	Inventaris Wob-verzoek W17-12								
		wordt verstrekt				**weigeringsgronden**			
nr.	**documenten NTS20171668**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
1	Aanvraagformulier				x		x	x	
2	Projectvoorstel				x		x	x	
3	Niet-technische samenvatting	x							
4	Bijlage beschrijving dierproeven 1				x		x	x	
5	Bijlage beschrijving dierproeven 2				x		x	x	
6	Bijlage beschrijving dierproeven 3				x		x	x	
7	Bijlage beschrijving dierproeven 4				x		x	x	
8	Bijlage beschrijving dierproeven 5				x		x	x	
9	Appendix				x		x	x	
10	DEC-advies				x		x	x	
11	Ontvangstbevestiging				x		x	x	
12	Verzoek aanvulling aanvraag				x		x		
13	Reactie verzoek aanvulling				x		x	x	
14	Advies CCD		x						x
15	Beschikking en vergunning				x		x	x	

19 MEI 2017

AVD 10400 2017 1668

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.zbo-ccd.nl of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

1 Gegevens aanvrager

1.1	Heeft u een deelnemernummer van de NVWA? Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.	[X] Ja > Vul uw deelnemernummer in	10400
		[ ] Nee > U kunt geen aanvraag doen	
1.2	Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt.	Naam instelling of organisatie	
		Naam van de portefeuillehouder of diens gemachtigde	[redacted]
		KvK-nummer	
1.3	Vul de gegevens van het postadres in. Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.	Straat en huisnummer	Akkermaalsbos 12
		Postbus	59
		Postcode en plaats	6700AW Wageningen
		Iban	NL10RABO0397066465
		Tenaamstelling van het rekeningnummer	Wageningen UR
1.4	Vul de gegevens in van de verantwoordelijke onderzoeker	(Titel) naam en voorletters	[redacted] [X] Dhr. [ ] Mw.
		Functie	onderzoeker
		Afdeling	[redacted]
		Telefoonnummer	
		Email adres	

1.5	(Optioneel) Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.	(Titel) naam en voorletters	[redacted]	[X] Dhr. [ ] Mw.
		Functie	onderzoeker	
		Afdeling	[redacted]	
		Telefoonnummer		
		Email adres		
1.6	(Optioneel) Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) naam en voorletters		[ ] Dhr. [ ] Mw.
		Functie		
		Afdeling		
		Telefoonnummer		
		Email adres		
1.7	Is er voor deze projectaanvraag een gemachtigde?	[ ] Ja > Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag		
		[X] Nee		

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	[x] Nieuwe aanvraag > Ga verder met vraag 3	
		[ ] Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het Dierenwelzijn	
		Vul uw vergunde projectnummer in en ga verder met vraag 2.2	
		[ ] Wijziging op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het Dierenwelzijn	
		Vul uw vergunde projectnummer in en ga verder met vraag 2.3	
2.3	Is dit een wijziging voor een project of dierproef waar al een vergunning voor verleend is?	[ ] Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier	
		[ ] Nee > Ga verder met vraag 3	

2.3	Is dit een melding voor een project of dierproef waar al een vergunning voor is verleend?	[ ] Nee > Ga verder met vraag 3 [ ] Ja > Geef hier onder een toelichting en ga verder met vraag 6	

3 Over uw project

3.1	Wat is de geplande start- en Startdatum	1-8-2017	
	einddatum van het project?	1-8-2022	
3.2	Wat is de titel van het project?	Nutritional Physiology and Metabolic Health in human, mouse as a model	
3.3	Wat is de titel van de niet-technische samenvatting?	Voedingsfysiologie en Metabole Gezondheid in muis als model voor de mens	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC Wageningen UR
		Postadres	Droevendaalsesteeg 4, 6708 PB Wageningen
		E-mailadres	dec@wur.nl

4 Betaalgegevens

4.1	Om welk type aanvraag	[X] Nieuwe aanvraag Projectvergunning €	1827
	gaat het?	[ ] Wijziging €	
4.2	Op welke wijze wilt u dit bedrag aan de CCD	[ ] Via een eenmalige incasso	
		[X] Na ontvangst van de factuur	

voldoen.
Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

[X] Projectvoorstel + 5 bijlagen

[X] Niet-technische samenvatting

Overige bijlagen, indien van toepassing

[ ] Melding Machtiging

[X] Bestelorder WUR105947

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.6). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.

Centrale Commissie
Dierproeven Postbus 20401
2500 EK Den Haag

- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

Naam [redacted]
Functie
Plaats
Datum
Handtekening

2.

Form
Project proposal

- This form should be used to write the project propsal of animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed
- For more information on the project proposal, see our website(www.zbo-ccd.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400
1.2	Provide the name of the licenced establishment.	Wageningen University
1.3	Provide the title of the project.	Nutritional Physiology and Metabolic Health in human, mouse as a model

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

[X] Basic Research
[X] Translational or applied research
[] Regulatory use of routine production
[] Research into environmental protection in the interest of human or animal health or welfare dier
[] Research aimed at preserving the species subjected to procedures

[] Higher education or training

[] Forensic enquiries

[] Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Worldwide obesity has more than doubled since 1980. Recent World Health Organisation (WHO) data show that in 2014, more than 1.9 billion adults, 18 years and older, were overweight with a Body mass index (BMI) over 25. Of these over 600 million were obese (BMI over 30), and numbers keep increasing. Of note, most of the world's population live in countries where overweight and obesity kills more people than underweight. In The Netherlands, more than half of the population in the age group 30-70 years has overweight, and 13% is obese, which equals the worlds average. Obesity is a well-known risk factor for the metabolic syndrome, insulin resistance, type 2 diabetes, cardiovascular diseases, and several forms of cancer like colon cancer and breast cancer. Metabolic syndrome is defined as central obesity plus 2 of the following factors: hypertension, low HDL-cholesterol, elevated blood glucose level and/or elevated triglycerides level. In the Netherlands, according to this definition, 34% of men and 24% of women suffer from the metabolic syndrome (Blokstra et al., Eur J Cardiovasc Nurs 2012).
Not only a direct effect of higher energy intake (food) than energy output (energy expenditure) is underlying the increase in overweight, also metabolic programming plays an important role. Metabolic programming is defined as metabolic factors acting during limited and sensitive time periods of pre- and postnatal development which can induce lasting effects on health and disease risk in later life up to old age (Koletzko et al., Am J Hum Biol 2005). Indeed, increased weight gain in the first 2 years of life leads to increased adipogenic activity and increased long-term risk of later obesity and associated non-communicable diseases (NCD) such as type 2 diabetes (Koletzko et al., Am J Clin Nutr 2011).
Calorie restriction and even bariatric surgery are examples of approaches to combat overweight/obesity and its associated metabolic diseases, but unfortunately, they fail to be successful in reducing weight and improving metabolic health and flexibility, partly due to high relapse rates (e.g. Arterburn et al., Obes Surg 2013). Thus, there is an urgent need to improve metabolic health via alternative routes, of which nutrients offer several attractive aspects even though many knowledge gaps exist in this domain.
Fundamental research has identified several proteins that function as central hubs in the regulation of energy metabolism. These findings make such proteins attractive therapeutic targets in the treatment of the metabolic syndrome and metabolic diseases like type 2 diabetes. Importantly, for some of these targets it has indeed been shown that these can be modulated by nutrients. However, in most cases effects on metabolic health have not yet been proven, but claims are predominantly based on theory or in vitro studies. In our ongoing research, we regularly identify nutrients that are able to modulate such targets (leads), and thereby are hypothesized to improve metabolic health and/or flexibility.

Testing of nutrients that target metabolic syndrome and metabolic diseases is done in distinct steps. An essential step in this cascade is an in vivo assessment in a model organism to show effectiveness in targeting a NCD. Standard measures in this type of studies include determination of adiposity, insulin resistance, and metabolic rate. Also the use of indirect calorimetry is a proven accurate method to investigate energy expenditure and oxidative substrate preference. Indirect calorimetry is performed by a closed lid on top of the homecage. This lid contains an air-inlet and air-outlet connected to control units and sensors with a steady airflow through the cage; measuring oxygen consumption and carbondioxide production allows to determine energy expenditure (kJ/min) and substrate usage (ratio of carbohydrate versus lipid oxidation in case of non-protein respiratory quotient). Moreover, [redacted] indirect calorimetry system also has a food and water sensor to measure intake in a real-time mode, and infrared beams in the horizontal plane to measure real-time activity. More recently, [redacted] shown added value of testing metabolic flexibility, induced by a fasting-refeeding challenge or a hypoxia challenge, using the indirect calorimetry system, as sensitive measure for metabolic health ([redacted]). Using combined sensitive measures in one integrative manner (metabolic flexibility using indirect calorimetry, challenge test like an oral glucose tolerance test or the described challenge tests in indirect calorimetry, body composition using Echo-MRI, molecular responses on tissue level) and short assessment will provide high applicability and power. For Echo-MRI measures, an individual mouse is put within an open plastic tube -with normal air available- in which they can move freely. This tube is positioned within the Echo-MRI equipment and a measurement lasts from 30 to roughly 60 seconds. Primary output is given as lean mass (g) and fat mass (g).
In applied research, there is a need for a sensitive model that can be used to study the hypothesized beneficial effects of nutrients. Moreover, such studies will contribute to fundamental knowledge about detailed mode of action and molecular regulation of physiological processes. The combination of both applied and fundamental aims of this research will benefit the project via the following loop: improved fundamental insight might improve identification of new targets and leads for further applied research, which will lead to more fundamental and applied knowledge.

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The key aim of this research is: to study the effectiveness in a direct or programmed manner of nutrients in targeting metabolic diseases, using a validated integrative physiological relevant in vivo model.
Metabolic health and metabolic flexibility as marker for metabolic health, largely depend on (regulation of) cellular substrate metabolism and organ function.
The present proposal contains several animal studies to investigate this effectiveness, which are primarily in the field of fundamental knowledge when underlying mechanisms are investigated. Simultaneously, several studies can be considered as applied scientific research; early-life nutritional interventions resulting in later-life health improvement are the first step as pre-clinical studies in ultimately supporting future clinical studies. Moreover, pre-clinical studies are a prerequisite for clinical studies, especially when it involves toddlers and children of young age. Each study will answer a specific research question of applied and/or fundamental origin:

• Can selected nutrients beneficially modulate substrate metabolism and organ function as measured using our developed integrative in vivo model? (applied)
• Which molecular processes that relate to substrate metabolism and organ function are modulated by selected nutrients? (fundamental)

FEASIBILITY
The expertise required for this research (indirect calorimetry, challenge tests including oral glucose tolerance test and hypoxia challenge, body composition analysis (Echo-MRI); details in appendix) is state-of-the-art, operational and available at our facility. The present proposal will benefit from our significant experience and expertise in performing in vivo experimentation, which has resulted in a proven well-structured coordination of studies with laboratory animals. The present proposal will use our integrative in vivo model that is designed to investigate nutrients targeting metabolic diseases and thereby improving metabolic health. In this context, various nutrients will be examined. The precise concentrations that will be used depend on data generated in previous (primarily in vivo) experiments, with for each protocol a well-developed rationale. The nutrients used in physiological dose ranges will be selected from the following subgroups of nutrients: [redacted] dietary [redacted] dietary [redacted] differing in [redacted] of [redacted]), dietary [redacted] (varying in [redacted]), [redacted] with a role in metabolism [redacted] and [redacted] (single [redacted], especially [redacted].
Animal studies are subdivided in five different categories: 1) direct metabolic health-effects by nutrients, 2) metabolic programming effects by nutrients, 3) longitudinal effects by nutrients in tissue-specific [redacted] knockout mice, 4) dose effects - interaction between natural nutrients, especially [redacted] and 5) [redacted] using [redacted] and our [redacted] indirect calorimetry system for real-time detection. It can be envisaged that usage of such an [redacted] for non-invasive [redacted] oxidation measurements is part of a category 1-4 study to identify adaptations in [redacted]

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

The scientific value of this proposed research is that detailed fundamental insight will be obtained by studying nutrient-induced molecular regulation of metabolic-related processes in a direct fashion (category 1 study) and in a programmed manner into later-life (category 2 study). We will characterize these molecular effects in multiple target tissues primarily focusing on adipose tissue(s), but liver and muscle tissues are considered as important target organs as well, in order to improve our understanding of mechanistic regulation of nutrient-induced improvement of metabolic health. Also, our integrated in vivo test system increases the quality of information from our proposed studies, because paired data of multiple endpoint measures can be obtained. Next, confirmation of our hypothesis that the selected nutrients can modulate metabolic processes at the molecular level (in tissues obtained from these studies) and thereby target metabolic disease in either a direct manner or via metabolic programming into adulthood is essential and a prerequisite for further lead optimization, toxicity evaluation, and pre-clinical evaluation.
The social relevance of this proposal is that using our integrative testing strategy shortens the duration of the animal experiment, while at the same time it increases the number of measurements (paired data), which is a significant improvement from an ethical point of view. This will not only be observed as a direct effect of nutrient(s), but also by its metabolic programming effects resulting in later-life health improvement. The modulatory effect of nutrients on metabolic health will be evaluated, thus contributing to identification of nutrients that target metabolic health. These nutrients, including [redacted] (especially [redacted], or [redacted] (e.g. [redacted]), can ultimately be used in the treatment or prevention of (later-life) metabolic diseases.

3.4 Research Strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

In order to answer the aim of the present proposal, we will use our integrative in vivo model for sensitive identification of beneficial health effects as induced by the selected nutrient. Selection is, and will be, based on prior knowledge from either published or our own in vitro studies or non-overlapping in vivo studies. As an example, the [redacted] shows in silico an [redacted] of the [redacted] et al., [redacted]) which might resemble the anti-diabetic pharmacological compound [redacted]. In vivo, a direct effect has been described for [redacted] in rats ([redacted] et al., [redacted]), but effects on [redacted] as nutritional treatment of metabolic health remain elusive. In all cases, doses will be in the physiological range, not in the toxicological range, and will be discussed with the IvD.
Based on the research questions of the overarching (larger PhD) project(s), a choice selection will be made for the study category, that is for instance either analyzing a direct effect (e.g. [redacted], or a metabolic programming effect (e.g. [redacted] or [redacted]).
As the study design also depends on prior knowledge, it is hard to provide a full overview of study designs for different animal studies. As an example, for a category 2 study (metabolic programming), we will use breeding [redacted] mice to obtain offspring in standardized nests, being re-distributed at post-natal day (PN)[redacted] over the dams and remain in this setting until weaning. From PN[redacted] till PN[redacted] they will be fed the intervention diet(s) versus control in different groups, and during the last few days of intervention, continuously measured in our indirect calorimetry system. A subset of animals will be sacrificed thereafter for direct effects measurements, while the remainder of the animals will be fed for [redacted] a similar humanized high fat diet to induce fat mass and body weight gain, resulting in an adverse situation. If the intervention diet has programming potential, a beneficial health effect like reduced fat mass and body weight gain can be identified. If the beneficial health effect has a more metabolic origin without changes in fat mass or body weight, the indirect calorimetry measurements at the end of the high fat diet-feeding will be able to identify those. Moreover, a single challenge test around e.g. PN77 will add to the paired data measures and increases power to detect (programmed) metabolic differences. Such a programming study can include [redacted], as our indirect calorimetry system has [redacted] to sensitively detect [redacted] like [redacted]
In all situations, nutrient dose(s) will be at relevant physiological nutritional level. If a category 4 study (e.g. doses of [redacted]) is planned, precautions will be made in order to have [redacted] levels in diets and the level of different doses will be discussed beforehand with the IvD.
Overall, we aim to study beneficial effects of nutrient interventions, therefore the dose(s) will not cause any additional severity.
In studies, where applicable, we will include a basal group (sacrificed before intervention), and a control group. The control group consists of animals that receive the diet without the specific nutrient and run parallel to our intervention group(s). These animals are included to determine nutrient-mediated effects on studied parameters. However, as an example, within a programming study (category 2 study), sacrifices at PN[redacted] will not be of added value and are therefore not included.
Overall, selected nutrients have added interest based on for instance recent, as of yet unpublished, data; these include specific [redacted] (e.g. [redacted] versus [redacted], specific [redacted] versus [redacted]), the [redacted], and [redacted]. This latter class of nutrients is foreseen to be analyzed using a category 4 study, in which the interaction with a higher dietary [redacted] level or an altered dietary [redacted] will be used as different intervention groups. Usage of [redacted] can be foreseen in future clinical studies focusing on toddlers in order to support beneficial programmed metabolic health into adulthood (applied research).

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

Within this proposal we discriminate several categories of animal studies, although all belong to the same overarching aim: to study the effectiveness -in a direct or programmed manner- of nutrients in targeting metabolic diseases thus beneficially improving metabolic health, using a validated integrative physiological relevant in vivo model. Based on prior knowledge, we will select the specific type of intervention category or categories (see below) best fit to the research question. It is therefore eminent that every nutrient will be analyzed in only a subset of categorical studies, and not by all.
Overall, as mice and humans are 99% genetically similar (Ghunter and Dhand, Nature 2002), including most genes associated with (metabolic) disease, mice are widely accepted to be an experimental model to study human metabolic disease. In addition, C57BL/6J mice are recognized to have an eating behaviour similar to humans, e.g. overeating on a high-fat diet and thereby developing diet-induced obesity and insulin-resistance. The analysis in various tissues to study molecular regulation of metabolic health cannot be realized in humans (sampling of biopsies of liver, muscle etc. are not feasible). We have selected the C57BL/6J strain, and in more detail the [redacted] strain, because these mice are sensitive to weight gain and insulin resistance when exposed to a western high-fat diet [redacted]. In more detail, the [redacted] substrain contains -like humans- a functional [redacted] gene encoding the [redacted] metabolic relevant [redacted], while the standard [redacted] strain from [redacted] has a dysfunctional [redacted] protein. We will also make use of tissue-specific [redacted] knockout mice, which lack the [redacted] protein [redacted] metabolic processes. Knock-out mice for the two tissues we specifically focus on, [redacted] and [redacted] were generated using the Cre-Lox system, and are available on a [redacted] functional and non-functional background, all on an otherwise full [redacted] background. We focus on [redacted] and metabolic regulation as the pivotal function in (metabolic) health and its regulation.
Furthermore, the results of these studies can be used for comparison to the other animal experiments carried out in our group as these also used the C57BL/6J strain. Moreover, this also allows us to use (tissue-specific) knockout mice of the same background strain to be used for comparison and elucidation of a specific protein. Finally, the highly controlled conditions, also in our indirect calorimetry system, together with large enough sample size allows measurements of small but significant and relevant differences between dietary groups.
Several of the proposed studies (categories 2-4) will be performed in a mouse model of diet-induced obesity of which, besides non-invasive measures and minimal blood sampling during the study when an oral glucose tolerance test is included, [redacted], and [redacted] tissues will be harvested at the end of the intervention.

Specifics of the differences between the proposed animal studies are as per category:
1, direct effect: after a run-in reference diet from PN[redacted], an intervention period of [redacted] with appropriate control dietary groups, intervention dietary groups, and if possible, a positive control dietary group. Indirect calorimetry is performed at the start and end of intervention period. In this way, direct effects of a specific nutrient are investigated.
2, metabolic programming: standardized nests will be stratified at PN[redacted], and subsequent groups will be fed the intervention diets for [redacted]. A subgroup will be sacrificed at PN[redacted], while the remainder of the animals will switch to the high fat diet for another [redacted]. Indirect calorimetry will be performed at the end of the intervention and high fat diet periods. A challenge test, like oral glucose tolerance test, hypoxia challenge or a fasting-refeeding challenge in our indirect calorimetry system, is scheduled minimally after [redacted] feeding the high fat diet. In this way, beneficial health effects in adulthood -obtained by metabolic programming during early life- is investigated. This differs from a direct effect (category 1), as health effects were 'programmed' in early life with a [redacted] weeks intervention period, and all groups received the same high fat diet thereafter.

3, longitudinal effects: after weaning, mice will be stratified and fed a high versus low fat diet for another [redacted] weeks, or [redacted] weeks. Animals will undergo an oral glucose tolerance test in week [redacted] while in the latter case in week [redacted], a subgroup of mice fed the high fat diet will be switched to the low fat diet, a high fat restricted diet, or remain on the high fat diet for another [redacted] weeks. A fasting-refeeding challenge is scheduled around week [redacted] and an oral glucose tolerance test in week [redacted]. Subsets of mice will be sacrificed in week [redacted].
4, dose-interaction effects: after a run-in period, mice are subdivided over several groups and fed a control (e.g. low fat) versus adverse (e.g. high fat) diet with and without a specific nutrient (e.g. [redacted], or [redacted]) for up to [redacted] weeks. It can also be envisaged that for instance several doses of a specific [redacted] are compared against the same control diet, while keeping in all cases an [redacted] status.
5, [redacted]: as part of studies 1-4, [redacted] can be implemented in order to sensitively analyze [redacted] metabolic oxidation of [redacted]. Our indirect calorimetry system has [redacted] in order to be able to detect those [redacted] sensitively and in real-time while being non-invasive. If follow-up at organ level is required, this results in sacrifices shortly following the [redacted] bolus, which cumulatively is called [redacted]

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points

The experiments that we propose are closely linked since we will use the integrative in vivo model for testing the effects of nutrients. In all studies, the intervention period will start at PN[redacted], that is, the start of the post-weaning phase. This allows us also to study metabolic programming (category 2) when mice are switched to an adverse high fat diet shortly thereafter. Recently, we identified several nutritional interventions that program metabolic health in later life [redacted] et al., [redacted] et al., submitted, [redacted] et al., submitted, [redacted] et al., submitted), showing the power of such interventions in early life to combat metabolic diseases in later life.
Studies focusing on [redacted] will primarily be performed using category 4 studies, while a category 1 study will be selected to investigate e.g. the [redacted] for its in vivo [redacted], so the dietary background should contain specified type of [redacted] and appropriate control [redacted] diets should be taken along, as well as the positive control [redacted]. Category 1 studies start with intervention at post-weaning day PN[redacted], while category 4 studies might also be performed in adult mice.
Basal measurements versus challenge tests have shown the added value of such challenges in identifying underlying metabolic dysfunction (e.g. Duivenvoorde et al., PlosOne 2015; Duivenvoorde et al., Pflugers Arch 2015; Bardova et al., Biochemie 2016).
For the first sets of experiments, we have selected 1 nutrient to study its direct effects [redacted], category 1), and another nutrient for its metabolic programming effects [redacted] as [redacted] and [redacted], category 2), including [redacted] (category 5).
It is foreseen that for [redacted] or [redacted] studies, we will use a category 4 study setup.

Based on our ongoing research we will select nutrients from our focused subset of nutrients as described under point 3.2 Purpose, to test their beneficial modulation on metabolic health in our integrative in vivo model. For specifics, see appendices 1 - 5.

3.4.4 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	1. Direct nutritional effects on metabolic health and flexibility
2	2. Metabolic programming effects of metabolic health and flexibility

Serial number	Type of animal procedure
3	3. Longitudinal effects by nutrients in tissue-specific [redacted] knockout mice
4	4. Dose effects – interactions on metabolic health and flexibility: focus on [redacted]
5	5. [redacted] to investigate non-invasive [redacted]

4.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 1	Type of animal procedure 1. Direct nutritional effects on metabolic health and flexibility

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

Mice will receive from weaning at PN[redacted] onwards for [redacted] weeks the reference diet, whereafter mice are stratified by body weight/fat mass. Next, groups receive the different intervention diet(s) versus appropriate control diet(s). The intervention period lasts for [redacted] and food intake and body weights will be measured weekly. Body composition analysis using our non-invasive Echo-MRI is performed on a (bi)weekly schedule.
Both at the start and end of intervention period, indirect calorimetry measurements are scheduled for detailed analysis of basal whole body energy expenditure and substrate usage; metabolic flexibility will be measured only once at the end of the study. Therefore, mice will remain in the indirect calorimetry system for up to 3 to 5-7 days, depending on in- or exclusion of a specific challenge.
Mice will have ad libitum access to food and water.
At the end of the experiment, animals are sacrificed and blood and tissues will be harvested, weighted, snap frozen, and stored at -80°C until further analysis.
We aim to study beneficial effects of nutrient interventions, therefore the dose will not cause any additional severity. It is common to select two doses and in all situations, dose selection is within physiological ranges, and will be discussed with the IvD. Nutrient selection will be based on prior knowledge of published data (in vitro, in silico) and -as of yet- unpublished data (in vitro, in vivio). For example, the natural [redacted] has been shown to have [redacted] potential in silico [redacted] et al., [redacted]), and in vivo it was shown it has [redacted] ([redacted] et al., [redacted] et al., [redacted] This [redacted] resembles the pharmacological widely used anti-diabetic drug [redacted] which [redacted]

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

From our breeding pairs, offspring mice will be distributed at post-natal day PN[redacted] over standardized nests with similar ratio of males/females. If needed, surplus pups will be culled at PN[redacted]. After weaning, they receive for [redacted] weeks the standard reference diet ad libitum and are at the end stratified by body weight and distributed over the different intervention groups. Intervention diets are provided for [redacted] ad libitum. Water is always available ad libitum. At the start and end of the intervention period, our integrative test phase using our non-invasive indirect calorimetry system is key in this experiment. This benefits animal welfare as e.g. principally non-invasive measurements are used. Maximal discomfort is moderate due to individual housing. In principle, both males and females will be analyzed, as sex-dependent differences in metabolism are potentially present. If this is known from literature, we might select otherwise for a specific nutrient.

Indirect calorimetry measurements include whole body energy expenditure, substrate usage, and total activity continuously and in real-time mode measured. Moreover, both water and food intake are recorded real-time. The mice will remain in their home cage with bedding for this assessment, which lasts up to 5-7 days in case a challenge test is included. As an example, for the metabolic flexibility assessment, the mice will be fasted during

the inactive light phase and re-fed prior to the following active dark phase. The metabolic response to refeeding will be assessed in the indirect calorimetry system, and [redacted] shown that such a fasting-refeeding response is a sensitive measure to assess metabolic flexibility ([redacted] et al., [redacted]). Moreover, if we use [redacted] for refeeding, we are capable to measure [redacted] and in real-time mode the substrate usage by [redacted] the indirect calorimetry system. For instance usage of [redacted] from [redacted] instead of [redacted] already generates enough discrimination for such an analysis. Alternatively, when [redacted] like [redacted] or [redacted] are used, a single oral gavage is needed.
Measurements in the first week will give insight into immediate responses of the nutrient intervention, while measurements in the last week are essential to show long lasting nutritional responses. Those responses are mostly expected in the later stage, and therefore we will measure whole body energy expenditure and metabolic flexibility only once at the end of the study.
For proper scientific measurements of the animal responses in the indirect calorimetry system we will assess body weight and body composition (fat and lean mass) using our non-invasive Echo-MRI equipment thus excluding the necessity to use anesthetics which is needed when DEXA-scan is used; this is done before and after the indirect calorimetry measurements to be able to adjust energy expenditure based on lean mass, if different.
At the end of the experiment, animals are sacrificed and blood and tissues will be harvested, weighted, snap frozen and stored at -80°C, which will be used for detailed molecular studies.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Regarding the number of animals per group, we will use as few as possible, but as many as needed. Power calculation on our previous data shows that 12 mice per group are needed to obtain sufficient power to assess fasting-refeeding metabolic flexibility differences (measured using indirect calorimetry) induced by a nutritional intervention. For follow-up molecular analyses, e.g. global gene expression analysis, we extensively evaluated the number of samples needed and showed that n=12 is sufficient for nutritional intervention studies to detect significant and relevant differences at the level of 6-7% differential expression, taking into account the correction for multiple testing ([redacted] et al., [redacted]). Moreover, when all transcripts encoding enzymes within a single metabolic pathway are significantly regulated in the same direction (e.g. as we showed for cholesterol biosynthesis, [redacted] et al., [redacted]), this further supports the biological relevance and statistical power to detect significant differences. This is normal procedure during transcriptomics/bioinformatics analyses to analyze transcripts at the pathway level instead of at individual transcript level.
Since sex differences have shown to significantly affect whole body metabolism and physiology, and therefore also the response to intervention strategies (e.g. van Helden et al., Cell Mol Life Sci 2011), we will select both sexes in our studies, unless prior knowledge suggests otherwise. As an example, beta-carotene clearly showed opposite regulation in gene expression when males and females were compared (van Helden et al., Cell Mol Life Sci 2011). It is therefore crucial to analyze (molecular) effects in both males and females.
Regarding the number of groups needed, we aim to reduce the number of groups as much as possible. It is clear that that the setup of a specific animal experiment depends largely on the nutrient being investigated. If we are aware that a positive control exists for the proposed mechanism of action by the nutrient, such a positive control group is added, but as said, this largely depends on the choice of nutrient investigated. For example, we will study [redacted] for its [redacted] activity by adding [redacted] to a diet containing a high [redacted]. As control diets, we will use the same [redacted] without [redacted], the [redacted] diet supplemented with the positive control, the pharmacological drug [redacted] and a [redacted]. In this case, 3 control groups are used, but if we lack a positive control group, we will make use of only 2 control groups. So for the maximal six nutrients, we schedule maximal three nutrients to be studied using three control groups, and

maximal three nutrients with only two control groups. Together, using this approach we will limit the number of groups in this experiment as much as possible, thus limiting the number of animals needed.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

In our proposal we discriminate the following groups:
Control group(s): animals that receive 0 mg/kg of nutrient (negative control) and run parallel to our intervention groups (e.g. Low and High dose groups). These animals are essential to determine nutrient-mediated effects on studied parameters. If applicable, we might select two additional control groups besides the control background diet without the addition of the specific nutrient: one control group is then used as a natural 'positive' control -like a [redacted] diet in a [redacted] study- and the third control group as a pharmacological control group. Showing that the natural [redacted] has an in vivo beneficia[redacted] resembling a pharmacological compound provides scientific evidence for usage of this nutrient in future foods, and allows us to analyze its molecular mechanism in a highly focussed manner.
Low group: Animals that receive the low concentration of the selected nutrient.
High group: Animals that receive the high concentration of the selected nutrient.
For Control/Low/High groups: Section is at the end of the nutrient intervention period, and all measures will be identical in Control/Low/High groups. [redacted] mice will be used for breeding, and the offspring for the animal experiment; the run-in diet period starts after weaning and lasts three weeks. The intervention period starts at post-natal day [redacted] and runs for [redacted], so from young age until young to middle adult age. We have selected this substrain of mice because these mice are sensitive to diet-induced weight gain and insulin resistance when exposed to a western high-fat diet [redacted] Furthermore, the data of this experiment can be compared to available in-house data of our group, as these data is also from the C57BL/6J strain.
One of our critical parameters is metabolic flexibility as measure for metabolic health (measured using indirect calorimetry), which will be measured in the Control, Low and High group. The exact values used for the calculation below are based on data derived from a study in which relevant biological significance of the intervention was demonstrated, therefore the values used in the power calculations have a solid biological background. The calculation showed that in studies in which effects of dietary interventions on metabolic flexibility are analyzed, at least 12 animals per group are needed to obtain sufficient power (tested one-sided). This was calculated with Java Applet for Power and Sample Size, Lenth R.V., (http://homepage.stat.uiowa.edu/~rlenth/power) using SD1 (SD control group)=252 ml O2/6h, SD2 (SD intervention group)=350 ml O2/6h, True difference of means = 367 ml O2/6h power=0.8.
We also checked for effects on blood glucose levels and an in-house data set showed that using 12 animals gave statistical differences with p-value<0.01 being detected. If we take this latter finding along in our cumulative evaluation on optimal animal number per group, n=12 seems appropriate and sufficient for all parameters being analyzed. Since we also will perform molecular analyses (to be measured in all groups (Basal, Control, Low, High)), including global gene expression profiling, we checked the number of animals that are needed for such analyses. Multiple studies [redacted] have shown that for identification of differences the use of 12 animals are optimal. In the publication of [redacted] et al., we describe detailed calculations on basis of group sizes and identification of significantly regulated genes (1000 times random selection of animals (different group sizes) using a data set of 12 animals). The key conclusion of this calculation was that the accepted minimum group size for identification of significant differences on gene expression level is 12 animals/group.
In conclusion and cumulatively, 12 animals per group are essential in the present study in all groups in order to draw reliable conclusions from our

data.
Within this animal experiment we plan to evaluate maximal six nutrients for their direct modulatory effects on metabolic health; for three nutrients we will use only two control groups, for the other three experiments we will use three control groups. With a minimum of n=12 per group and two concentrations (0 (control), low, high) per nutrient + natural positive group (and pharmacological control), we will maximally need 648 (288+360) animals (as both sexes are analyzed). Doses are based on prior knowledge (peer-reviewed publications, preferably based on in vivo studies, and in house data not yet published) and will be discussed with IvD. Of note, we investigate here beneficial metabolic health effects, not toxicological effects of specific nutrients. Indeed, like for [redacted] we previously showed that a nutritional intervention with [redacted] showed an absence of hepatic genotoxicity which was also observed in the organ exposed to relative highest levels of dietary [redacted]. Moreover, [redacted] intake was associated with decreased, not increased, levels of biomarkers for liver damage being ALT and AST ([redacted] et al., [redacted]

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

In this proposed study, we aim to evaluate improvement of metabolic health, which is predominantly of interest for human metabolic health, but might also have its scientific effect on production animals. Also, we aim to unravel molecular modulation of nutrient-induced improvement of metabolic health. For this, analyses in various tissues (including multiple adipose tissues, skeletal muscle and liver) are essential. Since sampling of these tissues is not preferred in humans (for instance using biopsies), we will use animal models in this study. As mice and humans are 99% genetically similar (Gunthar and Dhand, Nature 2002), including most genes associated with disease, mice are widely accepted to be used as experimental model to study human disease. The C57BL/6J mice are recognized to have an eating behavior similar to humans, e.g. overeating on high-fat diet. Therefore, these animals are a good model for research on studies that aim to investigate metabolic health improvement. Because we

aim to investigate the effects of nutrient intervention on whole body metabolism and no alternative for such complex integrative processes and organ-organ interactions are available, it is not possible to use an in vitro system, which focuses only on a single individual cell-culture system without inter-organ communication. Prior knowledge of potential effects on individual cell-types might support the choice of nutrients selected to be analyzed in vivo. Based on statistical calculations, animal use will be reduced as much as possible. Our high expertise on studies in mice ensures that we are competent in designing animal studies that require the least amount of animals with the least amount of severity. As such, we have chosen mostly non-invasive but sensitive measures to answer our research questions (refinement). Mice are always housed in their homecage with bedding and cage enrichment (refinement). The methods for body composition analysis (Echo-MRI) and indirect calorimetry are both non-invasive and provide a wealth of data on respectively body composition and whole body energy metabolism. Because these measurements are non-invasive we are able to perform these measurements at more than one time point within the same animal throughout the study. This limits variation and subsequently increases the power to detect significant differences within our experimental groups in time because of paired data, leading to reduction of animal numbers. In order to study beneficial effects of nutrient intervention and subsequent analyses for optimal dose selection for metabolic health improvement, multiple doses are essential (Slob et al., 2002). Via careful dosing selection based on state-of the-art data (in house pre-screening data and/or scientific publications) we expect that using only two doses per nutrient (e.g. low and high dose) is suitable. The use of only two dose groups contributes to an important reduction of the animal number in the present study.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

We have selected sensitive mostly non-invasive measurements that will not cause pain to the animals. Importantly, we analyze beneficial health effects by nutrients, no toxicological effects. Moreover, in our indirect calorimetry system the animals will remain in their own homecage which minimizes stress. Animals will be checked daily by an experienced person to assess their health condition. Based on our animal experimental set-up we do not expect any extreme discomfort (maximal severity is moderate). In any unexpected situation that an animal suffers it will be sacrificed. Humane endpoints will be determined in individual protocols in consultation with the IvD; these include e.g. reduced appetite (reduced energy intake) leading to reduced lean mass, passive behavior not due to increased obesity, or presence of erected fur. Environment: No substantial negative effects for the environment are expected.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

A literature search in Pubmed (search term: "Metabolic Diseases"[Mesh] AND "Mice"[Mesh] AND "Metabolism"[Mesh] combined with ‘indirect calorimetry’, intervention, nutrient, or health improvement) did only reveal a few publications that investigated the use of an integrative protocol to assess improvement of metabolic health. Discussions within our international network and at conference meetings provide additional support that our

proposed research has not been performed earlier. Per nutrient, a careful literature search is part of the standardized procedure to formulate research questions related to specific nutrients, being unique and not yet published. Our work is novel and thus ensures high impact publications.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Mice will be individually housed as group-housing interferes with the study set-up by not being able to record individual food intake. Offspring mice will be individually housed as soon as they are post-weaning at PN[redacted], because it is essential to monitor individual food intake during the experiment, which is not possible if animals are grouped-housed. The first[redacted] weeks of the post-weaning period are used as control phase and by individual housing we reduce variation in food intake due to social aspects. The mice will get cage enrichment and bedding throughout the study and ad libitum food and water (except for short fasting during indirect calorimetry when a fasting-refeeding challenge is included). In such a challenge, animals will be fasted at day 4 of the indirect calorimetry measurement to ensure similar basal measurements and start values of energy metabolism of the animals. However, this fasting will be performed during the inactive phase of the animals (light phase) when mice normally do not eat; refeeding starts 1 hour prior to the dark, active phase. Therefore this adaptation is not expected to intervene with animal well-being.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[X] No > Continue with question I.
[] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[] No > Justify why pain relieving methods will not be used.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Summary of discomfort: During this proposed study the following discomfort is expected: individual housing, weighing, Echo-MRI, indirect calorimetry, and fasting if applicable as challenge test. Individual housing starts at post-natal day [redacted] and lasts for a maximum of [redacted] weeks. Adverse effects by the presence or absence of a specific nutrient are not expected, as we focus on beneficial metabolic health effects, not toxicological effects. We will make sure that all essential dietary nutrients are available at appropriate levels.

Explain why these effects may emerge.

Discomfort is caused by experimental approach as described in A. No additional discomfort expected.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

We aim to minimize the discomfort as much as possible by the following actions: Animals can stay in their homecage during the indirect calorimetry measurement, which will prevent stress. Animals will be fasted during daytime (light period), during this time span mice normally don’t eat, which minimizes potential discomfort. We will combine measures as much as possible, to prevent that animals are disturbed more than needed. For example, at days when weighing and Echo-MRI is scheduled we will do this in one run.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

If animals show less appetite (reduced energy intake), show passive behavior not due to increased obesity, or show erected fur, this will be considered as indications for humane endpoint. Importantly, as a result of our usage of the non-invasive Echo-MRI to measure lean mass and fat mass longitudinally, we will identify compromised health in an early phase, as loss of lean mass is always associated with disease. This is seen in an earlier phase than loss of body weight, so we can monitor in those cases the animals in more detail. Loss of 20% of body weight is considered as an unhealthy situation, although long term caloric restriction leading to an improved health condition, also shows loss of body weight which can reach a similar reduction in body weight (Duivenvoorde et al., J Mol Endo 2011). Moreover, when mice are placed within our indirect calorimetry system we measure continuously physical activity, which also allows us to monitor animals in a highly detailed manner. As in our studies all mice are growing or in adulthood, not being aged nor frail, the 20% body weight loss is calculated from the time-point where body weight changed from an increased or steady state into body weight loss. This is our primary criteria for humane endpoint.

Indicate the likely incidence.

As we focus on beneficial (metabolic) health effects, we do not expect that animals will suffer from humane endpoints. Nevertheless, we cannot exclude that maybe an animal might become unexpectedly ill not related to the study setup, even though the animals are all at most into their young to middle adult age. Therefore the incidence is considered very low, if at all present.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The discomfort of the individual assessments is classified as follows: Individual housing from post-weaning until end of study ([redacted] weeks): moderate Weighing: minor Echo-MRI: minor Indirect calorimetry in home cage: minor Fasting: minor We do not expect additional discomfort from the dietary intervention. For example, our experimental high-fat diets reflect both the human energy% and saturated versus unsaturated fatty acid composition. Also, when we evaluate effects of nutrients these are selected because of their suggested beneficial effects on metabolic health. During the intervention period the cumulative discomfort is moderate (because of individual housing). Collectively, the cumulative discomfort of the interventions is considered moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

To evaluate intervention-induced regulation of molecular processes on tissue level, tissues need to be dissected after sacrifice of the animals at the end of the intervention. These tissues will be used for detailed molecular analyses, such as global gene and protein expression profiling and mitochondrial activity measurements.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

5.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 2	Type of animal procedure 2. Metabolic programming effects of metabolic health and flexibility

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

As gestation and lactation periods have a large effect on offspring growth, we will use breeding pairs in our facility to keep those periods of early life as controlled as possible, since we are interested in the period of post-weaning to start the nutritional intervention as part of metabolic programming. Intervention will be from post-natal day (PN) [redacted] to PN[redacted], followed for all offspring mice by a [redacted] humanized high fat diet period to investigate beneficial health effects induced by metabolic programming. Such a strategy has been used with success in our facility.
In more detail: breeding-pairs will be time-mated and nests will be standardized based on size (4-8 pups per nest) and male/female ratio, and distribution over the dams. Previous studies showed that we did not have to cull surplus pups at his stage. After weaning at PN[redacted], offspring mice will be stratified by body weight/fat mass. From PN[redacted] onward for three weeks the different intervention diet(s) versus an appropriate control diet -if applicable- is given. All mice will thereafter receive the humanized high fat diet for [redacted] weeks. Food intake, body weight and body composition (lean and fat mass) will be measured weekly throughout the intervention period, and biweekly throughout the high fat dietary period. Body composition analysis is performed using our non-invasive Echo-MRI.
Both at the end of intervention period and the high fat diet feeding, indirect calorimetry measurements are scheduled for detailed analysis of basal whole body energy expenditure and substrate usage, physical activity and metabolic flexibility. Moreover, if we use [redacted] for refeeding, we are capable to measure [redacted] and in real-time mode the [redacted] by [redacted] in the indirect calorimetry system. For instance usage of [redacted] from [redacted] in stead of [redacted] already generates enough discrimination for such an analysis. Alternatively, when [redacted] like [redacted] or [redacted] are used, a single oral gavage is needed. Mice will have ad libitum access to food and water. If it is expected that the intervention diet effects insulin resistance at later life, an oral glucose tolerance test is scheduled once at PN77. At the end of the intervention period and the high fat diet feeding, subsets of mice will be sacrificed and blood and tissues will be harvested, weighted, snap frozen, and stored at -80°C until further analysis.
We aim to study beneficial effects of nutrient interventions, therefore the dose will not cause any additional discomfort. If a single nutrient is investigated we can select two doses which in all situations will be discussed with the IvD. From past experience, the timed-mating and subset of breeding-pairs used, together with the 12 cages available in our indirect calorimetry system, determine that we can use one reference control diet versus 2 intervention diets (or two doses of one nutrient) simultaneously. Every indirect calorimetric measurement will therefore contain 4 animals per intervention group to compare the measurements directly, and multiple batches will be measured longitudinally in order to have sufficient power to detect significant differences for which we need 12 animals in total per group.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

From our breeding-pairs, offspring mice will be distributed over standardized nests with similar ratio of males/females. After weaning, they are stratified by body weight and distributed over the different intervention groups. Intervention diets are provided for [redacted] weeks ad libitum. Water is

always available ad libitum. Thereafter the high fat diet is provided for [redacted], ad libitum. At the end of the intervention period and high fat diet feeding, our integrative test phase using our non-invasive indirect calorimetry system is key in this experiment. This benefits animal welfare (e.g. principally non-invasive measurements are used and the period with maximal discomfort (moderate due to individual housing) is as short as possible with maximal effectiveness in terms of effect). In principle, both males and females will be analyzed, as sex-dependent differences in metabolic programming studies are reported. If this is known from literature, we might select otherwise for a specific nutrient.

Indirect calorimetry measurements include whole body energy expenditure, substrate usage, and total activity continuously and in real-time mode measured. Moreover, both water and food intake are recorded real-time. The mice will remain in their home cage with bedding for this assessment, which lasts up to 5-7 days in case a challenge test is included. As an example, for the metabolic flexibility assessment, the mice will be fasted during the inactive light phase and re-fed prior to the following active dark phase. The metabolic response to refeeding will be assessed in the indirect calorimetry system, and we have shown that such a fasting-refeeding response is a sensitive measure to assess metabolic flexibility ([redacted] et al., [redacted] Alternatively, if applicable, an oral glucose tolerance test will be performed once on 5hr-fasted mice around PN77. Blood will be sampled from the tail (3x 20 µl in 30 minutes) to measure circulating glucose and insulin levels.
Measurements at the end of the intervention period will give insight into immediate and direct responses of the nutrient intervention, while measurements at the end of the high fat feeding are essential to show long lasting, metabolically programmed, nutritional responses into adulthood.
To measure the animal response to the indirect calorimetry measurements we will assess body weight and body composition before and after the indirect calorimetry measurement to be able to adjust energy expenditure based on lean mass, if different. At the end of the intervention period and the high fat feeding, subsets of mice will be sacrificed and blood and tissues will be harvested, weighted, snap frozen and stored at -80°C, which will be used for detailed molecular studies.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Regarding the number of animals per group, we will use as few as possible, but as many as needed. Power calculation on our previous data shows that 12 mice per group are needed to obtain sufficient power to assess fasting-refeeding metabolic flexibility differences (measured using indirect calorimetry) induced by a nutritional intervention. For follow-up molecular analyses, e.g. global gene expression analysis, we evaluated the number of samples needed and showed that n=12 is sufficient for nutritional intervention studies to detect significant differences ([redacted] et al., [redacted]).
Since sex differences have shown to significantly affect whole body metabolism and physiology, and therefore also the response to intervention strategies (e.g. van Helden et al., Cell Mol Life Sci 2011), we will select both sexes in our studies, unless prior knowledge suggests otherwise.
Regarding the number of groups needed, we aim to reduce the number of groups as much as possible. It is clear that that the setup of a specific animal experiment depends largely on the nutrient being investigated.
Together, using this approach we will limit the number of groups in this experiment as much as possible, thus limiting the number of animals needed.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

In our proposal we discriminate the following groups:
Control group(s): animals that receive 0 mg/kg of nutrient (negative control) from PN[redacted]-PN[redacted] and run parallel to our intervention groups (e.g. Low and High dose groups). These animals are essential to determine nutrient-mediated effect on studied parameters.
Low/high groups: Animals that receive the low/high concentration of the selected nutrient.
For Control/Low/High groups: Section of a subset of mice is at the end of the nutrient intervention period, and all measures will be identical in Control/Low/High groups. The remainder of the mice will continue on the high fat diet and be sacrificed at the end of the experiment.
C57BL/6JRccHsd mice will be used for breeding and offspring for the animal experiment. We have selected this strain because these mice are sensitive to diet-induced weight gain and insulin resistance when exposed to a western high-fat diet [redacted]. Furthermore, the data of this experiment can be compared to available in-house data of our group, as these data is also from the C57BL/6J strain. We will analyze both males and females, as clear differences in effects between the sexes are reported, also in the field of metabolic programming (e.g. reviewed by Aiken and Ozanne, Reproduction 2013; [redacted] et al., in preparation), as well as in molecular regulation by nutrients (e.g. Van Helden et al., Cell Mol Life Sci 2011).
One of our critical parameters is metabolic flexibility as marker for metabolic health (measured using indirect calorimetry), which will be measured in the Control, Low and High group (intervention period, and high fat diet feeding period). The exact values used for the calculation below are based on data derived from a study in which biological significance of the intervention was demonstrated, therefore the values used in the power calculations have a solid biological background. The calculation showed that in studies in which effects of dietary interventions on metabolic flexibility are analyzed, at least 12 animals per group are needed to obtain sufficient power (tested one-sided). This was calculated with Java Applet for Power and Sample Size, Lenth R.V., (http://homepage.stat.uiowa.edu/~rlenth/power) using SD1 (SD control group)=252 ml O2/6h, SD2 (SD intervention group)=350 ml O2/6h, True difference of means = 367 ml O2/6h power=0.8. Twelve animals per group are thus sufficient to detect relevant effects with significance, and we will per selected nutrient determine whether we need those 12 per group or that significance can be obtained with less animals. We also checked for effects on blood glucose levels and an in-house data set showed that using 12 animals gave statistical differences with p-value<0.01 being detected. If we take this latter finding along in our evaluation on optimal animal number per group, n=12 seems appropriate and sufficient. Since we also will perform molecular analyses (to be measured in all groups (Control, Low, High)), including global gene expression profiling, we checked the number of animals that are needed for such analyses. Multiple studies [redacted] have shown that for identification of differences the use of 12 animals are optimal. In the publication of [redacted] et al., we describe detailed calculations on basis of group sizes and identification of significantly regulated genes (1000 times random selection of animals (different group sizes) using a data set of 12 animals). The key conclusion of this calculation was that the accepted minimum group size for identification of significant differences on gene expression level is 12 animals/group.
In conclusion, 12 animals per group are essential in the present study in all groups in order to have sufficient statistical power and draw reliable conclusions from our data.
Within this animal experiment we plan to evaluate nutrients for their metabolic programming for maximal six consecutive experiments. Our first studies will focus on different [redacted], including different [redacted] and different [redacted]. Results can be discussed as an interim report before next studies are scheduled, if needed. Preliminary results suggest that these [redacted] display indeed different metabolic programming effects resulting in a different metabolic health status ([redacted] et al., submitted; [redacted] et al., in preparation). With a

minimum of n=12 per subgroup and 2 concentrations (0 (control), low, high) per nutrient, we will maximally need 864 (432 at PN[redacted] 432 at end of experiment) animals (as both sexes are analyzed).

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

In this proposed study, we aim to evaluate improvement of metabolic health, which is predominantly of interest for human metabolic health, but might also have its scientific effect on production animals. Also, we aim to unravel molecular modulation of nutrient-induced improvement of metabolic health. For this, analyses in various tissues (including multiple adipose tissues, skeletal muscle and liver) are essential. Since sampling of these tissues is not preferred in humans (for instance using biopsies), we will use animal models in this study. As mice and humans are 99% genetically similar (Gunthar and Dhand, Nature 2002), including most genes associated with disease, mice are widely accepted to be used as experimental model to study human disease. The C57BL/6J mice are recognized to have an eating behavior similar to humans, e.g. overeating on high-fat diet. Therefore, these animals are a good model for research on studies that aim to investigate metabolic health improvement. Because we aim to investigate the effects of nutrient intervention on whole body metabolism and no alternative for such complex integrative processes and organ-organ interactions are available, it is not possible to use an in vitro system, which focuses only on a single individual cell-culture system without inter-organ communication. Prior knowledge of potential effects on individual cell-types might support the choice of nutrients selected to be analyzed in vivo. Based on statistical calculations, animal use will be reduced as much as possible. Our high expertise on studies in mice ensures that we are competent in designing animal studies that require the least amount of animals with the least amount of severity. As such, we have chosen mostly non-invasive but sensitive measures to answer our research questions (refinement). Mice are always housed in their homecage with bedding and cage enrichment (refinement). The methods for body composition analysis (Echo-MRI) and indirect calorimetry are both non-invasive and provide a wealth of data on respectively body composition and whole body energy metabolism. Because these measurements are non-invasive we

are able to perform these measurements at more than one time point within the same animal throughout the study. This limits variation and subsequently increases the power to detect significant differences within our experimental groups in time because of paired data, leading to reduction of animal numbers. In order to study beneficial effects of nutrient intervention and subsequent analyses for optimal dose selection for metabolic health improvement, multiple doses are essential (Slob et al., 2002). Via careful dosing selection based on state-of the-art data (in house pre-screening data and/or scientific publications) we expect that using only two doses per nutrient (e.g. low and high dose) is suitable. The use of only two dose groups contributes to an important reduction of the animal number in the present study.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

We have selected sensitive mostly non-invasive measurements that will not cause pain to the animals. The only action that may cause pain is the oral glucose tolerance test, if included. Use of anaesthesia affects blood glucose homeostasis and levels, which is one of the key parameters in this study. Importantly, we analyze beneficial health effects by nutrients, no toxicological effects. Moreover, in our indirect calorimetry system the animals will remain in their own homecage which minimizes stress. Animals will be checked daily by an experienced person to assess their health condition. Based on our animal experimental set-up we do not expect any extreme discomfort (maximal discomfort is moderate due to individual housing). Implementation of new insights in this scientific field of metabolic programming will be used, if applicable, to minimize the duration of the intervention period and thus the individual housing. In any unexpected situation that an animal suffers it will be sacrificed. Humane endpoints will be determined in individual protocols in consultation with the IvD; those include passive behavior not due to obesity, or reduced energy intake leading to loss of lean mass. Environment: No substantial negative effects for the environment are expected.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

In nutrition, we are one of the few that extensively and non-invasively measure beneficial health effects of nutrients. Discussions within our international network and at conference meetings provide additional support that our proposed research has not been performed earlier. Per nutrient, a careful literature search is part of the standardized procedure to formulate research questions related to specific nutrients, being unique and not yet published. Our work is novel and thus ensures high impact publications.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Mice will be individually housed as group-housing interferes with the study set-up by not being able to record individual food intake. Offspring mice will be individually housed as soon as they are post-weaning at PN[redacted] because it is essential to monitor individual food intake during the experiment, which is not possible if animals are grouped-housed. The mice will get cage enrichment and bedding throughout the study and ad libitum food and water (except for short fasting during indirect calorimetry when a fasting-refeeding challenge is included). In such a challenge, animals will be fasted at day 4 of the indirect calorimetry measurement to ensure similar basal measurements and start values of energy metabolism of the animals. However, this fasting will be performed during the inactive phase of the animals (light phase) when mice normally do not eat; refeeding starts 1 hour prior to the dark, active phase. Therefore this adaptation is not expected to intervene with animal well-being.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.

H. Pain and pain relief

[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

The only action that may cause pain is the oral glucose tolerance test, if included in the experiment. Use of anaesthesia affects blood glucose homeostasis and levels, which is one of the key parameters in this study.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Discomfort is caused by experimental approach as described in A. No additional discomfort expected.

Explain why these effects may emerge.

Summary of discomfort: During this proposed study the following discomfort is expected: individual housing, weighing, Echo-MRI, indirect calorimetry, fasting, oral gavage of glucose during oral glucose tolerance test, blood sampling from the tail during OGTT (3x 20 µl in 30 minutes).

Indicate which measures will be adopted to prevent occurrence or minimise severity.

We aim to minimize the discomfort as much as possible by the following actions: breeding animals at our facility will be used for breeding to prevent buying and transportation of very young mice (interventions start at post-weaning age post-natal day [redacted]. Animals can stay in their homecage during the indirect calorimetry measurement, which will prevent stress. During a fasting-refeeding challenge, if applicable in the experiment, animals will be fasted during daytime (light period), during this time span mice normally don't eat, which minimizes potential discomfort. We will combine measures as much as possible, to prevent that animals are disturbed more than needed. For example, at days when weighing and Echo-MRI is scheduled we will do this in one run.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

If animals show less appetite (reduced energy intake), show passive behavior not due to increased obesity, or show erected fur, this will be considered as indications for humane endpoint. Importantly, as a result of our usage of the non-invasive Echo-MRI to measure lean mass and fat mass longitudinally, we will identify compromised health in an early phase, as loss of lean mass is always associated with disease. This is seen in an earlier phase than loss of body weight, so we can monitor in those cases the animals in more detail. Loss of 20% of body weight is considered as an unhealthy situation, although long term caloric restriction leading to an improved health condition, also shows loss of body weight which can reach a similar reduction in body weight (Duivenvoorde et al., J Mol Endo 2011). Moreover, when mice are placed within our indirect calorimetry system we measure continuously physical activity, which also allows us to monitor animals in a highly detailed manner. As in our studies all mice are growing or in adulthood, not being aged nor frail, the 20% body weight loss is calculated from the time-point where body weight changed from an increased or steady state into body weight loss. This is our primary criteria for humane endpoint.

Indicate the likely incidence.

As we focus on beneficial (metabolic) health effects, we do not expect that animals will suffer from humane endpoints; although the control group will show high fat diet-induced weight gain and likely develop insulin resistance, but this will not further develop into diabetes due to the specific strain of mice used. Nevertheless, we cannot exclude that maybe an animal might become unexpectedly ill not related to the study setup, even though the animals are all at most into their young to middle adult age. Therefore the incidence is considered very low, if at all present.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The discomfort of the individual assessments is classified as follows: Individual housing (starting at PN[redacted]): moderate Oral gavage of glucose during OGTT: minor Weighing: minor Echo-MRI: minor Indirect calorimetry in home cage: minor Fasting: minor Oral glucose tolerance test (OGTT)(20 µl blood sampling, 3x in 30 minutes): minor We do not expect additional discomfort from the dietary intervention. Our experimental humanized high-fat diet reflects both the human energy% and saturated versus unsaturated fatty acid composition. Also, when we evaluate effects of nutrients these are selected because of their suggested beneficial effects on metabolic health. Collectively, the cumulative severity of the interventions is considered moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

To evaluate intervention-induced regulation of molecular processes on tissue level, tissues need to be dissected after the intervention. These tissues will be used for detailed molecular analyses, such as gene and protein expression profiling and [redacted] activity measurements.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 3	Type of animal procedure 3. Longitudinal effects by nutrients in tissue-specific [redacted] knockout mice

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

Whole body energy metabolism is at the cellular and molecular level regulated; this regulation underlies the usage of specific nutrients as energy substrates. Mitochondria play an pivotal role in cellular energy metabolism as they are the energy-, ATP-producing cellular organs, and one of its main [redacted] As the metabolic cofactor [redacted] has to be bound to the [redacted] located [redacted] to be functionally active, this shows the highly inter-connectivity of metabolism and regulation of cellular metabolism. [redacted] is present in cytoplasm and mitochondria, where it can accept protons (reduction) to become [redacted], which subsequently donates the protons (oxidation) to the mitochondrial electron transport chain, representing oxidative phosphorylation, to ultimately produce ATP. As oxidation of carbohydrates and lipids will give rise to a difference in total NADH and FADH, thus also have effects on ratios of mitochondrial NAD/NADH and FAD/FADH, it becomes clear that the [redacted] is so closely connected to mitochondrial and cellular metabolism, that those cannot be seen as separate entities. Therefore, fundamental knowledge on this mitochondrial regulation is crucial to understand nutritional effects on metabolic health and flexibility. Indeed, whole body [redacted] knockout mice show increased insulin resistance upon a high fat diet challenge [redacted] et al., [redacted] We have tissue-specific [redacted] knockout mice to investigate on a molecular level the mitochondrial function and regulation in a specific tissue and its contribution to whole body effects, and we will analyze this longitudinally upon a nutritional intervention based on a difference in fat content (exchanged with carbohydrates) to challenge mitochondria continuously.
The experimental approach for characterization of specific (novel) genotypes of [redacted] (dys)function will be a two step procedure.
The first step is as follows:
Per genotype: Offspring mice will be stratified by body weight/fat mass after weaning at post-natal day (PN) [redacted], of which one subgroup will be sacrificed (Basal group). Two intervention dietary groups will be used next: a low fat healthy diet group, and a humanized high fat adverse diet group. Diets will be provide for [redacted] weeks, ad libitum. Water is also available ad libitum. Food intake, body weights and body composition analysis using our non-invasive Echo-MRI will be measured weekly. In week [redacted], when mice are [redacted] weeks old, a standard oral glucose tolerance test is scheduled.
At the end of the experiment, blood and tissues will be harvested, weighted, snap frozen, and stored at -80°C until further analysis.
As an example for the choice of genotypes: we have the wildtype [redacted] mice, which harbor a functional mitochondrial [redacted] gene. In contrast, the [redacted] mice have a mutation in this [redacted] gene, rendering its protein non-functional at whole body level. However, those mice also have other mutations throughout the genome when compared to the [redacted] strain, so we constructed this [redacted] substrain with and without a functional whole body [redacted] gene to study its unique function on the same background. Moreover, we have constructed different tissue-specific [redacted] knockout mice. From our breeding colony, we can therefore obtain the wildtype (WT), [redacted] on WT and on [redacted] background (double Knockout, DKO). Elucidation of the role of different tissues in whole body metabolism and its molecular cellular and mitochondrial regulation is gaining more and more scientific attention, as it underlies ultimately the complex multi-organ dynamics in energy metabolism. We focus on mitochondrial regulation and dynamics underlying metabolic health.
As a second step, a similar approach is used, which is now extended from week [redacted] onwards. Focussing on this time-frame, the control low fat dietary group will continue on the low fat diet for another [redacted] weeks, while the subgroup on the high fat diet will be stratified on body weight/fat mass and

subdivided into three separate groups: 1) a subgroup continuing on the high fat diet, 2) a subgroup switched to the low fat diet, and 3) a subgroup receiving a HFD-caloric restricted diet (HFD-CR; adjusted for vitamin and mineral content); this group will receive a 20-30energy% restriction during the [redacted] weeks follow-up. This setup is analogous to previous studies (Duivenvoorde et al., J Mol Endo 2011; Hoevenaars et al., Genes Nutrition 2014) in reduction in energy versus a reduction in fat intake. In week [redacted] a fasting-refeeding challenge test in our indirect calorimetry system will be performed to measure (mitochondrial) metabolic flexibility, and in week [redacted] an oral glucose tolerance test will be performed.
Overall, this will result in quantitative measures of [redacted] (dys)function at tissue level and its role on whole body metabolism in a longitudinal manner.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

From our breeding colony, per genotype, offspring mice are stratified after weaning at PN[redacted]. They receive the intervention diets for [redacted] weeks, and food intake, body weight, and body composition analysis is performed weekly. Subsets of mice are sacrificed at start of intervention (PN[redacted]) and end of intervention ([redacted] weeks). Food and water are provided ad libitum. In principle, both males and females will be analyzed, as sex-dependent differences in metabolism are potentially present. In week [redacted] an oral glucose tolerance test will be performed. After sacrifice, blood and tissues will be harvested, weighted, snap frozen and stored at -80°C, which will be used for detailed molecular (mitochondrial) studies. In some cases, fresh muscle tissue will be used for direct mitochondrial functional analysis.
In step two, the same procedure is followed, but the experimental setup continues for subsets of mice: the low fat dietary group will continue for another[redacted] weeks, while the high fat dietary group will be stratified and subdivided over subgroups: one subgroup continuing on the high fat diet, one group switching to the low fat diet, and one group receiving the high fat diet in a calorie-restricted manner (20-30% restricted, with adjusted vitamin and minerals to have adequate intake levels). In week [redacted], a fasting-refeeding challenge in our indirect calorimetry system will be performed, and an oral glucose tolerance test in week [redacted].

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Regarding the number of animals per group, we will use as few as possible, but as many as needed. Power calculation on our previous data shows that 12 mice per group are needed to obtain sufficient power to assess fasting-refeeding metabolic flexibility differences (measured using indirect calorimetry) induced by a nutritional intervention. For follow-up molecular analyses, e.g. global gene expression analysis, we evaluated the number of samples needed and showed that n=12 is sufficient for nutritional intervention studies to detect significant differences ([redacted] et al., [redacted]).
Since sex differences have shown to significantly affect whole body metabolism and physiology, and therefore also the response to intervention strategies (e.g. van Helden et al., Cell Mol Life Sci 2011), we will select both sexes in our studies, unless prior knowledge suggests otherwise.
Together, using this approach we will limit the number of groups in this experiment as much as possible, thus limiting the number of animals needed.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

In our proposal we discriminate the following groups:
[redacted] mice are considered the wildtype background strain mice. Moreover, we will have whole body [redacted] knockout mice on this background (resembling the [redacted] mice but with exact same background as the [redacted] strain), plus additional specific tissue-[redacted] knockout mice with and without the functional [redacted] gene. All mice will thus be on the same background of the [redacted] substrain.
We have selected this strain because these mice are sensitive to diet-induced weight gain and insulin resistance when exposed to a western humanized high-fat diet [redacted] Furthermore, the data of this experiment can be compared to available in-house data of our group, as these data are also from the [redacted] or [redacted] strain.
One of our critical parameters is metabolic flexibility (measured using indirect calorimetry in the second step). The exact values used for the calculation below are based on data derived from a study in which biological significance of the intervention was demonstrated, therefore the values used in the power calculations have a solid biological value. The calculation showed that in studies in which effects of dietary interventions on metabolic flexibility are analyzed, at least 12 animals per group are needed to obtain sufficient power (tested one-sided). This was calculated with Java Applet for Power and Sample Size, Lenth R.V., (http://homepage.stat.uiowa.edu/~rlenth/power) using SD1 (SD control group)=252 ml O2/6h, SD2 (SD intervention group)=350 ml O2/6h, True difference of means = 367 ml O2/6h power=0.8.
We also checked for effects on blood glucose levels and an in-house data set showed that using 12 animals gave statistical differences with p-value<0.01 being detected. If we take this latter finding along in our evaluation on optimal animal number per group, n=12 seems appropriate and sufficient. Since we also will perform molecular analyses (to be measured in all genotypes), including global gene expression profiling, we checked the number of animals that are needed for such analyses. Multiple studies [redacted] have shown that for identification of differences the use of 12 animals are optimal. In the publication of [redacted] et al., we describe detailed calculations on basis of group sizes and identification of significantly regulated genes (1000 times random selection of animals (different group sizes) using a data set of 12 animals). The key conclusion of this calculation was that the accepted minimum group size for identification of significant differences on gene expression level is 12 animals/group.
In conclusion, 12 animals per group are essential in the present study in all groups in order to draw reliable conclusions from our data.
Within this animal experiment we plan to evaluate 2 tissues for which we have a tissue-specific [redacted] knockout. With a maximum of n=12 per group to be sacrificed, we will need per genotype a total of 72 animals for step 1 (per sex 12 basal, 12 high fat diet and 12 low fat diet till 12 weeks, so cumulatively for both sexes in total 72 mice). and 168 animals for step 2 (per sex 12 basal, 12 high fat diet and 12 low fat diet at 12 weeks, plus till 21 weeks after the high fat feeding 12 on high fat, 12 on high fat diet restricted, 12 on low fat diet, as well as 12 that continue on the low fat diet from week 12 till 21; cumulatively for both sexes 168 mice). Cumulatively, this is 240 animals per genotype. Analysing 4 genotypes (of a specific tissue) simultaneously, this represents 960 animals. As we will investigate 2 tissues in due time, we will need 1920 animals.

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

C. Re-use

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

In this proposed study, we aim to evaluate specific [redacted] proteins for their (protective) role in metabolic health, which is predominantly of interest for human metabolic health. Also, we aim to unravel molecular modulation of nutrient-induced alterations by these proteins with a role in metabolic health. For this, analyses in various tissues (primarily focus on multiple [redacted] tissues and [redacted] are essential. Since sampling of these tissues is not preferred in humans (for instance using biopsies), we will use animal models in this study. As mice and humans are 99% genetically similar (Gunthar and Dhand, Nature 2002), including most genes associated with disease, mice are widely accepted to be used as experimental model to study human disease. The C57BL/6J mice are recognized to have an eating behavior similar to humans, e.g. overeating on high-fat diet. Therefore, these animals are a good model for research on studies that aim to investigate metabolic health improvement. Because we aim to investigate the effects of nutrient intervention on whole body metabolism and no alternative for such complex integrative processes and organ-organ interactions are available, it is not possible to use an in vitro system. Based on statistical power calculations, animal use will be reduced as much as possible. We are competent in designing animal studies that require the least amount of animals with the least amount of severity, and our expertise is (inter)nationally seen. As such, we have chosen mostly non-invasive but sensitive measures to answer our research questions (refinement). Mice are always housed in their homecage with bedding and cage enrichment (refinement). The methods for body composition analysis (Echo-MRI) and indirect calorimetry are both non-invasive and provide a wealth of data on respectively body composition (lean and fat mass) and whole body energy metabolism. Because these measurements are non-invasive we are able to perform these measurements at more than one time point within the same animal throughout the study. This limits variation and subsequently increases the power to detect significant differences within our experimental groups in time because of paired data, leading to reduction of animal numbers. Control animals are for every animal study a necessity, and unfortunately, for peer-reviewed publications more and more reviewers require the control group also not to be published previously in other studies. Therefore, grouping of different studies using the same batch of control animals seems impossible.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

We have selected sensitive mostly non-invasive measurements that will not cause pain to the animals. Importantly, we analyze beneficial health effects by nutrients, no toxicological effects. Moreover, in our indirect calorimetry system the animals will remain in their own homecage which minimizes stress. For the tissue-specific knockout mice: the whole body [redacted] knockout mice are known to have no adverse effects under basal conditions [redacted] et al., [redacted] et al., [redacted]), so we therefore do not expect an increase in adverse effects from the tissue-specific knockout. Indeed, previously reported [redacted] specific and [redacted]-specific [redacted] knockout mice were shown to not manifest any overt metabolic phenotype under either chow or high fat diet conditions [redacted] et al., [redacted]). Of note, those tissue-specific knockout mice had a different genetic background than the mice in our studies, and we also focus on [redacted] knockout mice, for which we do not know what the metabolic effects can be. Animals will be checked daily by an experienced person to assess their health condition. Based on our animal experimental set-up we do not expect any extreme discomfort (maximal severity is moderate). In any unexpected situation that an animal suffers it will be sacrificed. Humane endpoints will be determined in individual protocols in consultation with the IvD. Environment: No substantial negative effects for the environment are expected.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

Discussions within our international network and at conference meetings provide support that our proposed research has not been performed earlier. Moreover, we are the first to start using the tissue-specific knockout mice, as well as the mutated [redacted] gene on the [redacted] background. Our work is novel and thus ensures high impact publications.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Mice will be individually housed as group-housing interferes with the study set-up by not being able to record individual food intake. Offspring mice will be individually housed as soon as they are post-weaning at PN[redacted], because it is essential to monitor individual food intake during the experiment, which is not possible if animals are grouped-housed. The mice will get cage enrichment and bedding throughout the study and in general ad libitum food and water. There are two situations when food availability is restricted: 1) During a short fasting as part of a fasting-refeeding challenge in our indirect calorimetry system. In such a challenge, animals will be fasted at day 4 of the indirect calorimetry measurement to ensure similar basal measurements and start values of energy metabolism of the animals. However, this fasting will be performed during the inactive phase of the animals (light phase) when mice normally do not eat; refeeding starts 1 hour prior to the following dark, active phase. Therefore this adaptation is not expected to intervene with animal well-being. 2) In a step 2 study, where the mice fed a high fat diet for[redacted] weeks, are subsequently divided into subgroups, of which only one subgroup will continue on a high fat diet-restriction regime at 20-30% restriction for another[redacted] weeks. This has previously been shown to result in beneficial health effects, even though body weight and adipose tissue mass decreases (Duivenvoorde et al., J Mol Endo 2011; Hoevenaars et al., Genes Nutrition 2014).

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

The only action that may cause pain is the oral glucose tolerance test. Use of anaesthesia affects blood glucose homeostasis and levels, which is one of the key parameters in this study.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Summary of discomfort: During this proposed study the following discomfort is expected: individual housing, oral gavage, weighing, Echo-MRI, indirect calorimetry (only step 2), fasting, blood sampling from tail during oral glucose tolerance test (3x 20 µl in 30 minutes). It is described that [redacted] whole body knockout mice are sensitized to high fat diet-induced obesity, insulin resistance, hyperlipidemia and steatohepatitis ([redacted] et al., [redacted] while [redacted]-specific [redacted] knockout mice showed no effects on these parameters ([redacted] et al., [redacted] Of note, those mice were on a slightly different background strain than the tissue-specific [redacted] mice we have, which might show small differences in metabolic regulation.

Explain why these effects may emerge.

Discomfort is caused by experimental approach as described in A. No additional discomfort expected.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

We aim to minimize the discomfort as much as possible by the following actions: Offspring animals are individually housed after weaning (PN[redacted]) when nutritional intervention period starts. They stay in their homecage during the indirect calorimetry measurement, which will prevent stress. Animals will be fasted during daytime (light period), during this time span mice normally don't eat, which minimizes potential discomfort. We will combine measures as much as possible, to prevent that animals are disturbed more than needed. For example, at days when weighing and Echo-MRI is scheduled we will do this in one run.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

If animals show less appetite (reduced energy intake), show passive behavior not due to increased obesity, or show erected fur, this will be considered as indications for humane endpoint. Importantly, as a result of our usage of the non-invasive Echo-MRI to measure lean mass and fat mass longitudinally, we will identify compromised health in an early phase, as loss of lean mass is always associated with disease. This is seen in an earlier phase than loss of body weight, so we can monitor in those cases the animals in more detail. Loss of 20% of body weight is considered as an unhealthy situation, although long term caloric restriction leading to an improved health condition, also shows loss of body weight which can reach a similar reduction in body weight (Duivenvoorde et al., J Mol Endo 2011). Moreover, when mice are placed within our indirect calorimetry system we measure continuously physical activity, which also allows us to monitor animals in a highly detailed manner. As in our studies all mice are growing or in adulthood, not being aged nor frail, the 20% body weight loss is calculated from the time-point where body weight changed from an increased or steady state into body weight loss. This is our primary criteria for humane endpoint.

Indicate the likely incidence.

As we focus on metabolic health effects, we do not expect that animals will suffer from humane endpoints induced by the nutritional intervention. Nevertheless, we cannot exclude that maybe an animal might become unexpectedly ill not related to the study setup, like development of elephant teeth, even though the animals are all at most into their young to middle adult age. Therefore the incidence is considered very low, if at all present. We observed in our recent studies 1 mouse out of ~ 400 mice (0.25%) development of elephant teeth.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The discomfort of the individual assessments is classified as follows: Individual housing: moderate Weighing: minor Echo-MRI: minor Indirect calorimetry in home cage: minor Fasting: minor Blood sampling from tail during Oral glucose tolerance test (OGTT)(20 µl, 3x in 30 minutes): minor We do not expect additional discomfort from the dietary intervention. Our experimental high-fat diet reflects both the human energy% and saturated versus unsaturated fatty acid composition. The high-fat diet restricted subgroup receives food restricted, but this results in beneficial health effects, and we therefore classify this as minor discomfort. Collectively, the cumulative discomfort of the interventions (in total, n=768) is considered moderate. OF NOTE: Animals in the basal groups (sacrificed at PN[redacted], in total n=192) will be sacrificed before the start of the intervention. These animals will only experience discomfort from determination of body weight and body composition. The total discomfort of basal groups is therefore minor.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

To evaluate intervention-induced regulation of molecular processes on tissue level, tissues need to be dissected after the intervention. These tissues will be used for detailed molecular analyses, such as gene and protein expression profiling and mitochondrial activity measurements.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

7.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 4	Type of animal procedure 4. Dose effects – interactions on metabolic health and flexibility: focus on [redacted]

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The experimental approach of this category 4 study resembles a category 1 experimental approach, although some minor, but important, differences exist. Therefore, we classified this category 4 study as 'Dose effects'. A category 4 study will primarily be used for investigation of [redacted] or [redacted]
Offspring mice will receive from weaning at PN[redacted] onward for three weeks the reference diet for [redacted] weeks, whereafter mice are stratified by body weight/fat mass. Next, groups receive the different intervention diet(s) versus appropriate control diet(s). The intervention period lasts for [redacted] and food intake and body weights will be measured weekly. Body composition analysis using our non-invasive Echo-MRI is performed on a (bi)weekly schedule. In general, a study will contain a control diet (e.g. low fat diet), a control diet + bioactive compound, an adverse diet (e.g. high fat diet) and an adverse diet + bioactive compound at same dose as provided to the low fat diet group. Alternatively, the control diet is provided, and the intervention groups receive the same control diet with added bioactive compound (e.g. [redacted]) in 3 different doses, or the same dose of this specific bioactive compound and another dietary alteration (e.g. higher[redacted], different [redacted] but at same energy % derived from fats). We showed previously that a difference in fat content, but in the same amount, of the diet has a clear effect on metabolic flexibility [redacted] et al., [redacted]), which might be enhanced by the addition of a specific [redacted]. Selection will be based on prior knowledge of peer-reviewed publications or as of yet unpublished data of our own group, and levels will be in the physiological, not toxicological range.
At the end of the intervention period, indirect calorimetry measurements are scheduled for detailed analysis of basal whole body energy expenditure and substrate usage, physical activity, and metabolic flexibility using a fasting-refeeding challenge test in our indirect calorimetry system. Therefore, mice will remain in the indirect calorimetry system for up to 5-7 days, depending on in- or exclusion of the challenge test. Moreover, if we use [redacted] for refeeding, we are capable to measure [redacted] the substrate usage by [redacted] in the indirect calorimetry system. For instance usage of [redacted] from [redacted] in stead of [redacted] already generates enough discrimination for such an analysis. Alternatively, when [redacted] like [redacted] or [redacted] are used, a single oral gavage is needed. Mice will have ad libitum access to food and water throughout the whole study period.
At the end of the experiment, animals are sacrificed and blood and tissues will be harvested, weighted, snap frozen, and stored at -80°C until further analysis.
We aim to study beneficial effects of nutrient interventions, not toxicity, therefore the dose will not cause any additional discomfort. It is common to select two-three doses and in all situations, dose selection will be discussed with the IvD.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

From our breeding-pairs, offspring mice will be distributed over standardized nests with similar ratio of males/females. After weaning, they receive for [redacted] weeks the standard reference diet ad libitum and are at the end stratified by body weight/fat mass and distributed over the different

intervention groups. Intervention diets are provided for [redacted] weeks ad libitum. Water is always available ad libitum. At the end of the intervention period, our integrative test phase using our non-invasive indirect calorimetry system is key in this experiment. This benefits animal welfare (e.g. principally non-invasive measurements are used and the period with maximal discomfort (moderate) is as short as possible with maximal effectiveness in terms of effect). In principle, both males and females will be analyzed, as sex-dependent differences in metabolism are potentially present. For instance, it is well described for beta-carotene that males show complete opposite effects compared to females at the molecular level (van Helden et al., Cell Mol Life Sci 2011). If it is known from literature to be absent, we might select otherwise for a specific nutrient or bioactive compound.

Indirect calorimetry measurements include whole body energy expenditure, substrate usage, and total activity continuously and in real-time mode measured. Moreover, both water and food intake are recorded real-time. The mice will remain in their home cage with bedding for this assessment, which lasts up to 5-7 days in case a challenge test is included. As an example, for the metabolic flexibility assessment, the mice will be fasted during the inactive light phase and re-fed prior to the following active dark phase. The metabolic response to refeeding will be assessed in the indirect calorimetry system, and we have shown that such a fasting-refeeding response is a sensitive measure to assess metabolic flexibility [redacted] et al., [redacted]
Nutritional bioactive responses are mostly expected in the later stage, and therefore we will measure whole body energy expenditure and metabolic flexibility only once at the end of the study.
To measure the animal response to the indirect calorimetry measurements we will assess body weight and body composition before and after this measurement to be able to adjust energy expenditure based on lean mass, if different. At the end of the experiment, animals are sacrificed and blood and tissues will be harvested, weighted, snap frozen and stored at -80°C, which will be used for detailed molecular studies.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Regarding the number of animals per group for this category 4 study, we align this to the category 2 studies.
We will use as few as possible, but as many as needed. Power calculation on our previous data shows that 12 mice per group are needed to obtain sufficient power to assess fasting-refeeding metabolic flexibility differences (measured using indirect calorimetry) induced by a nutritional intervention. For follow-up molecular analyses, e.g. global gene expression analysis, we evaluated the number of samples needed and showed that n=12 is sufficient for nutritional intervention studies to detect significant differences ([redacted] et al., [redacted] Since sex differences have shown to significantly affect whole body metabolism and physiology, and therefore also the response to intervention strategies (e.g. van Helden et al., Cell Mol Life Sci 2011), we will select both sexes in our studies, unless prior knowledge suggests otherwise.
Regarding the number of groups needed, we aim to reduce the number of groups as much as possible. It is clear that that the setup of a specific animal experiment depends largely on the nutrient being investigated. If we are aware of the existence of a positive control compound, a positive control group is added, but as said, this largely depends on the choice of nutrient investigated.
Together, using this approach we will limit the number of groups in this experiment as much as possible, thus limiting the number of animals needed.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

As the set-up of this type of experimentation (category 4, see 3.2 of overall description of full application) resembles the experimental setup for direct nutritional effects on metabolic health and flexibility (category 1 experimentation), it follows the same choices and justification of animals.
In our proposal we discriminate the following groups:
Control group(s): animals that receive 0 mg/kg of nutrient (negative control) and run parallel to our intervention groups (e.g. different dose groups, or the low fat and high fat dietary groups). These animals are essential to determine nutrient-mediated effect on studied parameters.
For all these groups: Section is at the end of the nutrient intervention period, and all measures will be identical in the groups.
[redacted] mice will be used for breeding and offspring for the animal experiment. We have selected this strain because these mice are sensitive to diet-induced weight gain and insulin resistance when exposed to a western high-fat diet [redacted]. Furthermore, the data of this experiment can be compared to available in-house data of our group, as these data is also from the [redacted] strain.
The power calculation for measurements in metabolic flexibility showed that at least 12 animals per group are needed to obtain sufficient power (tested one-sided). This was calculated with Java Applet for Power and Sample Size, Lenth R.V., (http://homepage.stat.uiowa.edu/~rlenth/power) using SD1 (SD control group)=252 ml O2/6h, SD2 (SD intervention group)=350 ml O2/6h, True difference of means = 367 ml O2/6h power=0.8.
We also checked for relevant effects on blood glucose levels and an in-house data set showed that using 12 animals gave statistical differences with p-value<0.01 being detected. If we take this latter finding along in our evaluation on optimal animal number per group, n=12 seems appropriate and sufficient. Since we also will perform molecular analyses (to be measured in all groups), including global gene expression profiling, we evaluated the number of animals that are needed for such analyses. Multiple studies ([redacted] have shown that for identification of differences the use of 12 animals are optimal. In the publication of [redacted] et al., we describe detailed calculations on basis of group sizes and identification of significantly regulated genes (1000 times random selection of animals (different group sizes) using a data set of 12 animals). The key conclusion of this calculation was that the accepted minimum group size for identification of relevant, significant differences on gene expression level is 12 animals/group.
In conclusion, 12 animals per group are essential in the present study in all groups in order to draw reliable conclusions from our data.
Within this animal experiment we plan to evaluate maximal 10 nutrients/bioactives for their direct modulatory effects on metabolic health; selection of nutrients is part of several PhD-projects in coming years. Independent of the exact study setup (control low fat and high fat diet, without and with bioactive versus setup of control diet and up to 3 doses of single bioactive), we need 4 groups per study, so 4*12 (animals)*2 (males and females) * 10 (different nutrients/bioactives) = maximally 960 animals.

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

In line with category 1 studies, here, in this proposed study, we aim to evaluate improvement of metabolic health, which is predominantly of interest for human metabolic health, but might also have its scientific effect on production animals. Also, we aim to unravel molecular modulation of nutrient-induced improvement of metabolic health. For this, analyses in various tissues (including multiple adipose tissues, skeletal muscle and liver) are essential. Since sampling of these tissues is not preferred in humans (for instance using biopsies), we will use animal models in this study. As mice and humans are 99% genetically similar (Gunthar and Dhand, Nature 2002), including most genes associated with disease, mice are widely accepted to be used as experimental model to study human disease. The C57BL/6J mice are recognized to have an eating behaviour similar to humans, e.g. overeating on high-fat diet. Therefore, these animals are a good model for research on studies that aim to investigate metabolic health improvement. Because we aim to investigate the effects of nutrient intervention on whole body metabolism and no alternative for such complex integrative processes and organ-organ interactions are available, it is not possible to use an in vitro system. Based on statistical calculations, animal use will be reduced as much as possible. Our high expertise on studies in mice and strong track-record in this field ensures that we are competent in designing animal studies that require the least amount of animals with the least amount of severity. As such, we have chosen mostly non-invasive but sensitive measures to answer our research questions (refinement). Mice are always housed in their homecage with bedding and cage enrichment (refinement). The methods for body composition analysis (Echo-MRI) and indirect calorimetry are both non-invasive and provide a wealth of data on respectively body composition and whole body energy metabolism. Because these measurements are non-invasive we are able to perform these measurements at more than one time point within the same animal throughout the study. This limits variation and subsequently increases the power to detect significant differences within our experimental groups in time because of paired data, leading to reduction of animal numbers. In order to study beneficial effects of nutrient intervention and subsequent analyses for optimal dose selection for metabolic health improvement, multiple doses are essential (Slob et al., 2002). Via careful dosing selection based on state-of the-art data (in house pre-screening data and/or scientific publications) we expect that using only two to three doses per nutrient is suitable. The use of only a low number of dose groups contributes to an important reduction of the animal number in the present study.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

We have selected sensitive mostly non-invasive measurements that will not cause pain to the animals. Importantly, we analyze beneficial health effects by nutrients, no toxicological effects. Moreover, in our indirect calorimetry system the animals will remain in their own homecage which minimizes stress. Animals will be checked daily by an experienced person to assess their health condition. Based on our animal experimental set-up we do not expect any extreme discomfort (maximal severity is moderate due to individual housing). In any unexpected situation that an animal suffers it will be sacrificed. Humane endpoints will be determined in individual protocols in consultation with the IvD. Environment: No substantial negative effects for the environment are expected.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

Discussions within our international network and at conference meetings provide support that our proposed research has not been performed earlier. Per nutrient or bioactive, a careful literature search is part of the standardized procedure to formulate research questions related to specific nutrients/bioactives, being unique and not yet published. Our work is novel and thus ensures high impact publications.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Mice will be individually housed as group-housing interferes with the study set-up by not being able to record individual food intake. Offspring mice will be individually housed as soon as they are post-weaning at PN[redacted], because it is essential to monitor individual food intake during the experiment, which is not possible if animals are grouped-housed. The first [redacted] weeks of the post-weaning period are used as control phase and by individual housing we reduce variation in food intake due to social aspects. The mice will get cage enrichment and bedding throughout the study and ad libitum food and water (except for short fasting during indirect calorimetry when a fasting-refeeding challenge is included). In such a challenge, animals will be fasted at day 4 of the indirect calorimetry measurement to ensure similar basal measurements and start values of energy metabolism of the

animals. However, this fasting will be performed during the inactive phase of the animals (light phase) when mice normally do not eat; refeeding starts 1 hour prior to the dark, active phase. Therefore this adaptation is not expected to intervene with animal well-being.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[X] No > Continue with question I.
[] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

As we do not expect pain to be present for the animals, we neither need to relief pain.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Summary of discomfort: During this proposed study the following discomfort is expected: individual housing, weighing, Echo-MRI, indirect calorimetry, and fasting as part of the fast-refeeding challenge. All diets will contain appropriate levels of essential dietary compounds, thus not inducing additional discomfort.

Explain why these effects may emerge.

Discomfort is caused by experimental approach as described in A. No additional discomfort expected.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

We aim to minimize the discomfort as much as possible by the following actions: animals are group housed until fully weaned, followed by stratification and intervention group assignment. From that point onward, mice are housed individually to measure individual food intake. Animals can stay in their homecage during the indirect calorimetry measurement, which will prevent stress. Animals will be fasted during daytime (light period), during this time span mice normally don't eat, which minimizes potential discomfort. We will combine measures as much as possible, to prevent that animals are disturbed more than needed. For example, at days when weighing and Echo-MRI is scheduled we will do this in one run.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

If animals show less appetite (reduced energy intake), show passive behavior not due to increased obesity, or show erected fur, this will be considered as indications for humane endpoint. Importantly, as a result of our usage of the non-invasive Echo-MRI to measure lean mass and fat mass longitudinally, we will identify compromised health in an early phase, as loss of lean mass is always associated with disease. This is seen in an earlier phase than loss of body weight, so we can monitor in those cases the animals in more detail. Loss of 20% of body weight is considered as an unhealthy situation, although long term caloric restriction leading to an improved health condition, also shows loss of body weight which can reach a similar reduction in body weight (Duivenvoorde et al., J Mol Endo 2011). Moreover, when mice are placed within our indirect calorimetry system we measure continuously physical activity, which also allows us to monitor animals in a highly detailed manner. As in our studies all mice are growing or in adulthood, not being aged nor frail, the 20% body weight loss is calculated from the time-point where body weight changed from an increased or steady state into body weight loss. This is our primary criteria for humane endpoint.

Indicate the likely incidence.

As we focus on beneficial (metabolic) health effects, we do not expect that animals will suffer from humane endpoints. Nevertheless, we cannot exclude that maybe an animal might become unexpectedly ill not related to the study setup, like development of elephant teeth, even though the animals are all at most into their young to middle adult age. Therefore the incidence is considered very low, if at all present.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

The discomfort of the individual assessments is classified as follows: Individual housing: moderate Weighing: minor Echo-MRI: minor Indirect calorimetry in home cage: minor Fasting: minor We do not expect additional discomfort from the dietary intervention. Our experimental high-fat diet reflects both the human energy% and saturated versus unsaturated fatty acid composition. Also, when we evaluate effects of bioactives or nutrients these are selected because of their suggested beneficial effects on metabolic health. Collectively, the cumulative severity of the interventions is considered moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

To evaluate intervention-induced regulation of molecular processes on tissue level, tissues need to be dissected after sacrifice of the animals at the end of the intervention. These tissues will be used for detailed molecular analyses, such as gene and protein expression profiling and [redacted] activity measurements.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

8.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10400	
1.2	Provide the name of the licenced establishment.	Wageningen University	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 5	Type of animal procedure 5. [redacted] to investigate non-invasive

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

Here, we will use [redacted] to investigate [redacted] of specific [redacted]. This is performed by providing mice a specific food as regular food, or a nutrient by gavage. In both cases, the food or the nutrient is [redacted], being a safe, [redacted] Examples of such nutrients are [redacted] or [redacted], while also corn starch versus wheat starch can be used, as [redacted] contains also [redacted]. In nature, [redacted] is normally and in the majority present as [redacted], while a very small amount is present as its [redacted]. In our indirect calorimetry system, we have [redacted], so we can sensitively and in real-time mode measure [redacted]. Using this technique, we are capable to determine [redacted] whole body [redacted] of e.g. [redacted] provided, and after sacrifice, tissue samples will also be used to determine [redacted] of [redacted] to investigate [redacted] of e.g. [redacted] or [redacted] derived from [redacted] into [redacted]. By doing so, we can perform [redacted] analyses, which largely depend on metabolic settings, which cumulatively help us to unravel underlying mechanisms.
Besides these animal studies, [redacted] to measure only [redacted] rates are also part of category 1, 2 or 4 studies as described in appendices 1, 2 and 4. In these latter cases, a single bolus (nutrient or meal) is given and [redacted] is measured in indirect calorimetry system without killing the animal.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Mice will be fed e.g. [redacted] diets as part of their regular diets, or alternatively, can be fed a single meal comparable to a single meal test as challenge test. When done in our indirect calorimetry system, we sensitively measure [redacted] the [redacted] of this single [redacted] by the [redacted].
Likewise, when we perform an oral glucose tolerance test by oral gavage of glucose, we are able to [redacted] part of the [redacted] by [redacted] and measure thereafter [redacted] of the [redacted] in the indirect calorimetry system. Moreover, after sacrifice (even after an extensive period) we are able to measure remaining [redacted] as [redacted] or even [redacted] or [redacted]
Simply said, mice will be fasted for 5 hours in the inactive light phase, whereafter they receive the single meal or [redacted] by gavage. Following breath analysis using our indirect calorimetry system is a non-invasive method to measure adequate [redacted]

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Pilot studies using [redacted], as part of the [redacted] given to young adult and middle aged male wildtype [redacted] mice, representing two age groups with a difference in metabolic flexibility as published [redacted] et al., [redacted], showed us that using minimally n=12 provides enough power to detect significant differences. Moreover, we also performed a pilot study using a [redacted] meal test [redacted] versus [redacted] component in the diet), which has much lower levels of [redacted], but again, results indicated that with minimal n=12 we will have enough statistical power to detect significant differences. Fortunately, if a tracer study is part of a category 1,2, or 4 study, those groups of mice also are based on n=12 and we are thus confident to detect significant differences based on the [redacted] of exhaled air, using our indirect calorimetry system.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Animals will be of the murine C57BL/6J strains, either with a functional [redacted] gene or the mutated [redacted] as present in the [redacted]
These mouse strains are sensitive to diet-induced overweight and obesity development, in conjunction with insulin resistance. This offers unique opportunities for non-invasive nutrient flux analysis under different metabolic situations.
Per study, we will use 12 animals per group and two groups will be compared. For example, [redacted] versus [redacted] will be compared, or [redacted] versus [redacted] (with differences in [redacted]). As sex differences are reported in metabolic studies (e.g. Van Helden et al., Cell Mol Life Sci 2011), we will analyze both males and females. Overall, ten such studies will be performed in the coming years, all as part of multiple ongoing PhD projects. Examples of those projects focus on different types of [redacted], different [redacted] and [redacted] on [redacted] digestion. Animals will be of young adult age. As supportive data for the observed differences in e.g. oxidation levels of the [redacted], we will apply transcriptomics on selected tissues, for which we evaluated the number of animals that are needed for such analyses. Multiple studies [redacted] have shown that for identification of differences the use of 12 animals are optimal. In the publication of [redacted] et al., we describe detailed calculations on basis of group sizes and identification of significantly regulated genes (1000 times random selection of animals (different group sizes) using a data set of 12 animals). The key conclusion of this calculation was that the accepted minimum group size for identification of relevant, significant differences on gene expression level is 12 animals/group.
After the first two studies, focusing on [redacted], an interim report can be discussed before continuation with the [redacted].

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

We will use as minimum number of animals as possible, and only scarcely use [redacted] for detailed tissue analysis. [redacted] whole body [redacted] using [redacted] is scheduled to be part of a category 1, 2 or 4 animal study (see appendices 1, 2 and 4). These [redacted] are minimally invasive, and only depend on the way how the nutrient or diet is provided to the animals: by oral gavage (e.g. [redacted] or [redacted] or as pelleted food having no invasive aspect. As we investigate whole body physiology, we cannot perform those studies in vitro by cell culture studies, and especially communication between organs is crucial for the way how an animal handles a specific nutrient.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

[redacted] or test meal will be given prior to the dark phase when animals are active and start eating much more than during their inactive light phase. No other adverse effects are expected.

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

A scientific literature search is a standard procedure to support defining a scientific research question for which a single tracer test is needed. To our knowledge, we are the first to have [redacted] indirect calorimetry system to detect e.g. [redacted] during mouse studies quantitatively and in real-time mode.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[] No
[X] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Animals are housed individually, especially when they are in the indirect calorimetry system in order to quantify food/nutrient intake at individual level, and more importantly, the individual whole body [redacted] levels based on [redacted].

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

H. Pain and pain relief

[X] No > Justify why pain relieving methods will not be used.

Potential pain is induced by oral gavage only. Use of anaesthetics is impossible as this increases blood glucose levels which interferes with measures of [redacted] when [redacted] or [redacted] are used.

[] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Mice will be individually housed, with bedding and cage enrichment. No additional adverse effects on welfare are expected by a single test meal challenge or [redacted]

Explain why these effects may emerge.

n.a.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Gavage is always performed by experienced personnel. Mice are housed in the indirect calorimetry system, which allows the researchers to sensitively measure welfare based on oxygen consumption and carbondioxide production. It is well known that stress induces increased glucose oxidation levels which is one of the parameters being continuously measured in the indirect calorimetry system.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

If animals show less appetite (reduced energy intake), show passive behavior not due to increased obesity, or show erected fur, this will be considered as indications for humane endpoint. Importantly, as a result of our usage of the non-invasive Echo-MRI to measure lean mass and fat mass longitudinally, we will identify compromised health in an early phase, as loss of lean mass is always associated with disease; if only a single dose by gavage is given or a 5-day study is executed it will be likely that such conditions will most likely not take place. A decrease in fat mass is seen in an earlier phase than loss of body weight, so we can monitor in those cases the animals in more detail. Loss of 20% of body weight is considered as an unhealthy situation, although long term caloric restriction leading to an improved health condition, also shows loss of body weight which can reach a similar reduction in body weight (Duivenvoorde et al., J Mol Endo 2011). Moreover, when mice are placed within our indirect calorimetry system we measure continuously physical activity, which also allows us to monitor animals in a highly detailed manner. As in our studies all mice are growing or in adulthood, not being aged nor frail, the 20% body weight loss is calculated from the time-point where body weight changed from an increased or steady state into body weight loss. This is our primary criteria for humane endpoint.

Indicate the likely incidence.

As we focus on beneficial (metabolic) health effects, we do not expect that animals will suffer from humane endpoints. Nevertheless, we cannot exclude that maybe an animal might become unexpectedly ill not related to the study setup, like development of elephant teeth, even though the animals are all at most into their young to middle adult age and the study duration is short. Therefore the incidence is considered very low, if at all present.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

Individual housing: moderate. As the oral gavage and measures in indirect calorimetry system are both minor, the cumulative classification is moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

L. Method of killing

At the end of a [redacted], animals are sacrificed in order to measure tissue content of [redacted]. For instance, when [redacted] is provided, the [redacted] will be partly [redacted], and partly [redacted] into mainly either [redacted] or [redacted] which are [redacted] into [redacted]

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

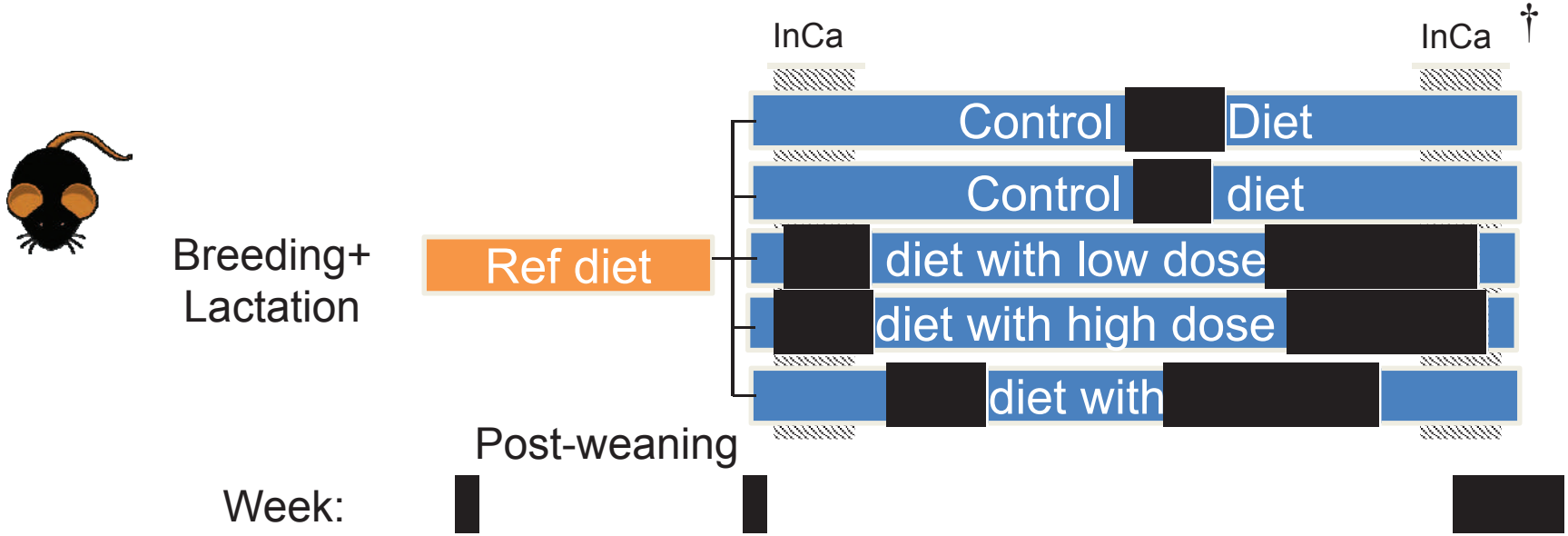
CCD application 2017.W-0024

Nutritional Physiology and Metabolic Health

[redacted]

Appendix 1: Direct nutritional effects on metabolic health and flexibility

This is detailed diagram for first study using Appendix 1 setup

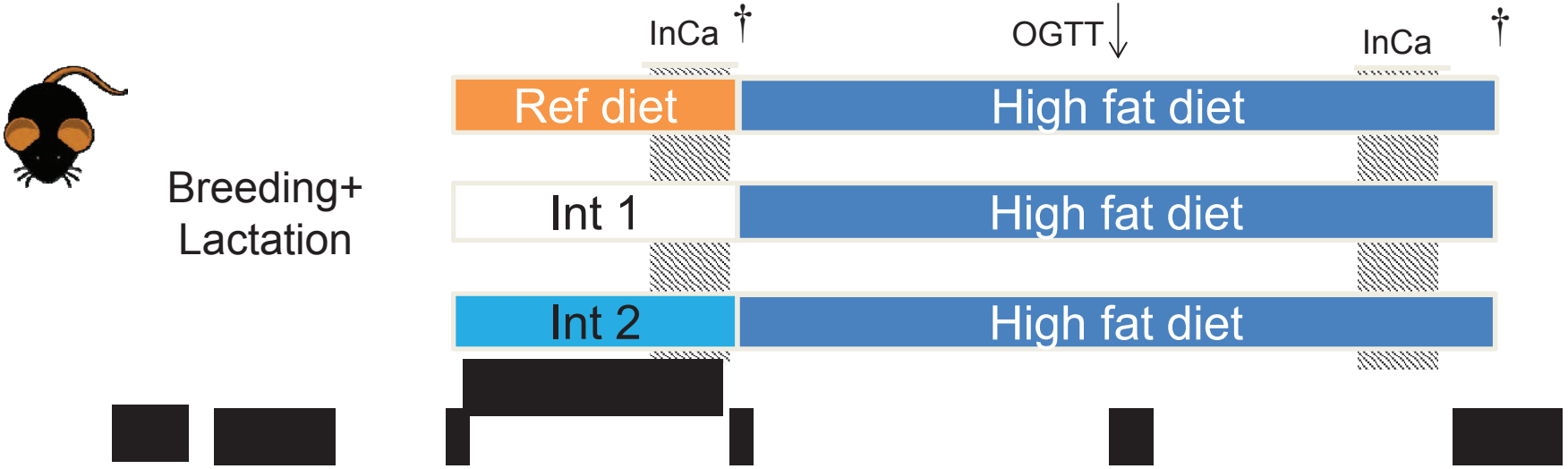


12 ♂, 12 ♀

InCa= Indirect Calorimetry, including a challenge like fasting-refeeding
†: section of mice for detailed molecular analyses

[redacted]-week Intervention diets : based on AIN93-G with ~20en% protein. [redacted] is a pharmaceutical drug and is used as a positive control to show [redacted] reflecting [redacted]
To enhance detection of [redacted] of [redacted], we will make use of our [redacted] in the InCa system. [redacted] and [redacted] levels will be based on previous published mouse studies.
This set-up is foreseen for in total 3 nutrients, while a set-up with only 2, not 3, control groups will be used for another 3 nutrients.

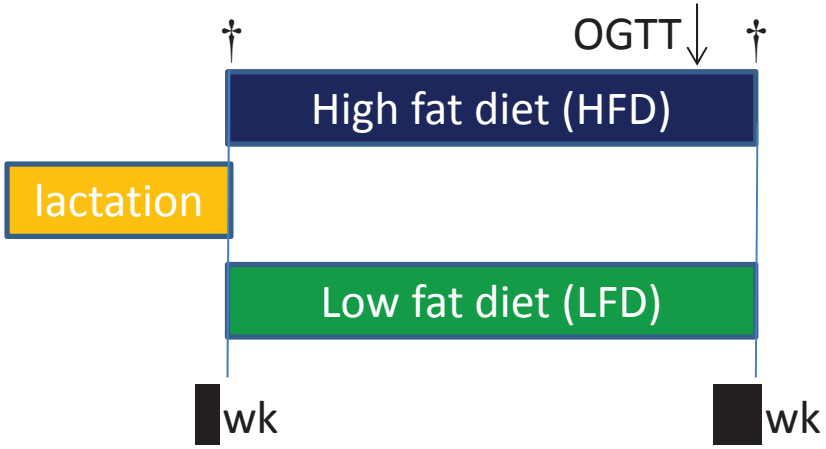
Appendix 2: Metabolic programming effects of metabolic health and flexibility



InCa= Indirect Calorimetry, including a fasting-refeeding challenge (with or without [redacted])
†: section of subset of mice for detailed molecular analyses
Int: intervention diet given [redacted]

[redacted]-week Intervention diets (en%): based on AIN93-G
Carbohydrate- and fat- amount and composition will depend on research question, but will always be based on physiological levels.

Appendix 3: Longitudinal effects by nutrients in tissue-specific [redacted] knockout mice

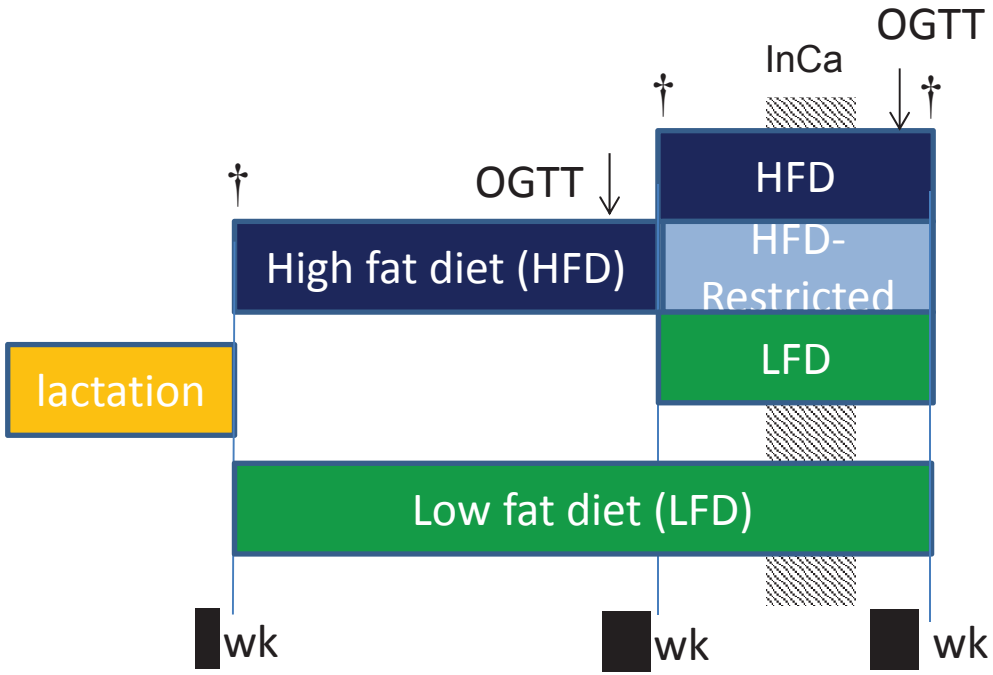


n=12 per group per genotype and per sex

Set-up 1.

OGTT = oral glucose tolerance test, week[redacted]

†: section of (subset of) mice for detailed molecular analyses



n=12 per group per genotype and per sex

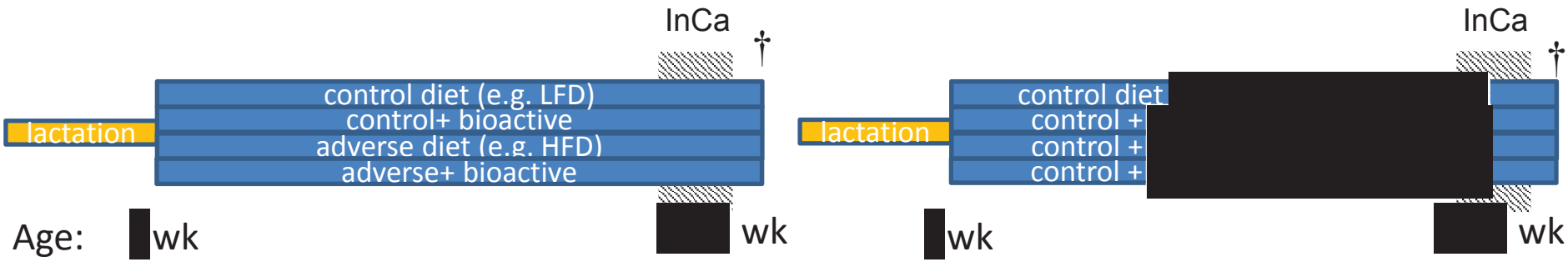
Set-up 2.

OGTT = oral glucose tolerance test, weeks [redacted] and [redacted]

InCa= Indirect Calorimetry, including a fasting-refeeding challenge (with or without [redacted])

† : section of (subset of) mice for detailed molecular analyses

Appendix 4: Dose effects – interactions on metabolic health and flexibility: focus on [redacted]



InCa= Indirect Calorimetry, including a fasting-refeeding challenge (with or without [redacted]), 1 week before sacrifice

OGTT = oral glucose tolerance test, week[redacted]

†: section of mice for detailed molecular analyses

[redacted] can either be a different concentration, a different [redacted] or [redacted] 1 plus other variation in the diet (e.g. higher [redacted] different [redacted]) [redacted] because the [redacted] is [redacted] may also be a [redacted] for example.

Overview of total animals requested for all studies

studie	n per subgroup	basal group	intermediate group	end group	total groups	genotypes	sex (1 or 2)	nutrients	tissues-KO	total:
1. direct effects	12	0	0	4.5	4.5	1	2	6	0	648
2. programming effects	12	0	3	3	6	1	2	6	0	864
3. longitudinal effects	12	2	2	6	10	4	2	1	2	1920
4. dose-effects	12	0		4	4	1	2	10	0	960
5. [redacted] effects	12	0		2	2	1	2	10	0	480
								Overall total:		4872
	total basal animals:	192								
	% basal:	3.9%	of overall total							

5. [redacted]:

Mice are kept within our indirect calorimetry system for adaptation for 24 hours, followed by basal measurements for 24-48 hours.
Thereafter, they receive a fasting-refeeding challenge by refeeding them a meal [redacted] via oral gavage.
In all these cases, a [redacted] is [redacted] thus enabling us to measure [redacted] and investigate [redacted]
Mice are killed subsequently, allowing us to study

1) tissue distribution of [redacted]
2) [redacted] by molecular analysis of [redacted] including [redacted] including [redacted] and [redacted]s, etc.

A. Algemene gegevens over de procedure

1. Aanvraagnummer: **AVD1040020171668**

2. Titel van het project: Nutritional Physiology and Metabolic Health in human, mouse as a model

3. Titel van de NTS: Voedingsfysiologie en Metabole Gezondheid in muis als model voor de mens

4. Type aanvraag: nieuwe aanvraag projectvergunning

5. Contactgegevens DEC:
   DEC-WUR
   [redacted]
   Secretaris: dec@wur.nl

6. Adviestraject
   Ontvangen door DEC: (CCD in:) 12-05-2017
   Aanvraag compleet: ja
   In vergadering besproken: 15-05-2017 (De DEC had de stukken informeel al ontvangen op 04-05-2017)
   Anderszins behandeld: n.v.t.
   Termijnonderbreking(en) van 19-05-2017 tot 02-06-2017 en van 12-06-2017 tot 13-06-2017
   Besluit van CCD tot verlenging van de totale adviestermijn met max. 15 werkdagen: n.v.t.
   Aanpassing aanvraag: 02-06-2017, 08-08-2017 en 13-06-2017
   Advies aan CCD: 21-06-2017

7. De Instantie voor Dierenwelzijn heeft een positief oordeel over de kwaliteit van de aanvraag uitgebracht en de DEC heeft dit in haar overweging betrokken.

8. Eventueel horen van aanvrager: n.v.t.

9. Correspondentie met de aanvrager:
   Datum vragen: 19-05-2017, 08-06-2017 resp. 12-06-2017
   Datum antwoord: 02-06-2017, 08-06-2017, resp. 13-06-2017 (redactioneel)
   Gestelde vragen *en antwoorden*:
   De DEC verzoekt u bij 3.4., mede met het oog op mogelijke vermindering, toe te lichten waarom de dierproeven van type 1 en 2 niet kunnen worden gecombineerd in een appendix (een voedingsinterventieproef waarbij soms wel en soms niet een OGTT wordt uitgevoerd en soms wel en soms niet individueel wordt gemeten).
   A category 1 study investigates the direct effects of a nutrient during and following the intervention period of [redacted] weeks. In contrast, a category 2 study has an intervention period of only [redacted] in early life (PN[redacted]-PN[redacted]), followed by a [redacted] of high fat diet feeding to investigate metabolic programming, resulting in beneficial health effects in adulthood, even though all animals are fed the same high fat diet from PN[redacted] onward.
   In 3.4.2, Outline it is textual adjusted into:
   "Specifics of the differences between the proposed animal studies are as per category: 1, direct effect: after a run-in reference diet from PN[redacted], an intervention period of [redacted] with appropriate control dietary groups, intervention dietary groups, and if possible, a positive control dietary group. Indirect calorimetry is performed at the start and end of intervention period. In this way, direct effects of a specific nutrient are investigated.
   2, metabolic programming: standardized nests will be stratified at PN[redacted], and subsequent groups will be fed the intervention diets for [redacted]. A subgroup will be sacrificed at PN[redacted], while the remainder of the animals will switch to the high fat diet for another [redacted]. Indirect calorimetry will be performed at the end of the interven-

tion and high fat diet periods. A challenge test, like oral glucose tolerance test, hypoxia challenge or a fasting-refeeding challenge in our indirect calorimetry system, is scheduled minimally after [redacted] feeding the high fat diet. In this way, beneficial health effects in adulthood -obtained by metabolic programming during early life- is investigated. This differs from a direct effect (category 1), as health effects were 'programmed' in early life with a [redacted] intervention period, and all groups received the same high fat diet thereafter.

Daarnaast verzoekt ze u een beknopte uitleg te geven over de indirecte calorimetrie (hoe werkt het en wat kun je ermee?).

We added the following explanation under '3.1 Background':

Also the use of indirect calorimetry is a proven accurate method to investigate energy expenditure and oxidative substrate preference. Indirect calorimetry is performed by a closed lid on top of the homecage. This lid contains an air-inlet and air-outlet connected to control units and sensors with a steady airflow through the cage; measuring oxygen consumption and carbondioxide production allows to determine energy expenditure (kJ/min) and substrate usage (ratio of carbohydrate versus lipid oxidation in case of non-protein respiratory quotient). Moreover, our indirect calorimetry system also has a food and water sensor to measure intake in a real-time mode, and infrared beams in the horizontal plane to measure real-time activity.

M.b.t. de appendices (voor zover van toepassing):

U geeft aan dat humane eindpunten (HEPs) worden toegepast als de haren overeind staan. De DEC acht het denkbaar, dat dit al in een vroeg stadium optreedt terwijl dit geen reden hoeft te zijn om de proef af te breken. De DEC verzoekt u scherper aan te geven, wanneer muizen daadwerkelijk uit de proef zullen worden gehaald. De DEC verzoekt u tevens in dit verband duidelijker aan te geven wat u bedoelt met "Loss of 20% of body weight is considered as an unhealthy situation" en ten opzichte waarvan de 20% wordt gemeten.

This is adjusted as follows (point 2, criteria Humane endpoints):

If animals show less appetite (reduced energy intake), show passive behavior not due to increased obesity, or show erected fur, this will be considered as indications for humane endpoint. Importantly, as a result of our usage of the non-invasive Echo-MRI to measure lean mass and fat mass longitudinally, we will identify compromised health in an early phase, as loss of lean mass is always associated with disease. This is seen in an earlier phase than loss of body weight, so we can monitor in those cases the animals in more detail. Loss of 20% of body weight is considered as an unhealthy situation, although long term caloric restriction leading to an improved health condition, also shows loss of body weight which can reach a similar reduction in body weight (Duivenvoorde et al., J Mol Endo 2011).

Moreover, when mice are placed within our indirect calorimetry system we measure continuously physical activity, which also allows us to monitor animals in a highly detailed manner. As in our studies all mice are growing or in adulthood, not being aged nor frail, the 20% body weight loss is calculated from the time-point where body weight changed from an increased or steady state into body weight loss. This is our primary criteria for humane endpoint.

Bovendien verzoekt zij u, met het oog op het ongerief, dat ermee gepaard gaat beknopt te beschrijven, hoe de Echo-MRI wordt uitgevoerd.

We included the following text (at '3.1 Background', analogous to explanation of indirect calorimetry):

For Echo-MRI measures, an individual mouse is put within an open plastic tube -with normal air available- in which they can move freely. This tube is positioned within the Echo-MRI equipment and a measurement lasts from 30 to roughly 60 seconds. Primary output is given as lean mass (g) and fat mass (g).

Daarnaast verzoekt ze u bij D. expliciet in te gaan op verfijning.

We altered text from "Since sampling of these tissues is not possible in humans (for instance using biopsies)" into "Since sampling of these tissues is not preferred in humans (for instance using biopsies)", as this represents better the (im)possibilities in human studies. Muscle and adipose tissue biopsies are allowed when ethical approval is obtained.

Moreover, we adjusted text into "…As such, we have chosen mostly non-invasive but sensitive measures to answer our research questions (refinement). Mice are always housed in their homecage with bedding and cage enrichment (refinement)…."
M.b.t. appendix 3:
Het in deze appendix beschreven onderzoek naar regulatoir belangrijke [redacted] eiwitten is naar het lijkt vooral belangrijk voor fundamenteel inzicht in het reguleren van de [redacted] functie. Het belang hiervan voor een nutritionele beïnvloeding van de metabole gezondheid en bestrijding van obesitasgerelateerde ziekten zoals metabool syndroom wordt aangestipt maar niet duidelijk gemaakt. Met de huidige tekst zou dit deel van het project heel goed als een aparte aanvraag voor fundamenteel onderzoek van [redacted] regulatie kunnen worden ingediend. De DEC verzoekt u de samenhang hiervan met de rest van de aanvraag te verduidelijken.
[redacted] is crucial for whole body metabolic health, as whole body knockout of the [redacted] leads to for instance increased diet-induced obesity and insulin resistance, but only when challenged by an adverse high fat diet [redacted] et al., [redacted]). This indicates a close relationship between metabolism, [redacted] and (metabolic) health. We included more information showing the indissoluble interconnectivity of metabolism and the [redacted] at several points in the application, e.g. A. and D. Surprisingly, in [redacted]-specific [redacted] knockout mice, no effects were seen when such a challenge was applied, which was also true for [redacted]-specific [redacted] knockout mice ([redacted] et al., [redacted] However, those mice were on a (partial) different background [redacted] lacking e.g. the functional [redacted] gene. These mice are also known to develop e.g. diet-induced obesity and insulin resistance much easier. This might have impact on the results obtained, and we therefore will use mouse strains that do not lack these genes. Additionally, we include [redacted]-specific [redacted] knockout mice to investigate whether the whole body SirT3 knockout phenotype can be explained by the absence of [redacted] in this metabolically important tissue. Finally, it has been suggested that caloric restriction, which is generally known to improve metabolic health, increases [redacted] expression. Altogether, our results will extend and provide new crucial knowledge on the role of [redacted] in both [redacted] and [redacted] tissue on metabolic health during diet-induced obesity and caloric restriction by including morphological and mechanistic information on [redacted] dynamics and functioning, which is missing in all previous mentioned research.
In more detail, we changed the text in A. as follows to accommodate clearness of integration with the remainder of the application:
"…[redacted]. As the metabolic cofactor [redacted] has to be bound to the [redacted] located [redacted] to be functionally active, this shows the highly inter-connectivity of metabolism and regulation of cellular metabolism. [redacted] is present in cytoplasm and mitochondria, where it can accept protons (reduction) to become [redacted], which subsequently donates the protons (oxidation) to the mitochondrial electron transport chain, representing oxidative phosphorylation, to ultimately produce ATP. As oxidation of carbohydrates and lipids will give rise to a difference in total NADH and FADH, thus also have effects on ratios of mitochondrial NAD/NADH and FAD/FADH, it becomes clear that the regulation by SirT3 is so closely connected to mitochondrial and cellular metabolism, that those cannot be seen as separate entities. Thus, fundamental knowledge on…"
"…Indeed, whole body [redacted] knockout mice show increased insulin resistance upon a high fat diet challenge [redacted] et al., [redacted]). We have tissue-specific [redacted] knockout mice to investigate on a molecular level the [redacted] function and regulation in a specific tissue and its contribution to whole body effects, and we will analyze this longitudinally…"
In 2.A geeft u eerst aan, dat de muizen ad libitum worden gevoerd en verderop (zowel bij A. als bij F.) dat een deel een caloriebeperkt dieet krijgt. De DEC verzoekt u dit zowel bij A. als bij F. te corrigeren.
To clarify this issue, we have a dual step approach in appendix 3: in the first step, animals are indeed fed ad libitum, but in the second step after [redacted] weeks ad libitum feeding a high or low fat diet, the high fat diet-fed mice are divided over three subgroups, of which only one thereafter receives a high fat diet-restriction during another [redacted] weeks. This was stated as follows in A:

"As a second step, a similar approach is used, which is now extended from week onward. Focussing on this time-frame, the control low fat dietary group will continue on the low fat diet for another weeks, while the subgroup on the high fat diet will be stratified on body weight/fat mass and subdivided into three separate groups: 1) a subgroup continuing on the high fat diet, 2) a subgroup switched to the low fat diet, and 3) a subgroup receiving a HFD-caloric restricted diet (HFD-CR; adjusted for vitamin and mineral content); this group will receive a 20-30energy% restriction during the weeks follow-up…."
and in A: "…In step two, the same procedure is followed, but the experimental setup continues for subsets of mice: the low fat dietary group will continue for another weeks, while the high fat dietary group will be stratified and subdivided over subgroups: one subgroup continuing on the high fat diet, one group switching to the low fat diet, and one group receiving the high fat diet in a calorie-restricted manner (20-30% restricted, with adjusted vitamin and minerals to have adequate intake levels)…."
Section F. was adapted as follows:
Mice will be individually housed as group-housing interferes with the study set-up by not being able to record individual food intake. Offspring mice will be individually housed as soon as they are post-weaning at PN, because it is essential to monitor individual food intake during the experiment, which is not possible if animals are grouped-housed. The mice will get cage enrichment and bedding throughout the study and in general ad libitum food and water. There are two situations when food availability is restricted:
1) During a short fasting as part of a fasting-refeeding challenge in our indirect calorimetry system. In such a challenge, animals will be fasted at day 4 of the indirect calorimetry measurement to ensure similar basal measurements and start values of energy metabolism of the animals. However, this fasting will be performed during the inactive phase of the animals (light phase) when mice normally do not eat; refeeding starts 1 hour prior to the following dark, active phase. Therefore this adaptation is not expected to intervene with animal well-being.
2) In a step 2 study, where the mice fed a high fat diet for weeks, are subsequently divided into subgroups, of which only one subgroup will continue on a high fat diet-restriction regime at 20-30% restriction for another weeks. This has previously been shown to result in beneficial health effects, even though body weight and adipose tissue mass decreases (Duivenvoorde et al., J Mol Endo 2011; Hoevenaars et al., Genes Nutrition 2014).
U geeft bij D. aan, dat de KO-muizen geen nadelige effecten ondervinden van de mutaties. De DEC verzoekt u dit met referenties nader te onderbouwen.
We added, as requested, several references as follows in D.:
"For the tissue-specific knockout mice: the whole body knockout mice are known to have no adverse effects under basal conditions (et al., ; et al.,), so we therefore do not expect an increase in adverse effects from the tissue-specific knockout. Indeed, previously reported liver-specific and specific knockout mice were shown to not manifest any overt metabolic phenotype under either chow or high fat diet conditions et al., Of note, those tissue-specific knockout mice had a different genetic background than the mice in our studies, and we also focus on knockout mice, for which we do not know what the metabolic effects can be."
To provide some more background information:
The references provide the first publication on knockout mice et al., , those mice on a high fat diet challenge et al.,) and to investigate tissue specific contributions to metabolic adaptations for and , resp. (et al.,).
Bij D. wordt gesteld dat analyse van en essentieel is, terwijl bij B. (laatste regel) staat dat er 2 weefsels zullen worden onderzocht. De DEC verzoekt u dit met elkaar in overeenstemming te brengen.
We removed in D. as primary focus, as we will focus here on and
Tot slot verzoekt ze u bij K. toe te voegen hoe de verdeling van ongerief is over de 2 klassen.
We added the following text to K:

"Collectively, the cumulative discomfort of the interventions (in total, n=768) is considered moderate.
OF NOTE: Animals in the basal groups (sacrificed at PN[redacted], in total n=192) will be sacrificed before the start of the intervention. These animals will only experience discomfort from determination of body weight and body composition. The total discomfort of basal groups is therefore minor.
M.b.t. appendix 5:
De DEC verzoekt u het aantal dieren uitgebreider te onderbouwen. Bij de overige appendices zijn de aantallen gebaseerd op diverse publicaties. Voor appendix 5 lijken deze niet van toepassing, aangezien het hier om andere uitleesparameters gaat (radioactiviteit) en de variatie anders is dan bij genexpressie. Het is de DEC niet duidelijk, waarom ook daar 12 muizen per groep nodig zijn.
First of all, we would like to stress that the use of [redacted] as part of the diet or individual nutrient, is [redacted] and safe. In fact, [redacted] is a [redacted] with a nucleus containing 1 neutron more than the natural and more common [redacted]. Being one of the [redacted] it makes up about 1.1% of all [redacted] on earth. Such compounds are safe because they are [redacted]. Our [redacted] in the indirect calorimetry system are however able to measure specifically [redacted] and [redacted], and thus we are able to measure metabolic [redacted] when [redacted] is provided. This has been tested in pilot studies based on both a [redacted], and as part of the [redacted] fraction of the normal diet, using [redacted]) versus [redacted]
We added this in A. as follows:
"In both cases, the food or the nutrient is [redacted] with [redacted], being a safe, [redacted]."
Moreover, in A. statistical methods, we altered the text as follows:
"Pilot studies using a [redacted], as part of the [redacted] bolus given to adult male mice, showed us that using n=12 provides enough power to detect significant differences. Moreover, we also performed a pilot study using a [redacted] test [redacted] versus [redacted] component in the diet), which has much lower levels of [redacted], but again, results showed that with n=12 we will have enough statistical power. Fortunately, if [redacted] is part of a category 1,2, or 4 study, those groups of mice also are based on n=12 and we are thus confident to detect significant differences based on the [redacted] of exhaled air using our indirect calorimetry system."
Naar aanleiding van dit antwoord (08-08-2017): U geeft m.b.t. de onderbouwing van 12 dieren per groep aan, dat uit pilotdata is gebleken dat u met 12 dieren per groep voldoende significantie verkrijgt. De (wettelijk verplichte) vraag is echter om aan te geven of het doel ook met minder dieren kan worden bereikt. De DEC verzoekt u hier nader op in te gaan.
Wat er beschreven was (n=12 geeft voldoende power om statistisch verschil significant aan te tonen) geeft ook de minimale groepsgrootte voor een dergelijke meting weer; tegelijkertijd realiseren we ons dat tijdens dergelijke metingen ook energy expenditure en substrate usage (RER), plus activity gemeten worden die ons ook informatie geven. Het gebruik van RER-metingen tijdens een metabole flexibiliteitstest in de indirecte calorimetrie geeft aan dat m.b.t. statistische power, minimaal n=12 noodzakelijk is (appendix 1). Cumulatief is er dus minimaal n=12 noodzakelijk, maar ook tegelijkertijd voldoende om significante verschillen waar te kunnen nemen.
De onderstreepte tekst is toegevoegd om dit duidelijker te maken.
"Pilot studies using a [redacted], as part of the [redacted] bolus given to <u>young adult and middle aged</u> male <u>wildtype</u> [redacted] mice, <u>representing two age groups with a difference in metabolic flexibility as published</u> [redacted] <u>et al.,</u> [redacted]), showed us that using <u>minimally</u> n=12 provides enough power to detect significant differences. Moreover, we also performed a pilot study using [redacted] test [redacted] versus [redacted] component in the diet), which has much lower levels of [redacted], but again, results <u>indicated</u> that with <u>minimal</u> n=12 we will have enough statistical power <u>to detect significant differences</u>. Fortunately, if [redacted] is part of a category 1,2, or 4 study, those groups of mice also are based on n=12 and we are thus confident to detect sig-

nificant differences based on the [redacted] of exhaled air, using our indirect calorimetry system".
Bovendien verzoekt de DEC u bij I. (adverse effects on welfare) toe te voegen dat de muizen individueel worden gehuisvest en de maatregelen ter beperking van ongerief die hieraan gekoppeld zijn te vermelden.
We added the following text in I.:
"Mice will be individually housed, with bedding and cage enrichment. No additional adverse effects on welfare are expected by [redacted] challenge or [redacted]."
Tot slot had de DEC enkele redactionele opmerkingen, die door de onderzoeker zijn verwerkt.

De antwoorden hebben geleid tot aanpassing van de aanvraag.

10. Eventuele adviezen door experts (niet lid van de DEC): n.v.t.

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig (dierproeven in de zin der wet).
2. De aanvraag is een nieuwe aanvraag.
3. De DEC is competent om over de aanvraag te adviseren vanuit het oogpunt van onafhankelijkheid, onpartijdigheid en beschikbare expertises.
4. Vanwege betrokkenheid bij het betreffende project is een aantal DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de behandeling van de aanvraag en het opstellen van het advies: n.v.t.

C. Beoordeling (inhoud)

1. De DEC heeft vastgesteld dat de aanvraag toetsbaar is. De samenhang lijkt door het afwezig zijn van concrete studiedoelen (welke stoffen gaan ze waarom testen) moeilijk te beoordelen, wel is de samenhang van het doen van de diverse proeven om stoffen te testen in voeding met het oog op een beter begrip van metabool syndroom duidelijk, in die zin toetsbaar. De DEC vraagt zich af of het mogelijk is een aantal bijlagen te combineren. Daar is over gesproken in de IvD, maar er zitten toch verschillende invalshoeken in, o.a. i.v.m. het gebruik van KO-muizen, de IvD achtte de keuze van de onderzoeker in die zin navolgbaar, ook met het oog op de follow-up van de experimenten en heeft dit aan de verantwoordelijkheid van de onderzoeker overgelaten. Met het oog op mogelijke vermindering is aan de onderzoeker evenwel gevraagd, waarom appendix 1 en 2 niet kunnen worden samengevoegd (zie A.9).
2. De DEC heeft geen tegenstrijdige wetgeving, gericht op de gezondheid en welzijn van het dier of het voortbestaan van de soort, gesignaleerd die het uitvoeren van de proef in de weg kan staan.
3. De DEC heeft vastgesteld dat de in de aanvraag aangekruiste doelcategorieën in overeenstemming zijn met de hoofddoelstellingen, hoewel het in haar ogen meer fundamenteel dan toegepast onderzoek lijkt, aangezien de toepassing nog ver weg lijkt. De start is wetenschappelijk, maar uiteindelijk is er interesse van partijen om het toe te passen.

Belangen en waarden

4. Het directe doel van de aanvraag is het testen van verschillende stoffen uit onze voeding in vijf verschillende voedingsproeven in muizen die gevoelig zijn voor overgewicht.
   Het uiteindelijke doel van de aanvraag is een causaal verband te vinden tussen inname van voedingsstoffen op jonge leeftijd en metabool syndroom.
   De DEC heeft vastgesteld dat er een directe en reële relatie is tussen beide doelstellingen is en dat het directe doel gerechtvaardigd is binnen de context van het onderzoeksveld.
5. De belanghebbenden en hun morele waarden in het project zijn:
   - mensen met metabool syndroom: gezondheid
   - proefdieren (muizen): ongerief door proefbehandelingen, hun gezondheid wordt niet bevorderd
   - onderzoekers: wetenschappelijke kennis, carrièrekansen
   - de maatschappij (op termijn): (kosten) volksgezondheid
   - levensmiddelenindustrie: economisch belang, vermarkting van gezonde producten

6. Voor zover de DEC dat kan inschatten is er geen aanleiding voor de DEC om de in de aanvraag beschreven effecten op het milieu in twijfel te trekken.

Proefopzet en haalbaarheid

7. De DEC heeft vastgesteld dat de kennis en kunde van de onderzoeksgroep en andere betrokkenen bij de dierproeven, afgaande op het geschreven voorstel en het oordeel van de IvD, voldoende gewaarborgd zijn. Er lijken redelijk wat publicaties aan ten grondslag te liggen.
8. De DEC heeft vastgesteld dat het project goed is opgezet, de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstelling. De gekozen strategie en experimentele aanpak kunnen in de ogen van de DEC leiden tot het behalen van de doelstellingen binnen het kader van het project. Het uitvoeren van de testen sluit goed aan bij de opzet. De keuze van de te testen voedingsmiddelen in relatie tot de daarvan te verwachten effecten op de langetermijnontwikkeling van metabool syndroom is nu niet duidelijk. Dat is in de ogen van de DEC echter niet nodig om een afweging te kunnen maken.

Welzijn dieren

9. Er is sprake van de volgende bijzonderheid op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren: in een type dierproef (appendix 5) wordt geen pijnbestrijding toegepast. Dit is voldoende beargumenteerd.
10. De dieren worden niet gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen om bijlage III van richtlijn 2010/63/EU. De dieren worden individueel gehuisvest, dat is voldoende onderbouwd.
11. De DEC stelt vast dat een cumulatieve inschatting van ongerief als "licht" of "matig" voor elk dier realistisch is ingeschat en geclassificeerd. De onderzoeker geeft aan dat er geen sprake is van ongerief van de KO-muizen door de [redacted] storing. Het ongerief bestaat (afhankelijk van het type dierproef) uit: individuele huisvesting, hanteren van de dieren (t.b.v. wegen, Echo-MRI, indirecte calorimetrie), vasten, orale gavage met geringe bloedafname tijdens de orale glucosetolerantietest.
12. Naast ongerief is er geen sprake van aantasting van integriteit van het dier anders dan door de proefbehandelingen.
13. De DEC heeft vastgesteld dat de criteria voor humane eindpunten goed zijn gedefinieerd en dat goed is ingeschat welk percentage van de dieren een humaan eindpunt zal bereiken.

3 V's

14. De DEC heeft vastgesteld dat de onderzoeker voldoende aannemelijk heeft gemaakt dat er geen alternatieven zijn om de doelstelling van het project te realiseren. Voor het vaststellen van het mechanisme achter effecten van voeding(stoffen) op complexe processen (zoals interactie tussen organen, energiemetabolisme/stofwisseling, aanpassingsvermogen) zijn (nog) geen proefdiervrije (in-vitro)methoden beschikbaar.
15. De DEC heeft vastgesteld dat de onderzoeker voldoende aannemelijk heeft gemaakt dat er optimaal tegemoet gekomen wordt aan de vereiste van vermindering van dierproeven. Een gefaseerde uitvoering van statistisch onderbouwde experimenten vormt de basis voor optimale uitvoering van dit wetenschappelijke onderzoek, mede ondersteund door ervaring in het voedingsdomein met dergelijke dierstudies. Zo min mogelijk muizen met zo min mogelijk ongerief zullen gebruikt worden, waarbij voldoende data verkregen worden om betrouwbare conclusies te trekken. Mannen en vrouwen kunnen verschillend op voeding reageren. Daarom worden mannetjes- en vrouwtjesmuizen in het onderzoek vergeleken. Inteeltmuizen worden gebruikt omdat dit variatie reduceert en er dus minder proefdieren gebruikt hoeven te worden.
    Afhankelijk van er precies wordt g test en van wat daarover bekend is qua seksegevoeligheid kan worden bepaald of het nodig is om beide seksen te onderzoeken en zo ja, of er in totaal met minder dieren kan worden volstaan. Dit moet per experiment worden onderbouwd en is naar de mening van de DEC ter beoordeling aan de IvD.
16. De DEC heeft vastgesteld dat het project in overeenstemming is met de vereiste van verfijning van dierproeven. De proeven worden zo humaan mogelijk uitgevoerd. Er is gekozen voor methoden die veel inzicht, maar zo min mogelijk ongerief geven voor de dieren geven, zoals de niet-invasieve ademhalingsluchtanalyses en het meten van het vet-

percentage m.b.v. de Echo-MRI, waarbij geen anesthesie nodig is. De muizen worden dagelijks gecontroleerd op welzijn. Muizen worden altijd in hun thuiskooi gehuisvest met bedding en kooiverrijking.
De DEC ziet geen extra mogelijkheden voor verfijning, anders dan die de onderzoeker nu toepast.

Dieren in voorraad gedood en bestemming dieren na afloop proef

17. De dieren worden in principe van beide geslachten in gelijke mate ingezet in de proeven. In geval er in de literatuur aanwijzingen zijn om hiervan af te wijken zal dit met de IvD worden overlegd.
18. De dieren worden gedood in het kader van het project. Er worden weefsels en organen geïsoleerd. De dieren worden gedood volgens een passende methode die vermeld staat in bijlage IV van richtlijn 2010/63/EU

NTS

19. De NTS is naar het oordeel van de DEC een evenwichtige weergave van het project, begrijpelijk geformuleerd en voldoet aan de vereisten in de herziene Wod Art. 10.a.1.7.

D. Ethische afweging

1. De centrale morele vraag van het project is: Rechtvaardigt het doen van onderzoek naar metabool syndroom en de onderliggende oorzaken, de inzet van 5500 dieren, die licht of matig ongerief ondergaan?
2. In de afweging heeft de DEC geconstateerd dat het hier gaat om een aanvraag met voldoende samenhang.

Zij heeft meegewogen dat er gezien het directe doel voor de onderzoekers/ het onderzoeksinstituut sprake is van een beperkt voordeel. Het gaat hierbij om carrièremogelijkheden en kennisvermeerdering.
Daarnaast zijn de waarden van de proefdieren in het geding. Een deel van de dieren zal maximaal matig nadeel ondervinden als gevolg van de handelingen binnen dit project.
Als het project zijn doel bereikt, zal in potentie er sprake zijn van een substantiële gezondheidswinst voor een aanzienlijk deel van de bevolking dat lijdt aan metabool syndroom. Wereldwijd gaat het om grote aantallen. Naast gezondheidswinst kan er voor de maatschappij op termijn ook een reëel voordeel worden behaald door verlaging van de kosten voor de volksgezondheid. Tot slot kan de levensmiddelenindustrie (economisch) voordeel hebben door vermarkting van de kennis uit dit project. De DEC heeft dit ingeschat als een beperkt belang in de ethische afweging.
Op basis van bovenstaande overwegingen is de DEC van mening dat het ethisch verantwoord is om onderzoek te doen naar 5.500 dieren met maximaal matig ongerief voor maximaal 5.280 dieren. De DEC ziet in dit stadium geen mogelijkheden op het terrein van vervanging, vermindering van het aantal dieren en verfijning van de aanvraag.

3. De centrale morele vraag kan met "ja" beantwoord worden.

E. Advies

1. Advies aan de CCD: De DEC adviseert de vergunning te verlenen.

2. Het uitgebrachte advies is gebaseerd op consensus.

3. Onderstaand dilemma is naar voren gekomen tijdens het beoordelen van de aanvraag en het opstellen van het advies: het project richt zich op een probleem (obesitas) dat als welvaartsziekte kan worden beschouwd, waarvan mensen zelf deels probleemeigenaar zijn en waar ze door gedragsverandering invloed op hebben. Dit blijkt in de praktijk niet gemakkelijk realiseerbaar. Het probleem is omvangrijker dan dat alleen. Bovendien heeft de WHO obesitas als een ziekte geïdentificeerd. Daarnaast richt het project zich op imprinting bij jonge kinderen die geen invloed hebben op hun voeding en het beperken van nadelige effecten van de welvaart op hun ontwikkeling. Binnen de context dat de DEC verwacht dat de welvaart niet zal afnemen en de neiging tot (over)consumptie ook niet, laat zij het belang van kennisontwikkeling en de zoektocht naar oplossingen hiervoor zwaarder wegen dan het matige ongerief bij 5.500 proefdieren.

11.

Centrale Commissie Dierproeven

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info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD1040020171668

Bijlagen
2

Datum 12 mei 2017
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted],

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 11 mei 2017. Het gaat om uw project "Nutritional Physiology and Metabolic Health in human, mouse as a model". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD1040020171668. Gebruik dit nummer wanneer u contact met de CCD opneemt.

Wacht met de uitvoering van uw project
Als wij nog informatie van u nodig hebben dan ontvangt u daarover bericht. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn bijgeschreven op de rekening van de CCD. U ontvangt binnen veertig werkdagen een beslissing op uw aanvraag. Als wij nog informatie van u nodig hebben, wordt deze termijn opgeschort. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Factuur
Bijgaand treft u de factuur aan voor de betaling van de leges. Wij verzoeken u de leges zo spoedig mogelijk te voldoen, zodat we uw aanvraag in behandeling kunnen nemen. Is uw betaling niet binnen dertig dagen ontvangen, dan kan uw aanvraag buiten behandeling worden gesteld. Dit betekent dat uw aanvraag niet beoordeeld wordt en u uw project niet mag starten.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Datum:
12 mei 2017
Aanvraagnummer:
AVD1040020171668

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Datum:
12 mei 2017
Aanvraagnummer:
AVD1040020171668

Gegevens aanvrager

Uw gegevens

Deelnemersnummer NVWA:	10400
Naam instelling of organisatie:	Wageningen University & Research
Naam portefeuillehouder of diens gemachtigde:	[redacted]
Straat en huisnummer:	Akkermaalsbos 12
Postbus:	59
Postcode en plaats:	6700 AW WAGENINGEN
IBAN:	NL10RABO0397066465
Tenaamstelling van het rekeningnummer:	Wageningen UR

Gegevens verantwoordelijke onderzoeker

Naam:	[redacted]
Functie:	Onderzoeker
Afdeling:	[redacted]
Telefoonnummer:	
E-mailadres:	

Datum:
12 mei 2017
Aanvraagnummer:
AVD1040020171668

Gegevens plaatsvervangende verantwoordelijke onderzoeker

Naam:	[redacted]
Functie:	Onderzoeker
Afdeling:	[redacted]
E-mailadres:	

Over uw aanvraag

Wat voor aanvraag doet u?	[x] Nieuwe aanvraag [ ] Wijziging op een (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn [ ] Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn

Over uw project

Geplande startdatum:	1 augustus 2017
Geplande einddatum:	1 augustus 2022
Titel project:	Nutritional Physiology and Metabolic Health in human, mouse as a model
Titel niet-technische samenvatting:	voedingsfysiologie en Metabole Gezondheid in muis als model voor de mens
Naam DEC:	Dec Wageningen UR
Postadres DEC:	Droevendaalsesteeg 4 6708 PB Wageningen
E-mailadres DEC:	dec@wur.nl

Betaalgegevens

De leges bedragen:	€ 1.827,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

Verplichte bijlagen:	[x] Projectvoorstel [x] Beschrijving Dierproeven [x] Niet-technische samenvatting

Ondertekening

Naam:	[redacted]
Functie:	
Plaats:	Wageningen
Datum:	11 mei 2017

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD1040020171668

Bijlagen
2

Datum 12 mei 2017
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 12 mei 2017
Vervaldatum: 11 juni 2017
Factuurnummer: 171668
Ordernummer: WUR1059476

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD1040020171668	€ 1.827,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL29INGB 070.500.1512 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 93144, 2509 AC te 's Gravenhage.

12.

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD1040020171668

Datum 11 juli 2017
Betreft aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 11 mei 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Nutritional Physiology and Metabolic Health in human, mouse as a model" met aanvraagnummer AVD1040020171668. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In deze brief leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

Welke informatie nog nodig
Wij hebben de volgende informatie van u nodig om uw aanvraag verder te kunnen beoordelen:

Niet technische samenvatting
- Het totaal aantal dieren in de verschillende bijlagen is 4892, niet de door u in de NTS aangegeven 5500. Graag dit aanpassen in de NTS (en indien nodig de percentages licht/matig ongerief aanpassen).
- De eerste twee zinnen lijken tegenstrijdige informatie te geven. Graag dit aanpassen.
- Paragrafen 3.1 en 3.4 zijn erg moeilijk beschreven. Graag deze aanpassen zodat deze duidelijk zijn voor de leek.

Onduidelijkheden
- De samenhang van uw aanvraag is niet volledig duidelijk. Kunt u een schematisch overzicht geven van de relatie tussen de verschillende onderdelen en de beslismomenten hiertussen?
- Het is ons nog niet duidelijk welke stoffen u zult gaan testen. Kunt u aangeven wat de criteria zijn waarop u uw te testen stoffen selecteert alvorens deze in vivo te testen?

- In bijlage 3.4.4.1 is onduidelijk hoe u aan de aangevraagde dieraantallen komt. Graag dit verhelderen.
- Bij bijlage 3.4.4.5 is vraag J niet ingevuld, gelieve dit nog aan te vullen.

Leges
De leges die u verschuldigd bent zijn nog niet door ons ontvangen of de betaling is nog niet verwerkt. Uw aanvraag is niet compleet als de leges niet zijn ontvangen.
Zonder deze aanvullende informatie kan de beslissing nadelig voor u uitvallen omdat de gegevens onvolledig of onduidelijk zijn.

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen veertien dagen na de datum van deze brief op. U kunt dit aanleveren via NetFTP. Stuurt u het per post op, gebruik dan het formulier dat u bij deze brief krijgt.

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Bijlagen:
- Melding bijlagen
- Niet technische samenvatting

Datum:
11 juli 2017

Aanvraagnummer:
AVD1040020171668

Below, we provide the questions and issued raised by the CCD, which we received on July 11th 2017, and we provide our responses. Overall, textual alterations in the proposal have been implemented and saved in IVention.

Welke informatie nog nodig

Wij hebben de volgende informatie van u nodig om uw aanvraag verder te kunnen beoordelen:

Niet technische samenvatting
- Het totaal aantal dieren in de verschillende bijlagen is 4892, niet de door u in de NTS aangegeven 5500. Graag dit aanpassen in de NTS (en indien nodig de percentages licht/matig ongerief aanpassen).

Response: We changed in the NTS the total number of mice as indicated to 4892, and apologize for this omission. The percentage of animals with welfare level 'licht' was and is based on the total of 4892 animals, so correctly shown in the NTS.

- De eerste twee zinnen lijken tegenstrijdige informatie te geven. Graag dit aanpassen.

Response: We altered text to exclude potential conflicting information as follows: "Steeds meer mensen kampen met (ernstig) overgewicht; in Nederland heeft al meer dan 50% van de populatie overgewicht en Wereldwijd al bijna 40%. Wereldwijd heeft 13% van de bevolking zelfs al ernstig overgewicht, oftewel obesitas. Overgewicht/obesitas is een erkende ziekte en is bovendien..."

- Paragrafen 3.1 en 3.4 zijn erg moeilijk beschreven. Graag deze aanpassen zodat deze duidelijk zijn voor de leek.

Response: We altered paragraphs 3.1 and 3.4 extensively in order to increase its accessibility for a non-scientific reader.

Onduidelijkheden
- De samenhang van uw aanvraag is niet volledig duidelijk. Kunt u een schematisch overzicht geven van de relatie tussen de verschillende onderdelen en de beslismomenten hiertussen?

Response: A schematic overview of the appendices that are part of this application are provided as attachment to the email, all centred around measuring metabolic health improvements using nutrients or food bioactives. This overview has previously also been send to the IvD, and we regret that it is not a standard possibility to include such an overview to the proposal. Which specific appendix is selected for an animal study is first of all based on the compound. For example, [redacted] as food bioactive is well described for its hepatic lipid oxidation enhanced effects, but [redacted] of [redacted] is suggested, but not yet proven, which might present a nutritional opportunity to improve glucose homeostasis. Therefore, we will use appendix 1 to study [redacted] direct effects on glucose homeostasis.
Likewise, preliminary data suggest metabolic programming by [redacted] and [redacted] in [redacted]. This will be further studied using an animal study setup described in appendix 2.
For the [redacted] tissue-specific knockouts, we will use appendix 3; [redacted] is a [redacted] of energy metabolism and thereby plays a crucial role in oxidative metabolism.
For studies focussing on [redacted], we will apply appendix 4. Again, based on the specific compound to be studied, both published and preliminary unpublished studies will provide background information for best study setup to fit scientific research question(s).
Finally, to determine [redacted] and (beneficial health) alterations induced by food bioactives or specific nutrients, we will apply appendix 5. To measure [redacted], we [redacted] our non-invasive indirect calorimetry system with [redacted] to measure specifically [redacted] which showed –preliminary- reproducible results using [redacted] and even [redacted].

- Het is ons nog niet duidelijk welke stoffen u zult gaan testen. Kunt u aangeven wat de criteria zijn waarop u uw te testen stoffen selecteert alvorens deze in vivo te testen?

Response:
Selection of nutrient or compound is, and will be, based on prior knowledge from either published or our own *in vitro* studies or non-overlapping *in vivo* studies. As an example, the [redacted] shows in silico an [redacted] of the enzyme [redacted] et al., [redacted]) which might resemble the anti-diabetic pharmacological compound [redacted]. *In vivo*, a direct effect has been described for [redacted] in rats [redacted] et al., [redacted]), but effects on [redacted] as nutritional treatment of metabolic health remain elusive. In all cases, doses will be in the physiological range, not in the toxicological range, and will be discussed with the IvD.
Based on the research questions of the overarching (larger PhD) project(s), a choice selection will be made for the study category, that is

for instance either analysing a direct effect (e.g. [redacted]), or a metabolic programming effect (e.g. [redacted] or [redacted]). In all situations, nutrient dose(s) will be at relevant physiological nutritional level. If a category 4 study (e.g. doses of [redacted]) is planned, precautions will be made in order to have [redacted] levels in diets and the level of different doses will be discussed beforehand with the IvD. Overall, selected nutrients have added interest based on for instance recent, as of yet unpublished, data; these include specific [redacted] (e.g. [redacted] versus [redacted]), specific [redacted] versus [redacted]), the [redacted], and [redacted]. This latter class of nutrients is foreseen to be analysed using a category 4 study, in which the interaction with a higher dietary [redacted] level or an altered dietary [redacted] will be used as different intervention groups. Usage of [redacted] can be foreseen in future clinical studies focusing on [redacted]s in order to support beneficial programmed metabolic health into adulthood (applied research), which might even underlie the beneficial health effects propagated by the 1,000-days window of good nutrition in the first 1,000 days of a child's life. This is largely described in paragraph 3.4.1.

- In bijlage 3.4.4.1 en 3.4.4.2 is onduidelijk hoe u aan de aangevraagde dieraantallen komt. Graag dit verhelderen.

Response: Unfortunately, we are unable to trace which paragraphs are exactly meant, as 3.4.4.1 and 3.4.4.2 do not seem to be present in IVention, nor in the resulting proposal. Nevertheless, we carefully checked every appendix for its description for usage of a specific number of animals under "Choice and justification animals". This is for appendices 1-4 described, including the power calculations for metabolic flexibility analysis, or global transcriptomics analysis, which underlie our choices. However, for appendix 5 it might have been under-described and we extended the description here.
By doing so, we hope the support for our reasoning to come to the indicated selection of number of animals is valued.

- Bij bijlage 3.4.4.5 is vraag J niet ingevuld, gelieve dit nog aan te vullen.

Response: For appendix 5, we qualified Humane endpoints as 'No', since the study length in appendix 5 is very short (e.g. single gavage and follow-up thereafter), especially when compared to the other appendices. Based on the request of the CCD, we altered this into a 'Yes' and described the criteria and incidence at points J.2 and J.3.

Leges
De leges die u verschuldigd bent zijn nog niet door ons ontvangen of de betaling is nog niet verwerkt. Uw aanvraag is niet compleet als de leges niet zijn ontvangen.

Response: We will make sure that payment is done in time, if it has not been done already.

15.

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD1040020171668
Bijlagen
1

Datum 25 juli 2017
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 11 mei 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Nutritional Physiology and Metabolic Health in human, mouse as a model" met aanvraagnummer AVD1040020171668. Wij hebben uw aanvraag beoordeeld.

Op 14 juli en 19 juli 2017. heeft u uw aanvraag aangevuld. Op ons verzoek is de NTS verduidelijkt en de dieraantallen in de NTS consistent gemaakt met die in de aanvraag, is de samenhang verhelderd en zijn in bijlage 3.4.4.5 de dieraantallen onderbouwd.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning.
De algemene voorwaarde(n) zijn opgenomen op grond van artikel 1d lid 4, artikel 10a1 lid 2, artikel 10 lid 2 en/of artikel 10a3 van de wet.

Omdat uit uw aanvraag niet blijkt welke stoffen u zal gaan onderzoeken, maar de uit te voeren handelingen wel omschreven zijn, is een voorwaarde opgenomen dat u de CCD moet terugkoppelen naar welke soort stoffen onderzoek plaats heeft gevonden. Deze voorwaarde is gesteld omdat de CCD graag een beeld wil krijgen van wat voor soort stoffen/experimenten worden uitgevoerd onder deze vergunning. Op deze wijze houdt de CCD zicht op het soort experimenten dat gedaan wordt en het soort stoffen dat getest wordt.

U kunt met uw project "Nutritional Physiology and Metabolic Health in human, mouse as a model" starten. De vergunning wordt afgegeven van 1 augustus

2017 tot en met 31 juli 2022. Deze termijn is anders dan in uw aanvraag, omdat een vergunning een maximale looptijd van 5 jaar kan hebben.

Overige wettelijke bepalingen blijven van kracht.

Procedure
Wij hebben advies gevraagd bij de Dierexperimentencommissie DEC Wageningen UR. Dit advies is opgesteld op 21 juni 2017. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet.
Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Dit advies van de commissie nemen wij over, inclusief de daaraan ten grondslag liggende motivering. Er worden aanvullende algemene voorwaarde(n) gesteld.
Het DEC-advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze nummers in de rechter kantlijn in deze brief.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Datum:
25 juli 2017
Aanvraagnummer:
AVD1040020171668

Centrale Commissie Dierproeven
namens deze:
[redacted]
ir. G. de Peuter
Algemeen Secretaris

Bijlagen:
- Vergunning
  Hiervan deel uitmakend:
  - DEC-advies
  - Weergave wet- en regelgeving

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	Wageningen University & Research
Adres:	Postbus 59
Postcode en plaats:	6700 AW WAGENINGEN
Deelnemersnummer:	10400

deze projectvergunning voor het tijdvak 1 augustus 2017 tot en met 31 juli 2022, voor het project "Nutritional Physiology and Metabolic Health in human, mouse as a model" met aanvraagnummer AVD1040020171668, volgens advies van Dierexperimentencommissie Dec Wageningen UR. Er worden aanvullende algemene voorwaarde(n) gesteld.
De functie van de verantwoordelijk onderzoeker is Onderzoeker.
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 11 mei 2017
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 19 juli 2017;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 19 juli 2017;
c Advies van dierexperimentencommissie d.d. 21 juni 2017, ontvangen op 21 juni 2017.
d De aanvullingen op uw aanvraag, ontvangen op 14 juli en 19 juli 2017.

Aanvraagnummer:
AVD1040020171668

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
3.4.4.1 Direct nutritional effects on metabolic health and flexibility				
	Muizen (Mus musculus) /	648	Matig	
3.4.4.2 Metabolic programming effects of metabolic health and flexibility				
	Muizen (Mus musculus) /	864	Matig	
3.4.4.3 Longitudinal effects by nutrients in tissue-specific [redacted] knockout mice				
	Muizen (Mus musculus) /	1.920	90% Matig 10% Licht	
3.4.4.4 Dose effects – interactions on metabolic health and flexibility: focus on [redacted]				
	Muizen (Mus musculus) /	960	Matig	
3.4.4.5 [redacted] to investigate non-invasive [redacted]				
	Muizen (Mus musculus) /	480	Matig	

Voorwaarden

Op grond van artikel 10a1 lid 2 van de Wet op de dierproeven zijn aan een projectvergunning voorwaarden te stellen

De vergunning wordt verleend onder de voorwaarde dat go/no go momenten worden afgestemd met de IvD.

Gedurende de looptijd van de vergunning, koppelt de aanvrager aan de CCD terug welk type/soort teststof is getest onder deze vergunning. Deze terugkoppeling moet uiterlijk 31 maart door de CCD ontvangen zijn

en rapporteert over het afgelopen kalenderjaar (1 januari - 31 december). Ook wanneer er geen dierstudies zijn uitgevoerd wordt dit gerapporteerd. De CCD kan op basis van deze terugkoppeling aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken. Wanneer u overtuigend en onbetwistbaar kunt aantonen dat er geen gegevens over de geteste stof kunnen worden vrijgegeven omdat de opdrachtgever deze als vertrouwelijke informatie heeft geclassificeerd kunt u deze informatie buiten de rapportage houden.

In artikel 10, lid 1 sub a van de wet, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning. Indien bovenstaande zich voordoet dient aanvrager dit in afstemming met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarde wijzigen of intrekken.

Aanvraagnummer:
AVD1040020171668

Weergave wet- en regelgeving

Dit project en wijzigingen
Volgens artikel 10c van de Wet op de Dierproeven (hierna de wet) is het verboden om andere dierproeven uit te voeren dan waar de vergunning voor is verleend. De dierproeven mogen slechts worden verricht in het kader van een project, volgens artikel 10g. Uit artikel 10b volgt dat de dierproeven zijn ingedeeld in de categorieën terminaal, licht, matig of ernstig. Als er wijzigingen in een dierproef plaatsvinden, moeten deze gemeld worden aan de Centrale Commissie Dierproeven. Hebben de wijzigingen negatieve gevolgen voor het dierenwelzijn, dan moet volgens artikel 10a5 de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b schrijft voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister een ontheffing is verleend.

Verzorging
De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn. In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

Pijnbestrijding en verdoving
In artikel 13 van de wet staat dat een dierproef onder algehele of plaatselijke verdoving wordt uitgevoerd tenzij dat niet mogelijk is, dan wel bij het verrichten van een dierproef worden pijnstillers toegediend of andere goede methoden gebruikt die de pijn, het lijden, de angst of de blijvende schade bij het dier tot een minimum beperken. Een dierproef die bij het dier gepaard gaat met zwaar letsel dat hevige pijn kan veroorzaken, wordt niet zonder verdoving uitgevoerd. Hierbij wordt afgewogen of het toedienen van verdoving voor het dier traumatischer is dan de dierproef zelf en het toedienen van verdoving onverenigbaar is met het doel van de dierproef. Bij een dier wordt geen stof toegediend waardoor het dier niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn

kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

Einde van een dierproef
Artikel 13a van de wet bepaalt dat een dierproef is afgelopen wanneer voor die dierproef geen verdere waarnemingen hoeven te worden verricht of, voor wat betreft nieuwe genetisch gemodificeerde dierenlijnen, wanneer bij de nakomelingen niet evenveel of meer, pijn, lijden, angst, of blijvende schade wordt waargenomen of verwacht dan bij het inbrengen van een naald. Er wordt dan door een dierenarts of een andere ter zake deskundige beslist of het dier in leven zal worden gehouden. Een dier wordt gedood als aannemelijk is dat het een matige of ernstige vorm van pijn, lijden, angst of blijven schade zal blijven ondervinden. Als een dier in leven wordt gehouden, krijgt het de verzorging en huisvesting die past bij zijn gezondheidstoestand.
Volgens artikel 13b moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.
Uit artikel 13d volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13c is vastgesteld dat proefdieren geadopteerd kunnen worden, teruggeplaatst in hun habitat of in een geschikt dierhouderijsysteem, als de gezondheidstoestand van het dier het toelaat, er geen gevaar is voor volksgezondheid, diergezondheid of milieu en er passende maatregelen zijn genomen om het welzijn van het dier te waarborgen.
De Minister heeft vrijstelling ontheffing verleend volgens artikel 13c, die de afwijkende methode van doden op basis van wetenschappelijke motivering ten minste even humaan acht als de in de richtlijn opgenomen passende methoden.