https://doi.org/10.1017/S0007114525000236 Published online by Cambridge University Press

Accepted manuscript

Absorption, accumulation and metabolism of cetoleic acid from dietary herring oil in tissues of diabetic male Zucker Diabetic Sprague Dawley rats

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Sources of support:

This research was funded by The Norwegian Seafood Research Fund (FHF, grant number 901769). The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Short title: Accumulation and metabolism of cetoleic acid

Conflict of interest: The authors declare no conflict of interest.

This peer-reviewed article has been accepted for publication but not yet copyedited or typeset, and so may be subject to change during the production process. The article is considered published and may be cited using its DOI

10.1017/S0007114525000236

The British Journal of Nutrition is published by Cambridge University Press on behalf of The Nutrition Society

https://doi.org/10.1017/S0007114525000236 Published online by Cambridge University Press

Abstract

Accumulation of exogenous fatty acids such as the long-chain n-11 MUFA cetoleic acid (CA, C22:1n-11) may induce functional changes, through direct effects or by affecting the amounts of other fatty acids through changes in catabolic and anabolic processes including desaturation of fatty acids or by other processes. The primary aim of the present study was to investigate if dietary CA was absorbed and accumulated in a TAG-rich tissue for storage (white adipose tissue), a stable phospholipid-rich tissue (brain), metabolically active tissues (liver and skeletal muscle), or circulating in the blood (blood cells) and metabolised. Secondary aims included to investigate any effects on the levels of EPA and DHA. Eighteen male Zucker Diabetic Sprague Dawley rats were fed diets with herring oil (HERO) containing 0.70 % CA or anchovy oil (ANCO) devoid of CA, or a control diet with soyabean oil for 5 weeks. The HERO and ANCO diets contained 0.35 and 0.37 wt% EPA+DHA, respectively. Data were analysed using one-way ANOVA. CA from dietary HERO was absorbed, and CA and its chain-shortened metabolites were found in blood cells, liver, white adipose tissue and (WAT) and muscle, but n-11 MUFAs were not found in the brain. The concentrations of EPA and DHA were similar in liver lipids (TAG, cholesteryl esters and NEFA) as well as in WAT, muscle and brain from rats fed the HERO or ANCO diets. To conclude, CA was taken up by tissues but did not affect levels of EPA and DHA in this diabetic rat model.

Keywords: cetoleic acid, n-11 MUFA, EPA, DHA, desaturases

List of abbreviations

7OA, 7-octadecenoic acid; CA, cetoleic acid; CE, cholesteryl ester; DPA, n-3 docosapentaenoic acid; FADS1, fatty acid desaturase 1; FADS2, fatty acid desaturase 2; GA, gadoleic acid; PL, phospholipid; SCD1, stearoyl-CoA desaturase 1; T2D, type 2 diabetes; ZDSD, Zucker Diabetic Sprague Dawley rats.

https://doi.org/10.1017/S0007114525000236 Published online by Cambridge University Press

Introduction

Vertebrates have limited capacity to biosynthesize the long-chain MUFA cetoleic acid (CA, C22:1n-11), however CA is found in high levels in fish species feeding on zooplankton such as copepods [1]. Unlike the well-known and much-studied long-chain n-3 PUFAs that are also found in fish and other marine species, few studies have investigated the metabolism of CA or the metabolic effects of CA intake in vertebrates. Research on the metabolism of ingested CA in humans and other animals is limited, but studies in grey seals (*Halichoerus grypus*) and minks (*Mustela vison*) demonstrate that CA is chain-shortened in peroxisomes by oxidation to C20:1n-11, C18:1n-11 and C16:1n-11 and is further oxidised in mitochondria to acetyl groups to be used for *de novo* fatty acid synthesis [2]. Findings in cell culture studies suggest that a beneficial effect of CA may be through stimulation of the endogenous synthesis of EPA and DHA from alpha-linolenic acid by upregulating fatty acid desaturases 1 and 2 (FADS1 and FADS2) in the liver [3]. EPA and DHA that are incorporated in phospholipids in membranes may affect the fluidity and thus the function of the cells, and they are precursors for anti-inflammatory prostaglandins, leukotrienes, resolvins and protektins [4]. Others have suggested that CA has a beneficial effect on the metabolism by stimulating the oxidation of other fatty acids, based on the observation of a higher oxidation *ex vivo* in peroxisomes [5], but not in mitochondria [6], from liver cells isolated from Wistar rats fed a diet with a high content of long-chain MUFA.

We recently summarised and meta-analysed the available literature investigating the effects of consumption of fish oils or fish oil concentrates with a high content of CA but low or no content of EPA and DHA on the circulating cholesterol concentration in rodents, showing that CA-rich fish oils and concentrates prevent high cholesterol concentration [7]. The articles included in the systematic review did not provide any details on the mechanism(s) behind the lower cholesterol concentration after CA intake [7]. A recently published paper from our group showed that when Zucker Diabetic Sprague Dawley (ZDSD) rats were fed a diet containing herring oil, which contains a high amount of CA but has relatively low contents of both EPA and DHA compared to anchovy oil, this resulted in a lower serum cholesterol concentration compared to a control group fed a diet containing soy oil [8], indicating that CA and possibly its metabolites were taken up by the tissues to induce metabolic effects. This is an interesting finding since elevated circulating cholesterol concentration is a major risk factor for CVD [9, 10], the leading cause of disease burden globally [11]. The lower serum cholesterol concentration was accompanied with a higher faecal

https://doi.org/10.1017/S0007114525000236 Published online by Cambridge University Press

excretion of bile acids, whereas markers of the cholesterol production in the liver, the hepatic secretion of VLDL, and the liver's capacity to take up cholesterol were similar to controls [8].

The knowledge on the metabolism of CA is scarce, and more information on where CA is stored in the body is warranted to better understand any impact of CA consumption on biochemical and physiological processes, including the reported lowering of cholesterol [7, 8]. Therefore, the main aim of the present study was to investigate if CA and any elongated or chain-shortened metabolites of CA are accumulated in a TAG-rich tissue for storage (WAT), a stable phospholipid (PL)-rich tissue (brain), metabolically active tissues such as the liver and skeletal muscle, or circulating in the blood (blood cells) in ZDSD rats fed a diet containing fish oil with CA. Our hypothesis was that CA would be recovered in all examined tissues. The secondary aims were to investigate any effects on the levels of other fatty acids including EPA and DHA in these tissues and liver lipids, and to explore any differences in the levels of the desaturases FADS1 and FADS2 in liver and skeletal muscle in ZDSD rats consuming diets containing fish oils with or without CA. To achieve this, the fatty acid compositions in adipose tissue (mainly TAG for storage), the brain (mainly PL and cholesterol, quite stable composition), skeletal muscle (relatively high in phospholipids but also contains other lipids) and blood cells (mainly phospholipids, less stable) were mapped in rats consuming diets added herring oil or anchovy oil, containing 0.35 and 0.37 wt% EPA+DHA, respectively. In addition, PL, TAG, cholesteryl esters (CE) and NEFA in liver were separated before the fatty acids were quantified, and the levels of desaturases in liver and skeletal muscle were measured. Since herring oil contains n-3 long-chain-PUFAs, we used anchovy oil with a similar EPA+DHA content to control for dietary intake of EPA and DHA and the endogenous synthesis of the long-chain n-3 PUFAs.

Methods

Ethical statement

The study protocol was approved by the National Animal Research Authority (Norway) in accordance with the Animal Welfare Act and the Regulation of animal experiments (Approval No. 29717). All applicable international, national and institutional guidelines for the care and use of animals were followed.

https://doi.org/10.1017/S0007114525000236 Published online by Cambridge University Press

Animals and diets

The design of this study including housing conditions and the diets has been described in detail in Rimmen et al. [8], and the present study presents secondary analyses of this experiment. Twenty-four male Zucker Diabetic Sprague Dawley rats (ZDSD/PcoCrl) were obtained from Charles River). The ZDSD rat model was generated by crossing the Zucker Diabetic Fatty (Lean +/+) with Sprague Dawley Crl:CD rats [12]. The ZDSD rat has an intact leptin signalling pathway and develops polygenetic metabolic disturbances with insulin resistance resulting in type 2 diabetes (T2D) that progresses similarly to the disease in humans including destruction of pancreatic β-islet cells [12, 13]. The rats were 81-95 days old at arrival, and were housed in pairs in 1500U Eurostandard Type IV S cages (IVC Blue Line, Tecniplast) with temperature 22-23°C, in a room with controlled light/dark cycle (dark 20:00-06:00). The rats were fed standard chow as used in our animal facility (V1536, containing 19.1% protein, 3.6% fat, 4.8% sugar, from ssniff Spezialdiäten GmbH, Soest, Germany) until they were approximately 16 weeks of age. Rats were fed a higher fat diet to synchronise the onset of T2D, as recommended by Charles River [14], using an in-house diet containing 20wt% protein (casein), 10wt% sucrose, 16.2wt% maltose dextrin, 32wt% lard and 7wt% soybean oil. See Rimmen et al. [8] for more details.

Eighteen of the rats (75%) developed diabetes (blood glucose > 13.9 mmol/L) and were randomly assigned to receive one of the three experimental diets by drawing paper lots from a jar. Each group consisted of six rats, and the rats were given random numbers that could not be linked to the experimental group. The rat cages were randomly placed in the rack. The experimental semi-purified diets were modified versions of the American Institute of Nutrition's recommendation for growing laboratory rodents (AIN-93G) [15] with the addition of 1.6 g methionine/kg diet as recommended by Reeves [16], and differed only in their lipid sources (**Table 1**). All diets contained 20wt% protein (casein), 10wt% fat and 11.8wt% sucrose, and had comparable energy content [8]. We chose to use the AIN-93G diet instead of the AIN-93M diet for maintenance containing 15wt% protein since the ZDSD rats will develop insulin resistance. Insulin resistance leads to increased muscle protein breakdown in both rodents and humans [17, 18]. The two intervention diets contained either refined oil prepared from herring (*Clupea harengus*) residuals (the HERO diet) or refined oil prepared from whole anchovies (*Engraulidae*) (the ANCO diet), designed with a comparable content of EPA+DHA but different CA content. All diets contained adequate amounts of essential fatty acids according to Reeves et al. [15]. The control diet contained soybean oil as the only lipid

https://doi.org/10.1017/S0007114525000236 Published online by Cambridge University Press

source. All ingredients were purchased from Dyets Inc. (Bethlehem, PA, USA) except casein which was purchased from Sigma-Aldrich (Munich, Germany), herring oil from Pelagia AS (Bergen, Norway) and anchovy oil from Epax Norway AS (Ålesund, Norway). The rats had ad libitum access to feed and water in their home cage. The diets were stored at -15 °C, and daily portions were thawed in the morning.

The rats were housed in IVCs equipped with one gnawing block (Aspen brick, size 100 mm × 20 mm × 20 mm, TAPVEI® Harjumaa, Estonia OÜ), three paper sachets containing soft wood bedding for nesting material (2HK Nestpak, Datesand Ltd, Manchester, UK) and one red polycarbonate hut (Fat Rat Hut, size 150 mm × 165 mm × 85 mm, Datesand). Due to signs of behavioural changes during the second week of housing in IVC, rats had periodic stays in a large 'playcage' with the purpose to increase their well-being and to provide cognitive as well as physical stimulation, as described in detail elsewhere [19].

Design

Rats were weighed three times per week, The groups had similar body weight at baseline (mean 542 (SD 36) grams, P ANOVA 0.80) and the change in body weight during the intervention period was similar between the groups (mean -66 (SD 22) grams, P ANOVA 0.74), as previously presented [8]. At the end of the experimental period, that is, after 36-37 days with powder feed, the feed was withdrawn at 6:30 AM. The rats were fasted for 6 hours, with free access to drinking water, and were euthanized while anaesthetized with isoflurane (Isoba vet, Intervet, Schering-Plough Animal Health, Boxmeer, The Netherlands) mixed with oxygen. Blood was drawn from the heart and collected in BD Vacutainer K2EDTA tubes (Becton, Dickinson and Company) for separation of blood cells from plasma. The blood cell fraction was frozen at −80°C. Liver, epididymal white adipose tissues (WATepi), skeletal muscle from the thigh, and brain were carefully dissected.

The personnel handling the rats and conducting the analyses were blinded to the rats' group allocation. The rats were handled and euthanized in random order.

Determination of fatty acid compositions in diets and tissues

Lipids were extracted from diets, liver and thigh muscle by the method of Bligh and Dyer [20] using a mixture of chloroform and methanol, and extracts were added to heneicosanoic acid (C21:0) as internal standard and were methylated as described previously [21]. Lipid classes in liver and fish oils were separated by TLC on silica gel plates (250 um Silica gel 60 from

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Merck KGaA, Darmstadt, Germany) developed in hexane–diethyl ether–acetic acid (40:10:1, by vol) [22]. The liver TAG, PL, CE and NEFA spots were identified using Rhodamine G (Fluka Chemie AG, Buchs, Switzerland) and co-migration with known standards, and were scraped off, added to heneicosanoic acid (C21:0) as internal standard and were methylated as described previously [21]. Samples of blood cells, WATepi and brain were added to heneicosanoic acid (C21:0) as internal standard and were methylated without prior extraction of lipids as previously described [21]. After methylation, lipids in the samples were extracted twice with isooctane. The methyl ester samples were quantified in randomized order using an Agilent 7890 gas chromatograph equipped with a flame ionisation detector (Agilent Technologies, Inc.) and a BPX-70 capillary column (SGE Analytical Science) as described in Sciotto & Mjøs [23] with minor adjustments of the temperature programme to provide baseline resolution between n-9 and n-11 isomers. To assure accurate quantitative amounts, chromatographic areas were adjusted with empirical response factors based on the GLC-793 reference mixture (Nu-Chek Prep, Elysian, MN, USA). The reference mixture was run as every 8th sample (or more often) in the chromatographic sequences and each sequence included at least four samples of the reference mixture. The fatty acids in the reference mixture were identified by GC-MS using the methodology described in Wasta & Mjøs [24].

Protein analyses in liver and skeletal muscle

Liver and thigh muscle samples were homogenised in PBS, and protein was quantified with the Bradford dye-binding method [25] using protein assay dye reagent (Bio-Rad Laboratories, Munich, Germany) with bovine serum albumin (Bio-Rad Protein Assay Standard II, Bio-Rad Laboratories, Hercules, CA, USA) as the standard. Fatty acid desaturase 2 (FADS2, also known as delta-6 desaturase) was measured using the Rat Delta-6 Desaturase / FADS2 ELISA Kit (Sandwich ELISA) cat no LS-F7004 (LifeSpan BioSciences, Inc.). Fatty acid desaturase 1 (FADS1, also known as delta-5 desaturase) was measured using the Rat FADS1 ELISA Kit (Sandwich ELISA) cat no LS-F56186 (LifeSpan BioSciences, Inc.). Stearoyl-CoA desaturase 1 (SCD1, also known as delta-9 desaturase) was measured using the Rat SCD1 / SCD ELISA Kit (Sandwich ELISA) cat no LS-F32611 (LifeSpan BioSciences, Inc.). Plates were read at 450 nm on a SpectraMax Plus384 Microplate Reader. All samples were analysed simultaneously in the same plate from each of the ELISA assays, with CVs < 5%, Concentrations of FADS2, FADS1 and SCD1 in liver and concentrations of FADS2 and FADS1 in muscle are presented relative to protein content.

https://doi.org/10.1017/S0007114525000236 Published online by Cambridge University Press

Outcome measurements

The primary outcome was to investigate in which organs CA and its metabolites can be found after consuming a diet containing a fish oil with CA. The secondary outcomes were to investigate any effects on the levels of other fatty acids including EPA and DHA, and to explore any differences in the levels of the desaturases FADS1 and FADS2 in liver and skeletal muscle.

Statistical analyses

This study was primarily designed to investigate the effects of fish oil consumption in ZDSD rats [8]. Since this was the first study designed with this outcome in ZDSD rats, no data on effect size were available for sample size calculation or minimally detectable effect sizes. Based on studies conducted in rats and mice using CA-rich fish oils with group sizes of six to twelve rodents/group [7], the study was designed with 8 rats per experimental group. We expected all rats to become diabetic when fed the high-fat diet, however 25% of the rats did not develop diabetes. Therefore, statistical analyses are conducted with *n* 6 rats in each of the experimental groups.

Statistical analyses were conducted using SPSS Statistics version 28 (SPSS, Inc., IBM Company, Armonk, NY, USA). All data were evaluated for normality using the Shapiro–Wilks test, revealing that the majority variables, with the exception of a few fatty acids that were found in very low amounts, were normally distributed; therefore, one-way ANOVA was used to compare the experimental groups. Datasets with non-parametric distribution were log-transformed before analyses. Since this study is regarded as an exploratory study without the possibility of a proper calculation of the necessary sample size, when appropriate, the ANOVA analyses were followed by Tukey HSD post hoc test as recommended by Lee et al. [26]. The cut off value for statistical significance was set at a probability of 0.05.

Results

Fatty acids in the diets

CA and gadoleic acid (GA, C20:1n-11) were found solely in the HERO diet, whereas a small amount of 7-octadecenoic acid (7OA, C18:1n-11) was found in both the HERO diet and the ANCO diet (**Table 2**). The n-3 PUFAs stearidonic acid (C18:4 n-3), EPA, n-3 docosapentaenoic acid (DPA, C22:5n-3) and DHA were found in the HERO diet and in the ANCO diet, with comparable amount of EPA+DHA. Long-chain n-11 MUFAs and long-

chain n-3 PUFAs were not found in the Control diet. The essential fatty acids linoleic acid (C18:2n-6) and alpha-linolenic acid (C18:3n-3), as well as oleic acid (C18:1n-9), all of which mainly originate from the soy oil added to the diets, were found in all three diets. The contents of saturated fatty acids were in general similar between the diets, i.e., deviation less than 0.1g/100 g diet, with the exception of myristic acid (C14:0) which was lowest in the Control diet and highest in the HERO diet. The long-chain MUFA gondoic acid (C20:1n-9) was found in the highest amount in the HERO diet compared to the other diets, and the longer chain n-9 MUFAs erucic acid (C22:1n-9) and nervonic acid (C24:1n-9) were identified only in the HERO diet. The fish oils consisted mainly of TAG, with only 0.25 and 0.04 wt% of fatty acids esterified as PL in the herring oil and the anchovy oil, respectively.

CA and its metabolites

Liver lipids were separated into PL, TAG, CE and NEFA by using TLC. The n-11 MUFAs CA, GA and 7OA were identified in all lipid classes from rats fed the HERO diet, with the highest relative amounts of all three n-11 MUFAs found in liver-TAG (**Fig 1a, Supplemental tables 1-4**). We did not detect n-11 long-chain MUFA with chain length >22 carbons. GA and 7OA were not detected in liver lipids from rats in the Control group or in the ANCO group.

CA, GA and 7OA were recovered in blood cells, epididymal white adipose tissue, and skeletal muscle, but not in the brain, from rats fed the HERO diet (**Fig 1b, Supplemental tables 5-8**). In control rats and rats in the ANCO group, CA, GA and 7OA were not detected in blood cells, WATepi, skeletal muscle or in the brain. The relative amount of CA in extrahepatic tissues ranged from zero in the brain, to mean 0.543 (SD 0.240) g/100 FA in muscle.

Long-chain n-3 PUFAs

The relative amount (g/100 g FA) of EPA was higher in PL, TAG, CE and NEFA from liver of rats fed the HERO diet when compared to the Control group (**Fig. 2, Supplemental tables 1-4**). The relative amount of DHA was higher in liver-PL, liver-CE and liver-NEFA in the HERO group, whereas the DHA concentration in liver-TAG was similar to that of the Control group. The DPA amount was higher in liver-PL and liver-NEFA in rats fed the HERO diet and similar in liver-TAG to the Control group but DPA was not detected in liver-CE. The contents of EPA, DHA, and DPA were similar within the respective liver lipids between the HERO group and the ANCO group, with the exception of a higher DHA content in liver-PL

https://doi.org/10.1017/S0007114525000236 Published online by Cambridge University Press

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in the HERO group compared to the ANCO group. The absolute amounts of EPA, DPA and DHA in the liver were calculated by summarising the amounts of these fatty acids esterified as PL, TAG or CE, or as NEFA (**Supplemental Figure**). The total amounts of EPA and DPA in liver were higher in the HERO and ANCO groups compared to the Control group, with no difference between the HERO and ANCO groups. The absolute DHA amount in the liver was higher in the HERO group compared to both Control and ANCO groups, with no difference between the two latter groups.

The content of EPA was significantly higher in blood cells, WATepi, muscle and brain from rats fed the HERO diet or the ANCO diet, with no difference between the two groups, when compared to the Control group (**Fig. 3, Supplemental tables 5-8**). The DPA content was higher in blood cells and brain in both HERO and ANCO groups when compared to controls, whereas the level of DPA in muscle was similar for all three groups and significantly higher in WAT harvested from the ANCO group. The DHA content was higher in blood cells from the HERO group and in WAT from both HERO and ANCO groups, but was not affected by the fish oil diets in muscle and brain. The levels of EPA, DPA and DHA were similar between the HERO and ANCO groups for WAT, muscle and brain. In blood cells, the HERO and ANCO groups were similar with regard to the amounts of EPA and DPA, whereas the DHA amount was higher in the HERO group when compared to both the ANCO group and the Control group.

Other MUFAs of interest

Oils from fish that feed on copepods are rich in gondoic acid (C20:1n-9) in addition to CA [(1)], therefore both fatty acids may serve as markers for dietary intake of fish such as herring. C20:1n-9 was recovered in higher amounts in liver-CE, liver-PL, blood cells, muscle and WAT in the HERO group, whereas the C20:1n-9 concentration was similar between the ANCO group and the Control group for all analysed tissues (**Supplemental tables 1-8**). Saturated fatty acids can be delta-9 desaturated in many tissues, producing in particular C16:1n-7 and C18:1n-9 from C16:0 and C18:0, respectively. The relative concentrations of C16:1n-7 and C18:1n-9 were similar between all three groups in liver-TAG, liver-NEFA, muscle and WAT, whereas the amount of C16:1n-7 was higher in liver-PL, blood cells and brain, in the HERO group compared to controls.

https://doi.org/10.1017/S0007114525000236 Published online by Cambridge University Press

Desaturases in liver and skeletal muscle

The hepatic content of FADS2 was significantly higher in the ANCO group compared to the Control group and the HERO group, with no differences between the groups for the hepatic concentrations of FADS1 and SCD1 (**Table 3**). The muscle contents of FADS2 and FADS1 were similar between all three groups.

Discussion

In the present study we show for the first time that when ZDSD rats are fed a diet containing herring oil, this results in accumulation of n-11 MUFAs in liver lipids, blood cells, WATepi and skeletal muscle, but not in brain tissue. We also show that the level of EPA was higher in liver lipids (PL, TAG, CE and NEFA) as well as in blood cells, WATepi, muscle and brain from rats fed the HERO diet when compared to the Control group, with no differences between the HERO group and the ANCO group. The effects of herring oil intake was less systematic with regard to the DHA level in various liver lipids and extrahepatic tissues, but was higher in liver CE and NEFA and similar to that of the Control group in liver PL, TAG, and higher in blood cells and in WATepi but was not affected in muscle and brain. The levels of EPA and DHA were similar between the HERO group and the ANCO group with regard to liver lipids with the exception of DHA in liver-PL and all extrahepatic tissues with the exception of blood cells where the DHA level was higher in the HERO rats. The higher levels of EPA and DHA in rats fed the HERO diet compared to controls are probably not caused by an increased endogenous synthesis, since the protein concentrations of FADS2 and FADS1 in liver and muscle was not different from those of the Control group, and since EPA and DHA levels were comparable in the HERO diet and the ANCO diet.

Long-chain n-11 MUFAs may be excellent biomarkers for compliance in clinical studies and animals studies when studying effects of consumption of filets or oils from fish species such as herring. Herring contain relatively high amount of CA since they feed on zooplankton such as copepods which produce 22:1n-11 *de novo* [1], whereas vertebrates have limited capacity to biosynthesize CA. However, recent research indicate that long-chain n-11 MUFAs such as CA also may have beneficial health effects, including lowering of cholesterol [7, 8] and improved glucose metabolism [27] in rodents. Accumulation of exogenous fatty acids such as the long-chain n-11 MUFAs may induce functional changes in membrane composition thus affecting membrane fluidity and enzyme activities, either as direct effects or by affecting the amounts of other fatty acids through changes in catabolic and anabolic processes including desaturation of fatty acids, or affects metabolic pathways through other

https://doi.org/10.1017/S0007114525000236 Published online by Cambridge University Press

mechanisms of action. A comprehensive survey of the occurrence CA, GA and 7OA (and possibly other n-11 MUFAs) in various tissues and lipid classes have not been conducted previously, however, others have found CA in plasma [28-30], WAT [6, 28-31], liver [28-32], muscle [30], plasma-PL[5] and liver-PL [5] in rodents fed diets containing long-chain MUFA concentrates [5, 6, 28, 31, 32], pollock oil [29] or saury oil [30], and GA was found in plasma [31], liver [31, 32] WAT [6, 31] after long-chain MUFA consumption [6, 31, 32]. Accumulation of 7OA was shown only in one article; in WAT from rats fed a diet containing long-chain MUFA [6]. In line with this, the present comprehensive study demonstrates that CA and the shorter n-11 MUFAs GA and 7OA accumulate in liver lipids, blood cells, WATepi and muscle after intake of feed containing herring oil, but we found no n-11 MUFAs in the brain tissue. GA and 7OA were found in amounts that were comparable to, or higher, than CA in the tissues, and since the HERO diet contained very low amounts of GA and 7OA when compared to CA, it is likely that GA and 7OA are products of beta-oxidation of CA in the rats. We did not identify any n-11 MUFAs with <18 carbons or >22 carbons in any of the tissues analysed.

The knowledge about the health effects of CA and its n-11 MUFA metabolites are scarce. Based on findings in cell culture studies, it has been speculated that a beneficial effect of CA may be mediated through stimulation of the endogenous synthesis of EPA and DHA by upregulating the FADS1 and FADS2 mRNA levels in the liver [3]. Since most studies that explore the effects of CA has used fish oils, it is challenging to interpret if these oils induce higher synthesis of EPA and DHA since fish oils also contain long-chain n-3 PUFA, or if the higher EPA and DHA contents are originating from the diet. A study in LDLr -/- mice using diets containing an EPA- and DHA-free CA-concentrate did not find higher hepatic levels of EPA and DHA [32], whereas several studies using CA-rich fish oil reported of higher hepatic concentrations of EPA and/or DHA in rodents [7]. In the present study, the EPA+DHA content was comparable between the HERO diet and the ANCO diet, and we found no difference between the HERO group and the ANCO group in the relative amounts of EPA and DHA in liver, WAT, muscle or brain. However, we found a higher relative amount of DHA in liver-PL and blood cells as well as a higher absolute DHA amount in liver in the HERO group when compared to the ANCO group, which may be a reflection of the marginally higher DHA content in the HERO diet compared to the ANCO diet. Since the hepatic FADS level was lower in HERO rats compared to ANCO rats, combined with similar hepatic FADS1 concentration and of FADS2 and FADS1 in muscle, it is not likely that the endogenous production of EPA and DHA from alpha-linolenic acid was higher in rats in the HERO group.

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This is also supported by findings in another rat study, where the hepatic gene expressions of both FADS2 and FADS2 were lower in rats fed a diet containing sandeel oil compared to rats fed a control diet with soybean oil [33]. The higher FADS2 concentration (catalysing the delta-6 desaturation of 18:2n-6 to 18:3n-6, and of 18:3n-3 to 18:4n-3) in livers from rats fed the ANCO diet compared to those fed the HERO diet was not reflected in higher amounts of any of the desaturated and elongated products of 18:2n-6 and 18:3n-3 in any of the tissues investigated.

We recently presented data indicating that that neither HERO nor ANCO affected the *de novo* lipogenesis from glucose, the TAG synthesis or the rate of VLDL assembly in the liver from ZDSD rats [8]. Here, we show that in the same rats, SCD1 was affected by intake of HERO or ANCO diets, supporting the previous finding that the hepatic fatty acid synthesis was not affected by these diets. In line with this, the relative concentrations of the delta-9 desaturated MUFAs C16:1n-7 and C18:1n-9 were similar to controls in liver-TAG, liver-NEFA, muscle and WAT. The higher level of C16:1n-7 in BC, brain, liver-PL and liver-TC may be a reflection of the higher dietary content of this fatty acid rather than a result of increased endogenous synthesis, since the HERO diet also contained a considerable amount of C20:1n-9, originating from copepods consumed by the herring [1], which can be beta-oxidized to C18:1n-9. Rats fed the HERO diet had higher concentrations of C20:1n-9 in BC, liver-CE, liver-PL, muscle and WAT whereas the C20:1n-9 concentration was similar to controls in rats fed the ANCO diet, thus supporting the assumption that the higher levels of C16:1n-7, C18:1n-9 and C20:1n-9 results from dietary intake and not endogenous production.

The present study presents evidence that CA from the HERO diet is absorbed from the diet, and is accumulated and metabolised in tissues with diverse structure and function; in blood cells, liver, WATepi and skeletal muscle, but we did not find any form of n-11 MUFAs in the brain. Although the present findings in this rat study are not directly transferable to humans, the mechanisms for uptake and storage as well as the metabolism of CA might be similar to those in other animals such as humans and should be further investigated in clinical studies.

This study has some strength and limitations. Strengths include quantification of fatty acids in a range of tissues with different characteristics and functions. The presence of CA and its shorter metabolites GA and 7OA in liver, blood cells, WAT and muscle indicate that these n-11 MUFAs are integrated in cells and thus may affect biochemical and physiological processes. Limitations to the study include the choice of animal model used, as the observed

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effects of fish oil intake on CA uptake and desaturases in rats with overt T2D may be specific to the ZDSD rats, since diabetes and obesity influence the gene expressions and activities of the desaturases in liver of rats [34, 35]. In the present study we measured the amounts and not the activities of the desaturases, and since the elongases involved in the LC-PUFA synthesis, that is, ELOVL2 and ELOVL5, were not quantified, we cannot with absolute certainty conclude that CA did not affect the endogenous synthesis of EPA and DHA. The biological availability of CA from the HERO may be specific to this particular fish oil and cannot be generalised to other fish oils containing CA, hence, other CA-rich fish oil should be investigated in both humans and animal models.

Conclusion

In the present study we present evidence that CA from herring oil is absorbed, accumulated and metabolised in tissues with diverse structure and function; in blood cells, liver, WATepi and skeletal muscle, but not in the brain. In contrast, no n-11 MUFAs were found in the investigated tissues harvested from the Control group or in the ANCO group. Thus, n-11 MUFAs may be used as biomarkers for compliance in intervention studies with consumption of CA-rich fish or fish oil from species such as herring. More importantly, the accumulation of n-11 MUFAs may induce functional changes in membrane composition thus affecting membrane fluidity and enzyme activities, either as direct effects or by affecting the amounts of other fatty acids or through changes in catabolic and anabolic processes including desaturation of fatty acids. The dietary content of EPA+DHA was similar between the HERO diet and the ANCO diet, and we show that the concentrations of EPA and DHA were similar in liver (with the exception of DHA in liver PL), WATepi, muscle and brain from rats fed the HERO diet or the ANCO diet. This, combined with the similar FADS2 and FADS1 concentrations in liver and muscle between rats fed the HERO diet and the Control diet, indicates that CA does not stimulate the endogenous synthesis of EPA and DHA in this diabetic rat model.

Acknowledgements

This research was funded by The Norwegian Seafood Research Fund (FHF, grant number 901769). The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

The study was designed by E. V. R., S. A. M., E. S. and O. A. G. The rat study was conducted by O. A. G. Data analyses and data interpretation were performed by E. V. R, S. A. M. and O. A. G. O.A.G. drafted the paper and had primary responsibility for the final content, and all authors have contributed to the writing and approved the final version of the manuscript.

The authors declare no conflict of interest.

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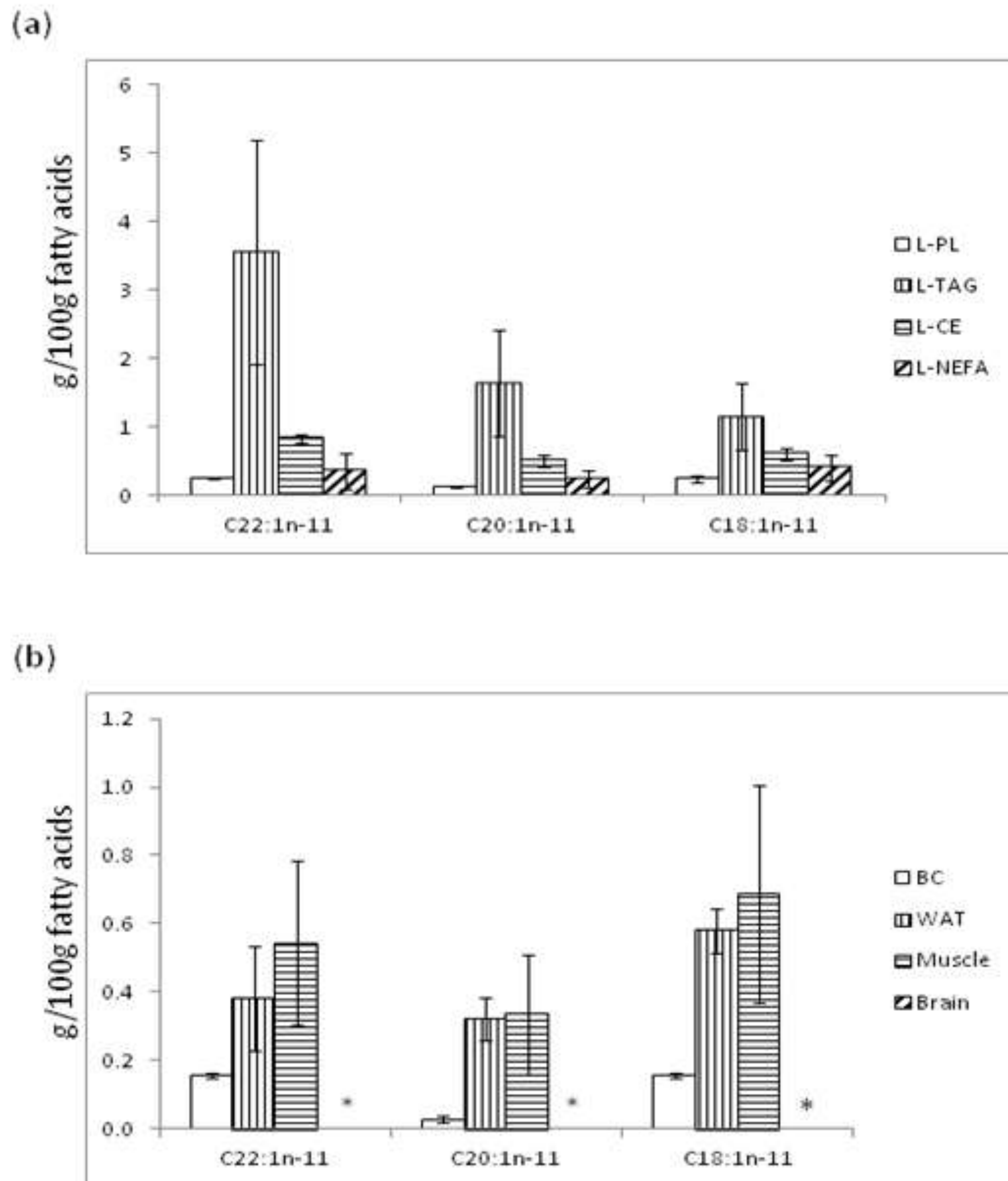

Fig 1: Relative weights of n-11 MUFAs in liver lipids (g/100g FAs) for phospholipids (PL), TAG, cholesteryl esters (CE) and as NEFA (a) and in total lipids from blood cells (BC), epididymal white adipose tissue (WAT), skeletal muscle (muscle) and brain (b) from rats fed the HERO diet, n-11 MUFAs were not recovered in rats fed the ANCO diet or the Control diet. Data are presented as mean and standard deviation for n 6 rats in each experimental group. HERO, herring oil; ANCO, anchovy oil. * CA, GA and 7OH was not detected in brain.

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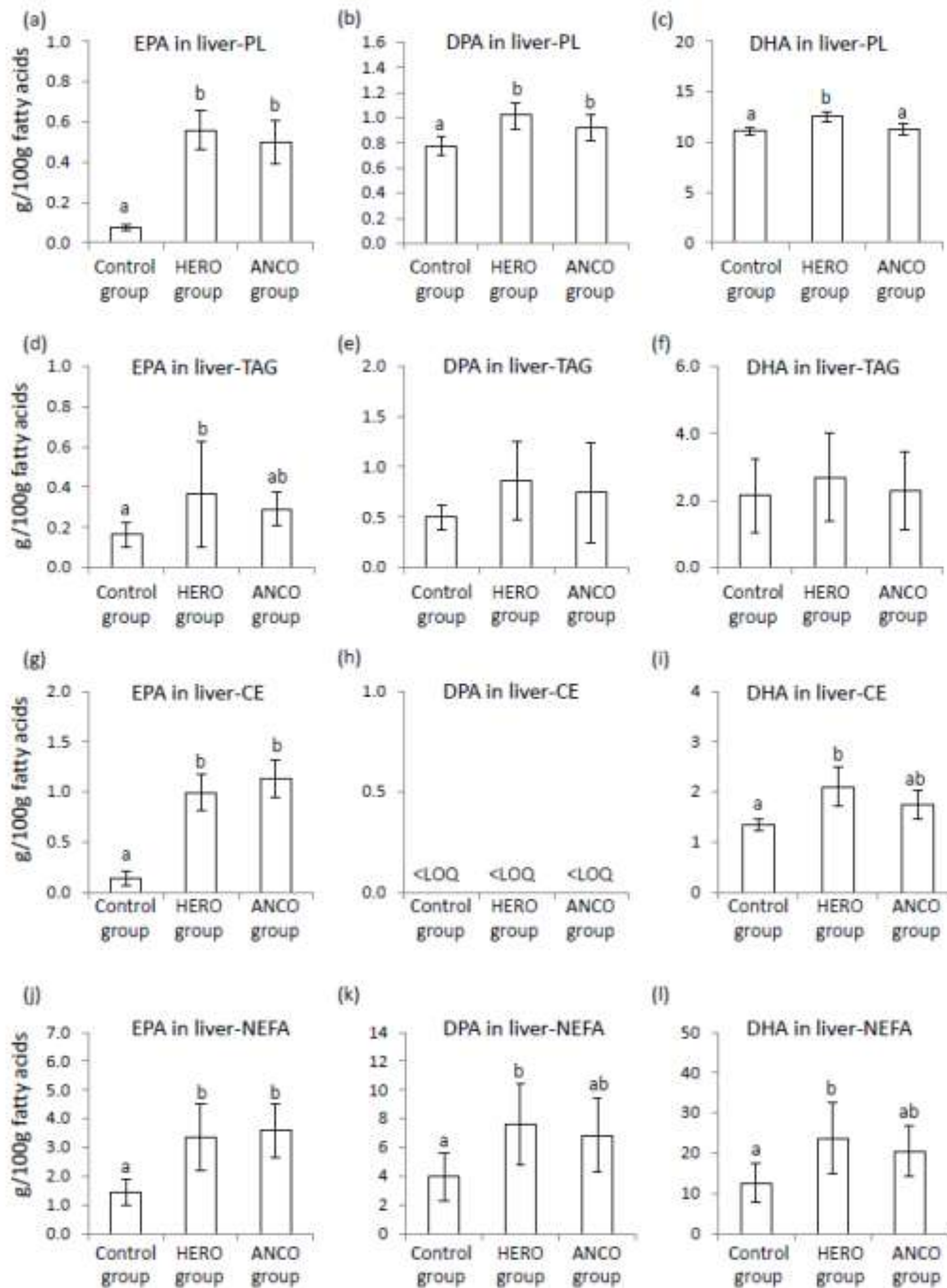

Fig 2: Relative weights (g/100g FAs) of EPA, DPA and DHA in liver lipids for phospholipids (L-PL) (a-c), L-TAG (d-f), cholesteryl esters (L-CE) (g-i) and as L-NEFA (j-l). Data are presented as mean and standard deviation for n 6 rats in each experimental group.

Groups are compared using one-way ANOVA followed by Tukey HSD post hoc test when appropriate. Bars with different letters are significantly different ($P < 0{\cdot}05$). HERO, herring oil; ANCO, anchovy oil. LOQ, level of quantification

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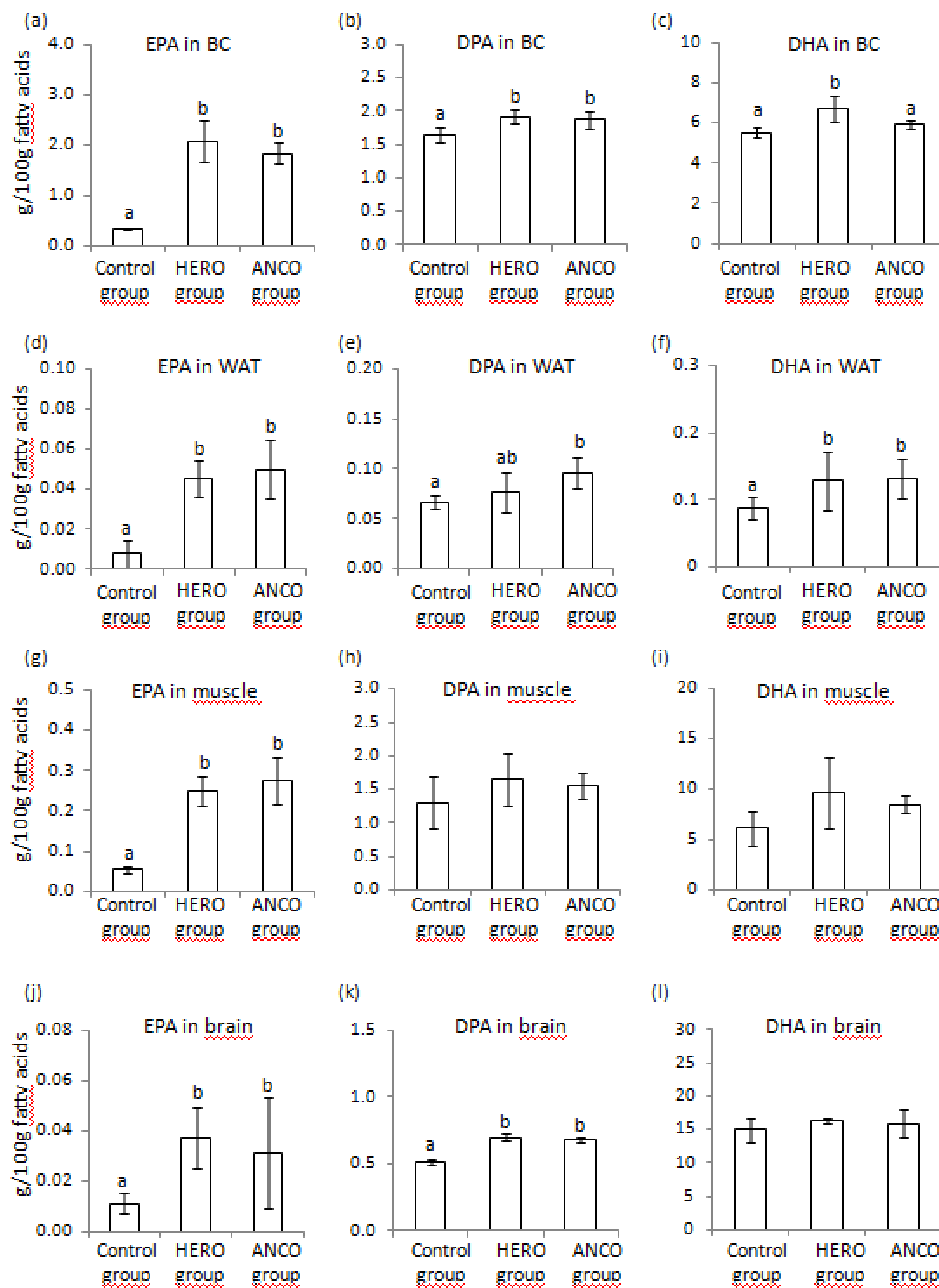

Fig 3: Relative weights (g/100g FAs) of EPA, DPA and DHA in total lipids from blood cells (BC) (a-c), epididymal white adipose tissue (WAT) (d-f), skeletal muscle (muscle) (g-i) and brain (j-l). Data are presented as mean and standard deviation for n 6 rats in each experimental group.

Groups are compared using one-way ANOVA followed by Tukey HSD post hoc test when appropriate. Bars with different letters are significantly different (P < 0·05). HERO, herring oil; ANCO, anchovy oil.

Table 1: Compositions of the experimental diets

Contents (g/100g diet)	Control diet	HERO diet	ANCO diet
Soybean Oil	10.00	6.30	8.40
Herring Oil	-	3.70	-
Anchovy Oil	-	-	1.60
Casein*	21.56	21.56	21.56
Sucrose	9.00	9.00	9.00
Cornstarch	48.23	48.23	48.23
Cellulose	5.00	5.00	5.00
t-Butylhydroquinone	0.0014	0.0014	0.0014
Mineral Mix (AIN-93-MX)†	3.50	3.50	3.50
Vitamin Mix (AIN-93-VX) ‡	1.00	1.00	1.00
Growth and maintenance supplement§	1.00	1.00	1.00
L-Methionine	0.16	0.16	0.16
L-Cystine	0.30	0.30	0.30
Choline Bitartrate**	0.25	0.25	0.25

*contains 91.9 % crude protein (high-fat diet) or 92.78 % crude protein (other diets)

†contains sucrose (221 g/kg)

‡contains sucrose (967 g/kg)

§contains vitamin B12 (40 mg/kg) and vitamin K1 (25 mg/kg) mixed with sucrose (995 g/kg) and dextrose (5 g/kg)

**contains 41 % choline

HERO, herring oil; ANCO, anchovy oil

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Table 2: Contents of fatty acids in the diets

g/100g diet	Control diet	HERO diet	ANCO diet
C14:0	0.02	0.26	0.12
C15:0	<LOQ	0.02	0.01
C16:0	0.92	0.95	0.98
C17:0	0.01	0.01	0.01
C18:0	0.31	0.24	0.30
C20:0	0.02	0.02	0.02
C22:0	0.02	0.02	0.02
C16:1 n-7	0.01	0.13	0.11
C18:1 n-5	<LOQ	0.01	0.01
C18:1 n-7	0.12	0.11	0.13
C18:1 n-9	1.71	1.31	1.51
C20:1 n-9	0.01	0.35	0.02
C22:1 n-9	<LOQ	0.03	<LOQ
C24:1 n-9	<LOQ	0.03	<LOQ
C18:1 n-11	<LOQ	0.04	0.03
C20:1 n-11	<LOQ	0.05	<LOQ
C22:1 n-11	<LOQ	0.70	<LOQ
C16:2 n-4	<LOQ	0.02	0.02
C16:3 n-4	<LOQ	0.01	0.02
C18:2 n-6	4.52	2.92	3.73
C18:3 n-3	0.57	0.39	0.48
C18:4 n-3	<LOQ	0.07	0.04
C20:5 n-3	<LOQ	0.17	0.23
C22:5 n-3	<LOQ	0.02	0.02
C22:6 n-3	<LOQ	0.18	0.14

The following fatty acids were below LOQ in all diets: C12:0, C23:0, C24:0, C14:1 n-5, C16:1 n-9, C17:1n-7, C17:1n-8, C18:1n-9t, C20:1n-7, C22:1n-7, C18:2n-6tc, C18:3n-6, C18:2 n-4, C20:2n-6, C20:3n-6, C20:4n-6, C20:3n-3, C20:4 n-3, C21:5 n-3, C22:4n-6, and C22:5n-6

HERO, herring oil; ANCO, anchovy oil

LOQ: level of quantification

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Table 3: Contents of desaturases in liver and desaturases in skeletal muscle (presented relative to protein content)

ng/mg protein	Control group	HERO group	ANCO group	*P* ANOVA
Liver FADS2	28.4 ± 8.3[a]	19.6 ± 9.5[a]	45.7 ± 11.1[b]	0.0010
Liver FADS1	201 ± 24	221 ± 22	234 ± 28	0.097
Liver SCD1	597 ± 109	583 ± 52	685 ± 126	0.20
Muscle FADS2	3.97 ± 2.89	4.02 ± 1.09	4.61 ± 8.21	0.91
Muscle FADS1	1.59 ± 3.13	1.42 ± 0.82	1.54 ± 1.32	0.97

ANCO, anchovy oil; FADS2, fatty acid desaturase 2, FADS1, fatty acid desaturase 1; HERO, herring oil; SCD1, stearoyl-CoA desaturase 1

Data are presented as mean ± standard deviation for *n* 6 rats in each experimental group. Groups are compared using one-way ANOVA followed by Tukey HSD post hoc test when appropriate. Means in a row with different letters are significantly different ($P < 0.05$).