Aquatic Toxicology

Interplay between dietary lipids and cadmium exposure in rainbow trout liver: influence on fatty acid metabolism, metal accumulation and stress response

--Manuscript Draft--

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Abstract:	The present study aimed at investigating interactive effects between dietary lipids and both short- and long-term exposures to a low, environmentally realistic, cadmium (Cd) concentration. Juvenile rainbow trout were fed four isolipidic diets (31.7 g/kg) enriched in either linoleic acid (LA, 18:2n-6), alpha-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) or docosahexaenoic acid (DHA, 22:6n-3). From the 4th week of this 10-week experiment, the lipid level of the diet was increased (120.0 g/kg) and half of the fish fed each diet were aqueously exposed to Cd (0.3 µg/L) while the other half were not exposed to Cd (control). Fish were sampled and their liver was harvested for fatty acid profile, hepatic Cd and calcium concentrations, total glutathione level and gene expression assessment, either (i) after 4 weeks of feeding and 24 h of Cd contamination (day 29) (short-term Cd exposure) or (ii) after 10 weeks of feeding and 6 weeks of Cd contamination (day 70) (long-term Cd exposure). We found that both dietary lipids and Cd exposure influenced fatty acid homeostasis and metabolism. The hepatic fatty acid profile mostly reflected that of the diet (e.g. n-3/n-6 ratio) with some differences, including selective retention of specific long chain polyunsaturated fatty acids (LC-PUFAs) like DHA and active biotransformation of dietary LA and ALA into LC-PUFAs. Cd effects on hepatic fatty acid profiles were influenced by the duration of the exposure and the nutritional status of the fish. The effects of diet and Cd exposure on the fatty acid profiles were only sparsely explained by variation of the expression pattern of genes involved in fatty acid metabolism. The biological responses to Cd were also influenced by dietary lipids. Fish fed the ALA-enriched diet seemed to be the least affected by the Cd exposure, as they showed a higher detoxifying ability against Cd with an early upregulation of protective metallothionein a (MTa) and apoptosis regulator BCL2-Like1 (BCLx) genes, an increased long-term phospholipid synthesis and turnover and fatty acid bioconversion efficiency, as well as a lower long-term accumulation of Cd in their liver. In contrast, fish fed the EPA-enriched diet seemed to be the most sensitive to a long-term Cd exposure, with an impaired growth performance and a decreased antioxidant capacity

	(lower glutathione level). Our results highlight that low, environmentally realistic aqueous concentrations of Cd can affect biological response in fish and that these effects are influenced by the dietary fatty acid composition.
Suggested Reviewers:	

Cover Letter

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Louvain-la-Neuve, 28th April 2020

Dr Mikko Nikinmaa
Editor
Aquatic Toxicology

Dear Dr Nikinmaa,

We are pleased to submit herewith a manuscript entitled *"Interplay between dietary lipids and cadmium exposure in rainbow trout liver: influence on fatty acid metabolism, metal accumulation and stress response"*. We would appreciate its consideration for publication in Aquatic Toxicology. This paper is a follow-up study of Ferain et al., 2016 (*Aquat. Toxicol. 177, 171-181*) and Ferain et al., 2018 (*Aquat. Toxicol. 205, 100-113*). In these papers, we showed that the phospholipid composition of rainbow trout liver cells (RTL-W1 cell line) modulates their tolerance to an acute cadmium challenge through complex mechanisms involving for instance a reduction of the oxidative stress induced by the metal and a differential induction of the eicosanoid cascade. We also highlighted that cadmium could modulate cellular fatty acid homeostasis and metabolism.

In the present *in vivo* study, we investigated interactive effects at the molecular level between dietary lipids and both short and long term exposures to a low, environmentally realistic, cadmium concentration in rainbow trout juveniles, with a focus on the liver.

We observed that both dietary lipids and cadmium exposure significantly influenced fatty acid homeostasis and metabolism. Cadmium effects were highly influenced by the duration of the exposure and the nutritional status of the fish. Overall, effects observed on the fatty acid profiles were not explained by variation of the expression pattern of the selected genes. On the other hand, the biological responses to cadmium were influenced by dietary lipids. Fish fed a diet enriched in alpha-linolenic acid (18:3n-3) and eicosapentaenoic acid (20:5n-3) seemed to be the least and most affected by cadmium exposure, respectively. The underlying mechanisms were diverse, with impacts on phospholipid synthesis and turnover, metal accumulation, antioxidant capacities and expression of genes involved in stress response.

The results coming from the present experiment highlight that cadmium can affect biological response in fish at low, environmentally realistic contamination level and that these impacts are influenced by the dietary fatty acid composition. Complex interactions as described in this study are probably encountered in the environment. We thus think that our study would be of interest to the readership of Aquatic Toxicology and we hope you will share this opinion.

Thank you for your interest,
We are looking forward to hearing from you,

Sincerely yours,
Aline Ferain
PhD Student

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Louvain-la-Neuve, 6 September 2020

Dr Hecker
Co-Editor-in-Chief
Aquatic Toxicology

Dear Dr Hecker,
Dear reviewers,

We are pleased to submit herewith a revised version of our manuscript entitled "*Interplay between dietary lipids and cadmium exposure in rainbow trout liver: influence on fatty acid metabolism, metal accumulation and stress response*".

You will find here under our answers to the reviewer's comments. Our answers follow each comment made by the reviewers, in-between #. For the sake of clarity, modifications have been highlighted in yellow in the revised manuscript.

We would like to thank you, as well as the reviewers, for your comments that enabled us to improve the quality of the manuscript,

Sincerely yours,

Aline Ferain

REVIEWER 1

Reviewer 1 : General comment

This study was extremely interesting and informative. The authors did so much work and it shows! I did have a few questions about the statistical analysis and have suggested changes to figures, data interpretation and analysis. I have also suggested a figure at the end, in the Discussion, and a couple of points on the mode of action of Cd that the authors might want to consider. I am suggesting major changes for these reasons, but no additional experiments.

We thank Reviewer 1 for having read and commented our manuscript.

Reviewer 1: Specific comments

1. *Abstract*

Please note the level of each lipid type in the diet for the first 4 weeks, then note the increase after this time. It appears to be a significant change to the experimental protocol and should be noted.

We clarified the text following Reviewer 1's advice. Note that the total lipid level of the diet was increased at the 4th week of the experiment, but the targeted fatty acid was recovered in the same proportions throughout the experiment.

"From the 4th week of this 10-week experiment, the lipid level of the diet was increased and half of the fish fed each diet were aqueously exposed to Cd (0.3 µg/L) while the other half were not exposed to Cd (control). Fish were sampled and their liver was harvested for fatty acid profile, hepatic Cd and calcium concentrations, total glutathione level and gene expression assessment, after 4 weeks of feeding and 24 h of Cd contamination (day 29) and after 10 weeks of feeding and 6 weeks of Cd contamination (day 70)."

This section is hard to follow. If the authors choose to give this much detail about the experimental protocol in the abstract, please break it down into 2 experiments, for clarity: experiment 1 - 4 weeks feeding (% FA) and 24h exposure to Cd; experiment 2: 4 weeks feeding (%FA) + 6 weeks (increased %FA)/Cd exposure. It is difficult for me to follow the way it was originally written. I hope these suggestions help, but please feel free to put your own spin on it. I only provided an example that I think would help with clarity, but it is certainly not the only way to write.

We have modified this section in order to improve its clarity. However, we could not refer to "two experiments" as the short-term and long-term exposures to Cd were conducted within the same experiment.

2. *Title*

Appropriate

We thank Reviewer 1 for the comment.

3. *Materials and Methods*

Section 2.1 - The authors used 14.4 g juvenile trout for their experiments, but here they state that all the fish were females. How did they determine this? Trout mature at 3+ years of age, depending on density, temperature and seasonality. It would be impossible to determine if a 14.4 g trout were male or female by visually examining the gonads.

We thank the Reviewer for the comment. We replaced "*Rainbow trout juvenile females"* by *"Rainbow trout juveniles"*#

Sections 2.3 and 2.4 - please replace "fishes" with "fish", since only one species was used ("fishes" refers to multiple species).

We thank the Reviewer for the comment. However, “fishes” has also to be used whenever the animals are specifically counted, as it is the case in here “25 fishes/tank”; “3 fishes per aquarium”; “3 fishes”

Section 2.7 - please make a note if the Cd numbers were corrected for %recovery (i.e. if recovery was 75%, were the Cd numbers obtained corrected by multiplying them by 1.25% or are the reported Cd numbers uncorrected?).

For metal analyses metal were not corrected by the recoveries. We added a note on that in the manuscript.

Section 2.8 - please provide specs on the cDNA kit.
- I agree with the method used by the authors to normalize their gene expression data. Running multiple housekeeping genes should be common practice, as more often than most of the genes commonly used as housekeeping genes change with treatment, life stage, stress exposure, etc.

Specifications were added in the text: “iScript cDNA Synthesis kit (Biorad Laboratories, Inc, USA)”

Section 2.9 - I am a bit confused about the statistical analysis used in this study. The authors did a two-way and two one-way ANOVAs (one for each Cd treatment and another for each diet) on the same data set, but are only reporting the one-way, from what I could tell. I am not sure why this approach was taken. A two-way ANOVA alone would have been the most appropriate statistical analysis to use, because it takes into account diet, Cd and interaction effects. It is more conservative, but it is the best approach here. I am also not sure which figures and/or table show the two-way ANOVA.
- The note made prior to this paragraph was written before I had a chance to go through the data. UP to this section, the authors appear to be setting up their data for a two-way ANOVA. However, the way they have presented the data in the Results section, it suggests that a 3-way ANOVA is more appropriate, with diet, Cd and time of exposure as dependent variables. Please check the statistical analysis and change the interpretation accordingly.

Statistical analyses were performed in collaboration with the Support in Methodology and Statistical Calculation (SMCS) technological platform of the University of Louvain (UCL), Belgium. We know that, in fully crossed experimental designs as we planned, the use of full factorial regression analyses or ANOVAs is recommended. We first considered it but opted for 2 separate 1-way ANOVAs performed at each time point for the following reasons:

- Full factorial ANOVAs or regression models require to have variability within each experimental group for their proper use, which we had not here. For example: Cd and some fatty acids analysed were not detected in some experimental conditions (0 ± 0), while they were quantifiable in other experimental conditions, (ii) the expression level of some genes were assessed at one time point only (0 ± 0). The use of full factorial ANOVAs was thus not possible for these parameters.

- The use of two separate one way ANOVAs at each time point appeared to be a good compromise to the full factorial model as, in fact, only the decomposition of the interactions from the full factorial model interested us. Indeed, we know from our previous experiments that there is an interaction between the two parameters studied (fatty acid profile and Cd) as we showed that the fatty acid profile modulated the sensitivity of rainbow trout liver cells and *Daphnia magna* to Cd. The purpose of the present experiment was to identify parameters that might explain interactions *in vivo*. Regarding the time, we also know from literature that such complex interactions depend on the exposure length. We were thus interested in describing the interactions between Cd and PUFA at two time points. Although the purpose was not to establish a kinetic of the effects across time. The latter would have required more time points and thus a much bigger experiment for being relevant. We thus “pictured” the interactive effects at two time points.

- Note that a Bonferroni-Sidak correction was applied to the p values in order to compensate for the inflation of the alpha level caused by multiple testing. The application of the Bonferroni-Sidak correction is often even more conservative than applying a full factorial model. #

4. *Tables*

This comment applies mostly to Table 2, but I suppose it can also be used for Table 1: please delineate with a vertical line between the 0-28 and 29-70 treatments. It is a small thing, but it would make a difference when visualizing Table 2. Table 1 already had a larger gap between the two time frames, so it is easier to follow visually.

We modified Table 2 : addition of a gap between the two time frames (similar to Table 1).

Table 4 - please check the note under the table. Cd concentration should be 0.3, not 0.03 (see bracket). The authors make a note about stars, stars in brackets and letters in brackets, but there are none in the table. It is unclear to me what the letters that are depicted in the table represent. My guess is they represent a diet effect in Cd-exposed fish at day 70, but I am not sure.

We thank the Reviewer for the comment. We corrected the note under the table 4. The effect was indeed a diet effect in Cd-exposed fish at day 70.

5. *Results: General comments*

I would recommend that the authors label their figures panels using letters (Fig 2A, B, C, etc). It would much easier to follow and report the data.

We thank the Reviewer for the suggestion. We modified the Fig 2-6 and the text according to the Reviewer's advice

An additional note would be related to the statistical analysis. It is my understand that the authors are comparing diet, Cd exposure and acute vs chronic Cd exposure. This is a 3-way ANOVA, not a 2 or a one-way. The way the data is presented by the authors also suggests that a 3-way ANOVA is appropriate. Please check the statistical analysis.

As explained in a previous comment: throughout the manuscript, we "pictured" the interactive effects between Cd and PUFA at two time points.

Figures 2,3,4,5,6 - I appreciate that the authors have put this data in figures. It is a quick look and one can spot the trends immediately. It would have been much more difficult if the data were presented in a table. Thank you!

We thank Reviewer 1 for the comment

Throughout the sections in the Results, the authors mention that some data is "significantly higher/lower" than other data, but they do not mention what that significance is. Is it 2-fold higher? 3-fold? Or is it 50% higher? Please add a note about the magnitude of the difference between significant data points.

We added magnitude of the effects in the sections 3.2.2.1, 3.2.2.2, 3.2.2.3, wherever they were missing. Note that the magnitude of the effects were already described in the other sections of the results.

6. *Results : Specific comments*

Section 3.2.1 - I am not sure which data the authors are referring to here. Which figure is part of this section? Please clarify.

Data used in this section are "Total phospholipids", "Total neutral lipids" and "Total free fatty acids" that can be found in Fig 2. Based on these "Totals", we calculated several % as follows:

- % phospholipids = Total phospholipids / (Total phospholipids + Total neutral lipids + Total free fatty acids) *100

- % neutral lipids = Total neutral lipids / (Total phospholipids + Total neutral lipids + Total free fatty acids) *100

- % free fatty acids = Total free fatty acids / (Total phospholipids + Total neutral lipids + Total free fatty acids) *100

As only the "Totals" and not the calculations of the % are reported in the Fig. 2, we did not refer the reader to any specific Fig for this section. We thus added *"data not shown"* in the text.) #

Section 3.2.2 - the authors mention here that Cd had no effect on FA composition, but I do see stars in Figure 2, denoting a Cd effect. Please check the data and change the text accordingly. If the authors noted changes in FA due to diet, what are these changes reported relative to? It is unclear what was compared to what in the study. I appreciate it is quite complex and there is a lot of data presented here, but it would be great to provide a bit more clarity on the statistical analysis that was performed.

Section 3.2.2 focusses on the impact **of the diet**. We wrote :*"Overall, Cd exposure did not influence **the impacts of dietary lipids on** hepatic fatty acid profiles (Fig. 2-6).)",* which does not mean that Cd had no impact. The impacts **of the Cd exposure** on the hepatic fatty acid profiles are described later on (in section 3.2.3). We slightly modified the sentence to clarify: *"Overall, Cd exposure did not modify the impacts of dietary lipids on the hepatic fatty acid profiles (Fig. 2-6)." #*

Section 3.2.2.1. - earlier the authors mention that there were no Cd effects, yet in Fig 2, phospholipids 70 days, the MUFA profile is quite different between 0 and 0.3 ug Cd. I have the same comment for n-3PUFA neutral lipids and free fatty acids day 29. Please revisit the statement that Cd exposure and length of treatment have no effect. What did the authors base this conclusion on (see statement in section 3.2.2)?

Again the section 3.2.2.1 focusses on the impact **of the diet**. The impacts of Cd are described later (in section 3.2.3). The effects pointed out by the Reviewer here, are thus described in section 3.2.3:

- Cd increased MUFA level in phospholipids at day 70 in fish fed the ALA- and DHA-enriched diets. This effect is described in the section 3.2.3.1 "*Cd significantly increased the total hepatic phospholipid level in fish fed the ALA-enriched diet by 13% (Fig. 2F, day 70). This increase mainly reflected a rise in the levels of MUFAs (Fig. 2B, day 70), among which mainly 18:1n-9 (Fig. 5C, day 70) and 20:1n-9 (Fig. 5D, day 70). Besides, Cd significantly increased by 49% the total MUFA level recovered in hepatic phospholipids of fish fed the DHA-enriched diet (Fig. 2B, day 70), among which mainly, 18:1n-7, 18:1n-9 and 20:1n-9 (Fig. 5B-D, day 70), without affecting significantly the total phospholipid level (Fig. 2F, day 70).*"

- The short-term exposure to Cd increased n-3 PUFA level in neutral lipids of fish fed the EPA-enriched diet. This effect is described in the section 3.2.3.2. *"In fish fed the EPA-enriched diet, the 24-h Cd exposure also significantly increased the hepatic neutral lipid content in PUFAs (+46%, Fig. 2E, day 29). The increase in PUFAs was essentially due to a rise of n-3 PUFAs (+41%, Fig. 2C, day 29), among which mainly DHA (+29%, Fig. 4I, day 29), but also, to a lesser extent, to an increase in 20:2n-6 (+63%, Fig. 3B, day 29)".*

- Cd (24h) decreased the n-3 PUFA level in the free fatty acid of fish fed the ALA-enriched diet. This effect is described in the section 3.2.3.3. "*The 24-h Cd exposure significantly decreased the total hepatic free fatty acid level by 16% in fish fed the ALA- enriched diet (Fig. 2F, day 29). This decrease mainly reflected a decline in both the n-3 (-36%, Fig. 2C, day 29) and n-6 (-35%, Fig. 2D, day 29) PUFA levels, including ALA (-49%, Fig. 4A, day 29), EPA (-45%, Fig. 4E, day 29), 22:5n-3 (-36%, Fig. 4F, day 29) and LA (-42%, Fig. 3A, day 29)."*

Section 3.2.2.2 - it is very difficult to follow this section without reference to a specific panel in Fig 2,3,4. Please consider adding letters to identify each panel.

We thank the Reviewer for the suggestion. We modified the Fig 2-6 and the text according to the Reviewer's advice

Section 3.6.1 - I disagree that the effects on FADS2 were modulated by Cd only, with no dietary effects. Please see the FADS2 panel in Fig 8, 70 days, controls. There is a diet effect that the authors are showing in the controls. The same applies for d9D. Please check the data.

We wrote in section 3.6.1 : *"The impacts of the diet on the hepatic FADS2 and d9D genes were only observed at day 70 and were modulated by the Cd exposure."* Thus, we totally agree with the Reviewer that there is a diet impact on these genes (as written) but this diet impact is only observed at day 70 (and not at day 29). And the diet impact is influenced by the Cd exposure. These diet effects and their modulation by Cd are detailed in the manuscript as well.

- Is there a reason why the authors are not showing data for day 29/70 for the other genes? This data is only shown for FADS2 in Fig 8.

The number of figures being limited we decided to present the relative expression of genes related to fatty acid metabolism that were affected neither by Cd nor by the diet in supplementary material (Fig. S2)

7. *Figures*

Figure 2 - the authors show significance on their figures, but I am not sure I understand relative to what. Is it relative to controls? Relative to day 0 sampling (0 day dotted line)? It is unclear here what was compared to what in the statistical analysis.

We thank the Reviewer for the comment. We clarified the caption as follows: *"For each sampling time and for each level of Cd exposure, letters indicate significant impact of the diet, with levels not connected to the same letter being significantly different from each other, ($p<0.05$). For each sampling time and for each diet, stars indicate significant impact of Cd exposure ($p<0.05$)".* We applied this modification at each caption.

- Please consider labeling the panels A,B,C, etc.

We thank the Reviewer for the suggestion. We modified the Fig 2-6 and the text according to the Reviewer's advice

Figure 3-6 - please label the panels with A, B, C. etc

We thank the Reviewer for the suggestion. We modified the Fig 2-6 and the text according to the Reviewer's advice

Figure 7 - please add an inset to denote what the different colored bars represent.

The light and dark grey represent the experimental conditions unexposed and exposed to Cd respectively, as stated in the caption of Fig. 7. *"....24 h of Cd exposure (0.0 µg/L in light grey or 0.3 µg/L in dark grey) ..."*

The stars denote a significant effect of Cd relative to what? Is it relative to controls within one diet? Please be specific.

We thank the Reviewer for the comment. We clarified the caption as follows. *"For each sampling time and for each diet, stars indicate significant impact of Cd exposure ($p<0.05$)."*#

Fig 8 - are data missing for days 70 and 29 for most genes? Only FADS2 is reported for both 29 and 70 days.

As explained above, the number of figures being limited we decided to present the relative expression of genes related to fatty acid metabolism that were affected neither by Cd nor by the diet in supplementary material (Fig. S2)

8. *Discussion :* General comments

This section was informative and all encompassing. However, I think it would benefit from an overall figure that shows the combined effects of diet x time x Cd on the FA utilization by fish (Figure 1 repeated here, but with the effects noted in the present study). This figure would be used to summarize all the findings of the present study and to put it into context for the reader. The authors have done a tremendous amount of work and I believe that showing such a figure will help the readers get an overall picture of the importance of diet in understanding sensitivity to metal contaminants.

We thank the Reviewer for the suggestion. Although, the number of figures being limited we could not add a summary figure. We thus modified Fig. 1 in order to present the major interactive results between Cd and dietary lipids coming from the present study (with different colour codes). We hope that this summary will help the reader.

A last point I will make here is that Cd is an uncoupler of mitochondria oxidative phosphorylation, by directly impacting complex III (CIII) in the electron transport chain (ETC). Have the authors considered the role of the various fatty acids in mitochondria function and how Cd affects that? I would suggest they have a paragraph that talks about this mode of action of Cd, in the context of the present findings (think about the roles of different FAs in mitochondria function and how that is impaired by Cd). Could various FAs be used as energy source to pump up the ETC, allowing it to maintain the proton gradient across the inner mitochondrial membrane and maintain ATP production if CIII is compromised by Cd?

We agree with Reviewer 1 that the electron transport chain is one potential site of interaction between cellular lipids and Cd. However, we did not investigate such interaction in the present experiment. We added a note on that in the discussion.

Specific comments

Section 4.2.1 - third paragraph - The authors mention effects of Cd on ELOVL2 at 24 h, but this is not shown in Fig 8. Only day 70 is shown in the figure for this gene. However, I did not have access to the supplementary figures for this manuscript. If the data was there, I apologize. I could not see the figures.

The number of figures being limited we decided to present the relative expression of genes related to fatty acid metabolism that were affected neither by Cd nor by the diet in supplementary material (Fig. S2). This is indeed the case for ELOVL2 at day 29

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Louvain-la-Neuve, 17th October 2020

Dr Hecker
Co-Editor-in-Chief
Aquatic Toxicology

Dear Dr Hecker,

We are pleased to submit herewith a revised version of our manuscript entitled "*Interplay between dietary lipids and cadmium exposure in rainbow trout liver: influence on fatty acid metabolism, metal accumulation and stress response*".

You will find here under our answers to your comments. Our answers follow each comment made, in-between #. For the sake of clarity, modifications have been highlighted in green in the revised manuscript.

We would like to thank you, for your comments that enabled us to improve the quality of the manuscript,

Sincerely yours,

Aline Ferain

EDITOR

General comment

Thank you for submitting your revised manuscript to Aquatic Toxicology. After carefully reading through the revised version of your manuscript, it is my opinion that you have successfully addressed the majority of the main concerns raised by the original referees. However, my own final review has identified several outstanding (mostly minor) issues that require your attention before I can accept your manuscript for publication. I have included a marked-up pdf version of your article with detailed comments and some suggestions for revisions to aid in revising your document. I invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by Nov 25, 2020..

We thank the Editor for having read and commented our manuscript.

Specific comments

1. *Introduction*

Sentence*: A mong fish, salmonids are very sensitive to Cd*
- Editor's comment*: Change to "Among"*
- # We applied the modifications suggested by the Editor. #

Sentence*: Lipids, and their constitutive fatty acids, are important for fish health as they provide energy, are determinants of the integrity, fluidity and permeability of the biological membranes, are precursors of bioactive molecules (e.g. eicosanoids) and act as nuclear receptor ligands (Tocher, 2003).*
- Editor's comment*: Delete "the"*
- # We applied the modifications suggested by the Editor. #

Sentence*: Fish oil, which is rich in the n-3 LC-PUFAs eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), has commonly been used in feed formulation in aquaculture as its fatty acid profile meets the dietary requirements and enables the farming of fish rich in valuable n-3 LC-PUFAs (FAO, 2018; Tocher, 2015).*
- Editor's comment*: Delete "the"*
- # We applied the modifications suggested by the Editor. #

2. *Material and method*

Sentence*: Cod liver oil (2.4 g/kg) was also added in diets during the last 6 weeks of the experiment.*
- Editor's comment*: Not entirely clear from the text as written whether this was done to all treatment groups and how this is different from your above cod liver oil treatments (e.g. DHA-rich cod liver oil)?*
- # We have modified the sentence in order to improve its clarity

Sentence: *Fish, with an initial mean body weight of 14.4 g, were reared at 14°C under a light cycle of 12h:12h light:darkness and were hand-fed twice a day, six days a week*
- Editor's comment: *Please add a measure of variation (SD).*
- # We added the measure of variation asked by the Editor. #

Sentence: *At each sampling time, 3 fishes per aquarium were taken, anesthetized (MS222, 120 mg/L), euthanized with an overdose of MS222 (240 mg/L) and death was confirmed by cervical dislocation*
- Editor's comments:
    - *The original referee was correct in that "fishes" is the inappropriate term here as it refers to multiple species! The plural of fish for the same species is "fish" so please change this as originally requested.*
    - *Please indicate whether you buffered your MS222! MS222 is an acid and causes sever distress in fish if not buffered, and which should be required as per animal ethics*

regulations/guidelines.

- # We applied the modifications suggested by the Editor. #

Sentence: *Equal amounts of ground liver from the 3 fishes from the same aquarium were mixed and subdivided into aliquots that were stored at -80°C until downstream analyses (fatty acid profile, Cd and calcium hepatic concentrations and gene expression).*

- Editor's comment: *Again, this should be changed to "fish".*
- # We applied the modifications suggested by the Editor. #

Section 2.5 Fatty acid profile analyses:

- Editor's comment: *In contrast to your subsequent sections this is too short. Even if the exact procedures have been published elsewhere you need to include the key steps.*
- # We further detailed this section as suggested by the Editor. #

Section 2.6 Glutathione level – second paragraph

- Editor's comment: *This paragraph reads like a protocol and not a methods paragraph in a peer reviewed paper. Most of this should go into the supplemental materias and only the key steps and information listed here.*
- # We shortened this section and referred the reader to supplementary material for further details as suggested by the Editor. #

Section 2.7 Cd and calcium quantification

- Editor's comment: *Same as previous comment.*
- # We shortened this section as suggested by the Editor. #

Section 2.8 Gene expression

- Editor's comment : *See previous comment*
- # We shortened this section as suggested by the Editor. #

3. Results

Sentence: *In fish fed the EPA-enriched diet, the 24-h exposure to Cd significantly increased the relative abundance of neutral lipids from 11% to 16% of the total hepatic lipids, at the cost of the two other fractions abundances (data not shown.*

- Editor's comment*: So are these values the same listed in the second sentence above? Or is this relative to the start of the experiment (0h vs 24h) in the same exposure group? Not entirely clear as written.*
- # Yes, they are the same values. We modified the section to improve its clarity #

Sentence: *This effect was not observed in fish fed the other diets and not maintained upon 6-week Cd exposure in fish fed the EPA-enriched diet abundances (data not shown).*

- Editor's comment*: Slightly awkward sentence. I recommend to split into two separate sentences/statements.*
- # We applied the modifications suggested by the Editor. #

Sentence: *Diet markedly modulated the fatty acid composition of the hepatic phospholipids, free fatty acids and neutral lipids.*

- Editor's comment: *Significantly? "Markedly" is relative.*
- # Yes, it is significant. We modified the sentence #

Sentence: *For the sake of clarity, we chose to develop here under the results for the condition without Cd exposure*

- Editor's comment*: There is an issue with language and clarity here. Please revise as not obvious as written.*
- # We modified the sentence to improve its clarity #

Sentence: *The diet had no impact either on the total phospholipid, neutral lipids and free fatty acid levels*

(Fig. 2F) or on their content in SFA (Fig. 2A) and PUFA (Fig. 2E) (day 29 and day 70).

- Editor's comment: *Recommend to not present in parentheses but instead change to "... on day 29 or 70*
- # We applied the modification suggested by the Editor. #

Sentence: *These effects were significant in every lipid classes, at days 29 and 70, except in neutral lipids where the total n-6 PUFA level in fish fed the LA-enriched diet was similar to the one of fish fed the ALA- and DHA-enriched diets, at days 29 and 70 (Fig. 2D)*

- Editor's comment: *This should be singular "class"*
- # We applied the modification suggested by the Editor. #

Sentence: *Bioconversion products derived from LA (i.e. 20:2n-6, 18:3n-6, 20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6) were observed in every lipid classes of fish fed the LA-enriched diet, but especially in phospholipids where the levels of 20:4n-6 and 22:5n-6 exceeded that of LA (Fig. 3A-G, day 29 and day 70).*

- Editor's comment *: This should be plural "those"*
- # We applied the modification suggested by the Editor. Besides, in this sentence, we changed "lipid classes" to "lipid class" #

Sentence; *Indeed, DHA level in phospholipids, neutral lipids and free fatty acids was respectively divided by 2.0, 2.1 and 2.7 between the start of the experiment and day 29, and again by 2.8, 1.9 and 4.1 between day 29 and day 70 (Fig. 4I).*

- Editor's comment: *Please specify what these #s represent as not obvious to the reader. Are these fold-reductions in levels of respective lipid classes? If yes, I recommend you express as % change.*
- # Yes these #s are fold-reductions in levels of respective lipid classes. We applied the modification suggested by the Editor #

Sentence: *In contrast, the total n-3 PUFA level was similar among the n-3-enriched diets, in every lipid classes (Fig. 2C, day 29 and day 70).*

- Editor's comment: *Should be singular "class"*
- # We applied the modification suggested by the Editor #

Sentence: *These effects were significant in every lipid classes, at days 29 and 70, with some exceptions regarding the 18:4n-3 level in neutral lipids (Fig. 4C).*

- Editor's comment *: This should be plural "those"*
- # There is no "those" in the sentence. We changed "classes" to "class" #

Sentence*: At day 29, the free EPA level recovered in the liver of fish fed the EPA-enriched diet was significantly higher (x2.9) than in the liver of fish fed the DHA-enriched only, while similar amounts of EPA were recovered in the hepatic neutral lipids of fish fed the n-3-enriched diets (Fig. 4E).*

- Editor's comment: *2.9-fold?*
- # We applied the modification suggested by the Editor #

Sentence: *A 24-h exposure to Cd just sparsely influenced the fatty acid composition of hepatic phospholipids.*

- Editor's comment: *This is subjective and relative!*
- # We modified the paragraph to remove subjectivity #

Sentence: *This increase mainly reflected a rise in the levels of MUFAs (Fig. 2B, day 70), among which mainly 18:1n-9 (Fig. 5C, day 70) and 20:1n-9 (Fig. 5D, day 70).*

- Editor's comment: *Language and grammar. Please revise, e.g. as "... among which predominantly 18:1n-9 ... were altered/increased."*
- # We applied the modification suggested by the Editor #

Sentence: *Besides, Cd significantly increased by 49% the total MUFA level recovered in hepatic phospholipids of fish fed the DHA-enriched diet (Fig. 2B, day 70), among which mainly, 18:1n-7, 18:1n-9 and 20:1n-9 (Fig. 5B-D, day 70), without affecting significantly the total phospholipid level (Fig. 2F, day 70).*

- Editor's comment: *Change to "..., mainly 18:11n-7 ..."*

- # We applied the modification suggested by the Editor #

Sentence: *The diet had no impact on hepatic FATP1, CPT1, FASN, ELOVL2 and ELOVL5 gene expressions (Fig. 8 and S2, day 29 and day 70).*
- Editor's comment: *Insert "at both 29 and 70 days" and remove "day 29 and day 70" from parentheses.*
- # We applied the modification suggested by the Editor #

Sentence: *Cd had no impact on the hepatic FATP1, CPT1, FASN, d9D and ELOVL5 gene expressions (Fig. 8 and S2, day 29 and day 70).*
- Editor's comment*: Same as previous comment.*
- # We applied the modification suggested by the Editor #

Sentence: *The 24-h exposure to Cd significantly upregulated the hepatic FADS2 gene (x1.7) in fish fed the ALA-enriched diet only (Fig. 8, day 29).*
- Editor's comments:
  - *Delete "the"*
  - *Change to "gene expression"*
- # We applied the modifications suggested by the Editor #

Sentence: *The diet had no impact on the hepatic MTa, MTb, CALR, BCLx and p53 gene expressions (Fig. 8 and S3, day 29).*
- Editor's comment: *Delete "the"*
- # We applied the modification suggested by the Editor #

Sentence: *Neither the diet nor the Cd exposure impacted the CYP20A1 gene expression (Fig. S3, day 29 and day 70).*
- Editor's comment*: Delete "the"*
- # We applied the modification suggested by the Editor #

4. *Discussion*

Sentence: *This effect was, however, only significant in presence of Cd, which suggests that Cd influences the impact that dietary fatty acids exert on fish growth.*
- Editor's comment: *Can you expand this and speculate/discuss how Cd could have affected this process?*
- # We added a potential explanation in the text as suggested by the Editor #

Sentence: *The influence of short-term Cd exposure on the fatty acid homeostasis and metabolism in fish has been poorly investigated*
- Editor's comment: *Insert "to date" after "investigated"*
- # We applied the modification suggested by the Editor #

Paragraph: *For instance, Cd (11.2 mg/L, 24 h) significantly increased the incorporation of n-6 PUFAs (AA, 22:4n-6) in neutral lipids of rainbow trout liver cells grown in a medium supplemented with AA at the expense of other fatty acids (mainly SFAs) while no impact was observed in cells grown in media enriched in LA, ALA, or EPA (Ferain et al., 2018b). It was suggested that these n-6 PUFAs originated from the cellular free fatty acid pool (Ferain et al., 2018b). This lipid fraction was, however, not analysed in that study. In the present experiment, Cd (0.3 µg/L, 24 h) decreased the levels of several free fatty acids in the liver of fish fed the ALA- and EPA-enriched diets, only.*
- Editor's comment: *This discussion is mostly descriptive and I miss a deeper analysis of the very different concentrations used (you used almost 5 orders of magnitude lesser concentrations of Cd, which will likely have had an effect here as well). This was also not included in your below discussion, which mostly relied on these other studies that tested these very great Cd concentrations.*
- # We modified the text to highlight the differences of concentrations used in the studies. Note that most of the existing literature to date focussed on much higher Cd concentrations than the ones tested in the present study. #

Sentence: *In fish fed the EPA-enriched diet, Cd (0.3 µg/L, 6 weeks) downregulated the FADS2 gene expression.*

- Editor's comment: *Remove "the"*
- # We applied the modification suggested by the Editor #

Sentence: *Still, interactions between long-term Cd exposure (5.7 µg/L, 8 weeks) and water temperature on FADS2 gene expression was reported in fathead minnow (Pimephales promelas), with either higher, lower or unchanged expression of the gene following Cd exposure, according to water temperature (15, 25 or 30°C) and the organ analysed (muscle or brain) (Fadhlaoui et al., 2018).*

- Editor's comment: *So did "higher, lower or unchanged" expression occur at 15, 25 or 30C, respectively? If yes, please add ", respectively" after "15, 25 or 30C".*
- # Unfortunately it followed a more complicated pattern. That is the reason why we initially did not detailed further. We added details in the revised manuscript #

Paragraph: *Cd (0.3 µg/L, 6 weeks) upregulated the hepatic ELOVL2 gene in fish fed the ALA-enriched diet only. This upregulation went, however, not along with a significant increase of LC-PUFAs. Upregulation of the ELOVL2 gene by Cd (5.7 µg/L, 8 weeks) was also observed in the brain of fathead minnow reared at 15°C, while no impact was observed in brain at 25 or 30°C or in muscle at 15°C, 25°C, 30°C (Fadhlaoui et al., 2018).*

- Editor's comments:
  - *So what? THis is mostly descriptive and I am missing an interpretation of these findings. What do they mean/indicate?*
  - *DO your mean "was not correlated"?*
  - *Should read "minnows"*
- # We modified this section as suggested by the reviewer. Note that we have not perform correlation analyses. #

Paragraphs: *Cd (0.3 µg/L, 6 weeks) increased total phospholipid level in the liver of fish fed the ALA-enriched diets, only. This may reflect an increased de novo phospholipid synthesis to replace damaged lipids by oxidative attacks under Cd exposure. The phospholipid synthesis and turnover seem to be particularly efficient in fish fed this diet, as it resulted in a net increase of the total phospholipid level. Phospholipids are composed of two fatty acids, typically one SFA or MUFA at the sn-1 position, and one LC-PUFA at the sn-2 position (Tocher, 2003). Accordingly, the increase of the total hepatic phospholipids level in fish fed the ALA-enriched diet was accompanied by an increase of MUFAs. The phospholipid synthesis and turnover seem to be also activated by the long-term Cd exposure, but probably to a lesser extent, in fish fed the LA-enriched diet as an increase of the 18:0 and total PUFA levels was observed in the phospholipid fraction, without, however, any impact on the total phospholipid level. Cd (0.3 µg/L, 6 weeks) induced a similar increase of phospholipid MUFA level in fish fed the DHA-enriched diet as observed in fish fed the ALA-enriched diet, but again without any impact on the total phospholipid level, due to a decrease in several n-3 PUFAs (ALA, 20:3n-3, 20:4n-3, EPA and 22:5n-3) in this fraction, possibly through oxidation. This resulted in a decrease in the unsaturation level of the hepatic phospholipids in fish fed the DHA-enriched diet, with possible consequences for membrane fluidity and permeability. Similarly, Cd (4 µg/L, 7 weeks) increased the phospholipid relative abundance of MUFAs while decreasing that of PUFAs in the muscle of yellow perch (Perca flavescens) reared at 9°C (Fadhlaoui and Couture, 2016). These effects were not observed at 28°C (Fadhlaoui and Couture, 2016), highlighting the complexity of the interactions.*
The long-term Cd exposure increased the hepatic free MUFA level, in fish fed the EPA-enriched diet. As this increase was not balanced by a decrease in any MUFA from neither the phospholipid nor neutral lipid fraction, this suggests an increased MUFA uptake, and/or an increased de novo synthesis though both FASN and d9D. However, genetic analyses revealed no direct impact of Cd (0.3 µg/L, 6 weeks) on the hepatic FATP1, FASN and d9D gene expressions. Cd was, however, reported to increase hepatic FASN activity and/or gene expression in javelin goby (Synechogobius hasta) (Song et al., 2014), zebrafish (Pan et al., 2018) and yellow catfish (Pelteobagrus fulvidraco) (Chen et al., 2013).

- Editor's comments:
  - *Again, most of this discussion is descriptive and lack any interpretation of the potential meaning of our findings.*
  - *The sentence "The phospholipid synthesis and turnover seem to be also activated by the long-term Cd exposure, but probably to a lesser extent, in fish fed the LA-enriched diet as an*

increase of the 18:0 and total PUFA levels was observed in the phospholipid fraction, without, however, any impact on the total phospholipid level." is a long and difficult to follow sentence. I recommend you split this into two separate statements for ease of reading and clarity.

- # We do not fully agree with the Editor. Indeed, we gave interpretations of the modifications observed in the fatty acid profiles:
    - increase of the phospholipid synthesis, turnover by Cd
    - oxidation of LC-PUFA by Cd
    - potential impacts on the membrane fluidity and permeability
    - complexity of the mechanisms involved
    - increase of MUFA uptake and/or de novo synthesis
- Expression levels of selected genes where however not impacted. Whether Cd modulated the concentration or activity of proteins/enzymes encoded by the selected genes cannot be concluded from the present experiment. An "omic" approach would have been a useful tool to unravel mechanisms of action, but we basically chose for a targeted approach. We added several specific notes on these aspects in the manuscript.
- Besides, we devided the above-mentioned sentence into two separate sentences, as suggested by the Editor #

Sentence: *The body weight was not influenced by Cd exposure in the present experiment. In accordance to this result, other authors reported that Cd (3 µg/L, 30-40 days) did not impact rainbow trout growth (Hollis et al., 2001; Hollis et al., 1999; McGeer et al., 2000).*

- Editor's comment: *Change to "Body weight"*
- # We applied the modification suggested by the Editor #

Sentence: *Differences of results may reflect differences in water chemistry, with a higher toxicity in softer water in the study of Heydarnejad et al. (2013).*

- Editor's comment: *Furthermore, you used a 3-times lesser concentration.*
- # We agree with the Editor and added a note on that in the manuscript #

Sentence: *In the opposite way, the decrease of the total hepatic glutathione level induced by Cd in fish fed the EPA- and DHA-enriched diets after 6 weeks of metal exposure (0.3 µg/L) could reflect a decreased total hepatic antioxidant capacity and thus higher susceptibility to oxidative stress in these fish.*

- Editor's comment: *Recommend to change to "in contrast"*
- # We applied the modification suggested by the Editor #

Sentence: *In the present study, Cd had no impact on p53 gene expression, as previously observed in RTLW1 cells (Ferain et al., 2018b).*

- Editor's comment: *So what? Again, please interpret this observation here and tell the reader what this may indicate.*
- # We added a potential explanation in the text as suggested by the Editor #

Highlights (3 to 5, 85 characters maximum each, including space)

- Dietary lipids and Cd influenced hepatic fatty acid homeostasis and metabolism
- Fish fed a ALA-enriched diet seemed to be the least affected by the Cd exposure
- Fish fed a EPA-enriched diet seemed to be the most affected by the Cd exposure
- Fish fed a ALA-enriched diet had higher detoxifying ability, lower hepatic Cd level
- Fish fed a EPA-enriched diet were lighter, had lower antioxidant capacity

Interplay between dietary lipids and cadmium exposure in rainbow trout liver: influence on fatty acid metabolism, metal accumulation and stress response

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KEY WORDS

Polyunsaturated fatty acids, cadmium, rainbow trout liver, gene expression

ABSTRACT

The present study aimed at investigating interactive effects between dietary lipids and both short- and long-term exposures to a low, environmentally realistic, cadmium (Cd) concentration. Juvenile rainbow trout were fed four isolipidic diets (31.7 g/kg) enriched in either linoleic acid (LA, 18:2n-6), alpha-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) or docosahexaenoic acid (DHA, 22:6n-3). From the 4th week of this 10-week experiment, the lipid level of the diet was increased (120.0 g/kg) and half of the fish fed each diet were aqueously exposed to Cd (0.3 µg/L) while the other half were not exposed to Cd (control). Fish were sampled and their liver was harvested for fatty acid profile, hepatic Cd and calcium concentrations, total glutathione level and gene expression assessment, either (i) after 4 weeks of feeding and 24 h of Cd contamination (day 29) (short-term Cd exposure) or (ii) after 10 weeks of feeding and 6 weeks of Cd contamination (day 70) (long-term Cd exposure). We found that both dietary lipids and Cd exposure influenced fatty acid homeostasis and metabolism. The hepatic fatty acid profile mostly reflected that of the diet (e.g. n-3/n-6 ratio) with some differences, including selective retention of specific long chain polyunsaturated fatty acids (LC-PUFAs) like DHA and active biotransformation of dietary LA and ALA into LC-PUFAs. Cd effects on hepatic fatty acid profiles were influenced by the duration of the exposure and the nutritional status of the fish. The effects of diet and Cd exposure on the fatty acid profiles were only sparsely explained by variation of the expression pattern of genes involved in fatty acid metabolism. The biological responses to Cd were also influenced by dietary lipids. Fish fed the ALA-enriched diet seemed to be the least affected by the Cd exposure, as they showed a higher detoxifying ability against Cd with an early upregulation of protective metallothionein a (*MTa*) and apoptosis regulator BCL2-Like1 (*BCLx*) genes, an increased long-term phospholipid synthesis and turnover and fatty acid bioconversion efficiency, as well as a lower long-term accumulation of Cd in their liver. In contrast, fish fed the EPA-enriched diet seemed to be the most sensitive to a long-term Cd exposure, with an impaired growth performance and a decreased antioxidant capacity (lower glutathione level). Our results highlight that low, environmentally realistic aqueous concentrations of Cd can affect biological response in fish and that these effects are influenced by the dietary fatty acid composition.

1. INTRODUCTION

Cadmium (Cd) is a non-essential metal that can be toxic to both human and wildlife. Anthropogenic pollution of aquatic ecosystems by Cd is a consequence of its direct or indirect use in industrial and agricultural applications (Pan et al., 2010). Through atmospheric deposition, runoff or leaching Cd ends up in the aquatic ecosystem where it can be taken up by living organisms, including fish (McGeer et al., 2011; Pan et al., 2010). Dissolved Cd concentrations in freshwater ecosystem are generally below 0.5 µg/L (around 1 µg/L or lower in European streams (Pan et al., 2010)). Among fish, salmonids are very sensitive to Cd. The liver and the kidneys are the major organs of Cd accumulation during acute and chronic exposure, respectively (Chowdhury et al., 2003). For example, Cd hepatic levels in wild salmonid populations from various parts of the world were reported to range from 0.07 to 50 µg/g dry weight (Jaffal et al., 2011). Toxicity is mainly linked to the ability of Cd to alter essential metal homeostasis, especially the calcium balance, and to induce oxidative stress (McGeer et al., 2011). Indeed, Cd decreases calcium branchial uptake through direct competition with transporters and decreases calcium transfer to the bloodstream through inhibition of sodium/potassium ATPase, which can both lead to hypocalcaemia (McGeer et al., 2011; Niyogi and Wood, 2004; Verbost et al., 1988; Zohouri et al., 2001). Cd also interferes with internal handling of calcium (e.g. tissue and subcellular distribution) (Adiele et al., 2012; McGeer et al., 2011; Thévenod and Lee, 2013; Wang et al., 2014). In addition, Cd has been shown to cause oxidative stress by weakening the enzymatic and non-enzymatic antioxidant defence systems, by displacing endogenous redox active metals, by increasing the electron leakage of the electron transport chain and by rising free cytosolic calcium levels, resulting in an accumulation of toxic reactive oxygen species (ROS) (Adiele et al., 2012; Matović et al., 2015; McGeer et al., 2011; Wang et al., 2014; Wang et al., 2004). ROS accumulation can damage DNA, proteins and lipids, especially long chain polyunsaturated fatty acids (LC-PUFAs), which are naturally present in high concentrations in the tissues of salmonids.

Lipids, and their constitutive fatty acids, are important for fish health as they provide energy, are determinants of the integrity, fluidity and permeability of biological membranes, are precursors of bioactive molecules (e.g. eicosanoids) and act as nuclear receptor ligands (Tocher, 2003). Fish oil, which is rich in n-3 LC-PUFAs eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), has commonly been used in feed formulation in aquaculture as its fatty acid profile meets the dietary requirements and enables the farming of fish rich in valuable n-3 LC-PUFAs (FAO, 2018; Tocher, 2015). For both economic (increased price) and environmental (overfishing) reasons, fish oil is progressively being replaced by more sustainable alternative lipid sources in feed formulations, mainly plant-

derived oils (FAO, 2018; Glencross, 2009; Tocher, 2015; Turchini et al., 2009). However, as plant-derived oils lack n-3 LC-PUFAs, the total or partial replacement of fish oil by plant-derived oils has considerably decreased the n-3 LC-PUFA level in aquaculture feeds (Glencross, 2009; Turchini et al., 2009). Linseed oil (rich in alpha-linolenic acid, ALA, 18:3n-3) and sunflower oil (rich in linoleic acid, LA, 18:2n-6) are two examples of plant-derived oils, widely studied as alternative lipid sources for salmonids (Francis et al., 2014; Glencross, 2009; Mellery et al., 2017; Thanuthong et al., 2011; Turchini et al., 2018). Salmonids, including rainbow trout (*Oncorhynchus mykiss*), have a naturally high fatty acid bioconversion ability and can produce EPA and DHA from ALA, and arachidonic acid (AA, 20:4n-6) from LA (see Fig. 1 for the biotransformation pathways) (Tocher, 2003). However, these bioconversion abilities are often overwhelmed, so that lower LC-PUFAs levels are usually found in tissues of fish fed plant-derived diets than in those of fish fed fish-derived diets (Bell et al., 2001; Mellery et al., 2017; Turchini et al., 2018).

In vitro and *in vivo* experiments have shown that the fatty acid composition of the diet or growth medium influences biological responses to Cd in fish (Cornet et al., 2018; Ferain et al., 2018b; Ferain et al., 2016), in invertebrates (Ferain et al., 2018c) and in mammals (Amamou et al., 2015; Linhartova et al., 2016; Linhartova and Sampels, 2015). Indeed, the nature of the fatty acid supply modulates Cd uptake, redox status, proteomic landscape, gene expressions, immune function and survival under Cd stress. On the other hand, *in vitro* and *in vivo* trials have shown that Cd influences the fatty acid homeostasis and metabolism in fish (Chen et al., 2013; Fadhlaoui and Couture, 2016; Fadhlaoui et al., 2018; Ferain et al., 2018b; Garg et al., 2009; Konar et al., 2010; Koskinen et al., 2004; Liao et al., 2019; Olsvik et al., 2016; Pan et al., 2018; Pierron et al., 2007; Song et al., 2014), in invertebrates (Filimonova et al., 2016) and in mammals (Amamou et al., 2015; Kudo and Waku, 1996; Larregle et al., 2008; Madejczyk et al., 2015; Sarmiento-Ortega et al., 2017; Zhang et al., 2015). Indeed, Cd influences the activity and genetic expression of key enzymes of the fatty acid metabolism, the levels of total lipid, lipid classes and individual fatty acids in cells and tissues.

We hypothesized that (i) both PUFAs and Cd would modulate fatty acid homeostasis and metabolism, and that (ii) PUFAs would modulate biological responses to Cd, in rainbow trout liver *in vivo*. Since molecular responses to Cd have been reported to vary in a time-dependent manner (Reynders et al., 2006), we speculated that the interactive effects between PUFAs and Cd observed after short-term (24 h) exposure to Cd (0.3 µg/L) would differ from those observed after long-term (6 weeks) exposure to Cd (0.3 µg/L).

2. MATERIAL AND METHODS

2.1. Experimental design

Rainbow trout juveniles were fed four diets containing the same amount of total lipids (isolipidic) but differing in their PUFA profile and enriched in either (i) LA, (ii) ALA, (iii) EPA or (iv) DHA (Supplementary data Fig. S1). From the 4th week of this 10-week experiment, (i) dietary lipids were increased and (ii) half of the fish fed each diet were aqueously exposed to an environmentally realistic Cd level (0.3 µg/L) while the other half were not exposed to Cd (control) (Supplementary data Fig. S1). Fish were sampled and their liver was harvested for fatty acid profile, hepatic Cd and calcium concentrations, total glutathione level and gene expression assessment at day 29, i.e. after 4 weeks of feeding and 24 h of Cd contamination, and at day 70, i.e. after 10 weeks of feeding and 6 weeks of Cd contamination (Supplementary data Fig. S1). In addition, the hepatic fatty acid profile was assessed at the beginning of the experiment (day 0).

2.2. Diets

Four isoenergetic, isoproteic and isolipidic diets differing in their PUFA profile, i.e. rich in either (i) LA, (ii) ALA, (iii) EPA or (iv) DHA, were formulated and prepared by adding one specific oil to a common basal diet devoid of fish meal, i.e. either (i) sunflower oil (Bio-time, Colruyt, Belgium), (ii) linseed oil (Vandeputte, Belgium), (iii) EPA-rich cod liver oil (Omegavie EPA 70 TG, Polaris, France), (iv) DHA-rich cod liver oil (Omegavie DHA 70 TH, Polaris, France). These specific oils were included in diets at levels of 31.7 g/kg for the first 4 weeks and 117.6 g/kg for the last 6 weeks of the experiment. Classical cod liver oil, i.e. not specifically enriched in either EPA or DHA, was also added in every diet (2.4 g/kg) during the last 6 weeks of the experiment. The gross composition of each diet and their fatty acid profiles are shown in Tables 1 and 2, respectively. Note that the vitamin E levels in the vitamin premix were adapted to compensate for the differences in vitamin E levels in the specific oils used in each diet (sunflower oil, linseed oil, EPA-rich cod liver oil and DHA-rich cod liver oil).

2.3. Animal housing, feeding and Cd exposure

The experimental design was approved by the local Ethic Committee for Animal Research of the University of Namur, Belgium (proposal number: 125 16272 KE, agreement number LA1900048). Animal housing, feeding and Cd exposure were performed as described by Cornet et al. (2018). Rainbow trout juveniles, obtained from the aquaculture farm Charles Murgat (Beaufort, France), were randomly distributed into 100 L polyethylene tanks (24 tanks, 25 fish/tank, 3 tanks/experimental condition) in a recirculating system according to dietary and Cd exposure conditions. Fish were acclimated for 14 days prior to the start of the experiment. Fish, with an initial mean body weight of 14.4 ± 0.3 g, were reared at 14°C under

a light cycle of 12h:12h light:darkness and were hand-fed twice a day, six days a week. Feed quantity corresponded to 3% of tank biomass during the first 4 weeks of the experiment and 2.5% during the last 6 weeks of the experiment. From day 28, half of the fish fed each diet were aqueously exposed to an environmentally realistic Cd level (0.3 µg/L) while the other half were not exposed to Cd (control). Exact contamination levels were measured by graphite furnace atomic absorption spectrometry (GFAAS) (see Section 2.7).

2.4. Sampling

Fish were sampled at day 29, i.e. after 4 weeks of feeding and 24 h of Cd contamination, and at day 70, i.e. after 10 weeks of feeding and 6 weeks of Cd contamination. At each sampling time, 3 fish per aquarium were taken, anesthetized (MS222, 120 mg/L, buffered with $NaHCO_3$ to reach pH 7.2), euthanized with an overdose of MS222 (240 mg/L, pH 7.2) and death was confirmed by cervical dislocation. Fish were weighed and their liver was sampled and weighed. A piece of liver was taken from each fish for total glutathione measurement. Those of the 3 fish from the same aquarium were pooled and homogenized in 600 µL of HCl (1%) at 4°C. After sedimentation, 480 µL of supernatant were transferred into an Eppendorf containing 120 µL of sulphosalicylic acid (SSA, 6.5%). After 3 min of incubation at 4°C the resulting solution was flash frozen in liquid N_2 and kept at -80°C until total glutathione analysis. The aliquots containing the liver pellets in 120 µL of HCL were flash frozen in liquid N_2 and kept at -80°C until protein analysis. The rest of the liver of each fish sampled was flash frozen in liquid N_2, freeze dried and ground individually. Equal amounts of ground liver from the 3 fish from the same aquarium were mixed and subdivided into aliquots that were stored at -80°C until downstream analyses (fatty acid profile, Cd and calcium hepatic concentrations and gene expression)..

2.5. Fatty acid profile analyses

Total lipids were extracted from liver homogenate (about 15 mg of freeze-dried weight) with chloroform:methanol:water (2:2:1.8, v:v:v) (Bligh and Dyer, 1959). Total lipid extract was separated into three lipid classes, i.e. free fatty acids, neutral lipids and phospholipids, by solid phase extraction (Schneider et al., 2012) and methylated (Ferain et al., 2016). Resulting fatty acid methyl esters (FAMEs) were extracted with hexane (Ferain et al., 2016) and separated by gas chromatography Ferain et al. (2018a). The gas chromatograph (GC Trace 1310, Thermo Fisher Scientific, Italy) was equipped with an autosampler TriPlus TP100 (Thermo Fisher Scientific, Italy). Separation was carried out on a RT2560 capillary column (biscyanopropyl polysiloxane, 100m x 0.25mm internal diameter, 0.2 µm film thickness; Restek, USA) with H_2 as carrier gas (200 kPa, 2 mL/min). Temperature was increased from 80°C to 235°C in four steps, to separate FAMES, as previously detailed (Ferain et al., 2018a). FAME detection was performed using a Flame Ionization Detector (Thermo Quest,

Italy) flowed by H_2 (35 mL/min), air (350 mL/min) and N_2 (40 mL/min), at a constant temperature of 255 °C. Chromatograms were analysed with the ChromQuest software (version 5.0, ThermoFinnigan, Italy). FAMEs were identified and quantified by comparing both retention times and peak areas with an external standard made from known concentrations of 44 FAMEs (Larodan, Sweden). The quality of the whole process was monitored through the use of internal standards, i.e. (i) 1,2-dipentadecanoyl-sn-glycero-3-phosphatydylcholine, triheptadecanoin and tridecanoic acid (Larodan, Sweden) and (ii) methyl-undecanoate (Larodan, Sweden), that were added at the extraction and injection steps, respectively. Concentrations that were below the limit of quantification were set at 0.

2.6. Glutathione level

Total hepatic glutathione level was determined by high performance liquid chromatography (HPLC) with fluorimetric detection, using the method described by Cereser et al. (2001) (adapted from Neuschwander-Tetri and Roll (1989)) with minor modifications. This method is based on the derivatization of reduced glutathione (GSH) with ortho-phtalaldehyde (OPA), a compound that reacts with both the sulfhydryl and the primary amino groups of GSH to form a highly fluorescent product (Cereser et al., 2001; Neuschwander-Tetri and Roll, 1989). To measure total hepatic glutathione, a reducing agent was added prior to the derivatization step to convert oxidized glutathione (GSSG) into GSH.

Analyses were performed in triplicates. A detailed protocol can be found in supplementary material. Briefly, aliquots stored for total glutathione analyses were thawed on ice and centrifuged. Supernatant was spiked with internal standard (glutathione ethyl ester, GEE), diluted in SSA/HCl, then neutralised with an excess of $NaHCO_3$. After centrifugation, supernatant was reduced by addition of DL-dithiothreitol (DTT, 25 mM) and TRIS buffer (100 mM, pH 8.5). After a 30 min-incubation at 4°C, samples were acidified by addition of metaphosphoric acid (MPA, 6%). Derivatization with OPA was performed by mixing the resulting suspension with OPA reagent and a subsequent 5 min-incubation at room temperature. Derivatized samples were finally neutralized with sodium phosphate buffer (500 mM, pH 7.0) before HPLC analysis. The HPLC system consisted of a degassing unit (DGU-20A5, Shimadzu, Japan), a pump (LC-20AT, Shimadzu, Japan), an autosampler (SIL-HT, Shimadzu, Japan), an oven (CTO-20A, Shimadzu, Japan), a XTerra MS C18 column (250 x 4.6 mm, 5 µm particle size, Waters, USA) and a fluorescence detector (RF-20A, Shimadzu, Japan). The column, maintained at 30°C, was flowed by mobile phase gradients at a constant rate of 0.7 mL/min. Fluorescence emitted by GSH-OPA and GEE-OPA adducts was detected at 420 nm following excitation at 340 nm. Spectra were processed with the LabSolutions software (version 5.32 SP1, Shimadzu, Japan). Quantification was performed through comparison of the peak areas with standard curves that were daily established for

GEE and GSH, under the same conditions as for the samples. Data were standardized by the protein level in the liver, which was determined using the Bicinchoninic acid (BCA) Protein Assay (Thermo Scientific Pierce, USA), with BSA as standard.

2.7. Cd and calcium quantification

Total hepatic Cd and calcium levels were quantified by GFAAS and inductively coupled plasma atomic emission spectrometry (ICP-AES), respectively. Liver homogenates (about 10 mg of freeze-dried weight) were first digested in HNO_3 (65%) at 80°C for 3 h and then diluted with deionized water. From this suspension, one aliquot was used for Cd quantification by GFAAS and another one was used for calcium quantification by ICP-AES. GFAAS analyses were run on an ICE3500 spectrophotometer (Thermo Scientific, UK) with argon as purge gas. A matrix modifier ($MgNO_3$, 5 µL, 4 g/L) was added to the samples (15 µL). Temperature was increased from 110°C to 2500°C in four steps (drying, pyrolysis, atomisation, cleaning), as previously described (Ferain et al., 2018b). During the atomisation step, Cd atomic absorption was measured at 228.802 nm, with a Zeeman background correction. ICP-AES analyses were run on an iCap 7200 duo spectrophotometer (Thermo Scientific, UK) with argon as plasma gas. Wavelength for calcium detection was 315.887nm, with the torch in the radial position. Cd and calcium concentrations were calculated thanks to calibration curves made from certified standards (Fluka, Germany). The accuracy of the whole process was controlled by the analysis of reference materials either from the destruction step, i.e. SRM 2977 (Mussel tissue, National Institute of Standards and Technology, USA), or from the quantification step, i.e. TM-28.4 (Fresh water sample, Environment Canada, Canada) for Cd in GFAAS and Cranberry-05 (Fresh water sample, Environment Canada, Canada) for calcium in ICP-AES. The recoveries obtained for SRM 2977 were 76.9 ± 1.8 % for Cd and 86.3 ± 1.2 % for calcium. TM-28.4 and Cranberry-05 yielded recoveries of 91.8 ± 1.6 % and 97.3 ± 1.1 %, respectively. In addition, standard additions were performed on liver homogenates for Cd analyses and yielded a recovery of 75.2 ± 1.2 %. Data were not corrected by recoveries. For each experimental condition from each biological replicate (aquarium), Cd and calcium quantifications were performed on two independent aliquots of liver homogenate, processed during two different days. Data from these technical duplicates were averaged. Quantification limits were 0.034 and 8.0 µg/g of freeze dried liver for Cd and calcium, respectively. Finally, dissolved Cd concentration was dosed in water by GFAAS. The measured contamination levels were 0.30 ± 0.07 µg/L for the Cd-exposed tanks and below the quantification limit (i.e. <0.1 µg/L) for the control tanks.

2.8. Gene expression

Liver homogenates (about 10 mg of freeze-dried weight) were lysed in TRIzol Reagent (1 mL, Life Technologies) and water (volume equal to the water lost through freeze-drying considering a mean freeze-dried liver weight of about 25% of fresh liver weight) by repeated passage through needles of decreasing diameters (1.1 then 0.7 mm) until fine suspensions were obtained. Lysates were centrifuged (10 min, 12000 g, 4°C). Chloroform (200 µL) was added to supernatants. Suspensions were vigorously shaken for 15 s, incubated 2 min at room temperature and centrifuged (15 min, 12000 g, 4°C). RNA was precipitated by adding ethanol (70%) to supernatants, homogenized and loaded on a RNA binding column (Aurum Total RNA mini kit; Biorad Laboratories, Inc, USA). Samples were then processed following the manufacturer's guidelines to obtain pure total RNA extracts. DNAse I treatment was always included. RNA quantity and purity were assessed spectrophotometrically (Nanodrop ND-1000 Spectrophotometer, Isogen, Life Science, The Netherlands) using the optical densities (OD) measured at 260, 280 and 230 nm (OD_{260}, $OD_{260/280}$, $OD_{260/230}$). Total RNA (1 µg) was reverse transcribed with the iScript cDNA Synthesis kit (Biorad Laboratories, Inc, USA), according to manufacturer's guidelines. cDNA was diluted (3 times) in DNA- and DNAse-free water, subdivided in aliquots and stored at -20°C until quantitative polymerase chain reactions (qPCR) were performed on a Step One Plus thermocycler (Applied Biosystems, Life Technologies, USA) as previously described (Ferain et al., 2018a). Each reaction well contained cDNA (1 µL), primer pairs and SYBR Select Master Mix I (10 µL, Applied Biosystems, Life Technologies, USA). Samples were run in duplicate (2 wells). Primer pairs sequences (Table 3) were either obtained from the literature or designed using rainbow trout transcript sequences available in the European Nucleotide Archive database (identifiers: Marancik et al. (2014)). The thermal profile used was 95°C for 2 min, then 95°C for 15 s followed by 1 min at the annealing temperature (60 or 62°C, Table 3) for 40 cycles. A melt curve analysis always followed the 40 cycles. The targeted genes (Fig. 1) are involved in fatty acid uptake (fatty acid transport protein 1 (*FATP1*)), fatty acid *de novo* synthesis (fatty acid synthase (*FASN*)), fatty acid bioconversion (fatty acid desaturase 2 (*FADS2*), Δ9 desaturase (*d9D*), fatty acid elongases 2 and 5 (*ELOVL2* and *ELOVL5*)), β-oxidation (carnitine palmitoyl transferase 1 (*CPT1*)) and stress response (metallothioneins a and b (*MTa* and *MTb*), apoptosis regulator BCL2-Like1 (*BCLx*), tumor protein 53 (*p53*), calreticulin (*CALR*)). The orphan cytochrome P450 20A1 gene (*CYP20A1*), whose expression was upregulated by specific PUFAs and either downregulated or not affected by Cd in rainbow trout liver cells enriched in specific PUFAs (Ferain et al. 2018), was also studied. Ct values from the technical duplicates were averaged. The $E^{-\Delta Ct}$ method was applied for relative gene expression analysis, using the geometric mean of *actin* and *18S* Ct values as housekeeping value (Vandesompele et al., 2002). Note that other genes and gene combinations were also

tested as housekeepers but proven to be unreliable (i.e., higher variation of Ct values across treatments, data not shown).

2.9. Statistical analyses

Graphs and statistical analyses were performed with the JMP PRO 14.1 software. Variance homoscedasticity and normality of residuals were checked with appropriate tests (Bartlett test, Levene test, Shapiro-Wilk test, skewness and kurtosis calculations) prior to performing parametric tests. Type I error (α) was set at 0.05. A Bonferroni-Sidak correction was applied to correct the p-values in any case of multiple testing (Sidak, 1967).

For each sampling time, two one-way ANOVAs or Kruskal-Wallis tests (if non-parametric tests were required) were performed to assess the impacts of the diet and of the Cd exposure. For each Cd exposure level, a first 1-way ANOVA (if parametric) or Kruskal-Wallis test (if non-parametric), followed by a post-hoc Tukey test (if parametric) or a Dunn's test (if non-parametric), was performed to assess the impact of the diet on the growth, the hepatosomatic index (HSI), the fatty acid profile of each lipid class analysed, the gene expression levels, the calcium and Cd hepatic concentrations and the glutathione status. For each diet, a second 1-way ANOVA (if parametric) or Kruskal-Wallis test (if non-parametric) was performed to assess whether Cd exposure influenced these parameters. Data were Log transformed prior to statistical analyses, except for glutathione data (no transformation), n-3/n-6 ratio data (square-root transformation) and for non-parametric tests (no transformation), which were required for 14:0 and 20:0 levels in phospholipids.

3. RESULTS

3.1. Growth and HSI

At day 29, fish weights were similar (Table 4). At day 70, fish fed the DHA-enriched diet were 13% and 14% heavier than fish fed the LA-enriched diet, in absence and in presence of a 6-week Cd exposure, respectively. This difference was, however, only significant upon Cd exposure (Table 4). Besides, at day 70, fish fed the DHA-enriched diet and exposed to Cd were 11% heavier than fish fed the EPA-enriched diet and exposed to Cd ($p<0.05$), while no impact was observed in fish fed these two diets but unexposed to Cd (Table 4). Cd had no significant impact on fish weight (Table 4, day 29 and day 70). Neither the diet nor the exposure to Cd had any significant impact on the HSI (Table 4, day 29 and day 70).

3.2. Hepatic fatty acid composition

3.2.1. Separation of the lipid classes: preponderance of the phospholipids

The phospholipids accounted for 74-81% of the total hepatic lipids (day 29 and day 70). Neutral lipids and free fatty acids represented 11-16% and 7-11% of the total hepatic lipids, respectively (day 29 and day 70). The diet had no impact on these relative abundances (data not shown). Cd had no impact on these relative abundancies in fish fed the LA-, ALA- and DHA-enriched diets. In fish fed the EPA-enriched diet, the 24-h exposure to Cd significantly increased the relative abundance of neutral lipids from 11% (in absence of Cd) to 16% (in presence of Cd) of the total hepatic lipids, at the cost of the two other fractions abundances (data not shown). This effect was not maintained upon 6-week Cd exposure (data not shown).

3.2.2. Diet impacts on hepatic lipids

Diet significantly modulated the fatty acid composition of the hepatic phospholipids, free fatty acids and neutral lipids. Overall, Cd exposure did not modify the impacts of dietary lipids on the hepatic fatty acid profiles (Fig. 2-6). We thus chose to develop here under the results for fish unexposed to Cd only.

3.2.2.1. Few or no impact on total saturated fatty acid (SFAs), monounsaturated fatty acids (MUFAs) and PUFAs

The diet had no impact either on the total phospholipid, neutral lipids and free fatty acid levels (Fig. 2F) or on their content in SFA (Fig. 2A) and PUFA (Fig. 2E) on day 29 and day 70. Some differences could be observed regarding the MUFA content of the phospholipid fraction at day 70. Fish fed the ALA- (+34%) and EPA- (+40%) enriched diets had a significantly higher MUFA content in their phospholipids than fish fed the DHA-enriched diet (Fig. 2B, day 70). Phospholipids of fish fed the EPA-enriched diet were also significantly richer in MUFA than those of fish fed the LA-enriched diet (+33%, Fig. 2B, day 70). The MUFA content of neutral lipids and free fatty acids was not affected by the diet throughout the experiment (Fig. 2B, day 29 and day 70).

3.2.2.2. Fish fed the LA-enriched diet: the richest in n-6 PUFAs and lowest in n-3 PUFAs

The hepatic lipids of fish fed the LA-enriched diet were richer in n-6 PUFAs (x1.9-11.7, Fig. 2D), lower in n-3 PUFAs (x0.1-0.5, Fig. 2C) and exhibited a lower n-3/n-6 ratio (x0.01-0.30, Fig. 2G) than those of fish fed the n-3-enriched diets. These effects were significant in every lipid class, at days 29 and 70, except in neutral lipids where the total n-6 PUFA level in fish fed the LA-enriched diet was similar to the one of fish fed the ALA- and DHA-enriched diets, at days 29 and 70 (Fig. 2D). Bioconversion products derived from LA (i.e. 20:2n-6, 18:3n-6,

20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6) were observed in every lipid class of fish fed the LA-enriched diet, but especially in phospholipids where the levels of 20:4n-6 and 22:5n-6 exceeded those of LA (Fig. 3A-G, day 29 and day 70). The hepatic DHA level gradually dropped throughout the experiment in fish fed the LA-enriched diet due to the exclusion of DHA and n-3 precursors from the diet during the first 4 weeks (Fig. 4I). The addition of cod liver oil (which contains n-3 PUFAs, including DHA) in the diet at day 29 provided small amounts of DHA but did not allow to stop the drop of hepatic concentrations. Indeed, DHA level in phospholipids, neutral lipids and free fatty acids underwent a drop of 49%, 52% and 63% between the start of the experiment and day 29, and another drop of 65%, 47% and 76% between day 29 and day 70 (Fig. 4I). Noteworthy, the relative abundance of DHA in hepatic phospholipids of fish fed the LA-enriched diet dropped from 42.2% at the start of the experiment to 20.7% at day 29 and to 8.1% at day 70.

3.2.2.3. Differences among the n-3-enriched diets: high bioconversion abilities

Feeding a diet enriched in either ALA, EPA or DHA influenced the total n-6 PUFA level in phospholipids (Fig. 2D, day 29 and day 70) and the n-3/n-6 ratio in every lipid classes (Fig. 2G, day 29 and day 70). In contrast, the total n-3 PUFA level was similar among the n-3-enriched diets, in every lipid class (Fig. 2C, day 29 and day 70). The phospholipid n-6 PUFA level was higher in fish fed the ALA-enriched diet than in fish fed the EPA- (x2.0) and DHA- (x1.4-1.5) enriched diets (Fig. 2D, day 29 and day 70). At day 70, the phospholipid n-6 PUFA level of fish fed the DHA-enriched diet became significantly higher than the one of fish fed the EPA-enriched diet (x1.4, Fig. 2D). The n-3/n-6 ratio of hepatic phospholipids and free fatty acids followed the same decreasing order as in the fish diet composition, with EPA > DHA > ALA (Fig. 2G, day 29 and day 70). The n-3/n-6 ratios of hepatic neutral lipids were similar in fish fed the EPA- and DHA-enriched diets, both being higher than in fish fed the ALA-enriched diet (Fig. 2G, day 29 and day 70).

The hepatic lipids of fish fed the ALA-enriched diet were richer in ALA (x4.6-39.3, Fig. 4A) and its direct elongation and desaturation products (20:3n-3 and 18:4n-3, Fig. 4B-C) than those of fish fed the other diets. These effects were significant in every lipid class, at days 29 and 70, with some exceptions regarding the 18:4n-3 level in neutral lipids (Fig. 4C). In this lipid fraction, the difference between fish fed the ALA-enriched diet and fish fed the EPA- and DHA-enriched diets was less important and only significant at day 29 and day 70, respectively (Fig. 4C). The level of 20:4n-3 (desaturation product of 20:3n-3) was significantly higher in fish fed the ALA-enriched diet than in fish fed the EPA-enriched diet in phospholipids (x4.6-7.0) and the DHA-enriched diet in every lipid classes (Fig. 4D, day 29 and day 70). The phospholipid level of EPA (desaturation product of 20:4n-3) was about 2.7

times higher in fish fed the ALA-enriched diet than in fish fed the DHA-enriched diet but about 2.0 times lower than in fish fed the EPA-enriched diet (Fig. 4E, day 29 and day 70). At day 29, the free EPA level recovered in the liver of fish fed the EPA-enriched diet was significantly 2.9-fold higher than in the liver of fish fed the DHA-enriched only, while similar amounts of EPA were recovered in the hepatic neutral lipids of fish fed the n-3-enriched diets (Fig. 4E). Similar differences were observed for 22:5n-3, the elongation product of EPA, except that the 22:5n-3 level recovered in the liver of fish fed the EPA enriched diet was similar to the one recovered in the liver of fish fed the ALA-enriched diet in the phospholipid fraction (day 29), and to the one recovered in the liver of fish fed the DHA-enriched diet in both the free fatty acid fraction (day 29 and day 70) and the neutral lipid fraction (day 70) (Fig. 4F). The elongation product of 22:5n-3, i.e. 24:5n-3, was only quantifiable in the neutral lipids of fish fed the EPA-enriched diet at day 29, in every lipid classes of fish fed the EPA-enriched diet at day 70 and in neutral lipids of fish fed the DHA-enriched diet at day 70 (Fig. 4G). Its desaturation product (24:6n-3) was found in significantly higher concentration in the phospholipids of fish fed the ALA- (x2.0-2.5) and EPA- (x3.0-3.4) enriched diets than in fish fed the DHA-enriched diet (Fig. 4H, day 29 and day 70). The neutral lipid level of 24:6n-3 was similar among fish fed the n-3 enriched diets (Fig. 4H, day 29 and day 70). In free fatty acids, 24:6n-3 was only quantifiable in fish fed the EPA- and DHA-enriched diets at day 70 (Fig. 4H). At day 29, the hepatic DHA level in phospholipids and neutral lipids did not differ between fish fed the n-3-enriched diets (Fig. 4I). By contrast, in the free fatty acid fraction, the DHA level was significantly higher in fish fed the DHA-enriched diet than in fish fed the ALA-enriched diet (x2.1, Fig. 4I, day 29). At day 70, the DHA level in fish fed the DHA-enriched diet was significantly higher than in fish fed the ALA- and EPA-enriched diets in the phospholipid (x1.4-1.6) and free fatty acid (x2.7-3.1) fractions and significantly higher than in fish fed the ALA-enriched diet in the neutral lipid fraction (x3.7) (Fig. 4I). Throughout the experiment, whatever the lipid class, the hepatic content in DHA of fish fed the ALA-enriched diet was similar to that of fish fed the EPA-enriched diet (Fig. 4I).

3.2.3. Cd impacts on hepatic lipids

3.2.3.1. Phospholipids

The 24-h exposure to Cd decreased by 14% the level of 20:2n-6 (Fig. 3B, day 29) and increased by 39% the level of 22:4n-6 (Fig. 3F, day 29) in hepatic phospholipids of fish fed the LA- and ALA-enriched diets, respectively. The 6-week Cd exposure influenced both the total level and the fatty acid composition of hepatic phospholipids. First, Cd significantly increased the total hepatic phospholipid level in fish fed the ALA-enriched diet by 13% (Fig. 2F, day 70). This increase mainly reflected a rise in the levels of MUFAs (Fig. 2B, day 70),

among which predominantly 18:1n-9 (Fig. 5C, day 70) and 20:1n-9 were increased (Fig. 5D, day 70). Besides, Cd significantly increased by 49% the total MUFA level recovered in hepatic phospholipids of fish fed the DHA-enriched diet (Fig. 2B, day 70), mainly 18:1n-7, 18:1n-9 and 20:1n-9 (Fig. 5B-D, day 70), without affecting significantly the total phospholipid level (Fig. 2F, day 70). In addition, Cd significantly decreased the hepatic phospholipid levels of ALA (-31%, Fig. 4A, day 70), 20:3n-3 (-33%, Fig. 4B, day 70), 20:4n-3 (-38%, Fig. 4D, day 70), EPA (-28%, Fig. 4E, day 70), 22:5n-3 (-37%, Fig. 4F, day 70) in fish fed the DHA-enriched diet. Besides Cd increased the level of 20:3n-3 in the phospholipids of fish fed the EPA-enriched diet (+29%, Fig 4B, day 70). Finally, Cd significantly increased the total PUFA (+20%, Fig. 2E, day 70) and the 18:0 (+24%, Fig. 6D, day 70) levels in phospholipids of fish fed the LA-enriched diet.

3.2.3.2. Neutral lipids

A 24-h exposure to Cd significantly increased the total SFA level in hepatic neutral lipids of fish fed the LA- (x2.2), EPA- (x2.3) and DHA- (x2.0) enriched diets (Fig. 2A, day 29). This increase was mainly due to enrichment in 16:0 (Fig. 6C, day 29). In fish fed the EPA-enriched diet, the 24-h Cd exposure also significantly increased the hepatic neutral lipid content in PUFAs (+46%, Fig. 2E, day 29). The increase in PUFAs was essentially due to a rise of n-3 PUFAs (+41%, Fig. 2C, day 29), among which mainly DHA (+29%, Fig. 4I, day 29), but also, to a lesser extent, to an increase in 20:2n-6 (+63%, Fig. 3B, day 29). As a result of these individual changes, the total hepatic neutral lipid level was 56% higher in fish fed the EPA-enriched diet and exposed to Cd during 24 h than in fish fed the same diet but unexposed to Cd (Fig. 2F, day 29). These effects were not maintained upon long-term Cd exposure (Fig. 2-4 and 6, day 70).

3.2.3.3. Free fatty acids

The 24-h Cd exposure significantly decreased the total hepatic free fatty acid level by 16% in fish fed the ALA- enriched diet (Fig. 2F, day 29). This decrease mainly reflected a decline in both the n-3 (-36%, Fig. 2C, day 29) and n-6 (-35%, Fig. 2D, day 29) PUFA levels, including ALA (-49%, Fig. 4A, day 29), EPA (-45%, Fig. 4E, day 29), 22:5n-3 (-36%, Fig. 4F, day 29) and LA (-42%, Fig. 3A, day 29). In fish fed the EPA-enriched diet, the 24-h Cd exposure induced a decline in 18:0 (-32%, Fig. 6D, day 29), 20:1n-9 (-49%, Fig. 5D, day 29) and DHA (-32%, Fig. 4I, day 29) in the free fatty acid fraction. These effects were not maintained upon long-term Cd exposure (Fig. 2-6, day 70). Instead, the 6-week Cd exposure induced a significant increase of the total hepatic free MUFA level in fish fed the EPA-enriched diet (x1.8, Fig. 2B, day 70). This increase mainly reflected a rise in 18:1n-9 (x2.2, Fig. 5C, day

70). The 6-week Cd exposure also induced a significant increase of the hepatic free 18:1n-9 level in fish fed the LA- (x2.3) and ALA- (x3.0) enriched diets (Fig. 5C, day 70).

3.3. Hepatic total glutathione level

The diet had no impact on the total hepatic glutathione level (Fig. 7A, day 29 and day 70). The 6-week Cd exposure significantly decreased the total hepatic glutathione level by 43% and 61% in fish fed the EPA- and DHA-enriched diets, respectively (day 70), while no impact was observed after an exposure of 24 h (Fig. 7A, day 29). Cd had no impact on the total hepatic glutathione level in fish fed the LA- and ALA-enriched diets (Fig. 7A, day 29 and day 70).

3.4. Hepatic calcium level

The diet had no impact on the hepatic calcium level (Fig. 7B, day 29 and day 70). The 24-h Cd exposure significantly increased the hepatic calcium level by 29% in fish fed the LA-enriched diet, while it had no impact in fish fed the other diets (Fig. 7B, day 29). This effect disappeared upon long-term Cd exposure (Fig. 7B, day 70).

3.5. Hepatic Cd level

The hepatic Cd level was below the quantification limit (i.e. <0.034 µg/g freeze dried liver) upon 24-h Cd exposure. Upon 6-week exposure, the hepatic Cd concentration was about twice lower in fish fed the ALA-enriched diet than in fish fed the LA- and EPA-enriched diets (Fig. 7C, day 70). The hepatic Cd level of fish fed the DHA-enriched diet did not significantly differ from the hepatic Cd level of fish fed the other diets (Fig. 7C, day 70).

3.6. Hepatic gene expression pattern

3.6.1. Fatty acid metabolism

The diet had no impact on hepatic *FATP1*, *CPT1*, *FASN*, *ELOVL2* and *ELOVL5* gene expressions at both 29 and 70 days (Fig. 8 and S2). The impacts of the diet on the hepatic *FADS2* and *d9D* genes were only observed at day 70 and were modulated by the Cd exposure. Indeed, in absence of Cd, the hepatic *FADS2* gene expression level was significantly lower in fish fed the DHA-enriched diet than in fish fed the LA- (x5.7) and EPA- (x3.4) enriched diets (Fig. 8, day 70). These differences were no longer observed upon long-term Cd exposure (Fig. 8, day 70). Instead, the hepatic *FADS2* gene expression level was significantly lower in fish fed the EPA-enriched diet than in fish fed the LA- (x2.3) and ALA- (x2.2) enriched diets (Fig. 8, day 70). Besides, in absence of Cd exposure, the hepatic *d9D*

gene expression level was lower in fish fed the DHA-enriched diet than in fish fed the LA-enriched diet (x6.0) (Fig. 8, day 70). Due to a higher variability of the data, this difference was not statistically maintained upon long-term Cd exposure (Fig. S2, day 70).

Cd had no impact on the hepatic *FATP1*, *CPT1*, *FASN*, *d9D* and *ELOVL5* gene expressions at both 29 and 70 days (Fig. 8 and S2). The 24-h exposure to Cd significantly upregulated hepatic *FADS2* gene expression (x1.7) in fish fed the ALA-enriched diet only (Fig. 8, day 29). This upregulation was maintained upon long-term Cd exposure (x1.5) (Fig. 8, day 70). In addition, the 6-week Cd exposure significantly downregulated (x0.5) the hepatic *FADS2* gene in fish fed the EPA-enriched diet (Fig. 8, day 70). Besides, the 6-week Cd exposure significantly upregulated the hepatic *ELOVL2* gene (x1.6, day 70) in fish fed the ALA-enriched diet only, while no impact of Cd was observed upon 24-h exposure (day 29) (Fig. 8).

3.6.2. Stress response

The diet had no impact on hepatic *MTa*, *MTb*, *CALR*, *BCLx* and *p53* gene expressions (Fig. 8 and S3, day 29). The 24-h Cd exposure significantly upregulated the hepatic *MTa* (x2.0) and *BCLx* (x1.3) gene expressions in fish fed the ALA-enriched diet only (Fig. 8, day 29). Cd had no impact on the hepatic *MTb*, *CALR* and *p53* gene expressions (Fig. S3, day 29).

3.6.3. CYP20A1

Neither the diet nor the Cd exposure impacted *CYP20A1* gene expression (Fig. S3, day 29 and day 70).

4. DISCUSSION

The present study aimed at investigating to what extent (i) dietary lipids and Cd influence the fatty acid homeostasis and metabolism (ii) dietary lipids modulate biological responses to Cd, in rainbow trout liver after both short-term (24 h) and long-term (6 weeks) exposure to an environmentally realistic Cd concentration (0.3 µg/L). A summary of the main interactive results between Cd and dietary lipids coming from the present experiment is presented in Fig. 1.

4.1. Dietary lipid impact on growth, fatty acid homeostasis and metabolism

Partial or total substitution of fish oil by plant-derived oil does not generally impair salmonid growth performances if fish meal, which contains n-3 LC-PUFAs, is included in the diet (Francis et al., 2014). However, the substitution of both fish meal and fish oil generally

reduces salmonid growth performances (Panserat et al., 2009). In contrast, fish fed diets devoid of both fish meal and fish oil (LA- and ALA-enriched diets) during 4 weeks grew similarly to fish fed diets devoid of fish meal but containing EPA- or DHA-rich cod liver oil (EPA- and DHA-enriched diets) in the present experiment. Differences of weights between dietary treatments were only observed at the term of the second feeding period, i.e. after 6 weeks of feeding diets devoid of fish meal (all diets), spiked with small amounts of cod liver oil (all diets) and enriched in a specific PUFA (LA, ALA, EPA or DHA). Fish fed the DHA-enriched diet were the heaviest and fish fed the LA-enriched diet were the lightest. This effect was, however, only significant in presence of Cd, which suggests that Cd influences the impact that dietary fatty acids exert on fish growth. The negative impact of sunflower oil, as sole dietary lipid source, on rainbow trout growth has been previously reported (Mellery et al., 2017) and could be related to a deficiency in ALA and n-3 LC-PUFA (Mellery et al., 2017) and/or a decreased lipid digestibility of the feed (Turchini et al., 2018). This negative impact might have been accentuated by exposure to Cd since this metal was reported to decrease both lipid and protein apparent digestibility while increasing energy expenditure for maintenance and detoxification in fish (Berntssen and Lundebye, 2001; Pierron et al., 2007). This might explain why the differences of weights between fish fed the LA- and DHA-enriched diets were only significant under Cd exposure.

Dietary lipids are known to modulate the fatty acid composition of salmonid tissues, with the fatty acid profile of the tissue generally reflecting that of the diet (Bell et al., 2001; Fonseca-Madrigal et al., 2005; Francis et al., 2014; Petropoulos et al., 2009). In accordance, the liver of fish fed a diet rich in n-6 PUFAs was richer in n-6 PUFAs and the reverse was observed for fish fed a diet rich in n-3 PUFAs. The n-3/n-6 ratio of the liver followed the same decreasing order as in the diet: EPA > DHA > ALA > LA. However, the individual fatty acid composition of the liver did not exactly correspond to the one of the diet, highlighting both an active *in vivo* fatty acid metabolism and a selective retention of specific fatty acids in the liver. Rainbow trout, like other salmonids, have high fatty acid bioconversion ability and successfully synthetize AA from LA as well as EPA and DHA from ALA (Tocher, 2003). In accordance, AA concentration was highest in the liver of fish fed the LA-enriched diet while this fatty acid was absent from the diet. Also, a high level of 22:5n-6 was observed in the liver of fish fed the LA-enriched diet, especially in the phospholipids. This high bioconversion of LA into AA and 22:5n-6 and their selective inclusion in phospholipids are most probably a strategy for maintaining a minimum unsaturation level of biological membranes in absence or deficiency of dietary DHA. The DHA level gradually dropped in the liver of fish fed the LA-enriched diet, but proportionally less in the phospholipid and neutral lipid fractions than in the free fatty acid fraction. The selective retention of DHA in phospholipids emphases, once

more, the importance of DHA as a structural component of biological membranes (Tocher, 2003). Besides, the bioconversion ability of ALA into DHA was striking in fish fed the ALA-enriched diet. Indeed, while similar amounts of DHA were present in the LA- and ALA-enriched diets, the DHA level in the liver of fish fed the ALA-enriched diet was between 1.6 and 5.3 times higher than in the liver of fish fed the LA-enriched diet, throughout the experiment. Similarly, rainbow trout juveniles fed a ALA-enriched diet during 3 weeks contained 3 times more DHA in their hepatic phospholipids than fish fed a low ALA diet, despite similar levels of n-3 LC-PUFAs such as EPA, 22:5n-3 and DHA in the diet (Gregory et al., 2016). An increased substrate level combined to a decreased LC-PUFAs level are usually suggested to be associated to increased desaturase and elongase activities as well as gene expressions, leading to an increased biotransformation ability in fish fed vegetable oil as compared to fish oil (Fonseca-Madrigal et al., 2005; Gregory et al., 2016; Mellery et al., 2017; Thanuthong et al., 2011; Tocher, 2015). Particularly, dietary DHA was reported to strongly downregulate the hepatic *FADS2* and *ELOVL2* genes in juvenile rainbow trout while having no impact on the expression of the *ELOVL5* gene (Gregory et al., 2016). In accordance, the hepatic *FADS2* gene expression level was higher in fish fed the LA- and EPA-enriched diets, than in fish fed the DHA-enriched diet at day 70 while *ELOVL5* gene expression was not impacted by the diet throughout the present experiment. However, the hepatic *FADS2* gene expression was similar in fish fed the ALA- and DHA-enriched diets and the diet had no impact the hepatic *ELOVL2* gene expression in the present experiment.

4.2. Cd impact on fatty acid homeostasis and metabolism

4.2.1. Short-term effects

The influence of short-term Cd exposure on the fatty acid homeostasis and metabolism in fish has been poorly investigated to date. Data from the literature highlight variations of effects according to the level and duration of exposure, the model used (Ferain et al., 2018b; Konar et al., 2010; Koskinen et al., 2004; Pan et al., 2018), but also the PUFA supply and the lipid fraction analysed (Ferain et al., 2018b).

In the present experiment, three lipid fractions, with specific biological functions, were analysed, i.e. the phospholipids, neutral lipids and free fatty acids. Phospholipids are important constituents of cell membranes, neutral lipids mainly serve as energy storage and free fatty acids are the metabolically available form of lipids (Tocher, 2003). The cellular free fatty acid pool is fuelled by (i) membrane phospholipid break-down by phospholipase, (ii) neutral lipid mobilisation by lipase, (iii) *de novo* synthesis by FASN, (iv) fatty acid uptake from the diet and the blood stream (Tocher, 2003). The cellular free fatty acid pool provides free

fatty acids for (i) the phospholipid turnover through either *de novo* synthesis or transacylation of existing phospholipids, (ii) lipid deposition under the form of neutral lipids for energy storage, (iii) energy (ATP) generation by β-oxidation, (iv) synthesis of bioactive molecules (e.g. eicosanoids) (Tocher, 2003). These processes can be influenced by both nutritional and environmental factors. For instance, high Cd concentrations (11.2 mg/L, 24 h) significantly increased the incorporation of n-6 PUFAs (AA, 22:4n-6) in neutral lipids of rainbow trout liver cells grown in a medium supplemented with AA at the expense of other fatty acids (mainly SFAs) while no impact was observed in cells grown in media enriched in LA, ALA, or EPA (Ferain et al., 2018b). It was suggested that these n-6 PUFAs originated from the cellular free fatty acid pool (Ferain et al., 2018b). This lipid fraction was, however, not analysed in that study. In the present experiment, a much lower environmentally realistic Cd exposure level (0.3 µg/L, 24 h) decreased the levels of several free fatty acids in the liver of fish fed the ALA- and EPA-enriched diets, only.

In fish fed the ALA-enriched diet, the decrease in the total hepatic free fatty acid level induced by Cd (0.3 µg/L, 24 h) mainly concerned n-3 and n-6 PUFAs (LA, ALA, EPA, 22:5n-3) and was not balanced by an increase in these fatty acids neither in the neutral lipids nor in the phospholipids. Oxidative stress induced by Cd may have resulted in loss of unsaturated fatty acids, especially LC-PUFAs (EPA, 22:5n-3). Besides, in an attempt to maintain a constant hepatic LC-PUFA level under oxidative stress induced by Cd, the rate of bioconversion of shorter chain PUFAs might have been increased. The increase of 22:4n-6 level by Cd (24 h) observed in phospholipids of fish fed this diet might be an example of such a defence mechanism. The upregulation of the *FADS2* gene by Cd (0.3µg/L, 24 h) observed in the liver of fish fed the ALA-enriched diet further supports this hypothesis. Nevertheless, the expression of the genes coding for the elongation enzymes, *ELOVL2* and *ELOVL5,* were not affected by the short-term Cd exposure (0.3 µg/L, 24 h). An *in vitro* exposure to higher Cd concentrations (5.6 or 11.2 mg/L, 24 h) had no impact on *FADS2* or *ELOVL5* gene expression in RTL-W1 cells grown in a medium enriched in ALA (Ferain et al., 2018b). The drop in free fatty acids level may also result from an increased β-oxidation rate to meet the energy requirement to initiate detoxification responses. There was, however, no corresponding increase in the gene expression of *CPT1*. In accordance, exposure to higher Cd concentrations (5.6 or 11.2 mg/L, 24 h) had no impact on the *CPT1* gene expression in RTL-W1 cells grown in a medium enriched in ALA (Ferain et al., 2018b).

In fish fed the EPA-enriched diet, Cd (0.3 µg/L, 24 h) decreased the free 18:0, 20:1n-9 and DHA levels. This loss of free fatty acids went along with an increase of the total hepatic neutral lipid level, among which mainly SFAs (essentially 16:0) and PUFAs (essentially DHA), but not MUFA. This suggests that, in the liver of fish fed the EPA-enriched diet, Cd

(0.3 µg/L, 24 h) induced an increased lipid deposition in the form of neutral lipids for energy storage, coupled with an increased uptake or *de novo* synthesis of fatty acids as the net deposition of SFAs in neutral lipids exceeded its disappearance from the free fatty acid pool. On the other hand, MUFAs might have preferentially been used for β-oxidation and ATP generation, which would explain the disappearance of 20:1n-9 from the free fatty acid pool without any increase in the other lipid fractions. The potential impact of Cd (0.3 µg/L, 24 h) on the fatty acid uptake, *de novo* synthesis and β-oxidation, was however, not confirmed by the genetic analyses as the expressions of *FATP1, FASN* and *CPT1* genes did not change upon Cd exposure. Similarly, Cd (5.6 or 11.2 mg/L, 24 h) had no impact on *FATP1*, *FASN* and *CPT1* gene expressions in RTL-W1 cells grown in a medium enriched with EPA (Ferain et al., 2018b). Nevertheless, Cd (0.56 mg/L, 24 h) was reported to increase lipid deposition through stimulating fatty acid *de novo* synthesis (upregulation of FASN activity) and decreasing fatty acid lipolysis and catabolism (downregulation of the *CPT1* gene expression) in primary cultures of zebrafish hepatocytes (Pan et al., 2018). The high difference in Cd exposure levels between these *in vitro* studies and the present *in vivo* study may explain divergence of results.

In the present study, an exposure to Cd (0.3 µg/L, 24 h) induced a similar increase of the neutral lipid SFA level in the liver of fish fed the LA- and DHA-enriched diets as observed in fish fed the EPA-enriched diet, without, however, any impact on both the total hepatic neutral lipid level nor the levels of any free SFA, which contrasts with what we observed in fish fed the EPA-enriched diet. This suggests a further increased fatty acid uptake or *de novo* synthesis potentially coupled to a lower use of SFAs for energy provision in fish fed these two diets. These hypotheses were not confirmed by the genetic analyses. Impacts of short-term Cd exposure on fatty acid metabolism and homeostasis might have been induced at the post-translational level, trough, for instance, modulation of protein concentrations, conformations and/or activities.

4.2.2. Long-term effects

The impacts of short-term Cd exposure (0.3 µg/L, 24 h) on lipid homeostasis and metabolism were not maintained upon long-term Cd exposure (0.3 µg/L, 6 weeks), except for the upregulation of the hepatic *FADS2* gene expression in fish fed the ALA-enriched diet. In fish fed the EPA-enriched diet, Cd (0.3 µg/L, 6 weeks) downregulated *FADS2* gene expression. Such interaction between long-term Cd exposure and dietary lipids on *FADS2* gene expression, i.e. up- or down-regulation depending on the diet, has never been reported. Still, interactions between long-term Cd exposure (5.7 µg/L, 8 weeks) and water temperature on *FADS2* gene expression was reported in fathead minnow (*Pimephales promelas*), where Cd

respectively up- and down-regulated *FADS2* gene expression in the brain of fish reared at 25°C and in the muscle of fish reared at 30°C (Fadhlaoui et al., 2018). On the other hand, no impact on the expression of that gene was observed in the brain of fish reared at 15, 30°C or in the muscle of fish reared at 15, 25°C, which highlights the complexity of the interactions (Fadhlaoui et al., 2018).

Cd (0.3 µg/L, 6 weeks) upregulated the hepatic *ELOVL2* gene in fish fed the ALA-enriched diet only. This upregulation went, however, not along with a significant increase of LC-PUFAs. In contrast, downregulation of the *ELOVL2* gene by Cd (5.7 µg/L, 8 weeks) was observed in the brain of fathead minnows reared at 15°C, while no impact was observed in brain at 25 or 30°C or in muscle at 15°C, 25°C, 30°C (Fadhlaoui et al., 2018), highlighting once again the complexity of the interactions between Cd exposure and fatty acid metabolism in fish.

Cd (0.3 µg/L, 6 weeks) increased total phospholipid level in the liver of fish fed the ALA-enriched diets, only. This may reflect an increased *de novo* phospholipid synthesis to replace damaged lipids by oxidative attacks under Cd exposure. The phospholipid synthesis and turnover seem to be particularly efficient in fish fed this diet, as it resulted in a net increase of the total phospholipid level. Phospholipids are composed of two fatty acids, typically one SFA or MUFA at the *sn-1* position, and one LC-PUFA at the *sn-2* position (Tocher, 2003). Accordingly, the increase of the total hepatic phospholipids level in fish fed the ALA-enriched diet was accompanied by an increase of MUFAs. The phospholipid synthesis and turnover seem to be also activated by the long-term Cd exposure, but probably to a lesser extent, in fish fed the LA-enriched diet. Indeed, the phospholipid content in 18:0 and PUFA was increased by Cd in fish fed the LA-enriched diet, without, however, any impact on the total phospholipid level. Cd (0.3 µg/L, 6 weeks) induced a similar increase of phospholipid MUFA level in fish fed the DHA-enriched diet as observed in fish fed the ALA-enriched diet, but again without any impact on the total phospholipid level, due to a decrease in several n-3 PUFAs (ALA, 20:3n-3, 20:4n-3, EPA and 22:5n-3) in this fraction, possibly through oxidation. This resulted in a decrease in the unsaturation level of the hepatic phospholipids in fish fed the DHA-enriched diet, with possible consequences for membrane fluidity and permeability. Similarly, Cd (4 µg/L, 7 weeks) increased the phospholipid relative abundance of MUFAs while decreasing that of PUFAs in the muscle of yellow perch (*Perca flavescens*) reared at 9°C (Fadhlaoui and Couture, 2016). These effects were not observed at 28°C (Fadhlaoui and Couture, 2016), highlighting, again, the complexity of the interactions.

The long-term Cd exposure increased the hepatic free MUFA level, in fish fed the EPA-enriched diet. As this increase was not balanced by a decrease in any MUFA from neither

the phospholipid nor neutral lipid fraction, this suggests an increased MUFA uptake, and/or an increased *de novo* synthesis though both FASN and d9D. However, genetic analyses revealed no direct impact of Cd (0.3 µg/L, 6 weeks) on the hepatic *FATP1*, *FASN* and *d9D* gene expressions. Whether Cd modulated the concentration or activity of these proteins/enzymes cannot be concluded from the present experiment. Cd was, however, reported to increase hepatic FASN activity and/or gene expression in javelin goby (*Synechogobius hasta*) (Song et al., 2014), zebrafish (Pan et al., 2018) and yellow catfish (*Pelteobagrus fulvidraco*) (Chen et al., 2013).

4.3. Dietary lipid impact on biological responses to Cd

Body weight was not influenced by Cd exposure in the present experiment. In accordance to this result, other authors reported that Cd (3 µg/L, 30-40 days) did not impact rainbow trout growth (Hollis et al., 2001; Hollis et al., 1999; McGeer et al., 2000). In another study, however, Cd (1 and 3 µg/L, 30 days) decreased rainbow trout body weight and body weight gain (Heydarnejad et al., 2013). Differences of results may reflect differences in water chemistry, with a higher toxicity in softer water in the study of Heydarnejad et al. (2013). Furthermore, differences may be explained by the difference in contamination levels, with a 3- to 10-times lower Cd concentration in the present study. Still, interactions between diet and Cd were observed on fish growth in the present experiment. Indeed, the body weight of fish fed the EPA-enriched diet was lower than that of fish fed the DHA-enriched diet after 6 weeks of Cd exposure (0.3 µg/L), while no impact was observed in absence of Cd challenge. This result suggests a higher sensitivity to long-term exposure to low Cd concentration in fish fed the EPA-enriched diet. In contrast, EPA was reported to protect liver cells against Cd acute toxicity in rainbow trout (Ferain et al., 2018b; Ferain et al., 2016) and human (Linhartova et al., 2016; Linhartova and Sampels, 2015). EPA-derived cytoprotection was notably linked to increased resistance to the oxidative stress induced by Cd (Ferain et al., 2018b; Linhartova et al., 2016). Such protection can be energetically costly, which upon long-term exposure can result in reduced fish growth.

Cd bioaccumulates in fish liver in the first place (Chowdhury et al., 2003). In hepatocytes, Cd is sequestered by low molecular weight thiols (MT, glutathione), by calcium binding proteins or in metal rich granules (McGeer et al., 2011; Wang and Rainbow, 2006). These sequestrations decrease the free cellular Cd level and thus the metal toxicity. Upregulation of *MT* genes is generally observed under Cd exposure in fish *in vivo* (Hollis et al., 2001; Qiang et al., 2017) and *in vitro* (Chen et al., 2014; Ferain et al., 2018b; Walker et al., 2008). In the present experiment, the 24-h Cd exposure (0.3 µg/L) had no impact on the hepatic *MTb* gene expression, but upregulated the hepatic *MTa* gene expression in fish fed the ALA-enriched

diets only, while the Cd hepatic level was still below the quantification limit. This very early response of the hepatic *MTa* gene expression might be the consequence of a more efficient short-term uptake of Cd and/or a faster detoxification response in fish fed the ALA-enriched diet. Under prolonged exposure, Cd is transferred from the liver to the kidneys through the bloodstream either as a free ion or as a complex with MT, glutathione, cysteine or serum proteins (Chowdhury et al., 2003; McGeer et al., 2011). In the present experiment, we observed that the diet influenced long-term Cd hepatic bioaccumulation. The Cd hepatic level was indeed twice lower in fish fed the ALA-enriched diet than in fish fed the LA- and EPA-enriched diets, after 6 weeks of Cd exposure (0.3 µg/L). This lower Cd hepatic level might reflect a lower long-term uptake rate and/or a higher/earlier transfer of Cd to the kidneys. Mechanisms have not been confirmed but PUFAs are known to modulate the fluidity and permeability of the biological membranes, which can influence metal uptake/excretion processes (Arts and Kohler, 2009; Tillman and Cascio, 2003). For instance, n-3 PUFAs (EPA and DHA mixture) increased Cd intracellular burden in human hepatocellular cells (Hep G2 cell line) following 24-h metal exposure (0.56 mg/L) (Linhartova and Sampels, 2015). In contrast, enrichments in ALA, EPA, LA or AA had no significant impact on Cd accumulation in RTL-W1 cells (Ferain et al., 2018b).

Cd induces oxidative stress through both increasing ROS production and weakening antioxidant defences (McGeer et al., 2011). In addition to their role as metal scavengers, MTs also act as ROS scavengers (Klaassen et al., 1999). The early upregulation of the hepatic *MTa* gene observed in fish fed the ALA-enriched diet could have increased the total hepatic antioxidant capacity in these fish. In contrast, the decrease of the total hepatic glutathione level induced by Cd in fish fed the EPA- and DHA-enriched diets after 6 weeks of metal exposure (0.3 µg/L) could reflect a decreased total hepatic antioxidant capacity and thus higher susceptibility to oxidative stress in these fish. This decrease in total hepatic glutathione level observed in fish fed the EPA- and DHA-enriched diets might also result from impairment of the glutathione synthesis or from enhancement of glutathione degradation, as previously suggested (Đukić-Ćosić et al., 2020; Jamwal et al., 2016; Ramakrishnan et al., 2017). Cd is also known to directly increase the mitochondrial production of ROS by decreasing the activity of the complex III of the transport electron chain (Wang et al., 2004) while n-3 PUFAs have been shown to induce the opposite effect (Hagopian et al., 2010). The latter interaction was, however, not investigated in the present experiment.

Oxidative stress induced by Cd can damage lipids, proteins and DNA. Cd has been reported to impair DNA repair mechanisms, notably through interaction with the transcription factor p53 (Antoniali et al., 2015). In the present study, Cd had no impact on *p53* gene expression, as previously observed in RTLW1 cells exposed to higher Cd concentrations during 24 h

(Ferain et al., 2018b). This may suggest that Cd does not modulate *p53* gene expression in rainbow trout liver. Whether Cd influenced p53 conformation, stability or activity was not investigated in the present study.

Cd is known to impair calcium homeostasis in fish (McGeer et al., 2011). For example, Cd decreased hepatic calcium concentration in rainbow trout exposed to 2.6 µg/L Cd during 20 days (Zohouri et al., 2001). However, no impact was observed after 35 days of exposure (Zohouri et al., 2001). In the present experiment, Cd (0.3 µg/L, 24 h) increased calcium hepatic level in fish fed the LA-enriched diets, while having no impact on fish fed the other diets. This effect was not maintained upon long-term exposure (6 weeks), which could reflect an adaptation to the metal contamination. At the subcellular level, Cd has been reported to induce calcium mobilisation from the endoplasmic reticulum and nucleus to the cytosol, which can lead to apoptosis (Thévenod and Lee, 2013; Wang et al., 2014). In the present experiment, Cd had no impact on the expression of the *CALR* gene, which codes for a calcium-dependent chaperone located in the endoplasmic reticulum, as previously observed in RTL-W1 cells (Ferain et al., 2018b). Cd can also induce cell apoptosis through increasing ROS level as well as modulating the expression of anti- and pro-apoptotic members of the BCL2 family. In our study, Cd upregulated the expression of the hepatic *BCLx* gene, which codes for an anti-apoptotic member of the BCL2 family, in fish fed the ALA-enriched diet. A similar upregulation of the *BCLx* gene expression has been reported in primary culture of Atlantic Salmon (*Salmo salar*) hepatocytes after short-term (48 h) exposure to a high Cd concentration (11.2 mg/L), but not to a low Cd concentration (0.1 mg/L) (Olsvik et al., 2016). In contrast, Cd (0.56 and 11.2 mg/L, 24 h) downregulated the *BCLx* gene expression in RTL-W1 cells (Ferain et al., 2018b). The upregulation by Cd of *BCLx* gene expression in fish fed the ALA-enriched diet suggests, again, a higher detoxification process in fish fed this diet. However, whether this protective effect is balanced by a similar upregulation of pro-apoptotic members of the BCL2 family (e.g. Bax) has not been investigated in the present study.

Cd and PUFAs have been reported to modulate the expression of various *CYPs* involved in cellular defence (Chen et al., 2019; Madejczyk et al., 2015; Olsvik et al., 2016; Reynders et al., 2006). We previously showed that the expression of the orphan *CYP20A1* gene was increased by enrichment in EPA and decreased by Cd exposure in RTL-W1 cells enriched in EPA (Ferain et al., 2018b). However, neither the diet nor the Cd exposure had any impact on the *CYP20A1* gene expression in the present experiment.

5. CONCLUSION

The present study highlighted molecular interactions between dietary lipids and both short-term and long-term exposures to a low, environmentally realistic, Cd concentration (0.3

µg/L). On the one hand, both dietary lipids and Cd exposure highly influenced fatty acid homeostasis and metabolism. The hepatic fatty acid profile reflected to some extent that of the diet (e.g. n-3/n-6 ratio), though with some differences. Selective retention of specific PUFAs (ex: DHA) and an active biotransformation of dietary LA into AA and 22:5n-6 and of ALA into EPA and DHA were indeed observed. These bioconversions successfully maintained a minimum unsaturation level in the biological membranes. Cd effects were highly influenced by the duration of the exposure and the nutritional status of the fish. Overall, effects observed on the fatty acid profiles were not explained by variation of the expression pattern of the selected genes. On the other hand, the biological responses to Cd were influenced by dietary lipids. Fish fed the ALA- and EPA-enriched diets seemed to be the least and most affected by Cd exposure, respectively. Fish fed the ALA-enriched diet showed a higher detoxifying ability against Cd with an early upregulation of protective *MTa* and *BCLx* genes, an increased long-term phospholipid synthesis and turnover and fatty acid bioconversion efficiency, as well as a lower long-term accumulation of Cd in their liver. In contrast, the long-term Cd exposure impaired growth performance and decreased antioxidant capacity (glutathione level) in fish fed the EPA-enriched diet. All together the results coming from the present experiment highlight that Cd can affect biological response in fish at low, environmentally realistic contamination level and that these impacts are influenced by the dietary fatty acid composition. It emphasizes the need to take into account feed composition in ecotoxicological studies. Focus should be put in future studies on genomic, proteomic and metabolomic analyses to provide a deeper understanding of the molecular action of individual PUFA enrichments towards environmentally realistic Cd stress.

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TABLE CAPTIONS

Table 1: Composition (g/kg dry diet) of the four experimental diets formulated to be enriched in a specific PUFA (LA, ALA, EPA or DHA) for the two feeding phases (day 0-28 and day 29-70).

Table 2: Fatty acid composition (% total identified fatty acids) of the four experimental diets formulated to be enriched in a specific PUFA (LA, ALA, EPA or DHA) for the two feeding phases (day 0-28 and day 29-70).

Table 3: Sequence, amplicon size, annealing temperature and optimal concentration in master mix for the primers of the genes investigated.

Table 4: Juvenile rainbow trout growth and hepatosomatic index after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 or 0.3 µg/L) (day 29) and after 10 weeks of feeding and 6 weeks of Cd exposure (day 70).

FIGURE CAPTIONS

Figure 1: Roles of the proteins encoded by the genes investigated and main interactive results between Cd and dietary lipids observed in the liver of rainbow trout juveniles after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 or 0.3 µg/L) (day 29, short-term Cd exposure, in orange), and after 10 weeks of feeding and 6 weeks of Cd exposure (day 70, long-term Cd exposure, in blue). The illustration (adapted from Ferain et al., (2018a) has been done using the “cell biology” package from Servier Medical Art. Abbreviations used: Δ5, Δ5 desaturation; Δ6, Δ6 desaturation; Δ9, Δ9 desaturation; ALA, alpha-linolenic acid; BCLx, apoptosis regulator BCL2-Like1; CALR, calreticulin; Cd, cadmium; CPT1, carnitine palmitoyl transferase 1; cs, peroxisomal chain shortening; d9D, Δ9 desaturase; DHA, docosahexaenoic acid; E, elongation; ELOVL2, fatty acid elongase 2; ELOVL5, fatty acid elongase 5; EPA, eicosapentaenoic acid; FADS2, fatty acid desaturase 2; FASN, fatty acid synthase; FATP1, fatty acid transport protein 1; LA, linoleic acid; FA, fatty acid; MT, metallothionein; MUFA, monounsaturated fatty acid; p53, tumor protein 53; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; Total, total fatty acids identified in the fraction.

Figure 2: Levels of lipid classes, i.e. SFA (A), MUFA (B), n-3 PUFA (C), n-6 PUFA (D), PUFA (E), Total fatty acids (F) (µmol/g freeze dried liver) and n-3/n-6 ratio (G) in rainbow trout juvenile hepatic phospholipids, neutral lipids and free fatty acids after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 or 0.3 µg/L) (day 29) and after 10 weeks of feeding and 6 weeks of Cd exposure (day 70). Data are expressed as means ± standard error of mean (n=3). The dotted lines refer to the fatty acid class level at the start of the first feeding phase (day 0). For each sampling time and for each level of Cd exposure, letters indicate significant impact of the diet, with levels not connected to the same letter being significantly different from each other, ($p<0.05$). For each sampling time and for each diet, stars indicate significant impact of Cd exposure ($p<0.05$). Abbreviations used: ALA, alpha-linolenic acid; Cd, cadmium; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; Total, total fatty acids identified in the fraction.

Figure 3: Levels of individual n-6 PUFAs, i.e. 18:2n-6 (A), 20:2n-6 (B), 18:3n-6 (C), 20:3n-6 (D), 20:4n-6 (E), 22:4n-6 (F), 22:5n-6 (G), (µmol/g freeze dried liver) in rainbow trout juvenile hepatic phospholipids, neutral lipids and free fatty acids after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 or 0.3 µg/L) (day 29) and after 10 weeks of feeding and 6 weeks of Cd exposure (day 70). For more details, refer to the legend of Fig. 2. Abbreviations used: ALA, alpha-linolenic acid; Cd, cadmium; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acid.

Figure 4: Levels of individual n-3 PUFAs, i.e. 18:3n-3 (A), 20:3n-3 (B), 18:4n-3 (C), 20:4n-3 (D), 20:5n-3 (E), 22:5n-3 (F), 24:5n-3 (G), 24:6n-3 (H), 22:6n-3 (I), (µmol/g freeze dried liver) in rainbow trout juvenile hepatic phospholipids, neutral lipids and free fatty acids after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 or 0.3 µg/L) (day 29) and after 10 weeks of feeding and 6 weeks of Cd exposure (day 70). For more details, refer to the legend of Fig. 2. Abbreviations used: ALA, alpha-linolenic acid; Cd, cadmium; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acid.

Figure 5: Levels of individual MUFAs, i.e. 16:1n-7 (A), 18:1n-7 (B), 18:1n-9 (C), 20:1n-9 (D), 22:1n-9 (E), 24:1n-9 (F), (µmol/g freeze dried liver) in rainbow trout juvenile hepatic phospholipids, neutral lipids and free fatty acids after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 or 0.3 µg/L) (day 29) and after 10 weeks of feeding and 6 weeks of Cd exposure (day 70). For more details, refer

to the legend of Fig. 2. Abbreviations used: ALA, alpha-linolenic acid; Cd, cadmium; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Figure 6: Levels of individual SFAs, i.e. 12:0 (A), 14:0 (B), 16:0 (C), 18:0 (D), 20:0 (E), (µmol/g freeze dried liver) in rainbow trout juvenile hepatic phospholipids, neutral lipids and free fatty acids after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 or 0.3 µg/L) (day 29) and after 10 weeks of feeding and 6 weeks of Cd exposure (day 70). For more details, refer to the legend of Fig. 2. Abbreviations used: ALA, alpha-linolenic acid; Cd, cadmium; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Figure 7: Total hepatic glutathione level (A), hepatic calcium level (B), hepatic Cd level (C) in juvenile rainbow trout after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 µg/L in light grey or 0.3 µg/L in dark grey) (day 29) and after 10 weeks of feeding and 6 weeks of Cd exposure (day 70). Data are expressed as means ± standard error of mean (n=3). For each sampling time and for each diet, stars indicate significant impact of Cd exposure ($p<0.05$). For each sampling time and for each level of Cd exposure, letters indicate significant impact of the diet, with levels not connected to the same letter being significantly different from each other ($p<0.05$). Abbreviations used: ALA, alpha-linolenic acid; Cd, cadmium, DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GSH, reduced glutathione; LA, linoleic acid; PUFA, polyunsaturated fatty acid

Figure 8: Relative expression of genes involved in fatty acid metabolism or in stress response in juvenile rainbow trout liver after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 or 0.3 µg/L) (day 29) and/or after 10 weeks of feeding and 6 weeks of Cd exposure (day 70). Data are expressed as means ± standard error of mean (n=3). For each sampling time and for each level of Cd exposure, letters indicate significant impact of the diet, with levels not connected to the same letter being significantly different from each other ($p<0.05$). For each sampling time and for each diet, stars indicate significant impact of Cd exposure ($p<0.05$). Abbreviations used: ALA, alpha-linolenic acid; BCLx, apoptosis regulator BCL2-Like1; Cd, cadmium; d9D, Δ9 desaturase; DHA, docosahexaenoic acid; ELOVL2, fatty acid elongase 2; EPA, eicosapentaenoic acid; FADS2, fatty acid desaturase 2; LA, linoleic acid; MTa, metallothionein a; PUFA, polyunsaturated fatty acid.

SUPPLEMENTARY MATERIAL - FIGURE CAPTIONS

Figure S1: Experimental design. Abbreviations used: ALA, alpha-linolenic acid; Cd, cadmium, DHA, docosahexaenoic acid, EPA, eicosapentaenoic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acid.

Figure S2: Relative expression of genes involved in fatty acid metabolism in juvenile rainbow trout liver after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 or 0.3 µg/L) (day 29) and/or after 10 weeks of feeding and 6 weeks of Cd exposure (day 70). Data are expressed as means ± standard error of mean (n=3). Abbreviations used: ALA, alpha-linolenic acid; Cd, cadmium; CPT1, carnitine palmitoyl transferase 1; d9D, Δ9 desaturase; DHA, docosahexaenoic acid; ELOVL2, fatty acid elongase 2; ELOVL5, fatty acid elongase 5; EPA, eicosapentaenoic acid; FASN, fatty acid synthase; FATP1, fatty acid transport protein 1; LA, linoleic acid; PUFA, polyunsaturated fatty acid.

Figure S3: Relative expression of genes involved in stress response and of CYP20A1 gene in juvenile rainbow trout liver after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 or 0.3 µg/L) (day 29) and/or after 10 weeks of feeding and 6 weeks of Cd exposure (day 70). Data are expressed as means ± standard error of mean (n=3). Abbreviations used: ALA, alpha-linolenic acid; CALR, calreticulin; Cd, cadmium; CYP20A1, cytochrome P450 20A1; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MTb, metallothionein b; p53, tumor protein 53; PUFA, polyunsaturated fatty acid.

Figure 1

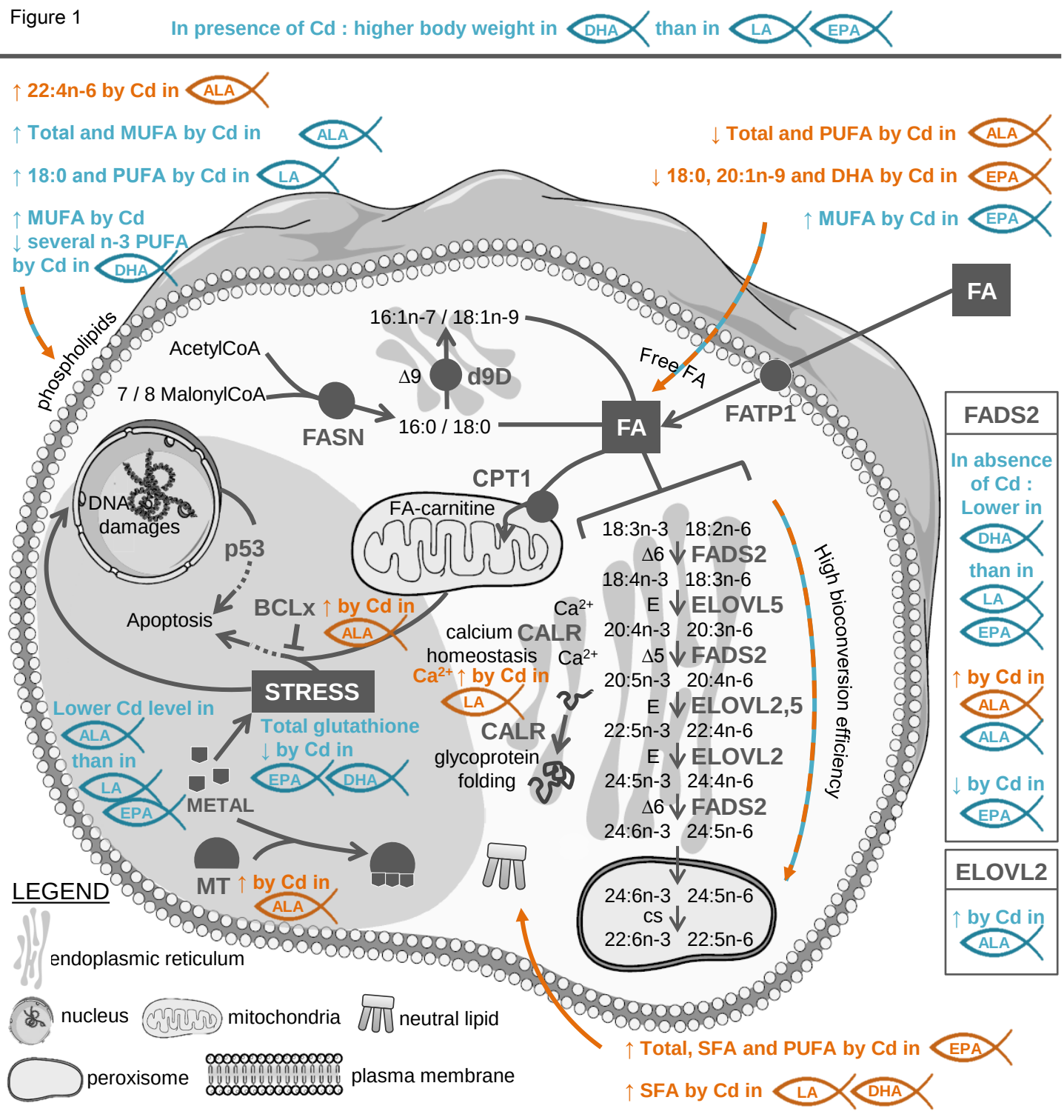
In presence of Cd : higher body weight in DHA than in LA EPA
↑ 22:4n-6 by Cd in ALA
↑ Total and MUFA by Cd in ALA
↑ 18:0 and PUFA by Cd in LA
↑ MUFA by Cd
↓ several n-3 PUFA
by Cd in DHA
↓ Total and PUFA by Cd in ALA
↓ 18:0, 20:1n-9 and DHA by Cd in EPA
↑ MUFA by Cd in EPA
FA
phospholipids
16:1n-7 / 18:1n-9
AcetylCoA
7 / 8 MalonylCoA
Δ9
d9D
FASN
16:0 / 18:0
FA
Free FA
FATP1
CPT1
FA-carnitine
DNA damages
p53
Apoptosis
BCLx ↑ by Cd in ALA
STRESS
Lower Cd level in ALA than in LA EPA
Total glutathione ↓ by Cd in EPA DHA
METAL
MT ↑ by Cd in ALA
Ca2+
calcium homeostasis
CALR
Ca2+ ↑ by Cd in LA
CALR
glycoprotein folding
18:3n-3 18:2n-6
Δ6 ↓ FADS2
18:4n-3 18:3n-6
E ↓ ELOVL5
20:4n-3 20:3n-6
Δ5 ↓ FADS2
20:5n-3 20:4n-6
E ↓ ELOVL2,5
22:5n-3 22:4n-6
E ↓ ELOVL2
24:5n-3 24:4n-6
Δ6 ↓ FADS2
24:6n-3 24:5n-6
24:6n-3 24:5n-6
cs ↓
22:6n-3 22:5n-6
High bioconversion efficiency
FADS2
In absence of Cd : Lower in DHA than in LA EPA
↑ by Cd in ALA ALA
↓ by Cd in EPA
ELOVL2
↑ by Cd in ALA
LEGEND
endoplasmic reticulum
nucleus
mitochondria
neutral lipid
peroxisome
plasma membrane
↑ Total, SFA and PUFA by Cd in EPA
↑ SFA by Cd in LA DHA

Figure 2

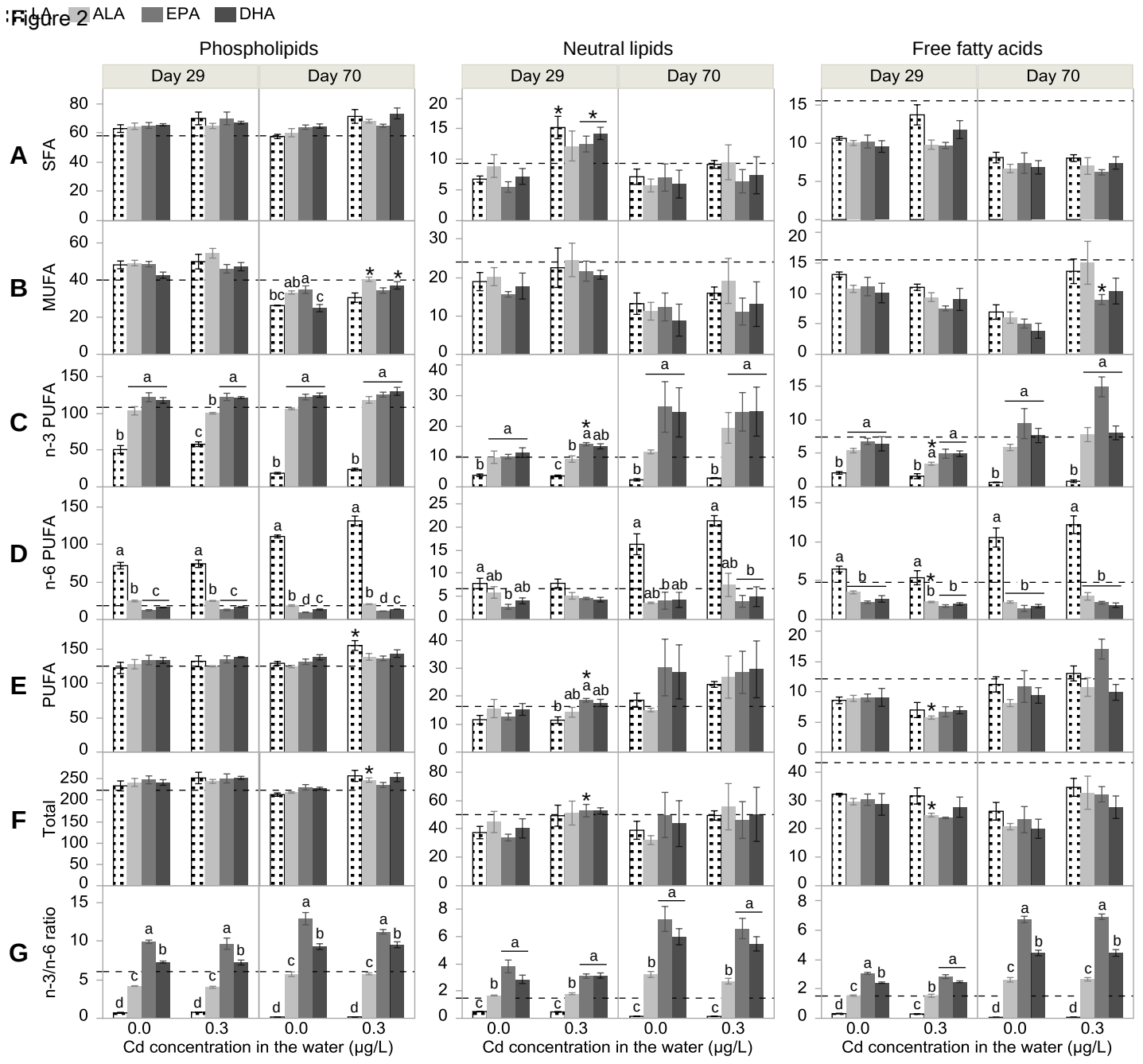
LA
ALA
EPA
DHA
Phospholipids
Neutral lipids
Free fatty acids
Day 29
Day 70
A
SFA
B
MUFA
C
n-3 PUFA
D
n-6 PUFA
E
PUFA
F
Total
G
n-3/n-6 ratio
0.0
0.3
Cd concentration in the water (µg/L)

Figure 3

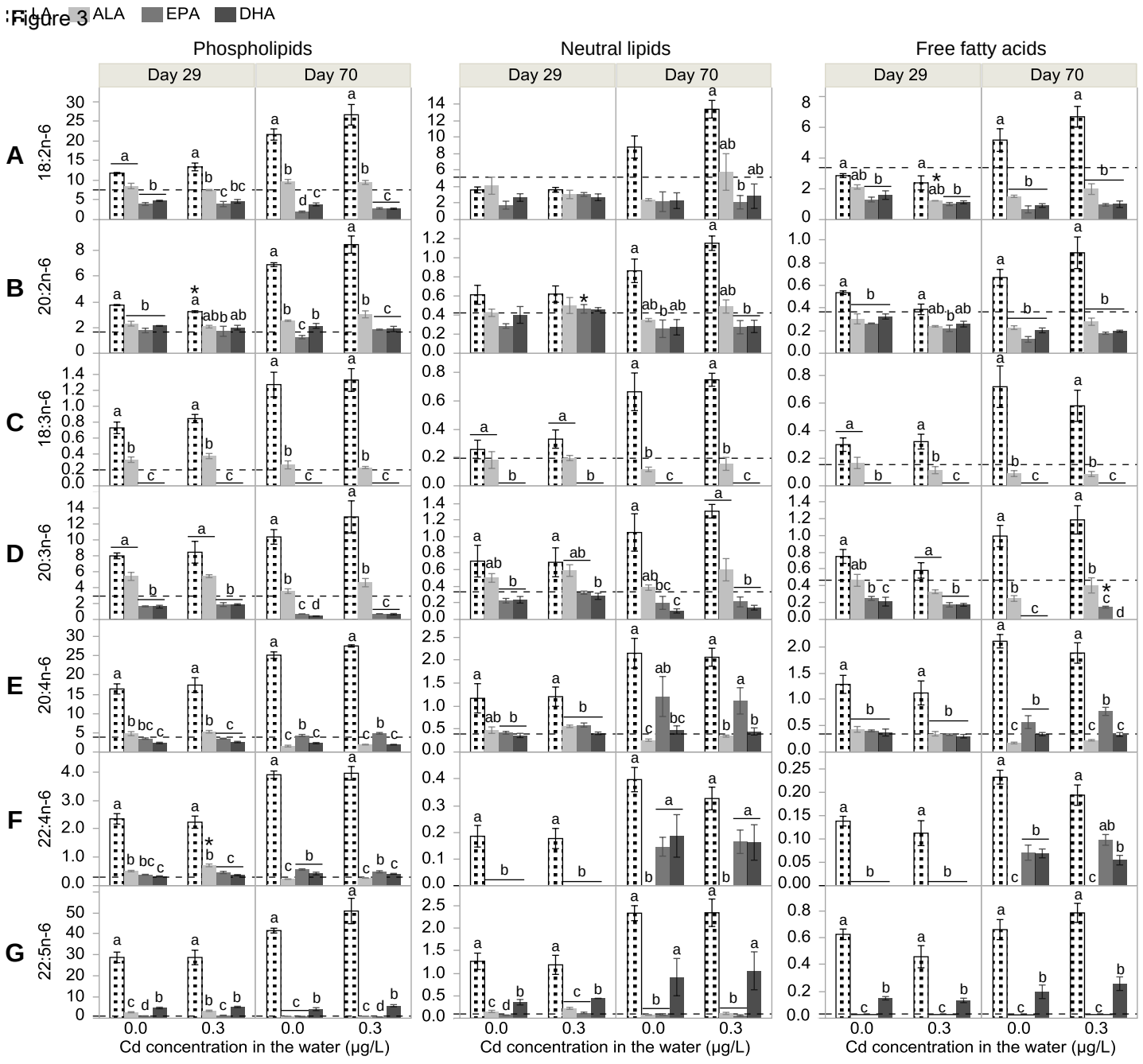
LA
ALA
EPA
DHA
Phospholipids
Neutral lipids
Free fatty acids
Day 29
Day 70
A
B
C
D
E
F
G
18:2n-6
20:2n-6
18:3n-6
20:3n-6
20:4n-6
22:4n-6
22:5n-6
Cd concentration in the water (µg/L)
0.0
0.3

Figure 4

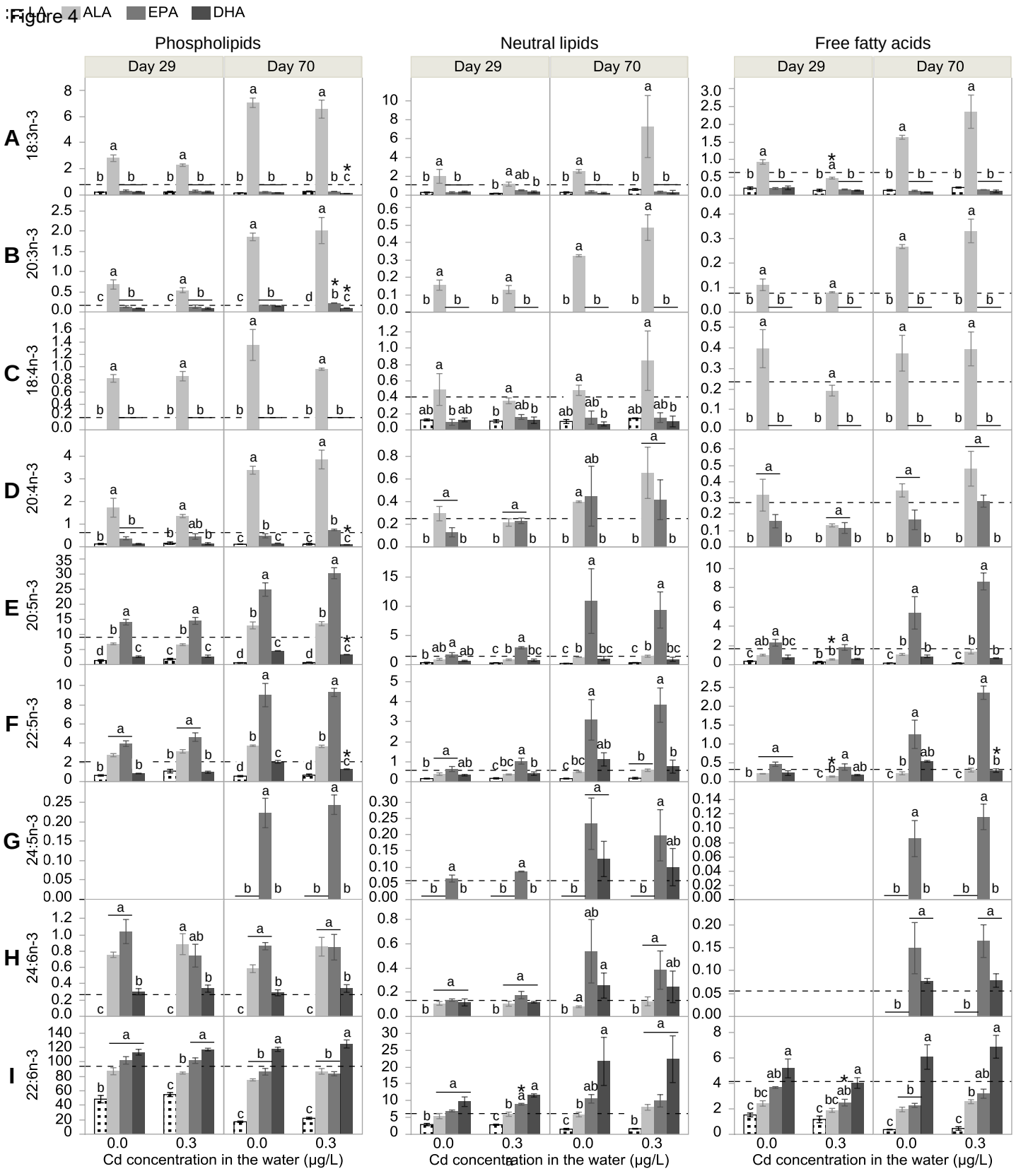
LA
ALA
EPA
DHA
Phospholipids
Neutral lipids
Free fatty acids
Day 29
Day 70
A 18:3n-3
B 20:3n-3
C 18:4n-3
D 20:4n-3
E 20:5n-3
F 22:5n-3
G 24:5n-3
H 24:6n-3
I 22:6n-3
0.0
0.3
Cd concentration in the water (µg/L)

Figure 5

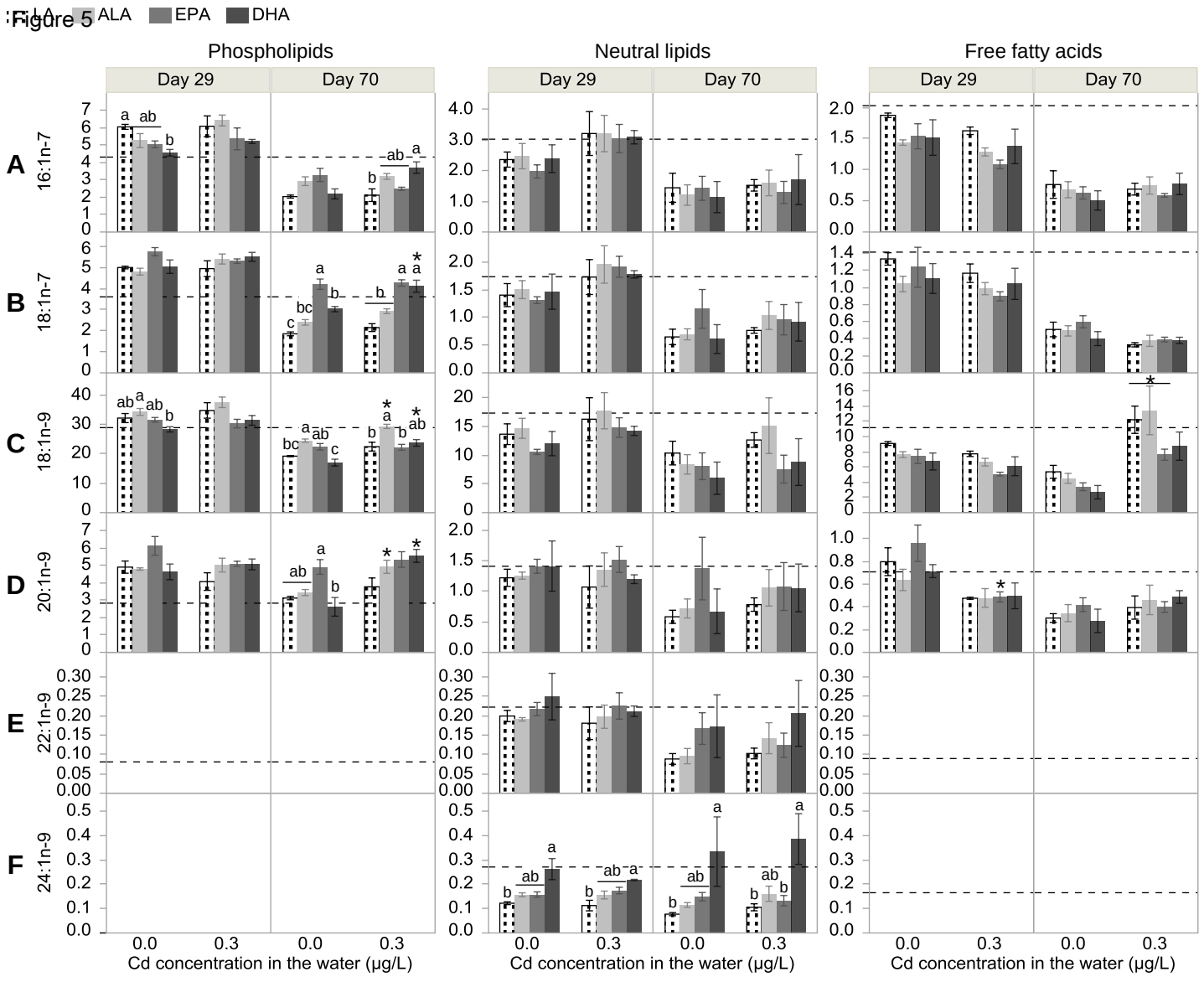
LA
ALA
EPA
DHA
Phospholipids
Neutral lipids
Free fatty acids
Day 29
Day 70
A
16:1n-7
B
18:1n-7
C
18:1n-9
D
20:1n-9
E
22:1n-9
F
24:1n-9
Cd concentration in the water (µg/L)
0.0
0.3

Figure 6

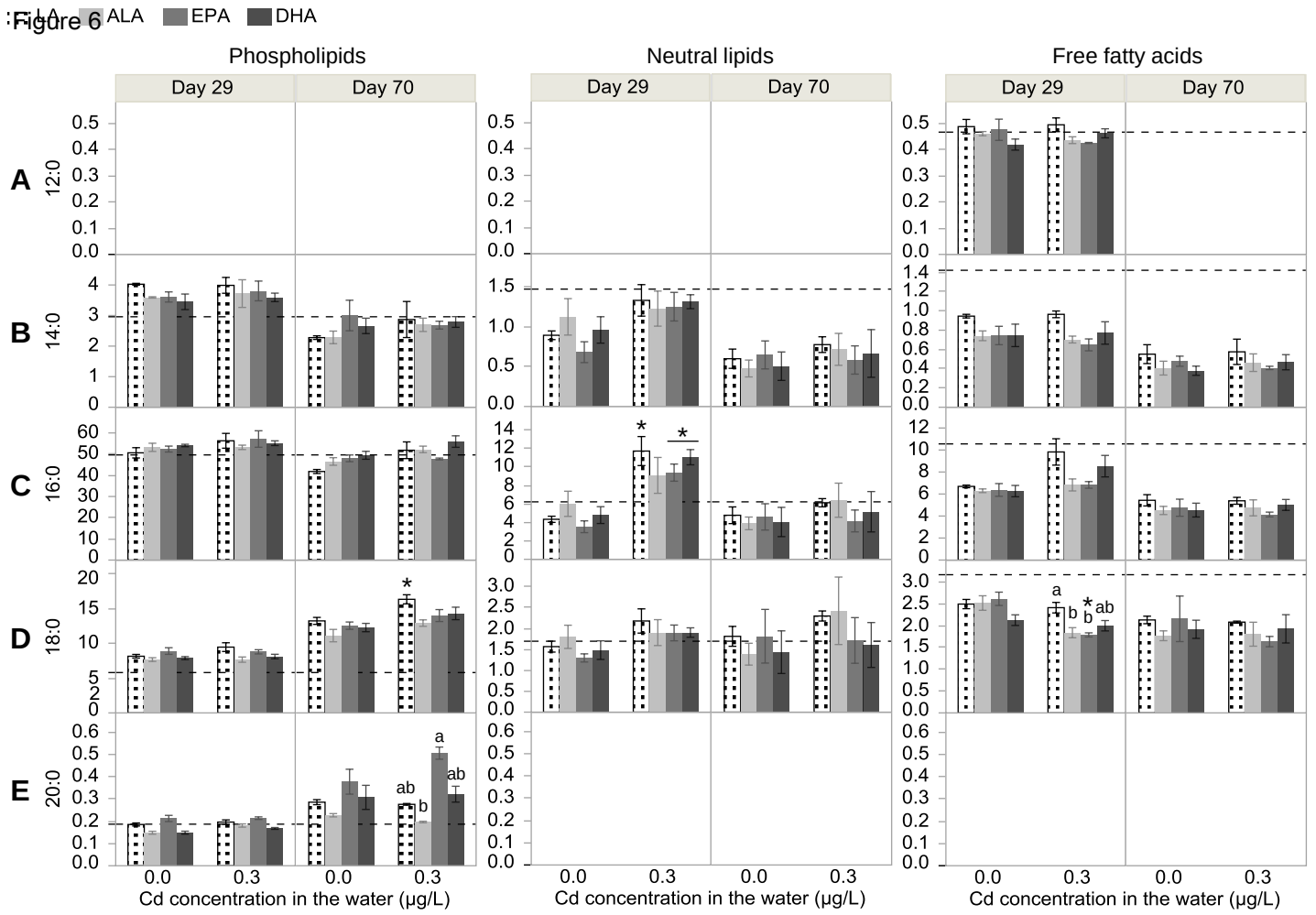
LA
ALA
EPA
DHA
Phospholipids
Neutral lipids
Free fatty acids
Day 29
Day 70
A
B
C
D
E
12:0
14:0
16:0
18:0
20:0
Cd concentration in the water (µg/L)

Figure 7

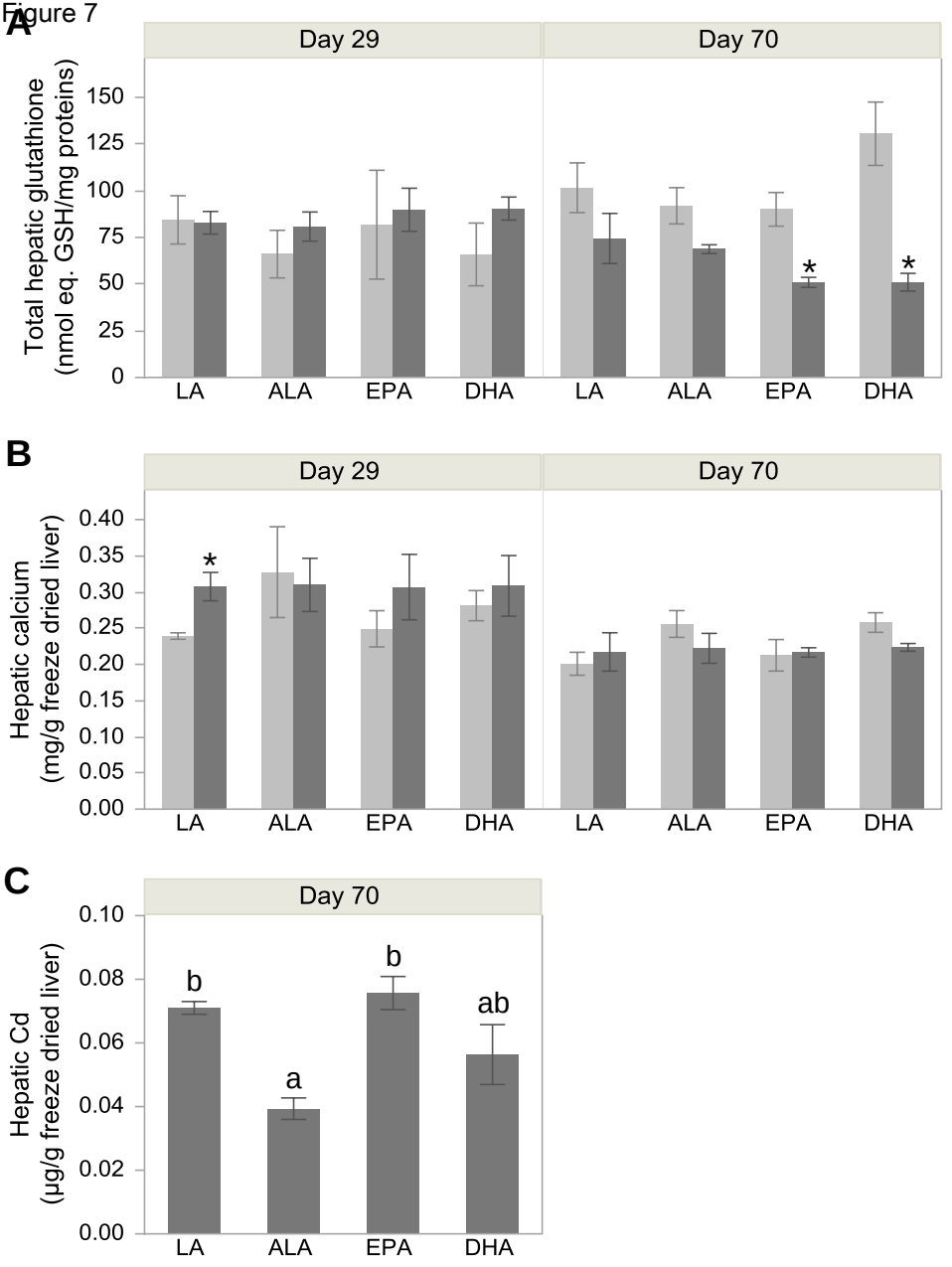
A
Day 29
Day 70
Total hepatic glutathione (nmol eq. GSH/mg proteins)
150
125
100
75
50
25
0
*
*
LA
ALA
EPA
DHA
LA
ALA
EPA
DHA
B
Day 29
Day 70
Hepatic calcium (mg/g freeze dried liver)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
LA
ALA
EPA
DHA
LA
ALA
EPA
DHA
C
Day 70
Hepatic Cd (µg/g freeze dried liver)
0.10
0.08
0.06
0.04
0.02
0.00
b
a
b
ab
LA
ALA
EPA
DHA

Figure 8

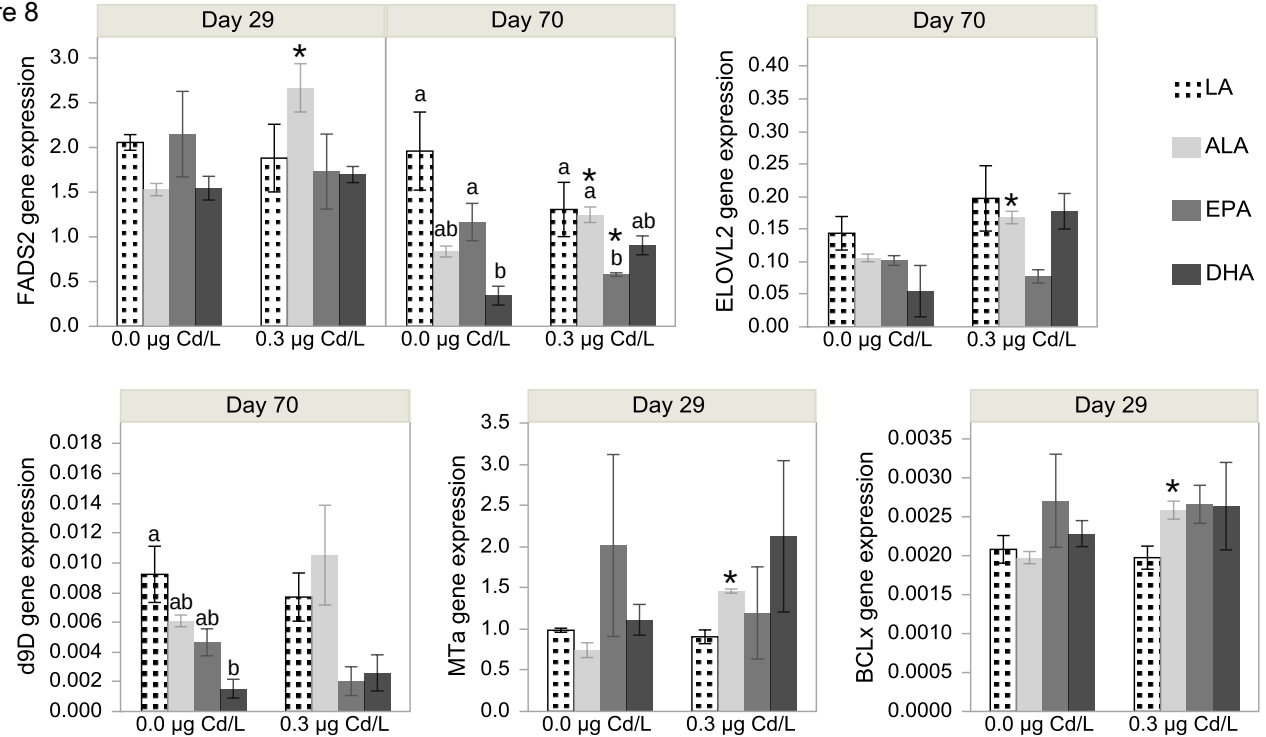
Day 29
Day 70
FADS2 gene expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
a
ab
a
b
a
*a
*b
ab
0.0 µg Cd/L
0.3 µg Cd/L
0.0 µg Cd/L
0.3 µg Cd/L
Day 70
ELOVL2 gene expression
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
0.0 µg Cd/L
0.3 µg Cd/L
LA
ALA
EPA
DHA
Day 70
d9D gene expression
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
a
ab
ab
b
0.0 µg Cd/L
0.3 µg Cd/L
Day 29
MTa gene expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
0.0 µg Cd/L
0.3 µg Cd/L
Day 29
BCLx gene expression
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
*
0.0 µg Cd/L
0.3 µg Cd/L

Table 1

Component	Day 0-28				Day 29-70			
	LA	ALA	EPA	DHA	LA	ALA	EPA	DHA
Casein	304.6	304.6	304.6	304.6	276.8	276.8	276.8	276.8
Gelatin	55.0	55.0	55.0	55.0	50.0	50.0	50.0	50.0
Wheat gluten	245.4	245.4	245.4	245.4	223.0	223.0	223.0	223.0
Dextrin	110.0	110.0	110.0	110.0	100.0	100.0	100.0	100.0
Sucrose	55.0	55.0	55.0	55.0	50.0	50.0	50.0	50.0
Cellulose	88.0	88.0	88.0	88.0	80.0	80.0	80.0	80.0
Carboxymethylcellulose	33.0	33.0	33.0	33.0	30.0	30.0	30.0	30.0
Mineral premix[a]	69.8	69.8	69.8	69.8	63.4	63.4	63.4	63.4
Hydrosoluble vitamin premix[b]	6.3	6.3	6.3	6.3	5.7	5.7	5.7	5.7
Liposoluble vitamin premix[c]	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1
Cod liver oil	0.0	0.0	0.0	0.0	2.4	2.4	2.4	2.4
Sunflower oil	**31.7**	0.0	0.0	0.0	**117.6**	0.0	0.0	0.0
Linseed oil	0.0	**31.7**	0.0	0.0	0.0	**117.6**	0.0	0.0
EPA-rich cod liver oil	0.0	0.0	**31.7**	0.0	0.0	0.0	**117.6**	0.0
DHA-rich cod liver oil	0.0	0.0	0.0	**31.7**	0.0	0.0	0.0	**117.6**

[a] Mineral premix (g/kg premix): $CaHPO_4$ 239.41 , $Ca(H_2PO_4)_2.H_2O$ 222.33 , $NaHCO_3$ 96.82 , $Na_2SeO_3.5H_2O$ 0.01, KCl 102.45, NaCl 176.63, KI 0.20, $MgCl_2$ 65.26, $MgSO_4.7H_2O$ 71.95, $MnSO_4.H_2O$ 1.55, $FeSO_4.7H_2O$ 12.72, $CuSO_4.5H_2O$ 0.41, $ZnSO_4.7H_2O$ 10.25; [b] Hydrosoluble vitamin premix (g/kg premix): ascorbic acid (Vit C) 56.0, thiamin (Vit B1) 6.27, riboflavin (Vit B2) 13.44, pyridoxine (Vit B6) 5.04, calcium pantothenate (Vit B5) 15.79, P-aminobenzoic acid (Vit H1) 44.79, cyanocobalamin (Vit B12) 0.03, niacin (Vit B3) 33.59, biotin (Vit H) 0.11, choline chloride 391.94, folic acid (Vit M) 1.68, inositol 55.99, canthaxanthin (E161g) 11.20, alpha-cellulose 364.01; [c] Liposoluble vitamin premix (g/kg premix): retinyl acetate (Vit A) 0.63, cholecalciferol (Vit D3) 0.16, menadione (Vit K3) 3.59, butylated hydroxyanisole 2.45, butylated hydroxytoluene 2.45, tocopherol acetate (Vit E) 10.46/21.01/10.29/9.00 in LA-/ALA-/EPA-/DHA-enriched diets. The differences in VitE levels in the premix compensated the different VitE levels in the specific oils used in each diet (sunflower oil, linseed oil, EPA-rich cod liver oil and DHA-rich cod liver oil).

Abbreviations used: ALA, alpha-linolenic acid; DHA, docosahexaenoic acid, EPA, eicosapentaenoic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acid

Table 3

Gene	Sequence 5'→3'	Size (bp)	Annealing T (°C)	Concentration (nmol L^{-1})	Reference
Actin *Housekeeping gene 1*	F: AAGACAGCTACGTGGGAGAC R: CAGCTCGTTGTAGAAGGTGTG	135	60	200	*Ferain et al. 2018a*
18S *Housekeeping gene 2*	F: CACGCGAGATGGAGCAATAA R: CGCAGAGTAGACACACGCTGAT	96	60	200	*Gagné et al. 2012*
FASN *fatty acid synthase*	F: TGATCTGAAGGCCCGTGTCA R: GGGTGACGTTGCCGTGGTAT	162	60	200	*Kamalam et al. 2013*
FATP1 *fatty acid transport protein 1*	F: CGAATGCAACTGTAGCATCG R: CTCYGTGGTCTCCTCATCC	122	60	300	*Ferain et al. 2018a*
CPT1 *carnitine palmitoyl transferase 1a*	F: CTCCTGGAAGAAGAGGTTCATACG R: ACTCACCACCACCACCAAAAAC	95	60	100	*Ferain et al. 2018a*
d9D *Δ9 desaturase*	F : GCCGTCCGAGGGTTCTTCTT R : CTCTCCCCACAGGCACCAAG	204	62	300	*Kamalam et al. 2013*
FADS2 *fatty acid desaturase 2*	F: AGGGTGCCTCTGCTAACTGG R: TGGTGTTGGTGATGGTAGGG	175	60	200	*Kamalam et al. 2013*
ELOVL2 *fatty acid elongase 2*	F: CCAGATCACCTTCCTGCAC R: GACAGGCCATAGTAGGAGTAC	151	60	200	*Ferain et al. 2018a*
ELOVL5 *fatty acid elongase 5*	F: CCAAGTACATGAGACACAGAC R: CACACAGCAGACACCATCTC	115	60	100	*Ferain et al. 2018a*
MTa *metallothionein a*	F: CATGCACCAGTTGTAAGAAAGCA R: GCAGCCTGAGGCACACTTG	75	60	300	*Ceyhun et al. 2011*
MTb *metallothionein b*	F: ATCCTGCAAGTGCTCAAACT R: ATGACTGCATTGTCACGGTA	139	60	300	*Ceyhun et al. 2011*
p53 *tumor protein 53*	F: GACAACCCTGGAGACCAAGA R: TCTCATCGTCACTCACAGCA	132	60	100	*Hecht et al. 2014*
BCLx *apoptosis regulator BCL2-Like1*	F: GGACTCAGTGGATGAGTTTGAGC R: GAACACTTCGTCCATCACACTCTC	122	60	200	*Ferain et al. 2018a*
CALR *calreticulin*	F: GTTCGGTCCAGACATCTGTG R: GTGTACTCATCATCCTTGC	115	60	300	*Ferain et al. 2018b*
CYP20A1 *cytochrome p450 20A1*	F: GCTGTCACTCAACTCGCTCTG R: CTGATGGAGCTCTTCTCCATG	137	60	300	*Ferain et al 2018a*

Transcript sequences used for primer design were gleaned from the litterature (see *"Reference"* column). Abbreviations used: bp, base pairs ; F, forward; R, reverse; T, temperature

Table 2

Fatty acid	Day 0-28				Day 29-70			
	LA	ALA	EPA	DHA	LA	ALA	EPA	DHA
14:0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1
15:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16:0	6.5	6.0	0.2	1.7	6.6	6.1	0.5	1.9
18:0	3.2	4.2	3.9	0.7	3.2	4.1	3.8	0.7
22:0	0.5	0.0	0.0	0.6	0.5	0.0	0.0	0.6
24:0	0.0	0.0	0.6	0.4	0.0	0.0	0.6	0.4
Total SFA	**10.1**	**10.2**	**4.7**	**3.3**	**10.3**	**10.4**	**4.9**	**3.7**
14:1n-5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16:1n-7	0.0	0.0	0.0	0.6	0.2	0.2	0.2	0.8
18:1n-7	1.0	0.9	2.0	0.3	1.1	0.9	2.1	0.4
18:1n-9	**26.3**	**18.5**	4.4	1.6	**26.1**	**18.4**	4.6	1.9
20:1n-9	0.0	0.0	2.3	0.3	0.2	0.2	2.5	0.5
22:1n-9	0.0	0.0	0.0	0.5	0.2	0.2	0.2	0.8
24:1n-9	0.0	0.0	0.0	1.2	0.0	0.0	0.0	1.2
Total MUFA	**27.3**	**19.4**	**8.7**	**4.6**	**27.8**	**20.0**	**9.6**	**5.5**
LA 18:2n-6	**62.6**	14.4	0.6	0.5	**61.4**	14.2	0.6	0.5
20:2n-6	0.0	0.0	0.4	0.0	0.0	0.0	0.4	0.0
20:3n-6	0.0	0.0	0.5	0.0	0.0	0.0	0.5	0.0
20:4n-6	0.0	0.0	3.8	0.7	0.0	0.0	3.7	0.7
22:4n-6	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.6
22:5n-6	0.0	0.0	0.0	4.8	0.0	0.0	0.0	4.8
Total n-6 PUFA	**62.6**	**14.4**	**5.3**	**6.6**	**61.4**	**14.2**	**5.3**	**6.5**
ALA 18:3n-3	0.0	**56.0**	0.6	0.1	0.0	**54.9**	0.6	0.2
18:4n-3	0.0	0.0	0.9	0.1	0.0	0.0	0.9	0.2
20:3n-3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20:4n-3	0.0	0.0	2.2	0.2	0.0	0.0	2.2	0.2
EPA 20:5n-3	0.0	0.0	**77.6**	2.0	0.2	0.2	**76.3**	2.1
22:5n-3	0.0	0.0	0.0	3.3	0.0	0.0	0.0	3.2
DHA 22:6n-3	0.0	0.0	0.0	**79.5**	0.2	0.2	0.2	**78.1**
24:5n-3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24:6n-3	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.2
Total n-3 PUFA	**0.0**	**56.0**	**81.3**	**85.5**	**0.5**	**55.4**	**80.2**	**84.3**
Total PUFA	**62.6**	**70.4**	**86.6**	**92.1**	**61.9**	**69.6**	**85.5**	**90.8**
n-3/n-6 ratio	**0.0**	**3.9**	**15.3**	**12.9**	**0.0**	**3.9**	**15.2**	**12.9**

Abbreviations used: ALA, alpha-linolenic acid; DHA, docosahexaenoic acid, EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 4

Parameter	LA		ALA		EPA		DHA	
	0.0 µg Cd/L	0.3 µg Cd/L	0.0 µg Cd/L	0.3 µg Cd/L	0.0 µg Cd/L	0.3 µg Cd/L	0.0 µg Cd/L	0.3 µg Cd/L
Weight (g/fish)								
Day 29	28.31 ± 0.15	27.76 ± 0.58	28.67 ± 0.42	28.95 ± 0.28	29.29 ± 0.26	28.38 ± 0.35	29.86 ± 0.70	29.73 ± 0.43
Day 70	72.98 ± 0.89	73.12 ± 1.06 [a]	76.34 ± 2.45	77.90 ± 0.42 [ab]	78.69 ± 2.30	74.98 ± 0.88 [a]	82.28 ± 3.25	83.39 ± 2.07 [b]
Hepatosomatic index								
Day 29	1.76 ± 0.02	2.02 ± 0.15	1.65 ± 0.18	1.68 ± 0.06	1.60 ± 0.07	1.56 ± 0.12	1.48 ± 0.02	1.47 ± 0.10
Day 70	1.55 ± 0.09	1.36 ± 0.06	1.47 ± 0.03	1.47 ± 0.09	1.59 ± 0.13	1.20 ± 0.05	1.56 ± 0.08	1.34 ± 0.14

Data are expressed as means ± standard error of mean (n=3). For each level of Cd exposure (0.0 or 0.3 µg/L), letters indicate significant impact of the diet, with levels not connected to the same letter being significantly different from each other ($p<0.05$). Abbreviations used: ALA, alpha-linolenic acid; Cd, cadmium; DHA, docosahexaenoic acid, EPA, eicosapentaenoic acid; LA, linoleic acid.

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CONFLICT OF INTERESTS: NONE

All the authors declare that no competing interests exist: i.e no financial or personal relationship with other people or organizations that could have inappropriately influenced our work.

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Yvan Larondelle: Conceptualization ; Methodology ; Resources ; Writing - Review & Editing ; Supervision ; Project administration ; Funding acquisition

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Cathy Debier: Conceptualization ; Methodology ; Resources ; Writing - Original Draft ; Writing - Review & Editing ; Supervision ; Project administration ; Funding acquisition