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Early performance, stress- and disease-sensitivity in rainbow trout fry (*Oncorhynchus mykiss*) after total dietary replacement of fish oil with rapeseed oil. Effects of EPA and DHA supplementation

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ABSTRACT

Different vegetable oils have been investigated as potential substitutes of fish oil in aquaculture feed and several of them have proven to be successful in terms of fish growth rates, survival rates, biometric indices or feeding efficiency, even when used as the only oils in the feed. However, final fish product composition (fish whole body or fish fillet) usually reflects feed composition, and fish products from fish fed with only vegetable oils usually show a deficiency in long chain polyunsaturated fatty acids (PUFAs). The welfare and health consequences for the fish of these alterations of the fatty acid profile are not well known. In the current study, three experimental diets using supercritically defatted fish meal as well as rapeseed oil as a 100% replacement of fish oil, and differing in essential fatty acid (eicosapentaneoid acid - EPA, docosahexaenoic acid - DHA) supplementation, were tested in rainbow trout, *Oncorhynchus mykiss*. The growth performance, feed conversion ratio and digestive enzyme profiles were evaluated. Further, stress-and infection-challenge experiments were performed to assess the robustness of the fish against stress and disease. The trout fry fed the experimental diets showed a different fatty acid profile than fish fed a commercial diet, with lower EPA and DHA levels but higher linolenic acid (18:3n-3) and oleic acid (18:1n-9) levels. No significant effects were found in growth performance, feed conversion ratio or digestive enzymatic activity among diets. The fish stress responsiveness (brain and plasma stress marker response) to acute and repeated stressors was in general also similar irrespective of the diet. Infection trials, however, showed a differential ability of the fish to survive upon a controlled infection with the bacterium *Flavobacterium psychrophilum*. Fish fed with the experimental diets displayed higher mortalities than fish fed a commercial trout fry diet. The current results highlight the need of further research on nutritional requirements to optimize the disease resilience in farmed fish. These results also demonstrate that the adaptation of the fish to new formulated feed (in this case associated to plant oil ingredients) should not be assessed solely based on the growth performance of the fish. Other aquaculture relevant welfare indicators, as disease or stress resilience, should be part of a more complete assessment of feed adequacy for fish farming purposes.

1. Introduction

During recent years, a global trend to substitute fish oil (FO) and fishmeal (FM) as main fat and protein sources for aquaculture feed ingredients has developed to increase the sustainability of the fish farming sector (Beheshti Foroutani et al., 2018; Bendiksen et al., 2011; Naylor et al., 2009; Turchini et al., 2009). While a significant substitution of fish protein by other sources has been achieved (Daniel, 2018; Sprague et al., 2017), a major substitution of FO requires finding appropriate alternative sources for n-3 (omega-3) long-chain polyunsaturated fatty acids (LC-PUFA), a challenge that still requires a definitive solution (Sprague et al., 2017; Tocher, 2015).

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Potential alternatives to FO include terrestrial plant-derived seed oils (Alhazzaa et al., 2018; Houston et al., 2017; Monge-Ortiz et al., 2018) but also other kind of oils such as those derived from terrestrial animal fats (Alhazzaa et al., 2018; Campos et al., 2019; Monteiro et al., 2018) or from microalgae and other microorganisms (Sprague et al., 2017). Substitution of FO with non-marine oils most often comes at the price of a reduction in the n-3 LC-PUFA content of the farmed fish and therefore, these essential compounds need to be added as dietary supplements (Tocher et al., 2019) in order to keep up with fish dietary requirements. In vertebrates, n-3 LC-PUFAs participate in functions commonly associated to fatty acids, such as being part of the structure of phospholipids and therefore, of cellular membranes, or serving as a metabolic energy source (Tocher, 2015; Tocher, 2003). Besides, LC-PUFAs and their eicosanoid derivatives have important roles as metabolic regulators (Tocher, 2010) and they modulate neural development and plasticity and have an important role as regulators of inflammatory processes (Tocher, 2015; Wall et al., 2010).

Regarding the effects of FO replacement on fish performance, most studies have focused in productive traits, namely fish growth rates. Fish growth rates have been shown to be reduced (Houston et al., 2017; Lazzarotto et al., 2018; Monteiro et al., 2018) or unaffected (Abbasi et al., 2020; Betiku et al., 2016; Campos et al., 2019; Reis et al., 2014; Torrecillas et al., 2017; Wang et al., 2018) by the replacement of FO by non-marine alternatives, depending on the type of oil used and the extent of the substitution, but also on the fish species and stage of development. Studies on salmonids have shown that it is possible to replace dietary FO by vegetable alternatives without affecting growth or feed efficiency when FM is included in the diet formulation (Bell et al., 2003; Lazzarotto et al., 2018; Richard et al., 2006), as the lipid content in the FM can partially meet the n-3 LC-PUFA requirements. Rainbow trout is capable of bioconversion of dietary 18:3n-3 (linolenic acid - ALA) to n-3 LC-PUFAs (Turchini and Francis, 2009), and this may explain their ability to cope with very low dietary levels without significant influence on performance. Despite the ability of salmonids for fatty acid elongation and desaturation, the bioconversion has proven insufficient to compensate for a decrease in n-3 LC-PUFA intake imposed by diets free of marine ingredients, which result in a significant reduction in tissue levels when compared to diets containing FO and/or FM (Turchini and Francis, 2009).

The substitution of FO may affect the welfare of farmed fish, beyond the potential effect on growth. Fish oil replacement has been demonstrated to affect stress and immune responses in fish (including salmonids), those effects being dependent on the species and type of vegetable oil (VO) used for the replacement (Montero and Izquierdo, 2010; Sadoul et al., 2016; Serradell et al., 2020). While some studies point to a lower stress resilience (more intense and/or more prolonged responses upon stress) in marine and freshwater fish fed diets rich in VO (Ganga et al., 2011b; Montero and Izquierdo, 2010; Sadoul et al., 2016), others show no effects or even a positive effect of VO-based diets on fish stress resilience (Bell et al., 1991; Conde-Sieira et al., 2018). Based on that, it has then been suggested that the effects of VO inclusion on fish stress physiology depend on the specific blend of VOs used in the diet (Conde-Sieira et al., 2018; Montero and Izquierdo, 2010). The lipid content of the diet is also known to affect both nonspecific and specific immune defenses in vertebrates. In this regard, dietary VO inclusion has also been shown to affect the immune competence of the fish, often in a negative way (Machado et al., 2019; Montero and Izquierdo, 2010). The effects of the dietary VO inclusion in fish stress and immune responses could be mediated, at least partially, by the dietary content of certain LC-PUFAs, particularly n-3 fatty acids such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), and n-6 fatty acids such as arachidonic acid (ARA). Inadequate dietary levels of these fatty acids, as well as an unbalance between n-3 and n-6 LC-PUFAs, have been shown to negatively affect stress and immune responses of fish (Carvalho et al., 2019; Lund et al., 2012; Montero et al., 1998; Oliva-Teles, 2012).

In the present study, we aimed at evaluating the effects of a 100% FO oil substitution (including supercritical defatting of FM) by rapeseed oil on rainbow trout fry performance. Specifically, the objective was to test the effects of FO substitution on fish early growth and digestive metabolism, but also on the stress- and disease-resilience of the fish. We hypothesized that the differential fatty acid profile of the diet would affect the growth of the fish. Secondarily, we also hypothesized that, independently of growth performance, the experimental diets would have an effect on the ability of the fish to cope with stress or disease. Three rapeseed oil-based diets, either with or without increasing n-3 LC-PUFA supplementation (EPA, DHA) were tested versus a commercial diet, in order to specifically address potential effects of those compounds during a three months period from first feeding. The growth of the fish and major digestive enzyme activity - gastric (pepsin), pancreatic (trypsin, α-amylase and lipase) and intestinal/brush border (alkaline phosphatase, aminopeptidase, N) - were assessed at regular intervals during the growth experiment. Furthermore, robustness of the fish against stress and an imposed bacterial infection was evaluated. Regarding the latter, one of the most important diseases in rainbow trout fry aquaculture is Rainbow Trout Fry Syndrome (RTFS), also known as Bacterial Cold Water Disease (BCWD). The cause of this disease, the bacterium *Flavobacterium psychrophilum* was therefore chosen as challenge pathogen for evaluation of robustness of disease in fry that had been fed the different diets.

2. Materials and methods

2.1. Fish and rearing system

Rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792) eyed eggs were purchased at a local supplier (Randbøldal, Vejle, Denmark). Eggs were hatched at DTU Aqua facilities (Hirtshals, Denmark) after incubation at 7–8 °C. At the end of the yolk sac stage, 500 fry were allocated into each of 12 tanks. Three tank replicates were randomly assigned to each of four diet groups (see below). All tanks were part of the same recirculation aquaculture system (RAS). The tanks were conical (d: 45 cm; h: 38 cm) with a perforated bottom grid, each holding approx. 40 L and connected to its own water inlet. Temperature was slowly increased from 10 °C to 12–14 °C during the first week after fry transfer. Oxygen content and temperature was monitored daily in each tank. Every second day, ammonia-N, nitrite-N and nitrate-N levels were measured in the system. Water exchange was adjusted to 40 L h^{-1} in each tank. The tank bottom was vacuum-cleaned daily to remove feed waste and faeces and to collect and count dead fry.

2.2. Ethics

The use of fish in this study complied with Danish and EU legislation (Directive 2010/63/EU) on animal experimentation and was approved by the Animal Welfare committee of DTU Aqua and by the Animal Experiments Inspectorate of Denmark (*Dyreforsøgstilsynet*, permission number 2013-15-2934-00976).

2.3. Diets and feeding

Three experimental diets were prepared and extruded (Sparos, Olhão, Portugal) in various sizes from 0.6 mm to 2 mm, and were formulated to be isonitrogenous and isoenergetic (Table 1). The three experimental diets differed in their relative content of n-3 LC-PUFAs DHA and EPA as by use of supercritical defatted fish meal (Eco Treasures, BVBA, Belgium) and subsequent supplementation of Incromega DHA, 500 TG, Croda Industrial Chemicals) and were named L (low), M (medium) or H (high) (Table 1) . A commercial rainbow trout fry diet in same size ranges (Aller Futura Ex.) from Aller Aqua (Christiansfeld, Denmark) was included as a control reference (CtrAF).

The four diets were supplied to replicate groups (three per diet) of post-yolk sac fry. The fry were initially fed by hand and after a weaning

Table 1
Feed formulation and proximate analysis of the three experimental diets; a commercial control diet (Aller Futura, Aller Aqua) was used as reference.

Diet Ingredients (g kg^{-1})	L	M	H	CtrAF
Fishmeal LT 70 (defatted)[a]	480	480	480	
Fish gelatin[b]	20	20	20	
Porcine blood meal[c]	60	60	60	
Soy protein concentrate[d]	60	60	60	
Wheat gluten[e]	150	150	150	
Wheat meal[f]	46	46	46	
Incromega DHA 500TG[g]	0.0	15	35	
Rapeseed oil[h]	170	155	135	
Vitamin & mineral premix PVO40.01[i]	10	10	10	
Antioxidant powder (Paramega)[j]	2	2	2	
Analyzed composition (% W.W.)				
Crude protein	58.8	58.8	58.5	52.5
Crude lipid	20.1	19.4	19.6	20.1
NFE + fibre (substracted)	4.7	5.2	5.2	10.9
Dry matter (DM)	93.5	93.3	93.0	92.0
Ash	9.8	9.8	9.7	8.6
EPA (mg g $d.w.^{-1}$)	0.5	1.8	3.6	16.0
DHA (mg g $d.w.^{-1}$)	0.3	2.0	5.1	3.1
(% protein) Lysine	3.25	3.26	3.23	
Methionine + Cysteine	1.91	1.93	1.92	

[a] Fish Meal LT70 Norvik, defatted[a]: 68.13% crude protein. 1.67% crude fat (Initial: 9.8%), moisture 12.12% ash 17.9%, EPA 0.09%, DHA 0.14%.
[b] Fish gelatin: 88% crude protein. 0.1% crude fat. LAPI Gelatine SPA. Italy.
[c] Porcine blood meal: 89.1% crude protein. 0.4% crude fat. Sonac BV. The Netherlands.
[d] Soy Protein concentrate: Soycomill, IL, USA.
[e] Wheat gluten: VITAL 83.7% crude protein. 1.4% crude fat. ROQUETTE Frères. France.
[f] Wheat meal: 11.7% crude protein. 1.6% crude fat. Casa Lanchinha. Portugal.
[g] Incromega DHA 500 TG: Croda chemicals; EPA 9.2%, DHA 71.9%.
[h] Rapeseed oil: Henry Lamotte Oils GmbH. Germany.
[i] PVO40.01 premix for marine fish (Premix Lda. Viana do Castelo. Portugal). Vitamins (per kg diet): 100 mg DL-alpha tocopherol acetate. 25 mg sodium menadione bisulfate. 20.000 IU retinyl acetate. 2.000 IU DL-cholecalciferol. 30 mg thiamin. 30 mg riboflavin. 20 mg pyridoxine. 0.1 mg B_{12}. 200 mg nicotinic acid. 15 mg folic acid. 1.000 mg ascorbic acid. 500 mg inositol. 3 mg biotin. 100 mg calcium panthotenate. 1.000 mg choline chloride and 500 mg betaine. Minerals (per kg diet): 2.5 mg cobalt sulfate. 1.1 mg copper sulfate. 0.2 g ferric citrate. 5 mg potassium iodide. 15 mg manganese sulfate. 0.2 mg sodium selenite. 40 mg zinc sulfate. 0.6 g magnesium hydroxide. 1.1 g potassium chloride. 0.5 g sodium chloride and 4 g calcium carbonate.
[j] Antioxidant powder: Paramega PX. KEMIN EUROPE NV. Belgium.

period of one week, feed was provided by means of clock belt feeders from 8 am to 3 pm in excess (visual feed waste). From 32 DPFF the feed ration was adjusted daily based on fish growth until 32 DPFF, initial biomass and an estimated feed conversion ratio (FCRs). Thus, in the growth periods from 32 DPFF -73 DPFF all groups were fed 3.5% of initial biomass with an expected FCR of 0.75. From 73 DPFF until DPFF 94 fry were fed 2% of biomass with an expected FCR of 0.85. Pellet size was gradually increased according to the size of the fry, starting with 0.6–0.8 mm and with use of 1.3 mm at the end of the study.

2.4. Experiment 1: Growth trial

The initial growth of the fish fed the different diets was monitored for a period of 94 days. During this period, fry were measured in a total of seven occasions, starting at 7 days post first feeding (DPFF) (Fig. 1). Fish were crowded by lowering the water level in each tank replicate and thirty fry from each tank were randomly sampled each time for individual weight (mg ind^{-1} or g ind^{-1}). Additionally, the total biomass in each tank was weighed three times, at intervals of 19 feeding days (i.e. starting at 32 DPFF, to avoid potential effects of weaning to dry diets) for measurement of specific growth rate (SGR) and FCR according to:

SGR calculated based on the overall biomass gain in the tanks for a given period, according to Eq. (1):

$$SGR = 100^{*}\left(lnW_t - lnW_{t_0}\right)^{*}d_t^{-1} \quad (1)$$

where W_t refers to weight at day t, W_{t0} refers to weight at day t_0 and d_t is the number of feeding days.

FCR calculated based on the gained biomass and the administered feed, according to Eq. (2):

$$FCR = administered\ feed/biomass\ gain \quad (2)$$

During the growth trial, fry were sampled from each tank for analysis of whole-body fatty acid content (at day 1, 16, 52 and 94) and digestive enzymatic activity (at day 7, 16, 24, 52 and 94). Sampled individuals for analysis were killed by an anesthetic overdose (benzocaine) and snap frozen at −80 °C. Six individuals per tank (18 per diet group) were sampled at each sampling point for fatty acid composition. For digestive enzymes activity, 10 individuals were sampled from each tank at each sampling point. Furthermore, 100 fish from each tank were removed at 52 DPFF to be transferred and submitted to the infection challenges described in section 2.5. The calculation of SGR and FCR took into account the fish biomass removed from each tank during all these samplings.

2.5. Experiment 2: Infection challenge

A total of 300 fish from each diet group were relocated to the facilities at DTU Aqua in Frederiksberg (Denmark). Fish individual mass was 1.5 g on average and they were allocated in replicate eight-liter aquaria, where they were later exposed to infection trials to assess their resilience against a bacterial pathogen. Stocking density was either 30 fish per aquarium or 60 fish per aquarium, depending on their later use on intraperitoneal (IP) or bath infection challenges, as described below. Fish were left to acclimate for two weeks before the start of the infection trials (at 68 DPFF) and were fed the corresponding experimental diets during this period.

The *Flavobacterium psychrophilum* strain used for the challenges was a well-characterized Danish strain 950,106–1/1 (serotype Fd, ribotype A, 3.3 kb plasmid, virulent) (Madsen and Dalsgaard, 2000; Madsen and Dalsgaard, 1999). The strain was stored at −80 °C in tryptone yeast extract salts (TYES) media (Holt et al., 1993) with 15 to 20% glycerol and was subcultured in agitated cultures at 15 °C. Strains were taken directly from −80 °C and incubated in TYES for a minimum of 48 h before further inoculations were made for liquid cultures in TYES. The incubation of bacterial cultures for experimental infection was done according to Madsen and Dalsgaard (1999).

Two challenge methods, an IP challenge and a bath challenge, were set up. The IP challenge, described by Madsen and Dalsgaard (1999) and used as challenge method in Gesto et al. (2018), was chosen to investigate the disease resilience to infection with *F. psychrophilum* in fry that had been fed the different diets from first feeding, as this method has been shown to be a reproducible infection method resulting in high mortalities when it comes to *F. psychrophilum*. Besides the IP challenge, a *F. psychrophilum* bath challenge with stressors was also set up and performed as described below. The use of concomitant stressors has been shown to increase the low mortality normally seen in connection with *F. psychrophilum* bath challenges (Madsen and Dalsgaard, 1999). Before, during (only the bath challenge groups) and after challenge the fish were kept in replicate eight-liter tanks supplied with water in a flow-through system (~2 L h^{-1}). The fish were fed the experimental diets at 1% biomass per day and kept at 12 °C. The condition of the fish was monitored at least three times per day, and moribund fish were collected and counted. Moribund fish were killed by an overdose of 3-aminobenzoic acid ethyl ester (MS-222, Sigma A-5040) in water, and samples from inner organs (spleen, kidney and brain) were streaked onto TYES agar and blood agar (BA) plates and incubated at 15 °C for five days up to three to four weeks. Yellow colonies that showed growth on TYES agar but not on BA was identified as *F. psychrophilum* by either a species-

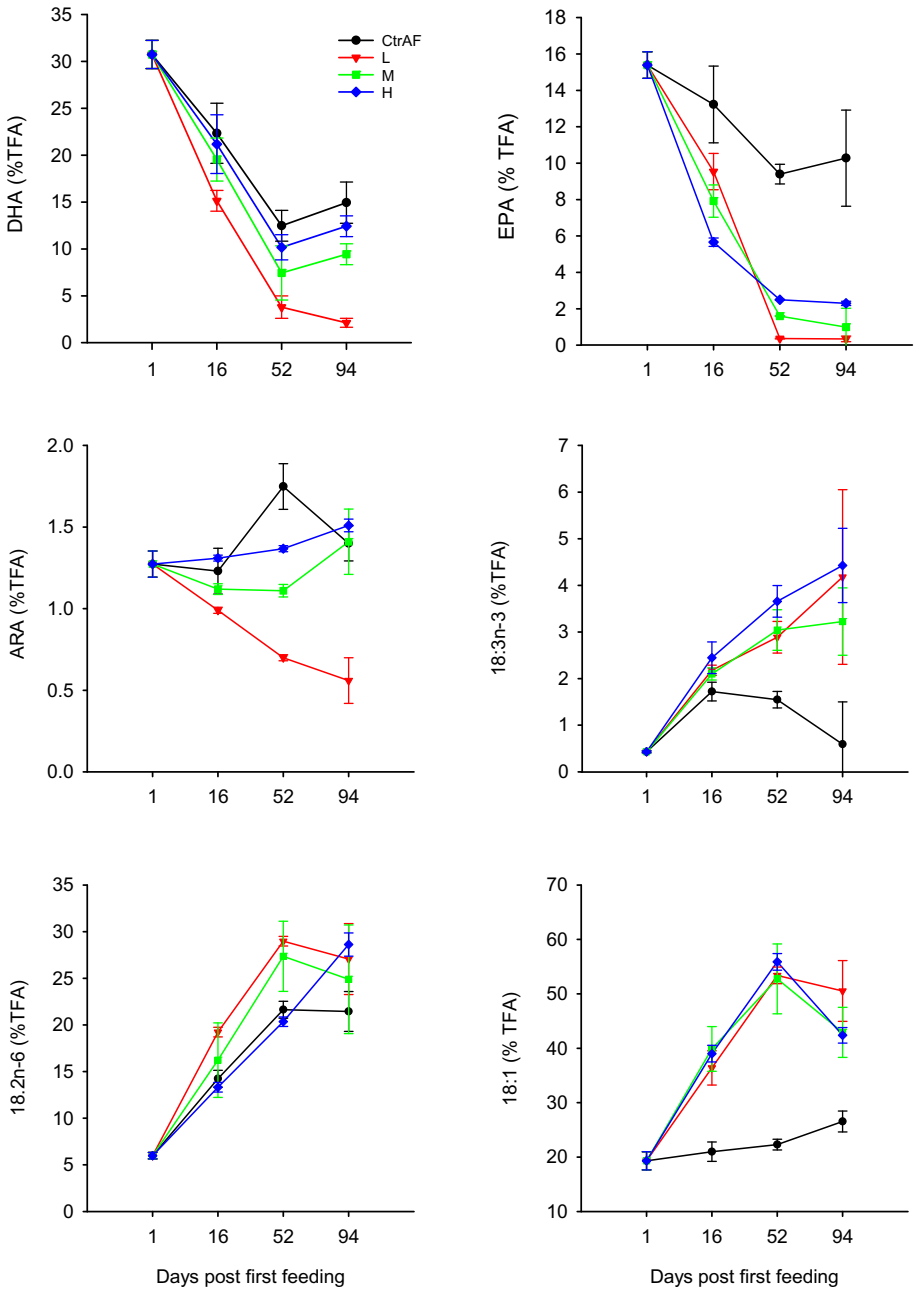

Fig. 1. Mean fatty acid composition of trout fry at 1, 16, 52, 94 DPFF when fed diets L, M, H and a commercial CtrAF. Mean values and standard deviations, $n = 6$.

specific PCR with DNA primers against a sequence of the *16SrRNA* gene (Wiklund et al., 2000) or by MALDI-TOF (Jansson et al., 2020). The IP challenge experiment and the bath challenge experiment were terminated after 34 and 55 days, respectively. The surviving fish were killed by an overdose of MS-222, and inner organs were sampled as described above.

For the IP challenge, a total of four replicates from each diet group (each replicate consisting of 30 fish) were used. One replicate served as a sham control where fish were injected with sterile TYES media, whereas in the other three replicates every fish was injected with *F. psychrophilum* in a concentration of 1.1×10^4 CFU (colony forming units)/fish. A total volume of 50 μL was injected into the peritoneal cavity of each fish, regardless of treatment type. Prior to injection all fish were anesthetized with MS-222.

For the bath challenge, short periods of air-exposure were used as concomitant stressors. The air-exposure was done by draining all the water through an opening in the bottom of the tank. Three replicated aquaria from each diet group (each replicate consisting of 60 fish) were used. Two of the replicates were challenged as follows: 1 min without water in the tank, bacterial challenge (7 h in 1.5 L with the bacterial concentration 1.4×10^8 CFU/mL, the fish being fed with 1% of the corresponding experimental feed after 3 h), finalizing with 1 min without water in the tank after the 7 h bath challenge. The third replicate was a sham control, in which the fish was similarly exposed to air and the bacterial inoculation was substituted by diluted sterile TYES media.

2.6. Experiment 3: Stress challenge

At the time the fish were reallocated for this experiment, one week after the end of the growth experiment (experiment 1), their average individual weight (SD) was 7.4 g (0.4 g), 6.5 g (0.4 g), 6.2 g (0.4 g) and

7.0 g (0.8 g) for the control, L, M, and H diets, respectively. The fish were distributed into 600 L tanks; three tanks were randomly assigned to each of the diets (control, L, M, H). In each of the 12 tanks, two groups of 90 fish were kept separated by using two plastic 48 L aquaria (with a metal grid at the bottom allowing for passage of water). Thus, a total of 24 paired aquaria, with 90 fish each, were used in the experiment.

The fish rearing tanks were part of the same RAS; water temperature was kept at 16 °C and water quality parameters (NO_3^- NO_2^-, NH_3/NH_4^+, pH) were measured every second day. Oxygen saturation levels were continuously monitored and kept at all times above 80%. The photoperiod was 14 h:10 h (light: dark). Fish were daily fed their corresponding experimental diets by means of clock belt feeders. Fish were kept in these conditions for two weeks before the start of the stress resilience experiment.

During the 15 day-period of the experiment, a repeated stressor was applied daily to one of the aquaria in each pair, always to the same aquarium and at the same time every day. The daily stressor consisted of lifting the plastic aquaria out of the water, exposing the fish to air (acute air exposure – the metal grid at the bottom of the tank allowed for the evacuation of the water). Fish were exposed to air for 45 s, returned to the water for 15 s, and exposed again to air for 45 s. This protocol was applied in the morning and feeding started 1 h after the stressor (belt feeders were switched on). After the 15 day period, all fish were exposed the next morning to an acute crowding stressor by lifting the plastic aquaria and leaving only a 4 cm water layer (stocking density of 200 kg m^{-3}). The crowding stress lasted for 30 min and then the water level was returned to normal by putting the plastic aquaria back to their normal position at the bottom of the tank. During this crowding trial, fish were sampled at 0 h (just before the crowding stress – controls), and at 1 h, 2 h, 4 h and 8 h after the start of the crowding. Fish groups not exposed to the repeated stress protocol served as controls for the influence of previous stress on the acute stress response of the fish. Fifteen fish per fraction and treatment (five from each aquarium) were sampled at every sampling point. During the sampling from each aquarium, five fish were netted together and quickly anesthetized in a benzocaine bath (200 mg L^{-1}). Fish were under deep anesthesia within 30 s and blood was collected from caudal vessels with ammonium-heparinized syringes. Then, the fish was decapitated and the head immediately frozen on dry ice and later stored at −80 °C. The sampling of each batch of five fish took approximately 4 min. The blood samples of each batch of fish were immediately centrifuged and the plasma was stored at −80 °C for subsequent analyses of cortisol, glucose and lactate.

2.7. Biochemical analyses

2.7.1. Fatty acid composition

Feed samples and fish fry (5 individuals sample $^{-1}$) were homogenized by a Tissue Tearor (probe diameter 4.5 mm, Biospec Products, Inc.; Bartlesville, USA). Fatty acid analyses were carried out as previously described (Lund et al., 2019). Briefly, lipids were extracted by a 2:1 (*v*/v) chloroform/methanol mixture (Folch et al., 1957) and 40 μL (1 mg mL^{-1}) of an internal 23:0 FAME standard (Sigma. St. Louis, MO, USA) was added. Samples were allowed to extract for 24 h at −20 °C followed by centrifugation. The supernatant was subsequently transferred to clean GC vials and allowed to dry out in a Pierce, reacti-therm heating module at 60 °C, under a continuous flow of nitrogen. Trans esterification of the lipids was done by addition of 1 mL methanol: Toluene: Acetyl chloride (40:50:10, HPLC grade) at 95 °C. The fatty acid methyl esters were analyzed by gas chromatography–mass spectrometry (GC–MS). Peaks on a given chromatogram were identified by comparison with the retention time of a commercial mix of a known FAME standard (SUPELCO 18919 4:0–24:0; SIGMA, St. Louis, MO, USA). Peaks were quantified by means of the target response factor of the fatty acids and using 23:0 as internal standard. Fatty acid concentrations were calculated (MSD Chemstation Data Analysis, G1710FA) based on the quantified peaks of the standard series and the samples as dry weight of feed and fry and expressed as ng sample^{-1}. A total of 34 fatty acids were analyzed, but only the most relevant are shown (Table 2).

2.7.2. Digestive enzymatic activity

To isolate the digestive segment, fry were placed on an ice-cold dissection glass and the stomach region was separated from the intestinal segments. Pooled samples from each tank (10 individuals) were homogenized in 10 volumes (*v*/*w*) of cold distilled water. Lipase activity was assayed according to the method by Iijima et al. (1998), using Tris-HCl 0.25 M, pH 9, *p*-nitrophenyl myristate (Sigma 70,124) 0.53 mM, 2-methoxyethanol (Sigma 284,467) 0.25 mM, Sodium cholate (Sigma C-1254) 5 mM as substrates and acetone/n-heptane as solvent.

Alkaline phosphatase (AP) and aminopeptidase N, two enzymes of brush border membrane, were assayed according to previous methods (Bessey et al., 1946; Maroux et al., 1973), using *p*-nitrophenyl phosphate (Sigma-Aldrich) and L-leucine *p*-nitroanalide (Sigma-Aldrich) as substrates, respectively. Pepsin was assayed according to Cuvier-Péres and Kestemont (2001). Trypsin and amylase activities were assayed according to Holm et al. (1988) and Métais and Bieth (1968), respectively, as described by Gisbert et al. (2009). Protein was determined using the Bradford procedure (Bradford, 1976). Enzyme activities are expressed as specific activities (mU/mg protein).

2.7.3. Cortisol, glucose, lactate, brain amines

A commercial ELISA kit (402,710, Neogen Europe, Ayrshire, UK) was used to quantify plasma cortisol concentration. The kit was previously shown to provide good linearity, parallelism and reproducibility (Gesto et al., 2018). Plasma glucose and lactate were analyzed with colorimetric kits (MAK263 and MAK064, respectively) from Sigma (St Louis, MO, USA).

Fish brains were dissected out from the frozen heads and immediately processed for the analysis of serotonergic activity. The forebrain,

Table 2
Analyzed total fatty acid (TFA) content (mg g^{-1} d.w. ± SD) and fatty acid composition (% of TFA ± SD) of the three experimental diets (L, M, H) and the commercial feed (Aller Futura, CtrAF; Mean values ± SD, $n = 2$).

Diet	L	M	H	CtrAF
TFA (mg g^{-1} d.w. ± SD)	210.1 ± 1.3^{a}	194.3 ± 12.3^{a}	189.0 ± 2.0^{a}	149.8 ± 3.1^{b}
FA				
14:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	5.3 ± 0.1
16:0	5.3 ± 0.2	5.1 ± 0.0	5.1 ± 0.1	17.3 ± 0.2
18:0	1.6 ± 0.1	1.8 ± 0.0	1.9 ± 0.0	2.5 ± 0.0
20:0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.3 ± 0.0
22:0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.1 ± 0.0
24:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
Total SFA	8.2 ± 0.3	8.2 ± 0.2	8.4 ± 0.1	26.5 ± 0.5
16:1(n-7)	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	5.3 ± 0.0
18:1(n-9)	59.0 ± 0.4^{c}	57.4 ± 0.1^{b}	54.8 ± 0.2^{a}	26.7 ± 0.1
20:1 (n-9)	1.4 ± 0.0	1.5 ± 0.0	1.6 ± 0.0	7.2 ± 0.0
22:1(n-9)	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.2	0.7 ± 0.1
24:1(n-9)	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Total MUFAs	61.4 ± 1.9	60.0 ± 1.7	57.2 ± 2.0	40.8 ± 7.5
18:2(n-6)	22.1 ± 0.2^{b}	21.7 ± 0.0^{ab}	21.3 ± 0.1^{a}	14.2 ± 0.1
18:3(n-6)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.
20:2(n-6)	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.4 ± 0.0
20:3(n-6)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
20:4(n-6) ARA	0.0 ± 0.0^{a}	0.2 ± 0.0^{b}	0.5 ± 0.0^{c}	0.6 ± 0.0
Total(n-6)	22.3 ± 0.3	22.1 ± 0.1	22.0 ± 0.1	15.5 ± 0.2
18:3(n-3)	7.7 ± 0.1	7.8 ± 0.1	7.7 ± 0.1	4.2 ± 0.0
20:3(n-3)	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
20:5(n-3) EPA	0.3 ± 0.0^{a}	0.9 ± 0.0^{b}	1.9 ± 0.0^{c}	10.7 ± 0.1
22:6(n-3) DHA	0.1 ± 0.0^{a}	1.0 ± 0.1^{b}	2.7 ± 0.2^{c}	2.1 ± 0.3
Total(n-3)	8.1 ± 0.2^{a}	9.7 ± 0.2^{b}	12.4 ± 0.3^{c}	17.2 ± 0.5
DHA/EPA	0.6 ± 0.3^{a}	1.1 ± 0.1^{ab}	1.4 ± 0.1^{c}	0.2 ± 0.0
(n-3)/(n-6)	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	1.1 ± 0.0

Experimental diets with a different superscript letter are significantly different ($p \leq 0.05$).

including olfactory bulb, telencephalon, optic tectum and hypothalamus, was homogenized in 0.5 mL of a 4% perchloric acid solution 0.1 mM EDTA. After centrifugation of the homogenate, a diluted aliquot of the supernatant was analyzed using high performance liquid chromatography with electrochemical detection (HPLC-EC) as previously described (Gesto et al., 2017). The levels of serotonin (5-HT) and its main oxidative metabolite 5-hydroxyindoleacetic acid (5-HIAA) were quantified by comparing peak areas with those of corresponding standards. The ratio between 5-HIAA and 5-HT was then calculated as an indirect measure of the activity of serotonergic neurons (Winberg and Nilsson, 1993).

2.8. Statistical analyses

In experiment 1, percent data were arcsine-transformed before analysis. Experimental data were subjected to a one-way ANOVA (fatty acid content; growth, enzymatic activity) for determining significant differences among experimental treatment groups (H, M, L); for analysis of the growth results CtrAF was included in the ANOVA. The Holm-Sidak post-hoc test was used for all pairwise multiple comparisons. For experiment 2, differences in survival between the groups were tested by one-way analysis of variance (ANOVA) and the Tukey multiple pairwise-comparison test, using the statistical analyzing software R version 4.0.2 (functions used were "aov()" and "TukeyHSD()"). In experiment 3, three-way ANOVA was used to analyze the stress markers using "diet (CtrAF, L, M, H), "repeated stress" (yes, no), and "time post-acute stress" (0, 1 h, 2 h, 4 h, 8 h), as factors. Tukey's HSD tests were used after the ANOVA to determine statistical differences among the groups. Data was analyzed using the tanks ($n = 3$) as experimental units. Sigmaplot v 14.0 (Systat Software, San Jose, CA, USA) was used for all analyses in experiments 1 and 3. Values throughout the text are generally expressed as the mean values with standard deviations. In all statistical tests used, $P \leq 0.05$ was considered statistically different.

3. Results

3.1. Dietary fatty acid composition

The analyzed total fatty acid content (TFA) was similar in all experimental diets ($P = 0.122$), while TFA was lower in the commercial control diet (CtrAF) (Table 2). For the n-3 LC-PUFA content, significant differences were observed in EPA and DHA ($p < 0.001$). For both n-3 LC-PUFAs the highest content was observed in diet H, followed by diet M and L. A similar difference was observed for the n-6 LC-PUFA ARA, while the opposite effect was observed for 18:1n-9 (oleic acid - OA) and 18:2n-6 (linoleic acid – LA). (Table 2). For the CtrAF diet, the content of EPA was more than five times higher than in any of the experimental diets.

3.2. Experiment 1: Fry fatty acid composition, growth performance and digestive enzyme activity

Yolk sac fry sampled before first feeding showed a DHA content of 30% and an EPA content of 15% (% TFA). At the end of the experiment at 94 DPFF (Table 3), EPA and DHA content in fry was significantly higher for trout fed diet H, with 2.3% and 12.4% content, respectively, and lower in fry fed diet L with about 0.3% EPA and 2.1% DHA (H > M > L, $p < 0.001$). In comparison, tissue EPA and DHA content was 10% - and 15% (% TFA), respectively, in fry fed the commercial CtrAF diet. The development in FA tissue content at 1, 16, 52 and 94 days post first feeding (DPFF) (Fig. 1) revealed a progressive significant ($p < 0.001$) steep decline in both DHA and EPA fry content until 52 DPFF for all treatment groups, most explicit for the L group. DHA content continued to decrease from 52 to 94 DPFF ($p \leq 0.04$) for diet L, but tended to increase in fry fed the other diets ($p \geq 0.194$); while EPA content for all groups remained at the same values as observed at 52 DPFF.

ARA tissue content at 94 DPFF reflected dietary composition and was lowest in juveniles fed diet L, significantly lower than for juveniles fed diet M or H ($p < 0.001$). ARA fry content was gradually depleted over the course of the experiment for fish on diet L, while for the M and H groups, it remained on a similar level as at DPFF 0.

Table 3
Analyzed total fatty acid (TFA) content (mg g-1 w.w.) and fatty acid composition (% of TFA ± SD) in trout fry at experimental start and at the end, at 94 days post first feeding (DPFF).

Fry age	DPFF 0	DPFF 94			
Diet		L	M	H	CtrAF
TFA (mg g^{-1} w.w.)	109.9 ± 27.5	56.2 ± 28.8	48.9 ± 11.0	53.2 ± 15.8	65.0 ± 29.1
FA (% TFA)					
14:0	0.1 ± 0.0	0.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
16:0	9.1 ± 0.8	7.8 ± 2.7	5.7 ± 0.7	5.3 ± 0.5	10.4 ± 1.9
18:0	2.7 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.4	0.6 ± 1.0
20:0	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:0	0.0 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
24:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total SFA	13.2	8.9	6.6	6.1	13
16:1(n-7)	4.0 ± 0.3	1.4 ± 0.7	0.9 ± 0.3	1.1 ± 0.1	4.3 ± 0.3
18:1(n-9)	19.3 ± 0.8	50.5 ± 5.6^{b}	42.9 ± 4.6^{ab}	42.4 ± 1.4^{a}	26.7 ± 1.9
20:1 (n-9)	2.1 ± 0.2	1.4 ± 0.6	2.2 ± 1.2	0.7 ± 0.3	1.8 ± 1.6
22:1(n-9)	3.7 ± 0.1	0.1 ± 0.0	0.5 ± 0.3	0.2 ± 0.1	3.4 ± 0.7
24:1(n-9)	2.2 ± 0.1	0.7 ± 0.2^{a}	3.1 ± 0.4^{c}	1.4 ± 0.2^{b}	0.7 ± 0.5
Total MUFAs	31.8	54.3	49.6	45.9	36.9
18:2(n-6)	5.9 ± 0.5	27.1 ± 3.8	24.9 ± 5.8	28.6 ± 1.3	21.5 ± 2.1
18:3(n-6)	0.0 ± 0.0	0.3 ± 0.5	1.1 ± 0.9	0.1 ± 0.2	0.1 ± 0.1
20:2 (n-6)	0.0 ± 0.0	0.4 ± 0.2	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.2
20:3(n-6)	0.6 ± 0.0	1.2 ± 0.5	1.4 ± 1.3	0.5 ± 0.0	0.2 ± 0.2
20:4(n-6) ARA	1.3 ± 0.0	0.6 ± 0.1^{a}	1.4 ± 0.1^{b}	1.5 ± 0.0^{b}	1.4 ± 0.1
Total (n-6) PUFA	8.7	30.3	29.2	31.4	23.7
18:3(n-3)	0.4 ± 0.0	4.2 ± 1.8	3.2 ± 0.7	4.4 ± 0.8	0.6 ± 0.9
20:3(n-3)	0.1 ± 0.1	0.1 ± 0.0	0.9 ± 0.4	0.1 ± 0.0	0.4 ± 0.3
20:5(n-3) EPA	15.4 ± 0.6	0.3 ± 0.2^{a}	1.0 ± 0.2^{b}	2.3 ± 0.4^{c}	10.3 ± 2.6
22:6(n-3) DHA	30.7 ± 1.2	2.1 ± 0.4^{a}	9.4 ± 1.1^{b}	12.4 ± 1.1^{c}	15.0 ± 2.2
Total (n-3) PUFA	47.1	7.1	14.9	18.5	27.5
DHA/EPA	2.0	7.0	9.4	5.3	1.5
(n-3)/(n-6)	5.4	0.2	0.5	0.6	1.2

Values represent means and standard deviations of $n = 6$ fry pools. Different superscript letters indicate significant differences ($p \leq 0.05$) among experimental diets at 94 DPFF.

The tissue content of OA, 18:1n-9; the most prevalent monounsaturated FA in fry tissues, showed a steep increase from 0 DPFF until 52 and then slightly decreased between 52 and 94 DPFF similar for all experimental groups ($p \geq 0.078$). For fry fed the experimental diets L, M and H, LA (18:2n-6) and ALA (18:3n-3) content revealed a steep significant ($p \leq 0.01$) increase over time until 52 DPFF. In the case of 18:2n-6 content, the curve flattened out at 94 DPFF for M and L, but continued to increase for H. Fry fed CtrAF showed a completely different time-related progressive development in tissue content of 16:0, 18:1n-9, 18:3n-3; ARA and EPA, as compared with the experimental diets, reflecting the use of other ingredients and oil sources.

During the experiment, DPFF 7 to DPFF 94 mean fry weight increased from 0.16 g to 5.5–6.3 g (Fig. 2A). Individual weight of sampled fry at 94 DPFF was similar between experimental treatment groups ($p \geq 0.568$), while fry weight for CtrAF was higher than for the three experimental groups ($p \leq 0.002$). Biomass growth measured as SGR in the period from 32 DPFF to 94 DPFF, (57 feeding days) ranged from 4.27 to 4.35% between groups ($p \geq 0.334$) and FCR from 0.75–0.78 ($p \geq 0.247$) (Fig. 2B and C). Results revealed, that during this growth period, CtrAF was not significantly different to any experimental group

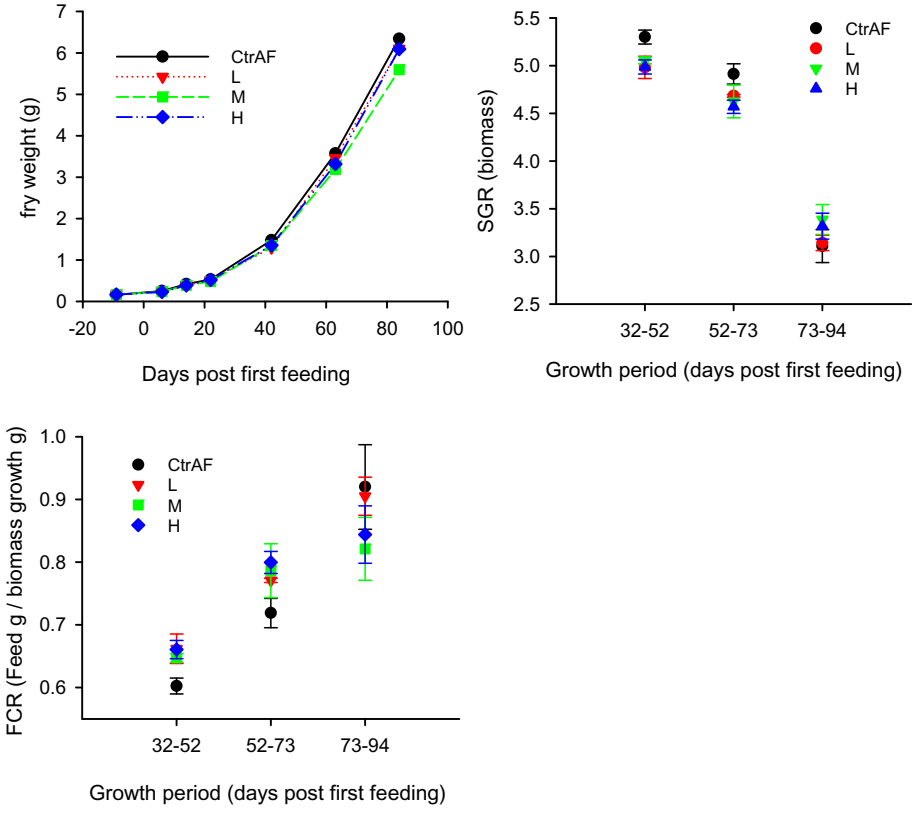

Fig. 2. Individual growth performance (individual mass, SGR and FCR) of rainbow trout fry during experiment 1 for the three experimental codes L, M, H and a commercial control diet (CtrAF), $n = 3$.

(SGR, $p = 0.052$; FCR, $p = 0.263$). When growth data was examined for each growth period, it revealed a higher SGR and lower FCR for CtrAF than any of the three experimental diets from 32 to 52 DPFF (SGR, $p \leq 0.022$; FCR, $p \leq 0.031$). Likewise, from 53 to 72 DPFF there was a higher SGR and lower FCR for CtrAF vs diet H (SGR, $p = 0.025$; FCR, $p = 0.031$), while in the last growth period (73–94 DPFF), no significant differences (SGR, $p = 0.112$; FCR, $p = 0.118$) were observed between treatments.

The development of digestive enzymatic activity revealed a similar pattern until DPFF 94 for all dietary groups (incl. CtrAF) despite the different dietary supplementation of n-3 LC-PUFAs (Fig. 3). For trypsin, AP, peptidase and lipase, no activity was revealed until 24 DPFF. Trypsin reached the highest activity at 52 DPFF, and a general increase in AP activity was seen from 24 DPFF. Lipase activity was not detected until 52 DPFF. Pepsin and amylase activity was detectable already from 7 DPFF and peaked for pepsin at 52 DPFF, while amylase activity increased until 16 DPFF, after which a steep decline was observed, followed by an increase until the end of the experiment for all dietary groups. Only lipase activity and amylase activity differed among experimental dietary groups. At 52 DPFF lipase activity was significantly higher ($p = 0.025$) for diet M than for L and H and similarly higher for M than L at 94 DPFF ($p = 0.028$). For amylase the activity was higher in L and M than for the control diet CtrAF at 7 DPFF ($p = 0.05$) and higher in L than H at 16 DPFF ($p = 0.03$).

3.3. Experiment 2: Infection challenge

In general, moderate survival of 57–78% was observed in the bath challenged fish, while low survival of 2–10% was observed in the IP challenged fish from the experimental treatments (Table 4). All control fish (i.e. fish challenged with bacterium free medium) survived the trials. For the bath challenge, the CtrAF-fed group had significantly higher survival of 92% compared to the M group with 57% survival ($p = 0.014$). The survival of the bath challenged groups fed the L and H feed (68 and 78%, respectively), was not significant lower than the group fed the CtrAF feed ($p = 0.056$ and $p = 0.213$, respectively). Concerning the IP challenged fish, there were statistically significant differences in survival between fish fed any of the L, M or H diet, with survivals of 6, 2 and 10%, respectively, compared to the CtrAF fed fish, with a survival of 30% ($p = 0.004$, $p = 0.001$ and $p = 0.010$, respectively). There were no statistical significant difference in survival among the fish delivered the three experimental feeds (L, M and H) in any of the two challenges. All survival values in Experiment 2 are final survivals recorded at termination of the experiments.

3.4. Experiment 3: Stress challenge

At the end of the experiment, fish from the CtrAF group had a higher individual mass than the L group (Table 5). However, neither the diet type, nor the daily stress protocol had any effect on the SGR of the fish.

An effect of post-stress time ($p < 0.001$) and diet ($p < 0.001$) was observed for cortisol (Tables 5 and 6). The kinetics of the cortisol profile was similar for all diets, with a cortisol peak at 60 min post-acute stress and at 120 min after stress exposure cortisol levels had recovered in all cases). The fish fed diet H had higher levels of cortisol globally (between 43% and 67% higher), when compared to the other groups. The repeated stress protocol had no effect ($p = 0.623$) on the cortisol response. No interactions among any of the three factors were found.

The three-way ANOVA demonstrated an effect of diet ($p = 0.002$) and post-stress time ($p < 0.001$), but not of repeated stress ($p = 0.887$) on the plasma glucose levels (Table 6). No interactions among any of the 3 factors were found. The fish fed the control feed showed 12% to 14% higher glucose values than the fish fed any of the other diets. In general, glucose levels increased at 60 min and 120 min after stress, and recovered to pre-stress levels at 240 min post stress.

For the plasma lactate levels, the three-way ANOVA demonstrated an effect of diet ($p < 0.001$) and post-stress time ($p < 0.001$), but not of the

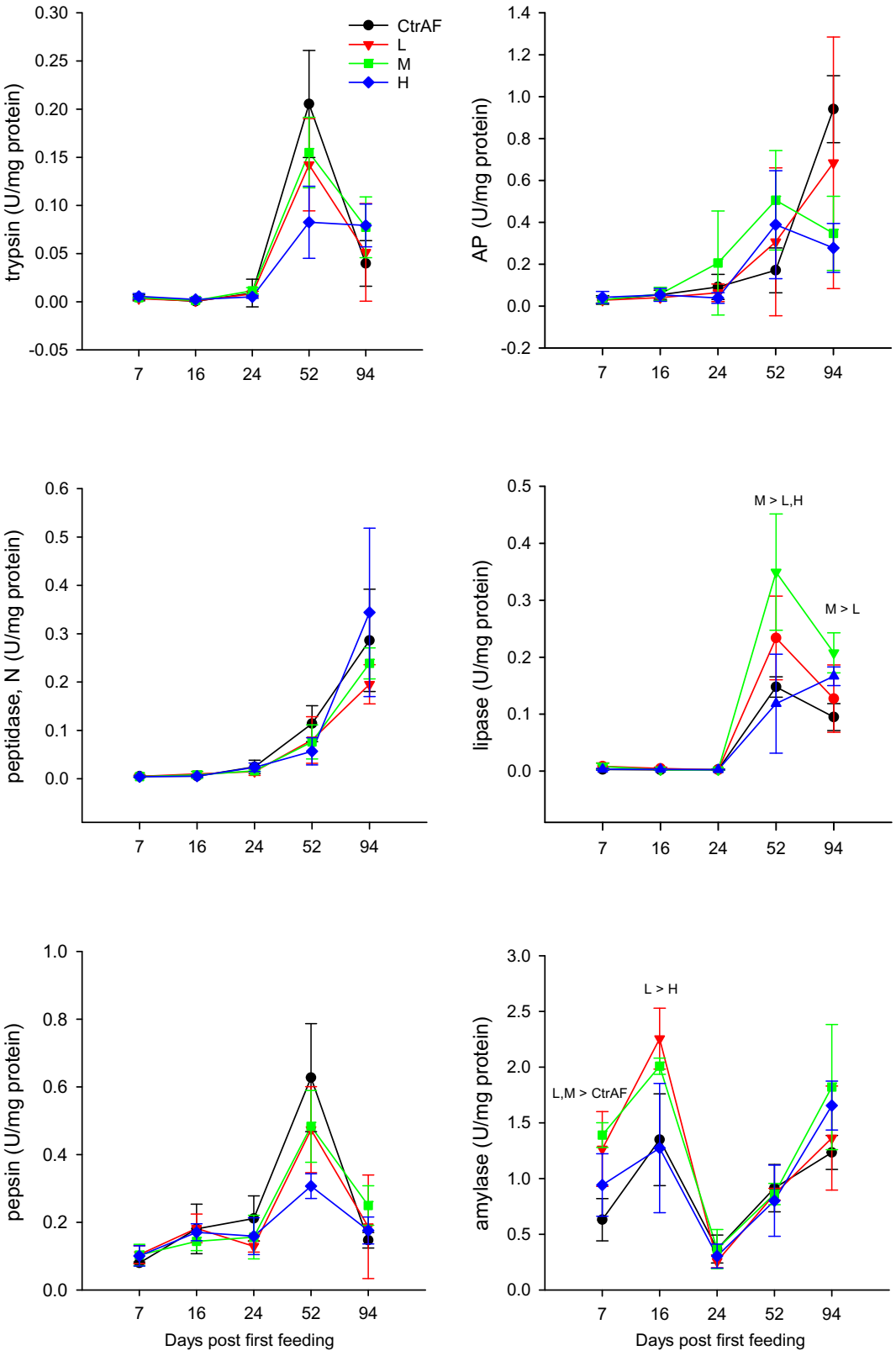

Fig. 3. Digestive enzymatic activity of trout at 7, 16, 52, 94 DPFF when fed diets L, M, H and a commercial CtrAF. Mean values and standard deviations, $n = 10$. Significant differences associated to the diets (only found for lipase and amylase) are indicated as superscripts at each sampling time.

repeated stress ($p = 0.113$) (Table 6). In general, lactate levels were higher in diet H and L than in diet M. Levels in diet H were also higher than in the control diet group. The repeated stress protocol interacted with the effect of the time post-stress on lactate levels ($p < 0.001$). Specifically, lactate levels increased 60 min after stress and then recovered to basal levels from 120 min after stress until the end of the experiment, but the lactate peak at 60 min was 31% lower in fish exposed to the repeated stress protocol than in non-exposed fish.

Table 4
Final survival of fish delivered four different feed types in two different challenge systems (bath and IP).

Feed type	L	M	H	CtrAF
Bath challenge (final survival % ± SD)	68 ± 5	57 ± 9*	78 ± 4	92 ± 4
IP challenge (final survival % ± SD)	6 ± 0*	2 ± 2*	10 ± 5*	30 ± 10

The IP challenge experiment and the bath challenge experiment were terminated after 34 and 55 days, respectively. The asterisk marks statistical significant difference ($p \leq 0.05$) in survival between the experimental diets and the CtrAF feed (controls). There were no statistical significant difference in survival among the fish delivered the three modified feed types.

Regarding the serotonergic activity in the forebrain, the ANOVA showed an effect of diet ($p = 0.007$) and of time post-stress ($p < 0.001$) but not of the repeated stress ($p = 0.449$) (Table 7). Fish fed the L and M diets showed higher serotonergic ability, than fish fed the H diet, whose levels were closer to those of the control group. The kinetics of the response to acute stress was similar to that of the glucose levels. Serotonergic activity increased after stress and was higher than basal levels at 60 min and at 120 min after stress. At 240 min after stress, serotonergic activity was back again at levels similar to pre-stress levels. This kinetics was similar to that found for the 5-HIAA levels (significant effect of time post-stress, $p < 0.001$), which were higher at 1 h and 2 h after stress and were again at basal levels at 4 h after stress. There was also an interactive effect ($p = 0.024$) of the diet and the repeated stress protocol on the 5-HIAA levels: In fish exposed to repeated stress, 5-HIAA content was lower in L and H diets than in M; in the case of L, levels were also lower than in the control (CtrAF). Only for the M diet, 5-HIAA levels were statistically higher in the fish exposed to repeated stress than in the unexposed controls. The forebrain 5-HT content was only affected by the diet ($p < 0.001$), and did not vary during the acute stress response. The 5-HT levels were higher in fish fed on CtrAF and H diets than in the L group.

4. Discussion

The aim of the present study was to evaluate dietary effects - of either total replacement of EPA and DHA by use of supercritically extracted FM and inclusion of rapeseed oil in first feeding rainbow trout - or by a gradual increased supplementation of EPA and DHA. The low dietary EPA + DHA content (0.4% TFA) in diet L and corresponding low values in fry fed this diet at 94 DPFF did not cause any significant effects on fry growth performance, feed utilization or survival, which were similar to results of highest supplementation of EPA + DHA (diet H: 4.8% TFA). The slightly better growth performance of the commercial CtrAF diet might be due to a higher dietary content of especially EPA than in the experimental diets, or reflect the use of high quality FM, FO and krill meal and a formulation better adjusted to the composition and requirement of yolk sac rainbow trout. It should also be noted that EPA and DHA levels were high (15% and 31%, respectively) in the fish at DPFF0, and they could have acted as a starting reservoir during the initial development after weaning. Dietary provision of ALA and LA in the experimental diets by vegetable rapeseed oil likely satisfied the physiological requirement for essential C18 PUFAs of the fry (Castell et al., 1972). The 18:3n-3 ALA and 18:2n-6 LA act as precursors for LC-PUFA biosynthesis, as rainbow trout possess the metabolic ability for upregulation and expression of long chain PUFA biosynthetic genes and PUFA bioconversion (Gregory et al., 2016; Mellery et al., 2017). It is also well known that this metabolic effort is insufficient to compensate for a decreased LC-PUFA intake (Turchini and Francis, 2009) and this corresponded well with EPA and DHA tissue levels in fry (94 DPFF) that were six- and eight-fold lower, respectively for diet L than for diet H. Fatty acid composition of fish will reflect that of the lipid dietary source (Bell et al., 2004; Bou et al., 2017; Wang et al., 2018) and a total PUFA requirement of salmonids (incl. 18:3n-3) has been reported to be in the range 1–2.5% of diet, depending on species, size and environmental conditions (Glencross, 2009). Rainbow trout fry used for the subsequent stress challenge and infection experiments most likely experienced a further depletion of EPA and DHA (i.e. for fish fed diet L) due to the prolonged experimental period, as indicated by an almost linear regression line (Fig. 1) but likely not having a metabolic deficiency due to the high tissue content of ALA.

Digestive enzymatic capacity is considered a good indicator of fry and larval digestive capacities and dietary ingredient composition has been reported to affect digestive enzymatic activity in several fish species. In red seabream (*Pagrus major*) digestive lipase activity related to both the acyl chain length and the degree of unsaturation, which has been shown to have a higher preference for PUFA as substrates (Iijima et al., 1998). In the present study, however, trout lipase enzymatic activity seemed to depend more on ontogeny and age/size, as no lipase activity was recorded at 7, 16 and 24 DPFF but only at 52 - and 94 DPFF with no clear trend in the effects related to EPA and DHA supplementation (activity was higher with the intermediate supplementation). Likewise, we observed similarity in the tissue activity of pancreatic proteases and amylase between dietary treatments, even for the commercial CtrAF diet, that had a different composition and showed periodic better growth and FCR than the experimental diets. In diets for which animal protein sources have been replaced by plant protein sources containing anti-nutritional factors and proteinase inhibitors, an effect on pancreatic protease has been reported (Sotoudeh and Mardani, 2018). For instance, trout proteases including trypsin are highly sensitive to plant protease inhibitors (Krogdahl et al., 1994). In this study, high quality, mainly marine animal proteins were used in all diets, which likely explain the similar digestive enzymatic activity.

Although growth and digestive enzymatic activity were not compromised by dietary depletion or additional supplementation of LC-PUFAs, there is a lack of knowledge concerning effects on rainbow trout fry ability to cope with stress and robustness towards infectious diseases. The addition of LC-PUFAs to vegetable-based diets may improve performance and robustness of the fish and act as immune-stimulants (Bou et al., 2017; Dawood, 2020).

Table 5
Growth performance of the fish in the stress-challenge trial.

Diet	C		L		M		H		two way ANOVA		
Repeated stress (RS)	no	yes	no	yes	no	yes	no	yes	DIET	RS	Diet x RS
Initial mass (g)[a]	7.37 ± 0.38	7.37 ± 0.38	6.46 ± 0.35	6.46 ± 0.35	6.20 ± 0.42	6.20 ± 0.42	7.01 ± 0.77	7.01 ± 0.77	$p = 0.240$	na	na
Final mass (g)	14.43 ± 0.83	14.32 ± 0.55	12.93 ± 0.39	12.53 ± 069	12.96 ± 0.43	13.63 ± 0.07	13.34 ± 0.51	13.19 ± 1.16	**$p = 0.018$, C > L**	$p =$ 0.992	$p =$ 0.631
SGR (% day-1)[b]	2.86 ± 0.11	2.83 ± 0.02	2.89 ± 0.15	2.76 ± 0.15	2.87 ± 0.29	3.07 ± 0.17	2.64 ± 0.25	2.57 ± 0.46	$p = 0.195$	$p =$ 0.954	$p =$ 0.713

Data indicate the average ± SD of $n = 3$ tanks per diet and stress condition. na: not applicable.
[a] Initial mass at the time of allocating the fish. Calculated per diet before assigning the stress treatment to the groups.
[b] SGR calculations englobe the 15 days of acclimation to experimental tanks plus 15 days of the repeated stress-challenge trial.

Table 6
Plasma stress markers in fish after an acute stress challenge in rainbow trout fed different diets (CtrAF, L, M, H), and that had been exposed (RS+) or not (RS-) to a daily-repeated stress protocol for 15 days.

Time post-acute stress (TPS)	0 h	1 h	2 h	4 h	8 h
Cortisol ng mL-1					
CtrAF - RS-	7.18 ± 2.79	16.01 ± 2.87	7.39 ± 1.78	5.47 ± 1.97	5.79 ± 1.84
CtrAF - RS+	4.07 ± 1.30	10.49 ± 2.78	5.21 ± 2.11	6.27 ± 0.71	6.04 ± 0.35
L - RS-	5.90 ± 1.11	19.66 ± 2.84	6.04 ± 0.67	8.47 ± 4.81	7.60 ± 5.41
L- RS+	6.16 ± 2.69	12.78 ± 0.75	5.44 ± 1.63	7.81 ± 1.00	6.37 ± 3.42
M - RS-	5.32 ± 2.69	12.29 ± 1.19	5.17 ± 1.89	5.06 ± 0.90	4.94 ± 3.38
M - RS+	4.54 ± 2.11	14.86 ± 2.66	7.95 ± 0.48	7.39 ± 1.18	6.47 ± 3.21
H - RS-	4.17 ± 0.37	26.11 ± 5.65	8.97 ± 1.48	9.23 ± 1.81	12.56 ± 3.97
H - RS+	8.82 ± 4.30	22.03 ± 4.15	10.35 ± 3.93	9.08 ± 5.73	12.47 ± 3.36
Glucose mM					
CtrAF - RS-	3.62 ± 0.42	4.27 ± 0.34	4.74 ± 0.58	3.88 ± 0.51	3.04 ± 0.32
CtrAF - RS+	3.65 ± 0.63	4.18 ± 0.63	4.43 ± 0.85	4.02 ± 0.73	3.43 ± 0.71
L - RS-	2.88 ± 0.05	4.02 ± 0.13	3.80 ± 0.08	3.39 ± 0.20	3.19 ± 0.21
L- RS+	2.89 ± 0.12	3.74 ± 0.19	3.75 ± 0.24	3.18 ± 0.14	3.40 ± 0.28
M - RS-	2.86 ± 0.11	3.71 ± 0.46	3.76 ± 0.33	3.09 ± 0.15	3.06 ± 0.38
M - RS+	3.13 ± 0.08	3.77 ± 0.13	3.71 ± 0.30	3.48 ± 0.21	3.22 ± 0.17
H - RS-	2.98 ± 0.21	3.85 ± 0.30	3.90 ± 0.28	3.26 ± 0.10	2.99 ± 0.17
H - RS+	3.25 ± 0.16	3.76 ± 0.31	3.37 ± 0.31	3.29 ± 0.35	2.98 ± 0.24
Lactate mM					
CtrAF - RS-	1.38 ± 0.20	3.26 ± 0.11	1.40 ± 0.31	1.25 ± 0.13	1.22 ± 0.09
CtrAF - RS+	1.13 ± 0.11	2.07 ± 0.11	1.40 ± 0.06	1.44 ± 0.12	1.36 ± 0.07
L - RS-	1.25 ± 0.05	3.55 ± 0.33	1.61 ± 0.32	1.16 ± 0.14	1.46 ± 0.19
L- RS+	1.40 ± 0.07	2.59 ± 0.48	2.05 ± 0.10	1.40 ± 0.32	1.78 ± 0.22
M - RS-	1.16 ± 0.16	2.45 ± 0.45	1.76 ± 0.05	1.27 ± 0.21	1.31 ± 0.20
M - RS+	1.39 ± 0.26	1.96 ± 0.08	1.26 ± 0.17	1.38 ± 0.12	1.53 ± 0.03
H - RS-	1.66 ± 0.26	3.77 ± 0.42	1.69 ± 0.22	1.30 ± 0.14	1.50 ± 0.11
H - RS+	1.70 ± 0.06	2.34 ± 0.24	1.64 ± 0.13	1.83 ± 0.29	1.57 ± 0.09

3-way ANOVA significant effects			
Variable	Factor	*P*-value	Effect
Cortisol	D	**< 0.001**	H > CtrAF, L, M
	TPS	**< 0.001**	1 h > 0 h, 2 h, 4 h, 8 h
Glucose	D	**0.002**	CtrAF > H, L, M
	TPS	**< 0.001**	1 h, 2 h > 0 h, 8 h
Lactate	D	**< 0.001**	H > CtrAf, M; L > M
	TPS	**< 0.001**	na
	RS x TPS	**< 0.001**	**In both RS groups**: 1 h > 0 h, 2 h, 4 h, 8 h; **At 1 h**: RS+ < RS-

When an interaction was found, main effects were not considered and appear as "not applicable" (na). Significant effects (3-way ANOVA) for the factors diet (D; CtrAF, L, M or H), repeated stress (RS; RS+ or RS-) and time post-acute stress (TPS; 0 h – unexposed controls, 1 h, 2 h, 4 h or 8 h) and/or their interactions are indicated.

Differently to growth performance, the resistance of the fish against infection with *Flavobacterium psychrophilum* was highly dependent on their diet. Dietary VO inclusion reduced survival in both bath and ip infection trials. Dietary VO inclusion had been previously shown to have different effects on fish mortality upon exposure to infectious diseases, depending on the fish species, the pathogen, and the nature and rate of inclusion of the VO (Ayisi et al., 2018; Geay et al., 2015; Montero and Izquierdo, 2010). The effects of VO inclusion on survival to infections are expected to be at least partly mediated by the alterations in fatty acid composition of the fish, which in turn are known to affect the fish immune system at different levels. We did not evaluate the immune response of the fish in this study, but it is known that dietary VO inclusion can affect different aspects of the fish immune system including immune cell fatty acid composition and activity, humoral immunity or eicosanoid production (Montero and Izquierdo, 2010; Nguyen et al., 2021; Nguyen et al., 2020; Nguyen et al., 2019). In our study, VO inclusion induced important alterations in the fatty acid composition of the fish, which were only partially compensated, in terms of body content of total n-3 PUFAs, by LC-PUFA supplementation in diets M and H. The observed reduced survival might have been mediated by an imbalance between n-3 and n-6 PUFAs in the VO-based diets. PUFAs are precursors for the production of eicosanoids, which exert hormone-like actions and are involved in different biological functions, including immune and inflammatory responses (Oliva-Teles, 2012; Rowley et al., 1995). The nature and activity of the eicosanoids differ depending on the type of precursor: It was observed that high levels of n-6 derived eicosanoids tend to be immunoactive and pro-inflammatory while high presence of n-3 eicosanoids, less active, tend to be immunosuppressive and/or anti-inflammatory (Bell and Sargent, 2003; Oliva-Teles, 2012; Wall et al., 2010). For example, in a previous study with large yellow croaker (*Larimichthys crocea*), total replacement of dietary FO by rapeseed oil induced an increase of the transcripts coding for pro-inflammatory cytokines, while the levels of transcripts for anti-inflammatory cytokines were reduced (Mu et al., 2020). Therefore, a proper balance between n-6 and n-3 PUFAs is important to adequately modulate the immune responses in vertebrates. In our study, fish fed the H diet, with the higher n-3 LC-PUFA supplementation, tended to show better survival than L and M diet groups, suggesting that the low dietary amount of n-3 PUFA might indeed be part of the reason behind the high mortality in VO diets. In this regard, different studies have shown a negative effect of insufficient dietary n-3 LC-PUFA content on the immune response of fish (Kiron et al., 1995; Montero et al., 1998). However, the mortality in the H group was far from that of the control group, suggesting that other factors might also be involved. In this regard, rapeseed oil contains high levels of OA (18:1n-9) and high levels this FA has been suggested to reduce the activity of immune phagocytic cells in

Table 7
Brain serotonergic activity in fish after an acute stress challenge in rainbow trout fed different diets (CtrAF, L, M, H), and that had been exposed (RS+) or not (RS-) to a daily-repeated stress protocol for 15 days.

Time post-acute stress (TPS)	0 h	1 h	2 h	4 h	8 h
5-HIAA/5-HT mass ratio					
CtrAF - RS-	0.103 ± 0.003	0.123 ± 0.011	0.107 ± 0.011	0.106 ± 0.005	0.100 ± 0.008
CtrAF - RS+	0.097 ± 0.004	0.128 ± 0.006	0.112 ± 0.004	0.114 ± 0.012	0.110 ± 0.005
L - RS-	0.106 ± 0.006	0.136 ± 0.005	0.123 ± 0.005	0.114 ± 0.003	0.102 ± 0.002
L- RS+	0.104 ± 0.008	0.123 ± 0.004	0.130 ± 0.004	0.109 ± 0.008	0.099 ± 0.007
M - RS-	0.100 ± 0.002	0.121 ± 0.004	0.114 ± 0.007	0.112 ± 0.005	0.112 ± 0.010
M - RS+	0.108 ± 0.008	0.141 ± 0.023	0.123 ± 0.007	0.112 ± 0.005	0.113 ± 0.006
H - RS-	0.091 ± 0.004	0.113 ± 0.002	0.127 ± 0.010	0.102 ± 0.005	0.099 ± 0.004
H - RS+	0.104 ± 0.007	0.113 ± 0.002	0.111 ± 0.004	0.101 ± 0.007	0.094 ± 0.005
5-HIAA ng g-1					
CtrAF - RS-	28.46 ± 0.83	33.64 ± 2.69	31.27 ± 1.43	31.15 ± 2.52	28.44 ± 2.21
CtrAF - RS+	23.96 ± 1.82	33.95 ± 0.96	33.75 ± 1.93	30.92 ± 2.01	31.38 ± 1.32
L - RS-	26.27 ± 1.96	36.06 ± 1.36	32.32 ± 3.16	28.63 ± 1.68	24.96 ± 1.79
L- RS+	24.58 ± 0.64	34.14 ± 2.23	27.26 ± 3.57	25.31 ± 0.73	26.45 ± 1.46
M - RS-	26.72 ± 1.12	31.82 ± 0.82	29.86 ± 0.45	28.39 ± 2.38	30.01 ± 3.38
M - RS+	29.28 ± 2.20	38.23 ± 3.60	33.96 ± 1.09	31.46 ± 1.89	29.79 ± 2.69
H - RS-	24.01 ± 1.79	30.48 ± 0.63	30.26 ± 1.43	26.19 ± 2.63	27.16 ± 0.76
H - RS+	28.28 ± 1.04	33.11 ± 0.97	28.90 ± 0.84	28.07 ± 1.35	25.86 ± 1.50
5-HT ng g-1					
CtrAF - RS-	277.27 ± 11.92	276.72 ± 5.90	298.27 ± 24.34	296.06 ± 15.62	288.61 ± 10.15
CtrAF - RS+	248.26 ± 16.53	280.21 ± 14.41	291.94 ± 12.55	284.10 ± 22.90	284.01 ± 26.81
L - RS-	247.99 ± 18.17	263.99 ± 13.29	264.38 ± 17.09	252.79 ± 13.39	241.94 ± 13.99
L- RS+	243.91 ± 22.63	275.86 ± 12.29	233.53 ± 6.28	243.62 ± 9.73	268.28 ± 16.10
M - RS-	266.33 ± 11.84	263.13 ± 12.37	266.40 ± 18.07	255.01 ± 12.73	267.34 ± 10.13
M - RS+	268.99 ± 1.72	278.16 ± 18.74	274.44 ± 9.13	277.95 ± 9.13	263.22 ± 12.05
H - RS-	263.40 ± 8.15	268.29 ± 0.96	245.85 ± 28.28	258.05 ± 17.20	274.10 ± 3.63
H - RS+	287.71 ± 0.30	292.21 ± 2.38	265.36 ± 10.03	280.42 ± 12.99	274.19 ± 4.37

3-way ANOVA significant effects			
Variable	Factor	P-value	Effect
5-HIAA/5-HT	D	**0.007**	L, M > H
	TPS	**< 0.001**	1 h > 0 h, 4 h, 8 h; 2 h > 0 h, 8 h
5-HIAA	D	**0.002**	na
	TPS	**< 0.001**	1 h > 0 h, 2 h, 4 h, 8 h; 2 h > 0 h, 8 h
	D x RS	**0.024**	**In RS+**: M > L, H; CtrAF > L **In diet M**: RS+ > RS-

When an interaction was found, main effects were not considered and appear as "not applicable" (na).
Significant effects (3-way ANOVA) for the factors diet (D; CtrAF, L, M or H), repeated stress (RS; RS+ or RS-) and time post-acute stress (TPS; 0 h – unexposed controls, 1 h, 2 h, 4 h or 8 h) and/or their interactions are indicated (5-HT: serotonin; 5-HIAA: 5-hydroxyindoleacetic acid).

fish (Montero et al., 2003; Mu et al., 2020). We cannot discard that the high levels of OA in the fish fed VO diets in our study (59 to 89% higher than in the control diet group) have had a deleterious effect at this level. However, a more plausible explanation for the better survival of fish fed the CtrAF commercial diet might reside in the latter containing specific nutrients and micronutrients with a role in immune regulation, possibly including additives specifically targeting immune stimulation.

No large differences were found among the different diets in relation to the responses to acute or repeated stress. The kinetics of the response of the different plasma and brain stress markers were similar for all diets, and were not affected by previous exposure to repeated stress. The stress markers were in general back to basal levels 2 h (cortisol, lactate) or 4 h (glucose, serotonergic activity) after stress exposure. The duration and amplitude of the response of the different stress markers were as expected for a mild stressor in rainbow trout (Gesto et al., 2013). It is, however, interesting that the cortisol and lactate levels were generally higher in fish fed diet H than in other diet groups. The reason behind this is unknown, but it does not appear to be mediated by VO dietary inclusion, since cortisol and lactate levels in L and M diet groups were similar to those of the fish fed the CtrAF diet. The effect could be mediated by the PUFA supplementation: In diet H, the content of ARA and EPA was higher than in diets L and M. EPA and ARA have been shown to stimulate cortisol release by interrenal tissue in fish, this stimulation being regulated by LC-PUFA-derived eicosanoids (Ganga et al., 2011a; Ganga et al., 2006). However, this does not explain why the fish fed the CtrAF diet, with even higher levels of ARA and EPA, also had lower blood cortisol concentration than diet H-fed fish. In spite of the higher cortisol levels, the forebrain serotonergic ratio in diet H was lower than in L and M diet groups. This is not in support of fish fed diet H being more stressed, since forebrain serotonergic activity is generally considered a reliable stress indicator in fish and other vertebrates (Gesto et al., 2013; Winberg and Nilsson, 1993). In this case, the interpretation of the serotonergic ratio requires caution, since it was suggested that diets rich in n-3 LC-PUFAs and with high n-3/n-6 ratio might have an effect on serotonergic activity (Winberg et al., 2016), maybe affecting is relevance as a stress indicator. In this regard, it was shown in mice that n-3 LC-PUFA supplementation induced increases of brain serotonin content (Vancassel et al., 2008). This is consistent with the results of the present study that showed higher serotonin levels in the fish fed the diets CtrAF and H, than in the L diet that had lower ARA, EPA and DHA levels. In the case of glucose, in spite of similar kinetics in the response to acute stress, levels were in general higher in the control diet than in the L, M or H diets. This was probably resulting from a differential composition in the experimental diets compared to the commercial control diet in terms of total protein and carbohydrate content, which are both known to affect blood glucose in fish (Polakof et al., 2012). Taken together, in contrast to previous studies showing important effects of VO dietary inclusion on fish stress responses and stress resilience, we found no clear signs indicating that fish fed FO-free diet had any deleterious effect on their stress resilience, which was similar to that of control fish. Besides, the supplementation of DHA and EPA did not seem to have relevant effects on stress resilience.

It is noteworthy that the fish exposed to repeated stress showed very limited signs of habituation in their stress response, independently of the diet group. The stressor used to evaluate the acute stress response of all groups was not identical, but was similar to the stressor that was applied repeatedly for 15 days. It was in fact expected to be a milder version, since the plastic aquaria were lifted reducing the water level, but without reaching the point to expose the fish to air. The only observed effect of the repeated stress protocol on the fish acute stress response was a smaller peak in the plasma levels of lactate, and this effect was independent of the diet. The same result was observed before in another study using a similar protocol (Gesto et al., 2018). The reduction in the

lactate response to the stressor suggests a faster lactate clearance rate or a reduced behavioral response to the stressor, resulting in less strenuous exercise and in a lower oxygen debt (Milligan and Girard, 1993). In spite of that difference in the lactate response, the serotonergic activity and the cortisol and glucose levels upon acute stress were similar in naïve than in repeatedly stressed fish, indicating that the fish were not able to habituate to the repeated stressor. In a similar stress challenge experiment, fish were shown to be highly diverse in their ability to habituate to repeated stress depending on their emergence time (Gesto et al., 2018). That large diversity might explain the lack of differences in the current study, where fish were not selected by emergence time.

In conclusion, the results of the present study showed that total replacement of FO by rapeseed oil in the feed did not compromise rainbow trout growth performance after an initial weaning period of several weeks, in spite of the altered FA composition of the juveniles. Furthermore, fish fed VO-based diets showed no large differences in their responses to mild acute and/or repeated stress when compared to fish fed a control, FO-based commercial diet. Dietary rapeseed oil inclusion had, in contrast, important deleterious effects on the ability of fish to survive infection challenges (bath and intraperitoneal), without significant improvements when supplementing the diet with the n-3 LC-PUFAs EPA and DHA up to a level of 1.9 and 2.7 mg g^{-1} dry weight, respectively. The results highlight the importance of using diversified protocols when assessing the impact of dietary alterations on fish physiology. Merely assessing performance traits such as growth might be insufficient to provide an accurate view of fish health and welfare status, since some diet-induced effects can become apparent only under pressing circumstances for the fish, as in the case of stress- or infection challenges. The specific mechanisms behind the differential disease resistance after replacing dietary FO by rapeseed oil remain to be clarified.

Significant effects (3-way ANOVA) for the factors diet (D; CtrAF, L, M or H), repeated stress (RS; RS+ or RS-) and time post-acute stress (TPS; 0 h – unexposed controls, 1 h, 2 h, 4 h or 8 h) and/or their interactions are indicated at the bottom.

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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