Paper III

Research Article

Lipid profile of mice fed a high-fat diet supplemented with a wax ester-rich marine oil

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Oil extracted from the marine copepod *Calanus finmarchicus* contains the long chain omega-3 fatty acids eicosapentaenoic acid and DHA in addition to stearidonic acid (18:4n-3). Unlike other marine lipids, the fatty acids in this oil are esterified with long chain fatty alcohols as wax esters. The aim of this study was to examine the fate of the wax esters in oil from *C. finmarchicus* when given as a 2% supplement in a high fat diet to C57BL/6J mice for 11 weeks. The study confirmed that feeding mice a high fat diet supplemented with a small amount of oil containing wax esters reduced the body weight gain. During digestion, wax esters were hydrolyzed and the fatty acids absorbed since the fatty acid composition of the adipose tissue and liver reflected the enrichment with the Calanus oil. The composition of the liver lipids demonstrated elongation and desaturation of the C18 omega-3 fatty acids from the feed and accumulation of longer chained omega-3 fatty acids. Elevated levels of FFA and FAOH in the feces suggest that the absorption process, not the hydrolysis, could be a rate limiting step in utilization of small amounts of wax esters included in high fat diets in mice.

Practical applications: The limited amount of available fish oil has led to extensive search for alternative sources of long-chain PUFA. One suggestion is to harvest at lower trophic levels, like small crustaceans, which may be abundantly present in the oceans. In this investigation, we have studied the effects of including the astaxanthin-rich oil from the marine copepod *C. finmarchicus* in a high fat diet in mice. This oil is different from other marine oils since most of the fatty acids are esterified to long-chain fatty alcohols, not in TAG, and that stearidonic acid is the major omega-3 fatty acid present. The results provide knowledge and understanding of aspects related to digestion and possible physiological effects of including small amounts of marine wax esters in diets.

Keywords: *Calanus finmarchicus* / Digestion / Mice / Omega-3 fatty acids / Wax esters

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Abbreviations: ALA, alpha linolenic acid; **+Cal,** inclusion of 2% calanus oil; **DPA,** docosapentaenoic acid; **EE,** ethyl ester; **EPA,** eicosapentaenoic acid; **eWat,** epididymal white adipose tissue; **FAOH,** fatty alcohol; **FFA,** free fatty acid; **HFD,** high fat diet; **HPLC-ELSD,** HPLC coupled with an evaporative light scattering detector; **LC-PUFA,** long-chain PUFA; **pWat,** perirenal white adipose tissue; **SDA,** stearidonic acid; **WE,** wax ester

1 Introduction

Nutritional interest in long-chain omega-3 fatty acids (LC-PUFA) has increased in the last decades because of their reported health benefits [1–5]. The intake of these fatty acids through eating seafood is recommended, however dietary supplements may be an alternative [6]. Many different types of dietary supplements containing the long chain omega-3 fatty acids eicosapentaenoic acid (EPA) and DHA, such as cod liver oils, whole body fish oils and products containing concentrated amounts of these fatty acids either as ethyl esters

(EE) or TAG, are available on the market. More recently oil from Antarctic krill (*Euphausia superba*), where most of the LC-PUFA are esterified in phospholipids (PL), has also become commercially available [7].

The calanoid copepod, *Calanus finmarchicus*, which is present in large amounts in the North Atlantic [8] and may be harvested in a sustainable way [9], has been suggested as a novel source of marine PUFA [10, 11]. In addition, oil from *C. finmarchicus* has a high content of stearidonic acid (SDA, 18:4n-3) similar to that of some specialized seed oils, such as *Echium* oil [12]. The oil extracted from *C. finmarchicus* is however biochemically different from krill oil and traditional fish oils since most of the fatty acids are esterified with long-chain fatty alcohols, mainly 20:1n-9 and 22:1n-11, as wax esters [13, 14]. *C. finmarchicus* is an herbivorous species with a life span of 1 year [15] and it is known that short-lived marine lipid-rich organisms at the base of the food web contain low levels of pollutants [16]. Analysis of Calanus Oil have shown that levels of heavy metals and organic pollutants are well below the limits specified for marine oils by the Commission Regulation (EC) No1881/2006 and 629/2008 (Commission of the European Communities) (manuscript in preparation). Recent studies suggest that inclusion of low levels of Calanus oil or isolated wax esters in feed to rodents on a Western type high fat diet (HFD), may reduce atherogenesis [17] and have other positive health effects such as reduced abdominal obesity, reduced adipose tissue inflammation and improvement in systemic glucose tolerance [18, 19].

Published results have shown that the ethyl esters of omega-3 fatty acids, which are structurally similar to wax esters, are hydrolyzed at a lower rate than TAG [20], and this may reduce the bioavailability of the fatty acids [21, 22]. Some studies have been carried out on the hydrolysis and bioavailability of plant derived or synthetic long-chain wax esters containing saturated or MUFA in mammals. Such wax esters are generally considered to be poorly digested in mammals and consumption of food containing large amounts of WE, including some fish species, is known to cause keriorrhea in mammals [23–26]. However, small amounts of WE are present in several common food products like whole cereal grains, nuts and seeds and they may be consumed without apparent adverse effects [27]. Gorreta et al. [28] studied the digestion and absorption of a synthetic wax containing EPA and DHA esterified with behenyl fatty alcohol (C22:0) by analysing the fatty acid composition of plasma phospholipids in rats after a 4-week feeding trial. The results indicated that the LC-PUFA level in plasma phospholipids were comparable to those of rats fed with equal amounts of LC-PUFA in the form of fish oil and ethyl esters. The aim of our work was to study the digestion of wax esters by determining the lipid profile in liver, adipose tissues and feces of mice fed a high fat diet supplemented with 2% oil from *C. finmarchicus*.

2 Materials and methods

2.1 Chemicals

Solvents (HPLC grade) were obtained from Sigma–Aldrich (Steinheim, Germany). Lipids used as standards were from Nu-Check-Prep Inc. (Elysian, MN, USA), Sigma–Aldrich and Supelco (Bellafonte, PA, USA). The commercially available oil added to the diet was provided by Calanus AS (Tromsø, Norway).

2.2 Diets and animals

Diet-induced obese mice were obtained by feeding 5–6 week old C57BL/6J mice (Charles Rivers, Sulzfeld, Germany) a lard-based high fat diet (HFD #58V8, Test Diet, IPS Ltd, Notts, UK) containing 18, 36, and 46% energy from protein, carbohydrate and fat, respectively. There were two groups; one receiving HFD and the second receiving HFD supplemented with 2.0% w/w Calanus oil throughout the whole 11-week feeding period. Addition of Calanus oil was compensated for by the removal of 2.0 g lard/100 g diet, so that the total fat content was unchanged and the diets remained isoenergetic. Apart from the difference in the fatty acid composition of the diets another noticeable difference is the higher content of cholesterol in the feed supplemented with Calanus oil compared to the control, 977 and 196 ppm, respectively as reported by the feed manufacturer. The mice in this study were part of a larger experiment where the animals were fed for 8 weeks and then given angiotensin for 3 weeks to provoke hypertension (to be published later). Only the control groups receiving saline for 3 weeks were used in our study. All animal experiments were approved by the local authority of the National Animal Research Authority in Norway (FOTS id 4438/2012). The mice were treated in accordance with 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. They were housed at 21°C on a 12 h light/dark cycle, three animals per cage. Mice received food ad libitum and had free access to drinking water. Body weight was recorded weekly and food intake was monitored at several time points throughout the feeding period. Feces were collected once every 2 weeks and frozen immediately. Prior to lipid extraction the dry weight of the feces was determined after drying triplicates at 110°C for 24 h. After 11 weeks the animals were sacrificed and the organs were carefully dissected out, weighed and snap-frozen in liquid nitrogen and stored at −80°C for later analysis.

2.3 Lipid analyses

Lipids were extracted from thawed tissue samples by using a modified version of Folch's procedure [29] were dichloromethane was used to replace chloroform. The lipid extracts

were dried under nitrogen, weighed, and redissolved in dichloromethane for further analysis.

The lipid classes were assessed by HPTLC using a modified method of Vaghela and Kilara [30]. Samples were dissolved in dichloromethane (10 μg oil/μL) and applied by capillary rods to silica gel HPTLC plates (Merck, Darmstadt, Germany) as 1 μL spots alongside lipid class standards. The plates were developed vertically in a solvent system of heptane/diethyl ether/acetic acid (80:20:2, vol/vol/vol). Lipids on the plate were charred with 10% cupric sulfate in 8% phosphoric acid for visualization by spraying and heating to 170°C in 15 min and documented by a Canon Scanner (Canon Europe Ltd., Middlesex, UK). Quantification of lipid classes was performed by an established method of HPLC-ELSD [31] using a monolithic silica column Chromolith Performance-Si, (100 mm 4.6 mm, 2 μm/13 mm, VWR, Darmstadt, Germany) and an Agilent 1200 Quarternary pump equipped with an Agilent 1200 degasser, thermostated autosampler and an column thermostat. The HPLC system wax coupled to an evaporative light scattering detector (Sedex ELSD Model 85LT, Sedere, France). The lipid classes were determined by external standard calibration and expressed as mg/g lipids.

Fatty acid composition was determined after methylation as described by Christie and Han [32]. The FAMEs were analyzed by capillary GLC using a Agilent 6890N (Agilent Technologies, Santa Clara, CA, USA) gas chromatograph with a 50 m × 0.25 mm Chrompack CP-Sil 88 CB capillary column (Varian Inc., Palo Alto, CA, USA). The injector temperature was 240°C, and the oven was programmed as follows: 80°C (1 min); 20°C/min to 170°C (0 min); 3°C/min to 200°C (0 min); 5°C/min to 240°C (15 min). The temperature of the FID was 270°C. The signal was analyzed by Agilent's Chemstation software and compared with standard mixtures of FAMEs. The individual FA in the samples were calculated by use of 17:0 as an internal standard and expressed as mg/g lipids. The identification was based on retention times.

2.4 Statistical analysis and calculations

Data presented as mean ± SD. Biochemical data was analyzed by SPSS (statistical software, IBM, NY, USA) for comparisons. Differences between the HFD and HFD + Cal group were analyzed by nonparametric *Kruskal–Wallis* and *One-Way ANOVA* test. Statistical significance was assumed with a $p < 0.05$.

3 Results

The analysis of fatty acid composition of commercial Calanus oil showed that the oil contained 180 mg omega-3 fatty acids/g lipid with 18:4n-3, 20:5n-3, and 22:6n-3 contributing with 70, 55, and 39 mg/g lipid, respectively (Table 1). The content of the long chain monounsaturated fatty acid cetoleic acid (22:1n-11) was determined to 43 mg/g lipid in Calanus oil. None of these fatty acids were detected in the lipids extracted from the high fat feed, but were present in the diet, which contained 2% Calanus oil. The Calanus oil contained mainly 20:1n-9 and 22:1n-11 fatty alcohols and these were the only ones that could be detected in the diet containing the Calanus oil.

As shown in Table 2, the supplementation of HFD with 2% Calanus oil resulted in a reduced body weight gain compared to the HFD group during the 11-week feeding period. The liver weights were not different between the two groups. The weights of the perirenal white adipose tissue

Table 1. Fatty acid and fatty alcohol content (mg/g lipid) of Calanus oil and experimental diets

Fatty acids	Calanus oil	High fat diet	High fat diet + Cal
14:0	64.42	10.41	11.95
16:0	45.05	173.65	149.83
18:0	2.42	106.63	92.16
20:0	0.40	1.70	1.37
Σ SFA	112,29	292,39	255.31
16:1*n-7*	17.17	10.95	9.98
18:1*n-7*	1.53	15.84	13.52
18:1*n-9*	15.54	243.82	208.42
20:1*n-9*	24.01	4.74	5.64
20:1*n-11*	3.90	nd	nd
22:1*n-9*	2.63	nd	nd
22:1*n-11*	43.33	nd	2.20
24:1*n-9*	2.81	nd	nd
Σ MUFA	110.92	275.36	239.76
18:2*n-6*	6.64	133.04	116.06
18:3*n-3*	13.72	12.49	11.67
18:4*n-3*	69.58	nd	4.54
20:2*n-6*	0.71	3.15	2.69
20:4*n-6*	1.39	0.48	1.15
20:5*n-3*	54.73	nd	3.35
22:5*n-3*	2.96	nd	nd
22:6*n-3*	39.35	nd	2.81
Σ PUFA	189.08	149.16	142.27
Σ *n-6*	8.74	136.67	119.90
Σ *n-3*	180.34	12.49	22.37
Σ *n-6/n-3*	0.05	10.94	5.36
Σ Fatty acids	412.29	716.91	637.34
Fatty alcohols	**Calanus oil**	**High fat diet**	**High fat diet + Cal**
14:0	4.50	nd	nd
16:1*n-7*	5.80	nd	nd
18:1*n-9*	10.40	nd	nd
20:1*n-9*	128.80	nd	9.65
22:1*n-9*	10.40	nd	nd
22:1*n-11*	188.10	nd	9.93
Σ Fatty alcohols	348.00	–	19.58

nd, not detected; SFA, saturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Table 2. Initial body weight, body weight gain and liver, pWat and eWat weights after 11 weeks of feeding

Biometric data (g)	HFD	HFD + Cal
Initial body weight	21.15 ± 0.25	20.97 ± 0.63
Body weight gain	14.37 ± 1.02	11.79 ± 0.47*
Liver weight	1.37 ± 0.13	1.44 ± 0.18
eWat	1.69 ± 0.73	1.15 ± 0.34
pWat	0.69 ± 0.27	0.51 ± 0.15
Lipid content in feces (% DM)	2.45 ± 0.08	3.09 ± 0.45

eWat, epididymal white adipose tissue; pWat, perirenal white adipose tissue; DM, dry matter.
Data are mean ± SD for $n = 10$ per group (body weight and weight gain mean ± SEM).
*HFD + Cal group significantly different from HFD using One-Way ANOVA test ($p < 0.05$).

and the epididymal white adipose tissue appeared lower in the HFD + Cal group. However, no significant differences were detected between the groups. No adverse effects were observed in the mice during the feeding period. The feces collected throughout the trial from the HFD and HFD + Cal groups had similar appearance with a lipid content of 2.45 and 3.09% (dry weight), respectively.

Stearidonic acid (18:4n-3), which is abundantly present in Calanus oil and therefore also present in the HFD + Cal diet were detected in pWat and eWat of mice fed this diet (Table 3). An increased level of stearidonic acid was observed in the livers of this group compared to the group fed HFD. Elevated content of docosapentaenoic acid DPA (22:5n-3) and DHA (22:6n-3) could be detected in pWat, eWat and livers of the HFD + Cal group. EPA (20:5n-3) was significantly increased only in the livers of HFD + Cal fed mice. The amount of AA (20:4n-6) was significantly lower in the adipose tissues from the HFD + Cal group. It is noticeable that the ratio of EPA + DPA + DHA/SDA was much higher in the liver lipids than in the adipose tissues and in the

Table 3. Fatty acid content (mg/g lipid) extracted from pWat, eWat, and liver

	pWat		eWat		Liver	
Fatty acids	HFD	HFD + Cal	HFD	HFD + Cal	HFD	HFD + Cal
14:0	8.55 ± 0.89	10.77 ± 1.34*	9.41 ± 0.79	11.55 ± 1.45*	3.15 ± 0.73	2.68 ± 0.83
16:0	178.40 ± 18.60	177.05 ± 18.23	193.85 ± 12.88	189.02 ± 9.23	192.82 ± 19.45	182.02 ± 13.49
18:0	30.42 ± 3.00	33.35 ± 7.43	35.15 ± 15.83	26.77 ± 11.14	75.97 ± 14.94	92.29 ± 14.58*
20:0	1.07 ± 0.06	1.13 ± 0.26*	0.22 ± 0.44	1.02 ± 0.16	2.82 ± 0.40	2.77 ± 0.49
22:0	nd	nd	nd	nd	1.71 ± 0.53	2.20 ± 0.50
Σ SFA	217.54 ± 21.98	221.88 ± 24.47	238.64 ± 25.37	227.78 ± 17.15	276.49 ± 16.90	281.95 ± 20.85
16:1*n-7*	45.53 ± 6.00	38.06 ± 3.34*	53.45 ± 10.43	49.15 ± 9.15*	15.34 ± 4.62	9.88 ± 2.16*
18:1*n-7*	24.58 ± 2.27	21.81 ± 2.86*	25.52 ± 1.15	23.48 ± 2.03	16.45 ± 2.93	10.50 ± 0.94*
18:1*n-9*	440.60 ± 36.86	411.44 ± 57.35	446.43 ± 26.46	428.10 ± 38.77	216.08 ± 44.77	154.85 ± 28.66*
20:1*n-9*	6.73 ± 0.72	11.25 ± 2.39*	5.59 ± 0.75	8.80 ± 0.89*	5.30 ± 0.83	5.07 ± 0.71
20:1*n-11*	nd	nd	nd	nd	nd	0.60 ± 0.17
22:1*n-9*	nd	2.24 ± 0.53	nd	1.66 ± 0.12	0.55 ± 0.10	0.55 ± 0.10
22:1*n-11*	nd	nd	nd	nd	0.55 ± 0.13	1.85 ± 0.31*
Σ MUFA	515.44 ± 44.13	484.80 ± 60.35	530.99 ± 34.55	511.21 ± 48.00	254.22 ± 52.47	183.30 ± 32.31*
18:2*n-6*	157.88 ± 14.41	152.74 ± 18.26	174.20 ± 15.31	175.32 ± 19.63	129.05 ± 6.46	123.73 ± 4.76
18:3*n-3*	6.66 ± 0.78	7.11 ± 0.82	8.54 ± 0.94	9.06 ± 1.09	3.89 ± 0.65	3.87 ± 0.67
18:4*n-3*	nd	1.60 ± 0.38	nd	1.12 ± 0.11	0.43 ± 0.12	0.77 ± 0.19*
20:2*n-6*	3.62 ± 0.33	3.39 ± 0.63	3.39 ± 0.19	3.15 ± 0.29	2.97 ± 0.23	2.44 ± 0.10*
20:4*n-6*	2.16 ± 0.30	1.46 ± 0.26*	2.97 ± 0.82	1.77 ± 0.33*	81.03 ± 16.49	70.18 ± 8.75
20:5*n-3*	nd	nd	nd	1.01 ± 0.08	2.12 ± 0.27	13.50 ± 1.74*
22:5*n-3*	nd	1.68 ± 0.57	0.10 ± 0.30	1.55 ± 0.17*	4.04 ± 0.66	7.90 ± 0.80*
22:6*n-3*	0.15 ± 0.33	3.33 ± 0.58*	0.99 ± 0.78	3.77 ± 0.47*	47.23 ± 8.46	89.98 ± 6.98*
Σ PUFA	170.46 ± 15.47	170.74 ± 19.03	190.18 ± 16.29	196.02 ± 20.82	270.67 ± 21.33	312.38 ± 16.88*
EPA + DPA + DHA/SDA	–	3.13	–	5.65	124.16	144.65
Σ Fatty acids	903.43 ± 79.33	877.42 ± 109.95	935.00 ± 71.75	959.78 ± 41.46	801.37 ± 49.72	777.63 ± 42.66

nd, not detected; SFA, saturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; pWat, perirenal white adipose tissue; eWat, epididymal white adipose tissue.
Values are mg FA/g lipids (mean ± SD of 8 mice/group).
*HFD + Cal group significantly different from HFD using one-way ANOVA test ($p < 0.05$).

feed given to this group of mice. Cetoleic acid (22:1n-11) was not detected in the adipose tissues, but showed a small increase in the livers of the group fed the diet containing Calanus oil. Palmitoleic acid (16:1n-7) were significantly reduced in pWat, eWat, and livers of the animals receiving the HFD + Cal diet, while oleic acid (18:1n-9) was only significantly lower in the livers.

Lipids were extracted from feces after 2, 4, and 6 weeks of feeding. The results of the analysis from each sampling gave a similar fatty acid composition and the data were therefore pooled. The dominating fatty acids in both groups were oleic acid (18:1n-9), stearic acid (18:0), palmitic acid (16:0), and linoleic acid (18:2n-6) (Table 4). Cetoleic acid was detected in the feces of the HFD + Cal group but not in the feces from the HFD group. DPA was found in the feces of both feed groups. EPA was not detected, while approximately 3 mg DHA/g lipid were present in the feces of the HFD + Cal group. The identified fatty acids in Table 4 constituted 42 and 30% of the total lipids extracted from the feces of the HFD and HFD + Cal fed mice, respectively. About 24% of the lipids in the HFD + Cal group were identified as fatty alcohols, mainly 20:1n-9 and 22:1n-11. A minor amount (0.2%) of the lipid was identified as the fatty alcohol 16:1n-7 in the feces of the HFD group.

Table 4. Fatty acid and fatty alcohol content (mg/g lipid) extracted from feces

	Feces	
Fatty acids	HFD	HFD + Cal
14:0	3.27 ± 1.04	3.40 ± 0.34
16:0	69.77 ± 8.87	47.86 ± 5.19
18:0	101.79 ± 12.19	76.62 ± 10.48
20:0	2.90 ± 0.84	2.33 ± 0.59
22:0	3.25 ± 3.39	1.86 ± 0.63
Σ SFA	180.98 ± 21.71	132.07 ± 16.00
16:1*n-7*	3.20 ± 0.76	1.71 ± 0.51
18:1*n-7*	12.19 ± 1.04	7.80 ± 0.52
18:1*n-9*	156.87 ± 20.46	97.61 ± 8.51
20:1*n-9*	13.01 ± 1.41	15.21 ± 1.24
20:1*n-11*	nd	nd
22:1*n-9*	1.71 ± 0.69	2.97 ± 0.79
22:1*n-11*	nd	12.11 ± 1.25
Σ MUFA	186.97 ± 21.81	137.43 ± 11.83
18:2*n-6*	39.07 ± 7.13	22.99 ± 1.95
18:3*n-3*	3.17 ± 0.68	1.62 ± 0.52
18:4*n-3*	nd	0.43 ± 0.57
20:2*n-6*	2.32 ± 1.15	1.68 ± 0.64
20:4*n-6*	1.78 ± 0.93	0.17 ± 0.26
20:5*n-3*	nd	nd
22:5*n-3*	11.96 ± 1.53	8.64 ± 3.41
22:6*n-3*	nd	3.09 ± 0.38
Σ PUFA	58.31 ± 8.74	38.62 ± 5.24
Σ Fatty acids	426.26 ± 23.47	308.11 ± 12.77
Fatty alcohols	HFD	HFD + Cal
14:0	nd	nd
16:1*n-7*	2.07	1.57
18:1*n-9*	nd	3.51
20:1*n-9*	nd	86.89
20:1*n-9*	nd	4.00
22:1*n-11*	nd	145.64
Σ Fatty alcohols	2.07	241.60

nd, not detected; SFA, saturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.
Values are mg FA/g lipids (mean ± SD $n = 6$).

The lipid classes present in Calanus oil, HFD, and HFD + Cal feeds and in the feces were assessed qualitatively by HPTLC and quantitatively by HPLC-ELSD. Together, the results confirmed that the dominating lipid class in Calanus oil was WE with as much as 857 mg/g lipids (Fig. 1, Table 5). The lipids from both diets consisted mainly of TAG, while 63 mg WE/g lipids were detected in HFD + Cal feed. Traces of cholesterol, fatty alcohols, and free fatty acids in the diets could be observed by the HPTLC analysis (Fig. 1, lanes B and C), but not detected by the HPLC-ELSD. The lipid classes in the feces appeared similar in both groups when analyzed by HPTLC, except for the presence of fatty alcohols in the HFD + Cal mice (lane E). Minor amounts of materials with the same mobility as fatty alcohols were apparently present in the feces of the HFD group (lane D). Cholesterol or cholesterol-like molecules and FFA were two of the major lipid classes present in the feces of both groups of mice. The feces from the HFD + Cal group contained 115 mg

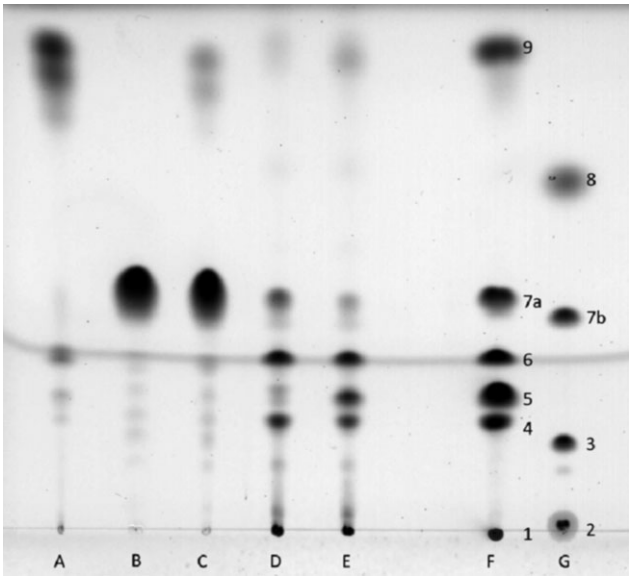

Figure 1. Lipid class composition of (A) Calanus Oil, (B) HFD, (C) HFD + Cal, (D) feces HFD, (E) feces HFD + Cal, (F and G) standards containing (1) PL, (2) MAG, (3) DAG, (4) Cholesterol, (5) FAOH, (6) FFA, 7(a) Triolein, (b) Trilinolenin, (8)ME, (9) WE, and CE.

Table 5. Lipid class composition (mg/g lipid) of calanus oil, diets, and feces

		Diet		Feces	
	Calanus oil	HF	HF + Cal	HFD	HFD + Cal
WE/CE[a)]	857.70	nd	63.06	33.59	53.93
TAG	16.96	970.33	936.04	105.95	42.88
FAOH	nd	nd	nd	nd	126,90
C	41.91	nd	nd	240.36	115.65
FFA	16.59	nd	nd	223.79	332.55
CL	nd	nd	nd	19,42	18.02
PC	nd	nd	nd	24,61	nd
$\sum$ lipid class	933.16	970.33	999.10	647.72	689.92

nd, not detected; WE, wax ester; CE, cholesteryl ester; TAG, triacylglycerol; FAOH, fatty alcohol; C, cholesterol; FFA, free fatty acid; CL, cardiolipin; PC, phosphatidylcholin.
[a)]WE and CE coelute.

cholesterol and 332 mg FFA/g extracted lipids, while the values for the HFD group were 240 mg cholesterol and 223 mg FFA/g lipids (Table 5). Neutral lipid classes such as TAG and wax ester/cholesteryl ester (WE/CE) could also be detected in the feces. Wax ester and cholesteryl ester are reported as one class since we were unable to separate these lipid classes. Small amounts of cardiolipin and polar lipid species with low mobility in the HPTLC system could also be detected in the feces. The lipid classes identified by HPLC-ELSD in the feces accounted for 647 and 689 mg/g extracted lipids in the HFD and HFD + Cal group, respectively.

4 Discussion

The analysis of fatty acid composition of Calanus oil demonstrated a high content of stearidonic acid in addition to EPA and DHA, resembling the fatty acid composition of *C. finmarchicus* published earlier [32–34]. The monounsaturated gondoic acid (20:1n-9) and cetoleic acid (22:1n-11) known to be abundantly present in WE of *C. finmarchicus* [14] were also detected in both Calanus oil and in the diet containing Calanus oil. The amount of omega-3 fatty acids in the HFD + Cal feed reflected the inclusion of 2% Calanus oil. The long chain monounsaturated fatty alcohols 20:1n-9 and 22:1n-11, the dominating wax ester alcohols in most herbivorous zooplankton [14], were detected in the oil and also in the supplemented feed.

The reduced body weight gain observed during feeding with the HFD where 2% of the lard was substituted with Calanus oil are in line with earlier published studies on the effects of including marine oils in diets for rodents [35–38]. Höper et al. [19] who included 1.5% Calanus oil in a high fat diet given to mice also observed reduced body weight gain. They also found reduced abdominal obesity, which we did not observe in our study. The reason for this could be that in the study of Höper et al. the feeding trial were carried out for 28 weeks while our study were carried out only for 11 weeks. Murota et al. [39] showed that including 2.5% pure oleyl alcohol in the diet to rats gave a lower amount of adipose tissue, but did not affect the body weight gain. They also reported a much higher content of excreted lipids in the group receiving the fatty alcohol diet. We did not observe any differences in the lipid content of the feces between the two treatment groups at the end of the feeding trial. This may be due to the lower amount of fatty alcohols present in the diet compared with the study of Murota et al. [39] or the fact that the fatty alcohols were esterified in WE in our study. In addition mammals are considered to utilize wax esters to only a small extent [26], although active intestinal hydrolysis and uptake of wax esters or of their hydrolytic products have been reported in rats, mice and dogs [17, 23–25, 28]. It is not known if it is digestion of the wax esters to fatty acids and fatty alcohols, the absorption of long chain fatty alcohols or the oxidation of fatty alcohols to the corresponding fatty acids in the enterocytes, which may regulate the nutritional value of wax esters.

Fatty acid composition of the depot fats (pWat and eWat) and liver lipids reflected to a large extent the enrichment of HFD with Calanus oil. The content of the omega-3 fatty acids in pWat and eWat were generally related, with the exception of EPA in pWat, to the amount present in the feed. However, some elongation and desaturation had apparently occurred resulting in the detection of DPA and increased amount of DHA compared to the HFD + Cal feed. It is interesting to note that the amount of the more pro-inflammatory AA is lower in livers and significantly reduced in pWat and eWat of the mice fed HFD + Cal. This may be due to the competition in interconversion of C18 n-6 and n-3 fatty acids to the longer chain variants [40]. The reduction in AA may have contributed to the reduced adipose tissue inflammation seen in mice fed diets supplemented with Calanus oil [19]. However, this requires further investigation including studies on the levels of AA in other tissues and in cell membranes.

The analysis of the liver lipids demonstrated pronounced elongation and desaturation of the C18 n-3 fatty acids from the feed and accumulation of the longer chain omega-3 fatty acids. This is shown by the high EPA + DPA + DHA/SDA ratio confirming that conversion of C18 n-3 fatty acids primarily occurs in this organ [40]. A significant higher amount of EPA, DPA and DHA were found in the livers of the HFD + Cal fed mice probably not only reflecting the content of these fatty acids in the feed, but also that the conversion of dietary SDA into EPA is more effective than conversion of ALA to EPA [41, 42]. Most of the omega-3 fatty acids in Calanus oil are esterified to long chain fatty alcohols and the results presented demonstrate that the wax esters were hydrolyzed and the fatty acids absorbed, reesterified and transported to the tissues examined.

It is known from classical studies that fatty alcohols may be oxidized to the corresponding fatty acids in the intestines of rats [43, 44]. It is therefore possible that some of the fatty alcohols from the hydrolyzed wax esters are absorbed and converted to fatty acids. Since TAG constitute a minor part compared to the wax esters in the Calanus oil it is quite possible that the significantly higher content of gondoic acid (20:1n-9) in pWat and eWat and cetoleic acid (22:1n-11) in the livers of HFD + Cal fed mice are originating from the fatty alcohols. The observation of the significantly reduced content of palmitoleic acid in the livers and adipose tissues of the HFD + Cal group may indicate a reduced activity of stearoyl-CoA desaturase (SCD; EC 1.14.99.5). A high activity of SCD has been associated with the development of obesity and metabolic disorders but this is not clarified [45–47].

Relatively small differences were found in the fatty acid composition of the lipids extracted from the feces of the two dietary groups. The presence of cetoleic acid (22:1n-11) in higher concentrations in the feces than in the feed from the HFD + Cal fed mice is difficult to explain, but may be due to impaired absorption of this fatty acid since it has been suggested that fatty alcohols may inhibit the gastrointestinal absorption of long chain fatty acids [39]. Analysis of lipids in feces showed that the identified fatty acids constituted 30 and 42% of the extracted lipids in the HFD + Cal and HFD groups, respectively. In addition, the former group contained about 24% fatty alcohols, mainly 20:1n-9 and 22:1n-11, reflecting the feed given to this group. The HPTLC results also indicated the presence of minor amount of fatty alcohols in the feces from the HFD group and this may be explained by the formation of fatty alcohols in the gastrointestinal tract as suggested by Bandi and Mangold [48]. The HPTLC and HPLC-ELSD results showed that free fatty acids were a major lipid class in feces from both groups indicating incomplete absorption of fatty acids in a high fat diet. Cholesterol or other sterols were present in relatively high amounts in the feces of both groups. Fecal excretion of such lipids is also known from the literature [49, 50]. The cholesterol content in the feces from the HFD + Cal appeared lower than in the HFD group. This finding is somewhat surprising and difficult to explain as the cholesterol content in the HFD + Cal diet is about five times higher than in the HF diet due to the relative high content of cholesterol known to be present in crustaceans. Phospholipids, MAGs, and microbial specific lipids like short chain fatty acids and cardiolipin, may contribute to the lipid species with the low mobility in the HPTLC analysis. The HPLC-ELSD and HPTLC analysis of the feces from the HFD group indicated the presence of steryl esters confirming results from previously feeding trials with mice [50]. Comparing the amount of WE/cholesteryl ester in the HFD + Cal group suggest that only a small amount of intact wax esters is excreted in this group. The relative amount of free fatty alcohols and FFA in the feces suggests that the absorption process may be the limiting step.

5 Conclusions

This study confirms that feeding mice a high fat diet supplemented with a small amount (2%) of Calanus oil containing wax esters reduces the body weight gain. Fatty acid analysis showed that the wax esters were hydrolyzed in the digestive tract and the fatty acids influenced the lipid profile of the animals. The hydrolysis was confirmed by analysis of the fecal lipids, which were mainly FFA and cholesterol. Fatty alcohols were present in the feces of the group supplemented with the Calanus oil. The medium chain omega-3 fatty acid SDA, which is abundantly present in the Calanus oil, was effectively absorbed and converted to long chain omega-3 fatty acids in the liver. The reduced amount of AA in adipose tissues may have contributed to lowering the inflammation status observed by others.

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