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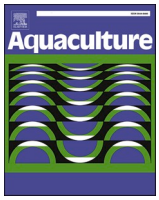

Intestinal health in Atlantic salmon post-smolt (*Salmo salar*) when fed low- and high HUFA diets

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ABSTRACT

It is well established that farmed Atlantic salmon (*Salmo salar*) need n3-highly unsaturated fatty acids (HUFA) in their diet to thrive and grow. However, the biological functions to the individual HUFAs may differ, implying that future supplementation could require fixed ratios for maximum benefit. The intestinal barrier is essential to fish health, and any disruption of the barrier can have detrimental effects. The current experiment was designed to examine the response of the intestinal hindgut when fed a low HUFA diet with 8.5 g/kg EPA + DHA (4.5% total fatty acid) or two high HUFA diets, with either high DHA (28.7 g/kg and 5.9% total fatty acid) or high EPA (25 g/kg and 14.2% total fatty acid). The diets were fed to Atlantic salmon post-smolt over 10 weeks and thereafter exposed to 3 weeks of chronic stress. After 10 weeks of feeding there were no differences in intestinal permeability and integrity, but intestinal morphology indicated increased intestinal health in the high EPA group. Gene expression also suggest that fish fed the high EPA diet had more regulation of pathways related to protein turnover compared to the high DHA fed fish. There was also indication of lower energy utilization in the low HUFA fed fish than high HUFA. Subjecting fish to 3 weeks of chronic stress led to a reduction in trans-epithelial resistance, increased ion flux and active L-lysine transport across the intestinal barrier in addition to a decrease in mucosal fold, enterocyte height and supranuclear vacuole density and an increase in thickness of the intestinal muscularis. After stress, the low HUFA group showed signs of inflammation with increased infiltration of MHCII positive cells. Gene expression also showed that low HUFA fed fish had a lower response to chronic stress compared to the high HUFA groups. Comparing fish fed either high DHA or EPA exposed to chronic stress showed few physical effects, but a lower density of supranuclear vacuoles and upregulation in immune-related gene expression indicate inflammation in the high DHA group.

1. Introduction

Atlantic salmon farming is a continuously expanding industry with a steady increase in production over time. In Norway, the sale of slaughtered salmon reached 1,377,185 metric tons in 2020 (round weight, Norwegian Directorate of Fisheries) This had led to an increased demand for feed resources to supplement the already exhausted supply of the traditional fishmeal and fish oil. Today, around 70% of the protein and oil sources used in salmon diets are from vegetable origin (Aas et al., 2019).There is a challenge with using terrestrial oilseeds in that they do not contain the marine HUFAs EPA and DHA, which are essential for optimum performance and health of the fish (Glencross, 2009; Løvmo et al., 2021; Sprague et al., 2019). The consequence of the ingredient replacement has been a reduction of EPA and DHA to a level that could eventually approach essential fatty acid deficiency in farmed salmon (Bou et al., 2017b). To avoid further reduction, there has been several approaches aimed at increasing availability of these fatty acids. Most efforts have been put into development of various algal sources (Santigosa et al., 2020; Sprague et al., 2015), but cost is generally very high and will limit their inclusion levels in the diets. More promising

Abbreviations: Fatty acid, FA; docosahexaenoic, DHA; eicosapentaenoic, EPA; linoleic acid, LA; alpha-linoleic acid, ALA; Highly unsaturated fatty acids, HUFA.

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approaches has been done with GMO (genetically modified organisms) altered oilseeds that are now engineered to produce EPA and DHA to the same levels as in fish oils (Betancor et al., 2017; Betancor et al., 2015; Sprague et al., 2017). Although the cost of these products is not very high, EU legislation currently prevents such use in commercial fish production. The introduction of algal or GMO oilseeds have also provided an unexpected opportunity to mix different oils to the optimum level needed for fish health and welfare, a possibility that was not available in the past.

There is accumulating evidence suggesting that both EPA and DHA are important for the fish, but also that they have some differential functions that justify the inclusions of different levels in the diets (Allam-Ndoul et al., 2017; Bou et al., 2017a; Durkin et al., 2021). It is also highly likely that different tissues respond differentially to altered dietary levels and ratios of EPA and DHA, especially when challenged by environmental challenges such as stress or temperature (Handeland et al., 1998; Overton et al., 2019). Commercial diets vary in their inclusion level of EPA from 1.3–38.6 g/kg, and DHA from 3 to 12.8 g/kg (Sele et al., 2019), and there is relatively little information on how salmon intestine responds under challenging conditions, and even less with regard to low HUFA levels and different EPA/DHA ratios. A recent study on Atlantic salmon midgut revealed differences in gene expression related to different EPA/DHA ratios (Løvmo et al., 2021), highlighting the need for more research on this area.

The fish intestine is a barrier forming a large interface separating the bodily internal and external milieu. More precise, it is a selective semipermeable barrier that absorbs nutrients, fluid and ions while effectively hindering bacteria and other harmful substances to pass. Importantly, it is also one of the organs that are highly affected by stress, which causes the barrier to become leakier and increases disease susceptibility (Olsen et al., 2005; Sundh et al., 2019; Sundh et al., 2010). This is again related to intestinal immune system and inflammation, where the hindgut is more sensitive with several relevant immune genes highly expressed in the region (Harstad et al., 2008; Koppang et al., 1998; Løkka et al., 2014). Studies also show that the hindgut barrier is more affected by inflammation (Bjørgen et al., 2019; Fuglem et al., 2010). There is also a limitation in relevant studies on intestinal gene expression, which is on one hand widely studied in relation to diet induced enteritis (Król et al., 2016; Øvrum Hansen et al., 2019; Sahlmann et al., 2013), however much less is known about changes to the intestinal transcriptome in relation to stress and dietary FAs.

In this study we have aimed to evaluate the impact of high and low dietary HUFAs and two EPA/DHA ratios on the Atlantic salmon post-smolt hindgut in the period after seawater transfer, under controlled conditions and chronic stress. We hypothesized that feeding a low HUFA diet would generate a more damaging response to stress and intestinal health, and that different EPA/DHA ratios would have alternating effects on the Atlantic salmon intestinal barrier. Hindgut health was assessed by intestinal fatty acid composition, morphology, barrier, transport function, inflammation, and transcription status after feeding 10 weeks of feeding and three weeks of chronic stress.

2. Material and methods

2.1. Experimental design

The experimental trial lasted from December to April 2017 and has previously been described in detail in Løvmo et al. (2021) and was approved by the Norwegian Experimental Animal committee (FOTS ID: 15041). In brief, Atlantic salmon post-smolts were divided into 4 groups fed experimental diets for 10 weeks, and subsequently challenged for 3 weeks with an unpredictable chronic stress (UCS) protocol. The diets were based on extruded pellets produced by Biomar and then vacuum-coated with different blends of Rapeseed oil, vevodar oil and Epax oil to achieve desired FA composition (Table 1). The final HUFA composition of the diets were: low HUFA (LHUFA) diet with EPA + DHA at 8.5 g/kg and 4.53% of total FA, high DHA diet (HDHA) with 28.7 g/kg and 15.86% of total FA, and high EPA (HEPA) with 25 g/kg and 14.17% of total FA (Table 2). At the start of the trial, fish weighing 149 ± 14 g were randomly sorted into 12,400 L tanks with 25 individuals in each tank. Fish were kept at 24 h light and feed twice a day until satiation with automatic feeders (Arvo-Tec T Drum 2000, Arvo.Tec Oy, Finland). Daily feed ratio in each tank was calculated from an expected feed conversion ratio (FCR) = 1.2 and was adjusted when needed to ensure satiation by having 10% or more excess feed in the tank outlet after 1 h feeding. During the three weeks of UCS the fish were feed continuously for 24 h. The UCS protocol consists of exposure to a randomly selected stressor 3

Table 1
Diet formulation.

	LHUFA	HDHA	HEPA
Fish Meal SA Superprime*	30	30	30
Fish Meal SA Krill*	20	20	20
Sunflower Expeller, low fiber*	21.4	21.4	21.4
Wheat Gluten*	127	127	127
Maize Gluten*	50	50	50
Pea Protein*	120	120	120
Wheat*	140	140	140
Oil blend (see below)*	192	192	192
Vitamins, minerals and additives*	35.7	35.7	35.7
Crystalline amino acids*	21	21	21
Lucantin Pink, BASF*	0.55	0.55	0.55
Rapeseed oil**	92	71	66
Vevodar**	2	0	0
Epax 1050**	3	29	0
Epax 4510**	3	0	34

* g/kg.
** % oil-blend for coting.

Table 2
Diet compositions (g/kg) and fatty acid composition (% total fatty acids).

	LHUFA	HDHA	HEPA
Crude lipid	20.2	19.5	19
EPA	4	6.5	25
DHA	4.5	28.7	8.1
ARA	2.7	2.1	1.7
EPA + DHA	8.5	35.2	33.1
Fatty acids:			
14:00	0.17	0.27	0.25
16:00	4.44	4.01	3.72
18:00	2.16	2.55	3.38
18:1n-7	2.60	2.30	3.26
18:1n-9	55.78	43.09	42.69
20:1n-9	1.39	1.51	1.77
18:2n-6	17.98	14.62	14.50
18:3n-6	0.08	0.04	0.06
20:2n-6	0.11	0.20	0.17
20:3n-6	0.12	0.09	0.14
20:4n-6	1.44	1.16	0.98
18:3n-3	6.73	5.61	5.70
18:4n-3	0.13	0.18	0.94
20:3n-3	0.01	0.08	0.06
20:4n-3	0.09	0.23	0.51
20:5n-3	2.16	3.58	14.17
22:5n-3	0.19	0.72	0.75
22:6n-3	2.37	15.86	4.60
SAT	7.89	8.17	8.31
MONO	60.55	48.30	48.93
n3	11.68	26.26	26.72
n6	19.88	17.27	16.04
n6/n3	1.7	0.66	0.6
n3 HUFA	4.82	20.47	20.09
n6 HUFA	1.81	2.61	1.48
n6/n3 HUFA	0.38	0.13	0.07
HUFA	6.63	23.08	21.57
EPA + DHA	4.53	19.44	18.77
DHA/EPA	1.10	4.43	0.32

times a day (Table 3). All tanks were treated with the same stressor at the same time. Samples of the hindgut, from the ileorectal valve to anus, were secured after 10 weeks of feeding and 3 weeks with UCS. All sampled fish were quickly collected with a net and transferred to a 50 L bucket containing an overdose of anesthetic Finquel.vet® (100 mg 1–1 tricainmesylat, Scanvacc, Hvam, Norway). Fish were sampled twice from each tank. For analysis of the intestinal barrier and transport functions, fed fish were sampled first and upon dissection, only fish with fecal matter was used for analysis. For morphology, FA composition and gene expression, fish were fed for two days and then starved for 24 h before sampling. Prior to the sampling of UCS treated fish, all tanks were exposed to the same stressor (crowding, Table 3) 24 h prior to sampling.

2.2. Lipid extraction

Polar lipids (PL) were extracted as described in Løvmo et al. (2021). Hindgut polar lipid fatty acids were analyzed from 5 individuals per group. Polar lipids were separated from natural lipids using 10 × 10 HPTLC silica plates on the solvent system hexane:diethylether:acetic acid (80:20:2) according to Olsen and Henderson (1989). Phospholipids remaining at the origin were scraped off and subjected to the sulfuric catalyzed transmethylation according to Christie and Han (2010), extracted into hexane and stored at −80 °C. Quantification of the fatty acid methyl esters were carried out with gas liquid chromatography according to Betancor et al., 2017.

2.3. Morphological evaluation

For each group, the hindgut intestinal morphology in 6 individuals was evaluated. After sampling, each tissue sample was fixed in buffered formaldehyde at 4 °C for 48 h, before dehydrated and embedded in paraffin using a tissue processor (TP1020, Leica Biosystems, Germany). Four μm tissue-sections were made on a microtome (RM2255, Leica Biosystems, Germany) and stained (Alcian Blu-Pas) before digitally scanned at 40× magnification (NanoZoomer, Hamamatsu photonics, Japan). Measurements for morphological scores were done manually on images using the image software NDP.view2 (NDP, Hamamatsu photonics, Japan). For each individual fish, one section was selected, and the height of mucosal folds (μm) and lamina propria width (μm) was measured 6–12 times. In addition, 6 folds with a longitudinal section through the lamina propria was randomly selected, and within each of these folds enterocyte height (μm) and goblet cell density (cells per 0.1mm^2) was measured. Muscularis thickness was measured (μm) at 3 random sites on each section. Enterocyte supranuclear vacuoles were assessed in 6 randomly chosen regions, with the same magnification and area for each preparation using density of supranuclear vacuoles from 1 to 4 as the main criteria. Score of 1 represent absence of vacuoles while 4 represent large vacuoles filling most of the space between the nucleus and the microvilli (Fig. 1).

Table 3
Stressors used in the UCS protocol.

Stressors	Description
Crowding	Lowering the water level down to the dorsal fin for 30 s.
Chasing with net	Chasing fish with the net for 2–5 min
Light	Turning of the light in the room and using a flashlight to chase the fish in the tank for 1 min x3
Sound	Mechanical noise in the lab, knocking on the tanks and opening the lid of the tanks
Chasing with cane	Chasing the fish with a cane for 1 min x3, with 2 min pause in between
Hypoxia	Lowering the oxygen level to 60% and then slowly returning to 80–90%

2.4. Immunohistochemistry

The density of hindgut MHCII expressing cells was assessed for 5 individuals per group as number of cells per length of epithelium and lamina propria. The primary antibody for MHCII was validated by Hetland et al. (2010). Five μm paraffine tissue-sections were mounted on silane coated glass slides and dried at 37 °C for 24 h. Slides were dewaxed and rehydrated and subjected to heat induced epitope retrieval by demasking in a pressure cooker with Tris/EDTA (10 mM/; pH 9.0) containing 0.1% Tween-20 for 5 min. Immunostaining was carried out according to the ImmPRESS® Horse Anti-Mouse IgG PLUS Polymer kit (Vector Laboratories). Accessing the number of positive MHCII-cells was done on digitally scanned sections at 40× magnification (NanoZoomer, Hamamatsu photonics, Japan) using an imaging software NDPview2 (NDP, Hamamatsu photonics, Japan). For each of the tissue sections, MHCII expressing cells were counted as in lamina propria and as intraepithelial, from the base of the fold to the top of the lamina propria, in 6 randomly chosen mucosal folds (Fig. 2). The results are presented as cells/μm lamina propria and cells/μm enterocytes, where the length of the enterocytes equals the length of the lamina propria x2.

2.5. Hindgut intestinal barrier and transport function

Barrier and transport functions were assessed using the Ussing chamber technology according to Sundell et al. (2003) and Sundell and Sundh (2012) and Løvmo et al. (2021). Hindguts from 12 individuals per group, 4 from each tank, were quickly dissected out, cleaned, opened longitudinally, and rinsed in cold Ringer modified for seawater acclimated salmonids. Intestinal sections were cleaned by removal of fatty tissue and serosa by gently peeling off under a stereo microscope before mounting onto the ussing half-chambers. Ringer was then added to both the serosa- and mucosa side of the chambers and the tissue was left to stabilize for 60 min. The trial started with the replacement of the Ringer on both sides of the chamber, with the Ringer on the mucosal side containing 0.07 MBq of the marker molecules ^{14}C-mannitol (PerkinElmer, MA, USA) and 0.07 MBq of ^{3}H-L-lysine (PerkinElmer, Boston, MA, USA) together with unlabeled L-lysine at a concentration of 0.5 mM. A 100 μl Ringer sample was taken from the mucosal side after 20, 30, 60, 80 and 90 min, and the mucosal side after 20 and 90 min. Five ml Opti-Fluor O (PerkinElmer, MA, USA) were added to the samples for quantification of radioactivity using a scintillation counter set with a dual label (^{14}C/^{3}H) protocol (Packard TRI-CARB 2300 TR Liquid Scintillation Analyzer, USA). Apparent permeability (Papp) for ^{14}C-Mannitol were calculated using Eq. (1) and transport-rate of L-lysine was calculated by Eq. (2). Transepithelial electrical resistance (TER) and transepithelial potential (TEP) was measured every five minutes. Data were given as the mean value from the last 30 min of recording in the results. The short circuit current (SCC) was calculated from the TER and TEP values (Amps = Volt/Ohm) and represent the electrical current needed to equalize the TEP and is equivalent to the sum of ion movement across the epithelium.

$$\text{Papp} = dQ/dt*1/AC0 \quad (1)$$

$$\text{Transport rate L} - \text{Lysine} = dQ/dt*1/A \quad (2)$$

2.6. Gene expression

The protocol for gene expression analysis is given in details in Løvmo et al. (2021). In brief, RNA was extracted from the hindgut according to instructions using RNeasy Plus Universal Mini kit (Qiagen, Hilden, Germany). All samples were quality checked (Agilent 2100 Bioanalyzer, Agilent Technologies, California, USA) and had RIN > 7. cDNA libraries were prepared according to instructions using TruSeq Stranded mRNA Sample prep HS protocol (Illumina, California, USA) and further sequenced at the Genomic Core Facility at the Norwegian University of

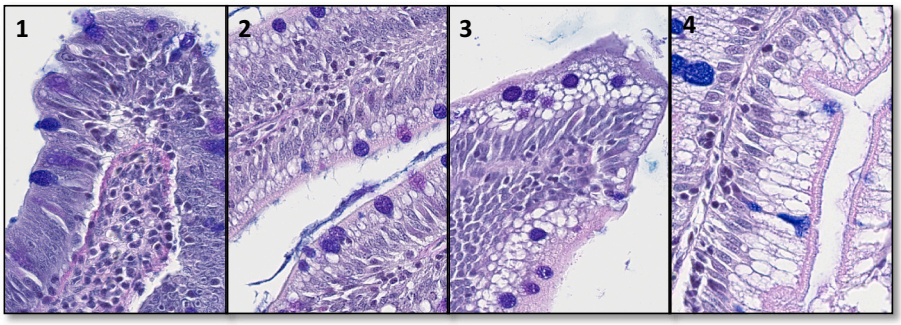

Fig. 1. Representative images of vacuolization score 1–4 in the hind intestine.

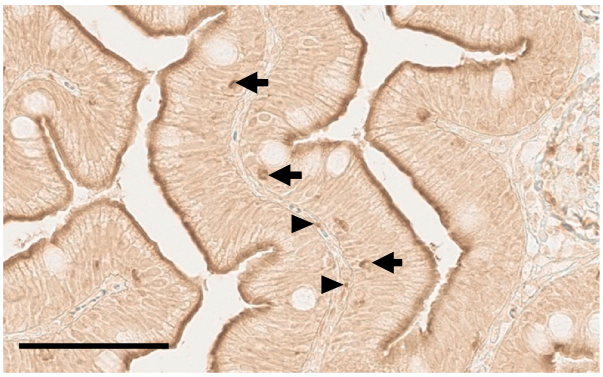

Fig. 2. Representative image of positive MHCII-expressing cells in the hindgut. Arrows mark positive intraepithelial cells and arrow heads mark positive cells in the lamina propria. Magnification at x40 and scalebar at 100 μm.

Science and Technology. Paired-end sequence reads were mapped to the Atlantic Salmon reference genome (ICSASG_v2. 6-10-2016) and a read table of count per gene for each sample was used for further analysis. Reads were mapped to the reference genome by HISAT2 (Kim et al., 2019) and a count table was generated using featuresCounts, v1.6.5, 18-07-2019, a Subread package (Liao et al., 2019). Outliers and batch effects was analyzed by PCA plots, pairwise distance dendogram and heatmap. Differential expression analysis was completed using the R package DESeq2, version 1.28.1 {Love, 2014 #339}. A functional annotation of Kyoto Encyclopeidia of Genes and Geneomes (KEGG) pathways using Gene Set Enrichment Analysis (GSEA) resulted in a list of pathways comparing the different dietary groups (Subramanian et al., 2005).

2.7. Statistics

Further statistical analysis was performed in R studio version 1.2.5033 (© 2009–2019 RStudio, Inc). Data distribution was inspected visually by Q-Q plots and calculated using Shapiro-Wilks normality test. Homogeneity of variance was tested using Levene's test. Differences between dietary groups were analyzed by one-way ANOVA after 10 weeks of feeding and 3 weeks of UCS. Effects of stress and diets, including interactions, were analyzed by two-way ANOVA. Significant interactions between diets and stress were further tested using Tukey's honest significance difference (HSD) *post hoc* test. When data distribution was not normal, as for vacuolization scoring data, the nonparametric Kruskal-Wallis rank sum test was used.

3. Results

3.1. Fish performance

As previously reported in Løvmo et al. (2021), all fish doubled their weigh from 149 g to 388 g in the LHUFA group and 392 g in the HDHA and HEPA groups over the 10-week feeding period. Weight gain per day (SGR) was calculated for the feeding and UCS period ($n = 6$, ± sd). No difference in fish growth performance was detected between groups. After 3 weeks of UCS the weight gain was significantly lower, with a weight increase to 392 g (2%) in the LHUFA group, 402 g (4.3%) in HDHA group and 410 g (4.6%) in HEPA group. No significant differences in growth performance were observed between the groups (Table 4).

3.2. Fatty acid composition

The hindgut FA composition generally reflected the same patterns as the diet composition, although the variation between diets were less pronounced in the tissue. After the feeding period, almost all FA were significantly affected by the diet ($p < 0.01$). The repeated pattern in dietary FAs can be seen in saturated FA, PUFA and HUFA levels and the DHA/EPA ratio in Table 5. One noteworthy difference from dietary composition was the high levels of DHA and DPA in LHUFA and HEPA, drastically decreasing the n6/n3 PUFA ratio in LHUFA. The n6 HUFA level in LHUFA was also significantly higher compared to HEPA and HDHA because of the high level of ARA in the tissue.

After UCS the FA still reflects the dietary composition, although the difference between diets was even less pronounced than after the feeding period. This was reflected in the low number of FA significantly affected by the UCS protocol. The pattern in n6/n3 and DHA/EPA ratios were not as affected by UCS, although UCS caused a significant decrease in n3 HUFA ($p < 0.05$). Specifically, the decrease was seen in the FAs 20:3n-3 ($p < 0.001$), 20:4n-3 ($p < 0.05$) and DPA ($p < 0.001$).

Table 4
Growth performance for all three dietary groups. Comparing average weight and weight gain at trial start, after 10 weeks of feeding and after 3 weeks of UCS. Letters in superscript mark significant difference between groups, $p < 0.05$.*.

	LHUFA	HDHA	HEPA
Start weight (g)	151 ± 13.3	149 ± 13.8	149 ± 15.0
Feeding			
Weight (g)	388 ± 49.7	392 ± 45.1	392 ± 49.0
Weight gain	157%	163%	163%
SGR	1.36	1.41	1.37
UCS			
Weight (g)	396 ± 56.2	402 ± 52.2	410 ± 60.6
Weight gain	2%	4.3%	4.6%
SGR	−0.04	−0.08	0.27

* One-way ANOVA and HSD *post hoc* test.

Table 5
Hindgut polar lipid fatty acids, % of total FA (mean of $n = 5 \pm$ sd). Differences between dietary groups were compared after 10 weeks of feeding (Feeding) and 3 weeks of stress (UCS). Differences between diets considered significant when $p < 0.05$ and a trend (t) when $p < 0.1$. Letters in superscript mark significant difference between groups, $p < 0.05$.

	Feeding				UCS				*p*-values	
FAs	LHUFA	HDHA	HEPA	Diets feeding*	LHUFA	HDHA	HEPA	Diets UCS***	Stress***	Diet x Stress***
16:00	16.90 ± 0.30	16.20 ± 1.17	16.20 ± 0.35	–	16.80 ± 1.16	16.10 ± 0.49	16.50 ± 0.88	–	–	–
18:00	7.60 ± 0.07	7.81 ± 0.79	8.41 ± 0.17	t	8.66 ± 0.63^{ab}	7.89 ± 0.34^{a}	9.13 ± 0.43^{b}	0.05	0.01	–
18:1n-7	2.15 ± 0.12^{a}	2.25 ± 0.06^{a}	2.63 ± 0.11^{b}	0.001	2.36 ± 0.25^{a}	2.37 ± 0.11^{a}	2.73 ± 0.12^{b}	0.05	0.05	–
18:1n-9	15.50 ± 0.83^{a}	13.00 ± 0.54^{b}	13.10 ± 0.79^{b}	0.001	15.90 ± 0.74	15.4 ± 1.02	14.5 ± 1.07	–	0.001	0.05
20:1n-7	0.23 ± 0.02^{a}	0.37 ± 0.07^{b}	0.44 ± 0.04^{b}	0.001	0.21 ± 0.04^{a}	0.24 ± 0.02^{a}	0.32 ± 0.04^{b}	0.01	0.001	0.05
20:1n-9	3.56 ± 0.45	3.63 ± 0.59	3.54 ± 0.31	–	2.72 ± 0.47	2.64 ± 0.19	2.45 ± 0.38	–	0.001	–
24:1n.9	1.74 ± 0.12^{a}	2.38 ± 0.15^{b}	1.91 ± 0.15^{a}	0.001	1.95 ± 0.47	2.07 ± 0.12	2.09 ± 0.28	–	–	t
18:2n-6	5.15 ± 0.39^{a}	3.29 ± 0.29^{b}	3.13 ± 0.44^{b}	0.001	4.73 ± 0.36^{a}	4.15 ± 0.25^{ab}	3.63 ± 0.67^{b}	0.05	t	0.01
18:3n-6	0.09 ± 0.03^{a}	0.32 ± 0.04^{b}	0.16 ± 0.07^{a}	0.001	0.11 ± 0.08	0.29 ± 0.15	0.23 ± 0.02	t	–	–
20:2n-6	3.51 ± 0.40	3.74 ± 0.50	3.38 ± 0.57	–	2.75 ± 0.88	3.06 ± 0.50	2.78 ± 0.58	–	0.01	–
20:3n-6	1.54 ± 0.33^{a}	0.23 ± 0.02^{b}	0.38 ± 0.0^{b}	0.001	1.22 ± 0.39^{a}	0.21 ± 0.04^{b}	0.36 ± 0.04^{b}	0.001	–	–
20:4n-6	5.02 ± 0.94^{a}	2.87 ± 0.82^{b}	2.63 ± 0.14^{b}	0.001	5.21 ± 1.36^{a}	3.40 ± 0.24^{b}	3.14 ± 0.30^{b}	0.01	–	–
22:4n-6	0.56 ± 0.13^{a}	0.38 ± 0.08^{b}	0.26 ± 0.03^{b}	0.001	0.49 ± 0.18^{a}	0.30 ± 0.02^{b}	0.24 ± 0.04^{b}	0.05	–	–
22:5n-6	0.80 ± 0.07^{a}	1.56 ± 0.05^{b}	0.35 ± 0.03^{c}	0.001	0.70 ± 0.17^{a}	1.52 ± 0.11^{b}	0.35 ± 0.04^{c}	0.001	–	–
18:3n-3	0.78 ± 0.06^{a}	0.6 ± 0.05^{b}	0.62 ± 0.12^{b}	0.01	0.78 ± 0.14	0.86 ± 0.08	0.74 ± 0.18	–	0.01	0.05
18:4n-3	0.15 ± 0.03^{a}	0.04 ± 0.01^{b}	0.07 ± 0.01^{c}	0.001	0.12 ± 0.05	0.08 ± 0.03	0.11 ± 0.05	–	–	0.05
20:3n-3	0.43 ± 0.05^{a}	0.62 ± 0.11^{b}	0.57 ± 0.04^{b}	0.01	0.42 ± 0.20	0.46 ± 0.06	0.40 ± 0.08	–	0.001	t
20:4n-3	0.44 ± 0.08^{a}	0.16 ± 0.02^{b}	0.35 ± 0.02^{c}	0.001	0.34 ± 0.04^{a}	0.16 ± 0.02^{b}	0.32 ± 0.03^{a}	0.001	0.05	t
20:5n-3	4.03 ± 0.39^{a}	3.02 ± 0.30^{b}	9.08 ± 0.37^{c}	0.001	4.54 ± 1.79^{a}	3.03 ± 0.17^{a}	8.16 ± 0.40^{b}	0.001	–	–
22:5n-3	2.08 ± 0.33^{a}	1.54 ± 0.26^{b}	5.15 ± 0.30^{c}	0.001	1.97 ± 0.47^{a}	1.23 ± 0.06^{b}	3.86 ± 0.54^{c}	0.001	0.001	0.01
22:6n-3	23.50 ± 2.43^{a}	33.00 ± 0.48^{b}	24.50 ± 1.51^{a}	0.001	23.90 ± 2.27^{a}	31.20 ± 0.51^{b}	24.7 ± 0.87^{a}	0.001	–	–
SAT	25.7 ± 0.28	25.50 ± 1.78	25.80 ± 0.40	–	26.7 ± 1.72	25.50 ± 0.74	27.0 ± 1.32	–	–	–
MONO	23.8 ± 0.81	22.3 ± 1.05	22.40 ± 0.58	t	23.5 ± 1.45	23.70 ± 1.19	22.9 ± 1.33	–	–	–
n6 PUFA	16.7 ± 1.05^{a}	12.4 ± 0.51^{b}	10.3 ± 0.72^{c}	0.001	15.2 ± 2.93^{a}	12.9 ± 0.54^{ab}	10.7 ± 0.95^{b}	0.05	–	–
n3 PUFA	31.4 ± 2.78^{a}	38.9 ± 0.15^{b}	40.7 ± 1.08^{b}	0.001	30.6 ± 2.26^{a}	37.0 ± 0.53^{b}	38.3 ± 1.03^{b}	0.001	0.05	–
n6 HUFA	11.4 ± 1.24^{a}	8.78 ± 0.49^{b}	7.0 ± 0.53^{c}	0.001	10.4 ± 2.27^{a}	8.49 ± 0.6a^{b}	6.87 ± 0.55^{b}	0.05	–	–
n3 HUFA	30.5 ± 2.82^{a}	38.2 ± 0.13^{b}	40.1 ± 1.19^{b}	0.001	29.6 ± 2.26^{a}	36.0 ± 0.58^{b}	37.5 ± 1.23^{b}	0.001	0.05	–
HUFA	41.9 ± 3.92^{a}	46.8 ± 0.19^{b}	46.9 ± 1.39^{b}	0.05	41.1 ± 3.24	44.5 ± 1.05	44.3 ± 1.44	t	t	–
PUFA	48.1 ± 3.56	51.1 ± 0.40	50.8 ± 0.95	–	47.0 ± 3.62	49.9 ± 0.76	49.1 ± 1.08	–	–	–
n6/n3 PUFA	0.53	0.32	0.25		0.47	0.35	0.28			
n6/n3 HUFA	0.37	0.23	0.17		0.33	0.24	0.18			
DHA/EPA	5.83	10.93	2.70		5.26	10.3	3			

* Dietary effects on FA-composition after Feeding or UCS was analyzed by One-way ANOVA.
*** Interaction between stress and diet analyzed by Two-Way ANOVA and HSD *post hoc* test.

3.3. Intestinal morphology

The intestinal morphology as affected by diets and UCS are presented in Table 6. Vacuolization was the only morphological parameter affected by diets after 10 weeks of feeding, which was significantly higher in the HEPA group ($p < 0.05$) (Fig. 3). After the period of UCS, the intestinal morphology showed a decreased mucosal fold height ($p < 0.01$), decreased enterocyte height ($p < 0.001$) and decreased vacuolization ($p < 0.001$). The decrease in mucosal fold height was a significant interaction between diets and UCS ($p < 0.05$), with the largest decrease in the HEPA group ($p < 0.001$). The drop in enterocyte height was largest in the HDHA group after UCS ($p < 0.001$). The increased thickness of the hindgut muscularis was also an interaction between diets and UCS ($p < 0.05$), and the largest increase was in the HDHA group ($p < 0.05$).

3.4. MHCII positive cells

The presence of MHCII positive cells was assessed in all diets after feeding and UCS (Fig. 4). The number of cells located in the intra-epithelial space was numerically lower after UCS in all diets, but not statistically significant. In the lamina propria, a higher number of positive cells was found in the LHUFA group after UCS (diet $p < 0.1$, diet x stress $p < 0.01$). The number of MHCII-positive cells increased

Table 6
Intestinal morphology scoring for all groups. Scores are presented from the two periods, 10 weeks of feeding and 3 weeks of UCS (in that order going downwards) for each category. Values are presented as mean ± sem. Letters in superscript mark significant difference between the dietary groups. Text in bold mark significant difference between the two period for one dietary group.

	LHUFA	HDHA	HEPA	Diet	Stress	Diet x Stress
Mucosal fold height*	855 ± 64.8	834 ± 39.1	**924** ± 35.9	–	0.001	0.05
	776 ± 18.7^{a}	736 ± 32.5^{ab}	**663** ± 31.5^{b}	0.05		
Enterocyte height*	53.5 ± 2.31	**55.0** ± 0.927	55.6 ± 1.37	–	0.001	t
	49.0 ± 1.41^{ab}	**45.1** ± 0.881^{a}	52.4 ± 1.40^{b}	0.01		
Muscularis*	184 ± 5.99	**169** ± 10.4	189 ± 8.72	–	0.01	0.05
	224 ± 8.17	**223** ± 13.0	185 ± 8.81	0.05		
Goblet cell*	36.1 ± 1.62	37.3 ± 3.67	39.2 ± 4.07	–	–	–
	33.5 ± 3.45	42.8 ± 4.80	44.0 ± 1.47	–		
Lamina propria*	7.22 ± 0.77	6.93 ± 0.58	6.02 ± 0.23	–	–	t
	6.97 ± 0.32	6.45 ± 0.58	8.04 ± 1.94	–		
Vacuolization**	3.37 ± 0.06^{ab}	3.36 ± 0.07^{a}	3.65 ± 0.09^{b}	0.05	0.001	–
	2.67 ± 0.25	2.87 ± 0.13	3.11 ± 0.16	–		

*/**Differences were considered significant when $p < 0.05$ and a trend (t) when $p < 0.1$.
* Two-Way ANOVA and HSD *post hoc* test.
** Kruskal-Wallis rank sum test.

significantly in the LHUFA from 10 weeks of feeding to after 3 weeks of UCS ($p < 0.05$). After UCS, the number of MHCII-positive cells was significantly higher compared HDHA ($p < 0.01$) and HEPA ($p < 0.05$).

3.5. Intestinal barrier and transport functions

The results show that the hindgut barrier was not significantly affected by the dietary groups, but UCS significantly reduced the intestinal TER ($p < 0.001$) (Fig. 5), and the reduction was significant comparing the feeding period to the UCS period in groups LHUFA ($p < 0.001$) and HDHA ($p < 0.05$).

Intestinal transport functions, evaluated by the intestinal short circuit current (SCC), transepithelial potential (TEP) and active transport of L-lysine, was significantly affected by UCS (Fig. 6). The results show an increase in SCC ($p < 0.001$), TEP ($p < 0.001$) and L-lysine transport ($p < 0.001$) across all dietary groups. SCC was significantly increased comparing the feeding period to the UCS period for LHUFA ($p < 0.05$), HDHA ($p < 0.001$) and HEPA ($p < 0.001$). TEP was significantly increased comparing the feeding period to the UCS period for LHUFA ($p < 0.05$), HDHA ($p < 0.001$) and HEPA ($p < 0.001$). L-lysine transport was significantly increased comparing the feeding period to the UCS period increased in HDHA ($p < 0.05$).

3.6. Hindgut gene expression

The hindgut transcription was run through a GSEA pathways analysis using KEGG. The data presented represent a summary of enriched pathways organized under different KEGG based categories: "Metabolism", which is a large category of reactions that sustain life in the organism and include sub-categories like energy metabolism, carbohydrate metabolism, amino acid metabolism and lipid metabolism among others. "Genetic information processing" represents pathways related to protein metabolism, like translation, transcription, replication and repair, protein folding, – sorting and - degradation. "Environmental information processing" represent pathways in membrane transport, signal transduction, signal molecules and signal interaction. "Cellular process" represents pathways in cellular transport, catabolism, growth, death, and community. "Organismal system" represents pathways in the immune system, endocrine system, and circulatory system. A selected number of pathways are mentioned here in the results and the complete

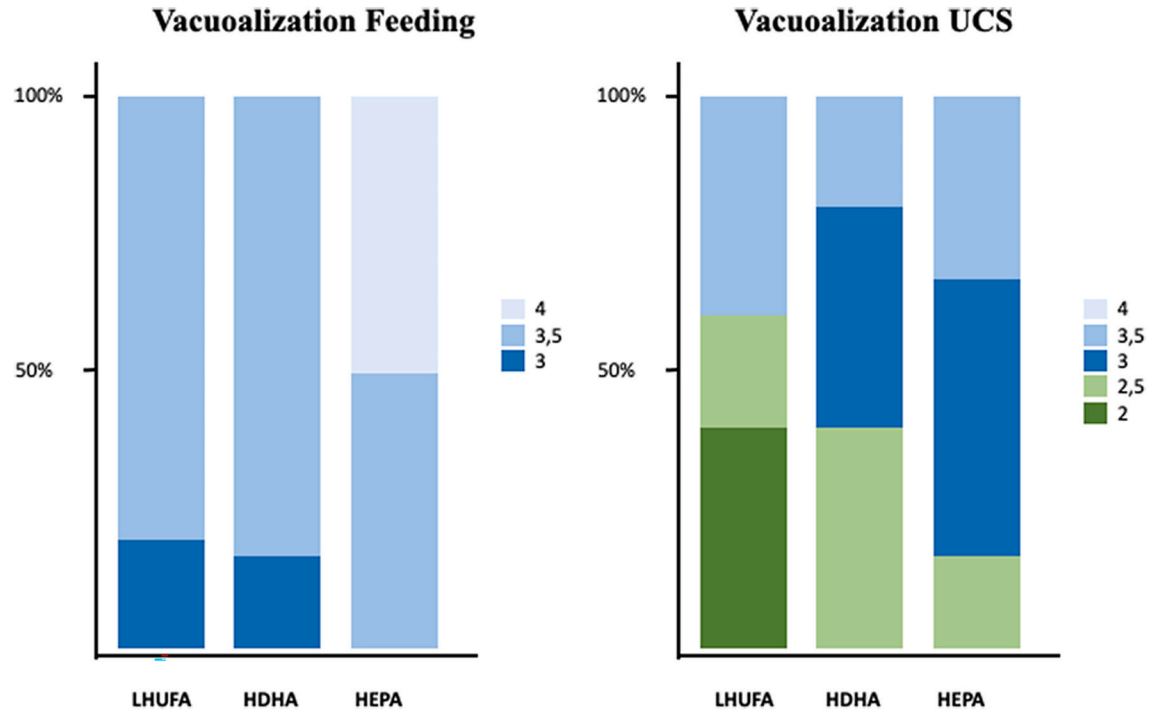

Fig. 3. Scoring of hindgut vacuolization after 10 weeks of feeding and 3 weeks of UCS. Scoring from 1 to 4 (with half-scores) where 1 represent low density of vacuoles and 4 represent high density.

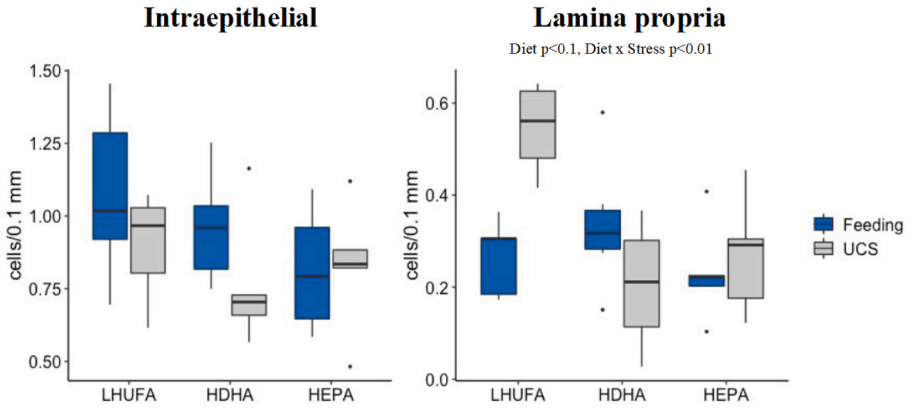

Fig. 4. Hindgut density of positive MHCII cells (cells/0.1 mm) in the intraepithelial space and lamina propria. Dietary differences were analyzed by One-Way ANOVA after 10 weeks of feeding or 3 weeks of UCS. Effects of stress and interaction between stress and groups were analyzed by Two-Way ANOVA and HSD *post hoc* test. Differences were considered significant when $p < 0.05$.

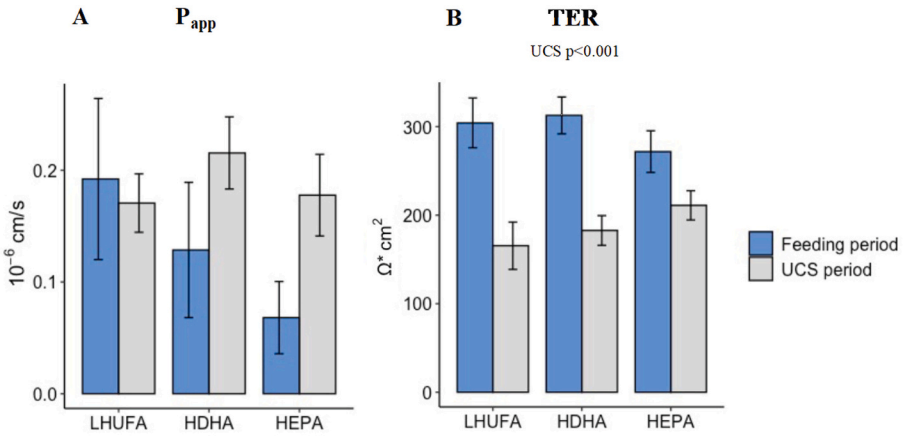

Fig. 5. Hindgut intestinal barrier function measured by P_{app} (A) and TER (B) after 10 weeks of feeding (blue fill) and 3 weeks of UCS (grey fill). Differences between dietary groups at the two sampling times were analyzed by One-Way ANOVA. Effects of stress and interaction between stress and groups were analyzed by Two-Way ANOVA and HSD *post hoc* test. Differences were considered significant when p < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

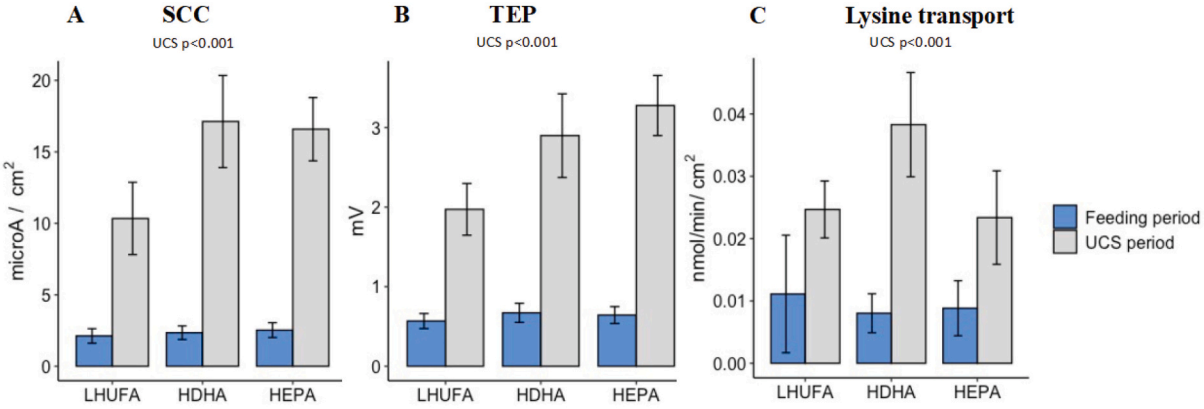

Fig. 6. Hindgut intestinal transport function measured by SCC (A), TEP (B) and L-Lysine transport (C) after 10 weeks of feeding (blue fill) and 3 weeks of UCS (grey fill). Differences between dietary groups at the two sampling times were analyzed by One-Way ANOVA. Effects of stress and interaction between stress and groups were analyzed by Two-Way ANOVA and HSD *post hoc* test. Differences were considered significant when p < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

list of all enriched pathways under each category are presented in Supplementary Tables 1–3.

3.7. Gene expression LHUFA

LHUFA hindgut gene expression was compared to both the HDHA and HEPA groups after 10 weeks of feeding and 3 weeks of UCS (Fig. 7). After feeding, the analysis resulted in a total of 14 enriched pathways between LHUFA and HDHA and 45 enriched pathways between LHUFA and HEPA. After UCS, the number of enriched pathways between LHUFA and HDHA increased to 43, and between LHUFA and HEPA decreased 34 (Fig. 8).

There were minor differences in gene expression between groups LHUFA and HDHA (Fig. 7A). In the HDHA group the larges upregulated

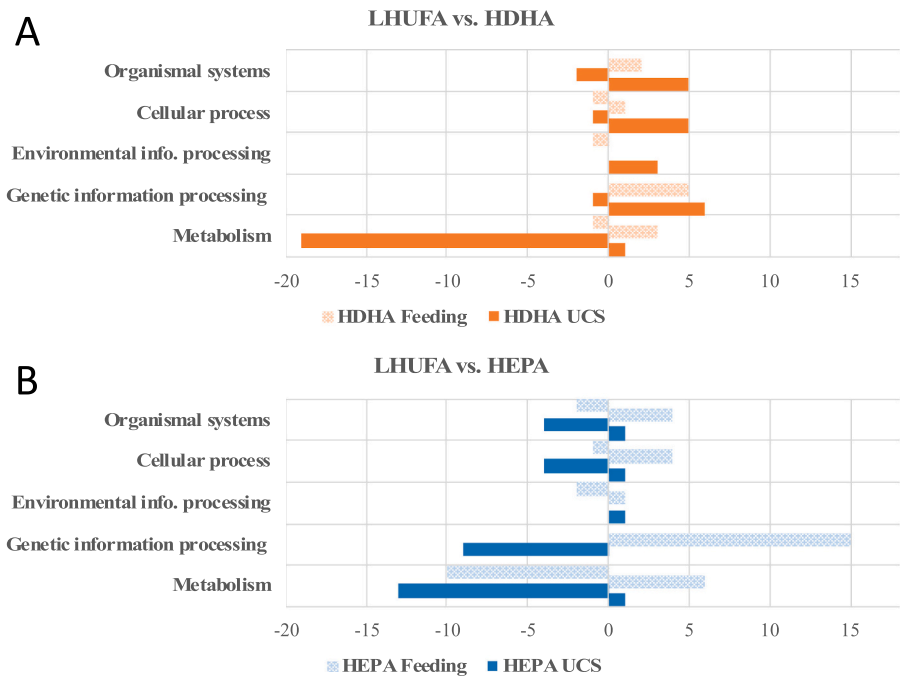

Fig. 7. Gene expression in the LHUFA group compared to HDHA (A) and HEPA (B) after 10 weeks of feeding (light blue textured fill) and 3 weeks of UCS (dark blue solid fill). Gene expression presented as GSEA where enriched pathways (up- and down-regulated as positive and negative on x-axis) were organized into KEGG categories; "organismal systems", "cellular process", "environmental information processing", "genetic information processing" and "metabolism". Complete list of enriched pathways can be found in Supplementary Tables 1 and 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

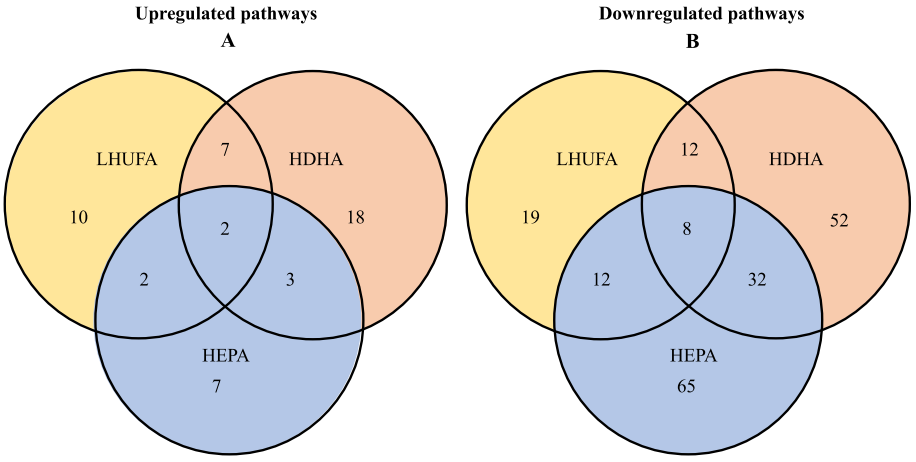

Fig. 8. Venn diagrams with the number of enriched pathways after 3 weeks of UCS in the three dietary groups LHUFA (yellow), HDHA (orange) and HEPA (blue). The two diagrams are divided between upregulated pathways (A) and downregulated pathways (B). Overlaps show enriched pathways shared by groups. Complete list of enriched pathways can be found in Supplementary Tables 1 and 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

category was 5 pathways in "Genetic information processing", indicating more protein metabolism. However, under the category "Metabolism" there were only 3 upregulated pathways, including oxidative phosphorylation. After 3 weeks of UCS there was a clear difference in gene expression related to "Metabolism" between the two group, with 19 downregulated pathways in the HDHA group. Downregulated metabolic pathways include oxidative phosphorylation, carbon metabolism, glycolysis, FA metabolism, FA biosynthesis, biosynthesis of unsaturated FAs and biosynthesis of amino acids. Further, in the HDHA group there was an increase of upregulated pathways related to "Genetic information processing" (6 pathways), "Environmental information processing" (3 pathways), "Cellular process" (5 pathways) and "Organismal systems" (5 pathways). All the upregulated pathways under "Organismal systems" represent immune pathways, with Toll-like-, NOD-like-, RIG-I-like and C-type lectin receptor signaling systems and cytosolic DNA-sensing pathways.

The difference in gene expression between LHUFA and HEPA after 10 weeks of feeding was greater (Fig. 7B), with a high upregulation of "Genetic information processing" (15 pathways) in the HEPA group, clearly indicating higher protein metabolism. Further, there was a high difference in "Metabolism" enriched pathways, with 6 upregulated and 10 downregulated pathways in HEPA group. The upregulated metabolic pathways include oxidative phosphorylation, fatty acid metabolism and amino acid metabolism, supporting higher protein metabolism and elongation of fatty acids in HEPA group. Four pathways were upregulated in "Cellular process", indicating higher cell turnover, and 4 pathways were upregulated in "Organismal systems". The latter pathways were mainly upregulation of the circulatory system. After UCS, several of the trends in pathway enrichment were reversed. "Genetic information processing" was downregulated with 9 pathways and "Metabolism" with 13 pathways in the HEPA group. Downregulated metabolic pathways include oxidative phosphorylation, carbon metabolism, TCA cycle, FA metabolism, FA biosynthesis. "Cellular process" was also downregulated with 4 pathways, and "Organismal system" with 4 pathways in the HEPA group. The latter includes three immune pathways, NOD-like and RIG-I-like receptor signaling pathway and Cytosolic DNA sensing pathway.

Subjecting the LHUFA group to 3 weeks of UCS caused the

downregulation of 10 "Metabolism" pathways, mainly in carbohydrate metabolism (6 pathways, Fig. 9A). In all other categories the response to UCS was low, with 3 downregulated and 4 upregulated pathways in "Genetic information processing ". In "Cellular process" 4 pathways were downregulated, including focal adhesions and tight junctions. In "Organismal system" 4 pathways were upregulated after UCS, including immune-related pathways Cytosolic DNA-sensing pathway and intestinal immune network for IgA production.

3.8. Gene expression HDHA and HEPA

The HDHA and HEPA groups gene expression were compared and resulted in 24 enriched pathways after 10 weeks of feeding and 34 enriched pathways after 3 weeks of UCS exposure (Fig. 10). After the feeding period, the main difference between hindgut gene expression was a clear upregulation of "metabolism" (12 pathways) in HDHA and upregulation of "genetic information processing" (7 pathways) in HEPA (Fig. 10A). In addition, the pathway amino acid biosynthesis was upregulated in HEPA, and together with a high number of "genetic information processing" this indicates higher protein turnover in the HEPA group. After UCS (Fig. 10B), there were few differences in "metabolism" pathways (2 upregulated in both diets) but upregulated in HDHA included Oxidative phosphorylation and TCA cycle. Further, a clear upregulation of "genetic information processing" was observed in HDHA (10 pathways). In addition, 6 pathways in "cellular process" and 6 in "organismal systems" were upregulated in HDHA. In the latter category, all were related to immunity. In the HEPA group the category "environmental information processing" was upregulated compared to HDHA with 4 pathways.

Considering the gene expression for the individual groups after UCS, there were 70 enriched pathways (18 up and 52 down) in the HDHA group and 72 enriched pathways (7 up and 65 down) in the HEPA group (Fig. 8). Of these pathways, 35 were shared between the HDHA and HEPA group (3 up and 32 down). In HDHA (Fig. 9B), 3 weeks of UCS resulted in a strong downregulation in "metabolic" pathways (45 pathways), including sub-categories like energy, carbohydrate, lipid, and amino acid metabolism. There was also a clear upregulation of 6 immune pathways ("organismal systems") and upregulation of three pathways in the sub-category cell growth and death ("cellular process"). In HEPA (Fig. 9C), 3 weeks of UCS also resulted in a strong downregulation of "metabolic" pathways (37 pathways), including sub-categories like carbohydrate, lipid, and amino acid metabolism. In addition, "genetic information processing" was downregulated with 16 pathways and "cellular process" with 9 pathways. The latter showing lower cell turnover in the hindgut. Upregulated pathways in the HEPA group were mainly related to environmental information processing (6 pathways).

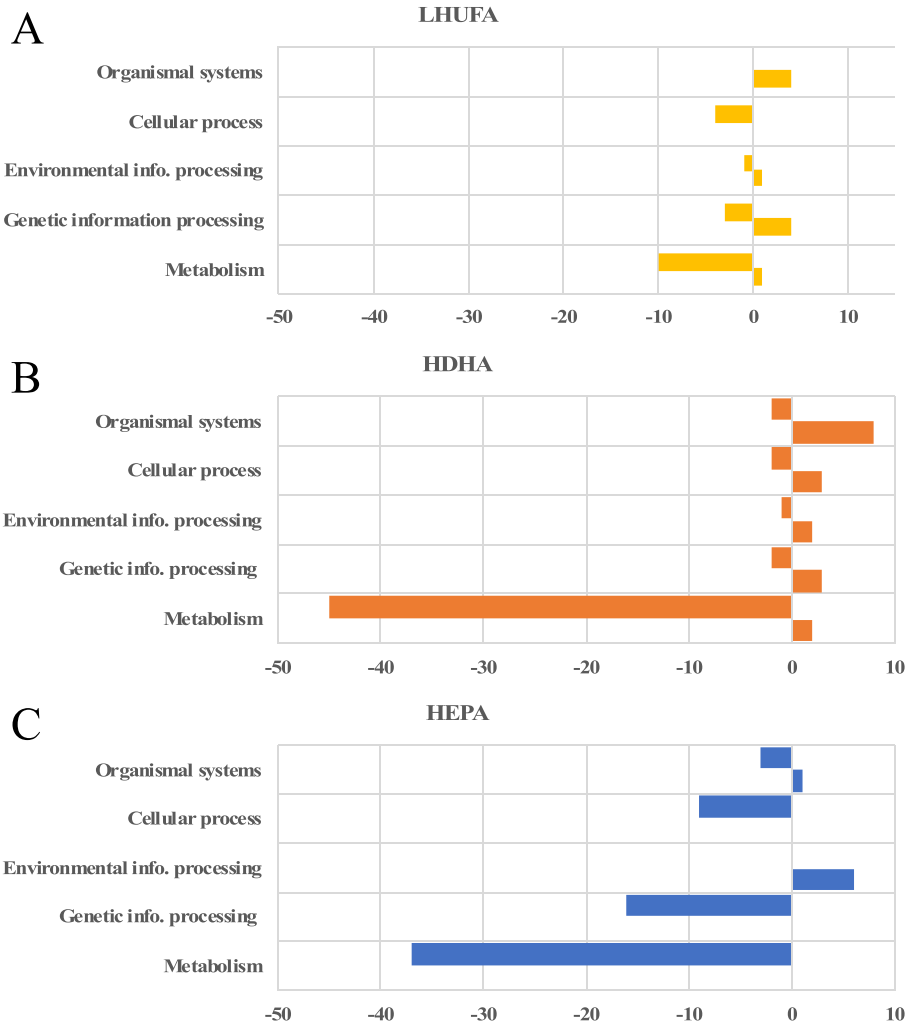

Fig. 9. Changes to gene expression in groups LHUFA (A, yellow), HDHA (B, orange) and HEPA (C, blue) after 3 weeks of UCS. Gene expression presented as GSEA where enriched pathways (up- and down-regulated as positive and negative on x-axis) are organized into KEGG categories; "organismal systems", "cellular process", "environmental information processing", "genetic information processing" and "metabolism". Complete list of enriched pathways can be found in Supplementary Table 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

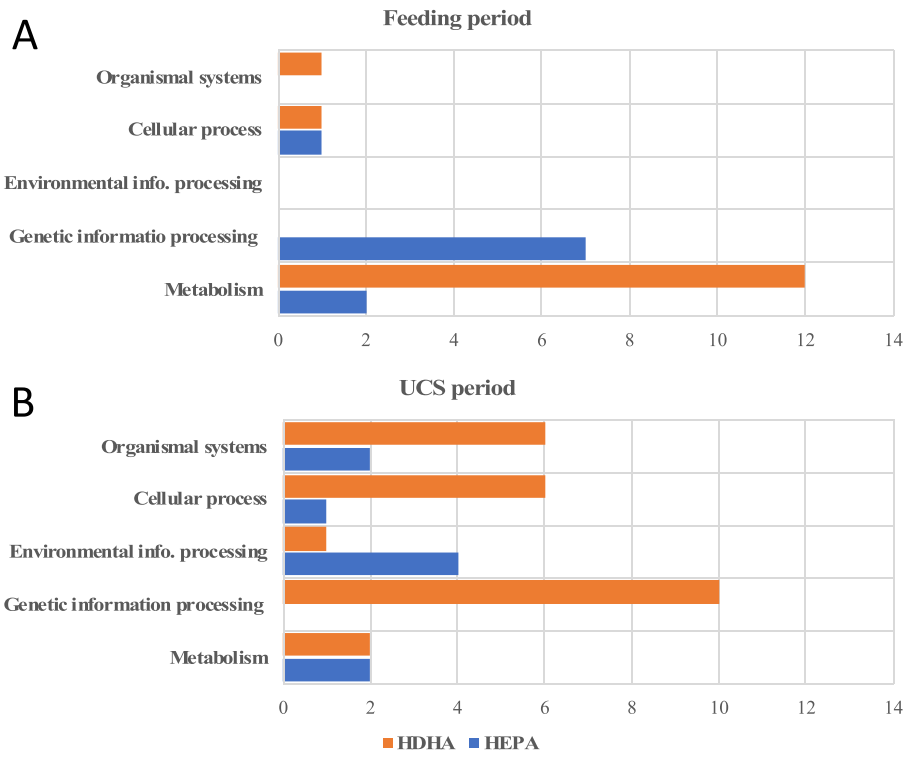

Fig. 10. Comparing gene expression between the dietary groups HDHA (orange) and HEPA (blue) after 10 weeks of feeding (A) and 3 weeks of UCS (B). Gene expression presented as GSEA where enriched pathways (up- and downregulated as positive and negative on x-axis) are organized into KEGG categories; "organismal systems", "cellular process", "environmental information processing", "genetic information processing" and "metabolism". Complete list of enriched pathways can be found in Supplementary Tables 1 and 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Low HUFA impact on intestinal health

In the first part of this study the intestinal health of the low HUFA fed group (EPA + DHA content of 8.5 g/kg feed and 4.5% of total FAs) was assessed before and after a period of chronic stress. The diet represent commercial diets with lower levels of EPA and DHA (g/kg) (Sele et al., 2019). Under normal feeding conditions there were no obvious negative effects on hindgut health as viewed through polar lipid FA composition, morphology, and integrity. Gene expression did however indicate a lower energy utilization in the LHUFA group compared to the high HUFA groups. Challenging the fish to 3 weeks of chronic stress had on the other hand a notable detrimental effect on the intestinal health in the LHUFA group, with signs of intestinal inflammation, reduced intestinal integrity and a lowered transcriptional response to stress.

In this study no reductions in growth performance, intestinal damage or increased inflammation under the feeding period was observed when comparing the HUFA diets. The dietary FA composition was clearly influenced by the polar lipid FA composition, but within a narrower range. Salmonids have a distinctive ability to upregulate HUFA incorporation and adjust lipid metabolism to compensate when dietary levels are low (Betancor et al., 2017; Olsen et al., 2003; Olsen et al., 1991; Olsen and Ringø, 1992; Oxley et al., 2010; Tocher et al., 2000). This is clearly seen through the higher levels of ARA, DHA and DPA synthesized from ALA and LA, giving a similar level of DHA in the LHUFA group as in the HEPA group. The fish was thus fully capable to maintain membrane and epithelial integrity in the LHUFA group. Salmonids have a limited capacity to synthesize HUFAs from precursors linolenic acid (LA, 18:2n-6) and alpha linolenic acid (ALA, 18:3n-3), but it is not sufficient to maintain good health and growth (Morais et al., 2009; Ruyter and Thomassen, 1999; Sprague et al., 2019; Tocher et al., 2000). Therefore, and in line with the limited availability of fish oils, there has been a considerable research activity into estimations of the minimum dietary requirement for HUFA in Atlantic salmon. Results are however variable and inconsistent. The intestinal tissue will maintain a minimum level of DHA even when the fish are fed a diet totally defiant of n3 HUFA, however with a clear negative impact on the fish health (Ruyter and Thomassen, 1999; Thomassen et al., 2017). Satisfactory growth has been achieved with levels of EPA + DHA down to 1–5 g/kg (Betancor et al., 2014; Bou et al., 2017a; Bou et al., 2017a; Dantagnan et al., 2017; Glencross et al., 2014). However, most studies point to levels around 10–15 g/kg diet for good growth and fish health (Bou et al., 2017b; Bou et al., 2017a; Xue et al., 2020).

The intestinal barrier and transport functions represent changes in "the flux of solutes and fluids across the epithelium", where TER reflects the paracellular permeability and tight junction integrity (Odenwald and Turner, 2013). After 3 weeks of chronic stress, the impairment of the intestinal integrity was evident in all diets by the increase in intestinal permeability (decreased TER) and higher net ion flux across the intestinal barrier (increased SCC and TEP). The reduction in intestinal integrity under chronic stress and inflammation is well documented in Atlantic salmon (Sundh et al., 2019; Sundh et al., 2010; Sundh et al., 2009). Stress can increase the permeability by opening and disrupting tight junctions in fish (Olsen et al., 2002; Sundell and Sundh, 2012) and create leakages of ions across the epithelium. The increase in SCC indicates increased net ion transport, presumable to compensate for the increased paracellular leakage (Sundell and Sundh, 2012). Comparing low HUFA to high, the LHUFA group responded to chronic stress in a manner similar to the high HUFA groups but with a significantly lower increase in SCC and TEP. This shows that the LHUFA group have a reduced capacity to counterbalance the resulting leaky gut generated from chronic stress.

Chronic stress had few effects on the intestinal morphology in the LHUFA groups, except for tendencies of lower vacuolization compared to the HEPA group. The results do however suggest an increased inflammatory load in salmon hindgut which agrees with the increased density of MHCII cells and upregulated immune pathways. Under the feeding period, LHUFA gene expression showed relatively few enriched pathways compared to the high HUFA groups, and most of the enriched pathways appeared to be more related to dietary HUFA sources than differential stress effects. The issues of EPA *vs.* DHA are discussed

separately below. However, we did note a distinct upregulation of protein and energy metabolism in the high HUFA group compared to the LHUFA group. Gene expressions following chronic stress showed a shift in gene expression towards metabolic downregulation and a shut-down of energy consumption in the hindgut of all dietary groups, but at a considerably lower rate in the LHUFA groups. This further strengthens the observations that the LHUFA group could not maintain a sufficient transcriptional response during chronic stress. This lack of metabolic responses may be related to a subclinical HUFA deficiency as suggested in Atlantic salmon midguts (Løvmo et al., 2021). As a farmed Atlantic salmon is likely to endure several stressful and potentially harmful treatments during the seawater phase this result could be of highly relevance for all fish sizes, and more research should be done on monitoring the intestinal integrity at all growth stages.

4.2. High DHA and EPA impact on intestinal health

The essential FAs DHA and EPA are often not differentiated upon when studying effects of dietary n3 HUFA on Atlantic salmon (Betancor et al., 2017; Sissener et al., 2016). The reason being that in marine oils both fatty acids are present in fairly equal amounts. However, with new technologies and products appearing it is now possible to create diets where only one of these fatty acids are dominating. This gives the possibility to tailor EPA/DHA ratios optimized to the need of the fish (Betancor et al., 2017; Betancor et al., 2015). In the second part of this study, the impact of high dietary level of either EPA or DHA on hindgut intestinal health was tested. The two diets have a higher EPA + DHA % of total fatty acids compared to commercial diets (Sele et al., 2019). The results indicate higher immune activity and possibly inflammation in the HDHA group and a difference in energy utilization between the two groups at the transcriptome level. While some studies have suggested a that inclusion of DHA alone is enough to satisfy the Atlantic salmon HUFA demand (Emery et al., 2016; Turchini et al., 2014) this study highlights the need for more knowledge of the individual role of the FAs, and that inclusion of dietary EPA can have several beneficial effects when Atlantic salmon are exposed to a challenging environment.

After the feeding period there was no physical difference in intestinal health between the two groups, except for a higher vacuolization in fish fed HEPA. After chronic stress the vacuolization was reduced in all groups with no difference between the HDHA and HEPA groups. The supranuclear vacuoles have been suggested to have a function in uptake and transport of macromolecules from the intestinal lumen to the portal vein in fish (Mclean and Ash, 1987; Sire and Vernier, 1992; UrÁn et al., 2008). A reduction of supranuclear vacuole density and transport function has previously been seen in relation to feed induced intestinal enteritis and inflammation (Baeverfjord and Krogdahl, 1996; De Santis et al., 2015; Knudsen et al., 2007; UrÁn et al., 2008). The results could therefore indicate compromised intestinal health in the HDHA group. In addition, chronic stress reduced the height of mucosal folds in the HEPA group and enterocytes in the HDHA group. Both can be viewed as signs of intestinal inflammation and a results of chronic stress (De Santis et al., 2015; Knudsen et al., 2007; Ringø et al., 2014). The effect of stress on the intestinal morphology were not as severe as one might expect from results of feed induced inflammation, however, this corresponds to Olsen et al., 2002 showing that acute stress had low impact on hindgut intestinal epithelia in Atlantic salmon. Low grade inflammation has also previously been observed after a chronic stress in Sundh et al., 2010 and Sundh et al., 2019.

The immune status of the intestine is interesting as DHA and EPA are precursors to important immune system mediators, like resolvins, protectins and maresins, resolving inflammation and maintaining homeostasis (Calder, 2015; Tocher, 2015). DHA is often regarded as more anti-inflammatory of the two (Serhan et al., 2008) In addition, chronic stress and prolonged elevation of cortisol is related to several mammalian inflammatory gastrointestinal diseases, like peptic ulcer, functional dyspepsia, inflammatory bowel disease and irritable bowel syndrome (Konturek et al., 2011). Chronic stress is also suggested to lead to intestinal inflammation in Atlantic salmon (Niklasson et al., 2011; Sundh et al., 2009), possibly related to disruption of the intestinal microbiome (Webster et al., 2019). A difference in immune-gene expression was evident between the two groups, as HDHA had a clear upregulation of several immune pathways compared to both the HEPA and LHUFA groups. Together with the higher vascularization in the HEPA group this further indicates that the HDHA group intestinal health was more affected by chronic stress resulting in higher inflammation in the intestine. The result from the present study suggests that DHA does not contribute to the anti-inflammatory action combined with EPA, opening for the possibility that EPA may be required in considerable amounts in fish diets.

Gene expressions also showed a clear difference in energy utilization between the two groups under the feeding period, with a high protein metabolism in the HEPA group and a high metabolism in the HDHA group. This indicates that the HDHA fish need to activate more pathways for energy utilization compared to the HEPA group. Moreover, this possibly reflects the role of EPA as an energy reserve that can more readily be used compared to DHA. Feeding Atlantic salmon with a high levels of a combination of DHA + EPA (12.6% of total FA) resulted in only mild effects on metabolism in hindgut transcriptome (Betancor et al., 2017), showing that the results are clearly related to the dietary levels of DHA and EPA.

To some extent, the transcriptome responded similarly to chronic stress in the two high HUFA groups with metabolic downregulation and protein turnover, showing a hindgut transcriptome that is more or less turned off under chronic stress. Compared to gene expression in Atlantic salmon parr hindgut subjected to chronic stress (Løvmo et al., 2020), the response was more severe in post-smolts. In parr, gene expression showed upregulation of apoptotic pathways, and reduced cell repair and immune capacity, but it was also suspected that the parr were starting to habituate to stress at the end of the experiment. The difference between the transcriptome level response from parr to post-smolt could be due to the large differences between the two Atlantic salmon stages and the extreme intestinal development of the intestine when moving from freshwater to saltwater (McCormick and Saunders, 1987). However, this could also be because the fish in this study was not habituated to the UCS protocol.

Previously, feeding high levels of DHA to Atlantic salmon under chronic stress caused an increased thickness to the midgut intestinal muscularis (Løvmo et al., 2021), and the results here show that the same response is seen in the hindgut during chronic stress. Further research is needed on the link between DHA and the impact on intestinal muscle, but studies on mammals have shown a positive link between dietary DHA and muscle growth (Ochi and Tsuchiya, 2018; Wei et al., 2013). Additionally, the results show that the thickness of the intestinal muscle layer does not affect the measured P_{app} when using the Ussing technology in fish. In contrast to salmon midgut during chronic stress (Løvmo et al., 2021), the hindgut barrier transport of L-Lysine increased under chronic stress and significantly so in the HDHA group. Considering that the pyloric and midgut sections are generally considered the main sites for amino acid absorption (Loretz, 1995), the results suggest that the increased transport is a result of the increased permeability and passive diffusion across the epithelium.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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