Aquatic Toxicology 196 (2018) 104–116

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

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

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ARTICLE INFO

Keywords:
Polyunsaturated fatty acids
Immunity
Ecotoxicology
Trace element

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.

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 their performance and facilitate pathogen infection (Dudgeon, 2010). Cadmium (Cd) is a non-essential 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 sublethal concentration of Cd on several innate immune genes (*e.g. cytokines*) in *Labeo rohita*'s head kidneys (Giri et al., 2016). In addition, a

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

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https://doi.org/10.1016/j.aquatox.2018.01.012
Received 18 October 2017; Received in revised form 8 January 2018; Accepted 15 January 2018
Available online 03 February 2018

recent study on zebrafish exposed to environmentally relevant concentrations of Cd (5 µg/L) during a full life-cycle exhibited an 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 injuries of cellular structure 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 alternatives 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 *etc.*) (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, leukocyte population and expression of immune genes were not detrimentally affected by dietary alpha-linolenic acid (ALA) enrichment strategy (Geay et al., 2015; 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.*, causing a decrease of the number of circulating leukocytes 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 in a realistic context of combined stressors in freshwater aquatic systems (unbalanced nutrient supply, pollutants and pathogenic bacteria), we investigated 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. Experimental design

The experiment was divided in three phases. Firstly, from day-28 to day 0 (D-28–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–D44) fish were challenged with *Aeromonas salmonicida achromogenes* to evaluate the response to pathogen.

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
Water soluble vitamin mix[b]	0,00
Lipid soluble vitamins in oil[c]	6,1
Day-28–0: Enriched Oil	28,8
Day 0–44: Oil mix[d]	120,0

[a] Mineral 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] Water soluble vitamin mix (in mg/kg of diet): 0.5 mg ascorbic acid (Vit C), 0.056 mg thiamine (Vit B1), 0.1200 mg riboflavin (Vit B2), 0.045 mg pyridoxin (Vit B6), 0.14 mg Ca pantothenate (Vit B5), 0.39 mg, P-aminobenzoic acid (Vit H1), 0.0003 mg cyanocobalamin (Vit B12), 0.29 mg, niacin (Vit B3), 0.001 mg biotin (Vit H), 3.50 mg, choline chloride, 0.0150 mg folic acid (Vit M), 0.49 mg inositol, 0.1 g canthaxanthin (E161 g), 3.25 g alpha-cellulose.

[c] Liposoluble vitamins in oil, 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.

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 100 L-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