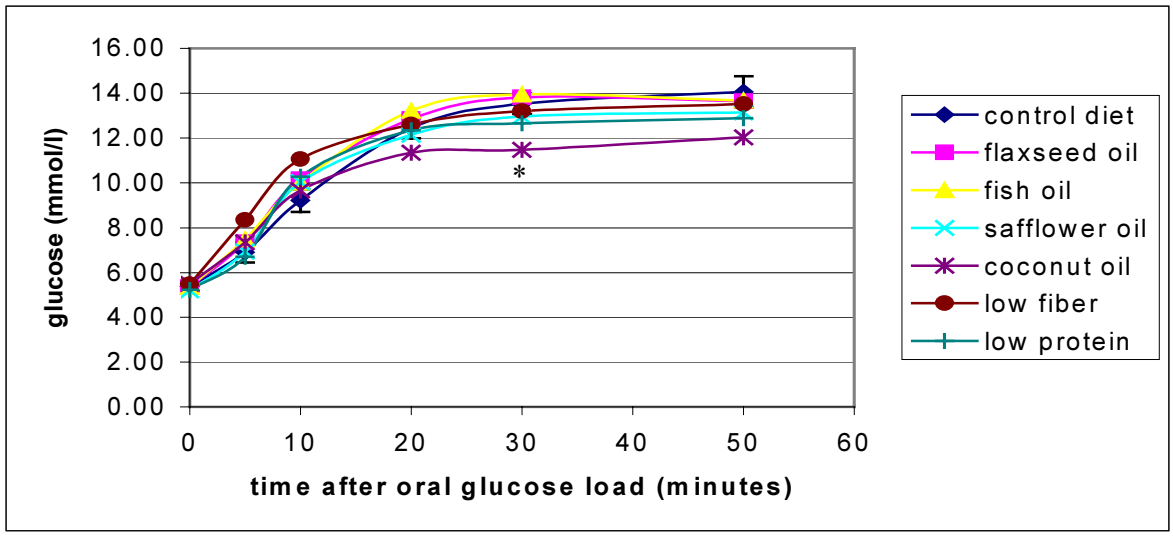

*Figure 9: Plasma glucose response after oral glucose load in male offspring of 12 weeks old fed different diets in utero and during weaning; n=8. Data are expressed as mean (± SEM for control group) and are compared to the control group. *: p<0.05*

3.4.4 Blood pressure and heart rate

No significant difference in systolic blood pressure (figure 10), in diastolic blood pressure (figure 11) or in heart rate was found compared to the control group (data shown in appendix 8). Remarkable was the low systolic and diastolic blood pressure in the low protein group. In figure 12 and 13, the effect of the infusion of the NO synthase inhibitor L-NAME on systolic and diastolic blood pressure is shown. No effects of maternal diet upon systolic or diastolic blood pressure response to L-NAME was noted in the offspring.

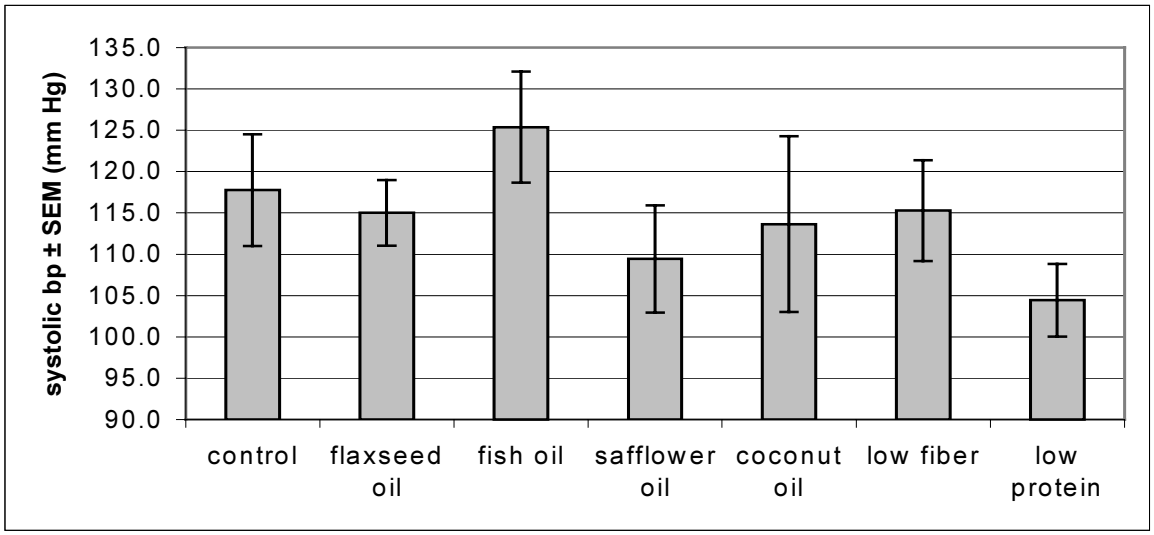

Figure 10: Basal systolic blood pressure (i.e. during physiologic saline injection) in male offspring of 12 weeks old fed different diets in utero and during weaning ; n=8. Data are expressed as mean ± SEM and are compared to the control group.

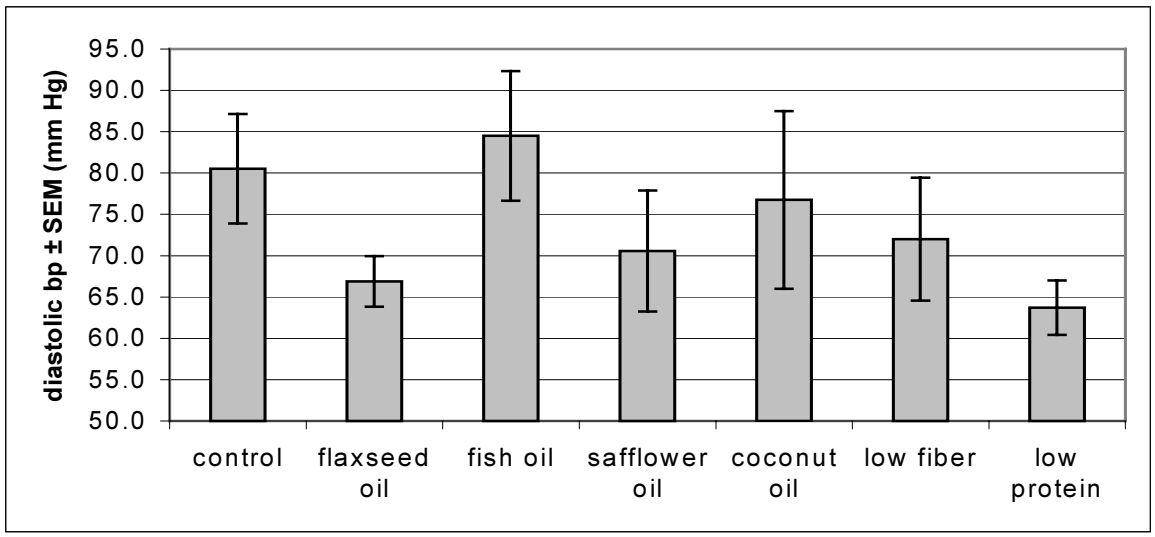

Figure 11: Basal diastolic blood pressure (during physiologic saline injection) in male offspring of 12 weeks old fed different diets in utero and during weaning ; n=8. Data are expressed as mean ± SEM and are compared to the control group.

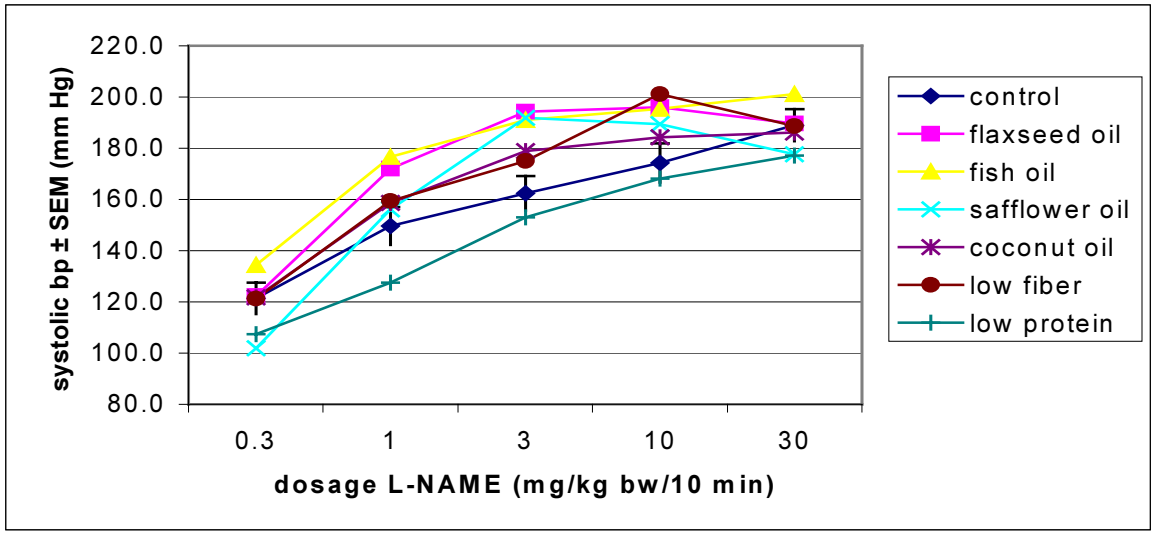

Figure 12: Systolic blood pressure after an L-NAME infusion in male offspring aged 12 weeks fed different diets in utero and during weaning, n=8. Data are expressed as mean (± SEM for control group) and are compared to the control group.

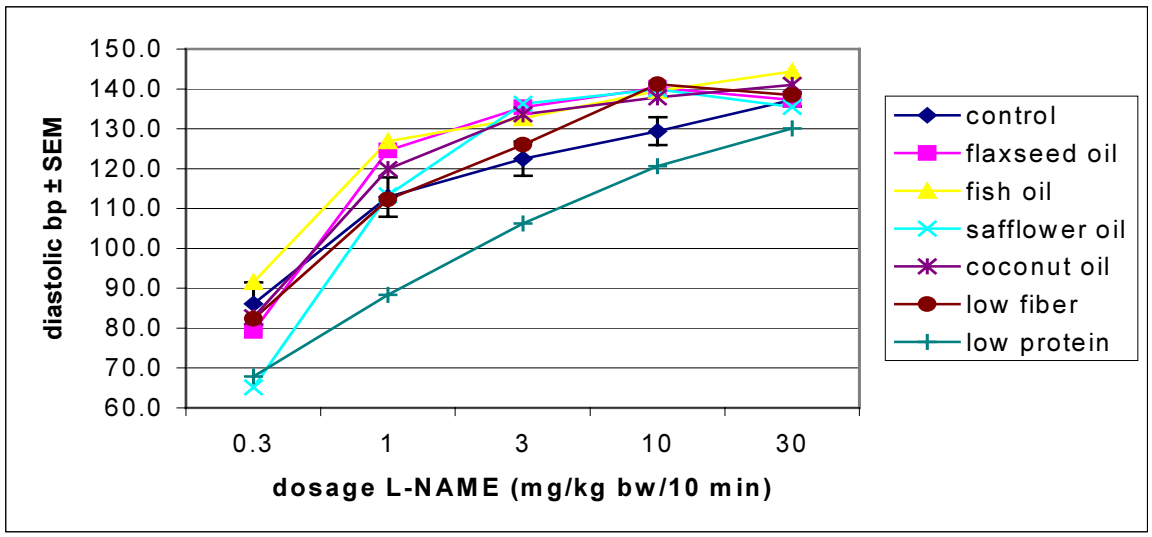

Figure 13: Diastolic blood pressure after a L-NAME infusion in male offspring aged 12 weeks fed different diets in utero and during weaning, n=8. Data are expressed as mean (± SEM for control group) and are compared to the control group.

3.4.5 Infection with *Listeria monocytogenes*

In figure 14, the number of colony forming units in the spleen four days after a *Listeria* infection is shown. An increased number of colony forming units indicates a reduced immunity.

The results show that the number of colonies was lowest in the flaxseed diet, although this did not reach statistical significance. A diet low in dietary fiber increased the number of bacterial colonies in the spleen compared to the control group, although not statistically significantly. Individual data are given in appendix 9.

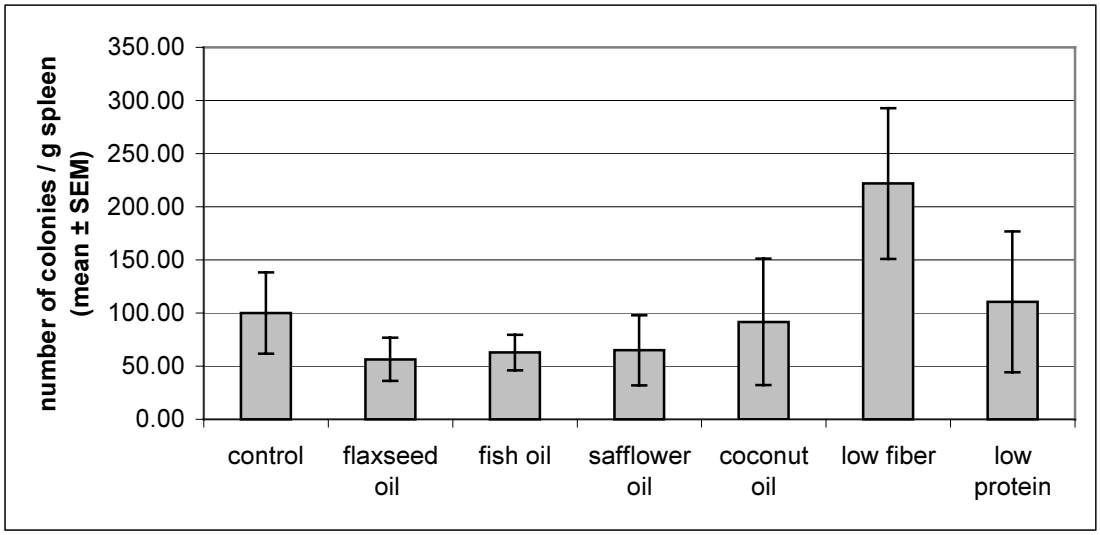

Figure 14: Number of colony forming units in the spleen after a Listeria infection (control: n= 8, flaxseed: n=7, fish oil: n=7, safflower oil: n=8, coconut oil: n=7, low fiber: n=7, low protein: n=4). Data are expressed as mean ± SEM and are compared to the control group.

3.4.6 Autopsy

3.4.6.1 Macroscopy

At autopsy no macroscopical abnormalities were observed. In appendix 10, absolute and relative organ weights are given for each diet group (average ± SEM). The offspring of mothers fed a fish oil diet during pregnancy and weaning had a significantly lower brain weight (1.66 g compared to 1.90 gram in the control group). Maternal protein restriction resulted in significantly smaller kidneys, compared to the control group (2.16 g vs 2.67 g, respectively). No statistically significant differences were observed between the relative organ weights of the experimental groups and the control group.

3.4.6.2 Microscopy

All histological lesions are summarized in appendix 11. In the control group a varying degree of vacuolisation (minimal to strong) of the zonae fasciculata and reticularis of the adrenals was seen. All control animals showed a small number of basophilic tubules in the kidney. In the liver of all control animals small lymphocytic aggregates were observed. In half the number of animals a slight to moderate quantity of glycogen was present in the periportal hepatocytes. In half the number of animals in the control group a small focus of pancreatic acinar cells with vacuolisation and inflammation was present. In the thymic cortex very small foci of starry sky macrophages were present. In heart, pituitary gland and spleen no changes were observed.

Except for the above mentioned lesions, also present in all other diet groups, other histopathological changes occurred in the respective different diet groups. Focal hyperplasia of the zona glomerulosa in the adrenals occurred in only one animal of the menhaden fish oil group. The presence of minimal to strong nephrocalcinosis in the outer stripe was restricted to only females of the menhaden fish oil, safflower, coconut oil, low fiber and low protein diet groups. Minimal nephritis occurred in a single animal in the flaxseed, menhaden fish oil, coconut and low fiber diet group, while slight nephritis was present in two animals of the low protein group. In one animal of the menhaden group strong nephropathy occurred consisting

of many basophilic tubules with thickened basement membrane, dilated tubules with proteinaceous casts and moderate nephritis. In some animals of the fish oil -, safflower oil -, coconut oil -, low fiber - and low protein diet, periportal lipid vacuolisation was noticed.
It was observed that the incidence of single cell vacuolisation and inflammation in the pancreas in the control group was nearly the same as in all other groups. However, the lesions were incidentally accompanied by focal slight degeneration or atrophy of acini, or by fybrotic islets. A small inflammatory lesion in a single animal occurred in the low protein group.

3.4.6.3 Morphometry

From morphometry (table 2, individual data are shown in appendix 12) it was evident that the volume estimate of the islets in the pancreas was statistically significantly decreased in the low protein group versus the control group. The number of pancreatic islets in the low protein group was not significantly different from the control number. However, the number of relatively large islets (area > 0.03 mm^2) was significantly reduced in the low protein group versus the control group. In the coconut oil group the number and volume estimates were not different from those in the control group.

*Table 2: Morphometry of the pancreas of control, low protein and coconut oil group (individual data are shown in appendix 12). Control group: n=9; coconut oil group: n=6; low protein group: n= 9. Data are shown as mean ± sd and are compared to the control group. *: p<0.05*

	volume of islets (mm^2)	n islets / 100 mm^2	n islets > 0.03 mm^2 / 100 mm^2
control group	1.39 ± 0.36	69 ± 14	21.0 ± 3.2
low protein group	0.96 ± 0.33 *	60 ± 13	6.8 ± 5.2 *
coconut oil group	1.09 ± 0.36	76 ± 17	6.5 ± 3.4 *

3.4.7 Corticosterone response after ACTH stimulation *in vitro*

Results from the *in vitro* adrenal sensitivity to ACTH are shown by diet group (figure 15a - 15g and per experimental day (figure 16a -16g). Data are shown in respectively appendix 13.1 and 13.2.
Variation in response between and within groups was large. In some cases, the response of the adrenal cells to ACTH was minimal. One might expect, with increasing ACTH concentration, a slightly increasing corticosterone concentration curve. When we look at the results according to testday, the response of corticosterone to ACTH seems to be less variable. This might indicate a methodological (i.e. protocol-derived) rather than a biological reason for the variation (figure 16a-16g).

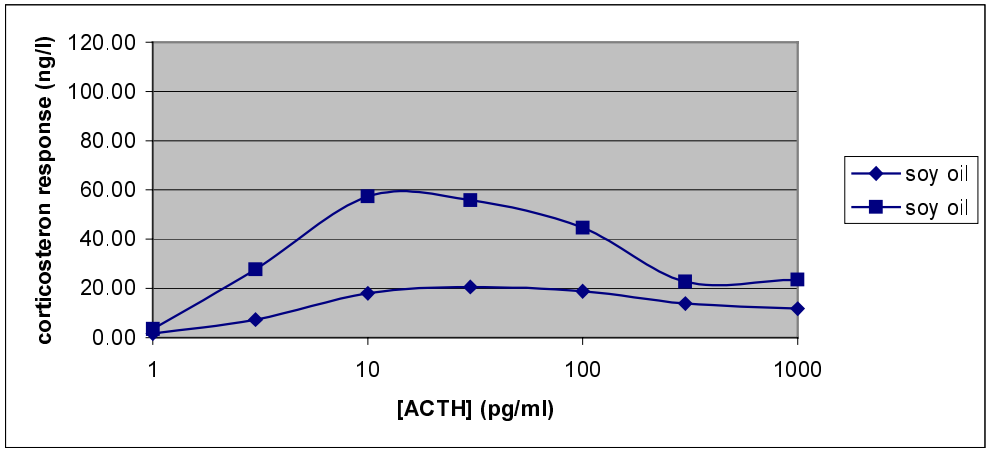

Figure 15a: Corticosterone response of adrenal cells to increasing [ACTH] in vitro of male rats aged 12 weeks fed the control diet (soy oil) in utero and during weaning. Data are expressed as mean, n=4 per curve.

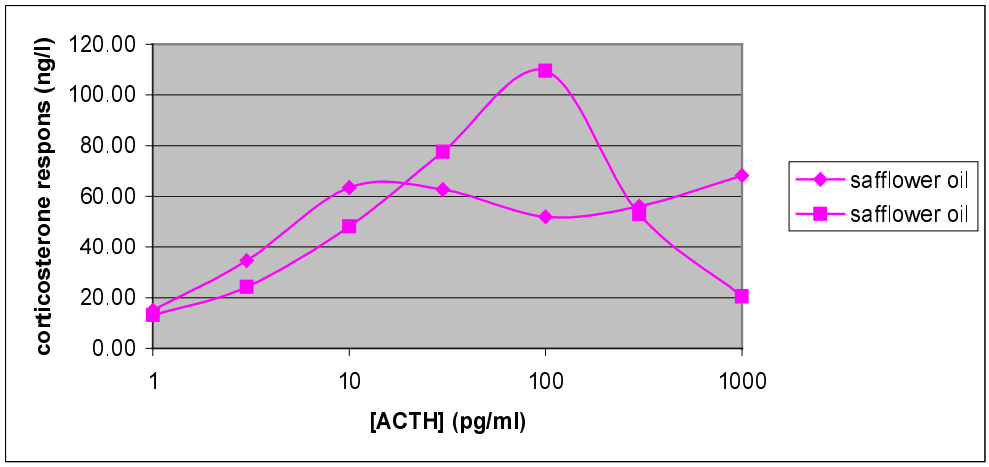

Figure 15b: Corticosterone response of adrenal cells to increasing [ACTH] in vitro of male rats aged 12 weeks fed a safflower oil diet in utero and during weaning. Data are expressed as mean, n=4 per curve.

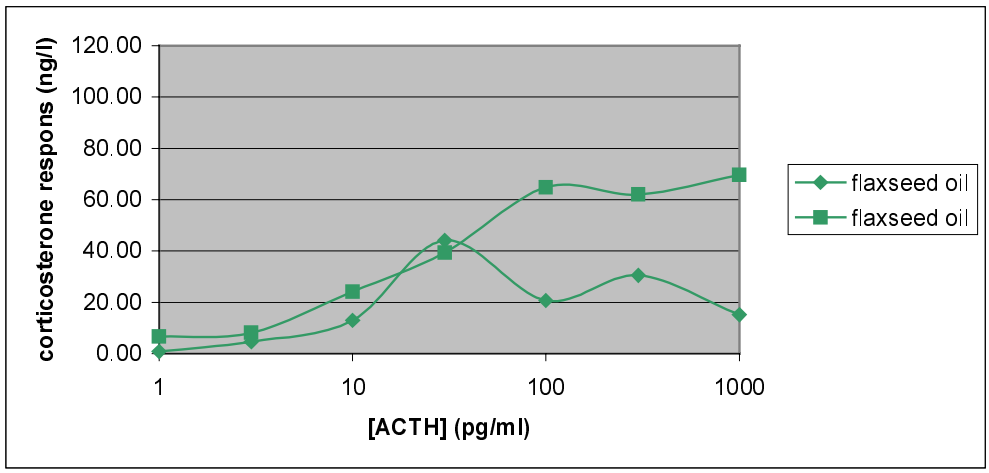

Figure 15c: Corticosterone response of adrenal cells to increasing [ACTH] in vitro of male rats aged 12 weeks fed a flaxseed oil diet in utero and during weaning. Data are expressed as mean, n=4 per curve.

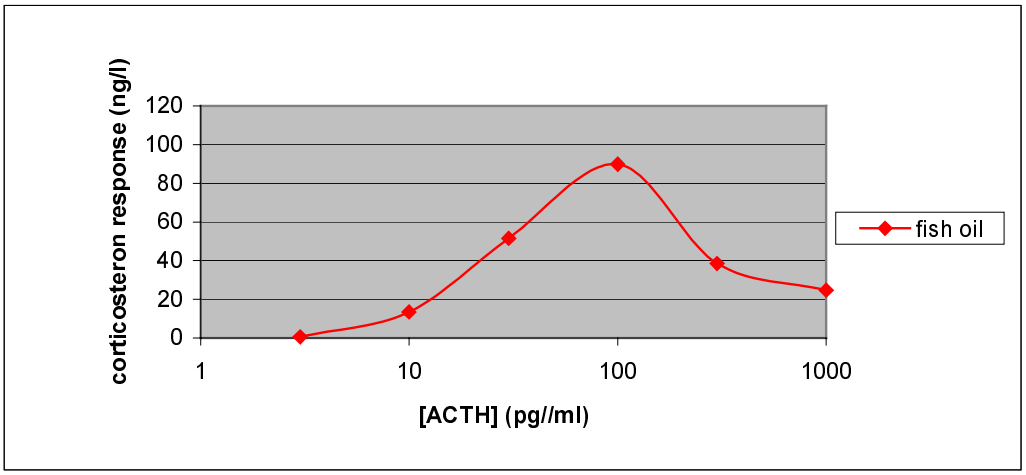

Figure 15d: Corticosterone response of adrenal cells to increasing [ACTH] in vitro of male rats aged 12 weeks fed a fish oil diet in utero and during weaning. Data are expressed as mean, n=4 per curve.

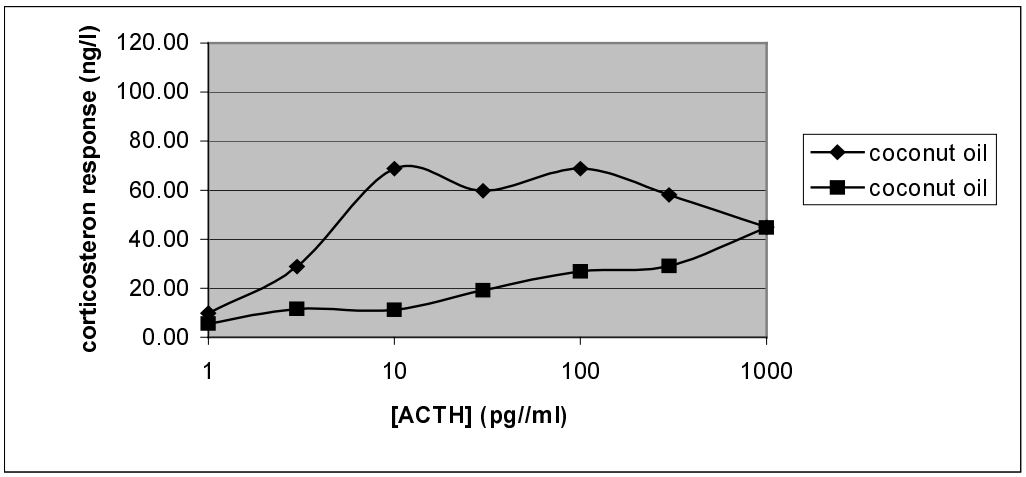

Figure 15e: Corticosterone response of adrenal cells to increasing [ACTH] in vitro of male rats aged 12 weeks fed the coconut oil diet in utero and during weaning. Data are expressed as mean, n=4 per curve.

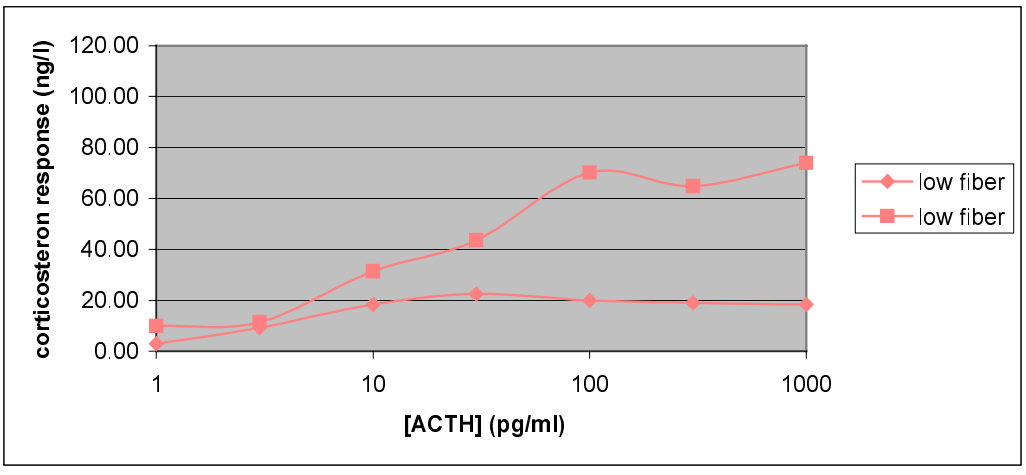

Figure 15f: Corticosterone response of adrenal cells to increasing [ACTH] in vitro of male rats aged 12 weeks fed the low fiber diet in utero and during weaning. Data are expressed as mean, n=4 per curve.

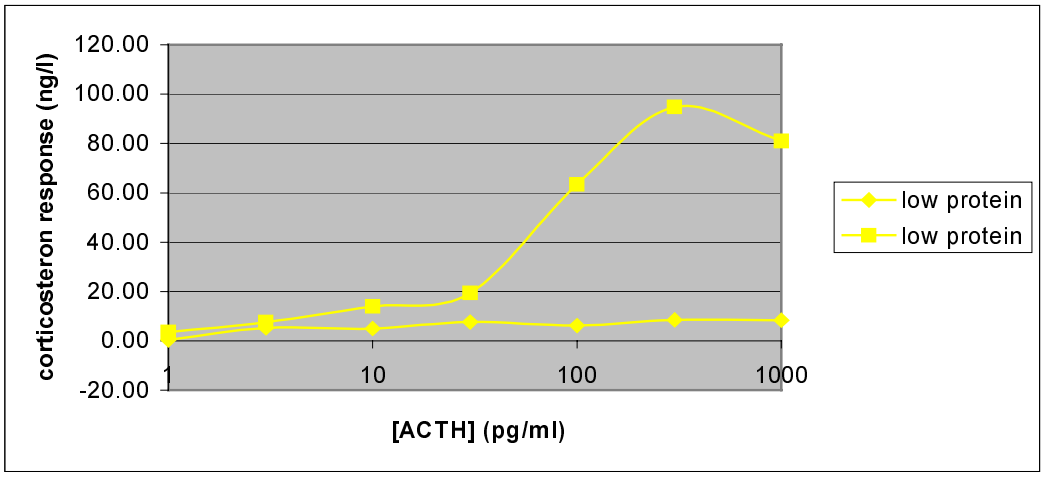

Figure 15g: Corticosterone response of adrenal cells to increasing [ACTH] in vitro of male rats aged 12 weeks fed the low protein diet in utero and during weaning. Data are expressed as mean, n=4 per curve.

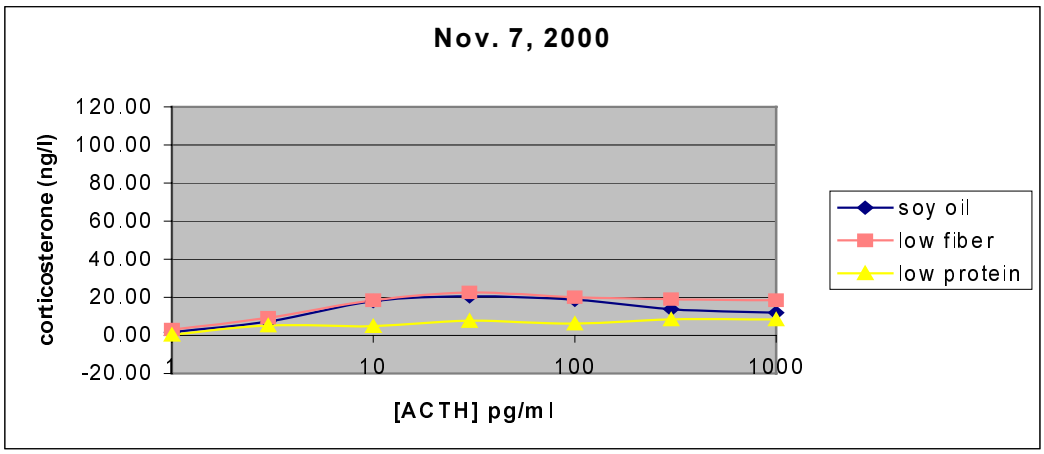

Figure 16 a: Corticosterone response of adrenal cells to increasing [ACTH] in vitro of male rats aged 12 weeks of different groups on different experimental days. Data are expressed as mean, n=4 per group.

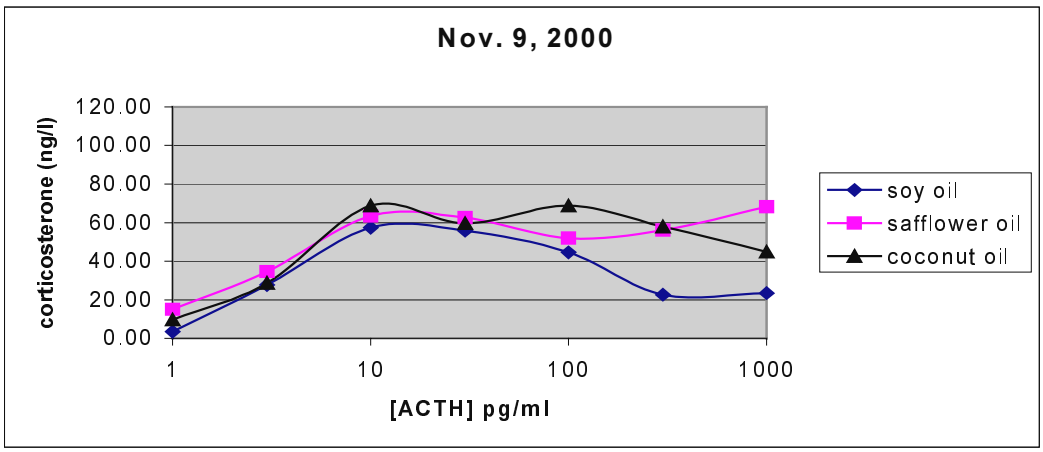

Figure 16 b: Corticosterone response of adrenal cells to increasing [ACTH] in vitro of male rats aged 12 weeks of different groups on different experimental days. Data are expressed as mean, n=4 per group.

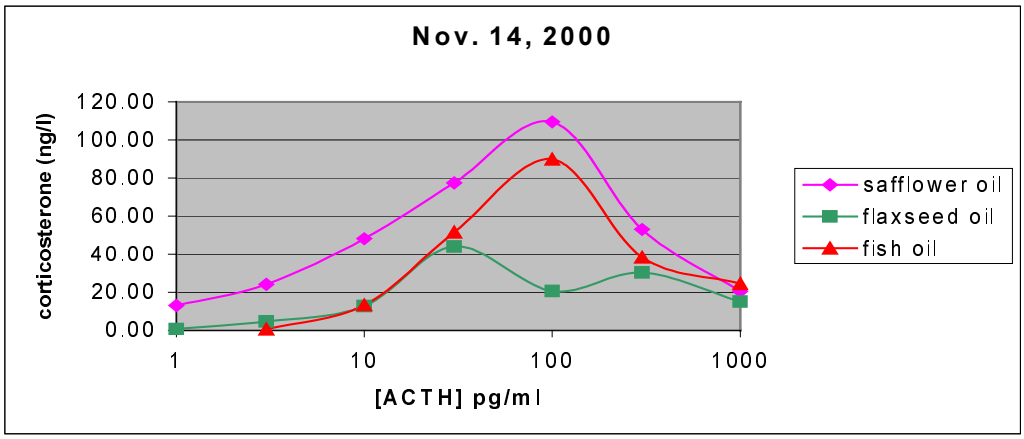

Figure 16 c: Corticosterone response of adrenal cells to increasing [ACTH] in vitro of male rats aged 12 weeks of different groups on different experimental days. Data are expressed as mean, n=4 per group.

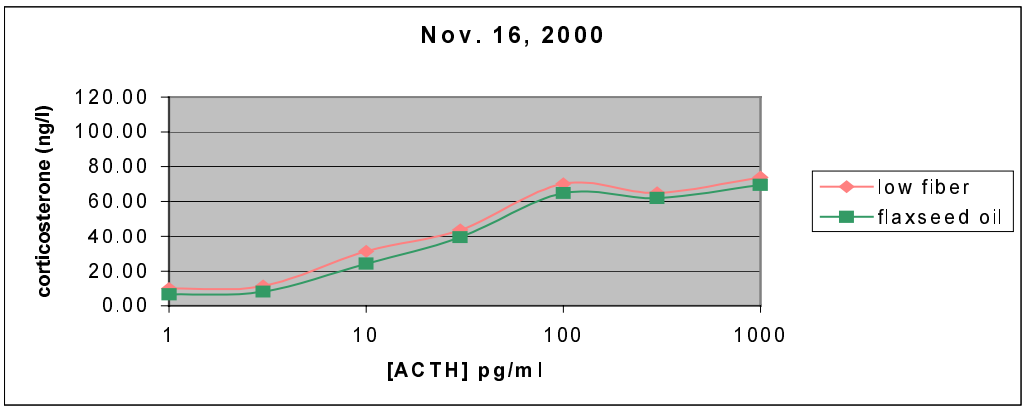

Figure 16 d: Corticosterone response of adrenal cells to increasing [ACTH] in vitro of male rats aged 12 weeks of different groups on different experimental days. Data are expressed as mean, n=4 per group.

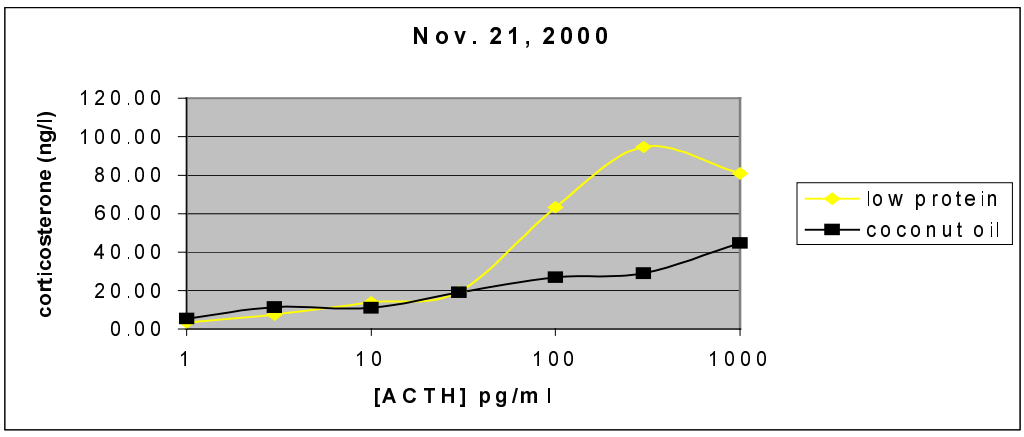

Figure 16 e: Corticosterone response of adrenal cells to increasing [ACTH] in vitro of male rats aged 12 weeks of different groups on different experimental days. Data are expressed as mean, n=4 per group.

3.4.8. Hexosamine pathway intermediates in rat muscle

In table 3, results of measurements of metabolites of the hexosamine pathway are given. No significant differences were found compared to the control group.

Table 3: Metabolites of the hexosamine pathway in rat muscle. Gal: UDP-galactose, GalNAc:UDP-N-acetyl-galactosamine, Glc: UDP-glucosamine, GlcNAC: UDP-N-acetyl-glucosamine.

		UDP nmol / ml homogenate			
	n	Gal	GalNAc	Glc	GlcNAC
control group	4	1.155 ± 0.2	4.49 ± 0.4	1.03 ± 0.5	9.99 ± 1.0
flaxseed oil	4	1.38 ± 0.3	5.12 ± 0.5	0.37 ± 0.1	10.57 ± 0.9
fish oil	4	1.14 ± 0.2	4.88 ± 0.3	0.34 ± 0.1	10.45 ± 0.3
safflower oil	4	1.73 ± 0.5	5.12 ± 0.5	1.48 ± 0.6	10.64 ± 0.8
coconut oil	4	0.96 ± 0.2	4.33 ± 0.3	0	8.62 ± 0.8
low fiber	4	0.77 ± 0.1	4.90 ± 0.6	0.11 ± 0.1	9.80 ± 1.5
low protein	4	1.07 ± 0.3	4.57 ± 0.3	0.041 ± 0.1	8.73 ± 1.0

4. Discussion

Recent epidemiological and experimental data have indicated a possible relationship between maternal diet and the susceptibility to adult disease in the offspring.
In this study, the effects of diets with different fatty acid composition on parameters of chronic disease were studied: blood pressure as a risk factor for cardiovascular disease, oral glucose tolerance as an early marker for diabetes mellitus and immune respons to an infection as a marker for infectious disease sensitivity. The proposed central mechanism underlying the association between maternal diet and disease risk in the offspring is an alteration in the sensitivity of the hypothalamus-pituitary-adrenal axis (HPA-axis). Therefore, we measured the corticosterone response to ACTH *in vitro* to detect differences in HPA-axis sensitivity.

Diet
Offspring of the mothers fed the coconut oil diet showed diffuse and reduced hairgrowth up to about age 8 weeks. Skin lesions and reduced hair growth are symptoms of an essential fatty acid (EFA) deficiency (23). The essential fatty acids are alpha-linolenic acid (omega-3 fatty acid) and linoleic acid (omega-6 fatty acid). The reduced hairgrowth raises the question whether the 1% soy oil added to preclude EFA deficiency in the dams supplied sufficient EFA to prevent a EFA-deficiency. Most evidence concurs that an alpha-linolenic deficient diet for one or more generations does not influence adult reproductive function, newborn body weight or subsequent growth. Increased mortality of the offspring from n-3 fatty acid deficient rodents in the first few days after birth has been reported in some (19;20) but not all studies (20). Analysis of the fatty acid composition of blood or brain samples may give an indication of the absorption of the diet and may give more insight into whether the dams were EFA deficient. These analyses are to be performed in the nearby future.
With respect to the rancidity of the diet, the peroxide level was the highest in the safflower oil diet (35.9 mmol O/kg compared to 2.4 mmol O/kg in the control diet). An upper limit for an acceptable peroxide level has not been established, although a peroxide level below 10 is regarded as appropriate (dr. M. Appel, TNO Nutrition, *personal communication*). It is unlikely that the high peroxide level in the safflower oil diet after 3 months of storage has influenced our results.

Placental - and fetal weight
At gestation day 21, fetuses of dams fed the low fiber diet were significantly heavier than the fetuses of the control group. We speculate that the higher glycemic index caused by the higher intake of carbohydrates in this group, as a compensation for the low fiber content of the diet, may have triggered higher insulin levels in the dam during and after a meal. Maternal hyperglycemia results in fetal hyperglycemia and, as a consequence, the fetal pancreas will excrete more insulin (38). Since insulin acts synergistically with growth hormone to promote growth (37), fetal growth may be stimulated.
However, the low protein diet contained slightly more carbohydrates than the low fiber diet whereas fetal weight was similar to the control group. Apparently, protein content of the maternal diet seems to play a more important role in determining fetal growth than carbohydrate content. Although fetal weight was not affected in this group, placental mass of fetuses on gestation day 21 was significantly lower compared to the control group.

Snoeck et al. (9) have used a similar protein restricted diet and found no difference in fetal weight or placental weight of fetuses at gestational day 21.

Postnatal growth

The lower body weight of the offspring fed the low protein diet and the fish oil diet *in utero* and during weaning might be related to the lower food consumption of the mothers in these groups during pregnancy or weaning. This lower food consumption may have influenced milk production or quality which might compromise growth later in life.
In the literature, controversial data exists about effects of a low protein diet *in utero* on postnatal growth. Using similar protein restricted diets *in utero*, Langley-Evans et al. showed that the offspring was significantly heavier at the age of weaning (10). Two other studies by this same group showed no effect of a 9% dietary protein content on body weight at age 9 weeks (14) and on postnatal growth up to age 21 weeks (15). Dahri et al. (13) also found no difference in body weight in male and female pups up to 12 weeks of age fed a 8% protein diet during pregnancy compared to the control group. However, when the exposure to the low protein diet continued into adulthood, a significantly reduced body weight gain was seen in this group compared to the control group and the group fed a normal diet after birth. In line with these results of growth retardation when exposure continued after pregnancy is our observation of a lower body weight in the low protein group with continued exposure during weaning. It is tempting to postulate that, besides maternal diet *in utero*, diet during the early postnatal period may also be of importance in determing growth rate later in life.

Blood pressure

No significant differences in systolic and diastolic blood pressure were found in male offspring at age 12 weeks. Remarkable is the absence of hypertension in our study in the offspring fed the low protein diet *in utero* and during weaning, whereas hypertension has been seen in studies from the research group by Langley-Evans et al. They found a highly significant elevation of systolic blood pressure in female rats at age 4 weeks (10) and in male offspring aged 9 weeks (15) after a similar protein restricted diet *in utero*.
We have several remarks regarding the low instead of the high blood pressure observed in the offspring of the low protein group in our study. Firstly, this study is unique with respect to the period of exposure to the diet (*in utero* and during weaning) compared to the other studies.We wanted to include the early postnatal period because in this period the brain and pancreas develops in the rat. Therefore, we expected programming effects of maternal nutrition to continue during this period. From our results it seems that early postnatal diet may have an attenuating or modulating role on the *in utero* programmed hypertension.
Secondly, we measured blood pressure by means of canulation of the artery carotis and under anesthesia instead of using the tail cuff method in conscious animals, applied in other studies. It is possible that the anesthesia may have influenced blood pressure.
Thirdly, we used a 20% fat diet whereas other research groups used a 10% fat diet. It is unlikely that the higher fat content might have concealed any elevation of blood pressure since a high fat diet in rats has been shown to elevate blood pressure via a weight-gain related mechanism (32, 33). However, since food intake in rats is determined by caloric content of the diet which is in turn determined mainly by fat content, a low fat diet should lead to a higher intake of food with, consequently, a higher intake of various associated nutrients. This difference in food intake is confirmed when we compare data on food intake from our study

with those of Langley-Evans et al. (16, 39). Considering this different intake over various associated nutrients, it might be possible that the overall balance and amount of nutrients rather than macronutrient composition as, for example protein content, may determine blood pressure in the offspring. It has been suggested that the composition of the carbohydrate source and the overall methionine content may also be of critical importance in determining whether or not a maternal diet leads to hypertension in the offspring (36).

Glucose tolerance and morphometric data of the Langerhans islets

Plasma insulin rose to a significantly higher concentration in the coconut oil group up to 10 minutes after the oral glucose load. However, this higher insulin concentration did not lead to a significantly lower glucose concentration. This might indicate that the animals of the coconut oil group might be slightly insulin resistent. However, insulin resistent animals should have a higher fasting insulin concentration with similar glucose concentrations, which was not the case. Moreover, twenty minutes after the oral glucose load, insulin concentrations were similar to the control group, so the feedback system seems to be still adequate.
Since the number of large islets of Langerhans in the pancreas was reduced in this group whereas the number and volume of islets were not affected, we speculate that this hypersecretion of insulin is preceding an exhaustion of the Langerhans islets. A deterioration in glucose tolerance with ageing in animals malnourished *in utero* and during weaning has been shown by Garofano et al. (40).

The offspring fed a 9% casein diet *in utero* had the lowest plasma insulin concentrations after an oral glucose load, while having similar glucose concentrations. Therefore, these animals seem to react most sensitive to the glucose load. A study by Dahri et al. (13) did not find any difference in blood glucose level after an oral glucose load in animals at the age of 10 weeks fed a 8% protein diet *in utero*. Langley (13) found a significant lower glucose level at some points after an intravenous dose of glucose in female rats aged 9 weeks fed 9% protein *in utero;* insulin concentrations were not determined.
The morphometric data show that in these animals of the low protein group the volume estimate of the pancreatic islets was statistically significantly decreased at an adult age, mainly caused by a reduction in the number of large islets. The same results were found by Petrik et al. (17) and Dahri et al.(15). Since the insulin response did not differ from the control group, the remaining volume area of islets seems to compensate for the lower volume estimate of islets.

Organ histopathology

Most of the histopathological phenomena observed in this study are very common in rats. Observed lesions occurred more or less randomly in the various experimental diet groups and had such a low incidence that they were considered as not treatment related.

Resistance to a Listeria infection

Our data on the number colony forming units (CFUs) in the spleen after an infection with *Listeria monocytogenes* show no statistically significant difference in resistance of diet groups compared to the control group.

No relationship between prenatal diet, the presence of aberrant thymus tissue (i.e. thymus tissue under parathyroid) and immune function in later life could be shown in contrast to some studies which showed abnormal thymus development and impaired immune function after prenatal exposure to exogenous agents (34,35).

Corticosterone response of adrenal cells to ACTH *in vitro*
Our data on responsivity of adrenal cells *in vitro* to an increasing ACTH dose are inconclusive due to 1) a large variation within and between groups and 2) limited data in each group. The variation may be partly protocol-derived and may be partly due to natural variation between individual rats and groups. In addition, we did not train the rats to minimize stress before decapitation. An other point which might have influenced the variation in stress responses is the fact that complete litters were housed together. It is known that plasma corticosteroid levels of rats housed in groups are higher and more variable than individually housed rats due to social structures within the group (18).

Overall, our study showed that prenatal and early postnatal diet influences physiological and morphological parameters of the offspring. Maternal diet affected fetal and placental weight and body weight of the pups. At an adult age, glucose tolerance was altered with permanent histological changes in the volume of pancreatic islets.
Effects were not consistently present in one diet group and, moreover, were at some points not in accordance with findings from other studies. This may in part be caused by the use of different methods or protocols. In oncoming studies, we will improve methodology to investigate the role of maternal diet in fetal programming of adult disease.

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Appendices

Appendix 1: Dietary composition

1.1 Composition of the experimental diets

g/100 g diet	Control Diet	Flaxeed oil diet	Fish oil diet	Safflower oil diet	Coconut oil diet	Low fiber diet	
ingredients:							
sour casein	18	18	18	18	18	18	9
L-cystein	0,3	0,3	0,3	0,3	0,3	0,3	0,3
wheat starch	51,45	51,45	51,45	51,45	51,45	**55,76**	**60,17**
cellulose	5	5	5	5	5	0,5	5
choline bitartrate	0,25	0,25	0,25	0,25	0,25	0,25	0,25
Ain93G minerals	3,09	3,09	3,09	3,09	3,09	**4,05**	3,09
KH2PO4	-	-	-	-	-	-	0,28
Ain 93 vitamins	1,1	1,1	1,1	1,1	1,1	1,14	1,1
soybean oil	20	-	-	-	-	20	20
coconut oil	-	-	-	-	19	-	-
safflower oil	-	-	-	20	1	-	-
flaxseed oil	-	20	-	-	-	-	-
menhaden oil	-	-	20	-	-	-	-

1.2 Macronutrient composition (analysed by TNO-Nutrition, Zeist)

diet	protein (g/100 g)	fat (g/100 g)	carbohydrates (g/100 g)	energy (kcal/100 g)
control diet	15,8	18,4	42,5	399
flaxseed oil diet	15	18,5	43,4	400
fish oil diet	15,5	18,1	44,5	403
safflower oil diet	14,9	18,1	43,5	397
coconut oil diet	15,4	18,4	43,3	400
low fiber diet	15,5	18,4	45,8	411
low protein diet	8,3	18,5	48,7	395

1.3 Fatty acid composition of the experimental oils (expressed as percentages of total fatty acids, analysed by TNO-Nutrition, Zeist)

fatty acid		control diet	flaxseed oil diet	fish oil diet	safflower oil diet	coconut oil diet	low fiber diet	low protein diet
C8:0	caprylic acid	-	-	-	-	5,6	-	-
C10:0	capric acid	-	-	-	-	4,8	-	-
C12:0	lauric acid	0,1	0,2	0,2	0,1	**42,3**	0,2	-
C14:0	myristic acid	11	-	6,9	-	17,1	-	-
C16:0	palmitic acid	-	5,9	17,9	7,2	10,3	10,5	10,4
C16:1 (n-7)	palmitoleic acid	0,2	0,1	9,2	0,2	0,1	0,2	0,1
C17:0		0,1	-	1,8	-	0,1	0,2	-
C18:0	stearic acid	4,5	4	3,3	2,5	4,7	-	-
C18:1 (n-9)	oleic acid	23,1	18	13	15,4	7,2	26,5	26,3
C18:2 (n-6)	linoleic acid	51,4	17	2,1	**71,6**	6,8	48,3	48,9
C18:3 (n-3)	linolenic acid	5,9	**53,6**	3,3	0,5	0,3	-	-
C20:0	arachidic acid	0,4	0,2	0,3	0,3	-	0,5	0,4
C20:4 (n-3)	arachidonic acid							
C20:5 (n-3)	EPA	-	-	**11,7**	0,2	-	-	-
C22:0	behenic acid	0,3	0,2	0,2	-		0,5	0,4
C22:1 (n-9)	erucic acid	-	-	?	-	-	-	-
C22:4 (n-6)	adrenic acid	-	-	0,2	-	-	-	-
C22:5 (n-6)	osbond acid	-	-	2,4	-	-	-	-
C22:6 (n-3)	DHA	-	-	**13,1**	-	-	-	-
C24:1 (n-9)	tetracosenoic acid	-	-	0,4	0,2	-	-	-

fatty acid	control diet	flaxseed oil diet	fish oil diet	safflower oil diet	coconut oil diet	low fiber diet	low protein diet
not identified	2,6	0,6	10,7	1	0,6	1,1	1,4

NB:
C4-C8: short chain fatty acids
C10-C14: medium chain fatty acids
C18-C22: long chain fatty acids

1.4 Peroxide levels in the experimental diet after 3 months of storage (analysed by TNO Nutrition, Zeist)

Experimental diet	Peroxide level (mm O / kg)
Control diet	2,4
Flaxseed oil diet	2,5
Fish oil diet	6,6
Safflower oil diet	35,9
Coconut oil diet	5,8
Low fiber diet	1,7
Low protein diet	1,3

Appendix 2: Food intake and body weight data of dams

2.1 Premating period

group	control	flaxseed oil	fish oil	safflower oil	coconut oil	low fiber	low protein
n	24	24	24	24	24	24	24
Body weight gain (g ± SEM)							
week 1	9.3 ± 2.5	9.0 ± 2.1	2.8 ± 1.1 *	21.3 ± 1.2	11.0 ± 2.0	12.5 ± 1.0	9.8 ± 1.3
week 2	7.6 ± 1.3	8.5 ± 1.7	7.1 ± 1.2	5.6 ± 1.2	3.7 ± 1.2	8.3 ± 1.5	5.3 ± 1.2
Food consumption (g ± SEM)							
day 1	15.7 ± 0.6	15.7 ± 0.5	12.4 ± 0.5 *	15.7 ± 0.4	15.0 ± 0.5	15.0 ± 0.4	16.2 ± 0.4
day 3	13.5 ± 0.6	13.0 ± 0.6	11.1 ± 0.5 *	12.5 ± 0.4	12.9 ± 0.4	12.5 ± 0.5	13.5 ± 0.5
day 8	14.7 ± 0.4	14.5 ± 0.6	13.3 ± 0.5	13.6 ± 0.5	12.9 ± 0.5 *	13.5 ± 0.4	13.9 ± 0.4
day 10	13.6 ± 0.6	14.4 ± 0.4	12.7 ± 0.4	13.6 ± 0.4	13.0 ± 0.4	13.1 ± 0.5	13.3 ± 0.5
total average food consumption per animal on 4 different days	57.5 ± 0.6	57.6 ± 0.6	49.5 ± 0.5 *	55.4 ± 0.4	53.8 ± 0.4	54.1 ± 0.4	56.9 ± 0.4

2.2 Pregnancy

group	control	flaxseed oil	fish oil	safflower oil	coconut oil	low fiber	low protein
n	16	16	16	16	16	16	16
Body weights gain (g ± SEM)							
day 1- day 6	33.9 ± 1.2	30.1 ± 1.1	29.3 ± 1.3	33.6 ± 1.8	33.1 ± 1.5	31.5 ± 1.9	28.9 ± 1.6
day 7-day 11	21.5 ± 0.8	20.5 ± 0.8	19.2 ± 1.4	22.9 ± 1.5	20.9 ± 1.3	21.3 ± 1.3	20.9 ± 1.2
day 12- day 16	24.9 ± 1.4	25.0 ± 1.3	21.5 ± 1.6	24.1 ± 1.5	26.2 ± 1.3	25.8 ± 1.6	23.2 ± 1.1
day 17- day 21	54.9 ± 2.9	54.9 ± 2.0	46.7 ± 1.9 *	55.8 ± 2.2	54.1 ± 2.7	54.6 ± 2.1	49.1 ± 1.4
day 1- day 21	135.2 ± 4.5	130.5 ± 3.1	116.7 ± 4.0 *	136.4 ± 5.3	134.2 ± 4.3	133.2 ± 5.3	122.0 ± 3.1
Food consumption (g ± SEM)							
day 1- day 6	99.9 ± 2.2	94.9 ± 1.4	92.3 ± 1.8	98.5 ± 1.8	98.1 ± 2.4	92.6 ± 2.6	97.0 ± 2.6
day 7-day 11	86.6 ± 1.7	80.9 ± 1.7	77.5 ± 1.5 *	87.2 ± 2.0	86.5 ± 2.6	80.9 ± 2.2	83.2 ± 1.8
day 12- day 16	89.0 ± 2.1	86.5 ± 1.8	82.7 ± 1.5	88.6 ± 2.6	88.8 ± 2.5	84.8 ± 1.9	88.4 ± 2.0
day 17- day 21	90.9 ± 2.4	93.4 ± 2.7	86.9 ± 2.0	91.2 ± 2.4	87.3 ± 2.6	86.8 ± 1.7	92.6 ± 1.6
day 1- day 21	366.4 ± 5.9	355.7 ± 5.9	339.4 ± 5.4 *	365.5 ± 7.2	360.8 ± 8.7	345.2 ± 6.7	361.3 ± 6.0

2.3 Lactation period

group **n**	control 8	flaxseed oil 8	fish oil 8	safflower oil 8	coconut oil 8	low fiber 8	low protein 8
food consumption (g ± SEM)							
day 1	22 ± 3	25 ± 2	19 ± 2	22 ± 2	22 ± 2	21 ± 1	23 ± 4
day 8	41 ± 2	42 ± 2	39 ± 2	39 ± 2	41 ± 3	43 ± 2	29 ± 2 *
day 15	55 ± 2	53 ± 2	41 ± 3 *	53 ± 1	50 ± 5	54 ± 2	36 ± 3 *
day 22	57 ± 2	57 ± 2	47 ± 5	58 ± 1	52 ± 6	58 ± 2	41 ± 3 *
total average food consumption per animal on 4 different days	175 ± 2	177 ± 2	146 ± 3 *	172 ± 1	165 ± 4	176 ± 2	129 ± 3 *

* $p < 0.05$

Appendix 3: Autopsy at gestation age 21 days

3.1 Number of fetuses	All fetuses	Males	Females
control	96	63	33
flaxseed oil	87	48	39
fish oil	83	42	41
safflower oil	87	35	52
coconut oil	85	47	38
low fiber	64	35	29
low protein	95	53	42

3.2 Litter size at birth

	live pups (mean ± SEM)	based on data from n litters	number of litters at birth n	all pups n	dead pups in week 0 - week 3 n	litters with dead pups: n	survival up to weaning age %
control	12.1 ± 4.3	8	9	72	0	0	100,0
flaxseed oil	12.7 ± 4.8	7	8	64	2	2	96,9
fish oil	7.8 ± 2.7 *	8	8	57	17	5	70,2
safflower oil	11.6 ± 4.4	7	8	56	1	1	98,2
coconut oil	11.8 ± 3.9	9	10	73	11	3	84,9
low fiber	11.1 ± 4.2	7	8	64	0	0	100,0
low protein	11.4 ± 3.8	9	10	80	17	3	78,8

3.3 Fetal weight

	All fetuses	Males	Females
control	4.14 ± 0.05	4.23 ± 0.05	3.95 ± 0.08
flaxseed oil	4.23 ± 0.03	4.32 ± 0.04	4.12 ± 0.04
fish oil	4.08 ± 0.05	4.14 ± 0.07	4.03 ± 0.07
safflower oil	4.26 ± 0.04	4.37 ± 0.06	4.2 ± 0.05 *
coconut oil	4.27 ± 0.05	4.39 ± 0.06	4.12 ± 0.06
low fiber	4.47 ± 0.04 *	4.5 ± 0.06 *	4.4 ± 0.05 *
low protein	4.15 ± 0.05	4.27 ± 0.06	4.01 ± 0.06

3.4 Placental weight

	All fetuses	Males	Females
control	0.56 ± 0.06	0.57 ± 0.00	0.54 ± 0.02
flaxseed oil	0.55 ± 0.06	0.56 ± 0.01	0.54 ± 0.01
fish oil	0.58 ± 0.06	0.60 ± 0.02	0.56 ± 0.01
safflower oil	0.54 ± 0.06	0.56 ± 0.01	0.52 ± 0.01
coconut oil	0.59 ± 0.06	0.61 ± 0.09	0.56 ± 0.02
low fiber	0.55 ± 0.07	0.55 ± 0.06	0.54 ± 0.02
low protein	0.49 ± 0.05 *	0.50 ± 0.07 *	0.48 ± 0.01 *

3.5 Ratio fetal/placental weight

	All fetuses	Males	Females
control	7.4 ± 0.09	7.4 ± 0.11	7.4 ± 0.19
flaxseed oil	7.8 ± 0.1 *	7.9 ± 0.15	7.7 ± 0.12
fish oil	7.1 ± 0.11	7.1 ± 0.18	7.2 ± 0.12
safflower oil	8.1 ± 0.13 *	7.9 ± 0.20	8.2 ± 0.17 *
coconut oil	7.4 ± 0.11	7.3 ± 0.14	7.5 ± 0.17
low fiber	8.2 ± 0.14 *	8.2 ± 0.17 *	8.3 ± 0.23 *
low protein	8.5 ± 0.11 *	8.6 ± 0.16 *	8.3 ± 0.14 *

3.6 Ratio body/head length

	All fetuses	Males	Females
control	2.1 ± 0.01	2.1 ± 0.02	2.1 ± 0.02
flaxseed oil	2.1 ± 0.02	2.2 ± 0.02 *	2.1 ± 0.02
fish oil	2.2 ± 0.02 *	2.2 ± 0.02	2.1 ± 0.02
safflower oil	2.1 ± 0.02	2.2 ± 0.02	2.1 ± 0.02
coconut oil	2.1 ± 0.02	2.2 ± 0.02	2.1 ± 0.02
low fiber	2.1 ± 0.02	2.2 ± 0.03	2.1 ± 0.03
low protein	2.2 ± 0.02	2.2 ± 0.02	2.1 ± 0.03

3.7 Maternal characteristics

number of:	fetuses per dam (mean ± SEM)	corpora lutea (mean ± SEM)	resorptions (mean ± SEM)	mean uterus weight (grams ± SEM)	ratio uterus weight/ number of fetuses
control	12.0 ± 1.1	14 ± 0.5	0.4 ± 0.2	71.5 ± 6.6	6.0 ± 0.1
flaxseed oil	12.4 ± 1.6	14.3 ± 1.7	0.9 ± 0.3	73.9 ± 3.9	5.9 ± 0.1
fish oil	10.5 ± 0.8	13.0 ± 5.6	1.8 ± 0.6	62.9 ± 4.2	5.9 ± 0.2
safflower oil	12.4 ± 0.5	14.1 ± 1.9	0.6 ± 0.4	74.3 ± 2.7	6.0 ± 0.1
coconut oil	12.0 ± 1.2	14.6 ± 0.7	1.3 ± 0.5	75.9 ± 5.7	6.4 ± 0.2
low fiber	12.8 ± 0.9	14.0 ± 2.7	0.4 ± 0.2	79.6 ± 5.2	6.2 ± 0.1
low protein	11.9 ± 0.4	14.3 ± 2.7	0.6 ± 0.2	70.1 ± 1.9	5.9 ± 0.1

* $p < 0.05$

Appendix 4: Visceral malformations of fetuses at gestation day 21

4.1 Absolute incidence of visceral malformations

	control	flaxseed	fish oil	safflower oil	coconut oil	low fiber	low protein
number of fetuses analyzed	23	20	15	21	20	15	22
THYMUS: malformed	9	10	7	7	13	6	8
KIDNEYS: slightly dislocated	0	2	1	0	2	2	2
KIDNEYS: increased renal pelvic cavitation	4	3	3	6	2	2	1
URETERS: kinked	3	6	1	4	1	2	0
URETERS: hydroureter	0	0	0	1	0	0	0
ADRENALS: haemorrhagic area	0	0	0	0	1	0	0
TESTES: slightly dislocated	0	0	0	0	0	0	1
RETINA: folded	1	0	0	0	0	0	0
BRAIN: dilatation of the lateral ventricles	1	0	0	0	0	0	1
THYROID: dislocated							

4.2 Relative incidence of visceral malformations

	control	flaxseed	fish oil	safflower oil	coconut oil	low fiber	low protein
	%	%	%	%	%	%	%
THYMUS: malformed	39	50	47	33	65	40	36
KIDNEYS: slightly dislocated	17	10	7	0	10	13	9
KIDNEYS: increased renal pelvic cavitation	13	15	20	29	10	13	5
URETERS: kinked	0	30	7	5	5	13	0
URETERS: hydroureter	0	0	0	0	0	0	0
ADRENALS: haemorrhagic area	0	0	0	0	5	0	0
TESTES: slightly dislocated	0	0	0	0	0	0	5
RETINA: folded	4	0	0	0	0	0	0
BRAIN: dilatation of the lateral ventricles	4	0	0	0	0	0	0
THYROID: dislocated	0	0	0	0	0	0	5

Appendix 5: Skeletal malformations of fetuses gestation day 21

5.1 Absolute incidence of skeletal malformations

	control	flaxseed oil	fish oil	safflower oil	coconut oil	low fiber	low protein
n	42	39	35	38	37	28	41
accessory ribs	2	1	1	5			3
zygoma fused	13	12	12	2	5	1	11
two or more ribs reduced size	2	1					
two ribs fused				1			
one ribs reduced size	2						
two sternebra fused	1			1	3		
one or two ribs not fused with sternum	8		4	10	6		9
one or two lumbar bodies missing	2	2					1
one or two lumbar arches missing	2	2					1
thoracal bodies: one or two bodies malformed					1		
one supernumeray sternebra		1		1			1
hyoid incompletely ossified			2	2			2
one sternebra unossified							1

5.2 Relative incidence of skeletal malformations

	control	flaxseed oil	fish oil	safflower oil	coconut oil	low fiber	low protein
	%	%	%	%	%	%	%
accessory ribs	5	3	3	13			7
zygoma fused	31	31	34	5	14	4	27
two or more ribs reduced size	5	3					
two ribs fused				3			
one ribs reduced size	5				8		
two sternebra fused	2		11	3	8		
one or two ribs not fused with sternum	19			26	16		22
one or two lumbar bodies missing	5	5					2

	control	flaxseed oil	fish oil	safflower oil	coconut oil	low fiber	low protein
	%	%	%	%	%	%	%
one or two lumbar arches missing	5	5			3		2
thoracal bodies: one or two bodies malformed							
one supernumerary sternebra		3		3			2
hyoid incompletely ossified			6	5			5
one sternebra unossified							2

5.3 Skeletal parameters

incidence (mean ± SEM) per group:	control	flaxseed oil	fish oil	safflower oil	coconut oil	low fiber	low protein
n	38	35	31	36	33	27	36
ossified caudal arches	1.2 ± 0.07	1.1 ± 0.08	1.2 ± 0.08	1.5 ± 0.08*	1.2 ± 0.07	1.5 ± 0.1	1.4 ± 0.08
ossified caudal bodies	3.8 ± 0.11	4.0 ± 0.11	4.6 ± 0.12*	4.0 ± 0.11	4.2 ± 0.11*	4.0 ± 0.11	3.9 ± 0.11
ossified metacarpals	7.9 ± 0.06	8.0 ± 0.0	7.9 ± 0.04	7.8 ± 0.12	8.0 ± 0.0	8.0 ± 0.00	8.0 ± 0.03
ossified front proximal bone	2.2 ± 0.27	2.8 ± 0.32	3.4 ± 0.28*	2.7 ± 0.3	3.3 ± 0.25*	3.9 ± 0.28*	3.0 ± 0.28
ossified front distal bone	9.3 ± 0.19	9.1 ± 0.4	9.3 ± 0.17	9.6 ± 0.14	9.6 ± 0.16	9.7 ± 0.11	9.3 ± 0.2
ossified metatarsals	8.2 ± 0.09	8.2 ± 0.1	8.2 ± 0.11	8.3 ± 0.12	8.2 ± 0.1	8.3 ± 0.11	8.1 ± 0.07
ossified hind distal	6.8 ± 0.74	3.4 ± 0.73	7.3 ± 0.85	6.2 ± 0.88	6.1 ± 0.81	8.9 ± 0.44	7.3 ± 0.75

Appendix 6: Body weight of the offspring

6.1 Mean body weight of males (in grams per group ± SEM)

	control	flaxseed oil	fish oil	safflower oil	coconut oil	low fiber	low protein
number of pups week 4-12	45	44	32	36	34	39	36
day 1	7.3 ± 0.1	7.4 ± 0.1	6.7 ± 0.1 *	7.6 ± 0.1	7.1 ± 0.1	7.5 ± 0.1	6.9 ± 0.1
day 7	19.2 ± 0.3	18.8 ± 0.4	16.7 ± 0.4 *	19.8 ± 0.3	17.8 ± 0.3 *	19.9 ± 0.3	13.5 ± 0.3 *
day 14	38.8 ± 0.5	38.1 ± 0.5	32.2 ± 0.5 *	39.5 ± 0.5	35.8 ± 1.0 *	39.9 ± 0.8	23.0 ± 0.4 *
day 21	60.8 ± 0.6	57.9 ± 0.5	49.8 ± 1.1 *	59.7 ± 1.0	58.2 ± 0.9	63.4 ± 1.1	34.2 ± 0.8 *
week 4	96 ± 1.0	91 ± 2.0	83 ± 3.0 *	94 ± 4.0	92 ± 5.0	99 ± 6.0	64 ± 7.0 *
week 5	143 ± 1.2	140 ± 0.9	129 ± 1.8 *	144 ± 1.6	139 ± 1.4	151 ± 1.3 *	108 ± 1.2 *
week 6	197 ± 1.9	190 ± 1.8	182 ± 3.1 *	195 ± 1.9	192 ± 2.0	207 ± 1.8 *	151 ± 2.1 *
week 7	250 ± 2.3	243 ± 2.0	231 ± 2.6 *	249 ± 2.7	246 ± 2.8	264 ± 2.2 *	199 ± 2.5 *
week 8	298 ± 2.7	290 ± 2.6	283 ± 3.2 *	298 ± 2.8	291 ± 3.3	312 ± 2.8 *	244 ± 3.3 *
week 9	340 ± 2.8	330 ± 3.2	322 ± 3.4 *	336 ± 3.4	328 ± 4.0	353 ± 3.6 *	277 ± 4.0 *
week 10	374 ± 3.2	360 ± 3.8	349 ± 4.0 *	367 ± 3.8	359 ± 4.4	383 ± 4.0	303 ± 4.4 *
week 11	400 ± 3.5	390 ± 4.2	380 ± 4.6 *	392 ± 4.2	386 ± 4.3	407 ± 4.8	326 ± 4.5 *
week 12	421 ± 4.2	406 ± 3.6	399 ± 5.3 *	411 ± 4.7	404 ± 4.5	434 ± 4.8	343 ± 5.0 *

6.2 Mean body weight of females (in grams per group ± SEM)

	control	flaxseed oil	fish oil	safflower oil	coconut oil	low fiber	low protein
number of pups week 4-12	18	18	16	19	17	25	27
day 1	6.9 ± 0.2	6.9 ± 0.2	6.6 ± 0.2	7.3 ± 0.1	7.1 ± 0.2	7.2 ± 0.2	7.0 ± 0.1
day 7	18.2 ± 0.5	17.9 ± 0.7	16.7 ± 0.4	19.1 ± 0.5	18.1 ± 0.5	18.1 ± 0.5	13.5 ± 0.4 *
day 14	38.0 ± 0.8	37.5 ± 1.1	32.1 ± 0.7 *	36.9 ± 0.9	37.4 ± 0.8	39.0 ± 1.1	21.3 ± 0.4 *
day 21	57.6 ± 0.9	55.8 ± 1.5	48.3 ± 1.4 *	54.3 ± 1.6	58.3 ± 1.5	61.0 ± 1.3	31.9 ± 0.6 *
week 4	89 ± 1.2	85 ± 2.3	77 ± 2.4 *	86 ± 1.7	89 ± 1.6	94 ± 2.1	58 ± 1.0 *
week 5	126 ± 1.9	124 ± 3.0	113 ± 3.3 *	124 ± 2.1	129 ± 2.3	127 ± 2.5	92 ± 1.4 *
week 6	156 ± 2.6	152 ± 3.7	139 ± 3.0 *	152 ± 2.6	156 ± 2.7	158 ± 3.1	118 ± 1.9 *
week 7	180 ± 2.8	176 ± 8.8	164 ± 4.7 *	176 ± 7.9	178 ± 5.8	181 ± 5.8	141 ± 8.0 *
week 8	200 ± 3.2	195 ± 4.2	190 ± 4.0	196 ± 2.9	196 ± 3.6	203 ± 3.5	161 ± 2.5 *
week 9	216 ± 3.7	210 ± 4.6	203 ± 4.3	212 ± 3.7	215 ± 4.0	220 ± 3.7	177 ± 3.7 *

	control	flaxseed oil	fish oil	safflower oil	coconut oil	low fiber	low protein
week 10	230 ± 4.2	223 ± 4.6	215 ± 4.7	221 ± 3.8	223 ± 4.8	232 ± 3.9	188 ± 4.4 *
week 11	242 ± 5.4	232 ± 5.3	227 ± 5.5	234 ± 4.5	237 ± 5.3	244 ± 4.9	199 ± 6.3 *
week 12	253 ± 5.6	244 ± 6.0	233 ± 6.2	246 ± 5.7	250 ± 5.4	251 ± 4.7	209 ± 7.0 *

* $p < 0.05$

Appendix 7: Glucose and insulin concentration during glucose tolerance test

7.1 Insulin concentrations (pmol/l)

	t (minutes)	0	5	10	20	30	50
	n						
control	7	105.0 ± 35.6	280.2 ± 63.6	409.8 ± 84.0	548.4 ± 126.7	468.5 ± 106.6	377.4 ± 71.8
flaxseed oil	8	41.0 ± 7.5	277.5 ± 42.5	449.4 ± 20.8	449.2 ± 32.7	367.4 ± 59.9	279.3 ± 27.6
fish oil	8	59.7 ± 11.9	244.6 ± 43.1	437.7 ± 98.0	492.8 ± 51.1	404.2 ± 49.6	284.8 ± 27.2
safflower oil	8	82.5 ± 20.2	329.4 ± 68.8	571.4 ± 111.8	547.9 ± 49.8	412.4 ± 48.4	279.8 ± 36.0
coconut oil	7	136.7 ± 28.8	563.2 ± 56.3 *	760.2 ± 72.0 *	613.7 ± 87.9	426.0 ± 46.7	441.3 ± 76.5
low fiber	8	101.2 ± 15.7	419.1 ± 111.1	552.7 ± 95.3	581.0 ± 60.6	477.0 ± 44.5	319.0 ± 63.4
low protein	8	50.3 ± 10.8	166.9 ± 50.6	342.6 ± 55.7	376.8 ± 74.5	303.1 ± 38.3	270.3 ± 40.7

7.2 Glucose concentrations (mmol/l)

	t (minutes)	0	5	10	20	30	50
	n						
control	7	5.36 ± 0.2	6.91 ± 0.5	9.21 ± 0.5	12.44 ± 0.4	13.53 ± 0.5	14.06 ± 0.7
flaxseed oil	8	5.4 ± 0.2	7.3 ± 0.4	10.2 ± 0.5	12.8 ± 0.6	13.8 ± 0.7	13.7 ± 0.6
fish oil	8	5.4 ± 0.1	7.5 ± 0.3	10.0 ± 0.5	13.2 ± 0.4	13.9 ± 0.6	13.7 ± 0.6
safflower oil	8	5.2 ± 0.2	6.9 ± 0.5	10.0 ± 0.3	12.1 ± 0.3	13.0 ± 0.4	13.1 ± 0.6
coconut oil	7	5.5 ± 0.2	7.3 ± 0.2	9.7 ± 0.3	11.3 ± 0.3	11.5 ± 0.5 *	12.0 ± 0.7
low fiber	8	5.5 ± 0.2	8.3 ± 0.5	11.1 ± 0.5	12.6 ± 0.5	13.2 ± 0.5	13.5 ± 0.5
low protein	8	5.2 ± 0.1	6.7 ± 0.3	10.3 ± 0.5	12.3 ± 0.5	12.7 ± 0.3	12.9 ± 0.4

7.3 Area under the curve

	insulin	glucose	relative AUC insulin (%)	relative AUC glucose (%)
control	21267,9	599,0	100,0	100
flaxseed oil	17998,6	614,8	84,6	102,6
fish oil	18861,7	618,4	104,8	100,5
safflower oil	20983,7	585,6	111,3	94,7

	insulin	glucose	relative AUC insulin (%)	relative AUC glucose (%)
coconut oil	25878,3	539,9	123,3	92,2
low fiber	23017,1	612,5	88,9	113,4
low protein	14774,4	580,0	64,2	94,7

* $p < 0.05$

Appendix 8: Blood pressure in male offspring at age 12 weeks

8.1 Blood pressure measured in a. carotis under anesthesia

	control	flaxseed oil	fish oil	safflower oil	coconut oil	low fiber diet	low protein diet
n	8	8	8	7	8	7	6
Basal systolic blood pressure (mm Hg)	117.8 ± 6.5	115.0 ± 4.0	125.4 ± 6.7	109.4 ± 6.5	113.6 ± 10.6	115.3 ± 6.1	104.4 ± 4.4
0.3 mg/kg L-NAME	121.4 ± 6.1	122.3 ± 4.0	134.4 ± 10.9	101.8 ± 5.9	121.8 ± 5.4	121.3 ± 8.3	107.4 ± 7.2
1 mg/kg L-NAME	149.6 ± 7.3	172 ± 10.0	176.6 ± 14.2	156.1 ± 12.6	158.6 ± 9.2	159.4 ± 14.0	127.5 ± 9.9
3 mg/kg L-NAME	162.4 ± 6.8	194.3 ± 10.2	191.0 ± 12.0	191.9 ± 8.6	178.9 ± 11.1	175.0 ± 9.7	153.0 ± 15.2
10 mg/kg L-NAME	174.3 ± 7.6	196 ± 7.3	195.4 ± 9.0	189.4 ± 5.1	184.3 ± 5.9	201.1 ± 4.3	168.1 ± 11.4
30 mg/kg L-NAME	189.0 ± 6.3	189.6 ± 7.5	201.1 ± 5.8	177.6 ± 5.7	186.1 ± 6.4	188.6 ± 6.7	177.1 ± 5.9
Basal diastolic blood pressure	80.5 ± 6.6	66.9 ± 3.1	84.5 ± 7.8	70.6 ± 7.3	76.8 ± 10.7	72.0 ± 7.4	63.7 ± 3.3
0.3 mg/kg L-NAME	86.1 ± 5.4	79.3 ± 4.4	91.6 ± 8.5	65.2 ± 8.4	82.8 ± 4.7	82.3 ± 9.3	67.9 ± 6.3
1 mg/kg L-NAME	112.9 ± 4.9	124.6 ± 5.1	126.9 ± 7.1	113.3 ± 9.1	119.9 ± 4.8	112.3 ± 8.8	88.4 ± 7.6
3 mg/kg L-NAME	122.5 ± 4.3	135.4 ± 5.4	132.6 ± 8	136.3 ± 4.9	133.6 ± 4.5	126.0 ± 6.1	106.3 ± 10.7
10 mg/kg L-NAME	129.4 ± 3.5	140.3 ± 4.0	139.5 ± 4.3	139.9 ± 3.5	137.9 ± 3.0	141.1 ± 2.4	120.6 ± 6.4
30 mg/kg L-NAME	137.3 ± 1.6	137.3 ± 3.0	144.4 ± 3.1	135.4 ± 2.8	141.0 ± 3.3	138.4 ± 2.5	130.1 ± 3.6
Basal mean arterial pressure	92.9 ± 6.6	82.9 ± 3.0	98.1 ± 7.4	83.5 ± 7.0	89.0 ± 10.7	86.4 ± 7.0	77.3 ± 3.3
0.3 mg/kg L-NAME	97.9 ± 5.6	93.6 ± 4.0	105.9 ± 9.2	77.4 ± 7.5	95.8 ± 4.7	95.3 ± 8.9	81.0 ± 6.3
1 mg/kg L-NAME	125.1 ± 5.6	140.4 ± 6.5	143.4 ± 9.2	127.6 ± 9.9	132.8 ± 6.1	128.0 ± 10.4	101.4 ± 8.1
3 mg/kg L-NAME	135.8 ± 5	155.0 ± 6.9	152.1 ± 9.1	154.7 ± 5.8	148.7 ± 6.6	142.3 ± 7.2	121.8 ± 12.0
10 mg/kg L-NAME	144.3 ± 4.7	158.8 ± 4.7	158.1 ± 5.7	156.4 ± 3.5	153.3 ± 3.8	161.1 ± 2.7	136.5 ± 8.0
30 mg/kg L-NAME	154.5 ± 3	154.7 ± 4.2	163.3 ± 3.9	149.5 ± 3.4	156.0 ± 4.0	155.1 ± 3.5	145.8 ± 4.1

Appendix 9: Individual data Listeria infection

Average number of colony forming units (CFU) per gram spleen compared with control group

	control	flaxseed oil	fish oil	safflower oil	coconut oil	low fiber	low protein
1	82,19	104,11	126,03	21,92	32,88	32,88	32,88
2	241,1	32,88	71,23	0	32,88	54,79	306,85
3	0	76,71	43,84	21,92	10,96	241,1	*43,84*
4	76,71	0	7,06	142	50,59	82,19	*313,73*
5	69,41	143,53	110,59	35,29	447,06	327,84	*11,76*
6	31,76	18,82	17,65	2,35	16,47	561,18	28,24
7	7,06	16,47	63,53	267,45	50,59	*200*	74,12
8	291,76	*265,88*		28,24		253,33	
mean	100	82,3	62,9	65	91,6	219,2	115,9
sem	38,2	31,5	16,8	33	59,5	61,4	50,1

cursivated: excluded from analysis because of incomplete injection of Listeria-solution in tail vein

Appendix 10: Data autopsy male offspring, age 12 weeks

10.1 Absolute organ weights (mean ± SEM)

	control	flaxseed oil	fish oil	safflower oil	coconut oil	low fiber	low protein
n	8	8	6	7	5	7	6
body weight (g)	409.6 ± 14.33	425.0 ± 15.64	391.5 ± 13.72	407.6 ± 11.28	397.2 ± 11.81	433.7 ± 14.58	345.2 ± 11.49
liver weight (g)	15.04 ± 0.68	14.76 ± 0.43	15.96 ± 1.61	51.16 ± 0.58	14.29 ± 0.49	16.09 ± 0.37	12.57 ± 0.82
pancreas weight (g)	1.22 ± 0.14	1.37 ± 0.13	1.25 ± 0.16	1.18 ± 0.08	1.39 ± 0.12	1.45 ± 0.13	1.25 ± 0.09
kidney weight (g)	2.67 ± 0.07	2.49 ± 0.04	2.49 ± 0.23	2.47 ± 0.1	2.31 ± 0.08	2.88 ± 0.10	2.16 ± 0.09 *
adrenal weight (g)	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.0	0.07 ± 0.00	0.07 ± 0.01	0.06 ± 0.01	0.04 ± 0.01
heart weight (g)	1.45 ± 0.09	1.48 ± 0.06	1.37 ± 0.08	1.36 ± 0.04	1.30 ± 0.03	1.50 ± 0.05	1.21 ± 0.05
thymus weight (g)	0.84 ± 0.08	0.76 ± 0.03	0.88 ± 0.07	0.87 ± 0.09	0.82 ± 0.06	0.99 ± 0.08	0.83 ± 0.06
brain weight (g)	1.90 ± 0.04	1.86 ± 0.03	1.66 ± 0.05 *	1.85 ± 0.05	1.88 ± 0.04	1.92 ± 0.03	1.81 ± 0.04
spleen weight (g)	0.68 ± 0.04	0.68 ± 0.03	0.66 ± 0.03	0.69 ± 0.04	0.55 ± 0.04	0.67 ± 0.04	0.64 ± 0.04

10.2 Relative organ weights (mean ± SEM)

	control	flaxseed oil	fish oil	safflower oil	coconut oil	low fiber	low protein
liver/body (%)	3.67 ± 0.08	3.49 ± 0.12	4.06 ± 0.33	3.72 ± 0.07	3.60 ± 0.05	3.72 ± 0.08	3.63 ± 0.17
pancreas/body (%)	0.3 ± 0.03	0.33 ± 0.03	0.32 ± 0.03	0.29 ± 0.02	0.35 ± 0.03	0.28 ± 0.05	0.37 ± 0.04
kidney/body (%)	0.65 ± 0.02	0.59 ± 0.01	0.64 ± 0.05	0.61 ± 0.01	0.58 ± 0.02	0.66 ± 0.01	0.62 ± 0.01
adrenals/body (%)	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
heart/body (%)	0.35 ± 0.02	0.35 ± 0.01	0.35 ± 0.02	0.33 ± 0.00	0.33 ± 0.01	0.35 ± 0.01	0.35 ± 0.01
thymus/body (%)	0.20 ± 0.02	0.18 ± 0.01	0.22 ± 0.01	0.21 ± 0.02	0.21 ± 0.01	0.23 ± 0.02	0.24 ± 0.01
brain/body (%)	0.47 ± 0.02	0.44 ± 0.02	0.43 ± 0.03	0.46 ± 0.02	0.48 ± 0.02	0.45 ± 0.02	0.53 ± 0.02
spleen/body (%)	0.17 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.18 ± 0.01

* $p < 0.05$

Appendix 11: Histological changes in organs of male rats aged 12 weeks

Incidence of histological lesions

Group		control	flaxseed oil	menhaden oil	safflower oil	coconut oil	low fiber	low protein
adrenals		8	8	7	8	7	8	7
no remarks		1	5	2	1	2	3	1
vacuolisation zonae fasc.and reticul.:								
minimal		2	-	-	2	-	-	4
slight		1	1	3	3	3	4	2
moderate		2	1	1	2	2	1	-
strong		2	1	1	-	-	-	-
hyperplasia z.glom.focal		-	-	1	-	-	-	-
heart		8	8	8	8	7	8	8
no remarks		8	8	8	8	7	8	8
kidneys		8	8	8	8	7	8	8
no remarks		-	-		1	-	-	-
basophilic tubules:	minimal	8	7	5	5	7	5	2
Slight		-	-	1	1	-	2	6
Moderate		-	-	1	-	-	1	-
nephrocalcinosis: cortex/med	minimal	-	-	-	1	-	-	2
	moderate	-	-	1	-	2	1	-
	strong	-	-	1	-		-	-
nephritis:	minimal	-	1	1	-	1	2	-
Slight		-	-	-	-	-	-	2
protein casts, slight		-	-	1	-	-	-	-
lymphoc.aggregates		-	-	1	-	-	-	-
chronic nephropathy		-	-	1	-	-	-	-

Incidence of histological lesions - continued

Group		control	flaxseed oil	menhaden oil	safflower oil	coconut oil	low fiber	low protein
no remarks								
lymphocytes aggregates:	minimal	7	5	6	7	6	6	7
slight		1	3	1	-	1	1	1
moderate		-	-	-	-	-	1	-
glycogen vacuolisation periportal:	minimal	-	1	-	-	-	1	-
slight		3	-	3	2	1	2	4
moderate		1	4		1	-	1	-
strong		-	-	1	-	-	-	-
lipid vacuolisation periportal	minimal	-	-	1	2	2	-	2
	slight	-	-		1	2	1	-
	moderate	-	-	2	1	2	-	-
	strong	-	-	-	-	-	2	-
pancreas		8	7	7	7	6	8	8
no remarks		4	2	5	6	4	4	7
pancreatitis focal	small	3	2	1	-	1	3	1
Moderate		-	-	-	1	-	-	-
single cell vacuolisation		4	5	2	1	1	1	-
degeneration of acini focal	slight	-	1	-		1	1	-
atrophy acini, focal		-	1	-	1	1	1	-
fibrotic islets		-	1	-	1	-	-	-
haemorrhage, focal small		1	-	-	-	-	-	-
pituitary gland		8	8	8	7	7	8	8
no remarks		8	8	8	7	7	8	8
spleen		8	8	7	8	7	8	8
no remarks		8	8	7	8	7	8	8
thymus		8	8	8	8	7	8	8
no remarks		1	2	6	3	6	3	2
starry sky macrophages	minimal	6	5	2	2	1	5	5
slight		1	1	-	-	-	-	1

Appendix 12: Individual morphometric data pancreas

Morphometric data of the pancreas of male offspring fed different diets in utero and during weaning

	surface area pancreas (mm2)	surface area islets (mm2)	% islets	n islets	n islets per 100 mm2	n islets > 0.03 mm2	n islets > 0.03 mm2 per 100 mm2
control group	100,44	1,39	1,38	60	60	11	11
	79,33	0,92	1,16	58	73	6	8
	112,28	1,56	1,39	94	84	13	12
	113,55	2,35	2,07	102	90	20	18
	88,89	1,26	1,42	58	65	10	11
	99,18	0,77	0,77	47	47	-	-
	124,31	1,67	1,34	92	74	12	10
	172,06	2,77	1,6	106	62	24	14
mean			1.39 ± 0.36		69 ± 14		
coconut oil	130,98	1,68	1,28	101	77	13	10
	115,92	0,81	0,7	64	55	3	3
	79,4	1	1,25	69	87	5	6
	114,54	1,86	1,62	103	90	13	11
	67,43	0,71	1,05	62	92	4	6
	146,17	0,99	0,67	82	56	5	3
mean			1.09 ± 0.36		76 ± 17		
low protein	13,73	0,07	0,51	8	58	0	0
	111,83	0,84	0,75	62	55	5	4
	94,95	1,04	1,09	50	53	11	12
	99	1,33	1,34	84	85	6	6
	78,26	0,62	0,79	37	47	4	5
	105,57	1,4	1,32	71	67	15	14
	65,09	0,67	1,02	46	70	4	6
	77,65	0,84	1,08	52	67	8	10
mean			0.96 ± 0.33 *		6.8 ± 5.2 *		

Appendix 13: Corticosterone concentration after ACTH stimulation in vitro

13.1 Corticosterone concentration (ng/l) after ACTH stimulation of an adrenal cell suspension according to group

ACTH (pg/ml)	7-Nov-00 ng	9-Nov-00	7-Nov-00 ng	16-Nov-00 ng	7-Nov-00 ng	21-Nov-00 ng	9-Nov-00 ng	21-Nov-00 ng	9-Nov-00 ng	14-Nov-00 ng	14-Nov-00 ng	16-Nov-00 ng	14-Nov-00 ng
	control diet	control diet	low fiber	low fiber	low protein	low protein	coconut oil	coconut oil	safflower oil	safflower oil	flaxseed oil	flaxseed oil	fish oil
0		0,81	0,90	6,74	0,68	11,06	11,23	3,95	4,34	9,64	9,89	5,00	10,64
1	1,65	3,49	3,05	9,95	0,32	3,46	9,77	5,46	15,03	13,11	0,79	6,58	
3	7,26	27,65	9,24	11,40	5,19	7,55	28,73	11,44	34,60	24,23	4,67	8,07	0,61
10	17,97	57,37	18,37	31,33	4,96	13,91	68,76	11,03	63,44	48,10	12,89	24,15	13,36
30	20,48	55,87	22,49	43,60	7,67	19,43	59,71	19,10	62,66	77,51	44,04	39,39	51,48
100	18,75	44,61	20,01	70,13	6,19	63,37	68,73	26,84	51,97	109,47	20,67	64,70	89,78
300	13,81	22,70	19,07	64,84	8,43	94,76	57,98	28,98	56,11	53,00	30,48	61,95	38,48
1000	11,80	23,51	18,39	73,94	8,41	80,93	44,81	44,75	68,19	20,52	15,17	69,52	24,58

13.2 Corticosterone concentration (ng/l) after ACTH stimulation of an adrenal cell suspension according to experimental day

ACTH (pg/ml)	7-Nov-00 ng	7-Nov-00 ng	7-Nov-00 ng	9-Nov-00 ng	9-Nov-00 ng	9-Nov-00 ng	14-Nov-00 ng	14-Nov-00 ng	14-Nov-00 ng	16-Nov-00 ng	16-Nov-00 ng	21-Nov-00 ng	21-Nov-00 ng
	control diet	low fiber	low protein	control diet	safflower oil	coconut oil	safflower oil	flaxseed oil	fish oil	low fiber	flaxseed oil	low protein	coconut oil
0		0,90	0,68	0,81	4,34	11,23	9,64	9,89	10,64	6,74	5,00	11,06	3,95
1	1,65	3,05	0,32	3,49	15,03	9,77	13,11	0,79		9,95	6,58	3,46	5,46
3	7,26	9,24	5,19	27,65	34,60	28,73	24,23	4,67	0,61	11,40	8,07	7,55	11,44
10	17,97	18,37	4,96	57,37	63,44	68,76	48,10	12,89	13,36	31,33	24,15	13,91	11,03
30	20,48	22,49	7,67	55,87	62,66	59,71	77,51	44,04	51,48	43,60	39,39	19,43	19,10

ACTH (pg/ml)	7-Nov-00 ng	7-Nov-00 ng	7-Nov-00 ng	9-Nov-00 ng	9-Nov-00 ng	9-Nov-00 ng	14-Nov-00 ng	14-Nov-00 ng	14-Nov-00 ng	16-Nov-00 ng	16-Nov-00 ng	21-Nov-00 ng	21-Nov-00 ng
100	18,75	20,01	6,19	44,61	51,97	68,73	109,47	20,67	89,78	70,13	64,70	63,37	26,84
300	13,81	19,07	8,43	22,70	56,11	57,98	53,00	30,48	38,48	64,84	61,95	94,76	28,98
1000	11,80	18,39	8,41	23,51	68,19	44,81	20,52	15,17	24,58	73,94	69,52	80,93	44,75

Appendix 14: Protocol corticosterone response after ACTH stimulation in vitro (in Dutch)

BIJNIERCELLEN: ISOLATIE, OPWERKING EN INCUBATIE EN CORTICOSTERONMETING: METHODE PER BIJNIERPAAR

Inleiding
Bijniercelisolatie en incubatie volgens methode van Goverde.

Materialen
- Krebs-Henseleit buffer modified (Sigma K3753, 10 x 1L, f.45,=).
- Fysiologisch zoutoplossing (SVM Z.2925c, 400 mL in 500 mL fles, 10 x f.9,95).
- Collagenase type I (Sigma C0130, 500 mg, f.309,70).
- Bovine Serum Albumin (Hoechst-Bering ORHD 20/21, 30 g, prijs onbekend).
- Adrenocorticotropic Hormone 1-24 (ACTH) (MRC 74/555, 100 ng, Pesman, Nijmegen).
- CaCl2.2H2O (Merck 2382).
- NADPH (Boehringer Mannheim 107816, 25 mg f.73,=).
- corticosteron (10,39 µg/mL, Pesman, Nijmegen).
- dichloormethaan (Merck 1.06050.2500, 2,5 L f.87,80).
- zwavelzuur 97 – 99 % (Merck 1.00713.1000, f.36,10).
- ethanol 96 % (Merck, => magazijn).
- 25 mL flesje.
- scalpelhouder en mesjes nr. 24 (Swann-Morton)
- plastic petrischalen
- 1 mL eppendorfcupjes.
- 2 mL pipetten plastic, Falcon 7507.
- 25 mL polystyreen verzendbuizen, Hospidex b.v. 55387.
- 12 mL polystyreen rondbodembuizen (Greiner 160101, afm. 16/100 mm, 1600st. f.36,=).
- gripstoppen voor buizen met diameter 16 mm (Greiner 310321, 100 st. f.25,90).

Oplossingen voor isolatie, opwerking en incubatie

- Krebs-Henseleit buffer (KHB):

1 potje Krebs-Henseleit buffer poeder oplossen in 900 mL demiwater.
0,373 g CaCl2.2H2O (2,5 mM) toevoegen.
2,1 g NaHCO3 toevoegen.
pH op 7,2 -7,4 brengen met 1 M HCl of 1 M NaOH.
aanvullen met demiwater tot 1 L.
Bewaren bij 2 - 8 graden Celsius.

- KHB met albumine 4 % (w/v) (KHB4A) (2 mL nodig):

voeg 0,2 g bovine serum albumine toe aan 5 mL KHB (op kamertemperatuur).
Bewaren bij 2 - 8 graden Celsius.

- Collagenasebuffer:

voeg 6,4 mg collagenase toe aan 2 mL KHB4A (op kamertemperatuur).

- KHB met hoog (7,65 mM) calcium (KHBCa) (50 mL nodig):

voeg 76 mg CaCl2.2H2O toe aan 100 mL KHB (op kamertemperatuur).

- KHBCa met albumine 0,5 % (w/v) (KHBACa) (7 mL nodig):

voeg 50 mg bovine serum albumine toe aan 10 mL KHBCa (op kamertemperatuur).
Bewaren bij 2 - 8 graden Celsius.

- KHBCa met hoog albumine 5 % (w/v) (KHB5ACa) (8 mL nodig):

voeg 0,5 g albumine toe aan 10 mL KHBCa (op kamertemperatuur).

- Fysiologisch zoutoplossing met albumine (0,5 % (w/v)) (FZA):

voeg 1,5 g albumine toe aan 300 mL fysiologisch zoutoplossing en breng de pH op 3,5 met 1 mol/L HCl.
Bewaren bij 2 - 8 graden Celsius.

ACTH-oplossingen

- concentraties ACTH zodat 0,1 mL aan de celsuspensie kan worden toegevoegd.
- ACTH 1-39 100 ng oplossen in 10 mL FZA in plastic buis;

=> stockoplossing van 10 ng/mL -> invriezen bij –20 °C in porties van 250 µL in eppendorfcupjes.

- 200 µL van stockoplossing verdunnen tot 400 µL => werkoplossing.
- elke concentratie in duplo => van elke concentratie 200 µL nodig => 250 µL maken in eppendorfcupjes.

NADPH-oplossing
- concentratie NADPH zodat 0,1 mL aan de celsuspensie kan worden toegevoegd.
- einconcentratie NADPH is 0,1 mg/cupje.
- oplossing maken van 1 mg NADPH/mL FZA.

Oplossingen voor corticosteronmeting
- Fluorescentie reagens:

300 mL ethanol koelen op ijs.
700 mL zwavelzuur zeer langzaam toevoegen.
Bewaren kamertemperatuur.

- Corticosteron standaard:

per dag vers maken
100 µL stockoplossing (= 1039 ng) in 12 mL buis pipetteren.
ethanol laten verdampen bij kamertemperatuur onder stroom lucht
verder behandelen als monsters.

Isolatie
- bijnieren van 1 Wistar rat worden bij CDL geïsoleerd.
- bijnieren worden in flesje met ca. 10 mL KHB op ijs verzameld.
- flesje met bijnieren wordt op ijs naar A3.111 getransporteerd.
- vanaf hier wordt alles met plastic materiaal uitgevoerd!
- in petrischaal vetresten van bijnieren verwijderen, op ijs houden en uitdrogen voorkomen.
- bijnieren in 6 - 10 stukjes snijden, hierbij zo min mogelijk druk erop zetten.
- in 2 mL collagenasebuffer in 25 mL buis op ijs overbrengen.
- 50 minuten incuberen bij 37 °C in een schudwaterbad onder carbogeen toevoer (een laag carbogeen boven de buis houden), schudstand 50.
- dispersie completeren door circa 50 keer de weefselstukjes met

een 2 mL pipet te pipetteren.
- de grote stukken laten uitzakken en het supernatant overpipetteren in een koude 25 mL buis.
- 2 mL KHBACa toevoegen aan de pellet en opnieuw circa 50 keer pipetteren.
- de grote stukken laten uitzakken en het supernatant in de 25 mL buis pipetteren.
- 10 minuten centrifugeren bij 100 x g bij 4 °C (700 rpm in Minifuge).
- het supernatant afzuigen, de pellet resuspenderen d.m.v. opwervelen met 2 mL KHBACa en centrifugeren als boven.
- deze wasstap herhalen en daarna de pellet voorzichtig resuspenderen in 1 mL KHBACa.
- 1 mL zeer langzaam en voorzichtig pipetteren op 8 mL KHB5ACa in een 12 mL buis.
- na 30 minuten de bovenste laag voorzichtig afzuigen (het onderscheid tussen beide lagen is moeilijk te zien).
- monster nemen voor celtelling (bij het bekijken met de microscoop moet de celsuspensie nauwelijks nog celdebris bevatten)
- celaantal per mL berekenen
- celsuspensie verdunnen met KHBCa, zodat 4.10exp4 cellen/mL wordt verkregen en de eindconcentratie BSA circa 0,5 % is.
- 2 mL KHBACa maken, waarbij KHBACa volgens verdunningsverhouding van celsuspensie is samengesteld uit KHBCa en KHB5ACa. Dit is je referentiebuffer

Incubatie
- 12 mL buizen vullen met 900 µL celsuspensie, aantal afhankelijk van te gebruiken scenario (zie pagina 4), en 2 buizen vullen met 900 µL referentiebuffer
- buizen 1 uur incuberen bij 37 °C in een schudwaterbad onder carbogeen toevoer (een laag carbogeen boven de buizen houden), schudstand 50.
- per buis 100 µL ACTH-oplossing of FZA of NADPH-oplossing toevoegen volgens schema.
- buizen incuberen bij 37 °C in een schudwaterbad onder

carbogeen toevoer (een laag carbogeen boven de buizen houden), schudstand 50.
- na 120 minuten de monsterbuizen uit het waterbad halen.
- monsters bij –20 °C bewaren tot corticocosteronmeting.

Meting corticosteron
- monsterbuizen, 2 lege 12 mL buizen en 1 buis met corticosteronstandaard nodig.
- aan elke buis 6,0 mL dichloormethaan toevoegen in de zuurkast.
- buizen goed afsluiten met doppen
- extraheren door 2 min. te schudden.
- buizen centrifugeren bij 200 x g gedurende 10 min..
- waterfase afzuigen
- 1,0 mL zwavelzuur-ethanol reagens per buis toevoegen in de zuurkast.
- buizen goed afsluiten met doppen
1 min. laten staan of 30 s schudden.
- organische fase afzuigen (hoeft niet zo nauwkeurig).
- buizen 50 min. laten staan.
- fluorescentie meten (ex 470 nm, em 520 nm)

Literatuur
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