Environmentally-realistic concentration of cadmium combined with polyunsaturated fatty acids enriched diets modulated non-specific immunity in rainbow trout

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Abstract

Nutrition is crucial to grow healthy fish particularly in a context of pollution, overcrowding and pathogen risks. Nowadays, the search for food components able to improve fish health is increasingly developing. Here, the influence of four dietary polyunsaturated fatty acids (PUFAs) that are alpha-linolenic acid (ALA, 18:3n-3), linoleic acid (LA, 18:2n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) on the sensitivity of rainbow trout (*Oncorhynchus mykiss)* juveniles to environmentally realistic cadmium (Cd, 0.3 µg/L) concentration was investigated. Fish diets were designed to ensure the specific abundance of one of these individual PUFAs, and were given for a 4-week pre-conditioning period followed by a 6-week Cd exposure period. Focus was put on growth performance and immune responses following a short (24 h) and a long-term (6 weeks) Cd exposure. For each experimental condition, some fish were submitted to a bacterial challenge

(24 h) with *Aeromonas salmonicida achromogenes* at the end of Cd conditioning period. DHA-enriched diet improved growth performances as compared to LA-enriched diet, but also increased ROS production (after short-term exposure to Cd) that could lead to a higher inflammation status, and some immunity-related genes (at short and long-term exposure). We notably highlighted the fact that even a low, environmentally-realistic concentration, Cd can strongly impact the immune system of rainbow trout, and that specific dietary PUFA enrichment strategies can improve growth performance (DHA-enriched diet), provide protection against oxidative stress (ALA- and EPA-enriched diet) and stimulate non-specific immunity.

Keywords

Polyunsaturated fatty acids, Immunity, Ecotoxicology, trace element.

Abbreviations

ALA: alpha-linolenic acid, LA: linoleic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, PUFAs: polyunsaturated fatty acids, Cd: cadmium, SGR: specific growth rate, FCR: feed conversion ratio, ACH50: alternative complement pathway, RBA: respiratory burst activity, tgf-b1: transforming growth factor β1, il-1β/6/8: interleukin 1 beta/6/8, lyz: lysozyme, mpo: myeloperoxidase, TLR: toll-like receptor, cox 2: cyclo-oxygenase-2, alox 5: lipoxygenase-5.

1. Introduction

The intensification of human activities leads to an overall degradation of aquatic ecosystems exposing organisms to multiple stressors like temperature rise, pollutant contamination, poor nutritional quality that can lower its performance and facilitate pathogen infection (Dudgeon, 2010). Cadmium (Cd) is a non-essential heavy metal intensively used for industrial, agricultural or artistic applications, widely distributed in the environment (IARC, 2012) and encountered in European rivers to concentration around 100 ng/L (Dudgeon, 2010). In fish,

Cd uptake occurs mainly through the gills and the gastro-intestinal tract. The main organs for Cd accumulation are kidneys, gills and liver (Kamunde and MacPhail, 2011; McGeer et al., 2011). Chronic exposure to sub-lethal or low Cd concentrations can affect swimming performance, reproduction, Ca^{2+} homeostasis, antioxidant and immune defenses (Avallone et al., 2015), (Luo et al., 2015) (Verbost et al., 1987), (Pathak and Khandelwal, 2006) (Guo et al., 2017; Krocova et al., 2000; Ninkov et al., 2015). Regarding the immune system, several studies described the immunomodulatory effects of Cd on aquatic organisms. Giri et al. demonstrated the immunosuppressive effect of a chronic sub-lethal concentration of Cd on several innate immune genes (*eg. cytokines*) in *Labeo rohita*'s head kidneys (Giri et al., 2016). In addition, a recent study on zebrafish exposed to environmentally relevant concentrations of Cd (5µg/L) during a full life-cycle exhibited in increase in splenic reactive oxygen species (ROS) levels (Guo et al., 2017). An over-production of ROS could result in an impairment of innate immune responses and cellular structure injuries leading to cell apoptosis and necrosis (Morcillo et al., 2015).

Further, in fish, the nutrition also represents a key element to ensure optimal fish growth performance and health (i.e. disease resistance, improved survival and reproductive capacities). An imbalance in the n-3/n-6 PUFAs (polyunsaturated fatty acids) supply can influence various physiological processes including immune response and stress resistance (Montero et al., 2015). The use of vegetable oils represents one of the best alternative to replace fish oil in the current problem of overfishing. However, changes in membrane phospholipid PUFA composition may affect immune cells in several ways (e.g., metabolic energy, functional properties of biomembranes, expression of genes involved in the production of lipid mediators…) (Holen et al., 2015; Knight and Rowley, 1995; Rueda et al., 2013). The use of linseed oil did not appear to deplete the innate immune system of rainbow trout or Eurasian perch *Perca fluviatilis*, as plasma enzyme activities, leucocytes population

and expression of immune genes were not detrimentally affected by dietary alpha-linolenic acid (ALA) enrichment strategy (Geay et al., 2015a; Kiron et al., 2011). However, in the European sea bass *Dicentrarchus labrax*, the substitution of fish oil with linseed oil altered the non-specific immune function (e.g., a decrease of the number of circulating leucocytes and of macrophage respiratory burst activity) (Mourente et al., 2005).

In the context of Cd contamination, fish nutrition and particularly the content of PUFAs can have its importance. Indeed, Cd can alter the activity of antioxidant enzymes, decreasing cell's ability to cope with oxidative stress (Matović et al., 2015). Resulting oxidative attacks, especially to lipids, could lead to oxidative damages to cell components such as membranes (Catalá, 2012). This might be of greater concern in the case of polyunsaturated fatty acids (PUFAs) and highly unsaturated fatty acids (HUFAs), which are very sensitive to oxidation. In a recent study, Ferain et al. (2016) modified the profiles of rainbow trout *Oncorhynchus mykiss* liver cells (RTL-W1) using 6 different PUFAs of the n-3 or n-6 series and highlighted that supply of several n-3 PUFA conferred protection of RTL-W1 cells towards Cd unlike the supply of some n-6 PUFA (Ferain et al., 2016).

To confirm or infirm the hypothesis of cyto-protection and in a realistic context of combined stressors in freshwater aquatic systems (unbalanced nutrient supply, pollutants and pathogenic bacteria), we aimed to investigate the influence of different PUFA enrichment strategies on the sensitivity to Cd of rainbow trout juveniles. Focus was put on fish growth related parameters and immune responses including expression of key-immune genes measured before and after a bacterial infection at the end of a long-term Cd exposure.

2. Materials and Methods

2.1. Design experimental

The experiment was divided in three phases. Firstly, from day -28 to day 0 (D-28 to D0), fish were preconditioned with an experimental diet containing low lipid levels in order to reduce

the general fat content (provided by commercial food) in all fish while keeping the contrasted fatty acid profiles (Xu and Kestemont, 2002). Then, at D0, Cd was added in the water of one of the two systems and fish of both conditions were conditioned with the experimental diets in order to magnify the changing in lipid membrane profile till day 42 (D42). Finally, from day 43 to day 44 (D43 to D44) fish were challenged with *Aeromonas salmonicida achromogenes* to evaluate the response to pathogen.

2.2. Feeding trial

Nutri-toxicological trials and bacterial challenge protocols were approved by the local Ethic Committee for Animal Research of the University of Namur, Belgium (Protocol number: 16272 KE). Rainbow trout juveniles, obtained from the aquaculture farm Charles Murgat pisciculture (Beaufort, France), were randomly distributed into 100L-polyethylene tanks (24 tanks, 25 fish/tank, 3 tanks/experimental condition/time point) in a recirculating system according to dietary and Cd exposure conditions. Fish were acclimated for 14 days prior to experimental trials. Fish with an initial mean body weight of 14 g were reared at 14 °C under a LD 12:12 photoperiod and were hand-fed twice a day, six days per week. Feed quantity corresponded to 3% of tank biomass during the preconditioning period (first 4 weeks; PUFA-enriched diets only) and 2.5% during the subsequent conditioning period (last 6 weeks; PUFA-enriched diets combined with Cd exposure). Four experimental diets varying in their PUFA composition only were formulated (see Table 1 for a detailed composition of the diets). The PUFA-enriched diets for preconditioning phase contained 28.8 g / kg of diet of one of the following oils: EPA-enriched cod liver oil (Omegavie EPA 70 TG, Polaris), DHA-enriched cod liver oil (Omegavie DHA 70 TG, Polaris), linseed oil (alpha-linolenic acid- or ALA-rich oil, Vanderputte), and sunflower oil (linoleic acid- or LA-rich oil, Bio-time, Colruyt) (see Table 2 for detailed diet fatty acid compositions). During the conditioning

period, similar PUFA-enriched diets were provided, except that their content was increased to 117.6 g/kg of diet of specific oil and 2.4 g/kg of cod liver oil.

2.3. Exposure to Cadmium

After 4 weeks of preconditioning (D0), in which trout juveniles received one of the 4 different PUFA-enriched diets, half of the population was subjected to waterborne Cd added from dissolved $Cd(NO3)_2.4H_2O$ (Sigma). Measured total dissolved Cd reached 0.0144 µg/L and 0.30 ± 0.11 µg/L for control and Cd-exposed tanks, respectively. Other water characteristics were: pH, 7.6; total hardness, 125 mg/L as CaCO3; calcium, 84.4 mg/L; magnesium, 11.3 mg/L; sodium, 23.8 mg/L; potassium, 3.1 mg/L.

After 24-h (D1) and 6-week (D42) Cd-exposure periods, 3 juveniles per tank (3 tanks per condition) were anesthetized with 120 mg/L of MS222 (Sigma Aldrich), and blood was sampled. Then, the head kidney (HK) was sampled after euthanasia by an overdose of MS222 (240 mg/L), and incubated at 4°C in Leibovitz's L15 medium for respiratory burst analysis. The mid kidneys and the spleen were also sampled, and were flash-frozen in liquid nitrogen and stored at -80°C until enzymatic and molecular analyses.

2.4. Bacterial challenge

The *A. salmonicida achromogenes* strain was provided by the Centre d'Economie Rurale (CER-groupe, Marloie, Belgium). Bacteria were grown in sterile brain heart infusion (BHI) liquid medium (Sigma Aldrich) at 26 °C for 18 h. Then, 1.2 x 10^9 CFU / 100 g fish weight were injected in the peritoneal cavity of selected fishes (3 fish per tanks) at D43. Mid kidneys, spleen and blood were taken 24 h (D44) post-challenge.

2.5. Growth performance

The growth performance and survival rate were calculated at D-28, D0 and D42 using the following formula:

Weight gain (g) = W_f (g) – W_i (g)

Specific growth rate (SGR, % day^{-1}) = 100 x (Ln W_f – LnW_i) / t

Feed conversion ratio (FCR) = dry feed intake (g) / weight gain (g)

Survival rate = 100 x (final number of fish) / (initial number of fish)

Where W_i is initial body weight (g) at day -28 or day 1, Wf is final body weight (g) at day 1 or 42; t is experimental duration or both conditioning periods in days.

2.6. Splenosomatic index

The splenosomatic index (= W_{spleen} (g) / W_{fish} (g); where W_{spleen} and W_{fish} are the weight of the spleen and corresponding fish) was used as an indicator of an abnormal condition in the spleen such as necrosis or swelling due to infection.

2.7. Immune enzymatic activities

Plasma lysozyme activity was assayed according to Ellis et al. (Ellis, 1990). Briefly, 7 µL of plasma were added to 130 µL of freshly prepared *Micrococcus luteus* (Sigma-Aldrich, Saint-Louis, USA) solution (0.6 mg/mL of Na_2HPO_4 0.05 M, pH 6.2) in technical triplicates. *Micrococcus luteus* lysis was measured kinetically at 450 nm for 30 min (5 min intervals). One unit (U) of lysozyme activity was defined as an absorbance decrease of 0.001 per min. Alternative complement pathway (ACH50) activity measurement was adapted from Milla (2010) (Milla et al., 2010). Briefly, 10 µL of 3% suspension of freshly sampled rabbit red blood cells (RRBC), diluted in veronal buffer, were mixed with serial dilutions of plasma (70 µL of total volume). Haemolysis and negative controls were obtained by adding 60 µL of distillate water or 60 µL of veronal buffer to 10 µL of RRBC respectively. After 120 min at 27°C (under constant agitation, 300 rpm), the absorbance was measured at 615 nm that reflect cell turbidity. Samples were measured in technical duplicates. The ACH50 value (U/mL plasma) was defined as the reciprocal of the plasma dilution inducing the haemolysis of half the RRBC population.

2.8. Leukocyte isolation and Respiratory burst activity (RBA)

Leukocytes were isolated from head kidney of each fish (9 replicates/condition) as described by Secombes (1990). Briefly, the HK was passed through a sterile nylon mesh (100 µm) and kept in Leibovitz's L15 medium (L15 medium, Gibco, Thermofisher Scientific) at 4°C. HK cell suspensions were then layered onto Ficoll gradient and centrifuged at 400 x g for 30 min at 4°C. Peripheral blood mononuclear cells (PBMC) were collected at the interface, washed with L15 medium and resuspended in L15 medium supplemented with 10% fetal bovine serum (Gibco, Thermofisher Scientific). PBMC were kept at 4°C overnight, then centrifuged again at 400 x g for 10 min and suspended in L15 medium at 4°C. Analyses were performed on whole leukocytes cells using FACSVerseTM (BD Biosciences). For each leukocyte sample, cellular mortality was assayed on 10,000 cells using propidium iodide (Sigma) as probe in flow cytometry. The RBA was evaluated according to the technique described by Chilmonczyk and Monge (Chilmonczyk and Monge, 1999). Using flow cytometry, intracellular hydrogen peroxide production was assayed by following the activation of Phorbol 12-Myristate 13-Acetate (PMA, Sigma). The stimulation index of respiratory burst was determined as the ratio of fluorescence of PMA-stimulated cells to that of unstimulated cells.

2.9. Immune gene expression

For each sampling time, the organs (kidneys or spleen) of the three fish sampled per aquarium were pooled. Total RNA was extracted from these organs using Tri Reagent solution (Ambion, Thermofisher Scientific) according to the manufacturer's instructions. The pellet, containing RNA, was dried and resuspended in 100 µl of RNase-free water. Total RNA concentration was determined by NanoDrop-2000 spectrophotometer (Thermo Scientific) and the integrity was checked with the bioanalyzer 2100 (Agilent). Genomic DNA was digested for 15 min at 37°C with 1U of rDNAse I (Thermofischer Scientific) and quantified again by NanoDrop-2000 spectrophotometer. Then, 1 µg of total RNA was

reverse-transcribed using RevertAid RT kit (Thermofischer Scientific) according to the manufacturer's instructions.

Resulting cDNA were used to measure the expression of 14 immune genes in Real-time quantitative polymerase chain reaction qPCR). Three housekeeping genes (β-actin, elongation factor 1α aka EF1α and 18S rRNA) were tested and their geometric mean chosen as reference. The gene expression of pro-inflammatory cytokines (*il-1β, il-6, il-8*), an antibacterial protein (*lysozyme*), a humoral component (*mIgM*), macrophages (*mcsfr-a*), neutrophils (*mpo*), and an anti-inflammatory response (*tgf-b*) biomarker were evaluated. The list of specific primers used is given in Table 3. Real-time qPCR was carried out with iTaq universal SYBR green supermix (Bio-Rad Laboratories) using a 1:40 dilution of the cDNA for target genes or 1:1000 dilutions for reference genes. Primers for target and reference genes were used at 500 nM. The thermal conditions were 3 min at 95ºC, followed by 40 cycles at 95ºC for 30 s and 60ºC for 30 s, and melting curves were analyzed to verify the absence of multiple amplicons. All reactions were performed using ABIprism 7300 (Applied Biosystem) and the relative gene expression was calculated according to the ΔCt method (Fellous et al., 2014). Values for each sample were expressed as normalized relative expression (NRE), calculated with the formula $NRE=2^{(CtRefgene-CtTargetgene)}$.

2.10. Statistical analysis

Data were expressed as means ± standard deviations (SD). Statistical analyses were performed using Rstudio software with car and multcomp packages (Team, 2015). Normality and homogeneity of variances of data were assessed respectively with Shapiro–Wilk and Bartlett tests. Effects of PUFA-enriched diets, waterborne Cd and their interaction on growth performances, enzymatic activities, respiratory burst and immune gene expressions were analyzed using Global Linear Model (using a Gaussian family). Post-hoc comparisons at a 5% significant level were performed with General Linear Hypotheses and multiple

comparisons for parametric models (multcomp R package). Data with no normality and/or homogeneity of variances were tested with Kruskall-Wallis tests and post-hoc pair-wise Wilcoxon comparison test.

3. Results

3.1. Growth performances and mortality

Growth performances, evaluated all along the experiment, are reported in Table 4. During the preconditioning and conditioning phase, rainbow trout juveniles fed with DHA-enriched diet had a significantly higher SGR than LA-enriched diet ($F(3,20)=0.0469$; $p=0.0469$). This was also reflected in similar changes of final body weight and weight/food intake between the two groups. The low FCR was related to higher food intake values for fish fed LA-enriched diet than those fed DHA-enriched diet ($F(3,20)=0.0045$; $p=0.0045$). Cd did not affect fish growth performance in any instance during the conditioning period. Neither PUFA-enriched diet nor Cd exposure induced a significantly higher mortality.

3.2. Splenosomatic index

The splenosomatic index was not impacted by 24 h of Cd-exposure (data not shown) but it was significantly decreased in fish exposed to Cd for 6 weeks ($p=0.0206$), regardless of PUFA treatment as shown in Fig. 1.

3.3. Plasma lysozyme and ACH50 activities

After the preconditioning phase and 24 h of Cd exposure (i.e., at D1), Cd-exposed fish exhibited significantly increased plasma lysozyme activity ($F(7,64)=1.84$, $p=0.00356$), independently of dietary treatment (Fig. 2A). After the conditioning phase and long-term exposure to Cd (i.e., day 42), plasma lysozyme activity was decreased in fish exposed to Cd, except for fish fed EPA-enriched diet ($p=0.0362$) (Fig. 2B).

After a short exposure to Cd (D1), plasma complement activity was significantly lowered (Fig. 3A) in Cd-exposed fish when fed ALA diet compared to LA-enriched diet

(F(7,64)=4.515, p=0.0064). In addition, after conditioning period to PUFA diet, ACH50 activity was higher in ALA-enriched groups compared to EPA-enriched ones (F(7,59)=2.14, p=0.0138) (Fig. 3B). After bacterial injection, no effects were observed (data not shown).

3.4. Respiratory burst activity (RBA)

At D1, no effect of PUFA-enriched diet was observed on RBA in leukocytes, but RBA of leukocytes values was affected by Cd in certain diet (Fig. 4). Indeed 24h after Cd exposure, ROS production of fish fed DHA-enriched diet was increased in all leukocytes population (*e.g* lymphocytes, macrophages and granulocytes). However, after long-term Cd exposure (*i.e.*, D42), neither diet nor Cd had any impact on that parameter whatever the diet and leukocyte type (data not shown).

3.5. Immune gene expression

Natural defenses against pathogen infection

After the preconditioning period and 24 h of Cd exposure (D1), no genes coding for proteins involved in opsonization and lytic activities were modulated neither by Cd nor diets but after 6 weeks of combined exposure to various PUFA-enriched diets and Cd, several genes were affected and the effects were even more pronounced after bacterial infection. In kidneys, *C3b component* gene was repressed by Cd (Fig. 5A) whatever the type of fatty acid (p=0.024). After bacterial infection (D44) in kidneys, in non Cd-exposed group, DHA-enriched diet induced a higher expression of *igm* gene than EPA-enriched diet (F(7,16)=3.733, p=0.0397) (Fig. 5B). In spleen, when fish were exposed to Cd, *igm* expression was upregulated in ALA-enriched diet groups compared to LA-enriched diet ones (F(1,22)=3.733, p=0.0367) (Fig. 5C). In kidneys, the exposure to Cd influenced the expression of lysozyme (*lyz*) and myeloperoxidase (*mpo*) genes in fish fed different diets at days 1, 42 and 44 (data not shown). At D1 in exposed groups, *lyz* expression was up regulated in fish fed LA-enriched diet and *mpo* expression was decreased in DHA-enriched diet groups compared to non Cd-exposed

fish. At day 42, Cd lowered the expression of *lyz* in fish fed both ALA- and DHA-enriched diets compared to unexposed fish (p=0.0495). Eventually, after bacterial infection, the expression of *lyz* in DHA-enriched diets was lowered by the exposure to Cd while the *mpo* mRNA quantity was higher in fish fed EPA-enriched diet and exposed to Cd compared to unexposed fish (p=0.0495).

Signaling pathways

In spleen, at D1, *Tlr3* expression was down-regulated with EPA-enriched diet compared to ALA- and LA-enriched diets in spleen ($F(3,16)=8.032$, $p=0.00172$) (Fig. 6A) while it was decreased in kidneys of fish fed DHA-enriched diet and exposed to Cd ($p<0.05$) (data not shown). In addition, at D1 in spleen, LA-enriched diets induced the highest splenic expression level of *tlr9* compared to others and EPA-enriched diet induced the lowest one ($F(3,16)=15.39$, $p<0.0001$) (Fig. 6B). At D44, in kidneys, *tlr9* expression was down regulated in fish fed DHA-enriched diets and exposed to Cd compared to unexposed group after bacterial challenge ($p<0.05$) (data not shown).

In addition, in kidneys, expression of the Myeloid differentiation primary response gene 88 (*Myd88*) was highly up-regulated in fish fed EPA-enriched diet and exposed to Cd compared to non-exposed group ($p<0.05$) (data not shown).

Then, the macrophage colony-stimulating factor receptor a (*mcsfr-a*) gene was affected by both the conditions in spleen after bacterial infection ($F(3,16)=2.838$, $p=0.0398$) (Fig. 6C). Cd enhanced the expression of *mcsfr-a* in DHA-enriched diet fed fish only ($p=0.031$) (Fig. 6C). Moreover, in Cd exposed fish, *mcsfr-a* was up regulated in fish fed DHA-enriched diet in comparison with those fed EPA-enriched diet ($p=0.00392$).

Inflammatory cytokines

The cytokine transforming growth factor β1 gene (*tgf-b1)* was affected by the dietary treatment after both the preconditioning and the conditioning periods in spleen (Fig. 7A/B),

and by the combined effect of diet and Cd exposure 24 h after bacterial infection (i.e., D44) in spleen and kidneys (Fig. 7C/D). The expression of *tgf-b1* was lower in fish fed ALA-enriched groups than LA-enriched ones at D1 ($p=0.026$) (Fig. 7A), and with ALA- and EPA-enriched diets as compared to DHA-enriched diet at D42 (respectively, $p<0.001$ and $p=0.0275$) (Fig. 7B). But at D44, its expression was upregulated in the spleen of non Cd-exposed fish fed with ALA-enriched diet than corresponding controls fed with DHA-enriched diet ($p=0.0143$) and Cd exposure induced an overexpression of this gene in the spleen of the infected fish fed with DHA-enriched diet ($p=0.0437$) (Fig. 7C). Besides, in absence of Cd challenge, *tgf-b1* expression level was lower in the kidneys of infected fish fed with DHA-enriched diets than in kidneys of infected fish fed with EPA-enriched or LA-enriched diets. (Fig. 7D).

The expressions of interleukin 1 beta gene (*il-1β*), *il-6* and *il-8* were modulated in the spleen or kidneys after bacterial infection, as a function of both diet and exposure (Fig. 7). Indeed, in the spleen of fish fed with EPA-enriched diet compared to those fed ALA-, DHA- ($p=0.0051$) and LA- ($p=0.0306$) enriched, *il-1β* was repressed diets while Cd exposure up-regulated its expression (Fig. 7E) and *il-8* expression level was lowered with EPA-enriched diet compared to those fed LA-enriched diet, under Cd challenge only ($p=0.0288$) (Fig. 7F). In kidneys, the cytokine *il-6* was over-expressed in fish fed ALA-enriched diet compared to DHA-and EPA-enriched diets (respectively, $p=0.033$ and $p=0.0048$) (Fig. 7G).

Prostaglandins and leukotrienes formation

After the preconditioning period, the gene coding for cyclo-oxygenase-2 (*cox 2*) was modulated by the dietary treatment ($F=4.37$, $p=0.0212$) (Fig. 8A). The renal expression of *cox-2* was significantly lower in fish fed ALA-enriched diet than those fed DHA- or LA-enriched diets (Fig. 8A) while DHA-enriched diet increased its expression in the spleen of fish fed DHA-enriched diets in response to Cd ($p<0.05$).

Finally, in spleen at D44, the expression of lipoxygenase-5 (*alox5*) ($F=8.713$, $p=0.00117$) was enhanced by DHA-enriched diet combined with Cd exposure compared to DHA-enriched diet without Cd ($p<0.001$), and EPA-and LA-enriched diets with Cd exposure (respectively $p<0.001$ and $p=0.0104$) (Fig. 8B).

4. Discussion

In the present study, the role of specific PUFA enriched diets and an environmentally plausible waterborne Cd concentration on growth performances, innate immune system status and response to pathogen infection of rainbow trout juveniles was investigated. The figure 9 summarizes the major findings of this study.

Growth performances and food efficiency

In fish, the imbalance in the n-3/n-6 PUFAs supply can influence various physiological processes including growth, immune response and stress resistance (Geay et al., 2015b; Montero et al., 2015). In this study, PUFA, diets did affect growth performances after both the preconditioning and the conditioning periods. DHA-enriched diet significantly enhanced growth rate of trout juveniles compared to LA-enriched diet, in association to a lower food intake and a better food efficiency. Our observation is consistent with previous studies combining high-LA and low n-3 PUFA dietary levels in coho salmon (*Oncorhynchus kisutch*) (Yu and Sinnhuber, 1979). The difference in growth performance between LA-enriched diet (C18:2 (n-6)) and DHA-enriched diet (C 22:6 (n-3)) could be found in physical properties, the length of the carbon chain and the number of unsaturation of these PUFAs. Indeed, n-3 PUFA and especially DHA-enriched diet play only a minor role as energy source but rather strongly influence membrane flexibility and fluidity while reducing its thickness (Hishikawa et al., 2017). Of note, Huster et al. (1997) demonstrated that membrane permeability to water was significantly higher when the cell membranes were composed of PUFA bilayers compared to mono-unsaturated bilayers (Huster et al., 1997). Thus, in our study, the trout fed

with DHA-enriched diet could have their membranes thinner, more flexible and fluid than those of trout fed LA-enriched diet, and so leading to a better assimilation of nutrients and, as a corollary, increased growth performances.

Further, salmonids are known to be particularly sensitive to heavy metal exposure, with adverse effects on the basal metabolism, food intake and food assimilation. This has potential to limit fish growth by reducing the efficiency of energy usage and by increasing metabolic maintenance costs (Heydarnejad et al., 2013; Waiwood and Beamish, 1978). But, our results demonstrated that growth performances were not influenced by the Cd contamination levels tested. This is consistent with what Hollis et al. (2000) previously reported on rainbow trout exposed to 0.11 µg/ L of Cd for 30 days (Hollis et al., 2000).

Influence of PUFA diet and Cd on immune system

In this study, although that neither mortality nor diseased animals were observed following the initiation of Cd-exposure (and nutritional diet), both PUFA diets and Cd exposure appeared to affect several immune functions in different ways. First of all, we showed the evidence of an adverse effect of Cd on splenosomatic index where the lowest values were observed in fish exposed to Cd after long-term exposure. Such reduction of spleen size by Cd exposure could indicate the decrease of health status since the spleen has an important role in blood cell mobilization, hematopoiesis and various mechanisms for production of antibodies (Kortet et al., 2003; Rohlenová et al., 2011). The immune system comprises many physiological functions and processes that all play different roles in host defense, the resulting effects of our experimental conditions are gathered by immune functions and further described here.

Natural defenses against pathogen infection: modulation of bactericidal effectors

Exposure to metals increases stress sensitivity in fish which may thereby stimulate anti-inflammatory and immune responses (Kim and Kang, 2016). Humoral components such as

lysozyme activity, alternative complement pathway and respiratory burst activity are essential components of innate immune responses. In our study, 24 h after the beginning of Cd exposure window, plasma lysozyme activity was increased in all diets while its gene expression was only upregulated in fish fed LA-diet, whereas plasma complement activity was reduced only in fish exposed to Cd and ALA-enriched diet compared to LA-enriched diet. In *Oreochromis sp*, Wu et al. (2007) observed an increase in cortisol level of hybrid tilapia, by cadmium exposure, which in turn stimulates the lysozyme activity and suppressed ACH50 activity (Wu et al., 2007). Hence, the modulation of these bactericidal parameters after 24h of Cd exposure might reflect the stress status of exposed fish more than immunostimulant properties of Cd. However, after the long-term exposure, plasma lysozyme activity decreased in fish exposed to Cd, concomitantly with the repression of its genes in DHA- and ALA-enriched diets while ACH50 was no more affected. The decrease of lysozyme activity was already observed by Giri et al. (2016) in the roho labeo *Labeo rohita* after exposure (28 days) to a sub-lethal concentration of Cd (Giri et al., 2016) and indicates that long-term Cd exposure can deplete bactericidal activity and cause immune function impairment.

Furthermore, two other genes coding for the myeloperoxidase (*mpo*) and immunoglobulin M (*IgM*) were also modulated by diet after bacterial infection. In fish, antibody levels are known to be influenced by environmental factors such as temperature, pH, oxygen, xenobiotic and pathogens (Dominguez et al., 2004). In this regard, after long-term Cd exposure and bacterial infection, *igm* gene expression was enhanced by DHA-enriched diet compared to EPA-enriched diet, suggesting that this PUFA could mitigate the negative Cd action and provide a more efficient response against pathogen infection by promoting the production of IgM. It appears, however, that Cd annihilated this beneficial effect. The capacity of fish oils, particularly DHA-rich ones, to stimulate IgM production was already demonstrated in

Sprague-Dawley rats (Hung et al., 1999). Moreover, the loss of DHA-mediated IgM stimulation is consistent with previous studies that showed immune function impairment including IgM level decrease after long-term exposure to Cd on roho labeo (Giri et al., 2016). In the same way, we observed that Cd affected *mpo* gene expression in fish fed EPA-enriched diet challenged with *A. salmonicida*, which could suggest compensatory processes that would induce different antimicrobial activities in those fish. Indeed, MPO from leukocyte is an important enzyme that participates to the regulation of the inflammatory process. It has been demonstrated to be an inflammatory marker, and the exposure of Cd was shown to increase MPO activity in rats (Ninkov et al., 2015) and has been reported to be a pro-inflammation factor in humans and rodents (Olszowski et al., 2012).

In addition, the dietary enrichment of DHA also affected several immune variables parameters. After 4 weeks of preconditioning with DHA-enriched diet and a short-term exposure to Cd (D1), reactive oxygen species (ROS) production in macrophages, granulocytes and lymphocytes, was found to be significantly affected by Cd whereas, for the three other diets, no differences were observed between control and Cd-exposed fish. In the present study, the tested PUFAs did not increase the production of ROS in the context of the stimulation of immune defense. Still, due to technical issues, respiratory burst activity after bacterial challenge could not be analyzed to strengthen or refute this statement (or hypothesis). Nevertheless, in fish fed DHA-enriched diet, the production of ROS appeared boosted following short-term Cd exposure. Cd is a redox-inactive metal and several studies reported to increase ROS production in different cell types (Hishikawa et al., 2017; Huster et al., 1997). In addition, DHA is also known to act as a pro-oxidant by producing moderate amounts of ROS through auto-oxidation (Lukiw and Bazan, 2008). In our study, the higher level of ROS observed in leukocyte of trout fed DHA-enriched diet exposed to Cd could partially be explained by the combination of oxidative stress induced by Cd contamination

and the auto-oxidation of DHA. In a context of pathogen infection, immune cells release ROS that exert biocidal properties and thus participate to the elimination of infectious agent (Kohchi et al., 2009). Thus, in our study, the high ROS production observed in fish fed DHA when exposed to Cd could suggest immunostimulant properties of Cd combined to DHA, and could represent an advantage to fight pathogens. However, it also reflects a higher sensitivity of those fish to Cd exposure and potentially to oxidative stress. Furthermore, ROS also act as immunotoxicant capable of causing apoptosis (Pathak and Khandelwal, 2006), then a high amount of ROS could also induce adverse effects and be deleterious for other cells.

Toll/NF-κB signaling pathway and inflammatory responses

In animals, the recognition of pathogens and induction of immune responses, including inflammation processes, are partly regulated by the Toll/NF-kB pathway (Cornet et al., 2015; Medzhitov and Janeway, 2000; Qi et al., 2017). In fish, ligands for the Toll-like receptors (TLRs) are type I transmembrane proteins recognizing a wide range of pathogen-associated molecular patterns such as lipopolysaccharides, bacterial DNA, double stranded RNA, but most of these TLRs ligands are not yet characterized in many fish species. In the fugu *Takifugu rubripes* TLR3 was associated to the recognition of relatively short-sized dsRNA (Matsuo et al., 2008) and TLR 9 was shown to be a receptor for CpG-rich bacterial DNA (Ortega-Villaizan et al., 2009). Few studies report the effects of PUFA on fish TLRs and its signaling pathway, but a recent study in Atlantic salmon *Salmo salar* showed that the leucocytes from low EPA- and DHA-enriched diets appeared less responsive towards immunostimulants, like TLR ligands (Arnemo et al., 2017). This suggests that low levels of dietary enrichments with EPA and DHA may have immunosuppressive effects in fish (Arnemo et al., 2017). Our results showed that in spleen, after the pre-conditioning period, EPA-enriched diet down regulated the expression of the two Toll-like receptors in comparison with other diets, while the n-6 PUFA increased both their transcription level.

Moreover, Toll/NF-κB pathway genes did not seem affected by Cd whereas in kidneys and especially after pathogen infection, Cd lowered *tlr9* expression in DHA-enriched diet while it enhanced *Myd88* expression in fish fed EPA-enriched diet. Overall, EPA-enriched diet induced down-regulation of Toll/NF-κB pathway that could benefit to the fish in the context of Cd contamination by suppressing the pro-inflammatory cytokines such as IL-1β and the deleterious damages caused to cells, but could also represent a disadvantage to face pathogen infection. Indeed, after infection, Cd appeared to trigger this pathway by the over-expression of *Myd88* in fish fed EPA-enriched diet and could support the hypothesis of the induction of compensatory immune processes observed in humoral responses with EPA. This result is in line with the observation made on blue mussel *Mytilus edulis* hemocytes that had several TLRs upregulated by Cd exposure (Granger Joly de Boissel et al., 2017). The activation of Toll/NF-κB cascade triggers the liberation of NF-κB transcription factor that translocates to the nucleus and promotes expression of genes involved inflammation processes, including interleukins (such as *il-1β, tgf-β*), iNOS and COX-2.

Cd is known to induce an up-regulation of some mediators and markers of inflammation, and to possess pro-inflammatory properties (Olszowski et al., 2012). In fish, Wang et al. (2016) highlighted that inflammation can be triggered by a waterborne sub-chronic exposure of Cd in rare minnow *Gobiocypris rarus* gills including the increase of pro-inflammatory cytokines TNFα and IL-1β (Wang et al., 2016). We observed that both the nature of the diets and the Cd exposure could modulate the cytokine molecules gene expression level of genes coding for cytokines such as *il-1β, il-8,* and *tgf-β* that play an essential role in the regulation of the inflammatory responses. The pro-inflammatory cytokine IL-1β stimulates the immune responses by activating lymphocytes and inducing the release of other cytokines that in turn will activate the immune cells such as macrophages, lymphocytes and natural killer cells. Then, the chemokine IL-8 induces the chemotaxis in neutrophils and granulocytes in order to

initiate their migration to the site of infection and induce phagocytosis once arrived there. Finally, TGF-β is an anti-inflammatory cytokine that acts as a regulator of inflammation response. In the present study, *il-1β* gene was enhanced by Cd contamination, mainly in spleen of fish fed DHA-enriched diet and infected with *A. salmonicida*, but EPA-enriched diet apparently repressed this cytokine. In addition, in fish fed EPA-enriched diet and exposed to Cd, *il-8* mRNA level was also lower than for LA-enriched diet/Cd exposed fish in spleen and *tgf-b1* was over-expressed by EPA-enriched and LA-enriched diets compared to DHA-enriched diet in kidneys. Together, these results suggest that after bacterial challenge, chronic exposure to a sub-lethal concentration of Cd could have immunostimulant properties. We also found that the inflammatory responses were modified with EPA-enriched diet in comparison with the other PUFAs as it promoted *mpo* gene expression while repressing *il-1β* and *il-8* pro-inflammatory interleukins and enhanced *tgf-b1*.

Prostaglandins and leukotrienes formation

Prostaglandins (PG) and leukotrienes (LT) are eicosanoids notably produced by cyclo-oxygenase and 5-lipoxygenase pathways. Incorporation of arachidonic acid (AA) and EPA in immune cell phospholipids could result in a higher production of AA/EPA-derived products such as PGE_2 and 4-series LTs (for AA) or PGE_3 and 5-series LTs (for EPA) with pro- or anti-inflammatory properties (Calder, 2007). These eicosanoids are involved in many complex physiological processes including the regulation of some immune functions such as inflammation and apoptosis (Funk, 2001; Gómez-Abellán and Sepulcre, 2016). In the present study, after 4-week of preconditioning diet and with or without Cd, LA-enriched and DHA-enriched diets appeared to induce a higher renal expression of *cyclo-oxygenase-2* gene compared to ALA-enriched diet. Hence, in comparison with ALA-enriched diet, both LA-enriched diet and DHA-enriched diet would have immunostimulant effect that would result in the increase in production of PGE_2 and PGE_3 respectively as described in gilthead seabream

Sparus aurata where lipopolysaccharides isolated from *A. Salmonicida* triggered a strong response of COX2 abundance concomitantly with high PGE_2 level in macrophages from head kidneys (Boltaña et al., 2014). Furthermore, both PGs exert different regulations on the immune system and thus a higher production of PG in LA-enriched and DHA-enriched diets could results in different immune response as described by Ganga and collaborators in the gilthead seabream (Ganga et al., 2005). The authors demonstrated that increased inclusion of vegetable oils (containing LA) in diet profoundly affected the fatty acid composition of plasma and leukocyte membranes, and that the resulting production of PGE3 partially altered the immune system and health status (Ganga et al., 2005). In addition, in spleen, Cd also appeared to enhance *cox2* gene expression in DHA-enriched diets which is consistent with the pro-inflammatory potential of COX2 and could be in correlation with the higher sensitivity of DHA-enriched diet to oxidative stress and induction of inflammatory responses (Funk, 2001). A previous *in vitro* study on human normal prostate epithelial cell line also pointed out an up-regulation caused by Cd on exposed cells (Bakshi et al., 2008). In the same way, Cd also raised the splenic expression of *lipoxygenase-5* gene in DHA-enriched diet compared with the other PUFAs reflecting a potential increase in LTs that are inflammatory mediators involved in phagocyte chemotaxis and increase of vascular permeability, and highlighting once again higher stimulation of DHA-enriched diet when fish are exposed to Cd. To our knowledge, there is no study that reported an effect of Cd on fish 5-lipoxygenase gene expression or activity. However, in plants, several studies indicated the upregulation of lipoxygenase as an important component of oxidative stress response to toxic Cd (Liptáková et al., 2013; Podazza et al., 2012).

Insight on the major responses induced by PUFA diets and Cd exposure

In this study, the results obtained allowed us to better understand the effect of some PUFA on the sensitivity to Cd by focusing on immune parameters and provide new evidence of cytoprotection properties observed *in vitro* by Ferain et al. (2016).

As for the DHA-enriched diet, we demonstrated that it improved growth performances compared to n-6 PUFA and that inflammation processes appeared to be magnified by Cd exposure as ROS production (at D28) and several genes like *igm, 5-lipoxygenase, cox2, il-1β* (after bacterial infection) were increased. The induction of a higher inflammation response can represent an advantage for the trout to fight bacterial infection as a higher amount of ROS is produced. However, in a context of a Cd-induced oxidative stress, the hyper sensitization to oxidative stress in leukocytes and potentially in other cell types could act as a negative factor ultimately leading to apoptosis of immune cells.

EPA-enriched diet also impacted immune responses in fish but in a way different from DHA-enriched diet. EPA-enriched diet repressed the expression of two Toll-like receptors (one mainly involved in response to virus and the other to bacteria) after the preconditioning period; and down-regulated *il-1β* and *il-8* pro-inflammatory interleukins while enhancing *tgf-b1* and *mpo* genes in the bacterial challenge. EPA-enriched diet thus appeared to trigger a different inflammatory response than the other diets and also favored humoral components (e.g., *mpo* gene expression) as an immune response in spleen. All together, the immune responses induced by EPA could be beneficial in a context of Cd contamination as it could help to reduce inflammation but still maintain the induction of humoral responses essential for host defense.

Then, concerning ALA-enriched diet, *cox2* expression and alternative complement activity were lowered after one month of preconditioning feeding. However, after long-term exposure and conditioning diet, ALA-enriched diet proved to be as good as other diets in terms of protection to Cd and confirm previous *in vitro* observations as very few of the measured

variables were affected by this heavy metal exposure except for *lysozyme* gene expression. Indeed, this PUFA seemed to be more efficient after long-term conditioning diet, what is not very surprising as this is the precursor of the n-3 PUFA bioconversion pathway. In rainbow trout, the bioconversion of n-3 PUFA is quite efficient. In our study, the beneficial effects observed after long-term conditioning diet could be due to the bioconversion of ALA to EPA and DHA that is efficient but might take time.

Finally, in our experiment, although LA-enriched diet did not appear to really change the sensitivity of the fish to Cd compared to other diet as it was observed with the *in vitro* study. However, it was clearly the less efficient PUFA diet, particularly considering the weak growth performance that it induced. Contrary to ALA, the bioconversion of LA cannot lead to EPA and DHA, explaining in part the difference with the n-3 PUFA, but it can be converted in arachidonic acid that is also the precursor of leukotrienes and prostaglandins. Indeed, we showed some interesting immunomodulatory properties as it could help to enhance the production of PGE2 by the induction of *Cox-2*.

5. Conclusions

To conclude, we highlighted that a low environmentally realistic concentration of Cd could influence the immune system of the rainbow trout *Oncorhynchus mykiss* dependent of their PUFA diets but not always, especially when fish were challenged with a bacterial pathogen. Indeed, we showed that DHA-enriched diet combined with Cd exposure exerted a higher stimulation of inflammatory processes, while EPA-diet appeared to trigger a different inflammatory response than the other diets as it favored humoral components like MPO and induced anti-inflammatory cytokines. After long-term, ALA-enriched diet appeared to be as efficient than other diets to protect fish against Cd adverse effects, moreover no immune function impairment related to the diet was observed making this vegetable oil a good alternative for fish oil replacement in aquaculture. Finally, unlike what observed with in-vitro

study, fish fed LA-enriched diet were not more affected by Cd than other PUFAs, however, this oil provided very poor efficiency in term of growth performances.

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Figures

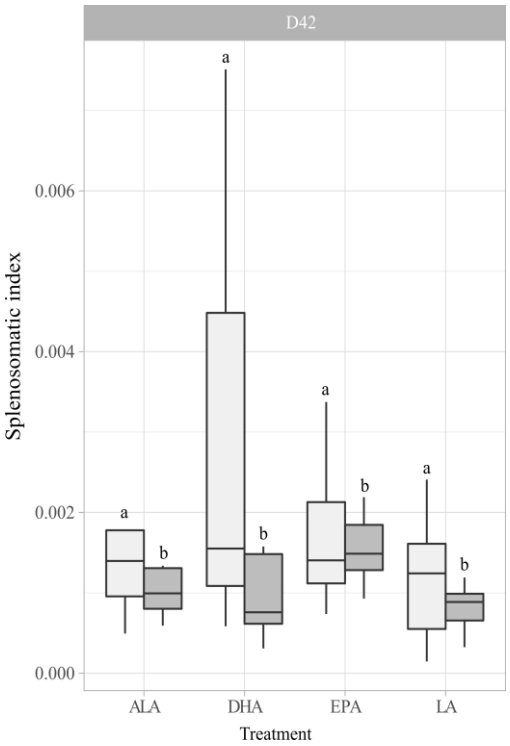

Figure 1. Boxplots represent the quartile distribution of the expression levels of spleno-somatic index of rainbow trout at day 42. Unexposed and exposed fish to Cd are respectively represented by lightgrey and darkgrey boxplots (n=9). Statistically significant differences between diet/exposed groups are denoted with a or b letters.

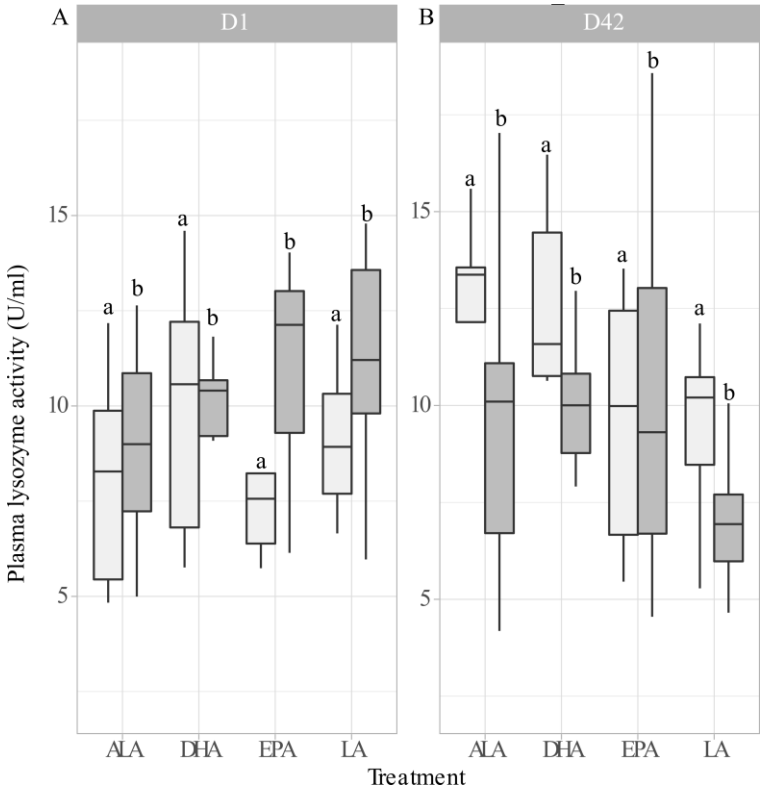

Figure 2. Plasma lysozyme activity at day 1 (A) and day 42 (B). One unit (U) of lysozyme was determined as an absorbance decrease of 0.001 per min. Boxplots represent the quartile distribution of the plasma lysozyme activity (n=9). Unexposed and exposed fish to Cd are respectively represented by lightgrey and darkgrey boxplots. Statistically significant differences between diet/exposed groups are denoted with a or b letters.

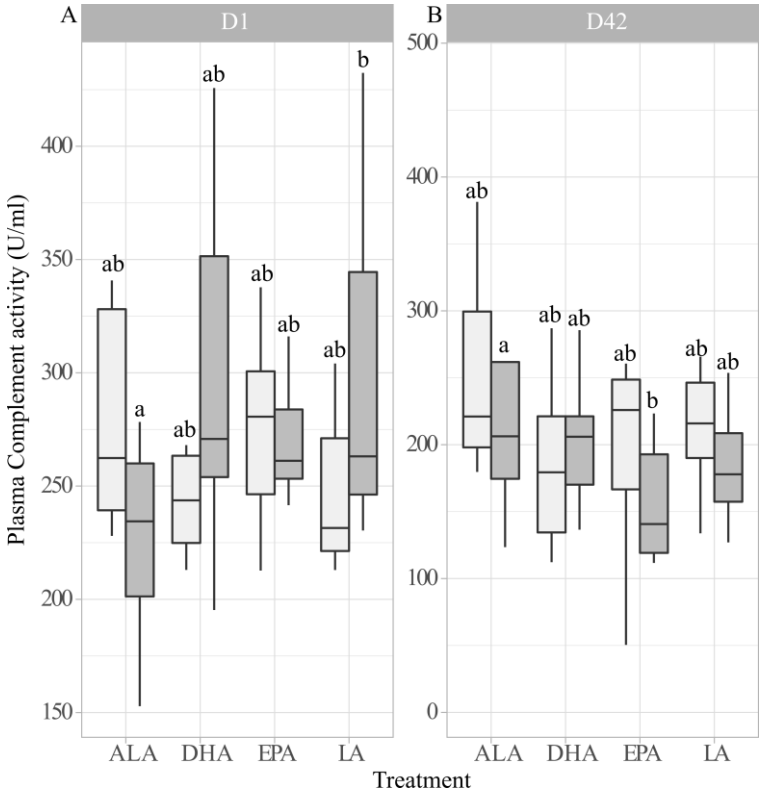

Figure 3. Plasma alternative complement activity at day 1 (A) and day 42 (B). The ACH50 value (unit/mL plasma) is defined as the reciprocal of the plasma dilution which induced the hemolysis of 50% RRBC. Boxplots represent the quartile distribution of ACH50 (n=9). Unexposed and exposed fish to Cd are respectively represented by lightgrey and darkgrey boxplots. Statistically significant differences between diet/exposed groups are denoted with a or b letters.

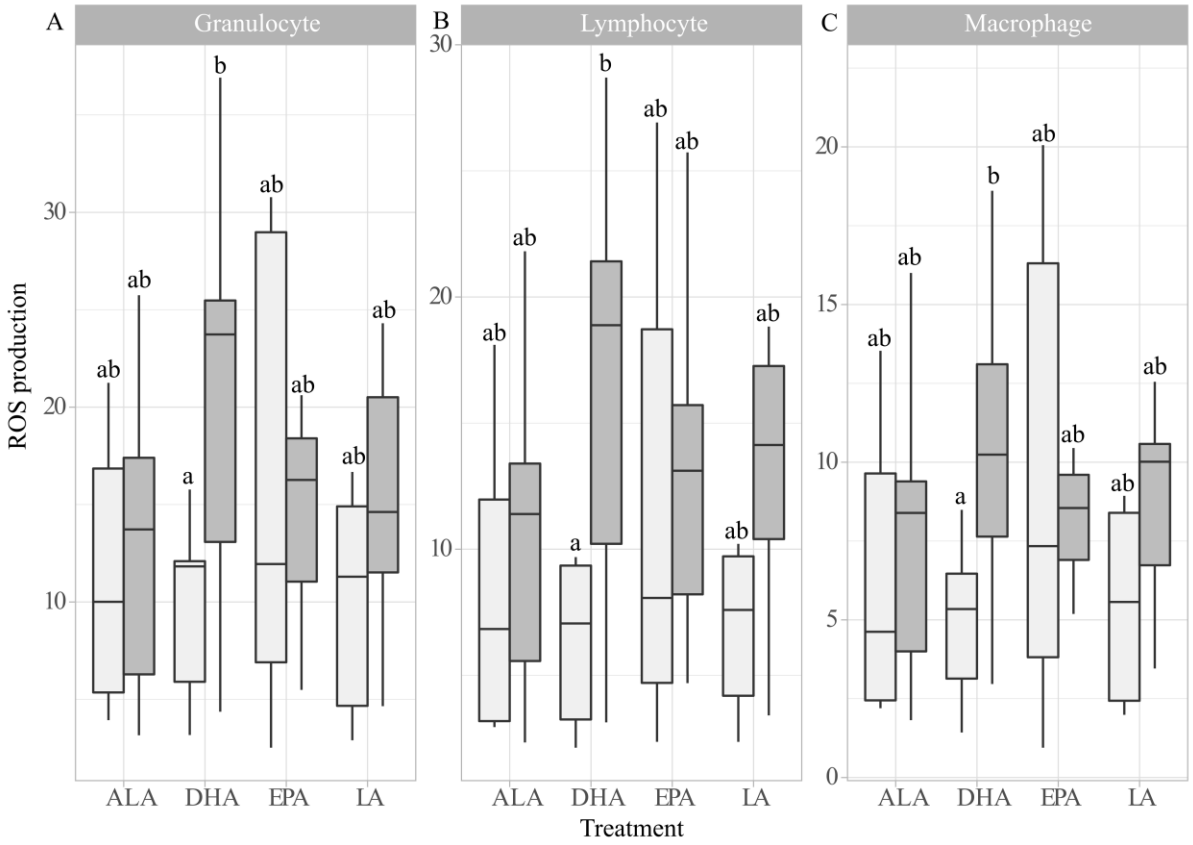

Figure 4. Respiratory burst activity of leukocyte cells as measured by oxidation of H_2DCFDA to DCF at D1. ROS production is expressed as the ratio of PMA-stimulated cells on unstimulated cells ± standard deviation (n=9). Boxplots represent the quartile distribution of ROS production of granulocytes(A), lymphocytes (B) and macrophages (C) respectively. Unexposed and exposed fish to Cd are respectively represented by lightgrey and darkgrey boxplots.

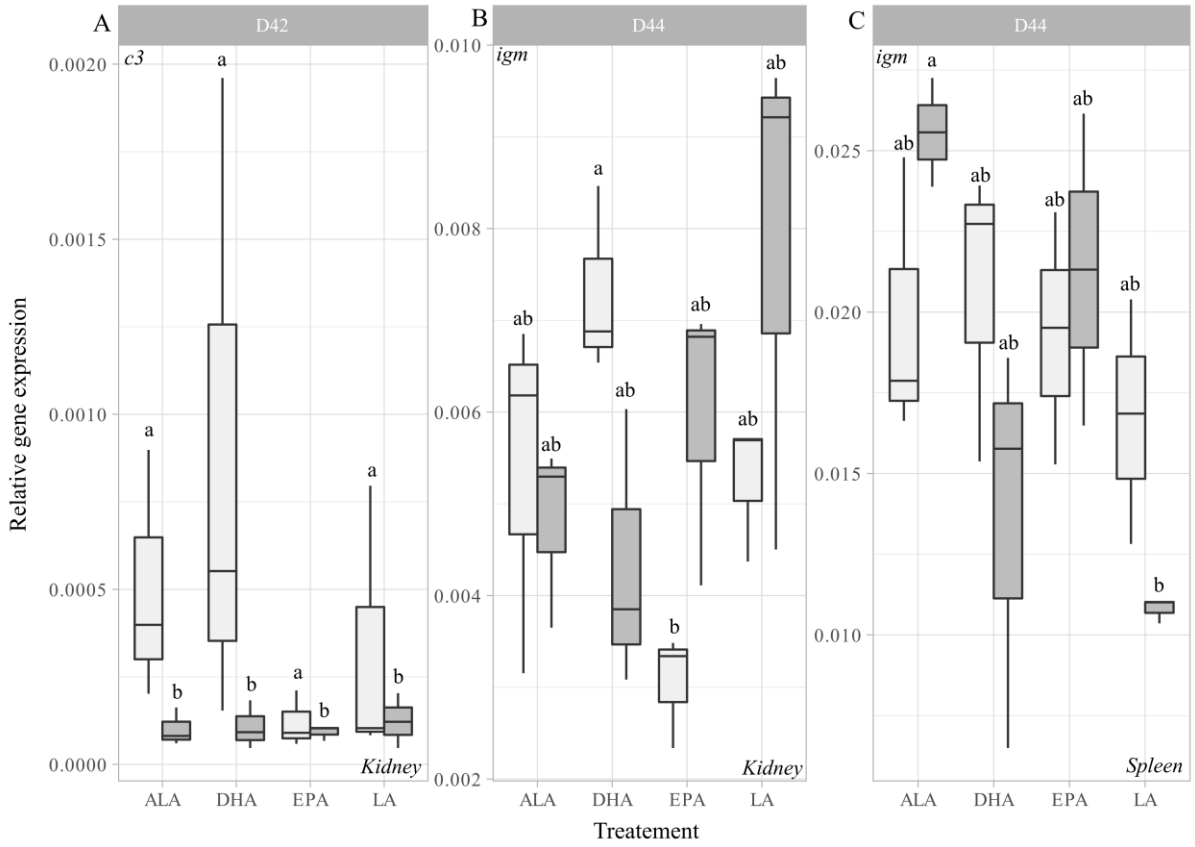

Figure 5. Relative expression of differentially expressed genes involved in natural defense against pathogen infection in spleen or kidneys after conditioning period (D42) or 24h after infection with *A. salmonicida achromogenes* (D44). (A) Relative expression of *C3 component* gene in kidney at D42. (B) and (C) Relative expression of *igm* gene in spleen and kidneys at D44 after bacterial infection. Boxplots represent the quartile distribution of target gene expressions (n=3). Unexposed and exposed fish to Cd are respectively represented by lightgrey and darkgrey boxplots. Geometric mean of EF-1a, 18S and β-actin was used as an internal control to calibrate the cDNA template for all samples. Bars represent the mean value of three biological replicates. Statistically significant differences between diet/exposed groups are denoted with a or b letters.

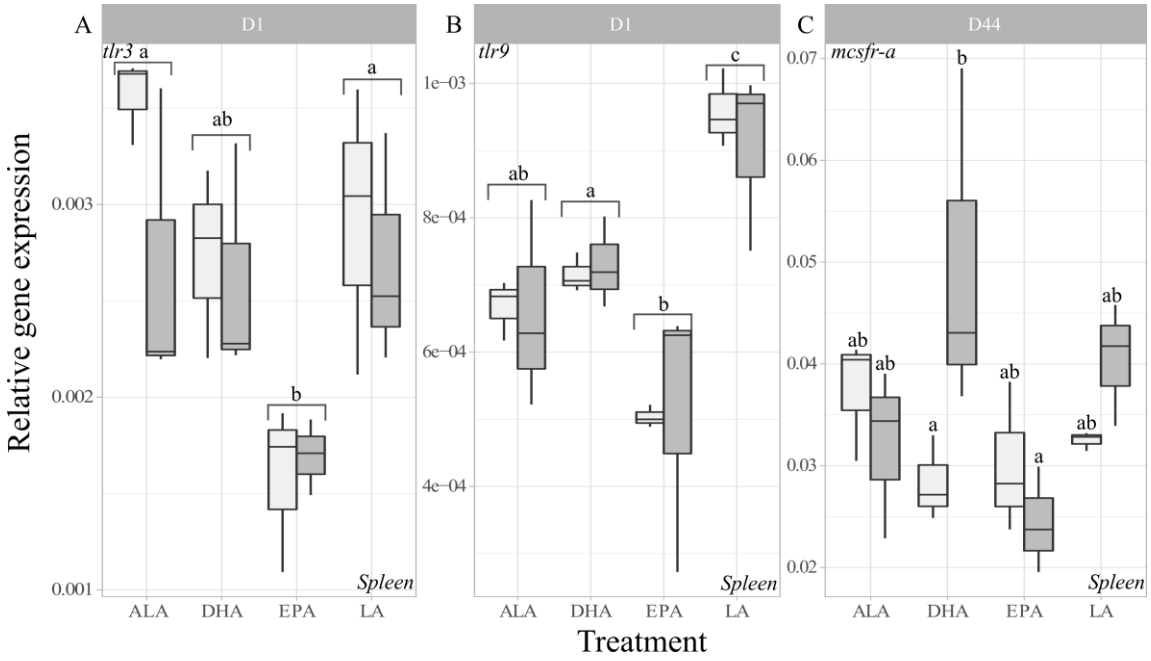

Figure 6. Relative expression of differentially expressed genes involved in signaling pathway in spleen after preconditioning period (D1). (A) Relative expression of *Toll-like receptor 3* gene at D1. (B) Relative expression of *Toll-like receptor 9* gene at D1. (C) Relative expression of *macrophage colony-stimulating factor receptor a* gene in spleen at D44. Boxplots represent the quartile distribution of target gene expressions (n=3). Unexposed and exposed fish to Cd are respectively represented by lightgrey and darkgrey boxplots. Geometric mean of EF-1a, 18S and β-actin was used as an internal control to calibrate the cDNA template for all samples. Statistically significant differences between diet/exposed groups are denoted with a or b letters.

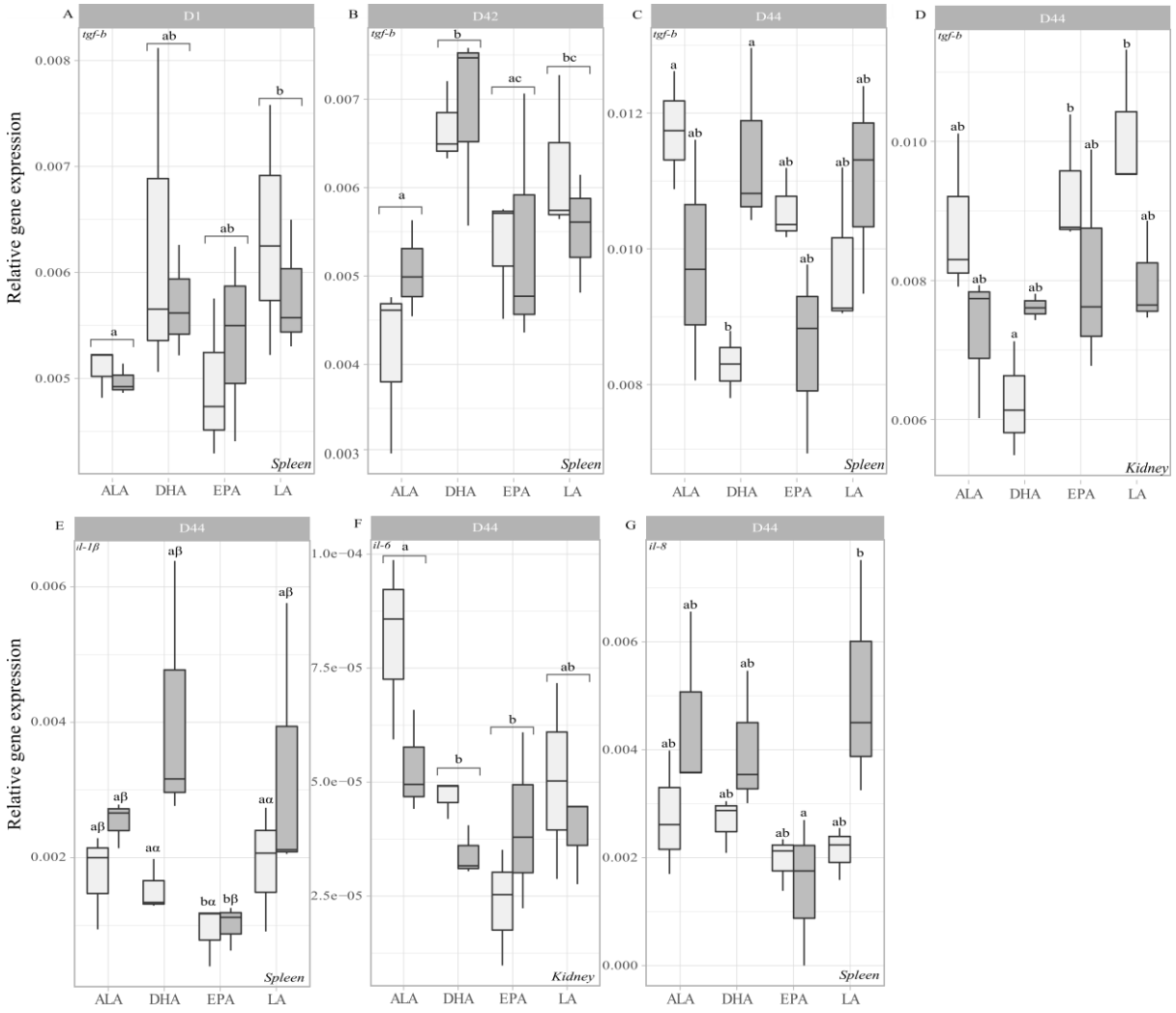

Figure 7. Relative expression of inflammatory response genes in kidneys and spleen after preconditioning (D1), conditioning period (D42) and 24h after infection with *A. salmonicida achromogenes* (D44). Pro-inflammatory responses are highlighted by the following genes *Tgf-b* (A) and (B), *il-1β* (C), *il-6* (D) and the anti-inflammatory response by *il-8* gene (E). Boxplots represent the quartile distribution of target gene expressions (n=3). Unexposed and exposed fish to Cd are respectively represented by lightgrey and darkgrey boxplots. Geometric mean of EF-1a, 18S and β-actin was used as an internal control to calibrate the cDNA template for all samples. Statistically significant differences between groups are denoted with a or b letters, and for (C) differences between diets and exposed or not groups are indicated with respectively by a or b letters of Greek letter α and β.

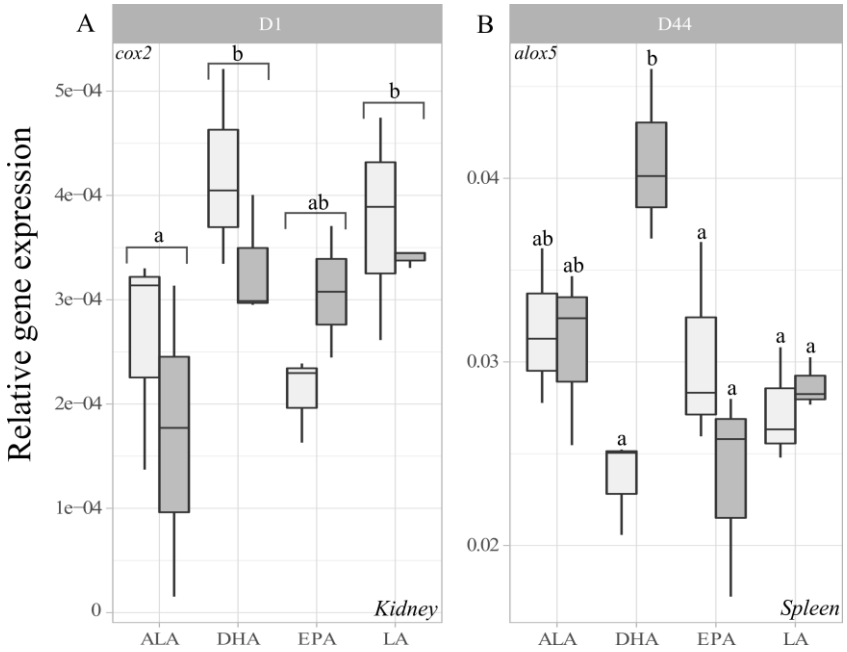

Figure 8. Relative expression of differentially expressed genes involved in prostaglandins and leukotrienes formation in kidneys or spleen after preconditioning period (D1) or 24h after infection with *A. salmonicida achromogenes* (D44). (A) Relative expression of *cyclo-oxygenase-2* gene at D1 in kidneys. (B) Relative expression of *5-lipoxygenase* gene at D44 in spleen. Boxplots represent the quartile distribution of target gene expressions (n=3). Unexposed and exposed fish to Cd are respectively represented by lightgrey and darkgrey boxplots. Geometric mean of EF-1a, 18S and β-actin was used as an internal control to calibrate the cDNA template for all samples. Bars represent the mean value of three biological replicates. Statistically significant differences between diet/exposed groups are denoted with a or b letters.

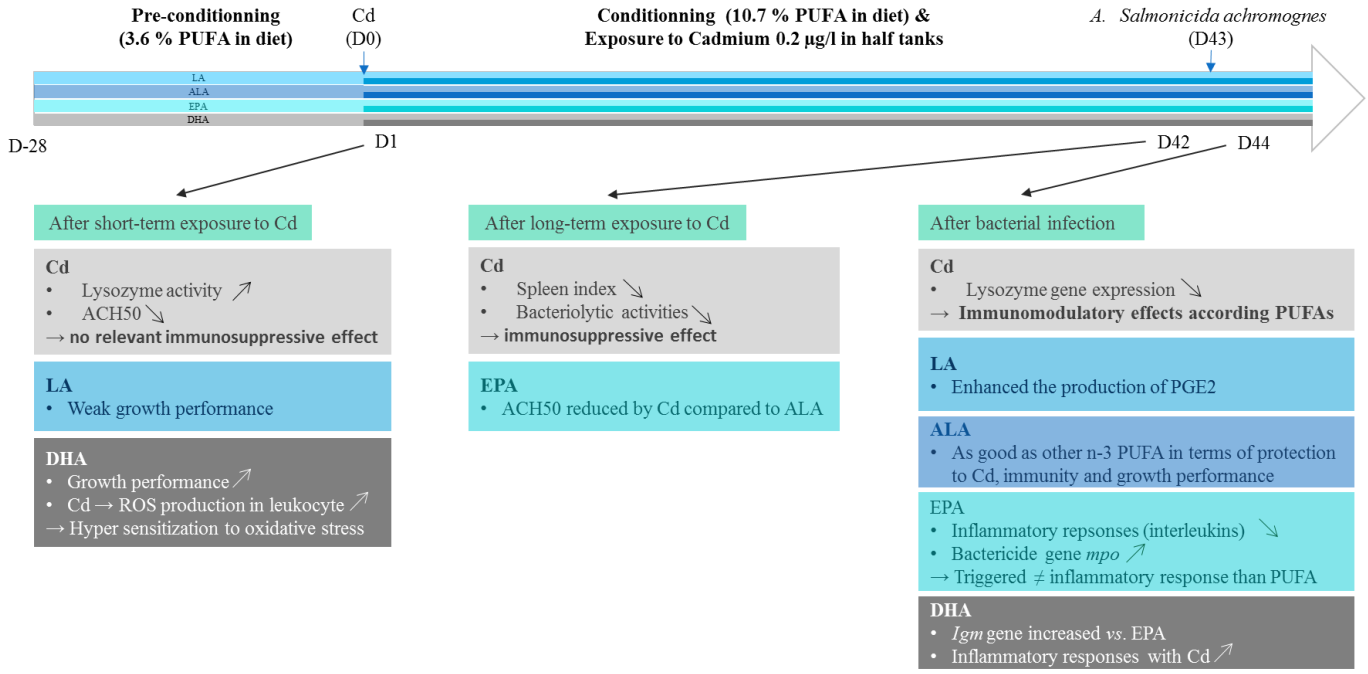

Figure 9. Diagram representing the experimental protocol and the key findings. The different days of sampling were identified by the letter “D” followed by the number of day since the beginning of Cd exposure.

Table 1. Composition of the four experimental diets for the preconditionning and conditionning period.

Products	g/kg of diet
Casein	276,8
Gelatin	50
Wheat gluten	223
Dextrin	100
Sucrose	50
Cellulose	80,0
Carboxymethylcellulose	30
Mineral mix[a]	63,443
Hydrosoluble vitamins mix[b]	0,00
Liposoluble vitamins in oil mix[c]	6,1
Day -28 to 0: Enriched Oil	28,8
Day 0 to 44: Oil mix[d]	120,0

[a]Minerals mix (quantity / kg of diet): 15.19 g CaHPO4, 14.11 mg Ca(H2PO4)2.H2O, 6.14 mg NaHCO3, 0.001 mg Na2SeO3.5H2O, 6,.5 mg KCl, 11.206 g NaCl, 0.013 mg KI, 4.14 mg MgCl2, 4.57 mg MgSO4.7H2O, 0.099 mg MnSO4.H20, 0.81 mg FeSO4.7H20, 0.026 mg CuSO4.5H20, 0.65 mg ZnSO4.7H2O; [b]Hydrosoluble vitamins mix (in mg/kg of diet): 0.5 mg acide ascorbique (Vit C), 0.056 mg thiamine (Vit B1), 0.1200 mg riboflavine (Vit B2), 0.045 mg pyridoxine (Vit B6), 0.14 mg pantothénate de Ca (Vit B5), 0.39 mg, P-aminobenzoic acid (Vit H1), 0.0003 mg cyanocobalamin (Vit B12), 0.29 mg, niacine (Vit B3), 0.001 mg biotine (Vit H), 3.50 mg, choline chloride, 0.0150 mg acide folique (Vit M), 0.49 mg inositol, 0.1 g canthaxanthin (E161g), 3.25 g alpha-cellulose ; [c]Liposoluble vitamins in oil mix, 3.86 mg retinyl acetate (Vit A), 0.1 mg cholecalciferol (Vit D3), 21.93 mg menadione (Vit K3), 14.95 mg butylated hydroxyanisole, 14.95 mg butylated hydroxytoluene, 5980.52 mg PUFA-enriched oil; [d] Oil mix is composed of 2,6 g of cod liver oil and 117,4 g of specific oils.

Table 2. Fatty acids composition of each diet (in mmol/kg of diet)

Fatty acids	Diet EPA+	Diet DHA+	Diet ALA+	Diet LA+
C14:0	0.271	0.271	0.271	0.271
C14:1 (n-9)	0.008	0.008	0.008	0.008
C15:0	0.002	0.002	0.002	0.002
C16:0	1.101	4.859	15.621	19.206
C16:1 (n-9)	0.031	0.031	0.031	0.031
C16:1 (n-9)	0.450	1.986	0.450	0.450
C18:0	9.034	1.884	10.477	9.280
C18:1 (n-9)	10.985	4.902	46.905	76.697
C18:1 (n-9)	4.889	0.934	2.407	3.106
C18:2 (n-6) = LA	1.522	1.248	36.115	180.936
C20:1 (n-9)	5.861	1.381	0.578	0.578
C18:3 (n-3) = ALA	1.359	0.437	139.926	0.083
C18:4 (n-3)	2.143	0.410	0.100	0.100
C20:2 (n-6)	0.928	0.017	0.017	0.017
C22:0	0	1.521	0	1.414
C20:3 (n-6)	1.154	0	0	0
C22:1(n-9)	0.578	1.936	0.578	0.578
C20:3 (n-3)	0.043	0.043	0.043	0.043
C20:4 (n-6)	8.841	1.813	0.019	0.019
C20:4 (n-3)	5.228	0.458	0.037	0.037
C20:5 (n-3) = EPA	180.29	5.536	0.425	0.425
C24:0	1.305	0.955	0	0
C24:1 (n-9)	0.014	3.153	0.014	0.014
C22:4 (n-6)	0	1.557	0	0
C22:5 (n-6)	0	12.426	0	0
C22:5 (n-3)	0.050	8.453	0.050	0.050
C22:6 (n-3) = DHA	0.504	204.313	0.504	0.504
c24:5 (n-3)	0.007	0.007	0.007	0.007
c24:6 (n-3)	0	0.643	0	0
Total	236.6	261.18	254.58	293.86

Table 3. Primer sequences used for analyses of selected immune related gene expression in *Oncorhynchus mykiss.*

Genes	Primer sequence (5'-3')	Accession number	Amplicon size
EF1a	Fw: 5'-ATGCCCCCAAGTTCCTGAAG-3'	NM_001124339.1	140
	Rv: 5'-AACAGCAACAGTCTGCCTCA-3'		
β-actin	Fw: 5'-GGACTTTGAGCAGGAGATGG-3'	NM_001124235.1	186
	Rv: 5'-ATGATGGAGTTGTAGGTGGTCT-3'		
18S	Fw: 5'-TGAGCAATAACAGGTCTGTG-3'	FJ710873.1	212
	Rv: 5'-GGGCAGGGACTTAATCAA-3'		
IL-1β	Fw: 5'-TGAGAACAAGTGCTGGGTCC-3'	NM_001124347.2	148
	Rv: 5'-GGCTACAGGTCTGGCTTCAG-3'		
IL-6	Fw: 5'-GAGTTTCAGAAGCCCGTGGA-3'	NM_001124657.1	149
	Rv: 5'-AGCTGGTACACTTGCAGACC-3'		
IL-8	Fw: 5'-CACAGACAGAGAAGGAAGGAAAG-3'	NM_001124362.1	162
	Rv: 5'-TGCTCATCTTGGGGTTACAGA-3'		
Lysozyme	Fw: 5'-TGCCTGTCAAAATGGGAGTC-3'	NM_001124716.1	152
	Rv: 5'-CAGCGGATACCACAGACGTT-3'		
mIgM	Fw: 5'-AAAGCCTACAAGAGGGAGACCGAT-3'	U04616.1	128
	Rv: 5'-AGAGTTATGAGGAAGAGTATGATGAAGGTG-3'		
MCSFR-α	Fw: 5'-ATCTCCACTCATGGCGACACA-3'	NM_001124739.1	177
	Rv: 5'-CATCGCACTGGGTTTCTGGTA-3'		
MPO	Fw: 5'-ATCCACACGGGCATCACCTG-3'	GBTD01119227	68
	Rv: 5'-GCAGAGTCACCAATGACACCA-3'		
TGF-β1	Fw: 5'-GCCAAGGAGGTCCACAAGTT-3'	NM_001281366.1	146
	Rv: 5'-GTGGTTTTGATGAGCAGGCG-3'		
C3	Fw: 5'-GAGATGGCCTCCAAGAAGATAGAA-3'	L24433.1	91
	Rv: 5'-ACCGCATGTACGCATCATCA-3'		
TLR3	Fw: 5'-AGCCCTTTGCTGCCTTACA-3'	NM_001124578.1	61
	Rv: 5'-GTCTTCAGGTCATTTTTGGACACG-3'		
TLR9	Fw: 5'-TCTTCATAGAGCTGAAGAGGCCTCA-3'	NM_001129991	137
	Rv: 5'-GTTCCCACTGAGGAGAAGTGTTTT-3'		
SOD	Fw: 5'-CCACGTCCACGCCTTCG-3'	NM_001160614.1	141
	RV: 5'-TCAGCTCCTGCAGTCACGTT-3'		
iNOS	Fw: 5'-CCAACCATGCACATCAAAAGTT-3'	NM_001124359.1	98
	Rv: 5'-CCTGAGGTAGGATTTCAAGAGTAGAAA-3'		
COX2	Fw: 5'-AGCACTTCACCCACCAGTTC-3'	NM_001124348.1	180
	Rv: 5'-GGTAGACCTCGCCGTTCAAA-3'		
Alox5	F: 5'-ATCTCAGAGCACTCCAGGGT-3’	NM_001165225.1	146
	R: 5'-CTGCGTTGGTGATCGTTTCC-3'		
MyD88	F: 5'-GACTGGATGTCTGTCGCAGAAA-3'	NM_001124421.1	109
	R: 5'-ACAACGGCTTGCCAGTCC-3'		

Table 4. Growth performances during preconditionning and conditionning period. Values are mean ± standard deviation (n=6 for D0 and n=3 for D42). Different superscripts letters in the same row stand for statistical differences between treatments.

	Day -28 to 0				**Day 0 to 42**							
	RI EPA+	**RII DHA+**	**RIII ALA+**	**RIV LA+**	**RI EPA+**		**RII DHA+**		**RIII ALA+**		**RIV LA+**	
Initial weight (g)	14,30 ±0,1	14,37 ±0,5	14,38 ±0,2	14,38 ±7,65	29,29 ±0,4	28,77 ±0,9	29,86 ±1,2	29,74 ±0,7	28,66 ±0,7	28,94 ±0,4	28,32 ±0,2	27,78 ±1,5
Final weight (g)	29,03 ±0,7^{ab}	29,80 ±0,9^{a}	28,80 ±0,5^{ab}	28,05 ±1,01^{b}	78,69 ±3,9^{ab}	74,98 ±1,5^{ab}	82,27 ±5,6^{a}	82,25 ±4,0^{a}	76,34 ±4,2^{ab}	78,26 ±1,0^{ab}	72,97 ±1,5^{b}	73,15 ±1,7^{ab}
Weight intake (%)	103,03 ±4,8^{ab}	107,43 ±5,9^{a}	100,34 ±3,6^{ab}	95,15 ±7,65^{b}	168 ±9^{ab}	160 ±9,7^{ab}	175 ±9,5^{a}	176 ±7,2^{a}	166 ±9,1^{ab}	170 ±3,0^{ab}	157 ±3,8^{b}	163 ±10,1^{ab}
SGR (%/day)	2,85 ±0,10^{ab}	2,92 ±0,15^{a}	2,80 ±0,0^{ab}	2,70 ±0,15^{b}	2,82 ±0,10^{ab}	2,73 ±0,10^{ab}	2,89 ±0,09^{a}	2,90 ±0,07^{a}	2,79 ±0,09^{ab}	2,84 ±0,04^{ab}	2,70 ±0,03^{b}	2,76 ±0,10^{ab}
Food intake (g)	330,56 ±2,2^{ab}	330,75 ±4,0^{a}	330,62 ±7,4^{ab}	337,44 ±9,5^{b}	905,24 ±27^{ab}	859,36 ±27^{a}	913,98 ±40^{a}	866,15 ±10^{a}	878,01 ±59^{ab}	841,89 ±27^{a}	937,14 ±29^{b}	917,22 ±38^{b}
Food conversion ratio (FCR)	0,9 ±0,04^{ab}	0,87 ±0,04^{a}	0,91 ±0,04^{ab}	0,99 ±0,07^{b}	0,97 ±0,07^{ab}	0,91 ±0,06^{a}	0,89 ±0,01^{a}	0,88 ±0,03^{a}	0,99 ±0,08^{ab}	0,87 ±0,05^{a}	1,07 ±0,02^{b}	1,06 ±0,08^{b}