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Metabolic effects of diet containing blue mussel (*Mytilus edulis*) and blue mussel-fed salmon in a mouse model of obesity

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ABSTRACT

Alternative feed ingredients for farmed salmon are warranted due to increasing pressure on wild fish stocks. As locally farmed blue mussels may represent an environmentally sustainable substitute with a lower carbon footprint, we aimed to test the potential and safety of substituting fish meal with blue mussel meal in feed for Atlantic salmon. Salmon were fed diets in which fish meal was partially replaced with blue mussel meal in increments, accounting for up to 13.1 % of the ingredients. Fillets from the salmon were subsequently used to prepare obesity-promoting western diets for a 13-weeks mouse feeding trial. In a second mouse trial, we tested the effects of inclusion of up to 8% blue mussel meal directly in a meat-based western diet. Partial replacement of fish meal with blue mussel meal in fish feed preserved the n-3 polyunsaturated fatty acid (PUFA) content in salmon fillets. The observed blue mussel-induced changes in the fatty acid profiles in salmon fillets did not translate into similar changes in the livers of mice that consumed the salmon, and no clear dose-dependent responses were found. The relative levels of the marine n-3 fatty acids, EPA, and DHA were not reduced, and the n-3/n-6 PUFA ratios in livers from all salmon-fed mice were unchanged. The inclusion of blue mussel meal in a meat-based western diet led to a small, but dose-dependent increase in the n-3/n-6 PUFA ratios in mice livers. Diet-induced obesity, glucose intolerance, and hepatic steatosis were unaffected in both mice trials and no blue mussel-induced adverse effects were observed. In conclusion, our results suggest that replacing fish meal with blue mussel meal in salmon feed will not cause adverse effects in those who consume the salmon fillets.

1. Introduction

Fish and seafood, in particular fatty fish such as Atlantic salmon (Salmo salar L.), are rich sources of n − 3 polyunsaturated fatty acids (PUFAs) and consumption of at least two portions of fish per week is commonly recommended, one of which should be fatty fish. Farmed Atlantic salmon is popular among consumers and currently contributes with more than 2700 thousand tonnes of fish available for human consumption (Fao, 2022). Traditionally, farmed salmon were fed diets with high levels of marine ingredients, and a single meal of 200 g Atlantic salmon would provide 4 g of n − 3 PUFAs, sufficient to cover more than the recommended weekly intake of n − 3 PUFAs (Reksten et al., 2022; Torstensen et al., 2005). Fish meal and fish oil traditionally used in aquaculture were generally based on pelagic fisheries (Péron et al., 2010; Tacon and Metian, 2009). Traditionally, 3–5 kg of fresh fish was used to produce 1 kg of farmed fish, but in a rapidly growing global aquaculture industry, this was not sustainable (Torstensen et al. 2008). Following the rapidly growing global aquaculture production (Fao, 2020), pressure on wild fish stocks led to limited access and increasing prices of marine ingredients, and consequently, current aquafeed contains more vegetable/terrestrial ingredients (Sissener et al., 2013; Tacon & Metian, 2008; Ytrestøyl et al., 2015; Aas et al., 2019). Feeding trials

have demonstrated that lower proportions of marine ingredients lead to reduced levels of marine n-3 PUFAs in the salmon fillets (Bell et al., 2002a; Bell et al., 2004; Liland et al., 2013; Menoyo et al., 2005; Sprague et al., 2010; Torstensen et al., 2005), and the n-3/n-6 PUFA ratio decreased in Scottish Atlantic salmon farmed between 2006 and 2015 (Sprague et al., 2010) and in Norwegian salmon farmed between 2005 and 2020 (Moxness Reksten et al., 2021). The observed steep decline in the contents of EPA and DHA between 2005 and 2011 corresponds in time with the concomitant reduction in the use of marine ingredients in the fish feed (Ytrestøyl et al., 2015; Aas et al., 2022).

The beneficial health effects of fatty fish such as salmon have largely been attributed to their high content of marine n-3 PUFAs. However, the use of plant ingredients in fish feed influences the fatty acid composition and contaminant content of farmed Atlantic salmon and a carryover to consumers may occur (Midtbø et al., 2013). Experimental replacement of fish oils with vegetable oils, soybean oil in particular, in fish feed leads to a reduced n-3/n-6 PUFA ratio in the salmon fillets. In addition, red blood cells from mice that consumed the salmon also showed a reduced n-3/n-6 PUFA ratio (Midtbø et al., 2013). This was accompanied by development of obesity, insulin resistance, and hepatic steatosis (Midtbø et al., 2015).

Blue mussel (*Mytilus edulis*) meal has been experimentally used as a partial substitute for fish meal in fish species, such as Tiger puffer, *Takifugu rubripes* (Kikuchi & Furuta, 2009) and juvenile Japanese flounder (Kikuchi & Sakaguchi, 1997), and may also represent a more sustainable substitute for fish meal also in feed for farmed salmon as mussel production has a relatively low greenhouse gas emission (Yaghubi et al., 2021). Blue mussels are mainly filter feeders with diets comprising a large amount of phytoplankton, rich in n-3 PUFAs (McLean and Bulling, 2005). In addition, mussel production may exert a beneficial effect on the environment by removing excess organic compounds from the surroundings, reducing the need to overharvest wild fish stocks, and providing marine-derived nutrients that are relatively similar to the natural diet of salmon. The total amount of fat in blue mussels is low, <1.5%, and hence the phospholipid/triacylglycerol ratio is high (Ahmmed et al., 2020). It has been reported that more than 80% of the fat in blue mussels is in the form of phospholipids, of which 20% and 11% of fatty acids moieties are EPA and DHA, respectively (Vaidya & Cheema, 2014), and the consumption of blue mussels is reported to improve the n-3 PUFA status in humans (Carboni et al., 2019; Lindqvist et al., 2019). Furthermore, blue mussels contain essential amino acids, n-3 PUFAs, as well as important micronutrients, such as iodine, iron, and vitamin B_{12} (Venugopal & Gopakumar, 2017; Yaghubi et al., 2021), and may serve as a nutritious food ingredient for humans. Additionally, due to their low trophic position and filter feeding, their levels of environmental contaminants such as methylmercury and persistent organic pollutants are relatively low, although some geographical variation may exist (Braune et al., 2005). As blue mussels can be sustainably produced, we sought to determine if replacing part of the fish meal with blue mussel meal would maintain the n-3 PUFA content in fish fillets without imposing adverse health effects for the consumers.

Since the feed ingredients affect the nutrient and contaminant status of salmon fillets, a carryover effect of partial blue mussel substitution on consumer's health could be envisioned. In the present study, we therefore also aimed to investigate if the intake of mussel-fed salmon affected metabolism and health of C57BL/6J mice, when added to their diet. While the inclusion of 5% blue mussel meal has been reported to cause feed aversion in mice, intake of blue mussel meal at low doses (2.5%) has been reported to protect C57BL/6J mice against high-fat high-sucrose-induced obesity (Vaidya et al., 2017a). Accordingly, we also examined the direct effect of blue mussel meal on mice by including blue mussel meal in a meat-based obesogenic western diet using a dose–response set-up.

2. Materials and methods

2.1. Fish experiment

The salmon experiment was conducted at LetSea land site aquaculture center, Bjørn, Dønna, Norway. Atlantic salmon (*Salmo salar* L), with a 1:1 sex ratio, were provided by LetSea AS in cooperation with the Lerøy Seafood Group and were placed in 12 separate experimental tanks. The fish were delivered by Grytoga Settefisk AS (Vefsn, Norway), and the stock was the Duo-IPN-Lice genetic strain provided by Salmobreed. Fish were approximately one year old and vaccinated with ALPHA JECT micro® 6 (PHARMAQ), which protects against furunculosis, vibriosis, cold water vibriosis, winter sores, and infectious pancreatic necrosis. The fish were examined upon arrival at LetSea AS facilities. Any fish showing signs of illness, deformity, or abnormal growth (according to the breed standard) were excluded from the experiments.

The fish were kept at 12 °C in 22 ppt salinity, until the beginning of the trial. The water was tested for nitrogen saturation level, UV-treated for sterilization, and adjusted to 14 °C and 90 % oxygen saturation in outflow at the start of the experiment. Water temperature and oxygen data were recorded daily for each tank in an environmental data log. The fish were selected based on weight for the experimental trial according to LetSea AS selection procedures and then randomized to ensure homogenous size distribution. To avoid the possible influence of parental effects, all juvenile salmon were randomized before distribution in such a way that each replicate tank received the same composition of fish with similar genetic background. The selected fish were acclimated for 2 weeks before the start of the trial. The fish weighed around 200 g at the start of the experiment, and on average 400g at the end of the experiment. Feeding treatments were randomly assigned to each tank. Each treatment was randomly assigned to three tanks at the lowest and highest inclusion levels, and two tanks at the intermediate inclusion levels with 160 fish per tank. Twelve glass fiber tanks of 2 m^3 were used for the experimental trial.

To prevent bias, both the staff at LetSea and the HoloFood partners involved in the sampling were blinded to the treatment groups by giving the treatment groups random code names. The fish tanks were all connected to a state-of-the-art recirculating aquaculture system (RAS) based on a 22 ppt mixture of freshwater and seawater from an isolated source tank. The experimental trial included an initial 2-week acclimation period followed by a 60-day growth phase. During the acclimation period, all fish were fed with "Energy X 75" (50 mg), a commercial feed from BioMar, which did not contain blue mussel meal. Fish were fed continually using 24-hour mechanical band feeders, based on well-established feeding protocols developed by LetSea AS. The daily feeding amount was regularly reassessed based on the amount overfed monitored from the previous days for each tank, adjusted for 10% overfeeding. All tanks were equipped with a collection system of uneaten feed, which allows ad libitum feeding and ensures 5 to 10% overfeeding. The feed recovery rate was estimated to be close to 100%.

Health performance and growth were assessed in fish fed the five distinct diets: five experimental diets with incremental doses of blue mussel meal (Table 1). The experimental diets were based on the commercial recipe of "Energy X 200" from BioMar. The blue mussel meal was produced in New Zealand from de-shelled blue mussels and spray dried. The blue mussel meal was included at five levels in the experimental diets from 0% (i.e. a control) to 13.1% of the ingredients to reflect common levels of fish meal in commercial diets (Table 1). All diets were in the form of small compact pellets, and the feed was provided ad libitum in every tank. The experimental diets were produced as extruded pellets by BioMar AS (Denmark). The recipe and chemical compositions of salmon feed are listed in Table 1.

All fish were euthanized using an overdose of Finquel, and the muscle samples were flash frozen on dry ice and subsequently stored at −20 °C. Tissue samples from five randomly selected fish per tank were sampled at day 0 (right before introducing the experimental diets) and

Table 1
Composition of the experimental salmon feed.

Ingredient (g/100 g)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Blue Mussel Meal	0	2.2	4.4	8.7	13.1
Fish Meal (LT quality)	30	27.8	25.6	21.3	16.9
Soy SPC	12	12	9.52	10.92	9
Wheat Gluten	9.26	10	10.28	10	13
Pea Protein	8.91	8.64	10.97	11.61	10.1
Wheat	16.39	15	14.56	11.16	11.16
Fish Oil	10.34	10.34	10.34	10.3	10.34
Rapeseed Oil	10.44	10.51	10.2	10.54	10.14
Lecithin Soy	0.8	0.95	1.12	1.36	1.66
Yttrium	0.05	0.05	0.05	0.05	0.05
Vitamins, minerals and AA	2.77	3.24	3.62	4.26	5.43
Total	100	100	100	100	100
Measured					
Energy (kJ/g ww)	22.7	22.7	22.3	22.2	21.9
Total fat (g/100 g ww)	24	24	23	23	23
Protein (g/100 g ww)	43	44	42	43	43
Ash (g/100 g ww)	7	7.3	7.6	8.2	8.8

AA; amino acids.

the remaining were sampled at day 60. In this study we are only focusing on the fatty acid composition of the diet and the salmon fillets before and after the trial, whereas all other results from the salmon growth will be reported in a separate study.

2.2. Mouse trials

Two mouse trials (A and B) were performed at the Institute of Marine Research (IMR), Bergen, Norway.

The commercially available low-fat AIN-93G Purified Rodent Diet (ssniff Spezialdiäten GmbH, SOEST Germany) and a high-fat high-sucrose diet (ssniff Spezialdiäten GmbH, SOEST Germany) were used as obesogenic control diets. The experimental diets were based on the 5TJN standard Western diet for rodents (Test Diet, St Louis, USA). In diets used in experiment A, 50% of the dietary protein was a mixture of meat and 50% of salmon protein collected in the fish trial. Hence, the mice diets contained meat and salmon fed either 0%, 2.2%, 4.4%, 8.7% and 13.1% blue mussel meal (Table 2). In experiment B, 5.3, 10.7, 16, and 21.3 % of the meat protein mix were exchanged with blue mussel meal (Table 2 and Supplementary Fig. 1).

The food sources used for the meat mix were steam cooked to a core

temperature of 75 °C, minced, freeze-dried, and pulverized. The meat mix comprised of 24.8 % beef, 7.3 % lamb, 29.6 % chicken, 0.6 % game, and 37.7 % pork following a typical Norwegian dietary pattern according to the report from the Norwegian Directorate of Health (Helsedirektoratet, 2019) on the development and changes in the Norwegian diet. The endogenous fat and nitrogen were measured in all food sources, to ensure equal amounts of protein and fat in all experimental diets. Different amounts of the fat mix were added to each diet to obtain an equal amount of 198 g total fat per kg diet. The fat mix contained vegetable shortening (30.3 %), milk fat (30.3 %), lard (30.3 %), soybean oil (6.4 %), and corn oil (2.7 %). The diets were blended using a Crypto Peerless EF20 blender and analysed for gross energy, total fat, protein and fatty acid profiles before use (Table 2, Supplementary Table 2, and 3).

For both trials A and B, seventy male C57BL/6J mice were received at 7–8 weeks of age from Charles River Laboratories (Charles River, Germany). They were individually caged in a thermoneutral room with a 12-hour light/dark cycle. The temperature in the individually ventilated cages (IVC) was kept between 28 and 30 °C and the humidity between 50 and 53%. The mice were acclimated to the new environment for seven days. During the acclimatisation period, all mice were fed a low-fat rodent diet (AIN-93G, Growth Purified Diet, TestDiet®) (ssniff Spezialdiäten GmbH, SOEST Germany). In both trials, the mice were allocated into seven diet groups (n = 10). To ensure similar group means of total, lean and fat mass in all experimental groups, the mice were subjected to a non-invasive scanning using a time-domain nuclear magnetic resonance (MR) system (Bruker Minispec LF50 Body Composition Analyzer mq7.5) (Bruker Optik GmbH, Ettlingen, Germany) and thereafter allocated into five experimental groups and two reference groups. The reference groups were fed standard low-fat AIN-93G Purified Rodent Diet (ssniff Spezialdiäten GmbH, SOEST Germany) or a high-fat high-sucrose diet from ssniff (ssniff Spezialdiäten GmbH, SOEST Germany) used as an obesogenic reference.

Body masses were registered once a week to monitor body weight development. Fresh food was provided three times per week (ad libitum feeding).The leftovers were weighed to estimate feed intake for each mouse, and the feed efficiency was calculated by dividing total weight gain by total energy intake. During week 6 and 7 of the experiment, the mice were placed in clean cages for faeces collection. Faeces samples were collected and weighed, and the total fat content in the faeces samples was gravimetrically determined after extraction with organic solvents before and after acidic hydrolysis, as described by Tastesen

Table 2
Composition of the mouse feed used in mouse trial A and B.

	Mouse trial A					Mouse trial B				
	% BM in salmon feed					% BM				
	0	2.2	4.4	8.7	13.1	0	2	4	6	8
Components added (g/kg)										
Total protein	180	180	180	180	180	180	180	180	180	180
Protein from meat-mix	90	90	90	90	90	180	170.4	160.8	151.2	141.6
Protein from salmon or BM	90	90	90	90	90	0	9.6	19.2	28.8	38.4
Total fat	198	198	198	198	198	198	198	198	198	198
Fat from meat mix	8	8	8	8	8	16	15.2	14.3	13.5	12.6
Fat from salmon or BM	42	44	44	41	42	0	0.2	0.4	0.6	0.8
Added fat mixture	148	146	146	149	148	182	182.6	183.3	183.9	184.6
Sucrose	192	192	192	192	192	192.2	192.2	192.2	192.2	192.2
Starch	330	330	330	330	330	327	317	307	297	287
Base mix*	101	101	101	101	101	101.5	101.5	101.5	101.5	101.5
Analyzed										
Fat (g/100 g)	19.5	19.3	19.1	19.3	19.3	19.7	19.6	19.8	19.6	20
Crude protein (g/100 g)	19	18	18	19	18	18	18	16.8	17	18
Energy (kcal/100 g)	500	500	500	500	500	507	507	504	504	502

* Base mix provided; Cellulose (50 g/kg), L-cysteine (3 g/kg), t-Butylhydroquinone (0.01 g/kg), AIN93G mineral mix (35 g/kg), AIM93VX vitamin min (10 g/kg) and Choline bitartrate (2.5 g/kg).

et al. (2014). Apparent fat digestibility was calculated using the formula:

$$Apparent\ fat\ digestibility = \frac{Amount\ of\ fat\ eaten - Amount\ of\ fat\ excreted}{Amount\ of\ fat\ eaten}$$
$$\times 100\%$$

In week 11 a second MR scanning was performed, and an oral glucose tolerance test (3 mg glucose/g lean body mass) was performed after 4.5 h of fasting. Blood glucose was measured before gavage and at 15, 30, 60, and 120 min after gavage. At baseline (0 min), 30 and 120 min after oral gavage, 20 μL of blood was collected in EDTA coated tubes to obtain plasma for insulin measurements using the Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc., USA).

In week 13, the mice were anesthetised with 4 % isoflurane (Isobavet, Schering-Plough A/S, Farum, Denmark) in a Univentor 400 Anesthesia Unit Apparatus (Univentor Limited, Sweden). Euthanasia was attained through cardiac puncture and blood was collected from each mouse in EDTA anticoagulant tubes. Organs and tissues were dissected out from each mouse, weighted, snap-frozen in liquid nitrogen, and stored at −80 °C.

2.3. Mouse liver histology

Sections of liver samples were placed in histology cassettes and fixated in 4 % formaldehyde in 0.1 M phosphate buffer. The samples were dehydrated, embedded in paraffin, sectioned, and stained at Molecular Imaging Center at Haukeland University Hospital, Bergen as earlier described (Midtbø et al., 2015). Histology of the hematoxylin and eosin-stained liver samples was investigated with a NanoZoomer S60 Digital slide scanner (Hamamatsu, Japan). Representative parts of the slides were examined using QuPath (Bankhead et al., 2017), and photographed at 20x enlargement. Histological evaluation or grading of hepatic steatosis was based on the percentage of hepatocytes affected by visible apparent fat accumulation. Grades 0, 1, 2, and 3 corresponded to <5 %, 5–33 %, 33–66%, and >66 %, respectively, of visible affected fat vacuoles (Hijona et al., 2010).

2.4. Biochemical analyses

The gross energy level in the diets was measured by bomb calorimetry following the manufacturer's instruction (Parr Instruments, Moline, IL, USA). Total fat content was determined gravimetrically after extracting samples with organic solutions as described by Tastesen et al. (2014). Protein content was estimated by measuring the nitrogen content multiplying it by 6.25, following the Dumas method, using a Leco FP-528 nitrogen analyzer (Leco Corp, MI, USA). The fatty acid profile (Supplementary Table 1) was determined by extraction of fatty acids with 2:1 chloroform: methanol (v/v). An internal standard (19:0 methyl ester) was added, and the samples were filtered, saponified, and esterified in 12 % BF_3 in methanol (v/v). Fatty acid methyl esters were separated on a gas chromatograph (GLC Trace GC 2000, Thermo Scientific, USA) and detected with a flame ionization detector (Thermo Scientific). The fatty acids were identified by retention time using standard mixtures of methyl esters (Nu-Chek-Prep, Elyian, MN, USA) and quantified by the internal standard method as described by Torstensen et al. (2004 and 2005). Haematology parameters in mouse blood samples (Supplementary Fig. 4a and b) were measured using an Abaxis Vetscan HM5 Haematology Analyser.

2.5. Statistical analyses

Comparison of means between groups was conducted using one-way ANOVA followed by Fisher's Least Significant Difference multiple comparison (LSD) posthoc test. For Principal Component Analysis (PCA) and heatmap analyses of fatty acid composition, variables with more than 20% below LOQ were removed and the remaining missing values were calculated using K-Nearest Neighbors (KNN) imputation. Differential analysis (linear regression analysis, q < 0.05 and p < 0.05) and hierarchical clustering analysis (HCA) were performed and significantly influenced fatty acids were visualized in the heatmap. Qlucore omics explorer 3.5 (Qlucore AB, Lund, Sweden) was used for PCA and heatmap analyses and the rest of the statistical analyses were performed using GraphPad Prism v9.4 (GraphPad Software Inc., San Diego, CA, USA).

Ethical statement

The fish experiment was conducted at LetSea land site aquaculture center, in Bjørn, Dønna, Norway, in cooperation with the Lerøy Seafood Group. The experiment followed the EU principles for animal care and experimentation and experimental procedures were approved by the Ethical Committees of LetSea. The mice trials were approved by the Norwegian Food Safety Authority (FOTS ID 23991). Mice were inspected daily, and experimental protocols were performed according to ethical guidelines for the use of animals in research from national authorities, and from the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. No adverse effects were observed during the experimental trials.

3. Results

3.1. Fish trial

3.1.1. Fatty acid composition in salmon muscle tissue

Increased use of plant-based ingredients in fish feed has led to reduced contents of the marine n-3 fatty acids, EPA, and DHA in farmed salmon (Moxness Reksten et al., 2021; Sissener, 2018). To investigate if partial substitution of fish meal with blue mussel meal would maintain a favorable n-3/n-6 ratio, fatty acid compositions were measured in individual muscles from all fish sampled at the start and at the end of the experiment (N = 360) and presented as mg/g wet weight and as relative amounts at tank levels (Supplementary Tables 4 and 5).

Increasing the inclusion of blue mussel in salmon feed did not change the level of n-3 PUFAs, but increased the level of sum n-6 PUFAs in the feed which subsequently was reflected in the salmon fillets (Fig. 2). To further investigate diet-related changes in the fatty acid composition of salmon fillets, fatty acid composition (% of total fatty acids) was examined through principal component analyses (PCAs). The PCA analysis including both samples from the start and end of the trial demonstrated that samples collected at the start of the experiment formed a cluster separate from samples collected at the end of the experiment (Fig. 1A), indicating that the feed affected the fatty acid composition in the salmon muscle tissue. Omitting the start samples from the analysis (Fig. 1B), PCA revealed a dose-dependent separation of samples along the first component (41% variance), indicating a direct effect of increasing inclusion of blue mussel meal in the feed on the fatty acid composition of the salmon fillet.

The heatmap in Fig. 1C demonstrates that the treatment led to significant changes in the fatty acid composition of salmon fillets. The most noticeable changes being an increase in polyunsaturated fatty acids (sum PUFA), saturated fatty acids (sum SFA), and omega 6 fatty acids (sum n-6) and a decrease in mono-unsaturated fatty acids (sum MUFA) as the amount of blue mussel meal in the fish feed increased.

The increased relative amounts of saturated fatty acids were mainly due to an increased amount of palmitic acid (16:0), whereas the reduced relative amounts of monounsaturated fatty acids were mainly due to decreasing relative amount of oleic acid (18:1n-9) (Fig. 3). The reduced n-3/n-6 ratio and relatively increased proportion of n-6 PUFAs in fish feed were mainly due to an increased proportion of linoleic acid (18:2n-6). The levels of the marine n-3 PUFAs, EPA, and DHA were unchanged, but increasing the levels of blue mussel in the fish feed led to a decreased relative proportion of alpha-linolenic acid (18:3n-3) in the salmon fillets.
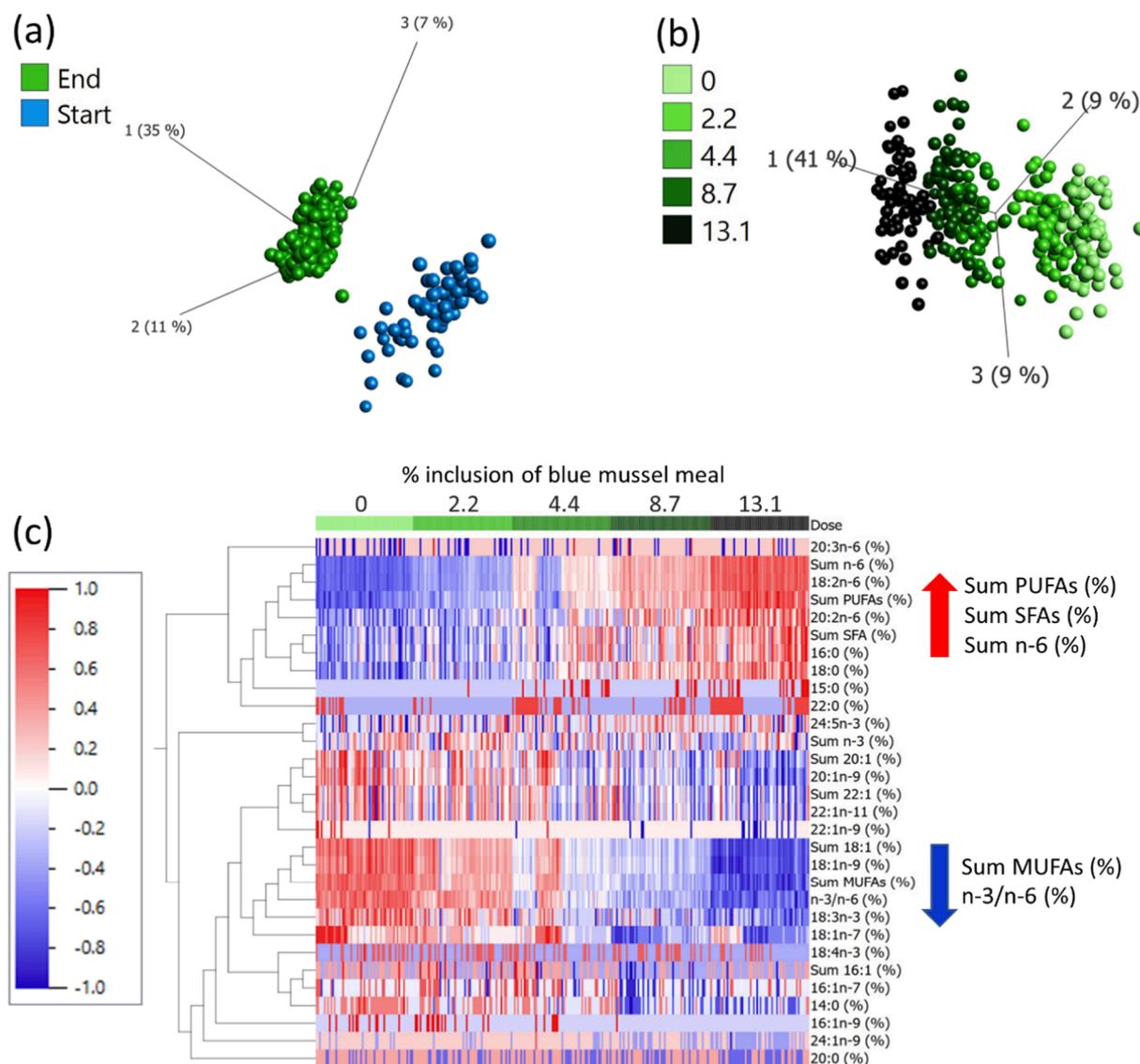
Fig. 1. (A) Principal component analyses (PCAs) of individual fatty acids composition (% of total fatty acids), investigating differences between Atlantic salmon muscle samples taken at the start (blue dots; n = 60) and end of the experiment (green dots; n = 300). (B) PCA investigating treatment-based effects in muscle samples taken at the end of the experiment with different inclusion of blue mussel meal in the diet (0%–13.1% blue mussel meal). (C) Differential analysis (linear regression analysis) and hierarchical clustering analysis (HCA) of fatty acids. The heatmap represents fatty acids composition significantly affected by the dietary treatment (% inclusion of blue mussel meal in the feed) after 60 days. Blue bars represent lower amounts of individual fatty acids, while red bars represent higher amounts of individual fatty acids in the muscle sample (n = 300). MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acids, SFA; saturated fatty acids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Mouse trials

3.2.1. Weight gain and organ masses

To investigate the potential spillover implications of using blue mussel meal in salmon feed for consumers, we prepared standard western diets (WDs) containing salmon filets, replacing 50% of the meat mix with the salmon fillets collected in the fish trial (Table 2). To further evaluate the safety and potential adverse health effects of blue mussel, we also prepared WD where the meat mix protein source was exchanged with blue mussel meal (Table 2). Obesity-prone male C57BL/6J mice were fed for 13 weeks, and the development of obesity and glucose intolerance were evaluated (Table 3, Fig. 3 and Supplementary Fig. 1).

All mice fed the WD developed obesity (Fig. 3). Obesity development was neither attenuated nor aggravated in mice receiving the blue mussel-fed salmon. Obesity development was not influenced by the addition of the blue mussel meal directly to the mice feed. Feed and energy intake (i.e. feed efficiency) were also not affected (Fig. 3).

Apparent fat digestibility was slightly reduced in mice fed high doses of blue mussel meal directly in the diet, but this was not sufficient to reduce obesity. Organ masses were not affected (Table 3) by blue mussel meal, and western diet-induced glucose intolerance (Supplementary Fig. 1) was neither aggravated nor attenuated.

To further evaluate the metabolic effect of blue mussel-fed salmon and blue mussel meal consumption *per se*, we performed liver histology. As expected, in mice fed western diets, blue mussel meal did neither attenuate nor aggravate western diet-induced accumulation of fat in the liver (Supplementary Fig. 3). Evaluation of the hematoxylin and eosin-stained liver sections and grading of hepatic steatosis confirmed that steatosis was not affected by either feeding mice blue mussel fed salmon (Supplementary Fig. 3a) or blue mussel meal directly (Supplementary Fig. 3b). Minor variations within hematological values were observed in both mice trials, but no dose-dependent effects were observed (Supplementary Fig. 4). Together our results suggest that blue mussel does not cause any significant adverse effect when included in salmon feed
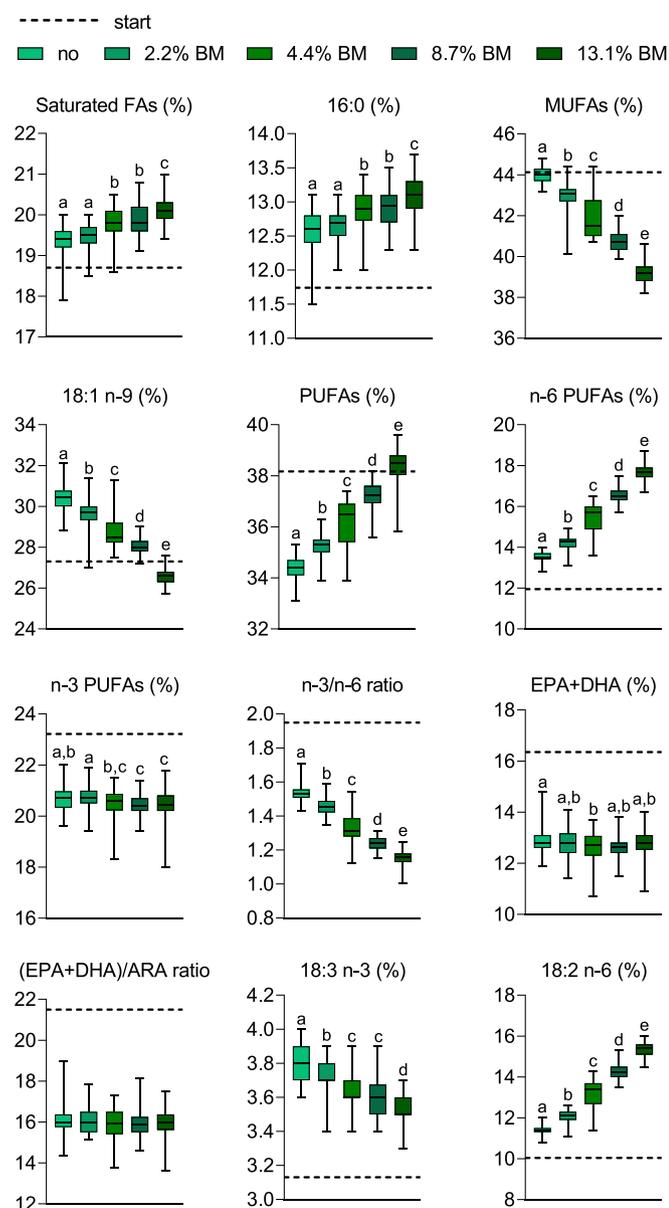
Fig. 2. Composition (% of total fatty acids) of selected fatty acids, investigating differences between muscle samples taken at the start (dotted line) and end in fish fed different inclusion levels of blue mussel meal (BM). Data from samples collected at the end of the experiment are presented as boxes and whiskers indicating min to max values. Different letters denote significant differences (p < 0.05) by one-way ANOVA using uncorrected Fisher's Least Significant Difference (LSD) multiple comparison. ARA; arachidonic acid, DHA; docosahexaenoic acid, EPA; eicosapentaenoic acid, FAs; fatty acids, MUFAs; monounsaturated fatty acids, PUFAs; polyunsaturated fatty acids. n = 360. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nor when consumed directly.

3.2.2. Fatty acid composition in mouse livers

Fatty acid compositions were measured in individual mouse livers from both mouse trials and are presented as mg/g wet weight (Supplementary Tables 6 and 7). To illustrate diet-related changes in the fatty acid composition of mouse livers, fatty acid compositions (as % of total fatty acids) were determined (Figs. 4 and 5). In line with analyses of the mice feed (Supplementary Table 2), the observed changes in the fatty acid profiles in salmon muscle were too small to directly translate into similar changes in livers from mice that consumed the salmon-

containing diet (Fig. 4). The relative levels of SFAs and MUFAs were not significantly changed. We did observe higher relative levels of both total n-6 PUFAs and 18:2n-6 in livers of mice fed salmon raised on the highest levels of blue mussel when compared with mice fed the salmon fed 4.4% blue mussel, but we did not observe a dose-dependent response. Importantly, the relative levels of the marine n-3 fatty acids, EPA, and DHA were not reduced and the n-3/n-6 ratios in livers from all salmon-fed mice were high but unchanged. Importantly, exchanging up to 21.3% of fish meal with blue mussel meal in fish feed did not reduce the contribution of salmon as a dietary source of n-3.

As blue mussel meal contains more than 3 mg EPA and DHA per gram (Supplementary Table 1), the levels of these fatty acids were expected to increase when blue mussel meal was included in the feed (Supplementary Table 3). However, the levels were below the LOQ when the percentage was <6%, and the hepatic levels of EPA + DHA in mice liver were only increased in mice which were fed 8% blue mussel (Fig. 5), and the levels were far below levels observed in salmon-fed mice. The relative proportions of n-6 PUFAs were within the range seen in the livers of salmon-fed mice. However, the n-3/n-6 PUFA ratios were, as expected, far lower in liver from mice fed a meat-based diet not supplemented with salmon (Fig. 5 and Supplementary Table 7). The inclusion of 4% blue mussels, corresponding to exchanging 10.7% of meat protein with proteins from blue mussels, increased this ratio, but to a magnitude far below the ratio observed in the livers of salmon-fed mice. In line with this, a dose-dependent increase in (EPA + DHA)/ARA ratio was observed with higher inclusion of blue mussel in the feed. We did not observe a consistent dose-dependent change in the relative amount of either sum n-6 PUFAs or 18:2n-6. Together our results suggest that exchanging meat with blue mussel meal may contribute to improving the n-3/n-6 status of the consumer, but far less efficiently than consumption of salmon.

4. Discussion

In this study, we show that feeding salmon with up to 13.1% blue mussel meal, representing a 43% exchange of fish meal with blue mussel meal, preserved the relative amounts of EPA and DHA in the fish fillets. However, we observed a dose-dependent decrease in the n-3/n-6 ratio, due to a higher relative proportion of n-6 PUFAs in the fish feed. In blue mussel meal, the relative proportion of n-3 PUFAs is higher than 35%, and the n-3/n-6 ratio is 6.1, but the total fat content is only about 11.5 mg/g. Hence, the amount of n-3 PUFAs from the blue mussel meal is relatively low. A close relationship between fatty acid composition in fish feed and fish fillets has been reported (Bell et al., 2002b; Liland et al., 2013; Torstensen et al., 2000). However, the addition of blue mussel meal at the expense of fish meal did not change the n-3 PUFAs in fish feed or in fish fillet as expected. Slight, but statistically significant increases in SFAs and n-6 PUFAs and reduction in MUFAs, however, were observed. The use of blue mussel meal, which has a lower fat content than the fish meal used in this study, led to an increase in the fraction of plant oil, which has a higher level of n-6 PUFAs than fish oil. Therefore, the reduced n-3/n-6 ratio in response to inclusion of blue mussel was due to increased levels of n-6 PUFAs originating from the plant oil added to the fish feed.

We have previously observed that changes in fatty acid composition in salmon fillets resulting from feeding salmon different vegetable oils, may translate into similar changes in liver fatty acid composition in mice consuming the salmon (Midtbø et al., 2015; Midtbø et al., 2013). However, the observed blue mussel-induced changes in the fatty acid profiles in salmon muscle were too small to directly translate into similar changes in livers of mice that consumed the salmon, and no clear dose-dependent responses were found in the mice. Importantly, the relative levels of the marine n-3 fatty acids, EPA, and DHA were not reduced and the n-3/n-6 PUFA ratios in livers from all salmon-fed mice were high but unchanged. We conclude that exchanging up to 21.3% of fish meal with blue mussel meal, corresponding to a total of 13.1% blue mussel meal in
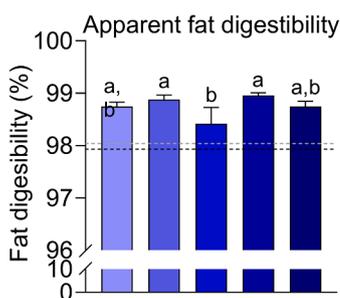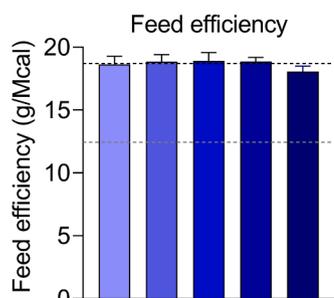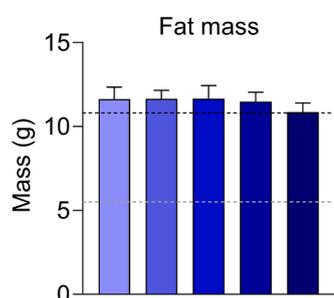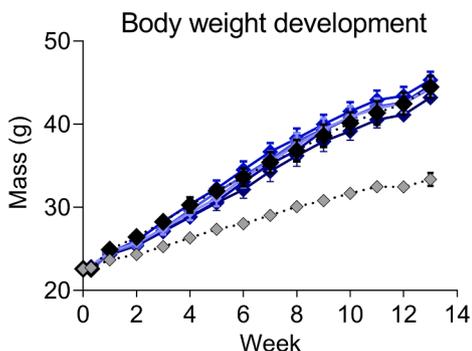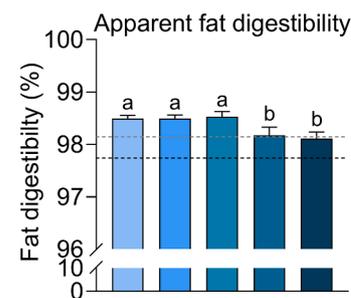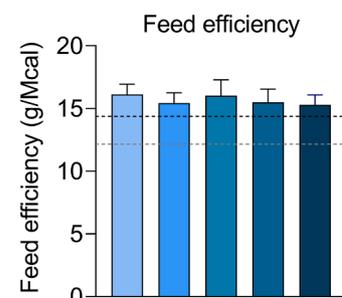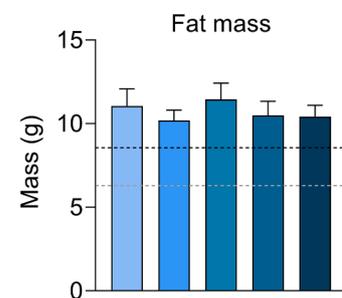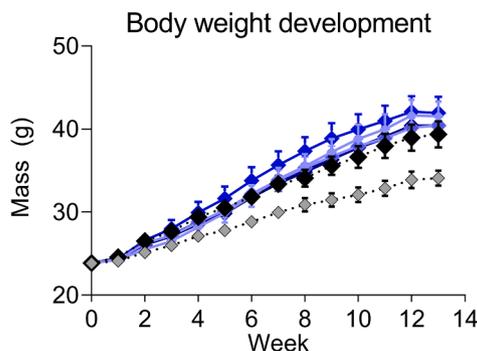
Fig. 3. Body weight development during 13 weeks of feeding, fat mass in week 11, feed efficiency and apparent fat digestibility in mice fed low fat or high-fat high-sucrose reference diets and different western diets where the protein source comprised a mixture of meat and salmon fed different levels of blue mussel meal (BM) (left) or the protein source comprised a mixture of meat supplemented with different levels of BM (right). Data from the experimental groups are presented as mean ± standard error of the mean and different letters denote significant differences (p < 0.05) by one-way ANOVA using uncorrected Fisher's Least Significant Difference multiple comparison. The dotted lines represent the standard low-fat (LF) and high-fat high-sucrose (HFHS) reference groups. n = 10 per experimental group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fish feed, will not reduce the contribution of salmon as a dietary n-3 source. It is worth mentioning that in this study salmon were sampled when they were approximately 400g and not grown to the commercial size, which may influence the fatty acid composition.

Exchanging meat from land-living animals with blue mussel meal in the mice feed led to a dose-dependent increase in (EPA + DHA)/ARA ratio in mice liver, and consumption of blue mussel may thus contribute to improving the n-3/n-6 status of the consumer. Increased consumption

of marine n-3 PUFAs is reported to attenuate high fat diet-induced non-alcoholic fatty liver disease and steatosis (Scorletti & Byrne, 2018). However, the improved n-3/n-6 PUFA status in blue mussel-fed mice was not associated with diminished steatosis in this study. However, it should be noted that the n-3/n-6 ratio increased from an average of 0.14 in liver from mice fed with meat and no blue mussel meal to averages of 0.28 and 0.27 in mice fed the highest doses of blue mussel. In comparison, the n-3/n-6 ratios observed in mice fed salmon were all above 0.67.
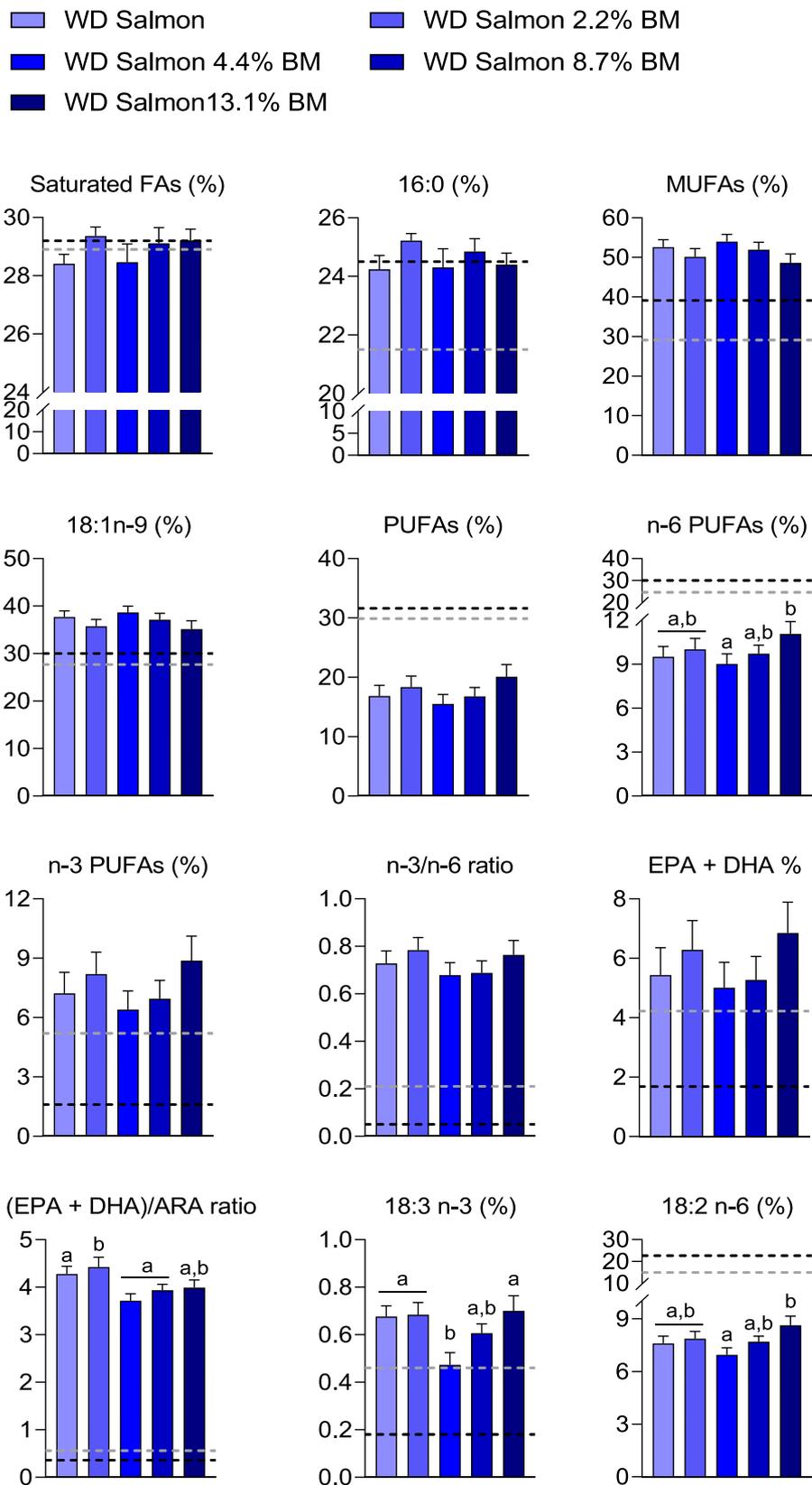
Fig. 4. Fatty acid composition (% of total fatty acids) of selected fatty acids in livers from mice fed low fat or high-fat high-sucrose reference diets and western diets where the protein source comprised a mixture of meat and salmon fed different levels of blue mussel meal (BM). Data from the experimental groups are presented as mean ± standard error of the mean and different letters denote significant differences ($p < 0.05$). $n = 10$ per experimental group. The grey dotted lines represent the standard low fat (LF) reference group and the black dotted lines represent high-fat high-sucrose (HFHS) reference group. ARA; arachidonic acid, DHA; docosahexaenoic acid, EPA; eicosapentaenoic acid, FAs; fatty acids, MUFAs; monounsaturated fatty acids, PUFAs; polyunsaturated fatty acids, SFAs; saturated fatty acids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Hence, feeding mice 8% blue mussel is less efficient than feeding mice salmon. However, feeding mice the salmon produced in this experiment was not sufficient to prevent hepatic steatosis. This is in line with an earlier experiment where mice were fed salmon in which a high proportion of fish oil in the salmon feed was replaced by soybean oil or rapeseed oil (Midtbø et al., 2015). Data from this experiment suggested that reducing the n-3 PUFA content in salmon feed was associated with reduced n-3/n-6 ratios in the liver of salmon fed mice accompanied by reduced efficiency to prevent hepatic steatoses. In this experiment, hepatic steatosis was prevented only in mice fed with salmon reared solely

Table 3
The body and tissue weights of mice after 13 weeks of feeding in trials A and B.

Mouse trial A	% BM in salmon feed				
	0	2.2	4.4	8.7	13.1
	mean±SD	mean±SD	mean±SD	mean±SD	mean±SD
Body mass (g)	44.4±3.3	44.3±3.1	45.3±3.2	44.7±2.3	43.2±2.3
Lean mass (g)	27.5±1.1	27.3±1.8	28.1±1	27.5±1.3	26.6±1.2
M. Tibialis (g)	0.12±0.01	0.12±0.01	0.12±0.01	0.12±0.01	0.13±0.03
Liver (g)	2.06±0.73	2.03±0.58	1.96±0.72	2.11±0.68	1.9±0.48
Kidneys (g)	0.31±0.03	0.32±0.05	0.34±0.03	0.32±0.03	0.31±0.02
Fat mass (g)	11.63±2.26	11.65±1.62	11.67±2.43	11.49±1.72	10.86±1.74
eWAT (g)	2.01±0.18	2.12±0.26	2.01±0.18	2.08±0.32	2.08±0.26
rWAT (g)	0.98±0.13	1.03±0.13	1.04±0.17	1.05±0.1	0.97±0.07
iWAT (g)	1.15±0.23	1.17±0.15	1.17±0.22	1.15±0.13	1.13±0.15
iBAT (g)	0.3±0.03	0.3±0.05	0.29±0.06	0.28±0.05	0.27±0.07

Mouse trial B	% BM				
	0	2	4	6	8
	mean±SD	mean±SD	mean±SD	mean±SD	mean±SD
Body mass (g)	43.2±5.9	42.3±3.3	43.6±6.3	42.3±4.1	42.2±4.2
Lean mass (g)	27±1.8	27.1±1.4	27.4±2.4	26.5±0.9	27.1±1.3
M. Tibialis (g)	0.12±0.01	0.13±0.02	0.12±0.01	0.12±0.01	0.13±0.01
Liver (g)	2.03±0.55	1.82±0.26	2.24±0.85	1.99±0.47	1.89±0.66
Kidneys (g)	0.33±0.04	0.35±0.03	0.35±0.05	0.34±0.03	0.35±0.04
Fat mass (g)	11.06±3.22	10.2±1.93	11.44±3.08	10.48±2.7	10.42±2.14
eWAT (g)	2.34±0.6	2.29±0.36	2.37±0.45	2.27±0.42	2.29±0.41
rWAT (g)	0.98±0.28	0.92±0.18	0.98±0.3	0.9±0.23	0.91±0.24
iWAT (g)	1.02±0.35	0.95±0.17	1.02±0.31	0.99±0.26	0.92±0.21
iBAT (g)	0.24±0.04	0.24±0.07	0.25±0.04	0.25±0.07	0.24±0.06

on fish oil, and the n-3/n-6 ratios in both mice feed and liver were >1.2.

Blue mussel meal is reported to influence fat accumulation in 3 T3-L1 cells (Vaidya & Cheema, 2014), and attenuate obesity and glucose intolerance in C57BL/6 mice (Vaidya et al., 2017a,b). Considering that Vaidya et al. found that the inclusion of 2.5% blue mussel meal in a high-fat high-sucrose diet attenuated obesity, whereas inclusion of 5% blue mussel meal caused feed aversion within four weeks (Vaidya et al., 2017a,b), it was surprising that inclusion of up to 8% blue mussel meal in our experiment affected neither feed intake nor weight development. Given that variation in the phytoplankton composition which mussels feed on may influence both n-3 PUFA levels and other micronutrients levels in the mussels (Ahmmed et al., 2020), geographical or seasonal variation in blue mussel nutrients is anticipated, and we cannot exclude the possibility that differences in the mussel meal used in this study and Vaidya et al. caused the observed differences. Furthermore, we used a mixture of fat sources and meat in this study, but Vaidya et al used lard and casein as fat and protein sources in the background diet. Casein has anti-obesogenic properties and is not a representative protein source (Madsen et al., 2018). We also housed our mice at thermoneutrality, which is claimed to be advantageous in aligning murine energy metabolism to human energy metabolism (Fischer et al., 2018). However possible effects of n-3 PUFAs on energy expenditure in brown or brown-like adipocytes (Kim et al., 2015; Sadurskis et al., 1995) may be masked, and it has been demonstrated that phospholipid-bound n-3 PUFAs have weak obesity-promoting effect in thermoneutrality housed mice when included in a meat-based diet (Fauske et al., 2018). Importantly, we observed no adverse health effects in mice consuming up to 8% blue mussel meal and feed aversion was not observed.

We found no adverse effect on metabolism in mice fed salmon raised on feed with inclusion of blue mussel meal or in mice fed a meat-based western diet containing up to 8% blue mussel meal. Together our results demonstrated that partial replacement of fish meal with blue mussel meal in salmon feed did not cause negative spillover effects in a mouse model of obesity, and hence, intake of such farmed salmon would not be expected to have negative effects on human consumers.

Fatty fish like salmon are known for their high content of marine n-3 PUFAs, which are believed to have various health benefits. One significant finding in this study is that, despite the fact that replacing fish meal with blue mussel led to a dose-dependent increase in the n-6 PUFA content in fish fillets, the n-3 PUFA content was maintained.

This study has certain limitations that could potentially impact the outcomes and conclusions. The salmon trial was terminated when fish were approximately 400g, which is lower than the typical industrial slaughter weight. This can have an effect on the fatty acid profile of the salmon fillets. An increased n-6/n-3 PUFA ratio in tissue and red blood cells are associated with inflammation as n-6 fatty acids are precursors of pro-inflammatory eicosanoids (Calder, 2015). In mice fed salmons, the fatty acid composition in both red blood cells and liver mirror the fatty acid composition in the salmon fillets used for feed preparation (Midtbø et al., 2015) but data on the n-6/n-3 ratio in red blood cells and inflammatory factors would have provided a more complete picture. A recent review on the health effects of a Mediterranean diet, indicated that the control of systemic inflammation is an important mechanism involved in the reduction of chronic diseases, cardiovascular disease and longevity (Tsoupras et al., 2018).

Blue mussel farming is suggested as a sustainable supply chain for both human consumption and as an alternative for fish meal replacement in aquaculture (Suplicy, 2018). Looking forward, the demonstrated potential to increasingly replace fish meal with more locally grown blue mussel protein paves the way towards a more sustainable aquaculture production in regions where mussels can be produced near the fish farms.

Author contributions

AMA: Planned, supervised and performed the mice trials, data analyses, writing and figure preparation; **AB:** Participated in planning of the salmon trial, participated in salmon trial sampling, data analyses; **AS:** Participated in mice trial A, data analyses; **LSM:** Participated in planning of the salmon trial, participated in mice and salmon sampling, data analyses; **AKL:** Participated in planning and sampling of the mice trials; **LAL:** Participated in planning and sampling of the salmon trials; **EF:** Participated in mice sampling, data analyses; **QTH:** Participated in data analyses; **HS:** Managed and designed the salmon trial; **KK:** Helped design study and structure manuscript; **MTL:** Managed salmon trial sampling; helped design study and structure manuscript; **LM:** Supervised the study, planning of the mice trials, writing and data-analyses, figure preparation.

Declaration of Competing Interest
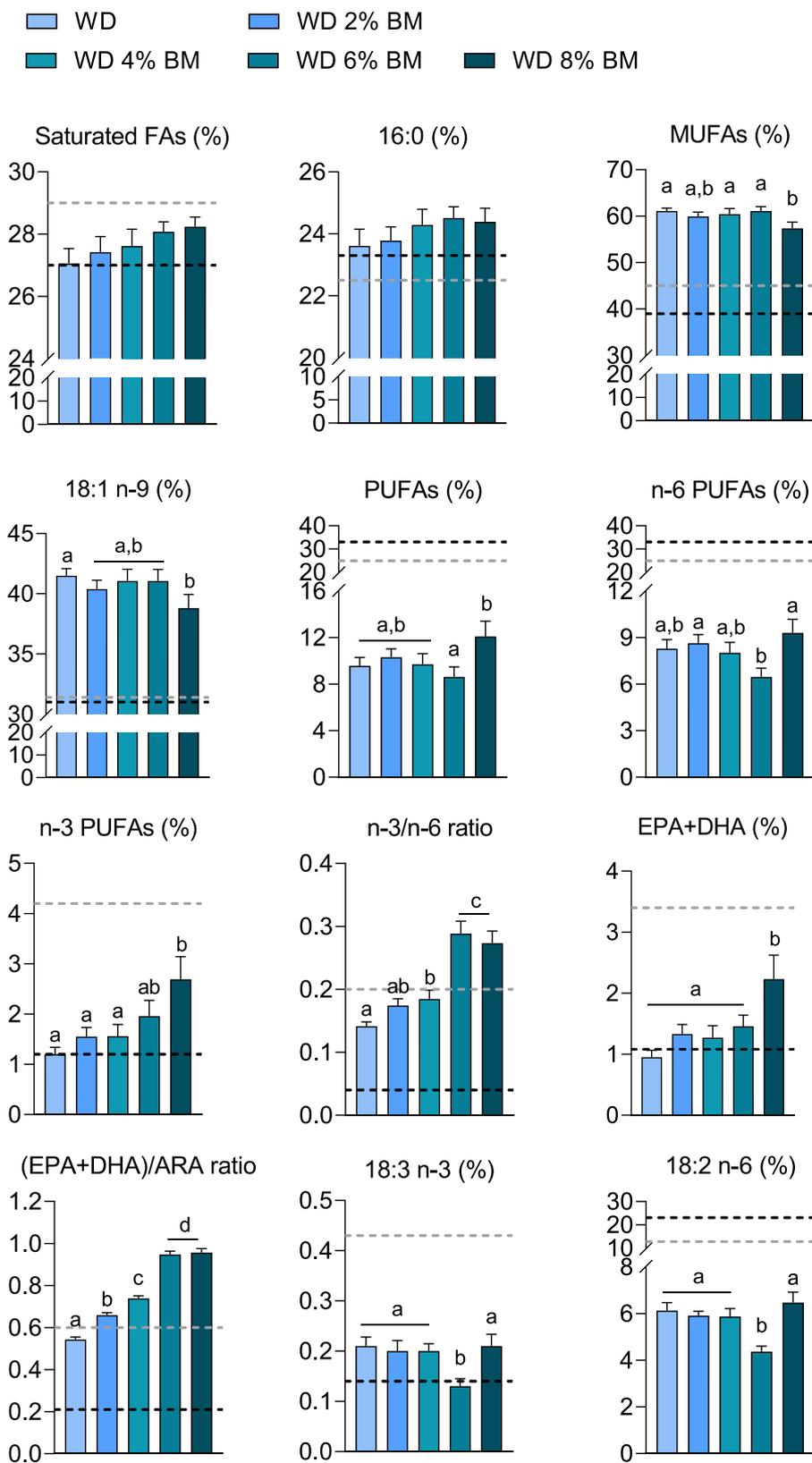
Fig. 5. Relative amounts (% of total fatty acids) of selected fatty acids in livers from mice fed low fat or high-fat high-sucrose reference diets and western diets where the protein source comprised a mixture of meat supplemented with different levels of blue mussel meal (BM). Data from the experimental groups are presented as mean ± standard error of the mean and different letters denote significant differences (p < 0.05) by one-way ANOVA using uncorrected Fisher's Least Significant Difference multiple comparison. n = 10 per experimental group. The grey dotted lines represent the standard low fat (LF) reference group, and the black dotted lines represent high-fat high-sucrose (HFHS) reference group. ARA; arachidonic acid, DHA; docosahexaenoic acid, EPA; eicosapentaenoic acid, FAs; fatty acids, MUFAs; monounsaturated fatty acids, PUFAs; polyunsaturated fatty acids, SFAs; saturated fatty acids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112927.

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