PAPER III

Chemical composition of Calanus oil and safety assessment based on physiological studies in rats

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Running title: Safety assessment of Calanus oil

Funding and conflict of interest statement: This study was mainly funded by Calanus AS. Other funding sources: The Norwegian Diabetes Association and the Norwegian Heart Foundation.

KST declares conflict of interest as an employee of Calanus AS. All other authors have a potential conflict of interest related to project support, according to ICMJE "Ethical Considerations in the Conduct and Reporting of Research" (http://www.icmje.org/ethical_4conflicts.html). It should be emphasized, however, that Calanus AS has not been involved in collection, analysis and interpretation of data or writing of the report from the animal studies.

Abstract

Benefical health effects of fish and marine oils, especially in relation to cardiovascular disease, have been ascribed mainly to the omega-3 polyunsaturated fatty acids (n-3 PUFAs), eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3).

Zooplankton acts as an important link between the primary producers of n-3 PUFAs (phytoplankton) and organisms at higher trophic levels, such as fish. As the world's fish stocks are in danger of being over-exploited, organisms at lower trophic levels represent good alternatives, especially when their productivity and biomass are taken into account. Zooplankton has also significantly lower concentrations of adverse compounds such as organic pollutants or heavy metals, compared to organisms higher up in the food chain.

Oil from the marine zooplankton *Calanus finmarchicus* (Calanus oil) has recently emerged as a product for the human market. In contrast to other n-3 PUFA-containing health supplements, this oil is mainly composed of monoesters of long-chain fatty acids and fatty alcohols, also called wax esters. In addition, it is rich in the potent anti-oxidant astaxanthin.

The present study shows the composition of Calanus oil with its high percentage of wax esters and its unique fatty acid composition, including high amounts of the n-3 PUFAs EPA, DHA and stearidonic acid (SDA, 18:4 n-3), as well as monounsaturated fatty acids (MUFAs), such as gondoic (20:1 n-9) and cetoleic acid (22:1 n-11). Toxicology analysis confirms low levels of heavy metals, organic and non-organic impurities. In addition, animal experiments revealed no adverse biological effects of Calanus oil supplementation. On the contrary, obese rats fed Calanus oil-supplemented high-fat diet showed reduced accumulation of abdominal and liver fat compared to rats fed a high-fat diet alone, indicating a lower risk for cardiovascular disease.

1. Introduction

During the past 30 years, seafood and marine oils have been shown to have a number of health benefits, which have first and foremost been associated with two typical marine omega-3 polyunsaturated fatty acids (n-3 PUFAs), namely EPA and DHA [1]. These n-3 PUFAs are the common denominator in health supplements such as fish oil, krill oil and other marine oils. Beneficial effects in primary and/or secondary prevention of atherosclerosis, thrombosis and embolic events, hypertriglyceridemia, hypertension, and autoimmune disease have been related to dietary intake of EPA and DHA [1-4]. Consumption of fish, fish oil or supplements containing these n-3 PUFAs is therefore recommended by nutritionists and health authorities [5].

The n-3 PUFAs in marine oils stem ultimately from phytoplankton, which effectively can desaturate and elongate its precursor α-linolenic acid [6]. Acknowledging the pivotal role of phytoplankton, there is a growing awareness of the unexploited potential, which is found at the base of the marine food web [7] . According to the Food and Agriculture Organization of the United Nations, most of the world's fisheries are either fully or over-exploited [8], and wild fish stocks are therefore insufficient to meet the increasing future demand for n-3 PUFAs both for direct human consumption and in feed for farmed fish. This implies a considerable drive for identifying alternative sources, and both terrestrial and marine possibilities are considered, including the use of biotechnology like the production of n-3 PUFAs in gene-modified plants [9-12].

Zooplankton acts as an important link between the primary producers (phytoplankton) and organisms at higher trophic levels, including predatory fish and marine mammals. Only 10-15 % of the energy produced at one trophic level is transferred to the next [7]. Thus, catching animals at lower trophic levels in a precautionary and sustainable manner provides a many-fold gain in potential harvestable biomass [13]. The position of herbivorous zooplankton in the marine food web is an advantageous feature also in other terms: The accumulation of persistent organic pollutants tends to increase with increasing trophic levels, often making it necessary to refine marine oils from organisms higher up in the food chain. On the contrary, in more primary sources of marine oils such as algae and zooplankton, adverse compounds are significantly lower [14]

The status of the world-wide plankton fisheries has been reviewed by Omori [15] in 1978 and was then estimated to be on the scale of 200.000 tons annually. Since that time marine oils from crustaceans have emerged with a certain market potential, following the development of sustainable harvesting technology for both krill [16] and copepods [17]. Until now the primary source of crustacean oil has been Antarctic krill (*Euphausia superba*). Krill is a pelagic shrimplike crustacean of up to 6cm length, which is found in oceans world-wide. *Euphasia superba* is the largest zooplankton stock in the Southern Ocean [18, 19], where this krill species is commercially harvested. Antarctic krill oil has a uniquely high content of phospholipids (PLs) [20].

Calanus oil is a marine oil which has recently emerged as a product for the human market. It is extracted from *Calanus finmarchicus*, the dominant marine zooplankton species in North Atlantic waters[21]. This copepod crustacean is 3-4 mm in body length. Its oil contains a unique combination of several high-valued long-chain fatty acids, fatty alcohols and natural antioxidants such as astaxanthin [22]. In contrast to krill oil and traditional fish oils, where lipids are predominantly bound as PLs and triacylglycerols (TAGs), respectively, lipids in Calanus oil are mainly comprised of esters of long-chain fatty acids and fatty alcohols, usually termed wax esters.

Due to an emerging market interest for using this novel oil for human consumption, a comprehensive assessment of the chemistry and environmental impurities (including the levels of heavy metals and organic or non-organic substances) in Calanus oil was carried out. In parallel, the safety of this oil at several relevant dosage levels were investigated in two separate rat studies (physiological and anatomical investigations).

2. Materials and methods

2.1. Outline of the study

An outline of the chemical analysis of Calanus oil, as well as the animal studies for examination of its biological effects, is shown in Figure 1. Regarding the animal studies, protocol A (rats) delineates in life observations where the effect of different dosages of Calanus oil supplementation (0.375, 0.75 and 1.5 % (w/w)) was evaluated. In protocol B, rats were fed only the highest dosage of Calanus oil (1.5 % w/w) for the assessment of in-life observations and physiological parameters, including data on clinical chemistry. The methodology and results are presented separately for each protocol.

CALANUS OIL

LIPID PROFILE

FATTY ACIDS AND FATTY ALCOHOLS

ASTAXANTHIN AND STEROLS

HEAVY METALS

DIOXIN AND DIOXIN-LIKE PCBs

POLYCYCLIC AROMATIC HYDROCARBONS PAHs

BENZO(A)PYRENE

PBDEs

ANIMAL SAFETY STUDIES ON RATS

Protocol A (in life observations, different dosages of Calanus oil):

- Food consumption
- Water intake
- Body weight

Protocol B (in life and physiological recordings, high dosage of Calanus oil):

- Body weight
- Abdominal and ectopic (liver, skeletal muscle, heart) fat
- Clinical chemistry
- Plasma lipids, glucose, insulin
- Glucose tolerance
- mRNA expression
- Cardiac function
- Substrate metabolism

Figure 1: Flow scheme outlining the various steps in the present safety study.

2.2. Chemical analysis

2.2.1 Lipid classes, fatty acids and fatty alcohols

Lipid classes, fatty acids and fatty alcohols were analysed by Unilab Analyse AS, Tromsø, Norway. Lipid classes were analysed by HPLC according to methods slightly modified from Graeve and Janssen [23], fatty acids and fatty alcohols were analysed by GC analysis.

2.2.2 Astaxanthin

The content of astaxanthin in the oil was measured by a method slightly modified from Schierle et al. [24]. The recorded amplitudes of all free and esterified astaxanthin, were calculated separately, using data from prior determined HPLC response factors (see [24] for further details).

2.2.3 Sterols

Analysis of sterols was done at the National Institute of Nutrition and Seafood Research (NIFES) in Bergen, Norway, by Method No 387 (Sterol determination-Saponification-extraction-silylation-GLC-FID detection).

2.2.4 Heavy metals

Heavy metal analysis of Cadmium (Ca), Lead (Pb) and Mercury (Hg) was performed using Agilent 7500c inductive coupled to plasma mass spectrophotometer (ICP-MS) coupled to a HP computer. For Arsen (As) only As (III) and As (V) are the inorganic forms, which are considered toxic and for all practical purposes are the only two being routinely monitored.

2.2.5 Pesticide and organic or non-organic impurities

Pesticides, organic or non-organic impurities were analysed by high resolution gas chromatography (HRGC) according to previously described analytical methods detailed by Borgå et al. [14]and Sandanger et al.[25]. The samples were analysed for the content of PCDD/PCDF and nonortho-PCBs; PBDE (TBA, DiBB, HexBB, the BDE congeners -28, --47, -66, -49+71, -77, -85, -99, -100, -119, -138, -153, -154, -183, -196, -206, -209); α-, β-, and γ-hexachlorocyclohexanes (ΣHCHs); the dichlorodiphenyltrichloroethane (DDT) compounds o, p'DDE; p,p'DDE; o,p'DDD; p,p'-DDD, and o,p'-DDT, p,p'-DDT (DDTs), and polychlorinated biphenyls (PCB congeners), and chlorinated pesticides. The analyses were carried out at the accredited analytical laboratory in Norway (Norsk institutt for luftforskning, NILU). For polycyclic aromatic hydrocarbons (PAHs) analyses were performed by Eurofins laboratories in Germany. All chemical analyses followed the directives given by the European

Commission for determination of dioxins for the official control of foodstuffs (2002/69/EC) and feedstuffs (2002/70/EC).

2.3 Animal studies

The safety of Calanus oil was assessed in rats fed a high-fat diet supplemented with up to 1.5% (w/w) Calanus oil .

The animal experiments were carried out at the Faculty of Health Sciences, Department of Medical Biology at UiT The Arctic University of Norway. The study included two experimental series with rats (protocol A and B). All animals were treated in accordance to the guidelines on accommodation and care of animals formulated by the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific purposes, and all experiments were approved by the local authority of the National Animal Research Authority in Norway (FOTS id 1343/2008 and 1853/2009).

2.3.1 Diets and experimental protocols

All animals had free access to food and water and were initially allowed to acclimatize for one week on normal rodent chow.

2.3.1.1 Rat protocols and animal in life observations

In protocol A, forty male Wistar Hanover rats (5-6 weeks old and weighing approximately 160 g; Charles River laboratories, Sulzfeld, Germany) were divided into 5 groups of 8 rats each (2 animals/cage) in a room maintained at 21 °C, 55% humidity and 12:12 h light-dark cycle. One group served as lean controls and received normal rat chow (Test Diet #58Y2, 10% energy from fat; IPS limited, Notts, UK). The other four groups received a high fat diet (Test Diet #58V8; 45% energy from fat; IPS limited, Notts, UK) supplemented with either 0, 0.375, 0.75 or 1.5% (w/w) Calanus oil (replacing equivalent amounts of lard in the diet). See Table 7 for macronutrient composition of the diets.

Protocol B included 36 male Wistar rats (5 weeks old, approximately 150 g) which were divided into 3 groups (each containing 12 rats, 2 rats/cage): a lean control group receiving normal rat chow and two groups receiving the high fat diet with and without 1.5% Calanus oil . Blood samples were taken two weeks before termination of the feeding period. At sacrifice, tissues were carefully excised and weighed, and relevant tissue samples were snap-frozen for later analysis.

The feeding periods for both protocols lasted 16-18 weeks. Food intake and body weight were recorded weekly, while water intake was recorded occasionally during the latter half of the experimental period. Gastro-intestinal function was assessed by the quality (texture, consistency) of the feces. The staff at the animal department observed the animals daily with special focus on any abnormal behavior or signs of discomfort, changes in posture, activity, as well as disorders in the skin and fur of the animals.

2.3.2 Triacylglycerol tissue content

Triacylglycerol (TAG) content from liver, heart and skeletal muscle (gastrocnemius) was extracted by the method of Folch et al. [25], dried and thereafter dissolved in a tert-butyl and Triton X-100/methyl alcohol mixture. The TAG concentration of the extracts was measured with a Triglyceride 25 kit from ABX Diagnostics (Montpellier, France), using a plate reader (Victor2 1420 Multilabel Counter, PerkinElmer, Oslo, Norway).

2.3.3 Blood samples and glucose tolerance test

Clinical chemistry parameters from rat blood were analysed by standard laboratory methods in the laboratory of the University Hospital of North Norway (UNN Tromsø) with a Cobas 8000 analyser from Roche (Oslo, Norway).

Plasma concentrations of glucose, non-esterified fatty acids (NEFA), triacylglycerol (TAG) and insulin were measured by standard biochemical kits from Wako Chemicals (Neuss, Germany), DRG (Germany) and ABX Diagnostics (Montpellier, France), respectively.

Glucose tolerance was assessed by an intra-peritoneal glucose tolerance test. The animals were fasted for 4-5 hours with free access to drinking water. Blood was collected from the saphenous vein before (0 minutes) and 15, 30, 60 and 120 minutes after intra-peritoneal administration of a glucose solution (1.5 g/kg body weight). Blood glucose was measured with a hand-held glucometer (Ascensia Contour, Bayer Healthcare, Berlin, Germany). Glucose tolerance tests and fasting blood samples in rats were taken from the vena saphena at the end of the feeding period. Non-fasting blood samples were taken from the abdominal aorta at sacrifice.

2.3.4 Gene expression

Total RNA was extracted according to the RNeasy Fibrous Tissue protocol (Qiagen Nordic, Nydalen, Norway) and quantified by real-time PCR (qPCR) as described previously [26]. Primers and probes were obtained from Eurogentech S.A. (Seraing, Belgium) and Roche Universal Probe Library (Roche diagnostics, Mannheim, Germany). The primer/probe

sequences for the genes studied are given in supplemental table 1. The expression of the target genes were normalized to the geometric mean of stably expressed reference genes based on testing by Normfinder (Aarhus University Hospital, Aarhus, Denmark) [27] of the possible reference genes GADPH, cyclophilin, HPRT, HMBS and SDHA.

2.3.5 Ex vivo cardiac function and metabolism

Cardiac function and metabolism were assessed using isolated perfused rat hearts. Briefly, the aorta and left atrium were cannulated to allow perfusion in the working-heart mode (for details, see [28]. Hearts were allowed to beat spontaneously, and heart rate, left ventricular pressure, aortic and coronary flow were recorded. Glucose and palmitate oxidation were determined by measuring $^{14}CO_2$ and $^{3}H_2O$ released by the oxidation of [U-^{14}C]glucose and [9,10-^{3}H]palmitate, respectively [28].

2.3.6 Statistical analysis

Values are represented as means with standard errors. Statistical analysis was performed using Sigma Plot 12.0 (Systat software, Alfasoft AS, Norway). 2-wayANOVA followed by Holm-Sidak post hoc tests was used for comparing means. $p<0.05$ was considered significant.

3. Results

3.1 Lipid profile of Calanus oil

The lipid profiles of 3 batches of Calanus oil are provided in Table 1 and confirm that the oil is primarily comprised of wax esters (>86%) with minor amounts of non-esterified fatty acids (NEFA), non-esterified fatty alcohols (FAOH), and glycerides. No individual minor component represents greater than 3% of the oil.

Table 1: Individual and mean values of the lipid classes of three different batches of Calanus oil. NEFA: non-esterified fatty acids, FAOH: (non-esterified) fatty alcohols

Lipid Class	**Analytical Data (% of oil)**			**Mean**
	Batch 1	**Batch 2**	**Batch 3**	
Wax ester	86.2	86.3	89.0	87.2
NEFA	0.7	1.9	1.6	1.4
FAOH	1.1	1.0	1.2	1.1
Triacylglycerol	3.3	2.7	2.4	2.8
Diacylglycerol	0.3	0.3	2.1	0.9
Monoacylglycerol	0.4	0.9	0.5	0.6
Cholesterol	0.3	0.3	0.5	0.4
Phospholipids	-	-	-	-
Sum of neutral lipids	>91	>91	>96	>91
Total lipids	92.3	93.5	97.3	-

3.2 Fatty Acid and Fatty Alcohol Profile

The fatty acid profiles of 3 different batches of Calanus oil are summarized in Table 2. Note the significant amounts of stearidonic acid (SDA, 18:4 n-3; ca. 7%), eicosapentaeonic acid (EPA, 20:5 n-3; ca. 7%), docosahexaenoic acid (DHA, 22:6 n-3; ca. 5%) and cetoleic acid (22:1 n-11, ca 5%). The fatty alcohol profiles of 3 batches of Calanus oil are provided in Table 3. The primary fatty alcohols identified are C20:1 n-9 (ca. 13%) and C22:1 n-11 (ca. 20%).

Table 2: Individual and mean values of fatty acids of three different batches of Calanus oil

Fatty Acid	**Analytical Data (% of oil)**			**Mean**
	Batch 1	**Batch 2**	**Batch 3**	
C14:0 (myristic acid[1])	8.3	8.2	7.9	8.1
C14:1 n-5	0.2	0.3	0.3	0.3
C15:0	0.3	0.4	0.4	0.4
C16:0 (palmitic acid[1])	4.6	5.3	4.7	4.9
C16:1 n-9	0.1	0.1	0.3	0.2
C16:1 n-7 (palmitoleic acid[1])	2.9	1.8	2.2	2.3
C16:1 n-5	0.2	0.3	0.3	0.3
C17:0	0.1	0.1	0.1	0.1
C17 undefined	0.2	0.2	0.2	0.2
C16:2 n-7	0.4	0.2	0.3	0.3
C16.3 n-4	1.1	0.3	0.4	0.6
C18:0 (oleic acid[1])	0.4	0.4	0.4	0.4
C16:4 n-1	1.8	0.5	1.1	1.1
C18:4 n-9	1.3	1.6	1.4	1.4
C18:1 n-7	0.4	0.3	0.3	0.3
C18:2 n-6	0.5	0.7	0.6	0.6
C18:3 n-6	0.5	0.3	0.5	0.4
C18:3 n-3	0.8	1.4	1.0	1.1
C20:0	0.1	0.1	0.1	0.1
C18:4 n-3 (stearidonic acid[1], SDA)	6.7	7.3	7.1	7.0
C20:1 n-11	0.8	0.7	0.7	0.7
C20:1 n-9 (gondoic acid[1])	2.5	2.6	2.4	2.5
C20:1 n-7	0.7	0.8	0.7	0.7
C18:3 n-3	-	-	0.4	<0.1
C20:2 n-6	0.1	0.1	0.1	0.1
C20:3 n-6	0.1	0.1	-	0.1
C20:4 n-6	0.2	0.1	1.3	0.5
C20:3 n-3	0.1	0.1	-	0.1
C22:0	-	-	0.1	<0.1
C20:4 n-3	0.6	0.6	0.7	0.6
C22:1 n-11 (cetoleic acid[1])	4.8	5.1	5.3	5.1
C22:1 n-9	0.2	0.2	-	0.1
C20:5 n-3 (eicosapentaenoic acid, EPA)	7.0	5.6	7.2	6.6
C21:5 (undefined, assumed n-3)	0.4	0.3	0.3	0.3
C24:0	-	-	0.1	<0.1
C24:1 n-9	0.3	0.3	0.5	0.4
C22:5 n-3	0.4	0.3	0.5	0.4
C22:6 n-3 (docosahexaenoic acid, DHA)	4.2	4.8	4.6	4.5
Sum of fatty acids	53.3	51.5	54.5	53.1
Sum of saturated fatty acids	14.0	14.7	14.1	14.3
Sum of monosaturated fatty acids	14.4	14.0	14.4	14.3
Sum of polyunsaturated fatty acids	24.7	22.6	26.2	24.5
Sum of n-3 fatty acids	19.8	20.0	21.5	20.4
Sum of EPA plus DHA	11.2	10.4	11.8	11.1

[1]Common name

Table 3: Individual and mean values of fatty alcohols of three different batches of Calanus oil

Fatty Alcohol	**Analytical Data (% of oil)**			**Mean**
	Batch 1	**Batch 2**	**Batch 3**	
C14:0	8.3	8.2	7.9	8.1
C16:0	3.6	2.8	2.7	3.0
C16:1 n-7	1.8	0.6	0.9	1.1
C18:1 n-9	1.1	1.0	1.0	1.0
C20:1 n-9	13.3	12.9	13.1	13.1
C22:1 n-11	17.3	18.8	22.3	19.5
C22:1 n-9	1.0	1.0	1.3	1.1
Sum of fatty alcohols	46.4	45.3	49.2	46.9
Sum of monosaturated fatty alcohols	34.5	34.3	38.6	35.8

3.3 Astaxanthin and sterol content

The total astaxanthin content of Calanus oil is > 750 µg/g, primarily in the esterified form (data not shown). The sterols in the oil are estimated to < 5 mg/g oil, mostly found as cholesterol and brassicasterol, while minor levels of campesterol and sitosterol are recorded (Table 4).

Table 4: Sterol profile of a representative batch (batch 2) of Calanus oil

Component	**Analytical Data**		
	% by weight of sterols	**mg/g of oil**	**% of oil**
Cholesterol	45.3	2.1	0.21
Brassicasterol	53.6	2.5	0.25
Campesterol	0.9	0.04	0.004
Sitosterol	0.2	0.01	0.001
Sum sterols (with cholesterol)	100	4.7	0.47

3.4 Water and Protein Content

The moisture of Calanus oil was found to be < 1% in the 3 batches analysed (data not shown). The protein content of Calanus oil has been investigated by gel electrophoresis and by high salt extraction followed by detection using the bicinchoninic acid (BCA) assay and gel electrophoresis techniques. No residues of protein were detected in Calanus oil in either of the methods described [29].

3.5 Heavy Metals

The results of heavy metals analyses for 3 batches of Calanus oil are presented in Table 5. All values reported for the metals tested are below detection limits or fall well below the maximum recommended limits (see legend of Table 5) of 0.1 mg/kg wet weight in fats and oils for lead, and 1.0 and 0.10 mg/kg wet weight respectively, for cadmium and mercury in food supplements.

Table 5: Content of heavy metals analysis for three different batches of Calanus oil.

Parameter	**Maximum Limits (mg/kg wet weight)[1]**	**Analytical Data (mg/kg of oil)**		
		Batch 1	**Batch 2**	**Batch 3**
Arsenic (inorganic)[2]	-	<0.1	<0.1	0.1
Cadmium	1.0[3]	0.026	0.29	0.73[4]
Lead	0.10[5]	<0.02	<0.02	<0.02
Mercury	0.10[3]	<0.02	<0.02	<0.02

[1] Maximum limits as specified under Regulation (EC) 1881/2006 (Commission of the European Communities, 2006) or its amendment, Regulation (EC) 629/2008 (Commission of the European Communities, 2008a) considered applicable to Calanus oil for the intended use as an ingredient in food supplements – limits are set for wet weight but given the reported values for Calanus oil fall well below the maximum levels no adjustment for the moisture content was considered necessary; [2] Only inorganic arsenic measured on the basis that this is the form of arsenic of toxicological concern, which provisional tolerable weekly intake of 2.14 µg/kg bw/day set by the Joint WHO/FAO Expert Committee on Food Additives (JECFA); [3] Limit applies to food supplements as sold; [4] In Calanus experience, levels are not usually this high with variability between batches typically ranging from 0.02 to 0.5 mg/kg (not adjusted for moisture content); [5] Limit applies to fats and oils, excluding milk fat.

Although, meeting legislative requirements for cadmium food supplements, it is noted that the levels of this metal in Calanus oil can vary between batches. Cadmium is known to concentrate in marine animals with bioaccumulation factors ranging from 5 to 3160 reported for saltwater aquatic species compared to their water (ASTER, 1994; EFSA, 2009). Reflecting the varying accumulation of cadmium in marine organisms, under Regulation 629/2008 (Commission of the European Communities, 2008a) fish species were re-grouped from previous legislation into different categories and maximum levels. Additionally, levels are recognised to be relatively high in certain marine products with maximum levels of 3.0

mg/kg wet weight set for food supplements exclusively or mainly comprising of dried seaweed or products derived from dried seaweed.

3.6 Pesticide and organic or non-organic impurities

Dioxins and Dioxin-Like Polychlorinated Biphenyls (PCBs): The results of dioxin and dioxin-like PCBs analyses for 3 batches of Calanus oil are presented in Table 6. The values reported for Calanus oil fall well below the limits specified for marine oils by Commission Regulation (EC) No 1881/2006 (Commission of the European Communities, 2006) of 2.0 pg/g fat for dioxins and 10 pg/g fat for dioxins and dioxin-like PCBs. Benzo(a)pyrene, as a marker for other Polycyclic Aromatic Hydrocarbons (PAHs) has been performed on 3 batches of Calanus oil, demonstrated values in the range from 0.27 to 0.33 µg/g wet weight, well below the limit specified for this substance in oils by Commission Regulation (EC) No 1881/2006 (Commission of the European Communities, 2006) of 2.0 µg/g wet weight.

Table 6: Content of dioxin, dioxin-like PCBs and benzo(a)pyrene in three different batches of Calanus oil .

Parameter	**Maximum Limits (pg/g fat)[1]**	**Analytical Data**		
		Batch 1	**Batch 2**	**Batch 3**
Sum of dioxins (WHO-PCDD/F-TEQ) [as pg/g]	2.0	1.55	1.09	0.74
Sum of dioxins and dioxin-like PCBs (WHO-PCDD/F-PCB-TEQ)	10.0	2.43	2.00	1.66
	Maximum Limits (µg/g wet weight) [2]			
Benzo(a)pyrene [as µg/g]	2.0	0.27	0.33	0.27

[1] Marine oils intended for human consumption. [2] Oils and fats intended for direct human consumption or as ingredients in food – limits are set for wet weight but given the reported values for Calanus oil fall well below the maximum levels no adjustment for moisture content was considered necessary.

A screen for residues of polybrominated dyphenyl ethers (PBDEs) has been performed on 3 batches of Calanus oil. The concentrations of PBDEs for all the batches tested are below detection limits or at levels which do not pose a toxicological concern under the conditions of intended use of Calanus oil. Furthermore a pesticide screen has been performed on 3 batches of Calanus oil. The screen included hexa-chlorocyclohexane (HCH) isomers, dichlorodiphenyltrichloroethane (DDT) and related substances/isomers and chlorinated

pesticides. The levels of residues in the batches tested were found to either be below detection limits or at levels which do not pose a toxicological concern, which act in concert with a natural product stemming from a lower trophic marine organism where the contamination is expected to be low.

3.7 Analysis of feed formulations

The macronutrient composition (energy %) of the diets is shown in Table 7. The energy content of HFD and CAL was approximately 20% higher than the CTR diet.

Table 7. Macronutritients and energy content of normal chow diet (CTR), high fat diet (HFD) and HFD supplemented with 1.5% (w/w) Calanus oil (CAL).

	CTR	HFD	CAL
Energy from fat (%)	10.2	45.7	46.1
Energy from carbohydrates (%)	71.5	35.5	35.8
Energy from protein (%)	18.3	18.3	18.1
% fat (w/w)	4.3	23.6	23.6[1]
% Calanus oil	0	0	1.5
Energy content, kJ/g	15.8	19. 4	18. 7

[1] Including Calanus oil

Analysis of the fatty acid composition of the diets (not shown) confirm earlier findings by Pedersen et al.(submitted); While the CAL diet showed increased levels of EPA (20:5 n-3), DHA (22:6 n-3), SDA (18:4 n-3), gondoic (20:1 n-9) and cetoleic acid (22:1 n-11), reflecting the addition of Calanus oil, these acids were not detected in the HFD.

3.8 Animal studies

Most parameters listed below were measured in both rat protocols, the exception being mRNA measurements which were measured only in protocol A. Body weight curves are shown from both protocol A and B, but otherwise only results from protocol B are listed below.

3.8.1 In life observations and biometric data

No treatment-related deaths occurred and the animals did not display any unusual behavior or appearance under feeding with Calanus oil .

Water intake and the calculated energy intake in rats (Table 8) were similar for all three groups. It should be noted that measurements were undertaken in normal cages and therefore can be slightly inaccurate due to a certain spillage.

Table 8: Food and water intake, as well as calculated energy intake of rats subjected to the various dietary regimens, i.e. normal chow diet (CTR), high fat diet (HFD) and HFD supplemented with 1.5% (w/w) Calanus oil (CAL). * $p<0.05$ vs HFD

	CTR	HFD	CAL
Food intake (g/animal/day)	19.6 ± 0.1*	16.6 ± 0.4	16.7 ± 0.3
Energy intake (kJ/animal/day)	309 ± 2	323 ± 8	312 ± 5
Water intake (mL/animal/day)	17.6 ± 0.5	17.3 ± 0.7	17.8 ± 0.7

In protocol A, high fat-fed rats (HFD) showed higher body weights than rats receiving normal chow (CTR), but this was not significant (Figure 2). Supplementation of the HFD with increasing amounts of Calanus oil (0.375% - 1.5%) had no clear effect on body weight development. However, we found that the variation in body weight was less in the group receiving HFD with 1.5% Calanus than in the other high fat-diets with and without supplementation (supplemental Figure S1 A-E).

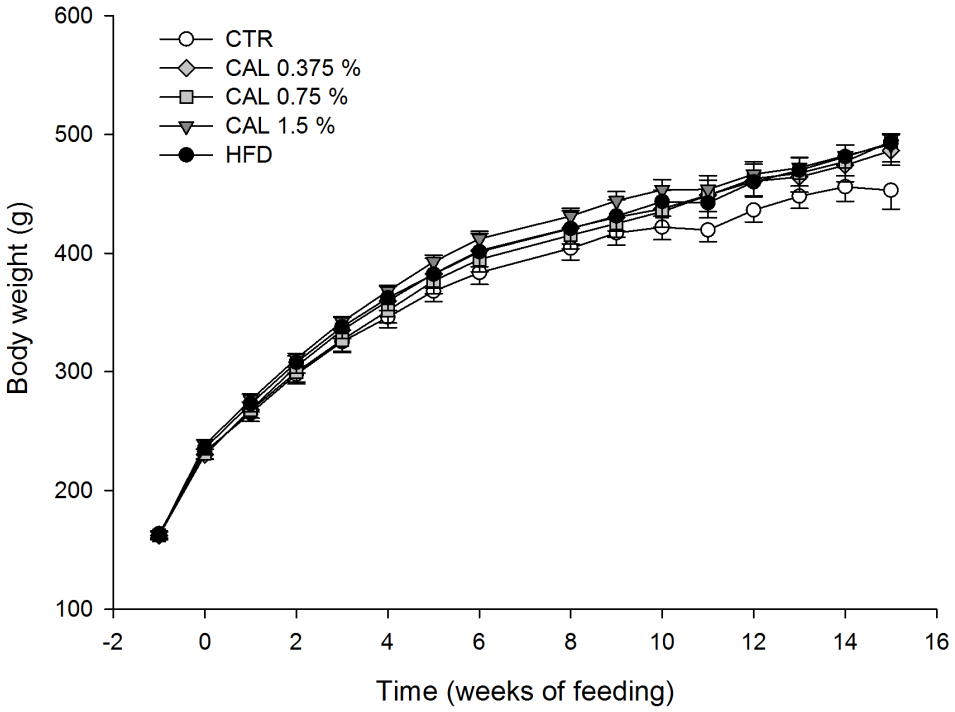

Figure 2: Body weight development of rats fed normal chow diet (CTR), high fat diet (HFD) or HFD supplemented with increasing amounts of Calanus oil (CAL). Values are means, with their standard errors represented by vertical bars (n = 8 in each group).

In protocol B, we observed a significant difference in body weight development between the HFD and CTR group as well as a tendency to lower body weight development in rats receiving Calanus oil -supplemented HFD compared to the group receiving non-supplemented HFD (Figure 3, Table 9).

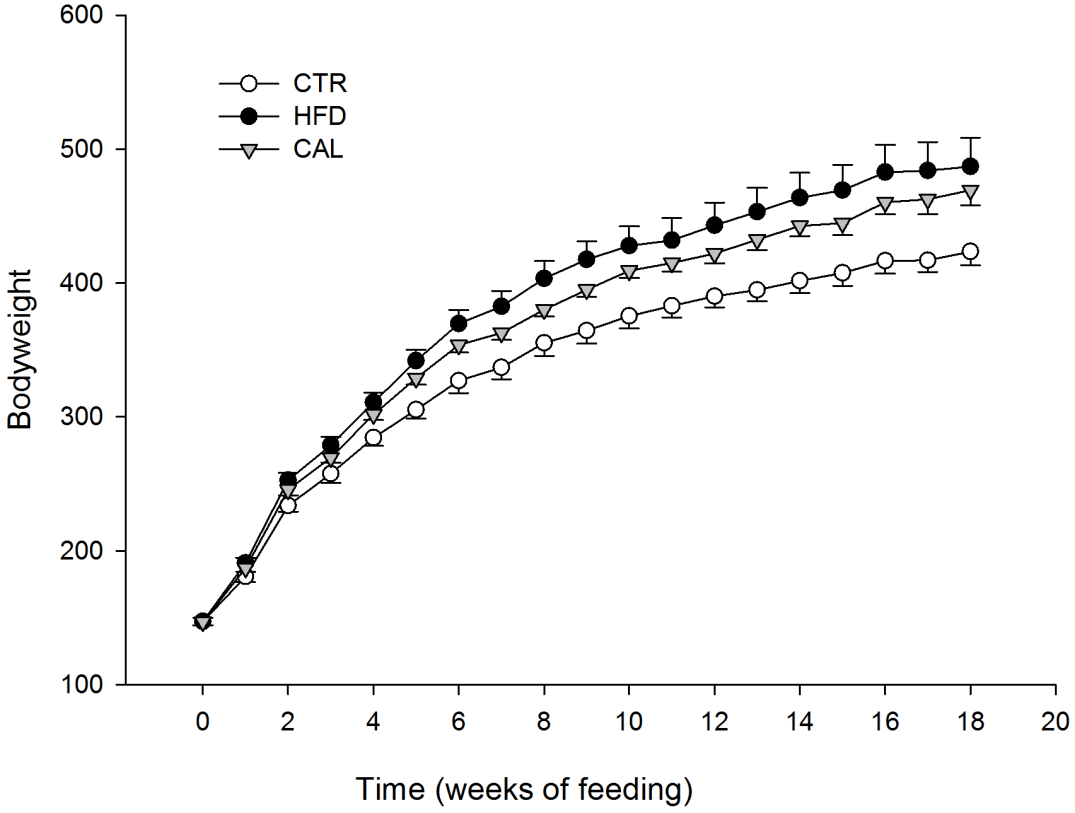

Figure 3: Body weight development of rats fed normal chow diet (CTR), high fat diet (HFD) or HFD supplemented with 1.5% (w/w) Calanus oil (CAL). Values are means, with their standard errors represented by vertical bars (n = 12 in each group).

Heart weight was significantly lower in CTR compared to HFD, when normalized to tibia length (Table 9), but there was no difference between HFD and CAL. No differences were observed between the groups with respect to liver weight (Table 9).

Table 9: Body weight gain over the 18 wk feeding period, as well as size of liver and heart at sacrifice, of rats fed normal chow diet (CTR), high fat diet (HFD) or HFD supplemented with 1.5% Calanus oil (CAL). *, $p<0.05$ vs HFD.

	CTR	**HFD**	**CAL**
Body weight gain (g)	276 ± 8 *	340 ± 21	323 ± 11
Liver wt/tibia length (g/cm)	29.5 ± 0.8	29.0 ± 1.7	29.2 ± 0.8
Heart wt /tibia length (g/cm)	2.6 ± 0.1 *	2.9 ± 0.1	2.8 ± 0.1

Abdominal fat depots, represented by perirenal (pWAT) and epidydimal fat (eWAT), were significantly higher in HFD compared to CTR (Figure 4 A,B). Calanus oil -fed animals had significantly reduced pWAT compared to HFD (Figure 4 A), as was obvious already by visual inspection upon opening the abdominal cavity (Figure 5). On the other hand, eWAT was only marginally affected by Calanus oil supplementation (Figure 4B).

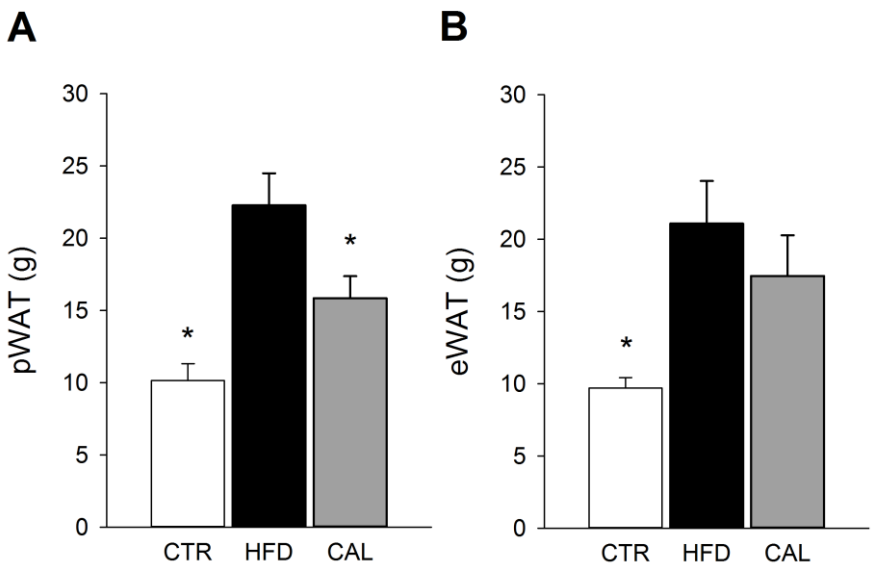

Figure 4: Size of perirenal (pWAT) and epididymal fat depots (eWAT) at the end of the feeding period of rats fed normal chow diet (CTR), high fat diet (HFD) or HFD supplemented with 1.5% (w/w) Calanus oil (CAL). *, $p<0.05$ vs HFD.

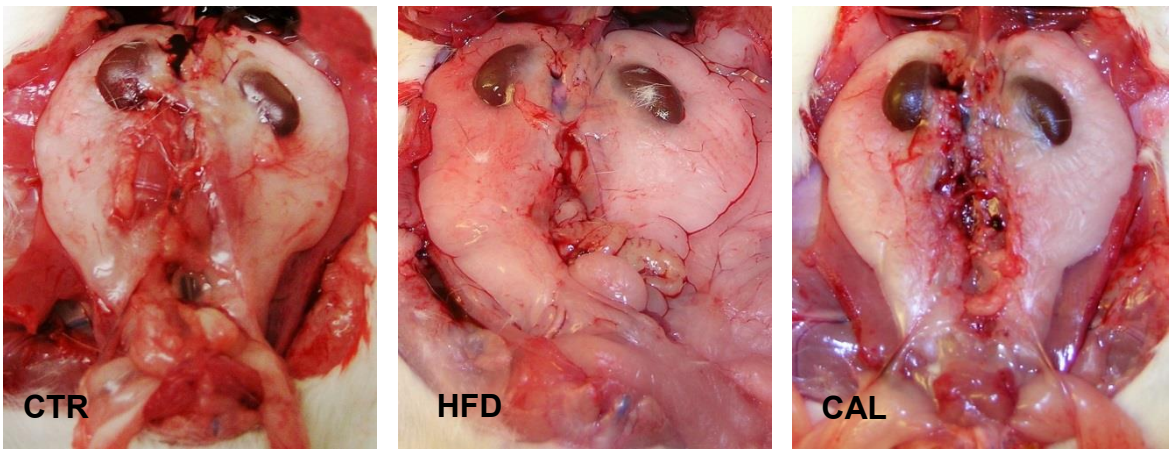

Figure 5: Representative pictures of abdominal (perirenal) fat depots of rats fed normal chow diet (CTR), high fat diet (HFD) or HFD supplemented with 1.5% (w/w) Calanus oil (CAL).

3.8.2 Tissue triacylglycerol (TAG) concentrations

Liver TAG concentration was lower in the CTR and CAL groups, relative to the HFD group, although the difference between CAL and HFD was not statistically significant (Figure 6A). There was no difference in skeletal or cardiac TAG concentration between the groups (Figure 6 B and C).

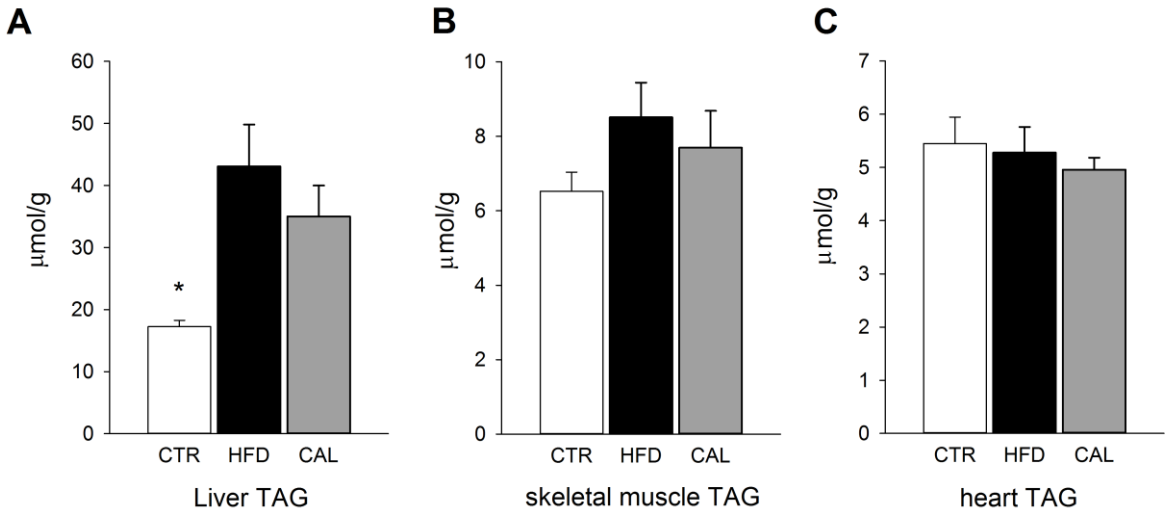

Figure 6: Triacylglycerol (TAG) content in liver, skeletal muscle and heart at the end of the feeding period of rats fed normal chow diet (CTR), high fat diet (HFD) or HFD supplemented with 1.5% (w/w) Calanus oil (CAL). *, $p<0.05$ vs HFD.

3.8.3 Blood chemistry and glucose tolerance

Analysis of routine clinical chemistry (electrolytes, liver enzymes, creatinine and protein) was only performed in the HFD and CAL groups. All values were within normal range or lower (Table 10). There were no differences between the groups, apart from a tendency of lower alkaline phosphatase (ALP) in CAL. We do not consider this to be important, as only elevated levels of ALP are of pathological importance.

Table 10: Clinical chemistry parameters of rats given high fat diet (HFD) or HFD supplemented with 1.5% (w/w) Calanus oil (CAL). ALAT = alanine amino-transferase, ALP = alkaline phosphatase, γGT = gamma-glutamyl transferase.

	HFD	CAL	REFERENCE VALUES[1]
Sodium (mmol/L)	145 ± 0.5	144 ± 0.3	139-154
Chloride (mmol/L)	104 ± 0.6	104 ± 0.4	97-109
Creatinine (µmol/L)	25 ± 1.0	25 ± 1.6	47-77
Urea (mmol/L)	7 ± 0.3	7 ± 0.4	4.5-10
γGT: (U/L)	<5	<5	<5
ALAT (U/L)	21 ± 5.9	16 ± 5.4	27-160
ALP (U/L)	93 ± 8.3	68 ± 3.9	140-503
Total protein (g/L)	53 ± 0.7	55 ± 0.6	63-81

[1]Reference values are from [30]

With respect to concentrations of plasma metabolic parameters (Table 11), we found that both the CTR and CAL group showed lower concentrations of TAG, though only CTR was statistically significant compared to HFD. There was no difference in the plasma concentration of glucose or NEFA between the groups, and plasma insulin concentration in CTR and CAL did not differ significantly from the HFD group.

Table 11: Plasma concentrations of glucose, non-esterified fatty acids (NEFA), triacylglycerol (TAG), and insulin of rats fed normal chow diet (CTR), high fat diet (HFD) or HFD supplemented with 1.5% (w/w) Calanus oil (CAL). *, $p<0.05$ vs HFD.

	CTR	HFD	CAL
Glucose [1](mmol/L)	10.4 ± 0.6	10.3 ± 0.5	10.3 ±0.4
NEFA[2] (mmol/L)	0.35 ± 0.03	0.38 ± 0.04	0.34 ± 0.04
TAG[2]	0.23 ± 0.02 *	0.42 ± 0.08	0.31 ± 0.08
Insulin[1] (pmol/L)	455 ±63	363 ± 64	277 ± 26

[1] measured in plasma from feed-deprived mice
[2] measured in plasma from fed mice

HFD-animals were clearly glucose intlolerant compared to control animals (Figure 7). Although Calanus oil-supplemented animals were slightly less glucose intolerant compared to HFD, this difference was not statistically significant.

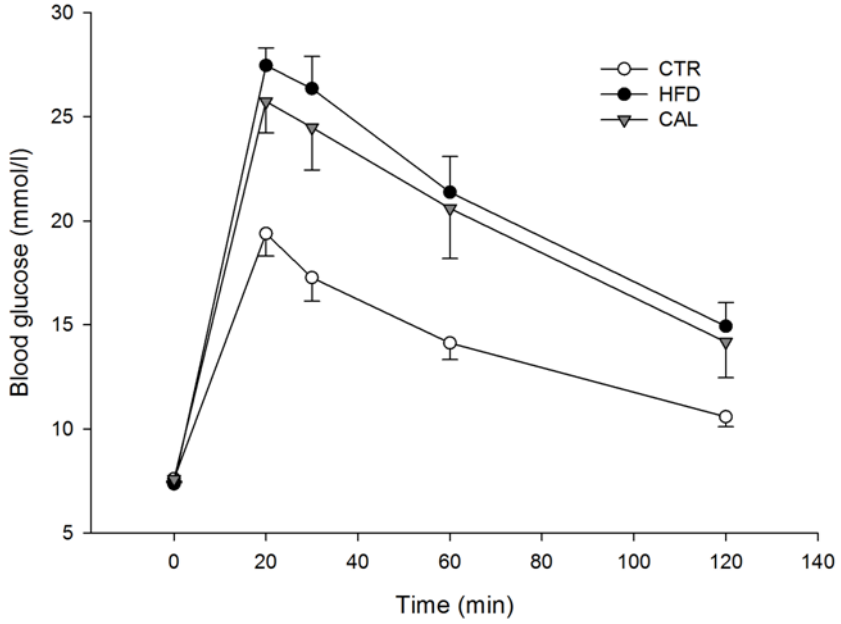

Figure 7: Changes in plasma glucose concentration during a standard glucose tolerance test in rats fed normal chow diet (CTR), high fat diet (HFD) or HFD supplemented with 1.5% (w/w) Calanus oil (CAL). The test was performed two weeks prior to the end of the feeding period.

3.8.4 mRNA expression of metabolic genes

CPT-1 expression in the heart was significantly lower in the CTR group than in the HFD and CAL groups. The same pattern was true for cardiac PDK4 (Table 12). There was no statistically significant difference between the groups in the expression of hepatic CPT-1 and PDK4. (m)CPT-1 and PDK4 in skeletal muscle showed the same pattern as in the heart, with lowest expression in the CTR group.

Table 12: mRNA expression of *carnitine palmitoyltransferase 1* (*CPT-1*) *and pyruvate dehydrogenase kinase 4* (*PDK4*) in various tissues of rats fed normal chow diet (CTR), high fat diet (HFD) or HFD supplemented with 1.5% Calanus oil (CAL). Values are normalized to the HFD group and given as means with standard errors (n = 5-8). *, $p<0.05$ vs HFD.

GROUP	GENE	ORGAN		
		Heart	Liver	Skeletal muscle
CTR	*CPT-1*	0.84 ± 0.06 *	1.06 ± 0.37	0.72 ± 0.04 *
	PDK4	0.47 ± 0.06 *	1.57 ± 0.30	0.72 ±0.08
HFD	*CPT-1*	1.00 ± 0.04	1.00 ± 0.16	1.00 ± 0.09
	PDK4	1.00 ± 0.15	1.00 ± 0.09	1.00 ± 0.16
CAL	*CPT-1*	1.03 ± 0.03	0.76 ± 0.13	0.9 ± 0.07
	PDK4	0.82 ± 0.08	1.06 ± 0.14	1.06 ± 0.16

3.8.5 Cardiac function and metabolism

Mechanical function assessment did not reveal any statistically significant differences between the three groups (Table 13). Analysis of cardiac metabolism showed significantly increased fatty acid oxidation for HFD compared to CTR with no effect of Calanus supplementation compared to HFD (Figure 8 A). There was a tendency to lower glucose oxidation in HFD and CAL compared to CTR, but again no difference between HFD and CAL (Figure 8 B).

Table 13: Parameters of left ventricular function of isolated perfused working hearts from rats fed normal chow diet (CTR), high fat diet (HFD) or HFD supplemented with 1.5% Calanus oil (CAL). LVSP = left ventricular systolic pressure, LVEDP = left ventricular end diastolic pressure.

	CTR	HFD	CAL
Aortic flow (mL/min)	55.8 ± 3.1	60.9 ± 3.8	57.4 ± 3.1
Coronary flow (mL/min)	22.3 ± 1.3	22.8 ± 1.4	22.4 ± 0.7
Cardiac output (mL/min)	78.1 ± 4.0	83.7 ± 3.3	79.8 ± 3.2
Heart rate (beats/min)	271 ± 10	264 ± 11	253 ± 13
LVSP (mmHg)	112.0 ± 3.4	120.7 ± 2.3	121.6 ± 4.0
LVEDP (mmHg)	12.3 ± 1.3	12.3 ± 0.8	13.4 ± 0.7
LVDP (mmHg)	100.4 ± 3.4	109.1 ± 1.4	109.3 ± 4.1

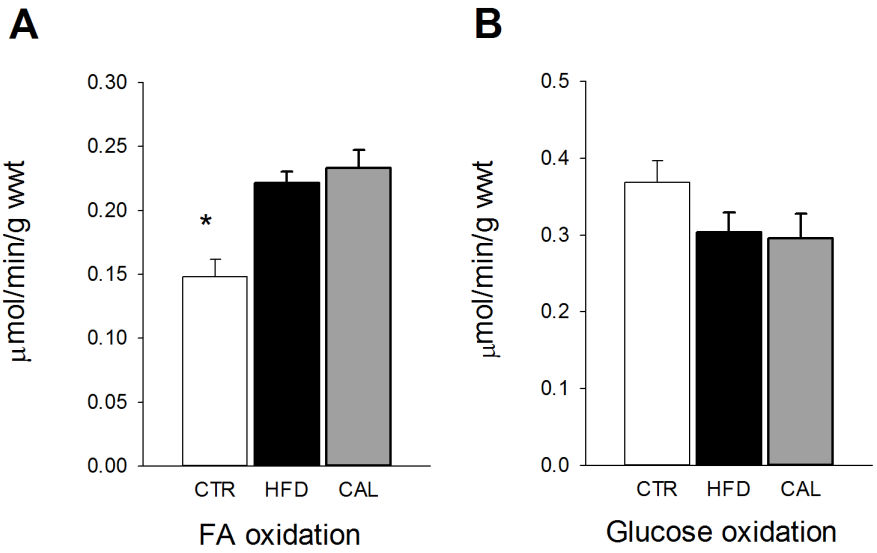

Figure 8: Myocardial fatty acid (A) and glucose (B) oxidation of isolated perfused working hearts from rats fed normal chow diet (CTR), high fat diet (HFD) or HFD supplemented with 1.5% Calanus oil (CAL). * $p<0.05$ vs HFD

4. Discussion

In the present study, the newly emerged oil from the crustacean *Calanus finmarchicus* has been thoroughly examined in terms of its chemistry as well as its physiological effects. Experiments in rats revealed no adverse biological effects of Calanus oil supplementation. No treatment-related deaths occurred, and the animals did not display any unusual behavior or appearance during the feeding experiments. This is in line with earlier studies on mice fed diets containing Calanus oil at concentrations of 1% and 1.5% (w/w) [31, 32]

Crustacean oils have certain features that make them attractive for commercial use; since they are obtained from organisms located near the bottom of the food chain, the available biomass for harvesting is large[18, 21]. Moreover, marine oils from lower trophic levels have generally low levels of organic environmental contaminants [14], but at the same time high quantities of marine lipids [33].

This study confirms the high quality of oil extracted from *Calanus finmarchicus*; all values reported for either heavy metals, dioxin, dioxin-like PCBs or polycyclic Aromatic Hydrocarbons (PAHs) fall well below the maximum limits specified by Commission Regulation (EC) No 1881/2006 (Commission of the European Communities, 2006) as amended by Commission Regulation (EC) No 629/2008 (Commission of the European Communities, 2008a). The values for polybrominated diphenyl ethers (PBDEs) and pesticides are below detection limits or at levels which do not pose a toxicological concern under the conditions of intended use of Calanus oil . No individual minor component contributed more than 3% of the oil, and no residues of protein were detected with either of the analytical methods used.

Some of the dominant fatty acids identified in Calanus oil are those typically obtained in species of marine origin, such as EPA and DHA. This, together with a high content of the strong anti-oxidant astaxanthin, makes it tempting to compare Calanus oil to another crustacean oil, Antarctic krill oil from the species *Euphasia superba* [20]. Krill oil has become popular as a health supplement and has recently shown a number of beneficial health effects [34-36]. However, in sharp contrast to both Antarctic Krill oil and other common marine oil products, Calanus oil comprises of more than 80% of wax esters (long-chain fatty alcohols esterified with long-chain fatty acids). In addition, Calanus oil also has a high concentration of stearidonic (18:4 n-3), gondoic (20:1 n-9) and cetoleic acid (22:1 n-11). Relatively little information is available regarding the biological effects of marine long-chain fatty alcohols or

the above mentioned fatty acids, but recent studies have shown beneficial health effects in high fat-fed mice receiving an oil rich in very long-chain mono-unsaturated fatty acids such as cetoleic acid [37, 38].

While health effects were not the scope of this paper, potentially health-promoting effects became apparent after supplementing a high-fat diet with 1.5% Calanus oil. Hence, reduced intra-abdominal fat deposition and reduced hepatic and plasma TAG in the Calanus oil-supplemented obese animals are signs of improved cardiometabolic health. Both the unique composition of fatty acids, as well as the high content of fatty alcohols could contribute to the apparent beneficial health effects, as discussed in detail in previous publications [32, 39] .

Similar effects as those observed with Calanus oil supplementation have previously been reported in animal studies with marine n-3 PUFAs and fish oil[40-43]. However, in many cases, n-3 PUFA products were administered in much higher (un-physiological) dosages. The concentration of 1.5% (w/w) Calanus oil in the present rat diet amounted to approximately 0.6 g/kg · day (450 g rat), which converts to approximately 6 g/day for a 70 kg human being [44]. The proposed daily dosage of Calanus oil for human consumption is approximately 2.3 g oil, based on the recommended daily value of 250 mg EPA plus DHA [45]. Thus, the currently applied dosage in our rat model was relatively higher than what is recommended for human use, but still within a physiological range, in particular when taken into consideration that larger doses are recommended for patients with cardiovascular disease as well as for hypertriglyceridemic subjects [46]. At these doses the oil seems very safe, and no deleterious effects were observed on nutritional (body weight, food and water intake), dietary (faeces quality) or clinical chemistry parameters, and no clinical or behavioural alterations were observed.

Rats receiving the high fat diet (HFD) showed both higher body weight as well as increased intra-abdominal fat compared to the controls. Intra-abdominal fat depots were significantly lower in the group receiving 1.5% of Calanus oil (CAL), which became evident both by visual inspection and by careful dissection and weighing of the perirenal fat depots. The reduction in abdominal fat was not reflected in a clear body weight reduction, which is in contrast to earlier findings in mice [32], but in line with other studies on high fat-fed Wistar rats supplemented with n-3 PUFA[40, 47]. We suggest that a potential reduction in body weight of the rats in our study was masked by the variation in body weight within the group.

High fat feeding in rats resulted in fat accumulation in the liver, which was antagonized to some extent by supplementation with Calanus oil. This ability of Calanus oil to reduce hepatic steatosis could possibly be due to increased fatty acid oxidation. However, mRNA expression of PDK4, which normally reduces glycolytic flux by inhibition of pyruvate dehydrogenase, was lower in liver of both the HFD and CAL group when compared to animals fed the control diet (CTR). This finding indicates a balance between fatty acid and glucose oxidation in favour of glucose, and therefore that reduced hepatic steatosis cannot be explained in terms of increased fatty acid oxidation.

High fat feeding – with and without Calanus oil supplementation, did not impair cardiac function. If anything, hearts from rats given HFD, as well as CAL showed slightly higher systolic pressure, compared to the CTR group. This observation could probably be explained by the modest hypertrophic response (increased heart weight) observed in the HFD group and, to a certain extent, also in the CAL group. It is tempting to suggest that the high fat-induced obesity is accompanied by a mild hypertension, which in turn elicits the hypertrophic response.

High fat feeding normally leads to a switch in myocardial substrate oxidation [26], causing overreliance of fatty acid oxidation at the expense of glucose. This was also the case in this study, although the reduction in glucose oxidation was not statistically significant. Increased expression of PDK4 and CPT1 (which are proteins favouring fatty acid oxidation) both in cardiac and skeletal muscle supports increased utilization of fatty acids as energy substrate in HFD compared to CTR animals.
Calanus oil supplementation did not reverse the HFD-induced switch in myocardial metabolism, as one might have expected in light of the fat-reducing effect of the oil. Other studies have reported that the fatty acid composition of the diet, rather than the fat content influences the activities of CPT I and PDK 4. Power and Newsholme [48] reported stimulation of skeletal muscle CPT I specific activity in rats receiving high-fat diet supplemented with menhaden (fish) oil. On the other hand, Fryer et al.[49], showed that dietary n-3 PUFA supplementation prevented high-fat induced increases in PDK 4 activity in cardiac and skeletal muscle. Differences in experimental conditions, especially PUFA supplementation, make interpretation and comparison to the present study difficult.

In summary, Calanus oil is a product of high purity with low levels of potentially toxic substances. It contains high amounts of mono- and polyunsaturated fatty acids and the anti-

oxidant astaxanthin and seems safe for the use as an oral supplement. In addition, Calanus oil might provide beneficial health effects in certain metabolic disorders.

Acknowledgement

The technical assistance of Elisabeth Børde, Knut E. Steinnes and the staff from the Department of Comparative Medicine of the University of Tromsø is greatly appreciated.

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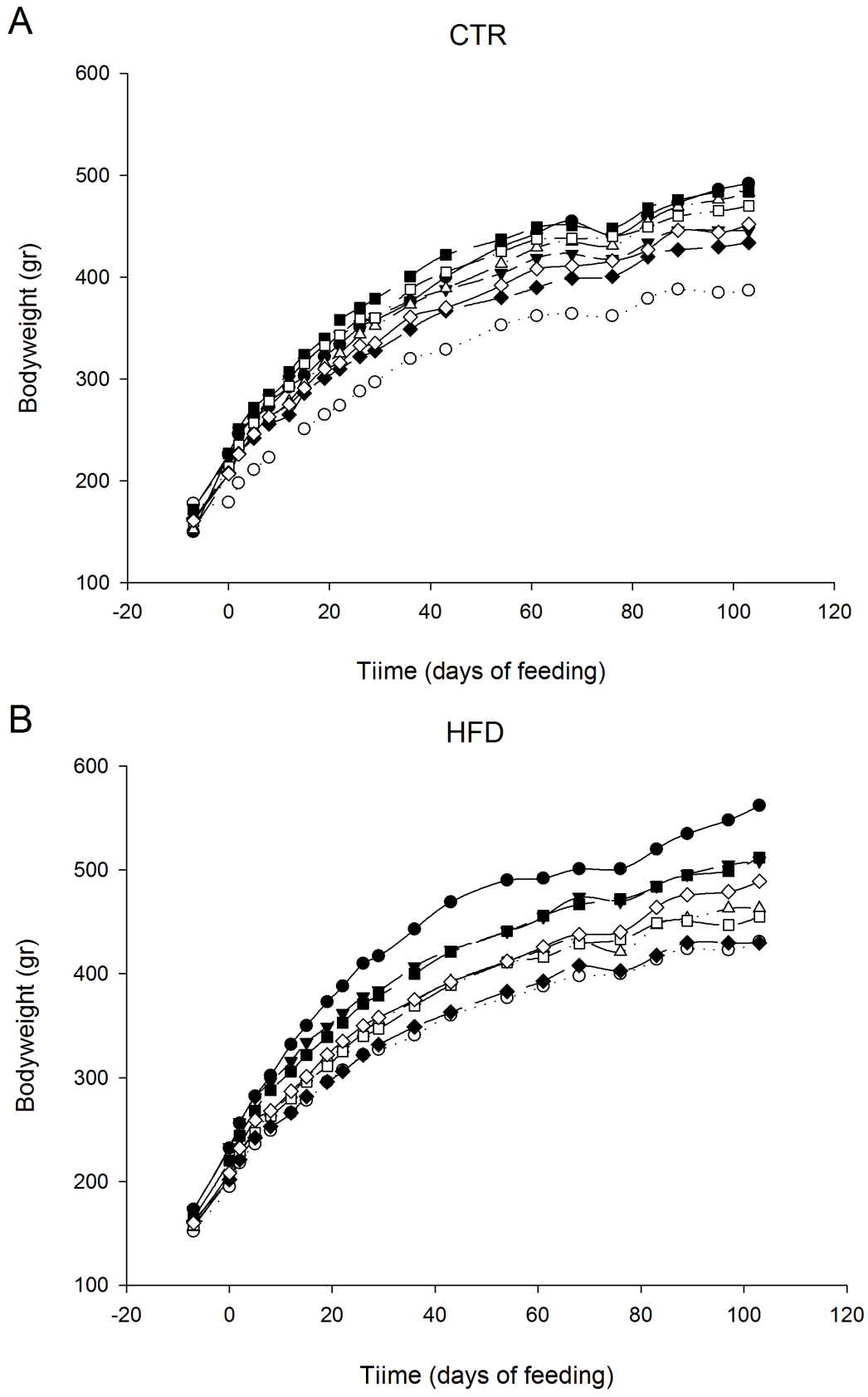
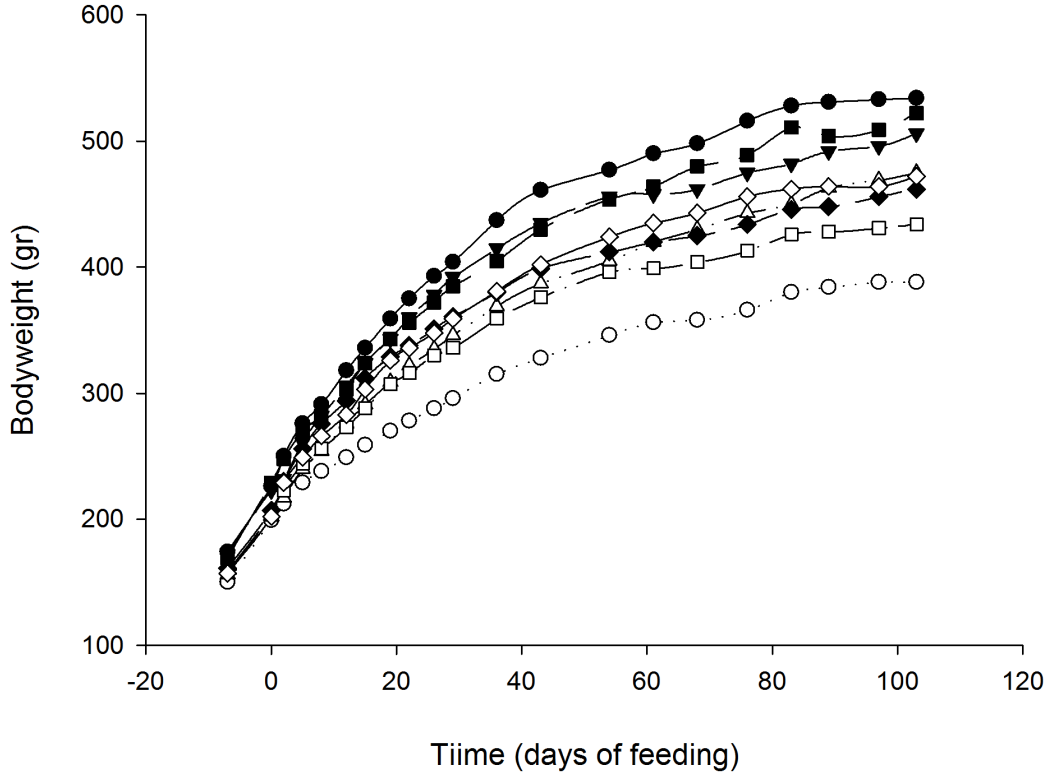
SUPPLEMENTARY DATA

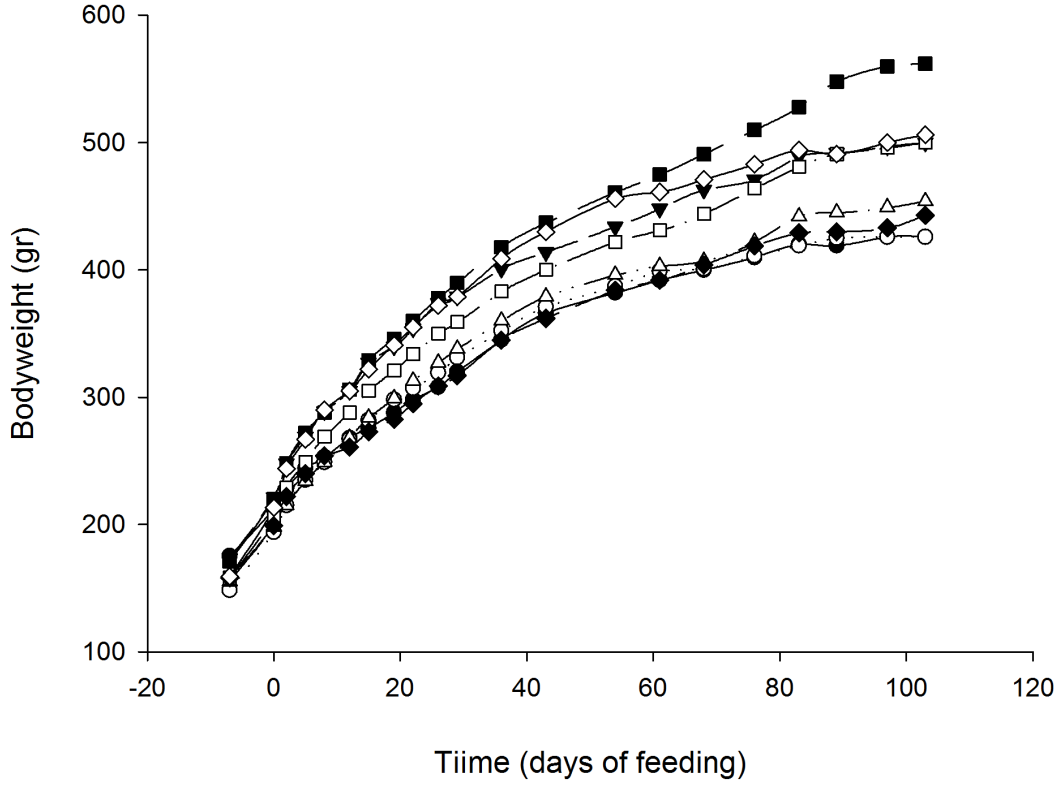
Figure S 1 – Individual body weights in protocol A



C


0.375% Calanus
Bodyweight (gr)
600
500
400
300
200
100
-20
0
20
40
60
80
100
120
Tiime (days of feeding)

D


0.75% Calanus
Bodyweight (gr)
600
500
400
300
200
100
-20
0
20
40
60
80
100
120
Tiime (days of feeding)

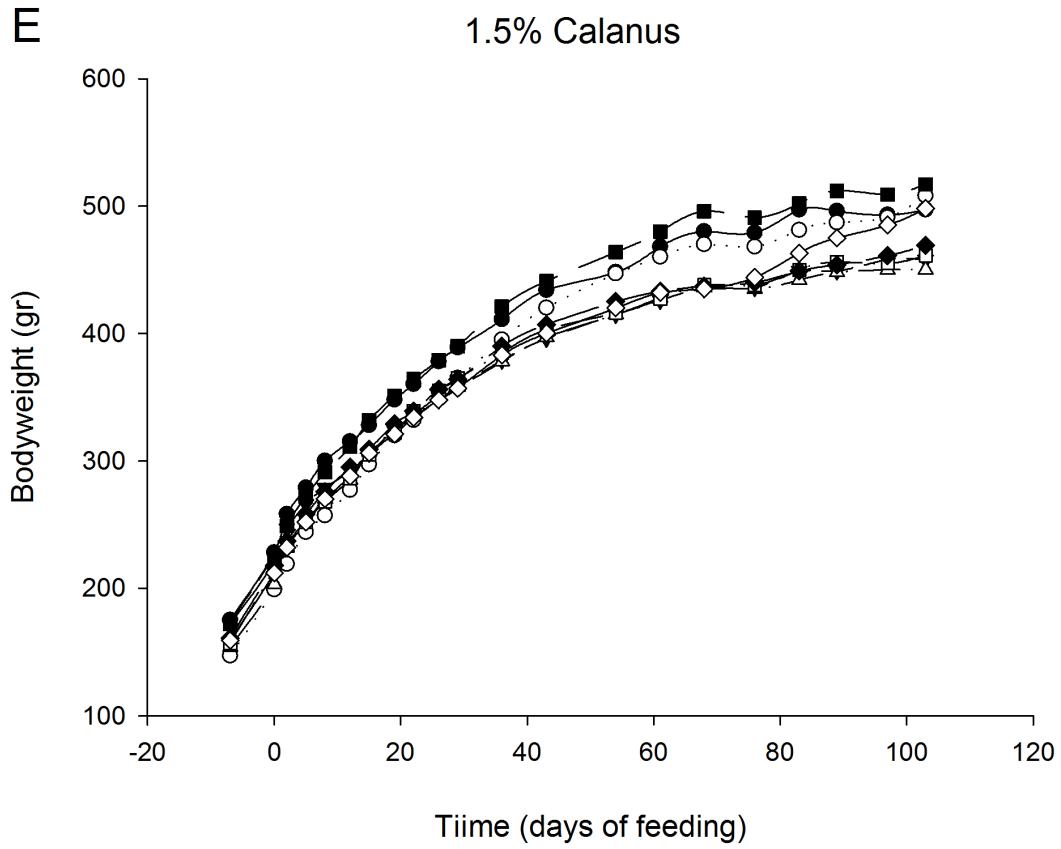


Table S1. Sequences of primers (5′–3′) and probes used for RT-PCR analysis.

GADPH: Glyceraldehyde-3phosphate dehydrogenase

Forward primer	CTG-CAC-CAC-CAA-CTG-CTT-AC
Reverse primer	CAG-AGG-TGC-CAT-CCA-GAG-TT
Probe	Roche Probe Library #9

Cyclophilin

Forward primer	CTG-ATG-GCG-AGC-CCT-TG
Reverse primer	TCT-GCT-GTC-TTT-GGA-ACT-TTG-TC
Probe	CGC-GTC-TGC-TTC-GAG-CTG-TTT-GCA

HPRT: Hypoxanthine phosphoribosyltransferase

Forward primer	GAC-CGG-TTC-TGT-CAT-GTC-G
Reverse primer	ACC-TGG-TTC-ATC-ATC-ACT-AAT-CAC
Probe	Roche Probe Library #95

HMBS: Hydroxymethylbilane synthase

Forward primer	TCC-CTG-AAG-GAT-GTG-CCT-AC
Reverse primer	ACA-AGG-GTT-TTC-CCG-TTT-G
Probe	Roche Probe Library # 79

SDHA: Succinate Dehydrogenase subunit A

Forward primer	CCC-TGA-GCA-TTG-CAG-AAT-C
Reverse primer	CAT-TTG-CCT-TAA-TCG-GAG-GA
Probe	Roche Probe Library # 80

CPT-1 (liver) carnitine palmitoyltransferase-1

Forward primer	ACA-ATG-GGA-CAT-TCC-AGG-AG
Reverse primer	AAA-GAC-TGG-CGC-TGC-TCA
Probe	(none, SYBR Green)

mCPT-1 muscle carnitine palmitoyltransferase-1

Forward primer	ATC-ATG-TAT-CGC-CGC-AAA-CT
Reverse primer	ATC-TGG-TAG-GAG-CAC-ATG-GGT
Probe	TCA-AGC-CGG-TAA-TGG-CAC-TGG-G

Modifications: 5’ 6-FAM; 3’ Darquencher

PDK 4 pyruvate dehydrogenase kinase, isoenzyme 4

Forward primer	TTC-ACA-CCT-TCA-CCA-CAT-GC
Reverse primer	AAA-GGG-CGG-TTT-TCT-TGA-TG
Probe	CGT-GGC-CCT-CAT-GGC-ATG-GCA-TTC-TTG

Modifications: 5’ 6-FAM; 3’ Darquencher