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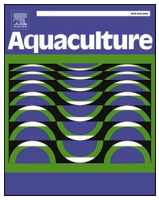

Effects of dietary lipid level on growth, digestive physiology and disease resistance in lumpfish (*Cyclopterus lumpus*)

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ABSTRACT

Lumpfish (*Cyclopterus lumpus*) aquaculture has expanded greatly in recent years due to demands for sea lice cleanerfish from the salmon industry. There are knowledge gaps in lumpfish digestive physiology, nutrient requirement and implications of nutrition for health and disease susceptibility. The present study, conducted to follow up our recent screening trials for estimation of optimal balance of protein, lipid and carbohydrate in diets for lumpfish, involved challenging the fish with *Aeromonas salmonicida* after a feeding period with diets varying in lipid composition. Three experimental diets were formulated to have similar content of digestible protein and carbohydrate but varying in content of lipid from 6.7 to 18%. Lumpfish with average body weight at start of 1.7 ± 0.03 g were fed the experimental diets in triplicate tanks each (110 fish per tank, in total 990 fish in 9 tanks) for a period of 90 days. After termination of the feeding trial and subsequent collection of biological samples, remaining fish were challenged with atypical *A. salmonicida.* No significant effects of diet were observed for growth performance. Carcass composition showed increasing content of lipid, protein, and energy with increasing dietary lipid level. Increasing dietary lipid also increased hepatic dry matter, lipid and energy levels, while crude protein decreased. Blood plasma nutrient levels and biomarkers of liver function showed few significant effects of diet, but dietary lipid level increased plasma cholesterol. Intestinal trypsin activity increased with increasing dietary lipid, whereas activity of other digestive enzymes and digesta bile salt levels were unaffected by diet. Increasing lipid level also increased lipid accumulation in the proximal and mid intestine. Expression profiling of genes related to digestive and immune function showed few effects of diet, but the nutrient transporters *fabp2* and *slc15a1*, as well as the immune genes *MHCII, igm*, and *nfkb* showed increases with dietary lipid levels, whereas the cholesterol transporter *npc1l1* was suppressed. Diet composition did not affect the lumpfish' resistance against *A. salmonicida*. To conclude, the variation in macronutrient composition induced modulations in metabolic, digestive and some immune functions. Modulations seemed however to be within normal ranges and did not produce clear compromises in immune responses to bacterial infection.

1. Introduction

The infestation by salmon louse (*Lepeophtheirus salmonis*) is a great challenge in Atlantic salmon production (Forseth et al., 2017; Kristoffersen et al., 2017). Lumpfish (*Cyclopterus lumpus*) has become an important species for co-culture with Atlantic salmon because of its capacity to remove sea lice, offering a biological alternative to chemicals and mechanical solutions (Stien et al., 2020). Initially, wild caught lumpfish were used. However, wild stocks are limited and may carry pathogens causing diseases, e.g., amoebic gill disease (Haugland et al., 2017), in the lumpfish as well as in the salmon they are meant to protect. Cultivated lumpfish have therefore become the most commonly used, and at present, lumpfish production is the third largest fish production in Norway with an annual production number of about 30 million

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Table 1
Diet levels of feed ingredients and estimated chemical composition.

	L	M	H
Ingredients (g/kg):			
Fish meal[1]	366.2	362.9	387.6
Wheat gluten[2]	183.1	181.5	193.8
Wheat meal[3]	188.0	125.0	40.0
Fish oil[4]	5.0	73.0	120.9
Codfish powder[5]	100.0	100.0	100.0
Krill hydrolysate[6]	20.0	20.0	20.0
Krill meal[7]	40.0	40.0	40.0
Krill oil[8]	10.0	10.0	10.0
Vitamin premix[9]	30.0	30.0	30.0
Organic mineral premix [10]	8.4	8.4	8.4
Mono sodium phosphate[11]	24.0	24.0	24.0
Biomos[12]	4.0	4.0	4.0
Cholesterol[13]	5.0	5.0	5.0
Choline chloride[14]	5.0	5.0	5.0
L-lysine[15]	6.0	6.0	6.0
Taurine[16]	2.0	2.0	2.0
Stay-C[17]	2.2	2.2	2.2
Carphyll pink (10% AX)[18]	1.0	1.0	1.0
Yttrium oxide[19]	0.1	0.1	0.1
Chemical composition			
Analyzed values, %			
Dry matter	90.5	92	91.4
Protein	54.1	52.7	53.3
Lipid	6.7	13.7	18
Ash	11.2	11.4	11.8
Starch	16	11.7	6.6

L, low lipid diet (6.7% lipid); M, medium lipid diet (11.7% lipid); H, high lipid diet (18% lipid).

[1] Fishmeal, Norse-LT, Vedde AS, Langevåg, Norway.
[2] Wheat gluten, Amytex 100, Tereos Syral, Aalst, Belgium.
[3] Wheat meal, Norgesmøllene AS, Bergen, Norway.
[4] Fish Oil, NorSalmOil, Pelagia, Egersund, Norway.
[5] Codfish powder, Seagarden AS, Avaldsnes, Norway.
[6] Krill 25ydrolysate, Rimfrost AS, Ålesund, Norway.
[7] Krill meal, Rimfrost AS, Ålesund, Norway.
[8] Krill oil, Aker BioMarine, Lysaker, Norway.
[9] Vitamin premix (per kg diet), 0.5% Nofima vitamin premix, Vilomix, Hønefoss, Norway. Vitamin A, 3000 IU; vitamin D3, 3800 IU; vitamin E, 300 mg; vitamin K3, 30 mg; vitamin B1, 30 mg; vitamin B2, 45 mg; vitamin B6, 38 mg; vitamin B12, 0.08 mg; niacin, 300 mg; Ca-D-pantothenate, 90 mg; biotin, 1.5 mg; folic acid, 15 mg; vitamin C, 300 mg.
[10] Mineral premix (per kg diet), 0.5% Nofima mineral premix, Vilomix, Hønefoss, Norway. Fe,60 mg; Mn, 30 mg; Zn, 130 mg; Cu, 6 mg; I, 5 mg; Co, 0.05 mg; Se, 0.3 mg.
[11] MSP (26% P), Mono sodiumphosphate, delivered by Vilomix, Hønefoss, Norway.
[12] Biomos, Alltech Norway AS, Førde, Norway.
[13] Cholesterol, Carbogen Amics B.V., Veenendaal, The Netherlands.
[14] Choline chloride, delivered by Vilomix, Hønefoss, Norway.
[15] L-lysine, delivered by Vilomix, Hønefoss, Norway.
[16] Taurine, VWR, Oslo, Norway.
[17] Stay-C 35%, delivered by Vilomix, Hønefoss, Norway.
[18] Carophyll pink (10% astaxanthin), delivered by Vilomix, Hønefoss, Norway.
[19] Yttrium oxide. Y_2O_3 (99.9%), delivered by Vilomix, Hønefoss, Norway.

individual fish (Stien et al., 2020). However, for successful cultivation of a new species, a solid knowledge basis regarding key physiological and health related aspects, and not in the least, nutrient supply required for normal function and disease resistance must be built. Regarding lumpfish, knowledge on nutrient requirement has substantial holes, and knowledge from other fish species cannot be expected to be relevant, at least not for a species with very different body composition than most fish for which nutrient requirements have been described so far (Council, N.R, 2011; Willora et al., 2021). We recently published findings from the first study to estimate optimal balance between

Table 2
Fish weights (g). length (mm). growth rates and mortality (% of wet basis, mean ± s.e.m., $n = 3$).

	L	M	H	*p*-value
Weight, day 0	1.73 ± 0.01	1.73 ± 0.02	1.71 ± 0.01	0.57
Weight, day 90	61 ± 4.2	62 ± 0.9	59 ± 2.6	0.76
Length1, day 90	90 ± 1.3	92 ± 0.4	90 ± 1.5	0.45
Length2, day 90	98 ± 1.3	104 ± 0.9	101 ± 2.2	0.10
SGR. % d^{-1}	3.9 ± 0.09	3.9 ± 0.01	3.9 ± 0.07	0.94
TGC	2.8 ± 0.10	2.9 ± 0.02	2.8 ± 0.07	0.87
Mortality. %	20 ± 2.2	22 ± 4.5	26 ± 2.0	0.4

L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%); Length 1, body length without caudal fin; Length 2, total length with caudal fin included.

Table 3
Chemical content in carcass ((% of wet basis, mean ± s.e.m., n = 3).

	L	M	H	p-value
Dry matter, %	11.3 ± 0.03^{c}	12.2 ± 0.1^{b}	12.9 ± 0.2^{a}	<0.0001
Ash, %	1.7 ± 0.03	1.7 ± 0.0	1.7 ± 0.0	0.42
Lipid, %	1.6 ± 0.00^{c}	2.4 ± 0.1^{b}	3.1 ± 0.1^{a}	<0.0001
Crude protein, %	7.8 ± 0.03^{b}	8.0 ± 0.1^{ab}	8.1 ± 0.1^{a}	0.04
Energy, MJ / kg	2.4 ± 0.01^{c}	2.7 ± 0.1^{b}	3.0 ± 0.1^{a}	0.006

L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%). Values with different superscript letters within the same row denotes significant difference ($p < 0.05$), $n = 3$.

Table 4
Chemical content in liver (mean ± s.e.m., n = 3).

	L	M	H	p-value
Dry matter, %	44.4 ± 0.3^{c}	46.4 ± 0.2^{b}	49.6 ± 0.1^{a}	< 0.0001
Ash, %	2.2 ± 0.2	2.3 ± 0.03	2.3 ± 0.1	0.85
Lipid, %	29.7 ± 0.3^{c}	32.7 ± 0.1^{b}	36.4 ± 0.2^{a}	< 0.0001
Crude protein, %	9.9 ± 0.1^{a}	9.8 ± 0.1^{a}	9.2 ± 0.1^{b}	0.01
Energy, MJ/kg	14.6 ± 0.1^{c}	15.7 ± 0.04^{b}	17.0 ± 0.1^{a}	< 0.0001

L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%). Values with different superscript letters within the same row denotes significant difference ($p < 0.05$).

macronutrients in diets for lumpfish (Hamre et al., 2022). Obtained results indicated that, based on observation of effects on growth rate and immune function biomarkers, the diet for lumpfish weighing 10–50 g should contain approximately 55% protein, minimum 10% lipid, and maximum 10% carbohydrate.

Dietary lipid plays vital roles in fish as they are the source of essential fatty acids, phospholipids, sterols (Oliva-Teles, 2012). Dietary lipid is a non-protein energy source for fish. When the lipid to protein ratio was optimal, increased weight gain, feed untilization and nitrogen retention were observed (Welengane et al., 2019). Optimal lipid level also resulted in high lipase activity (Sivaramakrishnan et al., 2017) and digestibility (Hansen et al., 2008) and therefore improved growth performance. Notably, dietary lipid level should be kept in a certain range. In intestine, excessive dietary lipid caused accumulation of lipid droplets in enterocytes (Morais et al., 2006; Bonvini et al., 2015), implying the disorder of intracellular lipogenesis. Excessive dietary lipid also influence immune system. For instance, increased content of ω3 polyunsaturated fatty acids (ω3-PUFA) by increasing fish oil in diet resulted in inhibited expression of immune relevant genes (Calder, 2008; Teitelbaum and Walker, 2001). This may further lead to reduction of disease resistance (Zuo et al., 2012) and therefore is harmful to fish.

Mortality is high in lumpfish production and use, often due to infection with atypical *Aeromonas salmonicida* (Ronneseth et al., 2017; Kverme et al., 2022). The close relationship between nutrition, immune function, and disease resistance, is well documented for humans and for most other production animals than fish (Maggini et al., 2018). As

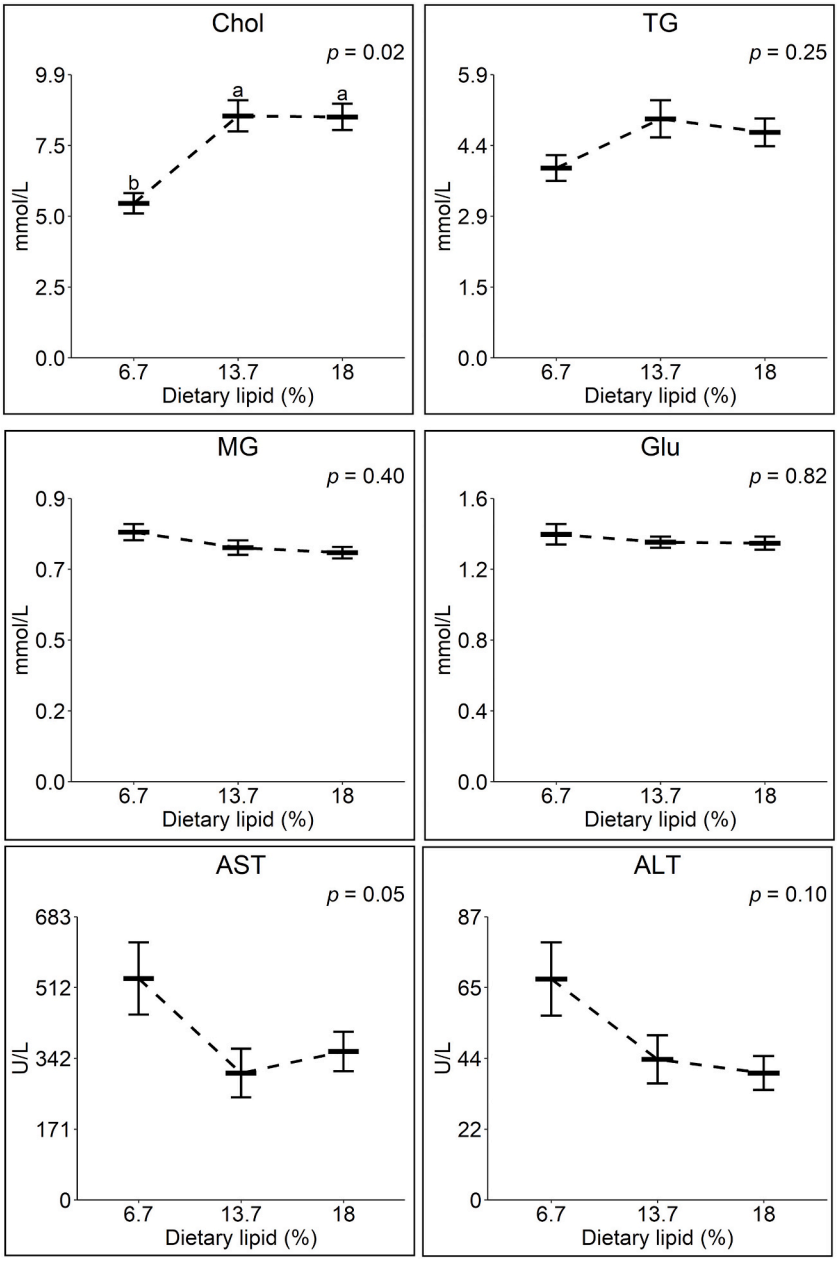

Fig. 1. Effects of dietary lipid variation (%) on blood plasma variables. Mean values are denoted as horizontal bars. Error bars indicates standard error. Dashed lines connecting means between groups are illustrated for understanding changing trends, without any statistical meanings.

nutrient requirement for optimal growth and optimal disease resistance is not necessarily the same (Wen et al., 2009; Wen et al., 2010), knowledge on both is necessary for formulation of optimal diets. Moreover, too high supply of certain nutrients may also be detrimental and should be investigated (Guo et al., 2017; Guo et al., 2018). Disease resistance should also be a criterion for estimation of nutrient requirement over a wide range of dietary inclusion levels, but no such information is available in the scientific literature for lumpfish. In the present study, the hypothesis was that high lipid level affects disease resistance, since high lipid level in diet was found to cause low expression of immune-relevant genes in our previous study (Zhou et al., 2022).The aim of the present work was to generate knowledge addressing effects of lipid level in the diet on disease resistance in lumpfish by challenging the fish with *A. salmonicida* after a feeding period with diets varying in lipid level.

2. Materials and methods

2.1. Diets

Three experimental diets were produced at Nofima's Aquafeed Technology Center in Bergen, Norway. The diets (Table 1) were formulated to have similar content of digestible protein and carbohydrate, and to vary only in level of digestible lipid. The varying level of lipid, 6.7, 13.7 and 18%, were chosen based on results regarding nutrient digestibilities from our previous studies on lumpfish (Hamre et al., 2022; Zhou et al., 2022). The particle sizes were adjusted accordingly to suit the fish size. During the challenge test a commercial diet (Lumpfish Grower, BioMar, 2.0 mm) was used.

Table 5
Brush border digestive enzyme capacity (EC) and specific activity (SA) in PC, MI, and DI (mean ± s.e.m., n = 3).

	Tissue	L	M	H	*p* value
Maltase SA (nmol/min/mg protein)	PC	4.6 ± 0.6	3.8 ± 0.6	7.0 ± 1.1	0.15
	MI	38 ± 3.3a	27 ± 2.7b	22 ± 1.5b	0.03
	DI	44 ± 3.5b	55 ± 2.6a	59 ± 3.3a	< 0.01
Maltase EC (μmol/min/kg fish)	PC	2.1 ± 0.3	2 ± 0.4	4.4 ± 0.7	0.08
	MI	1.9 ± 0.1	1.9 ± 0.1	1.8 ± 0.1	0.97
	DI	4.8 ± 0.3	5.2 ± 0.2	5.2 ± 0.3	0.78
LAP SA (μmol/h/mg protein)	PC	100 ± 5	92 ± 4.8	82 ± 5.4	0.31
	MI	268 ± 24[a]	209 ± 14[ab]	171 ± 12[b]	0.05
	DI	518 ± 43[b]	545 ± 30[ab]	613 ± 28[a]	0.04
LAP EC (mmol/h/kg fish)	PC	46 ± 1.7	48 ± 2.7	50 ± 2.7	0.52
	MI	13.1 ± 0.7	15.2 ± 0.8	14.5 ± 0.7	0.13
	DI	56 ± 2.5	51 ± 2.8	54 ± 2.4	0.78

L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%). Values with different superscript letters within the same row denotes significant difference ($p < 0.05$).

Table 6
Activity of trypsin and lipase and bile salt level in MI1. MI2 and DI (mean ± s.e.m., n = 3).

		Diet			
	Section	L	M	H	p value
Trypsin activity (U/mg dry matter)	MI1	7.6 ± 1.5	16.1 ± 0.9	16.5 ± 4.2	0.09
	MI2	5 ± 1.8	13.9 ± 3.4	16.2 ± 3.7	0.09
	DI	6.5 ± 1.4[b]	21.9 ± 1.6[a]	25.3 ± 2.1[a]	< 0.01
Lipase activity (U/mg dry matter)	MI1	1.7 ± 0	1.7 ± 0.3	1.6 ± 0.1	0.85
	MI2	1.6 ± 0.2	1.5 ± 0.2	1.4 ± 0.3	0.78
	DI	0.4 ± 0	0.4 ± 0.1	0.6 ± 0.1	NA*
Bile salt level (mg/g dry matter)	MI1	64.4 ± 11.7	72.5 ± 8	73.9 ± 3.9	0.71
	MI2	52.8 ± 7.9	50.4 ± 5.2	46.1 ± 3.2	0.72
	DI	6.8 ± 0.9	4.1 ± 1.3	2.6 ± 0.5	0.06

* The gut content of L group from DI was merely enough for analysis in one tank. The significant test was therefore not applicable. L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%). Values with different superscript letters within the same row denotes significant difference ($p < 0.05$).
* The gut content of L group from DI was merely enough for lipase activity analysis in one tank. the significant test was therefore not applicable (NA).

2.2. Experimental design

The trial was conducted according to the Norwegian experimental animal regulations and was approved by The Norwegian Food Safety Authority (FOTS ID 22076). The trial was comprised of two parts, a feeding period carried out at Nofima's research facility at Sunndalsøra and a challenge test conducted at the Aquaculture Research Station in Tromsø. At the start of the experiment, fish average initial weight was 1.7 ± 0.03 g. The fish were distributed to 150-l tanks, 110 fish per tank, and triplicate tanks per diet. The tanks were flat bottom with black walls, equipped with a flow-through system with seawater filtered to 10 μm and UV treated. Temperature and salinity were measured daily, while oxygen was measured twice a week. Temperature and salinity were measured in tank water while oxygen was measured in water from the outlet. The mean temperature in the tanks was 11.6 °C (max 12,4 and min 10,9 °C) and the mean salinity was 31.7 ppt (max 32.6 and min. 30.7 ppt). The oxygen varied from 79 to 96% but was adjusted daily with the aim to keep the saturation between 80 and 100%. Each tank was equipped with a LED light dimmed to 4% and fish were kept at 24 h light regime. The tanks were equipped with automatic belt feeders. Feed was distributed continuously, according to appetite. Feeding level was set according to growth tables and adjusted visually to have some overfeeding to ensure access to feed for all individual fish. Any dead fish were removed daily from the tanks, counted, and weighed. Total duration of the feeding trial was 90 days.

2.3. Data recording and sampling

All fish were weighed individually at start of the trial. Thirty fish were scored for welfare characteristics. These 30 fish were thereafter euthanized by an overdose of anesthetic (tricaine mesylate, Finquel vet® (MS-222)) and were frozen for whole body analyses. The fish in each tank were weighed in bulk after 4 and 8 weeks.

At termination of the feeding trial individual weight and length (both with and without caudal fin) were measured for all fish. Six fish per tank were randomly selected, anaesthetized and euthanized with 110 mg/l MS-222, for sample collection. Blood was collected in vacutainers containing lithium heparin from the caudal vein and stored on ice until centrifugation for plasma. Plasma was separated and immediately frozen in liquid nitrogen and stored at −80 °C until analysis. After blood withdrawal, fish were opened, the organ package was carefully removed from the abdominal cavity, and the organs separated. Liver and gonads (if present) were weighed. The intestine was cleaned free of mesenteric fat and divided into four segments, namely pyloric ceca (PC), mid intestine 1 (MI1), mid intestine 2 (MI2) and distal intestine (DI). Each segment was opened longitudinally, and the gut content was collected and snap frozen in liquid nitrogen and stored at −80 °C before further processing. The cleaned gut segments were weighed before tissue samples were taken for RNA extraction (kept in RNAlater solution, incubated at 4 °C for 24 h and stored at −20 °C), and for histological analysis (fixed in 4% phosphate-buffered formaldehyde solution for 24 h and transferred to 70% ethanol for storage). For each intestinal segment, the weight was recorded. Remaining tissue was put back with the carcass of the fish. Pooled samples per tank, of carcass and of livers, were frozen for later analyses.

2.4. Challenge test with atypical A. salmonicida

The remaining fish were transported by truck to the Aquaculture Research Station in Tromsø where a challenge test with atypical *A. salmonicida* was performed. Before the transport to Tromsø, all fish were individually tagged with electronic tags ((Unique 125 kHz, Sokymat tags, RFID solution) and sorted into two identical groups. Each group consisted of 340 fish with a mean body weight of 57 g. Each of the two groups consisted of a total of 113–114 fish from each of three diets, and the fish were collected from each of three parallel tanks per diet. Upon arrival in Tromsø the two fish groups were transferred to two 1800 l tanks and held under continuous light at 8 °C, i.e. the same temperature as in the transport truck at arrival. Acclimatization to 14 °C was done over a 7-days period. The fish started to eat immediately, and only one fish died during the first days after arrival. The fish were fed the same diet (Lumpfish Grower, BioMar, 2.0 mm) at maintenance level during the experimental period. The feeding was stopped for 24 h before start of the challenge test.

Table 7
Relative gene expression in PC and DI (mean ± s.e.m., n = 3).

Gene category	Gene symbol	PC			p value	DI			p value
		L	M	H		L	M	H	
Nutrient transport	***slc27a4***	$1.8 \pm 0.1 \times 10^{-1}$	$1.8 \pm 0.2 \times 10^{-1}$	$1.8 \pm 0.1 \times 10^{-1}$	0.88	$4.0 \pm 0.1 \times 10^{-2}$	$4.1 \pm 0.2 \times 10^{-2}$	$4\ 0.0 \pm 0.09 \times 10^{-2}$	0.81
	cd36	$2.5 \pm 0.2 \times 10^{-1}$	$2.7 \pm 0.1 \times 10^{-1}$	$2.5 \pm 0.2 \times 10^{-1}$	0.66	$0.5 \pm 0.1 \times 10^{-3}$	$1.5 \pm 0.7 \times 10^{-3}$	$0.6 \pm 0.1 \times 10^{-3}$	0.59
	fabp2	2.7 ± 0.1 **b**	2.9 ± 0.1 **b**	3.6 ± 0.2 **a**	< 0.01	$5 \pm 0.6 \times 10^{-2}$	$6.1 \pm 1 \times 10^{-2}$	$6.5 \pm 1 \times 10^{-2}$	0.54
	cav1	$4.1 \pm 0.3 \times 10^{-2}$	$4.4 \pm 0.2 \times 10^{-2}$	$4.2 \pm 0.2 \times 10^{-2}$	0.72	$2.4 \pm 0.1 \times 10^{-2}$	$2.7 \pm 0.2 \times 10^{-2}$	$2.7 \pm 0.07 \times 10^{-2}$	0.08
	npc1l1	$2.4 \pm 0.1 \times 10^{-1}$	$2.2 \pm 0.2 \times 10^{-1}$	$2.1 \pm 0.1 \times 10^{-1}$	0.27	$6.9 \pm 0.4 \times 10^{-2}$**a**	$5.2 \pm 0.4 \times 10^{-2}$**b**	$4.9 \pm 0.4 \times 10^{-2}$**b**	0.03
	slc15a1	$1.7 \pm 0.1 \times 10^{-1}$	$1.6 \pm 0.07 \times 10^{-1}$	$1.6 \pm 0.09 \times 10^{-1}$	0.29	$3.2 \pm 0.1 \times 10^{-2}$**b**	$4 \pm 0.2 \times 10^{-2}$**a**	$4.5 \pm 0.3 \times 10^{-2}$**a**	< 0.01
Disaccharide digestion	***si***	$4.3 \pm 0.2 \times 10^{-1}$	$4.0 \pm 0.1 \times 10^{-1}$	$3.9 \pm 0.1 \times 10^{-1}$	0.3	$2.9 \pm 0.08 \times 10^{-1}$	$3.1 \pm 0.1 \times 10^{-1}$	$2.9 \pm 0.1 \times 10^{-1}$	0.43
Ion exchange	***slc12a1***	$3.0 \pm 0.2 \times 10^{-1}$	$3.0 \pm 0.3 \times 10^{-1}$	$2.6 \pm 0.2 \times 10^{-1}$	0.34	$6.7 \pm 0.5 \times 10^{-2}$	$6.3 \pm 0.5 \times 10^{-2}$	$5.7 \pm 0.4 \times 10^{-2}$	0.33
Tight junction proteins	***occludin***	$1.7 \pm 0.09 \times 10^{-2}$	$1.8 \pm 0.08 \times 10^{-2}$	$1.9 \pm 0.09 \times 10^{-2}$	0.42	$7.9 \pm 0.1 \times 10^{-3}$	$8.1 \pm 0.3 \times 10^{-3}$	$7.3 \pm 0.2 \times 10^{-3}$	0.08
	tjp1	$7.5 \pm 0.4 \times 10^{-2}$	$7.9 \pm 0.5 \times 10^{-2}$	$7.4 \pm 0.4 \times 10^{-2}$	0.64	$2.1 \pm 0.1 \times 10^{-2}$	$2 \pm 0.08 \times 10^{-2}$	$2.0 \pm 0.07 \times 10^{-2}$	0.58
Immune regulation	***cox2***	$5.4 \pm 0.3 \times 10^{-3}$	$5.2 \pm 0.3 \times 10^{-3}$	$5.6 \pm 0.4 \times 10^{-3}$	0.76	$2.2 \pm 0.1 \times 10^{-3}$	$2.4 \pm 0.2 \times 10^{-3}$	$2.4 \pm 0.1 \times 10^{-3}$	0.47
	igm	$1.1 \pm 0.2 \times 10^{-3}$	$0.9 \pm 0.1 \times 10^{-3}$	$0.9 \pm 0.07 \times 10^{-3}$	0.53	$3.3 \pm 0.3 \times 10^{-4}$**b**	$4.9 \pm 0.5 \times 10^{-4}$**a**	$4.3 \pm 0.3 \times 10^{-4}$**ab**	0.03
	rela	$4.0 \pm 0.08 \times 10^{-1}$	$3.9 \pm 0.1 \times 10^{-1}$	$3.8 \pm 0.1 \times 10^{-1}$	0.5	$1.3 \pm 0.04 \times 10^{-1}$	$1.3 \pm 0.04 \times 10^{-1}$	$1.3 \pm 0.04 \times 10^{-1}$	0.69
	ikbkb	$8.1 \pm 0.5 \times 10^{-2}$	$6.9 \pm 0.2 \times 10^{-2}$	$7.3 \pm 0.2 \times 10^{-2}$	0.14	$4.2 \pm 0.1 \times 10^{-2}$	$4.3 \pm 0.2 \times 10^{-2}$	$3.9 \pm 0.2 \times 10^{-2}$	0.26
	nfkb	$1.7 \pm 0.03 \times 10^{-1}$	$1.6 \pm 0.03 \times 10^{-1}$	$1.6 \pm 0.06 \times 10^{-1}$	0.4	$5.2 \pm 0.09 \times 10^{-2}$**b**	$5.8 \pm 0.2 \times 10^{-2}$**a**	$5.5 \pm 0.2 \times 10^{-2}$**ab**	0.02
	c5	$1.3 \pm 0.2 \times 10^{-2}$	$1.1 \pm 0.1 \times 10^{-2}$	$1.3 \pm 0.2 \times 10^{-2}$	0.69	$2.6 \pm 0.4 \times 10^{-3}$	$2.3 \pm 0.2 \times 10^{-3}$	$2.2 \pm 0.3 \times 10^{-3}$	0.78
	cxcl19	$2.8 \pm 0.3 \times 10^{-3}$	$2.6 \pm 0.3 \times 10^{-3}$	$2.3 \pm 0.1 \times 10^{-3}$	0.58	$7.8 \pm 1.2 \times 10^{-4}$	$7.7 \pm 1.5 \times 10^{-4}$	$9.9 \pm 1.9 \times 10^{-4}$	0.65
	tnfa	$4.8 \pm 0.2 \times 10^{-3}$	$4.5 \pm 0.3 \times 10^{-3}$	$3.7 \pm 0.3 \times 10^{-3}$	0.17	$8.0 \pm 0.7 \times 10^{-4}$	$1.3 \pm 0.06 \times 10^{-4}$	$1.1 \pm 0.1 \times 10^{-4}$	0.09
	MHCII	$2.6 \pm 0.1 \times 10^{-1}$**b**	$3 \pm 0.2 \times 10^{-1}$**ab**	$3.5 \pm 0.2 \times 10^{-1}$**a**	0.02	$5.2 \pm 0.3 \times 10^{-2}$	$6.9 \pm 0.4 \times 10^{-2}$	$6.4 \pm 0.3 \times 10^{-2}$	0.07
	mmp13	$3.8 \pm 0.3 \times 10^{-2}$	$3.4 \pm 0.3 \times 10^{-2}$	$3.6 \pm 0.2 \times 10^{-2}$	0.81	$6.7 \pm 0.4 \times 10^{-3}$	$7.7 \pm 0.7 \times 10^{-3}$	$8.7 \pm 1 \times 10^{-3}$	0.49
Cell proliferation	***pcna***	$2.9 \pm 0.06 \times 10^{-1}$	$3.1 \pm 0.07 \times 10^{-1}$	$3.1 \pm 0.08 \times 10^{-1}$	0.14	$1.1 \pm 0.02 \times 10^{-1}$	$1.2 \pm 0.03 \times 10^{-1}$	$1.2 \pm 0.04 \times 10^{-1}$	0.16

L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%). Values with different superscript letters in bold within the same row denotes significant difference ($p < 0.05$).

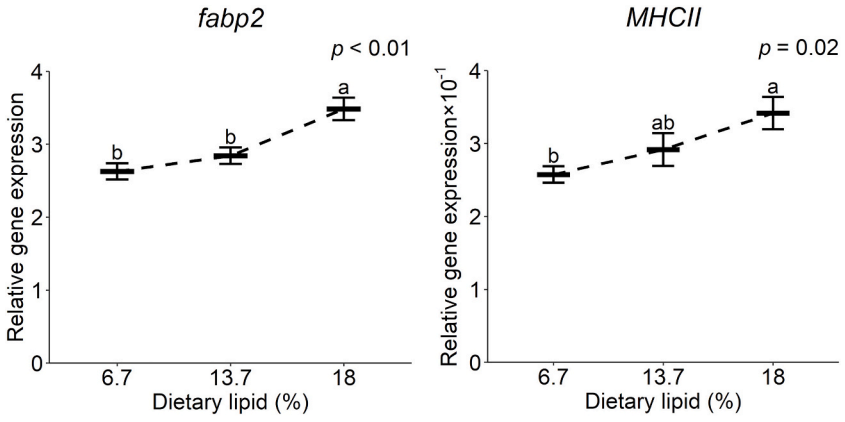

Fig. 2. Genes showing significant responses in PC. *fabp2.* fatty acid binding protein 2; *MHCII.* major histocompatibility complex class II. Mean values are denoted as horizontal bars. Error bars indicates standard error. Dashed lines connecting means between groups are illustrated for understanding changing trends, without any statistical meanings.

2.4.1. Bacteria cultivation

Atypical *A. salmonicida* newly passed and re-isolated from lumpfish was used in a bath infection experiment. The isolate originated from a diagnosed outbreak of atypical furunculosis, the disease caused by *A. salmonicida* infection, in lumpfish at a commercial production facility and was received from the Veterinary Institute in Harstad, Norway

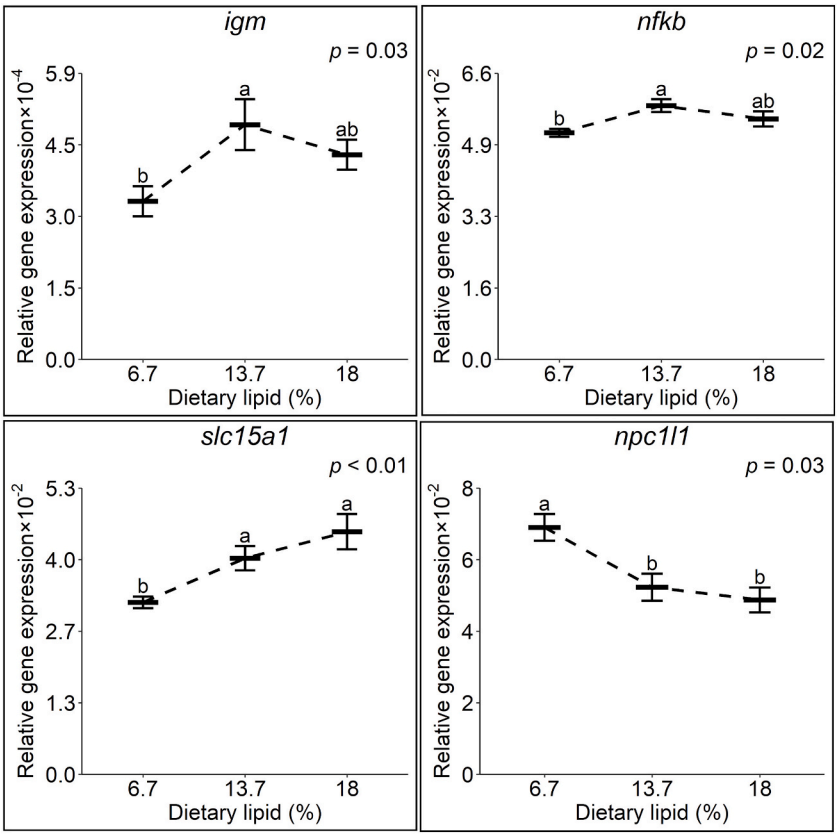

Fig. 3. Genes showing significant responses in DI. *igm:* immunoglobulin M; *nfkb*: nuclear factor kappa-light-chain-enhancer of activated B cells; *slc15a1*: peptide transporter 1; *npc1l1*: Niemann-Pick C1-Like 1. Mean values are denoted as horizontal bars. Error bars indicates standard error. Dashed lines connecting means between groups are illustrated for understanding changing trends, without any statistical meanings.

(2013–70-F-524.2/ A-layer type VI). The bacterial culture which was frozen with glycerol was thawed and transferred to blood agar (Blood agar base, Oxoid, with 5% human blood concentrate and 2% NaCl). The plates were incubated at 18 °C for 4 days until bacterial growth was visible. A single colony was transferred to 20 ml Brain Heart Infusion (BHI) medium (Oxoid) as pre-culture and incubated with gentle shaking for 24 h at 18 °C. OD520nm was measured and 10 ml from the pre-culture per flask was used to inoculate the 200 ml main cultures. After 24 h growth the OD520 nm was measured before harvesting the bacterial cultures. Titer (cfu/ml) was determined by titration on blood agar. The agar plates were incubated at 18 °C for 4 days before counting the colonies.

2.4.2. Infection procedure

The bath infection experiment was performed at 14 °C. The fish density was approximately 57 kg/m^3 during the infection procedure. The infection dose was 10^7 cfu/ml, based on experience from the established challenge model with lumpfish (previously present as conference poster (Johansen et al., 2017)). The water was oxygenated and monitored during this procedure which lasted for 2 h. Mortality was recorded daily for 49 days. The experiment was terminated when no dead or moribund fish had been recorded for three days and all remaining fish were euthanised by an overdose of the anesthetic metacaine (0.6 ml/l, Finquel vet®). Bacterial streaks from the head kidneys of the first moribund fish in each tank were used to confirm successful systemic infection by atypical *A. salmonicida*, the most likely cause of the morbidities and mortalities observed. This was done for 24 fish from both tanks. The plates were incubated at 18 °C until colonies were visible.

2.5. Chemical analyses

Feed samples were analyzed for dry matter (105 °C, until constant weight), ash (550 °C until constant weight), lipid after HCl hydrolysis (Soxtec HT6, Tecator, Höganäs, Sweden) and nitrogen (AOAC 2001.11; Kjeltec 8400 Analyzer Unit, Tecator, Höganäs, Sweden). Carcass and liver samples were analyzed for dry matter, ash, lipid, crude protein, methods as described above, and energy (Parr 63,000 Bomb calorimeter).

2.6. Blood plasma variables

Blood plasma was analyzed for cholesterol, monoglycerides, total triglycerides, glucose, activities of aspartate transaminase (AST) and alanine aminotransferase (ALT) at the Central Laboratory of the Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, according to standard, medical procedures.

2.7. Activity of digestive enzymes and bile salt concentration

The intestinal tissue samples were thawed and homogenized (1:20 *w/v*) in ice-cold 2 mM Tris/50 mM mannitol, pH 7.1, containing phenylmethyl-sulphonyl fluoride (P-7626, Sigma, Norway) as serine protease inhibitor. Tissue samples from MI1 and MI2 were homogenized together to represent the whole mid intestine (MI). The homogenates were sonicated, aliquoted and stored at −80 °C until analysis.

Leucine aminopeptidase (LAP) and maltase were the brush border digestive enzymes assessed. LAP activity was measured by employing the Sigma procedure no. 251, which was also used by Krogdahl et al. (Krogdahl et al., 2003), using l-leucyl-b-naphthylamide as the substrate. To measure maltase activity, the method described by Dahlqvist

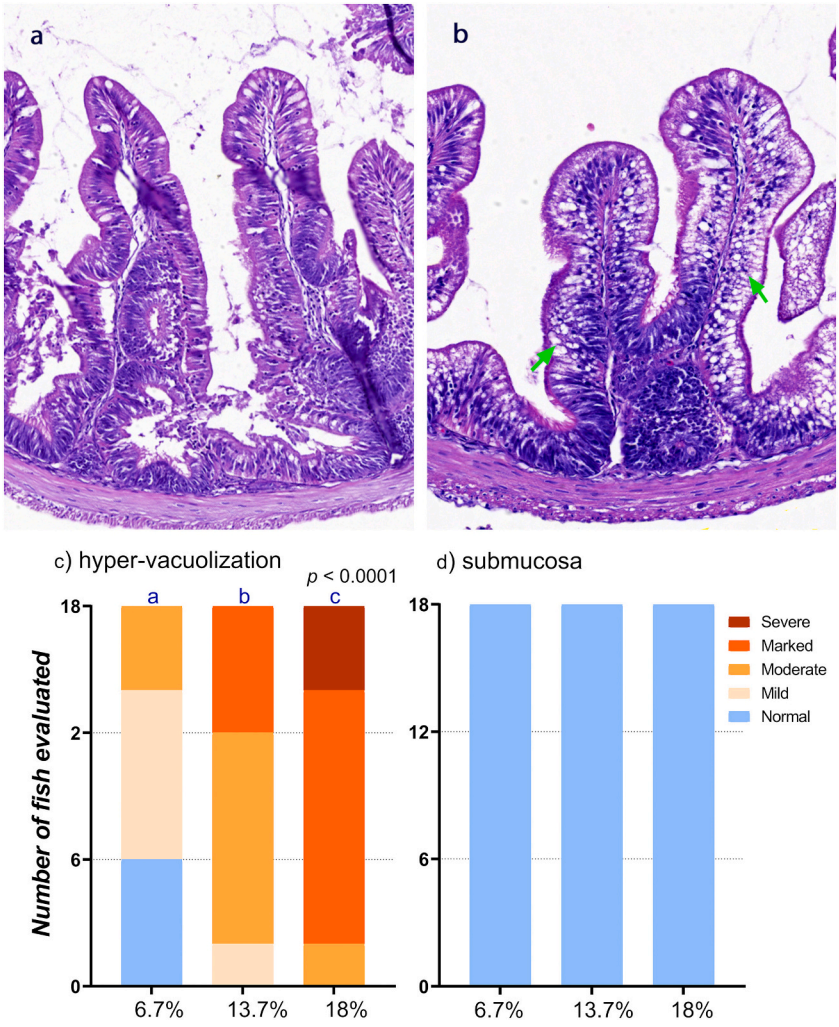

Fig. 4. Representative images of the pyloric caeca sections that were scored as a) normal and healthy and b) showing marked lipid accumulation (steatosis) appearing as vacuoles (green arrow) above the enterocyte nucleus; and the number of pyloric caeca tissue sections that were scored for c) enterocyte steatosis or hyper-vacuolization and d) increases in the width and inflammatory cell infiltration in the submucosa. The horizontal axis denotes dietary lipid in diet. *P*-values represent outcomes of an ordinal logistic regression for differences in the distribution of the histological scores between the diet groups. Columns not sharing a letter label are statistically different from the group 0 (reference group in the ordinal logistic regression analysis conducted). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Dahlquist, 1995) was applied, using maltose as substrate.

Trypsin activity and total bile salt level were measured in pooled freeze-dried intestinal contents from MI1, MI2 and DI. Trypsin activity was determined colorimetrically as described (Kakade et al., 1973), using the substrate benzoylarginine p-nitroanilide (Sigma no. B-4875, Sigma Chemical Co., St. Louis, MO) and a curve derived from standardized bovine trypsin solution. Lipase activity was determined as described (Brockman, 1981), using 4-Nitrophenyl myristate (Sigma 70,124, Sigma Chemical Co., St. Louis, MO) as substrate with sodium taurocholate hydrate as buffer (Sigma 86,339. Lot 0001428479).

Bile salt level was determined using the enzyme cycling amplification/Thio – NAD method (Inverness Medical, Cheshire, UK) in the ADVIA®1650 Chemistry System (Siemens Healthcare Diagnostics Inc.) at the Central Laboratory of the Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås. The assay measure total 3αOH of cholic acid, whereas the reported results indicate the corresponding level of bile salt as taurocholate.

2.8. Quantitative real-time PCR

Total RNA was extracted from tissue samples of PC and DI (~20 mg) of all fish using Trizol reagent (PureLink™ RNA Mini Kit, Thermo Fisher Scientific). RNA was purified by an on-column DNase kit (PureLink™ DNase Set, Thermo Fisher Scientific) according to the manufacturer's protocol. RNA purity and concentration were measured using the Epoch Microplate Spectrophotometer (BioTeK Instruments, Winooski, USA). The RNA integrity was verified by the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) in combination with RNA Nano Chip (Agilent Technologies, Santa Clara, CA, USA). First-strand complementary DNA (cDNA) was synthesized using 1.0 μg RNA from two fish of the same tank, namely 0.5 μg RNA of each two fish were combined as a unit of RNA sample for cDNA synthesis. The reaction volume was 20 μl, including 4 μl mastermix of the kit SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific). Negative controls were performed in parallel by omitting RNA or enzyme.

Primer information are shown in supplementary table S1. Lumpfish mRNA sequences were derived from NCBI database. The selected genes cover the functions of disaccharide digestion (*si*), nutrient transport (*slc27a4*, *slc15a1*, *npc1l1*), ion-exchange (*slc12a1*), tight junction forming (*tjp1*, *occludin*), immune regulation (*cox2*, *igm*, *ikbkb*, *c5*, *cxcl19*, *tnfa*, *nfkb*, *rela*, *il1b*, *MHCII*, *mmp13*) and cell proliferation (*pcna*). The qPCR primers were previously designed and used in our earlier published study(Zhou et al., 2022).

Expression of target genes were analyzed using the LightCycler 96 (Roche Diagnostics, Basel, Switzerland) with a 10-μl DNA amplification reaction. Each 10-μl DNA amplification reaction contained 2 μl PCR grade water, 2 μl of 1:10 diluted cDNA template, 5 μl LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics) and 0.5 μl (10 μM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no-template control. The three-step qPCR run included an enzyme activation step at 95 °C (5 min), 40 cycles at 95 °C (10 s), annealing temperature (10 s), and 72 °C (15 s) and a melting curve step. The mean normalized expression of the target genes was calculated from raw Cq values (Muller et al., 2002). Reference genes selected was done

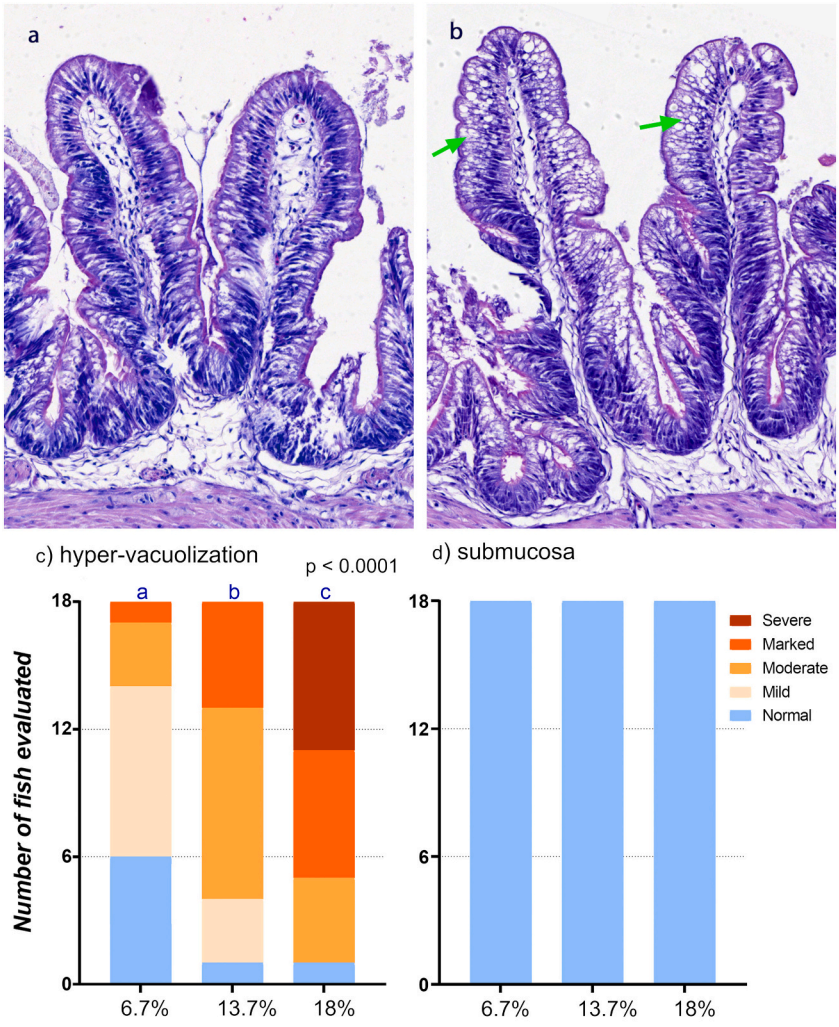

Fig. 5. The upper pictures show representative images of sections from the mid intestine scored as a) normal and healthy; b) with marked lipid accumulation (steatosis) indicated as enlargement of vacuoles (green arrow) above the nucleus in the enterocytes. The lower left graph (c) shows number of mid intestinal tissue sections scored as normal, mild, moderate, marked, or severe regarding enterocyte steatosis. The right graph (d) shows number of mid intestinal tissue sections scored as normal, mild, moderate, marked, or severe regarding increases in the width and inflammatory cell infiltration in the submucosa. Columns not sharing a letter label are statistically different. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in terms of its stability among different fish (Kortner et al., 2011). The chosen reference genes are hypoxanthine phosphoribosyltransferase 1 (*hprt1*), actin beta (*bactin*) and elongation factor 1 alpha (*ef1a*) for PC, and elongation factor 1 alpha (*ef1a*), actin beta (*bactin*) and tubulin beta chain (*tubb*) for DI.

2.9. Histological examination

The samples fixed for histological evaluation from all intestinal segments were processed using standard histological methods and stained with haematoxylin and eosin (H&E). The histological sections were evaluated to describe the general structure as well as observing for histological alterations associated with inflammatory reaction in the intestinal mucosa. The evaluated morphological characteristics included cellularity of the submucosa and lamina propria, enterocyte supranuclear vacuolization, and intra-epithelial lymphocytes. The degree of changes was graded as normal, mild, moderate, marked, or severe. The morphological assessment was guided by our experience with salmonid intestinal histopathology (Penn et al., 2011) as well as an examination of the histomorphology of the intestine in lumpfish (Zhou et al., 2022).

2.10. Calculations and statistical methods

$$\text{Specific growth rate } (\%\text{BW d}^{-1}); \text{SGR} = (\ln W_2 - \ln W_1)^{*} (t_2 - t_1)^{-1^{*}} 100$$

$$\text{Thermal growth coefficient; TGC} = \left(W_2^{1/3} - W_1^{1/3}\right)/((t_2 - t_1) \text{ x T})^{-1^{*}} 1000$$

where W_1 and W_2 are body weights (g) at time (days) t_1 and t_2, respectively, and T is average water temperature over the test period.

$$\text{Mortality per tank } (\%) : \text{total number of dead fish/initial number of fish}^{*} 100$$

Results recorded on group basis (mean per tank) were statistically tested by a one-way ANOVA, assessing the effect of diet, and using Duncan's multiple range test to rank the dietary treatments. For individual measured parameters (weight and length) from the final sampling, a mixed model was used, with diet as a fixed variable and tank as a random variable, using tank within diet as error term for testing.

Gut-related results and blood plasma results: Data were processed using R (version 3.5.2, 2018) in the integrated development environment Rstudio (version 1.1.463, 2018). A mixed model was used for testing, with diet as a fixed variable and tank as a random variable, followed by pairwise multiple comparison of estimated marginal means as post hoc analysis. When the singular fit was found, one-way ANOVA followed by Tukey's test as post hoc analysis was applied. Differences were considered significant at $p < 0.05$.

Differences in histological scores for the various evaluated morphological characteristics were analyzed using ordinal logistic regression. When score differences were only 2 levels, statistical significance was assessed using the Fisher exact test. Post hoc analysis for significant test results was conducted using the Chisq.post.hoc test (Fifer package in R).

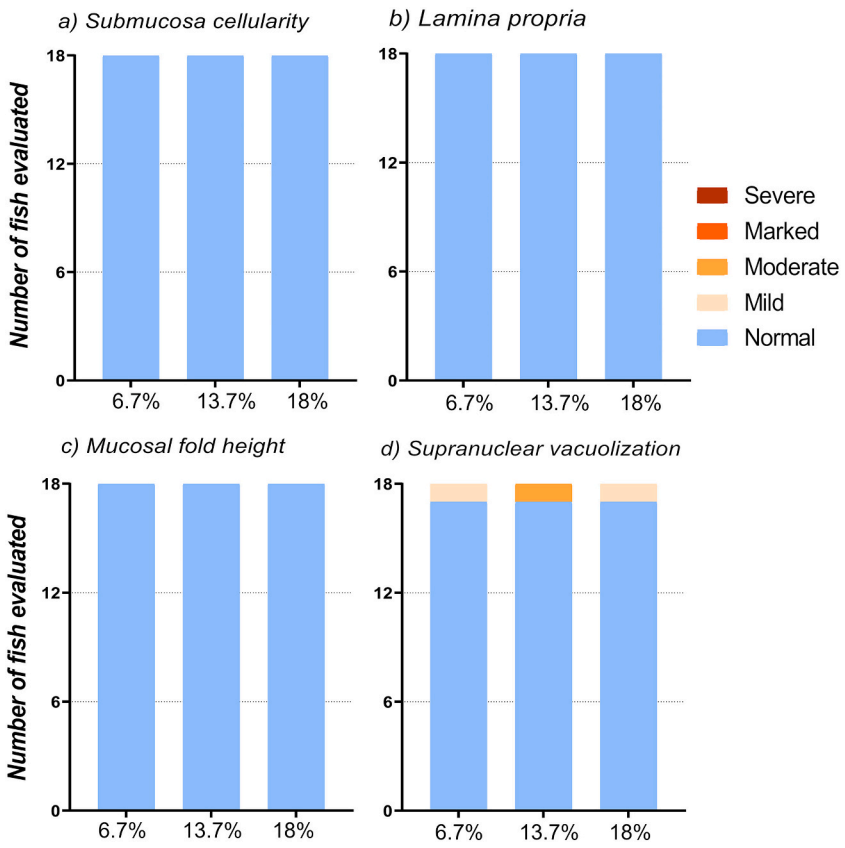

Fig. 6. Number of distal intestine tissue sections from the gut health assessment for (a) increase in width and inflammatory cell infiltration of the submucosa. (b) increase in width and inflammatory cell infiltration of the lamina propria. c) change of the mucosal fold height and d) loss in enterocyte supranuclear vacuolization.

Differences were considered significant at $p < 0.05$.

Results from the challenge test were evaluated employing the Chi-Squared test.

3. Results

3.1. Growth performance

Results on growth and mortality are shown in Table 2. The lumpfish grew from an average weight of 1.7 g to final weights around 60 g. There was no significant difference in final weight or body length of the fish. Specific growth rates (SGR) averaged 3.9, and thermal growth coefficients 2.8. No significant diet effects were observed. The total body length, with caudal fin included, tended to be lower ($p = 0.10$) in fish fed diet L. Mortality during the feeding period was highest (26%) in fish groups fed the diet H, and lowest (20%) in groups fed the diet L. However, the variation within diet was high, and no significant differences were revealed.

3.2. Body composition

Significant differences were found in composition of both carcass (Table 3) and liver (Table 4). The carcass showed increasing content of dry matter, lipid, crude protein and energy with increasing content of lipid in the diet. In the liver an increase with increasing lipid level in the diet was seen in dry matter, lipid and energy, while crude protein decreased.

3.3. Blood plasma variables

Results for blood plasma biomarkers are illustrated in Fig. 1. Cholesterol level was significantly elevated as lipid level increased from 6.7% to 13.7% but did not change with further increase in lipid level, i.e., from 13.7 to 18%. Monoglyceride, triglyceride, glucose and activities of AST and ALT did not show significant difference between diet groups. However, triglyceride and activities of AST and ALT tended to show responses to lipid level. Triglyceride level increased as lipid level increased from 6.7% to 13.7%, and its level slightly decreased when lipid level increased from 13.7% to 18%. Activities of AST and ALT shared a same trend, i.e., the activity decreased as lipid level increased from 6.7% to 13.7%, and it remained similar when lipid level increased from 13.7% to 18%.

3.4. Brush border digestive enzyme activities

Results of LAP and maltase specific activity and capacity are shown in Table 5. LAP specific activity was significantly affected by dietary lipid level in MI and DI. In MI, the activity decreased as lipid level increased from 6.7% to 18%. In DI, on the contrary, the activity increased as dietary lipid level increased from 6.7% to 18%. Although the activity was not significantly influenced in PC, it showed a decreasing trend as lipid level increased from 6.7% to 18%.

Specific activity of maltase showed significant diet effect in MI and DI but not in PC. In MI, the activity decreased when lipid level increased from 6.7% to 18%. In DI, the activity increased when lipid level increased from 6.7% to 13.7% but did not change with further increase in lipid level.

For capacity of LAP and maltase, there was no significant diet effect for any of the intestinal sections.

3.5. Trypsin and lipase activities and bile salt level in intestinal content

The results regarding trypsin and lipase activity are shown in Table 6. In MI1 and MI2 trypsin activity showed no significant treatment

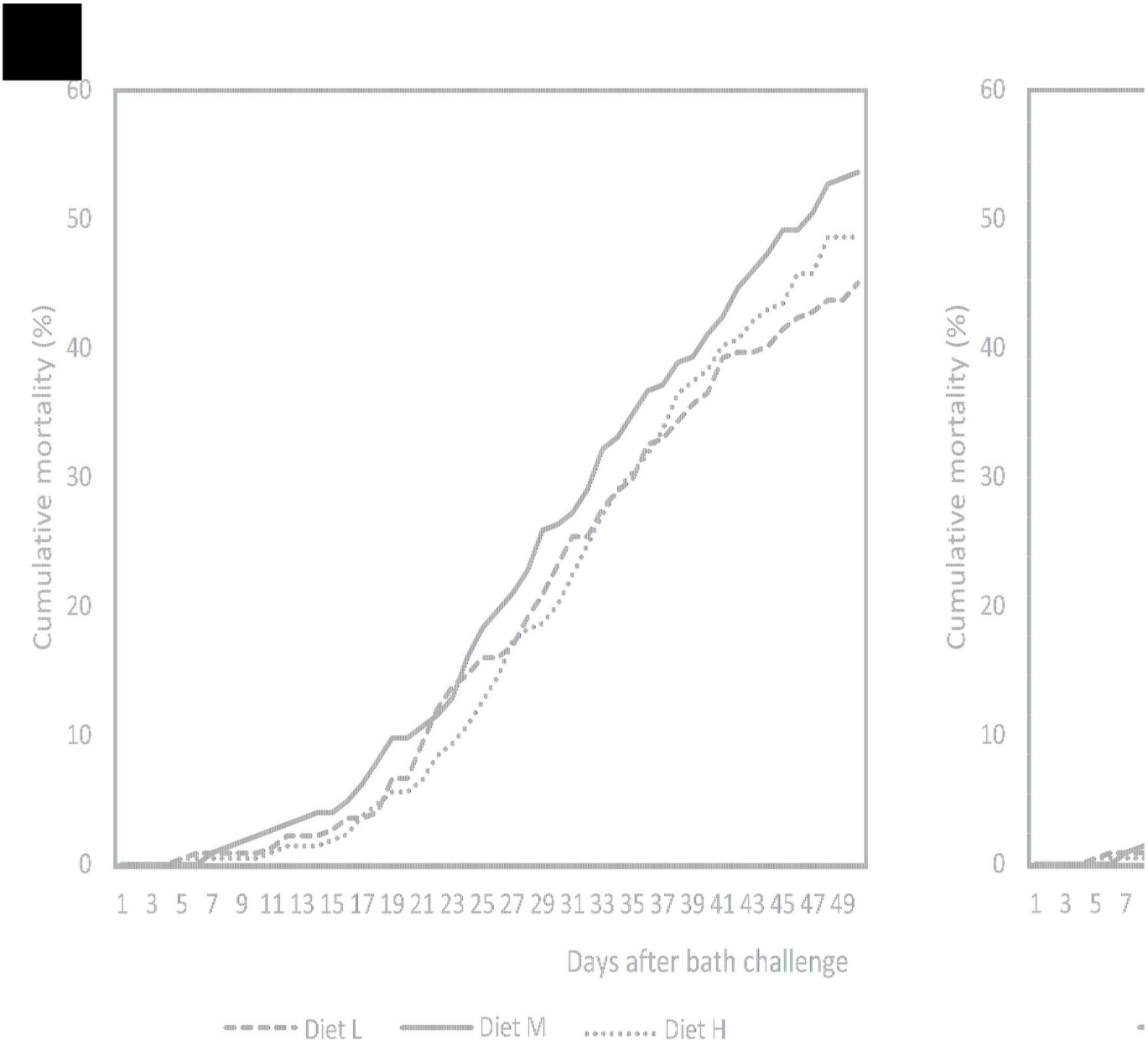

Fig. 7. Cumulative mortality (%) in lumpfish fed diet L, M and H after bath challenge with atypical *Aeromonas salmonicida.* L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%).Data are average from two parallel challenge tanks.

effects, however the mean value showed increasing trend as lipid level increased from 6.7% to 13.7%. In DI, trypsin activity was the lowest in diet of 6.7% and was increased to the highest as lipid level increased to 13.7% and 18%. Lipase activity did not show significant diet effect in any of the segments. The same trend observed regarding chyme bile salt level, however, a decreasing trend was observed in DI when lipid level increased from 6.7% to 18%.

3.6. Gene expression in PC and DI

Table 7 shows the gene expressions measured in PC and DI. In PC, the intracellular fatty acid transporter *fabp2* and the antigen-presenting peptide *MHCII* were the gene transcripts significantly affected by diet, and the response patterns were similar (Fig. 2). The expression of *fabp2* significantly increased when lipid level increased from 13.7% to 18% as the mean values were the lowest in L and M group and the highest in H group. The expression of *MHCII* significantly increased when lipid level increased from 6.7% to 18%.

In DI, the cholesterol transporter *npc1l1*, the peptide transporter *slc15a1*, immunglobulin *igm*, and the immune signalling *nfkb* were influenced by diet composition (Fig. 3). The expression of *igm* and *nfkb* increased as lipid level increased from 6.7% to 13.7%, and slightly decreased as lipid level reached 18%. The expression of *slc15a1* was increased with increasing lipid level. The expression of *npc1l1*, on the contrary, was decreased with increasing lipid level.

3.7. Histopathology

The pyloric caeca showed mild to severe lipid accumulation (steatosis) in the enterocytes (see Fig. 4 a and b for representative images). Fig. 4c illustrates the differences in enterocyte vacuolization between the groups. Fish from the L group (6.7% lipid) showed 66% prevalence of mild to moderate vacuolization. Fish from the two other diet groups were all scored with mild to severe steatosis, and the 18% lipid group

Table 8
Estimates for nutrient digestibility, content of digestible nutrient in the diets, and level of macronutrients in the faeces.[1]

Diet	L	M	H
Digestibility, %[1]			
Protein	84	82	83
Lipid	93	96	96
Starch	50	57	84
Dry matter	59	64	68
Level of digestible nutrient[1]			
Protein (DP), %	45	43	44
Lipid, %	6	11	15
Carbohydrates, %	7	8	9
Digestible energy (DE), MJ/kg	15	17	18
DP/DE, g/MJ	31	26	24
Proportion of macronutrients in faeces DM, % of sum			
Crude protein	52	63	85
Carbohydrates	46	34	9
Lipid	2.4	3.3	5.6

L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%).
[1] Estimated employing results from a previous experiment with diets based on the same formulation (Zhou et al., 2022).

showed the strongest responses. These apparent differences were statistically verified as indicated by different letters in Fig. 4c. None of the observed indicators of inflammation showed significant diet effects (Fig. 4d).

Mild to severe steatosis in the enterocytes was also observed in the mid intestine (see Fig. 5 a and b for representative images). The pattern for the MI was similar to that for the PC with the least changes observed in L (6.7% lipid) and the most changes for H (18% lipid) (Fig. 5c). No significant changes in morphological features of mucosal inflammation were observed in the MI (Fig. 5d).

The distal intestinal tissue was observed with largely normal and healthy morphology as observed in Fig. 6.

3.8. Challenge trial

Both external and internal symptoms of the infection are typical for fish suffering from infection with *Aeromonas salmonicida*. However, the evaluation of external signs of disease revealed only a few symptoms on a few of the fish. A few fish had small wounds and erosions around the cartilage peaks on the lateral sides. Likewise, no visible gross changes were observed in the internal organs. As total tank mortality at the end of the experiment did not differ significantly between replicate tanks ($p > 0.05$), the data were pooled. Mortality was 45% for diet L (6.7% lipid), 54% for diet M (13.7% lipid) and 49% for diet H (18% lipid) (Fig. 7). The statistical evaluation showed no significant diet effects ($p > 0.05$). However, there was a tendency to lower mortality in the fish group fed the low-fat diet (diet L).

4. Discussion

The observations made and issues raised in our study which deserve attention in this discussion are the following: a) The nutritional value of the diet indicated by analyses of proximate composition differed importantly from the value indicated by the observed level of digestible nutrients; b) Diet composition did not affect growth performance but changed body composition; c) Increasing dietary lipid level increased chyme trypsin activity along the intestine, but did not affect lipase activity, nor capacity of LAP and maltase in the brush border of MI and DI; d) In MI diet composition affected expression of two of the 21 observed genes, i.e. one involved in lipid transport, the other involved in antigen presentation, whereas in DI four of the genes were significantly affected, i.e. two involved in nutrient transport, and two in immune functions; e) Increasing lipid level increased lipid vacuolization of PC and MI; f) Increasing dietary lipid level increased plasma cholesterol and decreased plasma AST and ALT activities; g) Diet composition did not affect the lumpfish' resistance against *A. salmonicida*.

4.1. Considerations regarding diet composition, growth performance, and body and liver lipid content

The intention to formulate the diets with constant digestible protein and carbohydrate level and varying content of lipid was reached according to the chemical analyses which was made. However, animals sense and respond to the balance in supply of absorbable nutrients, which can be expressed as the balance between digestible protein (DP) and digestible energy (DE). Information on effects of dietary inclusion level on digestibility of macronutrients, generated in a study conducted in parallel to the present study showed great inverse effects for starch, only minor effects for protein and lipid (Zhou et al., 2022). The implication of this difference is that the diets varied more in protein value than indicated by the proximate composition. Table 8 shows estimated values for digestible macronutrients in the diets as well as estimated DP/DE ratio, which decreased from 32 to 27 g/MJ with increasing lipid level in the diet. In our recent study conducted to find optimal balance between macronutrient in lumpfish diet, the conclusion was that the best result was obtained with 55% protein, minimum 10% lipid and maximum 10% carbohydrate, as measured by proximate analyses (Hamre et al., 2022). A diet with these nutrient levels would contain, employing the same digestibility values as above, 29 g DP/MJ DE. If lipid level is increased to 18% and carbohydrate reduced to 6%, the ratio would become about 26 g/MJ DE. This indicates that the range of the DP/DE ratio in the present study was within the range suggested to support good performance. The lack of effects of diet composition on growth in the present study is in line with these considerations. The diet effects on body composition, with increase in dry matter, lipid as well as protein with increasing lipid level in the diet, are difficult to explain. In most situations when animals, including humans, are putting on fat, dry matter is increasing whereas protein level decreases (Sarett et al., 1966). The same was true for lumpfish in an experiment evaluating diets with the same nutrient content but differing nutrient sources, in which also growth performance differed (Sarett et al., 1966). However, lumpfish has a body composition deviating greatly from that of most other cultivated animals (Willora et al., 2020; Ageeva et al., 2021), i.e., with much higher water and hence, much lower protein content. Differences induced by variation in nutrient content of the diet without growth effects, may result in a different picture, than if the variation is due to differences in nutrient sources and growth rate is affected. Further investigations into the variation in body composition of the lumpfish, as well as causes and implications of such variations, is needed for better understanding of the lumpfish physiology.

The observation that liver lipid content increased with increasing dietary lipid level, is in agreement with the results of our previous screening study aiming to find optimal protein, lipid, and carbohydrate level of lumpfish diets (Hamre et al., 2022). However, in our previous study, with diets containing lipid in the same range as the present, the liver lipid content was lower, ranging from 10 to 25%, compared to the variation in the present study between 30 and 36%. A recent screening of 5200 lumpfish in four Faroese Atlantic salmon farming sites (Eliasen et al., 2020) showed values between 1.2% to 24.7%. Lumpfish is clearly a fish species which use the liver as a major lipid storage tissue. The observed liver lipid levels in the present study, were well below lipid levels observed in other species with the same characteristic, such as the cod, which may show levels above 80% (Kjaer et al., 2009; Weil et al., 2013).

4.2. Effects on activity of digestive enzymes

The general understanding of regulation of capacity of digestive

enzymes in the intestine is that an increase in content of protein, lipid, or carbohydrate, will induce an increase in activity in the chyme and brush border of the corresponding digestive enzymes (Krogdahl et al., 2011). However, the present results seem to indicate the opposite regarding trypsin activity in the chyme along the intestine, as the activity increased with increasing lipid level, decreasing protein level relatively. This was significantly so for DI and with the same trend for MI1 and MI2. However, as digestibility of the macronutrients differs greatly, the composition of the chyme dry matter changes continuously as it passes along the intestine. Information given in Table 8 intends to explain this development in numbers. Even though protein digestibility was high compared to starch digestibility, the proportion of protein in dry matter of the chyme increased along the intestine and was completely dominating towards the end. The explanation for the increasing trypsin activity with increasing lipid content in the diet, may therefore be, that in the chyme, the protein level increased, eliciting signals from the gut to the pancreas for more trypsin. Another consequence of high level of dietary protein in the chyme was, supposedly, decreased inactivation and digestion of the trypsin and other active proteases. As long as there is dietary protein in the gut, autolysis is slow, due to higher affinity of the protases for feed protein than endogenous functional proteins.

Similar considerations can be made regarding chyme lipase activity, which was not affected significantly by diet composition. The high lipid digestibility observed (Table 8), may indicate that most of the lipid was absorbed from the chyme in the proximal compartments of the intestine, and that very little lipid entered the more distal compartments. Hence, activity of lipase in the more distal intestinal segments was less affected by lipid level in the diet. Results of studies in other fish species may indicate that under other circumstances, e.g., with other lipid sources, the picture may differ (Infante and Cahu, 1999; Gomez-Requeni et al., 2013; Li et al., 2016; Chang et al., 2018). The high lipid digestibility observed in the previous trial (Zhou et al., 2022) indicate that lipase activity was not a limiting factor for efficient lipid digestion.

The decreasing effect of increasing dietary lipid level on specific activity of LAP, i.e. activity per weight of tissue protein, in the brush border in MI and the opposite effect in DI, are difficult to explain. However, total capacity was not significantly affected, indicating that the effects on specific activity was due to alterations in protein level in the tissue rather than enzyme activity. The same consideration is valid for the maltase activity and capacity.

4.3. Effects on nutrient transport

Among the genes involved in lipid transport, which were observed in the present study, only *fabp2* in PC showed significant diet effect, increasing with increasing dietary lipid level, as expected. The *fabp2* is highly expressed in the teleost intestine and shows a decreasing expression along the proximal to distal axis. The gene product is involved in transport of long chain fatty acids, saturated as well as unsaturated, across the mucosa (Storch and McDermott, 2009), forwards free fatty acids to different metabolic pathways, and modulate enzyme activity involved in lipid metabolism (Ordovas, 2001). The importance of the FABP2 protein is indicated by its abundance in the enterocyte, which, according to studies in humans, may exceed 3% of the enterocyte's cytoplasmic mass. It is also linked to mitochondrion β-oxidation and cholesterol uptake (Montoudis et al., 2008).

The signs of lipid accumulation (steatosis) in PC and MI which increased in severity with increasing lipid level in the diet, indicate that the capacity for lipid transport in the mucosa was insufficient for effective transfer from the gut to the systemic circulation. Similar results were seen in our previous digestibility study with similar diets (Zhou et al., 2022). In Atlantic salmon (Bou et al., 2017) and Arctic char (Olsen et al., 2000) steatosis has been associated with deficient supply of long chain ω-3 fatty acids. However, as supply of EPA and DHA was high in the present study, also other possible limiting factors should be considered. As lipid transport in the enterocytes is dependent on supply of choline, choline supply may have been insufficient for efficient transport in the present study. However, choline requirement of lumpfish has not been investigated. According to the diet formulation, choline level in the diets were about 5000 mg/kg, i.e. well above estimates of choline requirement in Atlantic salmon for efficient lipid transport across the intestine (Hansen et al., 2020b; Hansen et al., 2020a). Whether or not delayed lipid transport, causing signs of steatosis, is harmful for the fish cannot be concluded upon at present. Choline requirement of lumpfish should however be estimated in future studies, in which also effect on other functions of choline, such as donation of methyl groups in epigenetic processes, which are key elements in activation of immune function (Zeisel, 2017) should be observed.

4.4. Effects on cholesterol absorption and metabolism

The diet effect on expression of *npc1l1*, coding for a key transporter for intestinal cholesterol uptake, i.e. reduction with increasing lipid level, is in agreement with results in Atlantic salmon (Kortner et al., 2014) and mammals (den Bosch et al., 2008; Alvaro et al., 2010). This effect was likely a result of high content of n-3 polyunsaturated fatty acids (Alvaro et al., 2010; Yang et al., 2018), as fish oil was the ingredient varied for adjusting lipid level. Another explanation would be dietary cholesterol uptake correspondingly increased with the increased uptake of dietary fat, resulting in increased cholesterol levels in the blood. The increased blood cholesterol level thereafter suppressed expression of *npc1l1*, as a manner of reducing continuous increase of cholesterol level (Davis et al., 2004; Davies et al., 2005). However, our results differ from the experiment in lumpfish studying macronutrient requirement where plasma cholesterol level and *npc1l1* expression was not influenced (Hamre et al., 2022; Zhou et al., 2022). The difference could be caused by different experimental design. Protein, lipid, and carbohydrates were simultaneously varied in the study by Hamre et al. This may also influence other factors regulating cholesterol homeostasis and resulted in unchanged plasma cholesterol and *npc1l1* expression.

4.5. Effects on plasma biomarkers

Among the observed plasma biomarkers, all related to nutrient metabolism, cholesterol was the only showing significant effect of diet composition. Similar relationship with dietary lipid content has been observed also in other fish species (Yun et al., 2011; Kortner et al., 2014; Yan et al., 2015). Although the ANOVA did not show significant alteration in triglyceride level, the correlation with cholesterol level was high (Pearson correlation analyses: $p < 0.0001$; $r^2 = 0.78$). This result is as expected as increased lipid absorption increase plasma concentration of lipoproteins in which cholesterol and triglyceride are main components.

The about 50% drop in activity of AST and ALT also deserves attention. These aminotransferases are found in high concentrations in liver tissue, at low concentration in other tissues (Giannini et al., 2005). Increased activity in plasma may therefore, depending on the magnitude of increase, be a symptom of altered liver function, or disease and malfunction of the liver. However, in humans, alterations in the range > 5 times normal range, is considered to indicate moderately challenged liver. If lumpfish, in this regard, respond similarly, the observed decrease in AST and ALT with increasing dietary lipid level/decreasing protein level, indicates a physiologically normal response to changes in diet composition, and not necessarily an improvement in liver function. Relatively excess supply of protein, as was the case for fish fed the low lipid diet, represents a metabolic burden on the liver related to deamination of amino acids and production of NH_3. The relatively high value for these fish, may be reflecting such alterations in the liver metabolism.

4.6. Effects on immune functions and disease resistance

No information on expression of genes involved in immune functions

in lumpfish, induced by bath exposure to *A. salmonicida* has been found. However, as rainbow trout infected with this bacterium have shown effects on expression of a wide range of inflammatory, antimicrobial peptide and complement genes, similar functions would be expected also in lumpfish, and that the pathogen should be suitable for the present investigation (Castro et al., 2015). The low number of immune related genes showing effect of diet in the present study, i.e. two out of 11, indicates that immune function was not severely affected by the variation in macronutrient composition. Increasing lipid level induced pyloric caeca expression of *MHCII,* involved in antigen presentation. In the distal intestine induction was observed for *igm*, induced by initial exposure of antigens, and *nfkb,* a molecule characterized as a "rapid-acting" primary transcription factor. These responses indicate modulation by diet composition of processes in adaptive immune functions. However, as no structural or cellular alterations were observed, the response in these two genes was most likely not indicating important immunological effects. The absence of lymphocyte infiltration in intestinal tissues supports this consideration and are in line with results of our previous screening study (Zhou et al., 2022). The results of the challenge test with *A. salmonicida*, in which cumulative mortality did not differ significantly between treatments, supports this suggestion. It also indicates that the variation in nutrient composition of the diets did not compromise other important immune functions (Estensoro et al., 2012; Torrecillas et al., 2017).

5. Conclusions

The current study demonstrated effects of variation in dietary lipid level on fatty acid transport, cholesterol transport and metabolism, which macroscopically resulted in intracellular lipid accumulation and elevated level of blood cholesterol. Body and liver composition were also affected by dietary lipid variation, but growth performance was not affected. With variation in dietary lipid as in the present study (6.7% - 18%), we expected to observe immune-relevant changes. However, there was no significant change indicating that fish health was impacted during the normal feeding period, or after challenge by *A. salmonicida*. Lumpfish immune apparatus therefore seem tolerate high lipid diets, but too high lipid levels may result in metabolic disorders.

Author contributions

WZ analyzed samples and processed the data, drafted and completed the manuscript. ÅK and TK joined the planning of the experiment, overviewed the analyses, and joined the data processing. IL and GB were in charge of the experimental design and feed formulations, and as well as of the feeding trial. EC performed histological analysis and related statistics. LJ was in charge of the challenge trial. All authors contributed to the article and approved the submitted version.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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Appendix A. Supplementary data

The supplementary files including table S1. S1 listed primer information of genes involved in the present study. Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.739209.

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