Interplay between dietary lipids and cadmium exposure in aquatic biota: a multi-model study

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Every interaction is an opportunity to learn

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Qu'est-ce que tu dirais, toi, si t'étais là ?

Summary

The aim of this work was to investigate if and how (i) dietary lipids and Cd affect fatty acid homeostasis and metabolism, (ii) the individual/tissue/cell fatty acid profile modulates the Cd sensitivity, in rainbow trout (*Oncorhynchus mykiss*) and the large water flea (*Daphnia magna*), using both *in vitro* and *in vivo* approaches.

We successfully modulated the fatty acid profile of the experimental models. Overall, both the fatty acid supply and the Cd exposure influenced fatty acid homeostasis and metabolism. The fatty acid profile influenced individual/tissue and cell sensitivity to Cd.

In vitro enrichments in specific polyunsaturated fatty acids (PUFA) (e.g. alpha-linolenic acid (ALA, 18:3n-3) and eicosapentaenoic acid (EPA, 20:5n-3)) conferred protection against Cd while enrichment in others (e.g. linoleic acid (LA, 18:2n-6)) had no impact. PUFA-derived cytoprotection was not linked to a reduced cell Cd burden but would rather occur through a reduction of the oxidative stress induced by Cd and a differential induction of the eicosanoid cascade.

In vivo, rainbow trout fed the ALA- and EPA-enriched diets seemed to be respectively the least and the most affected by Cd. Fish fed the ALA-enriched diet had a higher detoxifying ability against Cd and a lower hepatic Cd burden. In contrast, fish fed the EPA-enriched diet had impaired growth performances and a decreased total hepatic antioxidant capacity under Cd exposure.

In vivo, liposome feeding increased adult daphnid tolerance to Cd. The presence of EPA in liposomes did not increase adult tolerance to Cd. Offspring's tolerance to Cd was influenced by the brood number and the maternal diet. It was positively correlated with the PUFA level in body neutral lipids, especially ALA.

Overall, this work provides new information about interactions between the nature of dietary lipids and the biological responses to Cd in aquatic biota.

List of abbreviations

5-CFDA,AM	5-carboxyfluorescein diacetate, acetoxymethyl ester
α	Type I error
AA	Arachidonic acid
ALA	Alpha-linolenic acid
ALOX5	Arachidonate 5-lipoxygenase
ALOX5	Arachidonate 5-lipoxygenase gene
AS	Atlantic salmon cell line
ASK	Atlantic salmon kidney cell line
ATGL	Adipose triglyceride lipase
B35	Rat B35-neuronal cell line
BCA	Bicinchoninic acid
BCLx	Apoptosis regulator BCL2-Like1 gene
BP	Base pairs
BSA	Bovine serum albumin
CAC	Carnitine-acylcarnitine
CALR	Calreticulin
CALR	Calreticulin gene
C6	Rat C6-glial cell line
Cd	Cadmium
CD36	Cluster of differentiation 36
CDP	Cytidine diphosphate
CMP	Cytidine monophosphate
CoA	Coenzyme A
COX	Cylcooxygenase
COX2	Cylcooxygenase 2
COX2	Cylcooxygenase 2 gene
cPLA2	Cytosolic phospholipase A2
cPLA2	Cytosolic phospholipase A2 gene
CPT1	Carnitine palmitoyl-CoA transferase 1
CPT1	Carnitine palmitoyl-CoA transferase 1 gene
CPT2	Carnitine palmitoyl-CoA transferase 2
CT	Control
CTB	CellTiter-Blue
CYP	Cytochrome P450
CYP20A1	Cytochrome P450 20A1

CYP20A1	Cytochrome P450 20A1 gene
CYPs	Cytochrome P450 genes
Cys	Cysteine
D[4,3]	Volumetric diameter
d9D	Δ9 desaturase
d9D	Δ9 desaturase gene
DC	Digestive caeca
DHA	Docosahexaenoic acid
DMT1	Divalent metal transporter-1
DOC	Dissolved organic carbon
DPA	Docosapentaenoic acid
DTT	DL-dithiothreitol
EC50	Median effect concentration
ECaC	Epithelial calcium channel
ELOVL	Elongation of very long chain fatty acid; also called fatty acid elongase
ELOVL	Elongation of very long chain fatty acid genes; also called fatty acid elongase gene
ELOVL2	Elongation of very long chain fatty acid 2; also called fatty acid elongase 2
ELOVL2	Elongation of very long chain fatty acid 2 gene; also called fatty acid elongase 2 gene
ELOVL5	Elongation of very long chain fatty acid 5; also called fatty acid elongase 5
ELOVL5	Elongation of very long chain fatty acid 5 gene; also called fatty acid elongase 5 gene
EPA	Eicosapentaenoic acid
F	Forward
FA	Fatty acid
FABP	Fatty acid binding protein
FABP7	Fatty acid binding protein 7
FADS1	Fatty acid desaturase 1 gene
FADS2	Fatty acid desaturase 2
FADS2	Fatty acid desaturase 2 gene
FAF-BSA	Fatty acid free-bovine serum albumin
FAME	Fatty acid methyl ester
FASN	Fatty acid synthase
FASN	Fatty acid synthase gene

FATP1	Fatty acid transport protein 1
FATP1	Fatty acid transport protein 1 gene
FBS	Foetal bovine serum
FID	Flame ionisation detector
FNRS	Fonds de la Recherche Scientifique
GC	Gas chromatography
GEE	Glutathione ethyl ester
GFAAS	Graphite furnace atomic absorption spectrometry
GSH	Glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HEK293	Human embryonic kidney cell line
Hep G2	Human hepatocellular cell line
HG	Hindgut
HPLC	High performance liquid chromatography
HSI	Hepatosomatic index
HSL	Hormone sensitive lipase
HSP	Heat shock protein
HSP30	Heat shock protein 30
HSP70-1	Heat shock protein 70-1
HSP70-2	Heat shock protein 70-2
HSP70a	Heat shock protein 70a gene
HSP70b	Heat shock protein 70b gene
HUFA	Highly unsaturated fatty acid
IAP	Interuniversity Attraction Pole
ICP-AES	Inductively coupled plasma atomic emission spectrometry
L15/ex	Restrictive medium that contains the salts, galactose and pyruvate of Leibovitz's L15 medium at the same concentration as Leibovitz's L15 medium
LA	Linoleic acid
LC-PUFA	Long chain polyunsaturated fatty acid
LDL	Low density lipoprotein
LOX	Lipoxygenase
LXR	Liver X receptor
MDA	Malondialdehyde
MeHg	Methylmercury

MG	Midgut
MPA	Metaphosphoric acid
MT	Metallothionein
MTa	Metallothionein a gene
MTb	Metallothionein b gene
mTOR	Mammalian target of rapamycin
MTP1	Metal transport protein-1
MUFA	Monounsaturated fatty acid
NOX	NADPH oxidase
$O_2^{\circ-}$	Superoxide radical
OD	Optical densities
OH°	Hydroxyl radical
OPA	Ortho-phtalaldehyde
p53	Tumor protein 53
p53	Tumor protein 53 gene
PBS-EDTA	Phosphate-buffered saline supplemented with 0.03% ethylenediamine tetraacetic acid
PC	Principal component
PCA	Principal component analysis
POPC	1-palmitoyl-2-oleoyl-phosphatidylcholine
POPG	1-palmitoyl-2-oleoyl-phosphatidylglycerol
PPAR	Peroxisome proliferator activated receptor
PPAR-alpha	Peroxisome proliferator activated receptor alpha
PPAR-gamma	Peroxisome proliferator activated receptor gamma
PPRE	Peroxisome proliferator response element
PUFA	Polyunsaturated fatty acid
Q_0 site	Ubiquinol oxidizing site
qPCR	Quantitative polymerase chain reaction
r^2	Coefficient of determination
R	Rectum or Reverse
RBMP	River Basin Management Plan
ROS	Reactive oxygen species
RTL-W1	Rainbow trout Liver Waterloo 1
SFA	Saturated fatty acid
SRE	Sterol regulatory element
SREBP-1C	Sterol regulatory element binding protein 1C
SREBP-1C	Sterol regulatory element binding protein 1C gene
SSA	Sulphosalicylic acid

T	Temperature
TBARS	Thiobarbituric acid reactive substances
TEAB	Tetraethylammonium bromide buffer
TK-TD	Toxicokinetic-toxicodynamic
TL	Trophic level
US EPA	United States Environmental Protection Agency
VLDL	Very low density lipoprotein
WFD	Water Framework Directive

Table of content

Foreword

Stress is a condition evoked in an organism by an external or internal stressor (environmental factor such as temperature change, sub-optimal nutrition, toxicants or infections) that moves the organism away from its optimum performance in terms of growth and reproduction (Van Straalen, 2003).

In aquatic ecotoxicology and aquaculture, limiting the impact of stressors on aquatic biota is of critical importance to preserve aquatic ecosystems and to guarantee a sustainable production of healthy and safe aquatic food for human consumption. Consequently, over the past decades, knowledge about biological and ecological responses across different levels of organisation (molecular to community level) to various stressors, considered individually, has been well developed in these two research disciplines. However, the focus on single stressors is in contradiction with reality, in which the combined action of multiple stressors on aquatic biota is the rule rather than the exception. The combined effects of multiple stressors may not simply be additive (i.e. equal to the sum of the effects of single stressors), but antagonistic (less-than-additive) or synergistic (more-than-additive). Those currently unpredictable interactions are confounding the decision-making process when dealing with multiple stressors in aquaculture and (aquatic) ecotoxicology (De Laender et al., 2009).

In this context, the Belgian science policy office funded an Interuniversity Attraction Pole (IAP) project, involving five Belgian universities (UGhent, KULeuven, UAntwerp, UNamur, UCLouvain) and two European partners from The Netherlands and Spain. The primary scientific objective of this project, which was called “AquaStress”, was to investigate multiple stressors (thermal, nutritional, chemical and infectious stress) in aquatic systems across multiple levels of biological organisation to verify to what extent multiple stressor effects occurring at higher levels of organisation can be predicted/explained based on observations of effects at lower levels.

Being a part of the AquaStress project, this thesis investigated the combined impacts of chemical stress, in terms of contamination by metals, especially cadmium (Cd), and nutritional stress, in terms of dietary lipid quality, on two aquatic organisms (i.e. rainbow trout, *Oncorhynchus mykiss*, a fish, and the large water flea, *Daphnia magna*, a filter-feeding zooplankton), using both *in vitro* and *in vivo* approaches.

Pollution of the aquatic ecosystems by Cd is of both natural and anthropogenic origins. Cd readily accumulates in and is highly toxic to aquatic biota. Toxicity is mainly linked to Cd ability to alter ion homeostasis and generate oxidative stress. Oxidative attacks to membrane phospholipids may lead to uncontrolled chain reactions of hydrogenation that can alter membrane functionality. This is of particular concern in aquatic organisms that are rich in long chain polyunsaturated fatty acids (LC-PUFAs), as they are very sensitive to oxidation.

In aquatic food webs, essential PUFAs are generated by primary producers and transferred to higher trophic levels, in which biotransformation can eventually occur. The PUFA composition of primary producers varies among taxa (Brett et al., 2009; Lang et al., 2011) and is modulated by environmental conditions, such as temperature (e.g. Hixson and Arts (2016)) and water pollution (including Cd contamination, e.g. Chia et al., (2013)). Shifts in dietary lipid quality are thus frequently encountered by primary consumers and predators in natural freshwater ecosystems. Dietary lipid quality shifts also occur in the aquaculture field, where fish oil, rich in n-3 LC-PUFA, is progressively replaced by plant-derived oils, that lack n-3 LC-PUFA (FAO, 2018; Glencross, 2009; Tocher, 2015; Turchini et al., 2009). Given the biological roles of fatty acids (energy provision, cell structure maintenance and signalling), these shifts may influence organism fitness and ability to cope with additional stress factors, such as Cd contamination.

The present thesis starts with an introduction summarizing the major concerns linked to contamination by metallic compounds, especially Cd, the fatty acid metabolism and the interplay between metallic compounds (especially Cd) and lipids, on aquatic biota, with a focus on fish (**Chapter 1**). This first chapter ends up with a presentation

of the experimental models used in the present research project. The aims of this research project as well as the experimental strategy are described in **Chapter 2**. The experimental results are then split into 4 chapters (**Chapters 3 to 6**). A general discussion, the main conclusions and the some perspectives are finally drawn **in Chapter 7**.

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Chapter 1

Introduction

Lipids, and their constitutive fatty acids, are key nutrients affecting organism fitness, while heavy metals are priority hazardous substances threatening organism fitness. This research project investigated the interactions between these two factors, i.e. dietary lipid quality, on the one hand, and metal contamination (especially cadmium (Cd)), on the other hand, in rainbow trout (*Oncorhynchus mykiss*) and in the large water flea (*Daphnia magna*), using both *in vitro* and *in vivo* approaches.

This first chapter summarizes the major impacts of metals (especially Cd), the fatty acid metabolism and the interplay between these two factors, on aquatic biota, with a focus on fish. It ends up with a brief presentation of the two reference models chosen, i.e. rainbow trout and the large water flea.

1.1. Metallic pollution

Metallic pollution of the hydrosphere is a global concern. Metallic compounds are released in the environment through events of both natural and anthropogenic origin and end up in the hydrosphere where they concentrate both in the sediments and biota. Zinc, copper, nickel, mercury and Cd are example of such toxic metals. Toxicity depends on the speciation. The most toxic form of Cd is free Cd^{2+} ion, while that of mercury is its organic form methylmercury (CH_3Hg^+, MeHg). These two metallic compounds where used as reference chemical stressors in the present research project, with a major focus on Cd (see section 1.2. for a detailed presentation of concerns linked to Cd pollution).

MeHg is an ubiquitous persistent pollutant resulting from methylation of mercury by sulphate-reducing bacteria at the oxic-anoxic interface of sediments (Selin, 2009). Total mercury concentrations in the freshwater ecosystems range from 0.3 ng/L to 450 µg/L, with higher levels found downstream of industrial discharges or mines (Kidd and

Batchelar, 2011; Rytuba, 2003). MeHg is prone to bioaccumulation and biomagnification throughout the aquatic food webs (Kidd and Batchelar, 2011; Lavoie et al., 2013), so that high contamination levels can be expected in carnivorous fish (Schwindt et al., 2008). MeHg impairs survival, growth and reproduction in wildlife and humans. This organo-metal acts as a neurotoxicant in mammals and fish, but is also toxic to other organs such as kidney and liver (Kidd and Batchelar, 2011). Cytotoxicity is mainly linked to MeHg ability to (i) alter homeostasis of some ions, such as calcium (Farina et al., 2011; Kidd and Batchelar, 2011); (ii) bind sulfhydryl and selenohydryl groups (thereby interfering with the activity of a wide variety of cellular proteins) (Farina et al., 2011; Kanda et al., 2014; Kidd and Batchelar, 2011); and (iii) generate oxidative stress (by both enhancing the direct production of reactive oxygen species and reducing antioxidant defences) (Farina et al., 2011; Kidd and Batchelar, 2011).

1.2. Cadmium

Cd is a non-essential metal of environmental concern. Sources of pollution of the aquatic ecosystems are both of natural and anthropogenic origins. Cd is prone to bioconcentration (i.e. accumulation of a substance in an organism at concentrations exceeding those found in the surrounding aquatic environment) and bioaccumulation (intake via all possible exposure routes, retention and concentration of a substance in an organism) and is highly toxic to aquatic organisms. Toxicity of Cd is mainly linked to its ability to alter ion homeostasis and generate oxidative stress. This section will further detail these aspects.

1.2.1. Occurrence

Cd levels in the geosphere are generally low (mean concentration in the Earth's crust = 0.2 mg Cd/kg) but high concentrations can be found locally (Lalor, 2008). Natural enrichments in Cd consist of greenockite (CdS) in connection with zinc, lead and copper sulfidic ore deposits. Cd also occurs naturally in some phosphate rock formations and in marine black shales. In such enrichments, Cd levels can reach 500 mg/kg (Lalor, 2008; McGeer et al., 2011; Pan et al., 2010).

Cd is extracted from the Earth' crust and released in the environment through events of either natural or anthropogenic origin. The former includes weathering, volcanic activity, windblown dust, aerosol from sea sprays and forest fires, while the latter includes mining and smelting of zinc, lead and copper ores, use of phosphate fertilizers, burning of fossil fuels, peat and woods and the manufacture of cement. These anthropogenic releases arise from the presence of Cd as an impurity, a waste-product. Cd has also been used to produce nickel-Cd batteries, pigments, stabilizers, alloys, nanomaterials and photovoltaic devices. Recycling programs to collect Cd-containing devices (e.g. batteries) have successfully lowered Cd release to the environment (McGeer et al., 2011; Pan et al., 2010).

Atmospheric deposition, leaching and runoff contribute to Cd incorporation in the aquatic ecosystems, where it concentrates both in the sediments and in the living organisms. Dissolved Cd concentrations are generally below 0.5 μg/L and 0.02 μg/L in freshwater and seawater, respectively. On the other hand, Cd concentrations in sediments and aquatic organisms can reach significantly higher levels. For example, the concentration of Cd dissolved in European streams ranges from less than 0.002 μg/L for the most pristine sites to 1.25 μg/L to the most contaminated sites while the Cd level in sediments can be as high as 43.1 mg/kg (Pan et al., 2010). While Cd readily accumulates in living organisms, evidences for both biodilution (i.e. decrease of concentration of a substance with the trophic level) and biomagnification (i.e. increase of concentration of a substance with the trophic level) are reported in the literature (McGeer et al., 2011). In a meta-analysis compiling laboratory, field and biokinetic modelling data from literature, Cardwell et al., (2013) demonstrated that Cd generally does not biomagnify in aquatic food chains consisting of primary producers, macroinvertebrate consumers and fish occupying the third trophic level (TL) and higher. Biodilution was even shown in freshwater food chains consisting of phytoplankton (TL1), cladocerans (TL2) and fish (TL3) (Cardwell et al., 2013). However, Cd biomagnification could be observed in specific freshwater invertebrate food chains consisting of epiphytes (TL1), macrophyte-dwelling invertebrates (TL2) and flatworms (TL3) and in specific marine food chains consisting of bivalves (TL1), herbivorous gastropods or barnacles (TL2) and carnivorous gastropods (TL3)

(Cardwell et al., 2013; Croteau et al., 2005). The authors pointed out the difficulty to accurately identify the food web structure in the field and thus to accurately assess the potential for biomagnification (Cardwell et al., 2013; Croteau et al., 2005). Finally, the magnitude of the trophic transfer factors could not be linked to the Cd toxicity level suggesting that trophic transfer factors are probably not useful predictors of effects of Cd on aquatic organisms (Cardwell et al., 2013).

1.2.2. Global issue

Cd pollution has been a global concern since the 1960s when a 30 year-consumption of contaminated rice and water was identified as the cause of the "itai itai" disease, an osteoporosis-like bone disease, in the Japanese prefecture of Toyama, downstream a zinc-lead mine (Pan et al., 2010). This catastrophe has raised awareness and concern about Cd toxicity and the need for risk assessment and legislation limiting its use and emission. Guidelines fixing maximum tolerable Cd levels in effluents, fertilizers, sewage sludge, food, air, soil, sediments and water have been established in many countries. Concerning European freshwater, Cd has been identified in the List I of dangerous substance discharged into (i) the aquatic environment in general and (ii) in ground water specifically, drawn by the Council of the European Communities in 1976 (76/464/EEC) and 1980 (80/68/EEC), respectively. In 2000, the European Parliament and Council adopted the Water Framework Directive (WFD) in order to ameliorate the quality of inland and coastal European waters in a harmonized way (2000/60/EC). The main objective of the WFD was to bring all European water bodies in good ecological status (biological, physicochemical and hydromorphological) by 2015 through implementing a River Basin Management Plan (RBMP), which includes a programme of measures. In 2001, a list of 33 priority substances in the field of water policy was established (2455/2001/EC), as first step of implementation of the WFD. Cd was classified as a priority hazardous substance in that list (2455/2001/EC). This list has been updated twice (2008/105/EC, 2013/39/EU) and contains in its last version 45 priority substances, among which 25 considered as priority hazardous substances (still including Cd). Environmental quality standards in the field of water policy (2008/105/EC) and technical specifications for chemical analysis and monitoring of water

status (2009/90/EC) were adopted as next steps of the WFD. Two environmental quality standards were defined: the annual average and the maximum allowed concentration, which fix the contamination level that should not be exceeded to ensure protection against long-term and short-term exposure, respectively. These standards provide a basis for assessing water body chemical health and controlling discharges or releases of pollutants. They were updated in 2013 (2013/39/EU). Threshold values for Cd in European freshwaters are listed in Table 1.1. For comparison, the recommended aquatic life ambient water quality criteria for both long-term (criteria continuous concentration) and short-term (criteria maximum concentration) exposure to Cd in freshwater, adopted by the United States Environmental Protection Agency (US EPA) are listed in Table 1.2. These standards are hardness-dependent (since aquatic Cd toxicity generally decreases with increased hardness) and expressed in dissolved Cd concentrations (since dissolved Cd ion is considered the bioavailable form of the metal and not particulate Cd).

Table 1.1. Environmental quality standards for Cd in European surface freshwater.

Water hardness (mg $CaCO_3$/L)	Annual average (µg/L)		Maximum allowable concentration (µg/L)	
	Inland	Other	Inland	Other
< 40	≤ 0.08	0.2	≤ 0.45	≤ 0.45
[40 – 50[	0.08	0.2	0.45	0.45
[50 - 100[	0.09	0.2	0.6	0.6
[100 – 200[	0.15	0.2	0.9	0.9
≥ 200	0.25	0.2	1.5	1.5

Data are expressed as dissolved cadmium concentrations (2013/39/EU).

Table 1.2. Aquatic life ambient water quality criteria for Cd in US freshwater.

Water hardness (mg $CaCO_3$/L)	Continuous concentration (µg/L)	Maximum concentration (µg/L)
25	0.25	0.49
50	0.43	0.94
75	0.58	1.40
100	0.72	1.80
150	1.00	2.60
200	1.20	3.40
250	1.40	4.20
300	1.60	5.00
350	1.80	5.80
400	2.00	6.50

Data are expressed as dissolved cadmium concentrations (EPA-820-R-16-002).

The WFD environmental objectives were not everywhere met in 2015. A second and then a third 6-year phase of RBMP were

implemented (2015-2021 and 2021-2027). In the case of Cd, nickel and lead, 943 surface water bodies improved their ecological status between the first and second RBMP, as a result of reduced emissions (Kristensen et al., 2018). However, 2137 out of 111062 surface water bodies still do not meet the environmental quality standards for these metals, among which 1014 for Cd only (Kristensen et al., 2018).

1.2.3. Uptake, transport, distribution and excretion

1.2.3.1. Uptake and transport

In fish and cladocerans, Cd is mainly absorbed by the gills or epipodites and gastrointestinal tract following waterborne and dietary exposure, respectively. The olfactory epithelium and the skin are two additional minor routes of Cd absorption during waterborne exposure in fish. In contrast, the chitinous exoskeleton of cladocerans constitutes an efficient barrier against Cd absorption (Chowdhury et al., 2004; McGeer et al., 2011; Klinck and Wood, 2013; Smirnov, 2017a; Tan and Wang, 2008).

Mechanisms of Cd uptake (and toxicity) rely on two major characteristics of Cd. First, Cd mimics divalent metals and compete with them at the binding sites of biomolecules including metal transport channels and carriers. Secondly, Cd has a high affinity for sulfhydryl groups (log K = 11.2-11.6) and thus binds cysteine (Cys), cys-containing oligopeptides and, more generally, thiol-containing biomolecules (Bridges and Zalups, 2005; McGeer et al., 2011; Moulis, 2010; Sóvágó and Várnagy, 2013; Zalups and Ahmad, 2003). Several candidate transporters of Cd were thus proposed.

In fish gills, Cd (free ion) uptake is believed to occur through divalent essential metal transport systems (e.g. calcium and iron), such as voltage-independent epithelial calcium channels (ECaC) and the iron-transporting divalent metal transporter-1 (DMT1, a proton/iron symporter) (Fig. 1.1A) (Cooper et al., 2006; Cooper et al., 2007; McGeer et al., 2011; Verbost et al., 1987). Similarly, Cd would be taken up by calcium transport systems (e.g. ECaC) in cladocerans epipodites (Tan

and Wang, 2011a; Tan and Wang, 2008). The gastrointestinal uptake of Cd in fish has not been fully characterized yet but is suggested to occur from the stomach until the posterior intestine (i) through divalent essential metal transport systems, such as L-type voltage-gated calcium channels, DMT1 or zinc transporters (e.g. the ZIP family which acts as zinc/bicarbonate symporters) as free Cd ion, or (ii) through transporters of amino acids and small peptides as conjugated Cd with Cys or with oligopeptides containing Cys (Fig. 1.1B) (Bridges and Zalups, 2005; Cooper et al., 2006; Franklin et al., 2005; Klinck and Wood, 2011; Klinck and Wood, 2013; Kwong and Niyogi, 2012; McGeer et al., 2011; Zalups and Ahmad, 2003). In cladocerans, Cd gastrointestinal uptake is believed to occur mainly in the diverticula of the midgut, through transport systems of calcium (e.g. ECaC) and nutrients (e.g. Cys) (Munger et al., 1998; Smirnov, 2017a; Tan and Wang, 2008).

While the rate of absorption depends on both water chemistry and diet composition, it is overall recognized that the gastrointestinal absorption of Cd is much less efficient than the branchial one in fish (Chowdhury et al., 2004; Franklin et al., 2005; Klinck and Wood, 2013).

A significant part of the absorbed Cd is sequestered in epithelial cells of the gills and gastrointestinal tract by intracellular low molecular weight thiols such as metallothionein (MT), glutathione (GSH), Cys and calcium-binding proteins or in metal rich granules while another part crosses the basolateral membrane to reach the bloodstream in fish or haemolymph in cladocerans either as free ion or as conjugated Cd (MT-Cd, GSH-Cd-GSH, Cys-Cd-Cys) (Bridges and Zalups, 2005; McGeer et al., 2011; Zalups and Ahmad, 2003). The basolateral extrusion of free Cd is thought to occur through high affinity calcium-ATPase and sodium/calcium exchanger in gills (Fig. 1.1A) (McGeer et al., 2011). Mechanisms of free Cd extrusion from epithelial cells of the gastrointestinal tract is largely unknown but the involvement of similar routes as in the gills and/or the metal transport protein-1 (MTP1) as in mammal enterocytes has been suggested (Fig. 1.1B) (Bridges and Zalups, 2005; Kwong and Niyogi, 2012; McGeer et al., 2011; Zalups and Ahmad, 2003). Extrusion of conjugated Cd from the enterocytes is suggested to occur through amino acid or peptide transporters or

through the damaged basolateral membrane (Bridges and Zalups, 2005; Zalups and Ahmad, 2003).

Once in the fish bloodstream, Cd is preponderantly transported by plasma proteins (mainly albumin) and, to a lesser extent, by MT, GSH and Cys (Bridges and Zalups, 2005; McGeer et al., 2011; Zalups and Ahmad, 2003). Cd can also be absorbed and transported by erythrocytes (Bridges and Zalups, 2005; Matović et al., 2015). The latter transport mechanism was suggested to predominate in cladoceran haemolymph.

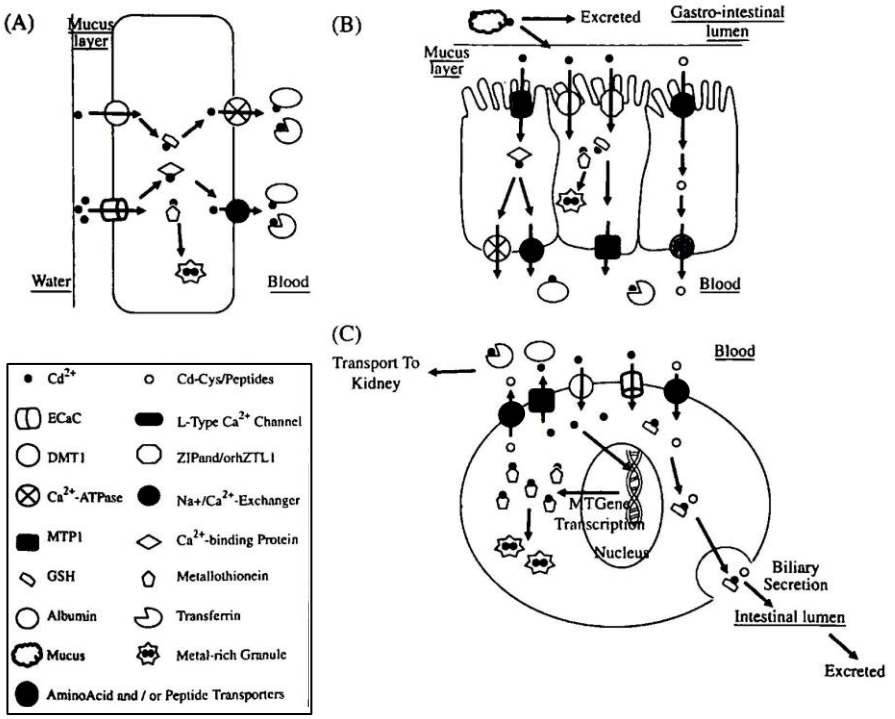


Fig. 1.1. Schematic representation of the mechanisms involved in Cd uptake and handling in fish (A) gill, (B) gastrointestinal tract and (C) liver (McGeer et al., 2011).

1.2.3.2. Distribution

In fish, plasmatic Cd is rapidly absorbed by the organs, especially the liver and the kidneys. Cd distribution changes over time, with the liver and the kidneys being the prime organs for short-term and long-term storage, respectively. Cd is transferred from the internal organs (including the liver) to the kidneys in case of long-term exposure or depuration events (Chowdhury et al., 2003). The cellular uptake and excretion of Cd by internal organs (e.g. liver) is thought to occur through

any of the mechanisms described in both the gills and the gastrointestinal tract (Fig. 1.1C) (McGeer et al., 2011). In freshwater fish, Cd accumulates in liver, kidney and epithelial cells of the gills, the gut and the olfactory system and, to a lesser extent, the blood (with a higher Cd level in erythrocytes than in the plasma), but not significantly in brain and muscle (Chowdhury et al., 2003; Chowdhury et al., 2004; Scott et al., 2003). The high level of cystine and methionine, that lack sulfhydryl groups in their structure, was suggested to decrease Cd-binding and thus accumulation in muscle (Sorensen, 1991). The relative importance between the accumulation sites varies with the route and duration of exposure, as mentioned above, as well as with the species. In cladocerans, Cd accumulates mainly in epithelial cells of gut diverticula and in the exoskeleton (Munger et al., 1998; Smirnov, 2017a; Yu and Wang, 2002).

At the subcellular level, Cd can be found in five separate analytical pools: (i) heat stable proteins (MT and MT-like proteins), (ii) metal-rich granules, (iii) cellular debris (membrane), (iv) heat sensitive proteins (e.g. enzymes) and (v) organelles (e.g. nucleus, mitochondria) (Wang and Rainbow, 2006). The two latter pools represent the metal sensitive fraction, while the two former ones represent the detoxification fraction (Wallace et al., 2003; Wang and Rainbow, 2006). Cd repartition among these compartments determines the toxicity level (Shekh et al., 2021). Subcellular partitioning is highly dynamic, organ-dependant and does not follow a priority order; i.e. there is no threshold below which Cd would be entirely stored in the detoxified fraction (Kamunde, 2009).

1.2.3.3. Excretion

Cd has a long biological half-life, which results from the absence of an efficient mechanism of excretion. In fish, Cd elimination from the body occurs through the gills (mucosal sloughing), the gut (excretion by the liver through the bile, and mucosal sloughing), the kidney (urine) and the ovaries (maternal transfer of Cd to the eggs) (Chowdhury et al., 2004; Sellin and Kolok, 2006). Mucosal sloughing is the most efficient existing excretion route in fish (McGeer et al., 2011). Urinary clearance rate has been estimated at less than 2% of the plasma clearance rate in fish (Chowdhury et al., 2004). In cladocerans, the identified elimination

routes are excretion into the water, molting and reproduction, with the former being reported to represent the dominant pathway. Deposition of Cd in the exoskeleton was also suggested to be an efficient means of depuration in *Daphnia magna*. In contrast, maternal transfer of Cd to the offspring seems to be limited in *Daphnia magna* (< 10%). This low maternal transfer of Cd might even be overestimated as Cd accumulation in the eggs may result from direct adsorption of the metal onto the eggs in the brood chamber upon water exposure instead of a real maternal transfer (Guan and Wang, 2006; Guan and Wang, 2004; Tan and Wang, 2008; Tsui and Wang, 2007; Yu and Wang, 2002).

1.2.4. Toxicity data and mechanisms

1.2.4.1. Variability

Cd sensitivity is highly influenced by environmental, nutritional and biological factors. First, Cd toxicity depends on its bioavailability, which, upon aqueous exposure is influenced by water chemistry (especially its richness in organic matter and hardness). Indeed, organic matter decreases Cd uptake by complexing the free Cd ion, the bioavailable form of Cd, from the solution (Niyogi et al., 2008). Cd uptake is also decreased by divalent ions such as calcium and magnesium that compete with Cd for the uptake sites at the gill surface (McGeer et al., 2011; Niyogi et al., 2008; Niyogi and Wood, 2004). Cd toxicity is thus generally lower in hard water rich in organic matter (Niyogi et al., 2008; Tan and Wang, 2011a). Second, Cd toxicity has been shown to be modulated by the diet content in specific trace elements (e.g. zinc and calcium that decrease Cd absorption through competition) and nutrients (e.g. lipids, see section 1.4.). Third, the sensitivity to Cd is highly influenced by the targeted species and its life stage (Jurgelėnė et al., 2019; Niyogi et al., 2004; Shekh et al., 2021; Shekh et al., 2018; Tan and Wang, 2011b; Traudt et al., 2017a, b). Salmonidae and daphniidae are among the most sensitive families of freshwater fish and zooplankton, respectively (EPA-820-R-16-002). The higher sensitivity was linked to a different subcellular Cd partition, a naturally higher calcium content, calcium influx rate and/or a lower ability to resist the calcium uptake inhibition by waterborne Cd exposure in these families (Niyogi and

Wood, 2004; Shekh et al., 2021; Shekh et al., 2018; Tan and Wang, 2011b).

1.2.4.2. Wide variety of toxic effects

A wide variety of biological processes have been reported to be impacted by Cd. Indeed, Cd impairs growth, reproduction and survival; causes developmental abnormalities as well as gill, liver and kidney histopathologies; disrupts the ionic homeostasis and the redox balance; disturbs the immune and endocrine systems; impairs the energy metabolism; is considered as a potential human carcinogen (goup 2B) for the US EPA and as a human carcinogen (group 1) for the International Agency for Research on Cancer of the World Health Organisation (Connon et al., 2008; Cuypers et al., 2010; Jurgelėnė et al., 2019; Matović et al., 2015; McGeer et al., 2011; Wang et al., 2021; Zheng et al., 2016b).

1.2.4.3. Ionic balance disturbance

Cd disturbs the homeostasis of essential metals (calcium, magnesium, selenium, copper, zinc and iron). Although specificities exist for each metal, the common mechanisms described in the literature are as follows. First, Cd decreases both branchial and gastrointestinal uptake of divalent cations leading to deficiencies (Niyogi and Wood, 2004; Shekh et al., 2018; Zohouri et al., 2001). Numerous cases of hypocalcaemia and anaemia have for example been linked to Cd contamination (Buha et al., 2019; McGeer et al., 2011; Moulis, 2010; Reynders et al., 2006; Silva and Martinez, 2014; Zohouri et al., 2001). Second, Cd impairs uptake of divalent cations in internal organs altering metal distribution. For example, Cd reduces calcium incorporation in bones, leading to osteoporosis (Bilal et al., 2022; Buha et al., 2019). Third, Cd displaces metals from their binding sites in biomolecules, leading to enzyme conformational changes and dysfunction (e.g. manganese superoxide dismutase) and unbuffered intracellular metal concentrations (e.g. release of zinc bound to MT) (McGeer et al., 2011; Moulis, 2010). Fourth, Cd alters subcellular partitioning of essential metals leading to adverse effects that are specific to each metal (e.g.

release of calcium from sarcoplasmic/endoplasmic reticulum leading to apoptosis) (Biagioli et al., 2008; Thévenod and Lee, 2013).

Impaired calcium homeostasis is one of the best described impacts of Cd in fish. Hypocalcaemia induced by Cd results from two major mechanisms. On the one hand, Cd decreases calcium branchial uptake by competing with calcium binding sites (Niyogi et al., 2015; Niyogi and Wood, 2004). On the other hand, Cd impairs calcium extrusion from the branchial cells to the blood by inhibiting calcium-ATPase at the basolateral side of the gill epithelial cells (Verbost et al., 1988). Cd also impairs calcium uptake by internal organs and tissues through competition and mimicry mechanisms (Choong et al., 2014; McGeer et al., 2011). Cd finally modulates calcium subcellular partitioning in various cell types. Under normal conditions, free calcium cytosolic level is maintained rather low through the action of plasma membrane and sarcoplasmic/endoplasmic calcium pumps that move calcium from the cytosol to the extracellular space and sarcoplasmic/endoplasmic reticulum lumen, respectively (Nelson et al., 2008). Cd has been reported to block the sarcoplasmic/endoplasmic calcium pump, resulting in a depletion of the endoplasmic reticulum calcium store and a rise in free calcium cytosolic level (Biagioli et al., 2008; Choong et al., 2014; Thévenod and Lee, 2013). Depletion of endoplasmic reticulum calcium store causes endoplasmic reticulum stress, with induction of the unfolded protein response, caspase-12 activation and apoptosis if endoplasmic reticulum chaperones buffering capacity is overwhelmed. Besides, a rise in cytosolic calcium can trigger its absorption by mitochondria, with, as possible successive consequences, unbalanced mitochondrial calcium equilibrium, a rise in mitochondrial reactive oxygen species (ROS) production, mitochondrial membrane depolarisation and permeabilisation, release of proapoptotic factors and apoptosis (Biagioli et al., 2008; Choong et al., 2014; Thevenod and Lee, 2013; Wang et al., 2014).

1.2.4.4. Oxidative stress

ROS, i.e. superoxide radical ($O_2^{\circ-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH°), are partially reduced oxygen forms that are generated through normal metabolic aerobic processes. ROS have short

half-life (ns to min) but are highly reactive and can damage macromolecules through inducing lipid peroxidation (see section 1.4.), protein carbonylation and oxidation of DNA bases. Under normal condition, ROS level is maintained low by the cellular antioxidant system, consisting of antioxidant molecules, e.g. GSH, ascorbic acid, vitamin E and their derivatives, and antioxidant enzymes, i.e. superoxide dismutase, catalase, glutathione peroxidase. Oxidative stress appears when the balance between pro- and anti-oxidant processes is disrupted in favour of the former (Lushchak, 2016).

Cd is a non-redox active metal that indirectly induces oxidative stress through both increasing ROS production and weakening cellular antioxidant defences.

On the one hand, Cd enhances ROS production through four identified mechanisms. First, as mentioned above, Cd rises free cytosolic calcium level which can lead to increased mitochondrial ROS production (Biagioli et al., 2008; Thévenod and Lee, 2013; Wang et al., 2014). Second, Cd displaces redox active metals (e.g. copper, iron) from cytoplasmic and membrane proteins, enabling them to enter the Fenton's reactions, with a net production of OH° (Matović et al., 2015). Third, Cd increases electron leakage from the electron transport chain, and thus the generation of $O_2^{\circ-}$, by decreasing the activity of the complex III of the electron transport chain (Adiele et al., 2012; Wang et al., 2004). Cd would bind to histidyl or sulfhydryl groups at the ubiquinol oxidizing (Q_0) site of the complex III, preventing electron transfer from the semiubiquinone to the heme b566, promoting thus accumulation of unstable semiubiquinones that are prone to donate electrons to molecular oxygen, with generation of $O_2^{\circ-}$ (Lorusso et al., 1991; Wang et al., 2004). Fourth, Cd was reported to upregulate NADPH oxidases (NOXs) at both enzymatic activity (Martínez Flores et al., 2013; Souza et al., 2009) and gene expression (Thijssen et al., 2007) levels. NOXs catalyse the reduction of O_2 into $O_2^{\circ-}$, which dismutates to H_2O_2 either spontaneously or through the action of superoxide dismutases (Cuypers et al., 2010). Mechanisms of upregulation have not been unravelled yet but have been suggested to rely on the mammalian target of rapamycin (mTOR) signalling pathway and the intracellular calcium signalling cascade (Chen et al., 2011; Thévenod and Lee, 2013; Xu et al., 2011).

On the other hand, Cd impairs both the molecular and the enzymatic antioxidant system. First, Cd decreases cellular GSH level (Jamwal et al., 2018; Ramakrishnan et al., 2017; Wang et al., 2015; Wang et al., 2014). GSH is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) that constitutes the majority of the cellular non-protein thiol pool. Under normal conditions, GSH is rapidly oxidized by ROS to limit oxidative damage in cells, a reaction catalysed by glutathione peroxidases. Oxidized glutathione (GSSG) is then reduced back into GSH by the action of glutathione reductase (Hellou et al., 2012). Cd is rapidly chelated by thiol-containing proteins, including GSH, when it enters cells (Cuypers et al., 2010; McGeer et al., 2011; Zalups and Ahmad, 2003). This chelation system represents a first line of detoxification as it decreases free ionic Cd level in cells, but it also results in the reduction of the total cellular antioxidant capacity (McGeer et al., 2011; Thévenod and Lee, 2013). In addition, Cd can impair GSH turnover through inactivation of glutathione reductase, probably through direct binding to sulfhydryl groups of the enzyme (Matović et al., 2015; Picaud and Desbois, 2006; Ramakrishnan et al., 2017; Tekman et al., 2008; Thévenod and Lee, 2013). Cd also enhances GSH degradation through upregulation of γ-glutamyltranspeptidase (Đukić-Ćosić et al., 2020). Second, Cd modulates both the activity and gene expression of a variety of antioxidant enzymes. Data reported in literature are, however, contradictive, i.e. either upregulation or downregulation or even no impact are reported (Banni et al., 2011; Casalino et al., 2002; Cuypers et al., 2010; Jamwal et al., 2018; Jamwal et al., 2016; Pan et al., 2018; Thijssen et al., 2007; Zheng et al., 2016a; Zheng et al., 2016b). These inconsistencies can be partly explained by differences in experimental procedures between studies (exposure duration and levels, tissue or cell type and targeted species), but also by the fact that Cd has been reported to have a bi-modal action on the antioxidant system. Indeed, as a result of its high affinity for the thiol groups, its similarity with their cofactors (e.g. manganese, selenium) or its direct interactions with their catalytic sites, Cd inhibits a variety of antioxidant enzymes (Casalino et al., 2002; Matović et al., 2015; McGeer et al., 2011; Thévenod and Lee, 2013; Wang et al., 2015). Consequently, ROS accumulates in cells; which leads to activation of defence systems via increased gene expression, eventually followed by an increase of antioxidant enzyme levels and activities (Banni et al., 2011; Cuypers et al., 2010).

1.2.4.5. Cellular détoxification

The first line of cellular detoxification is mediated by GSH and MT that act as metal and ROS scavengers (Cuypers et al., 2010; Faysse et al., 2006; Freisinger and Vašák, 2013; Klaassen et al., 1999; McGeer et al., 2011; Sóvágó and Várnagy, 2013). The second line of cellular detoxification is mediated by the antioxidant system (enzymatic and non-enzymatic) and general stress-defence proteins such as heat shock proteins (HSP) (McGeer et al., 2011). Activation of cellular defences has a non-negligible energetic cost, which can be further increased by Cd since this metal has been shown to impair ATP production (Adiele et al., 2012).

1.3. Fatty acid metabolism and functions

Lipids and their constitutive fatty acids play major roles in biota, especially in ectotherms, including energy provision, maintenance of biological membrane structure and functions as well as cellular signalling. Appreciation of these key functions requires understanding of the structure, biochemistry and metabolism of fatty acids. As there is little information available on fatty acid biosynthesis and metabolism in zooplankton, this section mainly focuses on fish. However, it seems likely that the molecular basis of the pathways described in this section would be similar.

1.3.1. Structure and classification

Fatty acids are carboxylic acids with hydrocarbon chains containing between 4 and 36 carbons (most commonly 12-24 carbons) (Nelson et al., 2008). In addition to their trivial and abbreviation names, fatty acids are termed and classified based on the length of the aliphatic chain, the number of unsaturation (ethylenic bond) and the position of the first unsaturation related to the methyl-end of the aliphatic chain (Table 1.3) (Glencross, 2009; National Research Council, 2011; Nelson et al., 2008; Sargent et al., 2002; Tocher, 2003).

In that way, saturated, monounsaturated and polyunsaturated fatty acids (SFA, MUFA and PUFA) possess, respectively, no, one or at least

two unsaturations. Among PUFAs, n-3 and n-6 PUFAs hold their first ethylenic bond respectively at 3 and 6 carbons from the methyl-end of the aliphatic chain (Glencross, 2009; National Research Council, 2011; Tocher, 2003). PUFAs with at least 20 carbons and 3 unsaturations are called highly unsaturated fatty acids (HUFAs) or long chain PUFAs (LC-PUFAs) (Glencross, 2009; Sargent et al., 2002; Tocher, 2003).

Fatty acids are the backbones of biological complex lipids. In contrast, simple lipids do not contain fatty acids. Simple lipids are mainly sterols, i.e. tetracyclic hydrocarbon alcohol compounds, among which cholesterol. Complex lipids are subdivided in two groups, the polar lipids (phospholipids, glycolipids, ether lipids) and the neutral lipids (triacylglycerols or triglycerides, cholesteryl esters, wax esters). Phospholipids and triglycerides are respectively the major polar and neutral lipids. (National Research Council, 2011; Nelson et al., 2008; Tocher, 2003). Neutral lipids of zooplankton may be rich in wax esters, which are composed of one fatty acid esterified to one fatty alcohol. Wax esters are converted to triglycerides during the digestion and absorption process in planktivorous fish. Although, some fish species contain considerable amounts of wax esters in their body tissues and eggs (Sargent et al., 2002; Tocher, 2003)

Phospholipids include phosphorus in their structure. Phospholipids are mainly phosphoglycerides, which are composed of one phosphate and two fatty acids, each esterified to one hydroxyl group of a glycerol molecule (Fig. 1.2A) (Nelson et al., 2008). The phosphate group can be esterified to either choline, ethanolamine, serine or inositol to generate the major phosphoglyceride classes found in fish tissues, i.e. respectively the phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol (Fig. 1.2A-E) (Nelson et al., 2008; Tocher, 2003).

Triglycerides are composed of three fatty acids, each esterified to one hydroxyl group of a glycerol molecule (Fig. 1.2F) (Nelson et al., 2008).

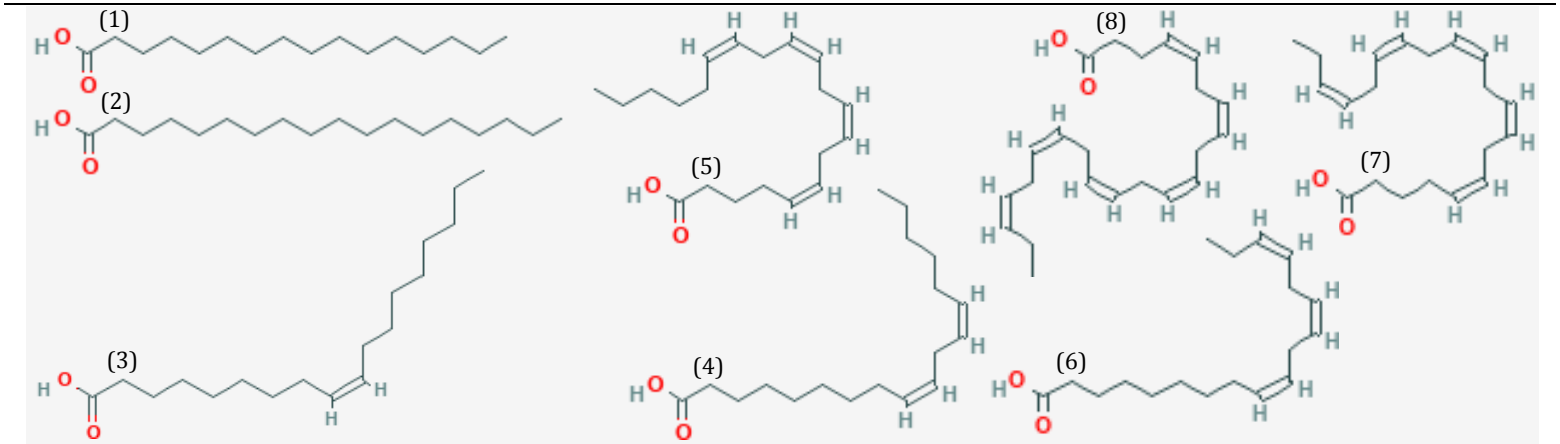
In fish, SFAs (mainly 16:0, 18:0) and MUFAs (mainly 16:1n-7, 18:1n-9, 20:1n-9, 22:1n-11) mainly occur at the sn-1 position of phospholipids and at both the sn-1 and the sn-3 position of triglycerides

while PUFAs (mainly linoleic acid (LA, 18:2n-6), alpha-linolenic acid (ALA, 18:3n-3), arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3)) mainly occur at the sn-2 position of phospholipids and triglycerides (Fig. 1.2A,F). However they are many exceptions to this general scheme. For example, triglycerides and phospholipids containing only DHA can be found in the eye lipids of some fish (Sargent et al., 2002; Tocher, 2003).

The two major freshwater zooplankton taxa, i.e. copepods and cladocerans, can be differentiated based on their fatty acid composition. On the one hand, copepods accumulate a higher proportion of DHA and are characterised by higher n-3/n-6 ratio. On the other hand, cladocerans accumulate a higher proportion of EPA and AA and are characterized by a lower n-3/n-6 ratio (Brett et al., 2009; Persson and Vrede, 2006). Besides, overall carnivorous cladocerans are richer in PUFA and LC-PUFA than the herbivorous ones (Brett et al., 2009; Persson and Vrede, 2006). In addition to these phylogenetic differences, environmental parameters (water temperature, contamination level and diet composition) influence zooplankton fatty acid composition (see section 1.4.).

Table 1.3. Nomenclature and structure of some naturally occurring fatty acids in aquatic organisms.

Carbon skeleton	Systematic name	Trivial name	Abbreviation	Group	2D-structure
16:0	Hexadecanoic acid	Palmitic acid	-	SFA	(1)
18:0	Octadecanoic acid	Stearic acid	-	SFA	(2)
18:1n-9	cis-9-Octadecenoic acid	Oleic acid	-	MUFA	(3)
18:2n-6	cis-9-12-Octadecadienoic acid	Linoleic acid	LA	n-6 PUFA	(4)
20:4n-6	cis-5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	AA	n-6 LC-PUFA	(5)
18:3n-3	cis-9,12,15-Octadecatrienoic acid	alpha-Linolenic acid	ALA	n-3 PUFA	(6)
20:5n-3	cis-5,8,11,14,17-Eicosapentanoic acid	Eicosapentaenoic acid	EPA	n-3 LC-PUFA	(7)
22:6n-3	cis-4,7,10,13,16,19-Docosahexaenoic acid	Docosahexaenoic acid	DHA	n-3 LC-PUFA	(8)



Abbreviations used: LC-PUFA, long chain polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid. 2D-structures were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/).

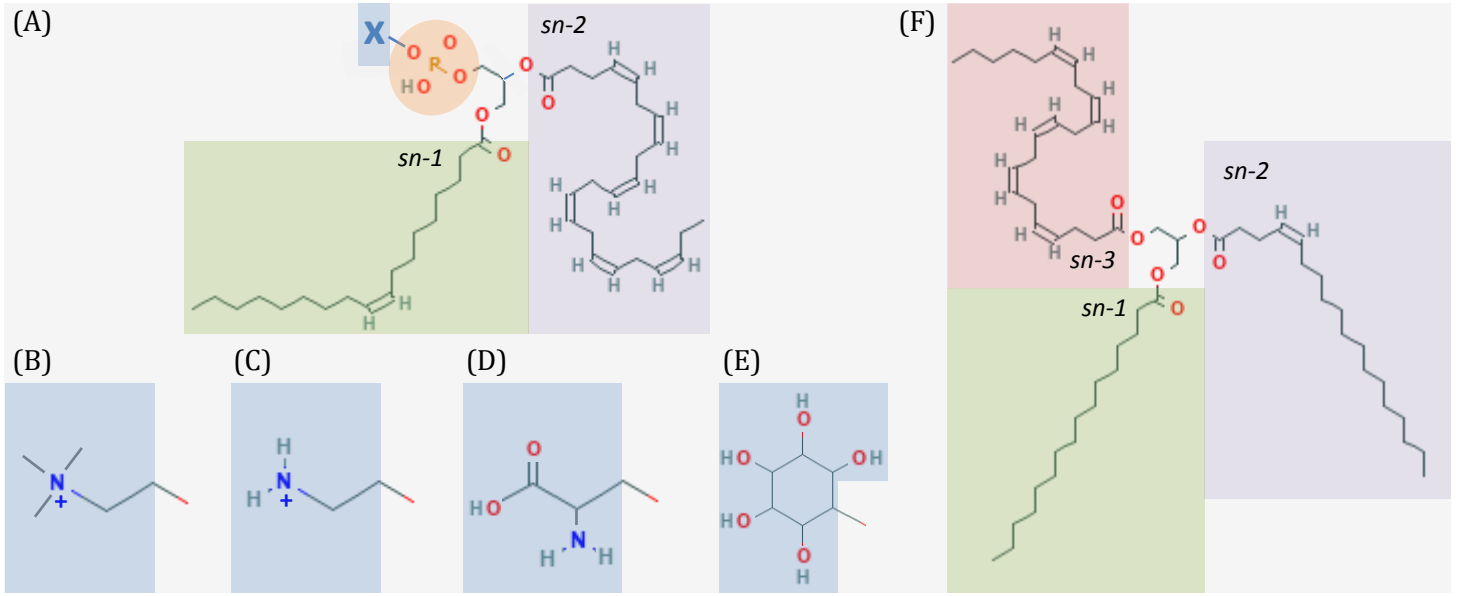


Fig. 1.2. General structure of phosphoglycerides (A) and triglycerides (F). Structure of head-groups (X) in major phosphoglyceride classes, i.e. (B) phosphatidylcholine, (E) phosphatidylethanolamine, (C) phosphatidylserine, (D) phosphatidylinositol. Built with the PubChem database (https://pubchem.ncbi.nlm.nih.gov/).

1.3.2. Digestion and absorption of dietary lipids

Dietary lipids contain five lipid classes: phospholipids, sphingolipids, triglycerides, wax esters and sterols. In fish, lipid digestion and absorption mainly occurs in the proximal part of the intestine and the pyloric caeca (Glencross, 2009; Tocher, 2003). In cladocerans, digestion occurs in the middle region of the midgut (mesenteron) and the absorption occurs in its anterior region, including the liver diverticula. This process relies on both antiperistalsis and peristalsis (Smirnov, 2017b). Overall, fatty acid digestibility increases with the number of unsaturation and decreases with the length of the aliphatic chain (Caballero et al., 2002; Francis et al., 2007; Glencross, 2009). Digestibility of PUFAs and LC-PUFAs is thus high (between 90 and 98% in Atlantic Salmon, *Salmo salar*) while digestibility of SFA is low (between 50 and 70% in Atlantic salmon) (Francis et al., 2007; Guillaume et al., 2001). The position of the first ethylenic bond in the aliphatic chain also influences fatty acid digestibility, which follows the decreasing order n-3 > n-6 > n-9 (Francis et al., 2007). Salmonids tolerate high amounts of lipids in their diets (up to 30%) (Glencross, 2009).

Dietary lipids are first solubilized by bile salts, amphipathic molecules produced from cholesterol in the liver, stored in the gallbladder (if present, like the majority of fish species, including salmonids) and released in the proximal midgut lumen upon intestinal stimuli induced by chime influx. Bile salts enable the formation of lipid emulsions, which increase the lipid fraction accessible to the digestive water-soluble enzymes (triacyglycerol lipases, phospholipases, esterases) secreted by the pancreas or hepatopancreas in fish. Bile salts also allow the formation of mixed micelles, which are small disk-shaped structures containing the major products of digestion, i.e. free fatty acids, mono- or di-acylglycerols, glycerol, lyso-phospholipids, cholesterol and fatty alcohols. Those final products of digestion are finally taken up by enterocytes mainly by passive diffusion, but also by facilitated transport aided by proteins, such as fatty acid binding proteins (Glencross, 2009; Guillaume et al., 2001; Kamalam et al., 2020; National Research Council, 2011; Nelson et al., 2008; Tocher, 2003;

Tocher et al., 2008). In daphnids, fat globules are carried from the gut lumen through the gut wall by blood corpuscles and via epithelial cells from the anterior region of the midgut, including the liver diverticula (Smirnov, 2017b).

1.3.3. Internal transport

Absorbed free fatty acids, acylglycerols and lyso-phospholipids are re-esterified into triglycerides and phosphoglycerides in the endoplasmic reticulum of enterocytes, before being packed with cholesterol and apolipoproteins into lipoproteins and sent through first the lymphatic system then the circulatory system to extrahepatic tissues and finally to the liver. A portion of small lipoproteins may also be transported from the intestine to the liver through the portal system (Glencross, 2009; Guillaume et al., 2001; Nelson et al., 2008; Tocher, 2003; Tocher et al., 2008). In daphnids, fat globules are absorbed and transported by blood cells or as minute fat particles (haemokonia) in the haemolymph (Smirnov, 2017b).

Lipoproteins are characterized based on their size and density, which depends on their composition. Chylomicrons, very low, low and high density lipoproteins (VLDL, LDL and HDL) are characterised by a decreasing lipid to protein ratio. The relative proportion of plasmatic lipoproteins depends on the species, age, nutrition and sexual cycle (Babin and Vernier, 1989; Guillaume et al., 2001; National Research Council, 2011; Tocher, 2003). Overall, HDL is the main plasmatic lipoprotein in trout, followed by LDL and VLDL (Richard et al., 2006; Tocher, 2003; Torstensen et al., 2004). Besides, in trout, triglycerides account for respectively 84%, 52%, 22% and 11% of the chylomicron-like molecules, VLDL, LDL and HDL weights while phosphoglycerides represented 8%, 19%, 27%, 32% of their respective weights (Babin and Vernier, 1989). Chylomicrons-like molecules and, to a lesser extent, VLDL transport the majority of dietary lipids from the intestine to extrahepatic tissues, first, and, finally, to the liver. Free fatty acids bound to albumin, on the one hand, and VLDL, on the other hand, can also be transported from the intestine through the portal system. The latter is, however, a minor transport, except in case of refeeding after a fasting period (Guillaume et al., 2001; National Research Council, 2011; Tocher,

2003; Tocher et al., 2008). Hepatocytes also supply the peripheral tissues with neutral lipids (triglycerides and cholesterol) packed in VLDLs while adipocytes release free fatty acids that are transported to peripheral tissues bound to albumin. The majority of plasmatic VLDL is thus of hepatic origin. VLDLs become LDLs as extra-hepatic tissues take up neutral lipids. Triglycerides present in lipoproteins are hydrolysed by extracellular lipoprotein lipase (extrahepatic tissues) or hepatic lipase (liver) into glycerol and free fatty acids, which are taken up by cells (Nelson et al., 2008; Tocher, 2003). Fatty acid uptake occurs either by flip-flop diffusion or by facilitated transport with membrane transport proteins and translocases such as the fatty acid transport protein 1 (FATP1) and the cluster of differentiation 36 (CD36) (Frohnert and Bernlohr, 2000; Nelson et al., 2008; Zhou et al., 2010). The former mechanism is, however, a very slow process (Nelson et al., 2008). In the cytosol, fatty acids are bound to fatty acid binding proteins (Tocher, 2003). In contrast to the other lipoproteins, HDL transport cholesterol from extra-hepatic tissues to the liver. Once relieved of most of the neutral lipid they carried (chylomicrons, VLDL, LDL) or once loaded with cholesterol (HDL), lipoproteins are finally taken up by the liver by endocytosis or pinocytosis where they are recycled (Nelson et al., 2008; Tocher, 2003).

1.3.4. Biosynthesis and bioconversion

1.3.4.1. *De novo* synthesis

De novo synthesis of fatty acids is an endergonic and reductive process which requires metabolic energy (ATP) and reduced electron carriers (NADPH) (Glencross, 2009; Nelson et al., 2008). This process mainly occurs in hepatocytes and adipocytes, with the former being predominant in rainbow trout (Tocher, 2003). It is a cycling process, in each cycle of which, a two carbon precursor, acetyl-Coenyme A (CoA), is extended two carbons by two carbons to generate 16:0 and 18:0 as major products. Each cycle consists in four steps, catalysed by the cytosolic multi-enzyme fatty acid synthase (FASN). It starts with the condensation of acetyl-CoA (or acyl-CoA from the second cycle) and malonyl-CoA moieties to generate a β-ketoacyl that is subsequently

hydrogenated in three successive steps (Glencross, 2009; Nelson et al., 2008).

The acetyl-CoA moiety is either of carbohydrate origin, i.e. mitochondrial oxidative decarboxylation of pyruvate, or of protein origin, i.e. mitochondrial oxidative degradation of some amino acids (Nelson et al., 2008). The latter predominates in rainbow trout (Tocher, 2003). The malonyl-CoA needed at each cycle is generated from another acetyl-CoA moiety, irreversible reaction requiring metabolic energy (ATP) and catalysed by the cytosolic acetyl-CoA carboxylase (Nelson et al., 2008). The electron donor required at second and forth step of the cycle is NADPH (Nelson et al., 2008).

Longer SFAs are synthesized in mitochondria and endoplasmic reticulum through a similar metabolic process, but catalysed by four different enzymes, i.e. one specific enzyme per step (Glencross, 2009; Nelson et al., 2008; Tocher, 2003). However, elongation of very long chain fatty acid (ELOVL), the specific the enzymes catalysing the condensation, are considered as the effective elongase enzymes since it is the rate-limiting step (Tocher, 2003).

Fatty acid *de novo* synthesis is tightly regulated. The generation of malonyl-CoA, catalysed by the cytosolic acetyl-CoA carboxylase, is the rate-limiting step of the fatty acid *de novo* synthesis, and thus an important point of regulation (Nelson et al., 2008). Acetyl-CoA carboxylase is inhibited by palmitoyl-CoA (negative feedback), inactivated by phosphorylation and stimulated by citrate (allosteric activation) (Nelson et al., 2008). Dietary lipids downregulate lipogenesis in rainbow trout (Alvarez et al., 2000; Panserat et al., 2008; Tocher, 2003).

1.3.4.2. Bioconversion

Bioconversion of fatty acids consists in elongation, i.e. extension of the aliphatic chain by two carbons, and desaturation, i.e. addition of an ethylenic bound in the aliphatic chain, of *de novo* synthesized or dietary fatty acids. These processes mainly occur in the endoplasmic reticulum of hepatocytes and enterocytes (Sargent et al., 2002; Tocher, 2003).

Desaturation of 16:0 and 18:0 generates 16:1n-7 and 18:1n-9. Those reactions are catalysed by Δ9 desaturase, also called stearoyl-CoA-desaturase, a NADPH- and oxygen-dependent enzyme located in the endoplasmic reticulum that inserts an ethylenic bound 9 carbons from the carboxyl-end of the molecule. Further elongations of 18:1n-9, catalysed by elongases, in the endoplasmic reticulum, generates longer chain MUFAs (Tocher, 2003).

Because they lack Δ12 and Δ15 desaturases, vertebrates, including fish, and zooplankton, including cladocerans, are not able to successively synthesize LA and ALA from 18:1n-9 (Parrish, 2009; Sargent et al., 2002; Tocher, 2003). In contrast, primary producers and some heterotrophic protists possess these enzymatic activities and are thus able to generate n-6 and n-3 PUFAs from MUFAs (Desvilettes and Bec, 2009; Parrish, 2009). ALA and LA must thus be found in fish and zooplankton diet. They are called essential fatty acids (Glencross, 2009; National Research Council, 2011; Nelson et al., 2008; Tocher, 2003).

Fish are able to desaturate and elongate dietary LA and ALA to generate the valuable LC-PUFAs: AA, EPA and DHA (Fig. 1.3). LA and ALA are indeed successively Δ6 desaturated, elongated and Δ5 desaturated to respectively generate AA and EPA (Glencross, 2009; National Research Council, 2011; Sargent et al., 2002; Tocher, 2003). Generation of DHA from EPA is performed through the Sprecher pathway. Indeed, EPA undergoes two successive elongations followed by a Δ6 desaturation to successively generate 22:5n-3, 24:5n-3 and 24:6n-3. The latter fatty acid is then translocated from the endoplasmic reticulum to the peroxisome to undergo one cycle of β-oxidation to generate DHA (Buzzi et al., 1996, 1997; Glencross, 2009; Sargent et al., 2002; Tocher, 2003). Similarly, AA can be converted into 22:5n-6 through the Sprecher pathway. However, the latter bioconversion occurs in minority as the main end-products of the n-6 and n-3 biotransformation pathways are respectively AA and DHA in fish (Sargent et al., 2002; Tocher, 2003).

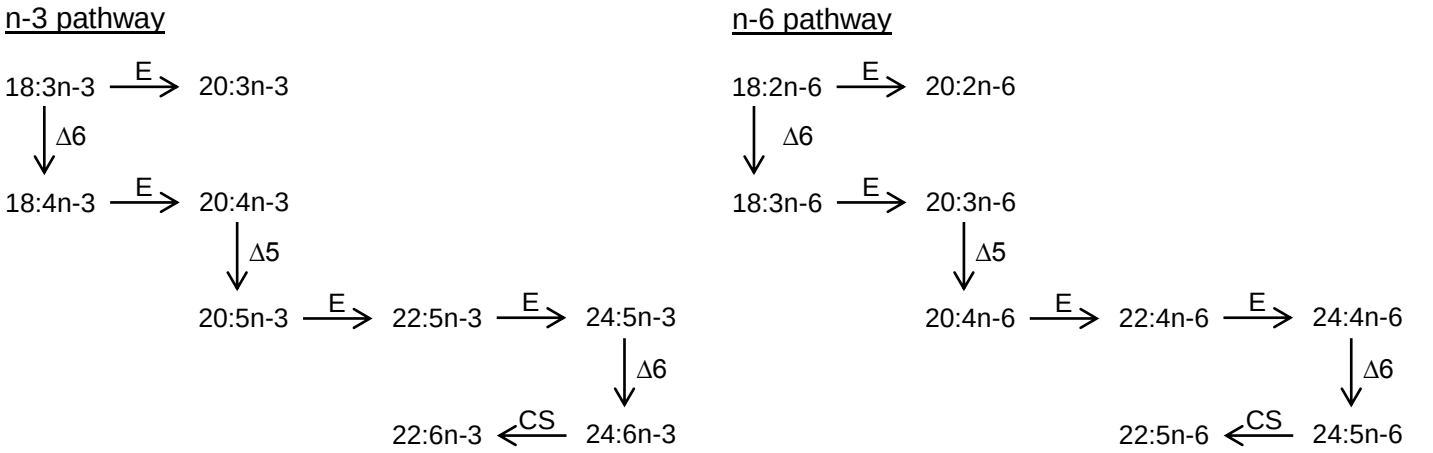


Fig. 1.3. Alpha-linolenic acid (left) and linoleic acid (right) biotransformation pathways in fish hepatocytes. Abbreviations used: Δ5 and Δ6, microsomal fatty acid desaturase enzyme mediated reactions; CS, peroxisomal fatty acid chain shortening; E, hepatic microsomal elongase mediated reactions.

As the same enzymes catalyse the n-3 and n-6 biotransformation pathways, competitions between substrates exist, with an overall higher affinity for n-3 over n-6 fatty acids (Glencross, 2009; Leaver et al., 2008a; Sargent et al., 2002; Thanuthong et al., 2011; Tocher, 2003; Zheng et al., 2004a). The affinity of the elongases and desaturase enzymes is also influenced by the unsaturation level and length of the aliphatic chain of the fatty acid. For example, Δ6 desaturase showed higher affinity for longer chain and more unsaturated fatty acids (Glencross, 2009). ELOVL5 has activity predominantly toward the 18 and 20 carbons fatty acids, while ELOVL2 has activity predominantly towards 20 and 22 carbons fatty acids (Gregory and James, 2014; Morais et al., 2009). These enzymes thus catalyse different steps of the biotransformation pathways. The Δ5 and Δ6 desaturase activities are either held by one single protein (e.g. zebrafish*, Danio rerio*) or several ones (e.g. rainbow trout), depending on fish species (Abdul Hamid et al., 2016; Hastings et al., 2001; Seiliez et al., 2001). In case of essential fatty acid deficiency, desaturase can use MUFAs as substrate to generate n-9 PUFAs. In that way, the 20:3n-9 level and the ratio of phospholipid 20:3n-9/DHA are two markers of essential fatty acid deficiency in freshwater fish and salmonids (Glencross, 2009; Tocher, 2010).

All fish are able to synthesize AA and DHA from their precursors LA and ALA. However, the efficiency of this process is highly variable among species. Overall, freshwater and anadromous fish, like salmonids, have a natural higher capacity of LC-PUFAs synthesis than marine fish. This difference would be due to the fatty acid composition of their diets, with a high level of n-3 LC-PUFA in marine phytoplankton and a high level of the shorter chain PUFAs (LA and ALA) in freshwater phytoplankton. As a result, marine fish would have progressively decreased their ability of LC-PUFA synthesis through downregulation of elongase and desaturase activity and genetic expression (Glencross, 2009; National Research Council, 2011; Sargent et al., 2002; Tocher, 2003; Tocher, 2010). Besides, the efficiency of the bioconversion pathway is rather low in cladocerans (Farkas et al., 1981; Martin-Creuzburg et al., 2010; Smirnov, 2017b; von Elert, 2002). The LC-PUFA content of these organisms is thus highly dependent on the lipid composition of their food (Brett et al., 2009). In contrast to the

bioconversion process, some cladocerans were shown perform retroconversion of LC-PUFAs. Indeed, using radiolabelled tracers, Strandberg et al. (2014) demonstrated that *Daphnia magna* retroconverted 22:5n-6 to AA more efficiently that they converted LA to AA. Retroconversion of DHA to EPA by *Daphnia magna* had also been suggested as being the underlying mechanism of predominant EPA accumulation in *Daphnia magna* fed diets enriched DHA (Martin-Creuzburg et al., 2010).

Dietary lipids are known to modulate the endogenous ability of LC-PUFA synthesis, with overall an increased efficiency when fish are fed a plant-derived diet in comparison to a fish-derived diet (see section 1.4.). This diet impact is, however, only observed in species that possess the genetic and enzymatic capacity of fatty acid bioconversion. Environmental parameters (e.g. temperature, pollutants (including Cd)) were also reported to modulate LC-PUFA synthesis, and, more generally, fatty acid metabolism in fish. Data up to know are however contradictory (see section 1.4.).

In teleost fish, like salmonids, Δ5 and Δ6 desaturases are encoded by the *FADS2* gene (Abdul Hamid et al., 2016; Hastings et al., 2004; Leaver et al., 2008a; Monroig et al., 2010; Seiliez et al., 2001; Zheng et al., 2004a). This contrasts with mammals, in which *FADS1* and *FADS2* genes respectively code for the Δ5 and Δ6 desaturases (Marquardt et al., 2000). In rainbow trout, *FADS2* gene expression was highest in the liver, followed by the intestine, brain (Abdul Hamid et al., 2016; Seiliez et al., 2001), while this gene was not expressed in the stomach, muscle and adipose tissue (Abdul Hamid et al., 2016). Besides, ELOVL2 and ELOVL5 elongases are respectively encoded by the *ELOVL2* and *ELOVL5* genes (Gregory and James, 2014; Hastings et al., 2004; Leaver et al., 2008a; Meyer et al., 2004; Morais et al., 2009). Expression was highest in the liver, intestine and brain of salmonids (Morais et al., 2009). *ELOVL5* gene expression level in rainbow trout liver was higher than that of *ELOVL2* (Gregory and James, 2014).

Transcription of genes coding for desaturases and elongases (FADS2, ELOVL2 and ELOVL5) is controlled by transcription factors: sterol regulatory element binding protein 1C (SREBP-1C) and peroxisome proliferator activated receptor alpha (PPAR-alpha), activate

genes involved in fatty acid anabolism and catabolism, respectively (Carmona-Antoñanzas et al., 2014; Carmona-Antoñanzas et al., 2016; Dong et al., 2017; Jump, 2008; Leaver et al., 2008a; Matsuzaka et al., 2002; Schmitz and Ecker, 2008; Vagner and Santigosa, 2011). SREBP-1C stimulates the transcription of *FASN, acetyl-CoA carboxylase, FADS2* and *ELOVL* genes through binding to a sterol regulatory element (SRE) in the promotor region of these genes (Carmona-Antoñanzas et al., 2014; Carmona-Antoñanzas et al., 2016; Dong et al., 2017; Jump, 2008; Kremmyda et al., 2011; Minghetti et al., 2011; Schmitz and Ecker, 2008; Vagner and Santigosa, 2011; Zheng et al., 2009). The inhibitory effect of PUFAs on the desaturase enzymes would be due to reduction of the nuclear form of SREBP-1C, reduction of the stability of SREBP-1C mRNA and/or inhibition of liver X receptor (LXR)-mediated activation of SREBP-1C (Carmona-Antoñanzas et al., 2014; Cruz-Garcia et al., 2009; Cruz-Garcia et al., 2011; Jump, 2004; Jump, 2008; Kremmyda et al., 2011; Leaver et al., 2008a; Ou et al., 2001; Schmitz and Ecker, 2008; Worgall et al., 1998; Xu et al., 2001). The heterodimer PPAR-alpha-retinoid X receptor activates the transcription of desaturase and β-oxidation related genes through binding a peroxisome proliferator response element (PPRE) in the promotor region of the genes (Dong et al., 2017; Kremmyda et al., 2011; Matsuzaka et al., 2002; Vagner and Santigosa, 2011). The heterodimerisation is only possible when a ligand is bound to the nuclear receptor PPAR-alpha. PUFAs and certain eicosanoids have been identified has PPAR-alpha ligands (Leaver et al., 2008a; Schmitz and Ecker, 2008; Vagner and Santigosa, 2011).

1.3.4.3. Complex lipid generation

Triglyceride and phosphoglyceride synthesis starts with two successive esterifications of activated fatty acyl-CoA units on the alcoholic functions of a glycerol-3-phosphate molecule. These two reactions are respectively catalysed by glycerol-3-phosphate acyltransferase and 1-acyl-glycerol-3-phoshate acyltransferase and generate a phosphatidic acid. In case of triglyceride synthesis, the phosphatidic acid is first hydrolysed into 1,2-diacylglycerol by the phosphatidic acid phosphatase. A third esterification of a fatty acyl-CoA moiety, catalysed by the diacylglycerol acyltransferase, finally leads to the formation of a triglyceride. In case of phosphoglyceride synthesis, a

phosphodiester bound is generated between the phosphatidic acid and a polar head group through two different pathways, depending on the type of phosphoglyceride synthesized. On the one hand, diacylglycerol is produced from phosphatidic acid by the action of phosphatase. Nucleophilic attack by the hydroxyl function of diacylglycerol on activated cytidine diphosphate (CDP)-choline or CDP-ethanolamine respectively generates phosphatidylcholine and phosphatidylethanolamine, with elimination of cytidine monophosphate (CMP). On the other hand, nucleophilic attack by the hydroxyl function of serine or inositol on activated phosphatidic acid (CDP-diacylglycerol) generates phosphatidylserine and phosphatidylinositol respectively, with elimination of CMP (Nelson et al., 2008; Tocher et al., 2008). Base and acyl exchange reactions on existing phospholglycerides enable constant remodelling of phosphoglyceride cellular composition which is called phospholipid turnover (Tocher, 2003; Tocher et al., 2008). On the one hand, phosphatidylserine decarboxylase, phosphatidylserine synthase and phosphatidylethanolamine methyltransferase respectively catalyse conversion of phosphatidylserine into phosphatidylethanolamine, phosphatidylcholine and phosphatidylethanolamine into phosphatidylserine and phosphatidylethanolamine into phosphatidylcholine (Nelson et al., 2008; Tocher et al., 2008). On the other hand, cycles of deacylation of phospholipids and reacylation of lyso-phospholipids catalysed respectively by phospholipases (mainly A1 and A2) and acyltransferase are an important mechanism defining the fatty acid composition of phosphoglycerides (Tocher et al., 2008).

1.3.5. Catabolism

Catabolism of fatty acid is called β-oxidation and takes place in the mitochondria and peroxisomes (National Research Council, 2011; Nelson et al., 2008; Tocher, 2003). Energy generated is used to produce ATP, in the former, or lost into heat, in the latter (Nelson et al., 2008). Fatty acids with aliphatic chain shorter than 14 carbons can enter mitochondria by passive diffusion but not the longer ones (Nelson et al., 2008). The transfer of fatty acids with aliphatic chains of 14 carbons or longer from the cytosol to the mitochondrial matrix is a three-step process (Fig. 1.4). First, acylcarnitine is generated from activated acyl-

CoA by the action of carnitine palmitoyl-CoA transferase 1 (CPT1), enzyme also called carnitine acyltransferase 1 and located in the outer mitochondrial membrane (Fig. 1.4). Then, the acylcarnitine and carnitine are respectively translocated to and out of the mitochondrial matrix, diffusion facilitated by the carnitine-acylcarnitine (CAC) carrier located in the inner mitochondrial membrane (Fig. 1.4). Through the action of CPT2, enzyme located in the inner mitochondrial membrane, acylcarnitine is cleaved into carnitine and acyl-CoA (Fig. 1.4), the latter of which is finally used as substrate for β-oxidation (National Research Council, 2011; Nelson et al., 2008).

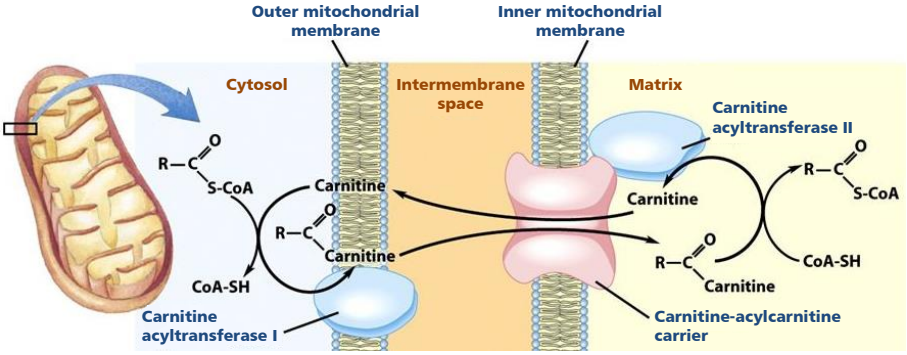


Fig. 1.4. Fatty acid entry into mitochondria. Abbreviations used: R, alkyl group; CoA or CoA-SH, Coenzyme A. Adapted from Nelson et al. (2008).

The β-oxidation is a cycling process that consists in sequential dehydrogenation, hydration, dehydrogenation, and cleavage of the acyl-CoA to release at each cycle an acetyl-CoA unit, NADH and $FADH_2$, resulting in a shortening of the aliphatic chain, two carbons by two carbons (Glencross, 2009; National Research Council, 2011; Nelson et al., 2008; Tocher, 2003). Generated acetyl-CoA units are metabolised through the citric acid cycle in the mitochondrial matrix (Glencross, 2009; Nelson et al., 2008; Tocher, 2003). NADH and $FADH_2$ released through both the β-oxidation and the citric acid cycle are then converted into metabolic energy (ATP) through mitochondrial oxidative phosphorylation (Glencross, 2009; National Research Council, 2011; Nelson et al., 2008; Tocher, 2003). The extent to which fatty acids are β-oxidized is positively correlated to their concentrations in diets. However, SFAs and MUFAs are generally preferred as substrates for β-oxidation over the PUFAs, at least in salmonids. Within PUFAs, shorter

chains and n-6 PUFAs are generally preferred as substrates for β-oxidation over the longer chains and the n-3 PUFAs, respectively (National Research Council, 2011; Stubhaug et al., 2005). DHA is particularly spared from β-oxidation as its Δ4 etylenic bound requires a peroxisomal oxidation to be cleaved (National Research Council, 2011; Sargent et al., 2002; Tocher, 2003).

Fatty acid catabolism is tightly regulated. The carnitine-mediated entry process is the rate-limiting step of the mitochondrial β-oxidation of fatty acids, and thus an important point of regulation (Nelson et al., 2008). In mammals, malonyl-CoA inhibits CPT1, which ensures that *de novo* synthetized fatty acid would be used for complex lipids generation rather than for energy provision through β-oxidation (Nelson et al., 2008). Other direct elements of regulation identified are the inhibition of β-oxidation related enzymes by increased NADH/NAD+ ratio and acetyl-CoA levels (Nelson et al., 2008). At the genetic level, β-oxidation-related genes are, overall, upregulated by the nuclear receptor PPAR-alpha (Jump, 2008; Leaver et al., 2008a; Nelson et al., 2008; Vagner and Santigosa, 2011).

1.3.6. Functions

1.3.6.1. Energy storage and provision

Triglycerides are the densest form of energy with about 38.5 KJ/g. In comparison, proteins and carbohydrate yield respectively 23.6 and 17.3 kJ/g (Glencross, 2009).

When energy available exceeds energy requirement, excess of neutral lipids are packed in VLDL and exported from the liver to the sites of lipid storage (Nelson et al., 2008; Tocher, 2003). In salmonids, lipids are stored in the perivisceral adipose tissue, mainly, and, to a lower extent, in muscles (Corraze et al., 2010; Tocher, 2003). In cladocerans, lipids accumulate in the fat cells, principally located ventrally along the gut (Smirnov, 2017b).

When energy requirement exceeds energy available, triglycerides stored in the adipose tissue are hydrolysed into glycerol and free fatty

acids (Nelson et al., 2008). Those reactions are catalysed by three enzymes: the adipose triglyceride lipase (ATGL) that catalyses the hydrolysis of triglyceride into diglyceride and free fatty acid, hormone-sensitive lipase (HSL) that catalyses the hydrolysis of diglyceride into monoglyceride and free fatty acid and monoglyceride lipase that catalyses the hydrolysis of monoglyceride into glycerol and free fatty acid. Released fatty acids are then exported bound to albumin-like proteins to sites where they are required as fuel (Ruggles et al., 2013; Tocher, 2003; Zimmermann et al., 2009). Fatty acid catabolism (β-oxidation, see section 1.3.5.) is the major source of metabolic energy in fish for reproduction, embryogenesis, development, growth and swimming (Tocher, 2003). In cladocerans, the body lipid reserve varies along with the reproduction cycle. Indeed, fat cells accumulate lipids until their mobilisation to the ovaries at the start of the yolk formation (Smirnov, 2017b; Tessier and Goulden, 1982).

1.3.6.2. Maintenance of biological membranes

Phospholipids and sterols (cholesterol) are fundamental components of biological membranes (Nelson et al., 2008; Sargent et al., 2002; Tocher et al., 2008). The fatty acid composition of membrane phospholipids modulates the physicochemical properties of the lipid bilayer, i.e. fluidity, permeability, which influence its functionality (Arts and Kohler, 2009; Kremmyda et al., 2011; Nelson et al., 2008; Tocher, 2015). Changing the fatty acid composition of the biomembrane enables the homeostatic adaptation to environmental changes. The best described homeostatic response in ectotherms is the increase of the LC-PUFA level in phospholipids to maintain sufficient membrane fluidity under exposure to low temperature (Arts and Kohler, 2009).

Although essential for biomembrane functionality, PUFAs and LC-PUFAs are very sensitive to oxidative attacks by ROS and organic radicals (Catalá, 2009, 2012; Sargent et al., 2002; Tocher, 2003). The richness in n-3 LC-PUFAs of fish biological membranes makes them particularly susceptible to oxidative stress induced by environmental stressors, such as Cd (see section 1.4.).

1.3.6.3. Cellular signalling

PUFAs with 20 and 22 carbons are precursors of eicosanoids, protectins and resolvins, auto- or para-crine modulators with a short half-life. These PUFA-derived signalling molecules are modulators of several processes such as reproduction, inflammation, ion transport and cellular redox status in both vertebrates and invertebrates (Arts and Kohler, 2009; Calder, 2015; Glencross, 2009; Heckmann et al., 2008; Kremmyda et al., 2011; Nelson et al., 2008; Schlotz et al., 2016; Schlotz et al., 2012; Schmitz and Ecker, 2008; Tocher, 2003). Eicosanoid production starts with the liberation of 20 carbons fatty acid precursors (mainly AA but also EPA and, to a lower extent, 20:3n-6) from the phospholipid membranes, while protectin production starts with the release of 22 carbon fatty acids (22:5n-3 and DHA) and resolvin production starts with the release of either 20 or 22 carbon fatty acids (EPA, 22:5n-3, DHA). These first steps are catalysed by phospholipases (mainly the cytosolic phospholipase A2 (cPLA2)). The released precursors will then be oxidized to generate eicosanoids (prostanoids, leukotrienes, lipoxins), protectins or resolvins. These oxidation steps are catalysed by cyclooxygenases, such as COX2, and lipoxygenases, such as ALOX5 (Arts and Kohler, 2009; Calder, 2015; Kremmyda et al., 2011; Nelson et al., 2008; Tocher, 2003). As the same enzymes catalyse the synthesis of eicosanoids from the n-3 and n-6 family, competitions between substrates exist. The AA/EPA ratio of membrane phosphoglycerides, which in turn is influenced by both nutritional and environmental factors, determines the nature of the eicosanoid generated. However, enzymes have an overall higher affinity for n-6 over the n-3 PUFAs. Consequently, AA is the major precursor of eicosanoids in fish cells despite the excess of EPA in fish tissues (Arts and Kohler, 2009; Sargent et al., 2002; Tocher, 2003). Eicosanoids derived from n-3 PUFAs are overall less bioactive than those from n-6 PUFAs (Arts and Kohler, 2009; Calder, 2015; Tocher, 2003). Besides, n-3- and n-6-derived lipid mediators often play antagonistic roles in cells. For example, prostaglandins and leukotrienes derived from AA and EPA have respectively pro- and anti-inflammatory properties (Arts and Kohler, 2009; Calder, 2015; Kremmyda et al., 2011; Schmitz and Ecker, 2008). Resolvins and protectins derived from n-3 LC-PUFAs also have

potent anti-inflammatory and pro-resolving properties (Kremmyda et al., 2011).

Fatty acids, and especially PUFAs, are regulators of gene transcription. PUFAs interact with PPARs, LXR and SREBP, transcription factors regulating genes involved in fatty acid metabolism. For example, PUFAs are ligand of the nuclear receptor PPAR-alpha, which regulates genes involved in fatty acid biotransformation and catabolism. Specific eicosanoids have also been identified as natural PPAR ligands (Jump, 2004; Jump, 2008; Kremmyda et al., 2011; Leaver et al., 2008a; Schmitz and Ecker, 2008; Tocher, 2003; Vagner and Santigosa, 2011).

Biological lipids vary a lot in chemical structure, so do their biological role as cellular signalling molecules. For example, phosphoinositides, which are phosphorylated derivatives of phosphatidylinositol, play roles in golgi, lysosome and endosome trafficking, ion homeostasis and cell proliferation, survival and migration (Kremmyda et al., 2011; National Research Council, 2011; Tocher et al., 2008). Besides, phosphatidic acid is involved in cell proliferation, transformation and survival. In addition, ceramide and sphingomyelin regulate protein kinases, cell division, differentiation, migration and apoptosis (National Research Council, 2011).

1.4. Interplay between dietary lipids, fatty acid metabolism and metallic pollution

Dietary lipids and metallic coumpouds can both influence fatty acid homeostasis and metabolism in aquatic biota. Besides, dietary lipids can modulate biological response to metal (e.g. Cd and MeHg) stress. This section will further detail these interactions, with a major focus on Cd.

1.4.1. Impact of dietary lipids on fatty acid homeostasis and metabolism

Fish oil and fish meal, which are rich in the n-3 LC-PUFAs EPA and DHA are classically 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 (Bell and Tocher, 2009; FAO, 2018; Sargent et al., 2002; Tocher, 2015). In 2012, aquaculture used 75% of the global fish oil supply. Over 80% of the fish oil used by this sector was incorporated in aquafeeds, mainly for salmonids (62%) (Tocher, 2015). Fish oil is, however, a finite marine source that has become rare and expensive. For mainly economic (increased price) but also environmental (overfishing) reasons, fish oil has thus been progressively replaced by more sustainable alternative lipid sources in feed formulations, mainly plant-derived oils (FAO, 2018; Sargent et al., 2002). As a result of this replacement, global fish oil consumption has been stable since the last decade (0.8 million metric tons/year) despite the constant expansion of the aquaculture (+ 6.2% annual increase between 2000 and 2012) (Tocher, 2015). 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 aquafeeds (Bell and Tocher, 2009; Glencross, 2009; Sargent et al., 2002; Turchini et al., 2009). Rapeseed and olive oils (both rich in 18:1n-9), palm oil (rich in 16:0 and 18:1n-9), linseed oil (rich in ALA), sunflower oil (rich in LA) and soybean oil (rich in LA) are examples of plant-derived oils, widely studied as alternative lipid sources for salmonids (Francis et al., 2014; Glencross, 2009; Mellery et al., 2017; Sargent et al., 2002; Thanuthong et al., 2011; Turchini et al., 2018). However, some plant-derived alternatives (e.g. linseed oil) are still expensive, which limits their use in aquaculture farming (Turchini et al., 2009). As described in section 1.3., salmonids, including rainbow trout, have a naturally high fatty acid bioconversion ability and can produce EPA and DHA from ALA, and AA from LA (Tocher, 2003). This natural ability is further increased when salmonids are fed plant-derived diets. Desaturases and elongases are indeed generally upregulated (from two to three times) at both the gene expression and enzymatic activity level in salmonids fed a plant-derived diet as compared to fish fed a fish-derived diet (Dong et al., 2017; Fonseca-Madrigal et al., 2005; Morais et al., 2009; Stubhaug et al., 2005; Tocher, 2015; Vagner and Santigosa, 2011; Zheng et al., 2004b; Zheng et al., 2005a; Zheng et al., 2005b). The enhancement of the LC-PUFA synthesis is suggested to be the consequence of both the lower LC-PUFA (especially DHA) and higher PUFAs (ALA and LA) dietary levels, as they are known to respectively downregulate and upregulate desaturase and elongase genes (Gregory

et al., 2016; Leaver et al., 2008b; Minghetti et al., 2011; Tocher, 2015; Vagner and Santigosa, 2011; Zheng et al., 2009). The latter upregulation is believed to be triggered by the binding to the SREBP transcription factor (Carmona-Antoñanzas et al., 2014; Dong et al., 2017; Minghetti et al., 2011). However, despite this stimulation of bioconversion pathways, 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 and Tocher, 2009; Bell et al., 2001; Dong et al., 2017; Leaver et al., 2008b; Mellery et al., 2017; Turchini et al., 2018). As a result, the fatty acid composition of fish tissues after a feeding trial globally reflects that of the experimental diet (Bell et al., 2004; Bell et al., 2006; Bell et al., 2001; Caballero et al., 2002; Fonseca-Madrigal et al., 2005; Francis et al., 2014; Mourente and Bell, 2006; Petropoulos et al., 2009). Different feeding strategies are thus studied and implemented in order to grow fish of acceptable quality (n-3 LC-PUFA richness of the flesh) at acceptable production cost. For example, salmonids are commonly fed a plant-derived diet (cheaper but lacking n-3 LC-PUFAs) throughout the grow-out period followed by a finishing diet containing fish oil (more expensive but rich in n-3 LCPUFAs) before slaughter (Bell and Tocher, 2009; Glencross, 2009).

The body fatty acid profile of the cladoceran *Daphnia magna* overall mimics that of its diet, without, however, being exactly similar. Specific fatty acids, such as EPA and AA, are indeed preferentially retained while others, such as DHA are not significantly accumulated (Brett et al., 2009; Martin-Creuzburg et al., 2010; Taipale et al., 2011). Instead, *Daphnia magna* was shown to retroconvert dietary DHA to EPA and dietary 22:5n-6 to AA (Martin-Creuzburg et al., 2010; Strandberg et al., 2014; Taipale et al., 2011). Fatty acid composition in *Daphnia magna* was shown to be highly plastic, with a very fast turnover. Indeed, *Daphnia magna* replaced more than 50% of their fatty acids within 2 days of diet switching. A new steady state was reached after 6 days of diet switching, with replacement of up to 95% of initial body fatty acids (Taipale et al., 2011). *Daphnia magna* provisioned their offspring with different amounts of PUFAs depending on dietary availability, with, however, a preferential allocation of LC-PUFA, especially EPA and AA, to eggs than to somatic tissues (Schlotz et al., 2013; Sperfeld and Wacker, 2015; Wacker and Martin-Creuzburg, 2007). Finally, a preferential allocation

of ALA into eggs in case of low dietary PUFA levels was also reported (Sperfeld and Wacker, 2015).

1.4.2. Impact of MeHg and Cd on fatty acid homeostasis and metabolism

It is increasingly recognized that pollutants, such as heavy metals, can affect cellular fatty acid homeostasis (i.e., uptake, synthesis and use for signalling, structural or energetic purposes) (Yadetie et al., 2016; Zhou et al., 2022). Evidences from *in vitro* and *in vivo* studies highlighted an influence of Cd and MeHg on lipid accumulation, mobilisation, synthesis and oxidation at organismal, tissue and cellular levels. However, reported effects are highly variable and depend on exposure characteristics (concentration, duration) (e.g. Koskinen et al., 2004), on the tissue investigated and on environmental conditions (water temperature during fish growth) (e.g. Fadhlaoui et al., 2018), highlighting the complexity of mechanisms.

Long-term exposure to Cd dose-dependently increased hepatic lipid deposition in zebrafish (Pan et al., 2018; Zheng et al., 2016b) and in yellow catfish (*Pelteobagrus fulvidraco*) (Chen et al., 2013), through stimulation of fatty acid *de novo* synthesis (Chen et al., 2013; Pan et al., 2018), decrease of lipolysis and fatty acid catabolism (Pan et al., 2018) and/or increase of lipid import into the liver (Chen et al., 2013). Indeed, increased hepatic triglyceride (Chen et al., 2013) or total lipid (Pan et al., 2018) levels induced by Cd went along with upregulation of lipogenic enzymes, including FASN gene expression and activity (Chen et al., 2013; Pan et al., 2018), downregulation of expression of genes coding for enzymes involved in lipolysis and fatty acid catabolism, such as triglyceride lipase, hormone-sensitive lipase and CPT1 (Pan et al., 2018), and/or upregulation of hepatic lipoprotein lipase activity, involved in lipid import (Chen et al., 2013). Similarly, long-term exposure to Cd increased hepatic FASN activity in javelin goby (*Synechogobius hasta*), without however affecting hepatic lipid content (Song et al., 2014). Although, Cd either downregulated (Chen et al., 2013) or had no impact on (Pan et al., 2018) hepatic lipoprotein lipase gene expression. Hepatic CPT1 was not affected by Cd in yellow catfish (Chen et al., 2013). Interestingly, an overall opposite pattern was reported for MeHg in

Atlantic cod (*Gadus morhua*) (Yadetie et al., 2013) and fathead minnow (*Pimephales promelas*) (Klaper et al., 2008), with genes involved in hepatic fatty acid catabolism and anabolism being respectively up- and downregulated by the organo-metal. In contrast, MeHg downregulated hepatc *CPT1* gene expression in Atlantic salmon (Olsvik et al., 2011). This impact was, however, only observed in fish fed a fish oil based diet, which highlights, once more, the existing interaction between dietary lipid and biological responses to metallic compounds. MeHg was finally reported to increase the hepatic expression of an apolipoprotein in fathead minnow, suggesting a potential impact of the organo-metal on lipid and cholesterol transport (Klaper et al., 2006). The latter effect was, however, only observed in females, pointing out again complex interactions (Klaper et al., 2006).

A different pattern was observed in fish muscles. On the one hand, Cd dose-dependently decreased the efficiency of muscular lipid accumulation in European yellow eel (*Anguilla anguilla*) (Pierron et al., 2007), rainbow trout (Konar et al., 2010) and carps (*Labeo rohita, Cirrhinus mrigala* and *Catla catla*)(Garg et al., 2009). However, Cd had no impact on muscular lipid accumulation in yellow catfish, which was attributed to concomitant downregulation of genes involved in lipogenesis (including *FASN*), lipid import (lipoprotein lipase) and fatty acid catabolism (*CPT1*)(Chen et al., 2013). On the other hand, MeHg upregulated genes involved in lipid transport (e.g. apolipoprotein Eb, high-density lipoprotein-binding protein, fatty acid binding protein 10) in muscle of zebrafish males (Cambier et al., 2010).

The adipose tissue has key role in lipid and energy storage. MeHg has been resported to display pro-adipogenic properties in rainbow trout preadipocyte (Tinant et al., 2021). Indeed, MeHg dose-dependently increased neutral lipid, especially n-3 PUFAs, accumulation at least partly through upregulating fatty acid transport/intake (upregulation of *FABP11a* and apolipoprotein EB gene expressions), *de novo* synthesis (upregulation of *FASN* gene expression) and adipocyte maturation (upregulation of perilipin 2 gene expression) (Tinant et al., 2021). MeHg also modulated the expression of other target genes playing role in fatty acid metabolism (e.g. *FATP1*, lipoprotein lipase, glycerol-3-phosphate dehydrogenase) (Tinant et al., 2021). However the impacts varied with

experimental conditions (MeHg concentration, exposure duration, presence/absence of hormonal cocktail of differenciation) (Tinant et al., 2021), highlighting once more the complexity of results and the need for taking caution while comparing results from different experiments). In contrast to MeHg, the impact of Cd on fish adipose tissues has never been investigated yet. Still, Cd was reported to dose-dependently decrease adipose tissue weight and adipocytes size, impair adipo- and lipogenesis and induce lipolysis notably by downregulating adipose tissue differentiation markers (CCAAT/enhancer-binding protein and PPAR-gamma), adipokines (adiponectin and leptin) and perilipin gene expression in mice white adipose tissue (Attia et al., 2021; Kawakami et al., 2013; Kawakami et al., 2010; Lee et al., 2012).

Whole body analysis yielded contrasting results, which might reflect different response patterns of internal compartments. For instance, in rainbow trout fry, a Cd challenge of a low intensity (0.05 or 0.025 mg/L, 24 h) upregulated genes related to fatty acid biosynthesis while a Cd challenge of a higher intensity (0.5 mg/L, 96 h) downregulated these genes (Koskinen et al., 2004). Reduction of the body lipid reserves was observed in juvenile daphnids exposed to Cd (2 and 5 µg/L, 48 h) (Barata et al., 2005) and in adult daphnids exposed to Cd (10-100 µg/L, 48-96 h) (Soetaert et al., 2007). On the other hand, no impact was observed in daphnids exposed to Cd (1 and 5 µg/L, 7-21 days) despite reduction of the feeding rate and growth under metal exposure (Bodar et al., 1988). Downregulation of filter-feeding rate, lipid absorption and transport as well as upregulation fatty acid β-oxidation to meet increased energy demand were suggested as possible mechanisms of Cd-induced reduction of lipid reserves in daphnids (Muyssen et al., 2010; Poynton et al., 2011; Poynton et al., 2007).

Cd impacts on hepatic lipid deposition and metabolism was suggested to be mediated by oxidative stress and mitochrondrial dysfunction in zebrafish (Pan et al., 2018). Indeed, pretreatment with N-acetylcystein, a precursor of GSH and a ROS scavenger, reversed the impacts of Cd on lipid accumulation, lipolytic and lipogenic enzyme activities or gene expressions while preventing hepatocytes from Cd-induced lipid peroxidation, reduction of antioxidant enzyme activities

(glutathione peroxidase, superoxide dismutase) and reduction of mitochondrial ATP production (Pan et al., 2018).

Cd and MeHg indirectly induce oxidative stress through both increasing ROS production and weakening cellular antioxidant defences. Oxidative stress can cause lipid peroxidation. Lipid peroxidation is a three step process. In the initiation step, peroxide radical is generated by electrophilic attack of a highly energetic and instable molecule, such as a ROS, on the ethylenic bound of a PUFA. The peroxide radical can then react with another PUFA to generate a second peroxide radical, leading to uncontrolled chain reaction of lipid peroxidation (propagation step). This finally ends up (termination step) either by reaction between two radical species or by intervention of the antioxidant system (molecular or enzymatic), with generation of hydroperoxides, which can then be cleaved and give rise to stable but cytotoxic compounds with aldehyde, cetone or alcool functions (Catalá, 2009, 2012; Sargent et al., 2002). Increased of malondialdehyde (MDA) or thiobarbituric acid reactive substances (TBARS), the most common endpoints to assess lipid peroxidation, were observed in aquatic organisms exposed to Cd (Jamwal et al., 2018; Li et al., 2011; Pan et al., 2018; Wang et al., 2015; Zheng et al., 2016a; Zheng et al., 2016b) and MeHg (Berntssen et al., 2003, Mozhdeganloo et al., 2015). Accumulation of oxidized phospholipids in biological membranes impairs lipid-lipid and protein-lipid interactions within the bilayer with consequences on transmembrane proteins functions and membrane integrity (Catalá, 2009, 2012). Peroxidation of mitochondrial membrane lipids can lead to mitochondrial depolarisation and cellular death, which has been shown under exposure to Cd (An et al., 2014; López et al., 2006; Wang et al., 2014) and MeHg (Nøstbakken et al., 2012a). Besides, Cd (5 µM, 24 h) decreased the proportion of cardiolipin, a phospholipid generally rich in DHA and exclusively located in the inner mitochondrial membrane, in cellular phospholipids of the human hepatocellular cell line Hep G2, possible through oxidation (Linhartova and Sampels, 2015). This effect was, however, not observed at lower Cd concentrations (1 µM, 24 h) (Linhartova and Sampels, 2015).

Unsaturated fatty acids (especially LC-PUFA) are prone to oxidation (Catalá, 2012; Sargent et al., 2002). Oxidative stress induced by Cd and

MeHg would thus decrease the MUFA, PUFA and LC-PUFA tissue or cellular levels. However, so far, data so far suggest that MeHg has few impacts on the relative amounts of PUFAs in liver, brain and muscle of Atlantic salmon (Amlund et al., 2012; Olsvik et al., 2011) as well as in mouse and rainbow trout liver phospholipids (Ferain at al., 2018; Zeng et al., 2016). On the other hand, Cd reduced the relative abundance of PUFA in muscle of rainbow trout (Konar et al., 2010) and yellow perch (*Perca flavescens*) under specific growth conditions (Fadhlaoui and Couture, 2016). However, Cd was also reported to increase the MUFA (Fadhlaoui and Couture, 2016) and PUFA (Fadhlaoui et al., 2018) relative abundances in fish (under specific growth conditions), showing more complex mechanisms of interactions, such as modulation of desaturase, elongase activities and/or gene expressions as well as the induction or repression of the eicosanoid cascade by Cd.

Cd (0.5-1 mg/L, 10 days) decreased the hepatic 18:1n-9 relative abundance (lowest dose tested), Δ9 desaturase activity (lowest dose tested) and gene expression (both doses tested) in silver pomfret (*Pampus argenteus*) (Shi et al., 2020). Similarly, Cd (0.5-5 µM, 1-3 days) decreased levels of 18:1n-9 in both triglycerides and phospholipids of in primary cultures of rat hepatocytes, notably through inhibition of Δ9 desaturase (Kudo and Waku, 1996). However, Cd had no direct impact on Δ9 desaturase activity in isolated liver microsomes of rats. The authors thus suggested that the inhibition observed in primary cultures of rat hepatocytes would be due to reduction of the Δ9 desaturase content, possibly through modulation of gene expression, rather than to direct impairment of enzyme functionality (Kudo and Waku, 1996). Cd effects on Δ9 desaturase in other tissues varied. Cd (1 mg/L, 10 days) increased the intestine 18:1n-9 relative abundance, Δ9 desaturase activity and gene expression in silver pomfret while a challenge of a lower intensity (0.5 mg/L, 10 days) had no impact (Shi et al., 2020). Cd (5.7 µg/L, 8 weeks) decreased Δ9 desaturase gene expression level in muscle of fathead minnow reared at 30°C while no impact was observed in muscle of fish reared at 15°C or 25°C (Fadhlaoui et al., 2018), neither on muscle of silver pomfret exposed to Cd (0.5-1 mg/L, 10 days) (Shi et al., 2020). The downregulation of Δ9 desaturase gene expression in muscle of fathead minnow went, however, not along with a reduction of MUFA or 18:1n-9 relative abundances. Cd (5.7 µg/L, 8 weeks) had no

impact on Δ9 desaturase gene expression in brain of fathead minnow (Fadhlaoui et al., 2018). MeHg impacts on Δ9 desaturation ability has been poorly assessed. Still, MeHg (intraperitoneal injection of 2 µg/g body mass, 4 days) downregulated hepatic Δ9 desaturase gene expression in fathead minnow (Klaper et al., 2008). In line with these results, MeHg slightly decreased the level of 18:1n-9 and the total MUFA content in RTL-W1 cells enriched in ALA (Ferain et al., 2018). However, no impact was observed in control and LA-, EPA and AA-enriched cells (Ferain et al., 2018), showing interaction between fatty acid supply and cellular MeHg impacts on fatty acid homeostasis.

Data available to date on the impact of Cd and MeHg on bioconversion of PUFA are highly dynamic and no common pattern could be identified. On the one hand, Cd (5.7 µg/L, 8 weeks) respectively up- and down-regulated *FADS2* gene expression in the brain of fathead minnow reared at 25°C and in the muscle of the fish reared at 30°C, while having no impact on the expression of that gene in the brain of fish reared at 15, 30°C neither in the muscle of fish reared at 15, 25°C. In fish reared at 15°C, Cd downregulated *ELOVL2* gene expression in the brain and upregulated *ELOVL5* gene expression in the muscle. Cd had no impact on the expression of *ELOVL2* and *ELOVL5* genes in both organs in fish reared 25, 30°C. Cd-induced modifications in membrane fatty acid composition of these fish was complex and not systematically correlated with transcription levels of desaturase and elongase, suggesting that post-transcriptional regulation of elongases and desaturases by Cd and/or other membrane biosynthetic processes may be involved (lipid oxidation, phospholipid turnover). Similarly, Cd (0.5 and 1 mg/L, 10 days) decreased the relative abundance of n-3 PUFA (mainly DHA) in brain of silver pomfret while upregulating *FADS2* and *ELOVL5* gene expressions (Liao et al., 2019). The authors suggested that the upregulation of desaturase and elongase genes may be a coping mechanism for the reduction of DHA caused by Cd-oxidative stress in the brain (Liao et al., 2019). This upregulation was organ-specific as no impact was observed in liver and in intestines (Liao et al., 2019). On the other hand, MeHg was reported to upregulate hepatic *FADS2* gene expression in juvenile flounder (*Paralichtys olivaceus,* 1 µg/L, 30 days)(Ren et al., 2022) while decreasing the expression of that gene in zebrafish larvae (1 µM, 4 days) (Lu et al., 2017). Interestingly, MeHg (5

mg Hg/kg, 84 days) downregulated hepatic Δ5 desaturase gene expression in Atlantic salmon fed a vegetable oil based diet but not in fish fed a fish oil based diet (Olsvik et al., 2011). MeHg (5 mg Hg/kg, 84 days) had no impact on hepatic Δ6 desaturase gene expression irrespective of the diet fed to fish (Olsvik et al., 2011). Responses depend thus on targeted species, tissues, metal concentrations and quality of the diets.

Cd and MeHg were both reported to induce the eicosanoid cascade. On the one hand, Cd (1-5 μM, 2-24 h) was reported to induce AA release from membrane phospholipids and increase prostaglandine E2 production by induction of cPLA2 activity and gene expression and *COX2* gene expression in primary mouse osteoblastic cells (Miyahara et al., 2001). Upregulation of *cPLA2* gene expression was also observed in the liver of rat 24 h after intraperitoneal injection of Cd (1.25 mg/body weight) (Madejczyk et al., 2015). Besides, Cd (1 mg/L) upregulated COX2 in zebrafish liver (Zheng et al., 2016a). Effects were first observed at the transcriptional level, i.e. after 24 h exposure, then at the post-translational level (protein content and enzyme activity), i.e. after 96 h exposure (Zheng et al., 2016a). A similar pattern was observed in the brain and ovary of fish exposed to Cd, except that Cd had no impact on COX2 protein content and gene expression in the brain and ovaries of fish, respectively (Zheng et al., 2016a). On the one hand, MeHg induced AA release from membrane phospholipids in rat astrocytes (Shanker et al., 2002), rat neurons (Shanker et al., 2004), bovine pulmonary artery endothelial cells (Mazerik et al., 2007; Sherwani et al., 2013) and rainbow trout preadipocytes (Tinant et al., 2021). Similarly, chronic MeHg intoxication (5 mg Hg/kg in the diet during 3 months) decreased the relative amounts of AA in brain phospholipids of Atlantic salmon (Amlund et al., 2012). The AA release went along with a significant increase of the cellular cPLA2 level in rat astrocytes (Shanker et al., 2002) and in rat neurons (Shanker et al., 2004), upregulation of *cPLA2* gene expression in rat astrocytes (Shanker et al., 2002) and with a higher production of prostaglandine E2 and lipoxygenase products (5-, 12- and 15-hydroxyeicosatetraenoic acids) in bovine pulmonary artery endothelial cells (Sherwani et al., 2013). MeHg was also reported to upregulate *COX2* gene expressions in the Atlantic salmon kidney cell line (ASK) (Nøstbakken et al., 2012a). Finally, MeHg tended to upregulate

COX2 and *ALOX5* expression in RTL-W1 cells, in a PUFA-supply dependent manner (Ferain et al. 2018). Consequences of the induction of the eicosanoid cascade by Cd and MeHg may be multiple as these PUFA-derived molecules are modulators of a wide variety of biological processes such as inflammation, ion transport and cellular redox status (Kremmyda et al., 2011).

1.4.3. Impact of dietary lipids on biological response to MeHg and Cd

Knowing the biological functions of lipids and fatty acids, i.e. energy storage and provision, membrane physico-chemical structure maintenance, cellular signalling (either as nuclear receptor ligand or as precursors of signalling molecules) (Tocher, 2003), it appears plausible that they could play key roles in organismal, tissue and cellular response to pollutants such as MeHg and Cd, in fish and zooplankton.

Such interaction has, however, either poorly (for MeHg) or never (for Cd) been investigated in aquatic experimental models.

On the one hand, preincubation of ASK cells in growth media supplemented with specific PUFAs modulated MeHg toxicity. Ideed, EPA protected against while DHA potentiated and AA had no impact on MeHg-induced cell apoptosis. PUFA impacts were not linked to a differential cellular uptake (Nøstbakken et al., 2012a). Proteomic analyses highlighted potential interaction sites between PUFAs and MeHg, however, only a few targets enabled descriminition between DHA and EPA opposite effects (Nøstbakken et al., 2012b). EPA protection was suggested to be mediated through modulation of calcium intracellular regulation and/or the eicosanoid cascade (Nøstbakken et al., 2012b). Besides, a change in dietary fatty acid composition, obtained by substituting fish oil (rich in n-3 LC-PUFAs) with vegetable oils (deprived of n-3 LC-PUFAs) in diets was found to modulate the transcriptional response to MeHg in liver, white muscle and brain of Atlantic salmon, with caspase-dependent apoptotic response, calcium intracellular regulation and the antioxidant system being suggested as possible interactive pathways between PUFAs and MeHg (Olsvik et al., 2011). Extension to research with mammalian models strengthens the body of

evidence about the existing interaction between dietary lipids and MeHg toxicity. Overall, data showed that dietary lipids can modulate metal uptake, ion homeostasis, redox status, energy metabolism, cell signalling and survival under MeHg stress (Grotto et al., 2011; Jayashankar et al., 2012; Jin et al., 2008; Jin et al., 2007; Kaur et al., 2008; Kaur et al., 2007; Nøstbakken et al., 2012a; Oguro et al., 2021; Pal and Ghosh, 2012; Remø et al., 2011; Takanezawa et al., 2019; Zhang et al., 2021). However, results were sometimes contradictive (e.g. DHA increased (Kaur et al., 2007; Takanezawa et al., 2019) vs decreased (Kaur et al., 2008; Oguro et al., 2021; Zhang et al., 2021) MeHg-induced oxidative stress) and derived from studies testing fatty acid mixtures (blends or oils) which makes the isolation of the effects of individual fatty acids harder.

On the other hand, short-term Cd toxicity was increased by the enrichment of yeast (*Saccharomyces cerevisiae*) cellular lipids in either LA or ALA (Howlett and Avery, 1997). Indeed, viability loss and potassium efflux (as marker of plasma membrane permeabilisation) induced by Cd exposure (50-100 µM, up to 24 h) was accentuated by LA- or ALA-enrichments (Howlett and Avery, 1997). In contrast, a mixture of EPA and DHA protected human hepatocellular cells (Hep G2 cell line) against short-term Cd exposure (Linhartova and Sampels, 2015). Indeed, supplementation of growth media with n-3 LC-PUFAs (EPA and DHA mixture) increased Hep G2 cellular tolerance to Cd (5-20 µM, 24-48 h). PUFA-derived cytotoxicity (in yeast) or cytoprotection (in Hep G2 cells) was not explained by modulation of Cd uptake as PUFA-enrichments either had no impact on Cd burden in yeast (Howlett and Avery, 1997) and in Hep G2 cells (1-2.5 µM Cd, 24 h) (Linhartova and Sampels, 2015) or even increased Cd burden in Hep G2 cells (5 µM Cd, 24 h) (Linhartova and Sampels, 2015). The ALA- and LA-derived cytotoxicity was suggested to be linked to the sensitivity of these PUFA to oxidation (Howlett and Avery, 1997). In contrast, enrichments with EPA and DHA, i.e. two LC-PUFAs more prone than ALA and LA to oxidation, protected Hep G2 cells against the oxidative Cd-induced damage to lipids and proteins (Linhartova et al., 2016). Interestingly, this protective effect was associated with stable levels of superoxide dismutase activity and total non-enzymatic antioxidant capacity in cells enriched in these two LC-PUFAs while these two markers were significantly increased by Cd (5 µM, 24 h) in control cells (Linhartova et

al., 2016), suggesting that LC-PUFA-derived protection was not associated with a stimulation of the antioxidant markers analysed. Further research is needed to unravel mechanisms of protection. Besides, dietary crude palm oil protected rabbit against the oxidative damage (lipid peroxidation, impaired superoxide dismutase, catalase, sodium/potassium-ATPase, magnesium-ATPase, calcium-ATPase activities), induced by Cd in retina (2 mg/kg, given daily as eye drops during 4 weeks) (Eriyamremu et al., 2008). However, protection was related to crude palm oil richness in β-carotene instead of its fatty acid composition (Eriyamremu et al., 2008). Later on, Amamou et al. (2015) showed that inclusion of olive oil or colocynth oil in diets of rats prevented from the generation of plasmatic and hepatic lipid and protein oxidation and from the depletion of the hepatic GSH level induced by Cd exposure (50 mg/L in drinking water during 8 weeks), while inclusion of sunflower oil in diets (control) did not confer such protection. Besides, plasmatic and hepatic catalase and superoxide dismutase activities were less affected by Cd when olive oil or colocynth oil were included in diets as compared to sunflower oil (Amamou et al., 2015). However, again, the protective effect of olive and colocynth oils could not be specifically related to their fatty acid compositions as they also contained other valuable components, such as vitamins and phenolic compounds, in varying concentrations (Amamou et al., 2015). Finally, feeding mice with Euterpe oleracea oil during 42 days after 7 days of Cd exposure (4.28 mg/kg) restored the structural biochemical changes induced by Cd in testicular parenchyma. The oil richness in oleic acid, which has antioxidant properties, was suggested to be responsible for the regenerative potential of the Euterpe oleracea oil (Mouro et al., 2020). However, the antioxidant properties of Euterpe oleracea oil may also result from its richness in vitamin E. Still, inclusion of oleic acid in feeds of rats increased survival rate and decreased the oxidative damage (lipid peroxidation, impaired superoxide dismutase activity, ROS production) induced by Cd (30 mg/kg, 3-7 days) in different organs (including liver), with, however, varying efficiencies depending of the organ and inclusion level in the feed (Wang et al., 2019). Protection was linked to the ROS scavenging activity of oleic acid (Wang et al., 2019).

Altogether, these *in vitro* and *in vivo* results suggest that the growth media or dietary fatty acid composition may modulate organismal, tissue and cellular response to MeHg and Cd. However, data available to date are contradictive, focus either on a narrow set of individual fatty acids (ALA vs LA, EPA vs DHA, oleic acid) or on mixtures (EPA+DHA, oils), which precluded a detailed mechanistic understanding of the effect of individual fatty acids in the elicited responses. Finally, it should be noted that most of the research data cited in this section have been published during or even after the experimental part of the present research project.

1.5. Reference models

Rainbow trout and large water flea are the two reference models used in this work.

Rainbow trout is a fish species belonging to the *Oncorhynchus* genus, from the *Salmonidae* family, from the *Salmoniformes* order, from the *Teleostei* class. Rainbow trout is native to coastal waters and cold-tributaries of the Pacific basin, from the West coast of the USA to the Kamchatkan Peninsula (Russia) (Guyomard and Vandeputte, 2010; Hardy, 2002; Purser, 2019). Since 1870, rainbow trout have been introduced for both recreational and economic reasons in many countries and is nowadays found in many cold, clear and well-oxygenated freshwater bodies throughout the world, except in Antarctica (Crawford and Muir, 2008; Guyomard and Vandeputte, 2010). Rainbow trout strains are either freshwater or anadromous strains. The latter ones live a few years in the Pacific Ocean before returning to their freshwater birthplace to spawn. Rainbow trout is iteroparous and spawn between autumn and spring (Bobe et al., 2010; Purser, 2019). Rainbow trout is carnivorous. Natural preys are mostly invertebrates (zooplankton, crustaceans and insects) but also vertebrates (small fish) (Hardy, 2002). Rainbow trout is used in intensive aquaculture, especially in Europe, due to its high nutritional value, fast growth, large dietary tolerance (dry formulated diets), high resistance against disease and stress, easy reproduction method and high tolerance to high fish density culture (Woynarovich et al., 2011). Rainbow is classified as an intermediate fish (2-10% of lipids). Lipids

accumulate in the perivisceral adipose tissue and in the filet (Corraze et al., 2010; Fontagné-Dicharry and Médale, 2010; Médale, 2010). The gastrointestinal tract is subdivided into the foregut with mouth, pharynx, short muscular oesophagus and J-shaped stomach (split into cardiac, intermediate and pyloric regions), the midgut with pyloric caeca and the hindgut ending by the rectum (Fig. 1.5) (Kamalam et al., 2020). Accessory organs consist of the liver and a diffuse exocrine pancreas (National Research Council, 2011). The liver is a key organ for both lipid metabolism and contaminant response. Rainbow trout is commonly used as model species in nutrition and toxicology studies. The existence of a rainbow trout liver cell line, i.e. the Rainbow Trout Liver Waterloo 1 (RTL-W1) cell line (Lee et al., 1993), also allows both screening and mechanistic investigations. The RTL-W1 cell line has been developed from the non-tumorous liver of a four-year-old male trout. The first and second trypsinization of the cell culture were performed respectively 5 and 9 months after cell isolation for primary culture. About 100 passages were performed within 8 years of culture. Time to reach confluence progressively decreased to reach a periode of 7-10 days (Lee et al., 1993). The origin of the RTL-W1 has been debated. Based on cytological, immunocytochemical, ultrastructural and growth characterization, it was suggested that the RTL-W1 cell line was originally preductular epithelial cell. Those cells are are considered as stem cells in teleost liver capable of differentiating into both hepatocytes and biliary cell lineages (Lee et al., 1993; Malhao et al., 2013). RTL-Wl cells exhibit a number of important liver-typic functions including CYP450 and ABC transporters activities and has thus been widely used as a model for studying the physiology and the xenobiotic metabolism of the fish liver (Bols et al., 2017; Dayeh et al., 2005; Dimastrogiovanni et al., 2015; Heinrich and Braunbeck, 2020; Kienzler et al., 2015; Lammel et al., 2019; Lee et al., 1993; Malhão et al., 2013; Önlü and Saçan, 2017; Pannetier et al., 2018; Perovic et al., 2012; Schnell et al., 2009).

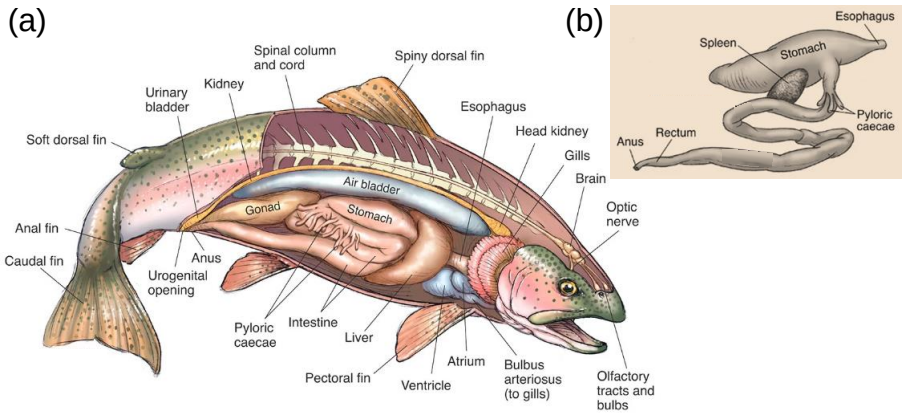


Fig. 1.5. Morphological overview of rainbow trout (a) female and (b) digestive tract. Adapted from the *illustration of the external and internal anatomy of a typical bony fish, the rainbow trout* by Dave Carlson.

The large water flea is a crustacean species belonging to the *Daphnia* genus from the *Daphniidae* family, from the *Cladocera* suborder, from the *Branchiopoda* class (Fig. 1.6) (Ebert, 2005). The large water flea inhabits all types of standing freshwater bodies throughout the world, especially ephemeral ponds deprived of fish predators (Altshuler et al., 2011; Ebert, 2005; Miner et al., 2012). Under favourable conditions, reproduction is cyclically parthenogenetic (asexual) (Fig. 1.7) (Ebert, 2005; Smirnov, 2017c). Diploid parthenogenetic eggs are laid and develop in the brood chamber. Embryos are released while moulting (Smirnov, 2017c). Under unfavourable conditions (ex: habitat desiccation, thermal or chemical stress, changes in food quality or quantity) males are released and females produce two large haploid eggs. After fertilization by males, the diapausing eggs are enclosed in a capsule, called the epiphippium, and laid. Resting eggs hatching is under environmental control and gives rise to females (Fig. 1.7) (Ebert, 2005; Smirnov, 2017c). The large water flea is a non-selective pelagic filter-feeder. Food particles suspended in the outer medium are filtered by filtering fans consisting of setae on thoracic limbs (Geller et al., 1981; Smirnov, 2017b). Its natural diet is composed of a wide variety of microorganisms and particulate suspended matter with size ranging from 0.6 to 40 µm (Geller et al., 1981). The gastrointestinal tract is subdivided into the foregut with mouth and oesophagus, the midgut with a pair of blind diverticula, also called the digestive caeca, and the

hindgut ending by the rectum (Fig. 1.6) (Ebert, 2005; Smirnov, 2017b). *Daphnia sp.* occupies a central position in freshwater food webs as it transfers biomass and macronutrients, such as lipids, from the primary producers to higher trophic levels (Ebert, 2005; Miner et al., 2012). Lipids are stored in fat cells principally located ventrally along the gut (Smirnov, 2017b). The large water flea is commonly used as model species in ecotoxicological studies as it (i) has a short generation time, (ii) can easily be handled and maintained in controlled laboratory conditions and (iii) has a parthenogenetic reproduction cycle, which ensures genetic stability of the clones (Altshuler et al., 2011; Miner et al., 2012).

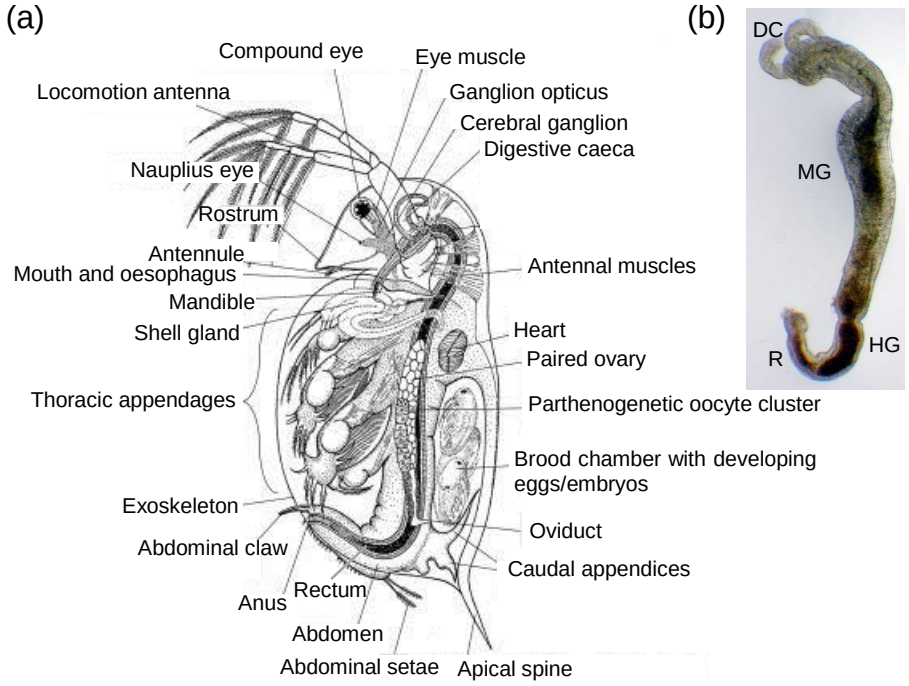


Fig. 1.6. Morphological overview of *Daphnia magna* (a) parthenogenetic female and (b) dissected gut. Abbreviation used: DC, digestive caeca; HG, hindgut; MG, midgut; R, rectum. Adapted from Ebert (2005).

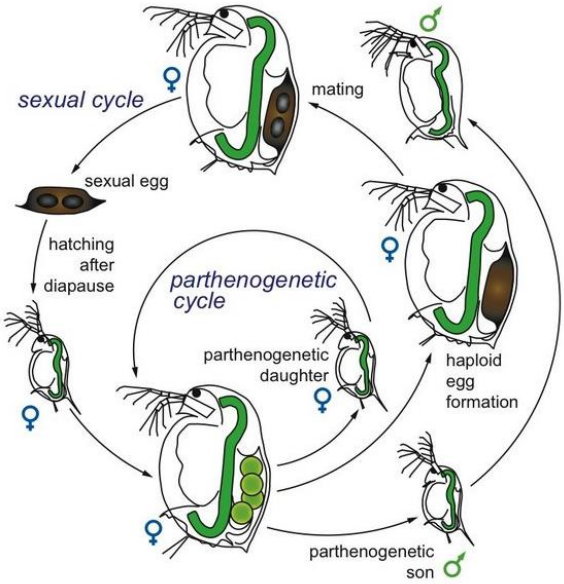


Fig. 1.7. *Daphnia magna* sexual and asexual (parthenogenetic) life cycles (Ebert, 2005).

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Chapter 2

Thesis aims and scientific strategy

The general objective of this research project is to characterize the interactions between dietary lipids, and especially the nature of their constitutive fatty acids, and the biological responses to chemical stressors, especially cadmium (Cd), in aquatic biota. Two aquatic organisms, belonging to two different trophic levels, were selected to study these interactions: a primary consumer, the large water flea (*Daphnia magna*, a planktonic crustacean), and a secondary consumer/predator, the rainbow trout (*Oncorhynchus mykiss*, a fish). These two reference models are widely used both in the ecotoxicology and in nutrition research fields. We first used an *in vitro* approach (rainbow trout liver cell line, RTL-W1, Lee et al. (1993)) for screening and mechanistic investigations and then two *in vivo* approaches (the large water flea and rainbow trout juveniles).

The specific aims of the present research project are to investigate (i) if and how a tissue's fatty acid profile modulates the sensitivity of aquatic organisms to chemical stressors, especially Cd and (ii) if and how Cd is able to affect lipid homeostasis and metabolism in aquatic organisms.

To address these questions, we followed a four-step strategy (Fig. 2.1).

In **Chapter 3**, we assessed whether specific polyunsaturated fatty acids (PUFAs) could modulate the tolerance of the RTL-W1 cell line to heavy metals. RTL-W1 cells were incubated in growth media enriched in specific PUFAs during one week, in order to modify their fatty acid profiles, before being acutely challenged with increasing doses of two metallic compounds, i.e. methylmercury (MeHg) and Cd. The metabolic activity and membrane integrity of the cells were assessed as endpoints. Median effect concentrations (EC50) were calculated from dose-response curves and compared to each other. This screening step

allowed us to select one metallic compound (Cd) and specific PUFAs for further investigations.

In **Chapter 4**, we assessed (i) how specific PUFAs modulate the sensitivity to Cd and (ii) whether Cd influences the fatty acid homeostasis and metabolism, in the RTL-W1 cell line. RTL-W1 cells with different fatty acids profiles were acutely challenged with Cd (two doses and a control). Cellular viability, fatty acid profile, Cd burden, proteome landscape and expression of specific genes involved in fatty acid metabolism, PUFA-derived signalling and stress response were determined as endpoints.

In **Chapters 5 and 6**, we assessed whether the interactions observed *in vitro* also occurs in two *in vivo* models of different biological complexities: the large water flea (**Chapter 5**) and the rainbow trout (**Chapter 6**).

In **Chapter 5**, *Daphnia magna* neonates were raised into adulthood on diets specifically enriched in one specific PUFA, using the liposome supplementation technique, in order to modify their fatty acid profiles. Adult daphnids from each dietary treatment as well as the offspring they generated during this life-history experiment (3rd to 5th brood) were then challenged with Cd during 48 h. The immobilisation of the daphnids was assessed as toxicity endpoint. EC50s were calculated from dose-response curves, compared to each other and linked to individual fatty acid profiles.

In **Chapter 6**, rainbow trout juveniles with different fatty acid profiles, obtained through feeding diets enriched in specific oils, were exposed to an environmentally realistic Cd concentration during both short and long term periods. Hepatic fatty acid profile, Cd and calcium concentrations, total glutathione level and expression of specific genes involved in fatty acid metabolism, PUFA-derived signalling and stress response were determined as endpoints.

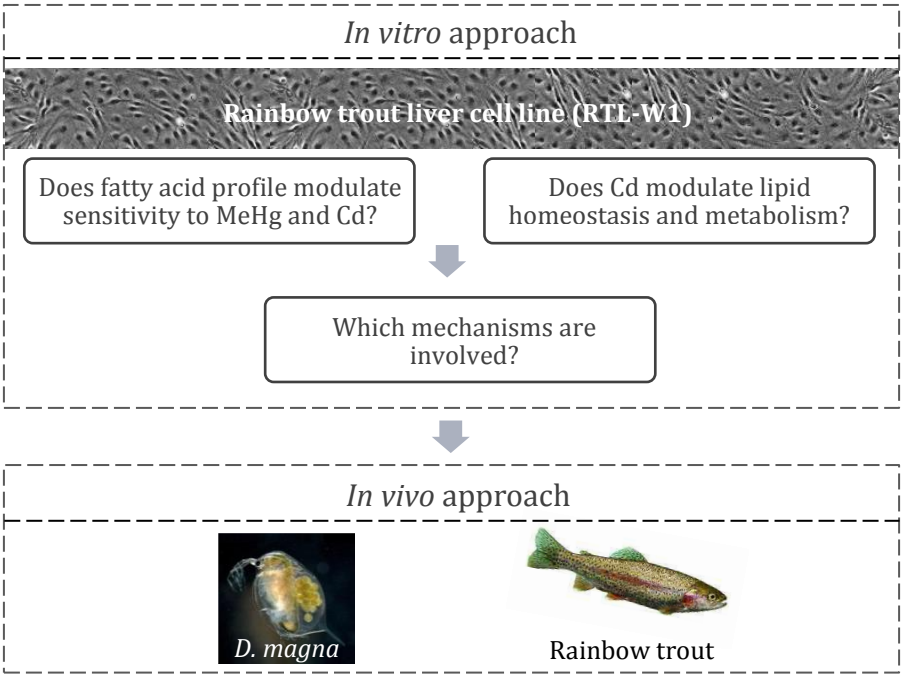

Fig. 2.1. Specific objectives and scientific strategy of the research project. Abbreviations used: Cd, cadmium; MeHg, methylmercury.

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Chapter 3

The fatty acid profile of rainbow trout liver cells modulates their tolerance to methylmercury and cadmium

Foreword

As presented in the general introduction of this thesis, a growing body of evidence suggests that the fatty acid profile may influence individual, tissular and cellular response to metallic compounds, such as methylmercury (MeHg) and cadmium (Cd). However, data available to date are contradictive, target one single metallic compound and focus on either on a narrow set of individual fatty acids or on mixtures which precluded a detailed and comparative mechanistic understanding of the effect of individual fatty acids in the elicited responses. In the present chapter we assessed whether specific polyunsaturated fatty acids (PUFAs) could modulate the tolerance of rainbow trout liver cells (RTL-W1 cell line) to MeHg and Cd. Cells were incubated in growth media enriched in specific specific PUFAs during one week, in order to modify their fatty acid profiles, before being acutely exposed to increasing doses of MeHg or Cd. The metabolic activity and membrane integrity of the cells were assessed as endpoints. Median effect concentrations were calculated from dose-response curves and compared to each other. This screening step will allow us to select one metallic compound and specific PUFAs for further investigations. The present results were published in Aquatic Toxicology.

The fatty acid profile of rainbow trout liver cells modulates their tolerance to methylmercury and cadmium

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Abstract

The polyunsaturated fatty acid (PUFA) composition of fish tissues, which generally reflects that of the diet, affects various cellular properties such as membrane structure and fluidity, energy metabolism and susceptibility to oxidative stress. Since these cellular parameters can play an important role in the cellular response to organic and inorganic pollutants, a variation of the PUFA supply might modify the toxicity induced by such xenobiotics. In this work, we investigated whether the cellular fatty acid profile has an impact on the *in vitro* cell sensitivity to two environmental pollutants: methylmercury and cadmium. Firstly, the fatty acid composition of the rainbow trout liver cell line RTL-W1 was modified by enriching the growth medium with either alpha-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), linoleic acid (LA, 18:2n-6), arachidonic acid (AA, 20:4n-6) or docosapentaenoic acid (DPA, 22:5n-6). These modified cells and their control (no PUFA enrichment) were then challenged for 24 h with increasing concentrations of methylmercury or cadmium. We observed that (i) the phospholipid composition of the RTL-W1 cells was profoundly modulated by changing the PUFA content of the growth medium: major modifications were a high incorporation of the supplemented PUFA in the cellular phospholipids, the appearance of direct elongation and desaturation metabolites in the cellular phospholipids as well as a change in the gross phospholipid composition (PUFA and monounsaturated fatty acid (MUFA) levels and n-3/n-6 ratio); (ii) ALA, EPA and DPA enrichment significantly protected the RTL-W1 cells against both methylmercury and cadmium; (iv) DHA enrichment significantly protected the cells against cadmium but not methylmercury; (v) AA and LA enrichment had no impact on the cell tolerance to both methylmercury and cadmium; (vi) the abundance of 20:3n-6, a metabolite of the n-6 biotransformation pathway, in phospholipids was negatively correlated to the cell tolerance to both methylmercury and cadmium. Overall, our results highlighted the importance of the fatty acid supply on the tolerance of fish liver cells to methylmercury and cadmium.

Key words (6): methylmercury, cadmium, polyunsaturated fatty acids, fish, RTL-W1 cells, cytotoxicity

3.1. Introduction

The release of various pollutants in the environment, the degradation and fragmentation of habitats, the overexploitation of ecosystems as well as global warming have increased the number and intensity of external stressors on aquatic organisms (Dudgeon, 2010; Dudgeon et al., 2006). Shifts in water quality, flow, temperature and nutrient availability are relevant examples of such stressors. By moving an organism away from its optimum performance in terms of growth and reproduction, these stressors may influence survival and fitness (Van Straalen, 2003). While many studies focus on the effect of one stressor in otherwise optimal conditions, responses to a stressor such as chemical pollution are known to be influenced by environmental conditions (e.g. temperature, nutritional status) (Holmstrup et al., 2010).

Chemical pollutants are ubiquitous stressors that can strongly degrade aquatic ecosystems (Peters et al., 2013). Among these, methylmercury (MeHg) and cadmium (Cd) occur at elevated concentrations in many aquatic environments and can pose significant problems to organisms (Boening, 2000; Pan et al., 2010; Rytuba, 2003). For example, anthropic release of MeHg and Cd in the environment was historically associated with human health and ecological effects in the Japanese Minamata Bay (Harada, 1995) and in the Japanese prefecture of Toyama (Sebastian and Prasad, 2014), respectively. However, the current stock of MeHg and Cd in the environment is mainly due to their direct and indirect use in numerous industrial, agricultural, medical and artistic applications, the combustion of fossil fuels and the incineration of waste (Kidd and Batchelar, 2011; McGeer et al., 2011; Pan et al., 2010; Rytuba, 2003).

MeHg and Cd are persistent in the environment and can bioaccumulate in most aquatic organisms (Kidd and Batchelar, 2011; McGeer et al., 2011). Whilst the aqueous route is most important for Cd exposure, the dietary route accounts for 90% of the MeHg exposure (Hall et al., 1997). MeHg is biomagnified throughout aquatic trophic webs (Lavoie et al., 2013; Rolfhus et al., 2011). In fish, Cd is mainly accumulated in liver and kidneys (McGeer et al., 2011), whereas MeHg is

stored in a wide variety of organs including muscle, liver, kidney, spleen, gills, intestine and brain (McGeer et al., 2011). While Cd^{2+} has been shown to enter cells through facilitated diffusion (McGeer et al., 2011), both passive and active transport have been suggested for MeHg cellular uptake (depending on the cell type and on the metal speciation) (Heggland et al., 2009; Kidd and Batchelar, 2011). The two metallic compounds impair growth, reproduction and survival (Kidd and Batchelar, 2011; McGeer et al., 2011; Sevcikova et al., 2011). They alter divalent ions metabolism, induce oxidative stress and trigger apoptosis (Kidd and Batchelar, 2011; McGeer et al., 2011). For instance, MeHg and Cd can alter the activity of many antioxidant enzymes, decreasing the cell ability to cope with reactive oxygen species (Kidd and Batchelar, 2011; Matović et al., 2015; McGeer et al., 2011). Oxidative attacks to lipids may lead to uncontrolled chain-reactions propagating the oxidative damage throughout cell membranes (Catalá, 2012). This is particularly the case with fatty acids with a high degree of unsaturation (polyunsaturated fatty acids (PUFAs) and highly unsaturated fatty acids (HUFAs)) as they are very prone to oxidation.

In fish, the cellular fatty acid profile of tissues such as muscle and liver is highly influenced by both dietary and environmental parameters. Firstly, the fatty acid profile globally reflects that of the diet (Bell et al., 2004; Bell et al., 2001; Bell et al., 2006; Caballero et al., 2002; Fonseca-Madrigal et al., 2005; Mourente and Bell, 2006; Petropoulos et al., 2009). Secondly, environmental conditions such as temperature have also been reported to modulate the fish tissue fatty acid composition (Caballero et al., 2002; Snyder et al., 2012; Wijekoon et al., 2014). These shifts may alter the organism fitness and ability to cope with additional stressors, such as chemical pollutants, as lipids play a central role in fish metabolism. Indeed, they provide metabolic energy for maintenance, growth, reproduction and locomotion, maintain the structure and function of biological membranes and are the precursors of highly bioactive molecules such as the eicosanoids (Tocher, 2003).

As suggested by previous literature, the toxicity of MeHg and/or Cd could target cell properties modulated by PUFA content such as susceptibility to oxidative stress or membrane characteristics. The cellular lipid profile is thus likely to affect sensitivity to Cd and MeHg,

particularly in animals with variable lipid profiles such as fish. Despite the potential sites of interaction between lipids and pollutants, the influence of dietary lipids on metallic compounds toxicity has received little attention. A few authors have assessed whether the quality of the lipid supply can modulate the biological response to MeHg (Grotto et al., 2011; Jayashankar et al., 2012; Jin et al., 2008; Jin et al., 2007; Kaur et al., 2008; Kaur et al., 2007; Nøstbakken et al., 2012a; Nøstbakken et al., 2012b; Olsvik et al., 2011; Pal and Ghosh, 2012b; Remø et al., 2011) and Cd (Amamou et al., 2015). Overall, these authors pointed out a protective effect of fish oil and n-3 PUFAs against MeHg or Cd toxicity. In general those studies focussed on only few selected n-3 PUFAs and on one metallic compound. For instance, the impact of alpha-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) has been assessed on MeHg toxicity *in vitro* (mouse and rat neural cells, fish and human renal cells) and *in vivo* (brain, plasma, liver and kidneys of rats) but not on Cd toxicity (Kaur et al., 2008; Kaur et al., 2007; Nøstbakken et al., 2012a; Nøstbakken et al., 2012b; Pal and Ghosh, 2012a, b). To date, the impact of n-6 PUFAs on metallic compounds sensitivity has received very little attention as only one study investigated the impact of arachidonic acid (AA, 20:4n-6) on the cell resistance to MeHg in fish and human renal cells (Nøstbakken et al., 2012a). Comparisons between these studies are difficult as the models and the tested PUFAs and concentrations varied between studies.

In this study, we investigated the effect of n-3 and n-6 PUFAs on the toxicity induced by both MeHg and Cd, using a cellular model. Liver being an important site of interaction between lipids and metallic compounds as it is the key organ for both detoxification and fatty acid metabolism, we conducted an *in vitro* experiment based on rainbow trout liver cells (RTL-W1 cell line (Lee et al., 1993)). Cells were artificially enriched with a specific PUFA from either the n-3 (ALA, EPA or DHA) or the n-6 (linoleic acid (LA, 18:2n-6), AA or docosapentaenoic acid (DPA, 22:5n-6)) pathway (Fig. 3.1) before being exposed to MeHg or Cd during 24 h. After exposure, cell viability was assessed and linked to the fatty acid profile of the cells.

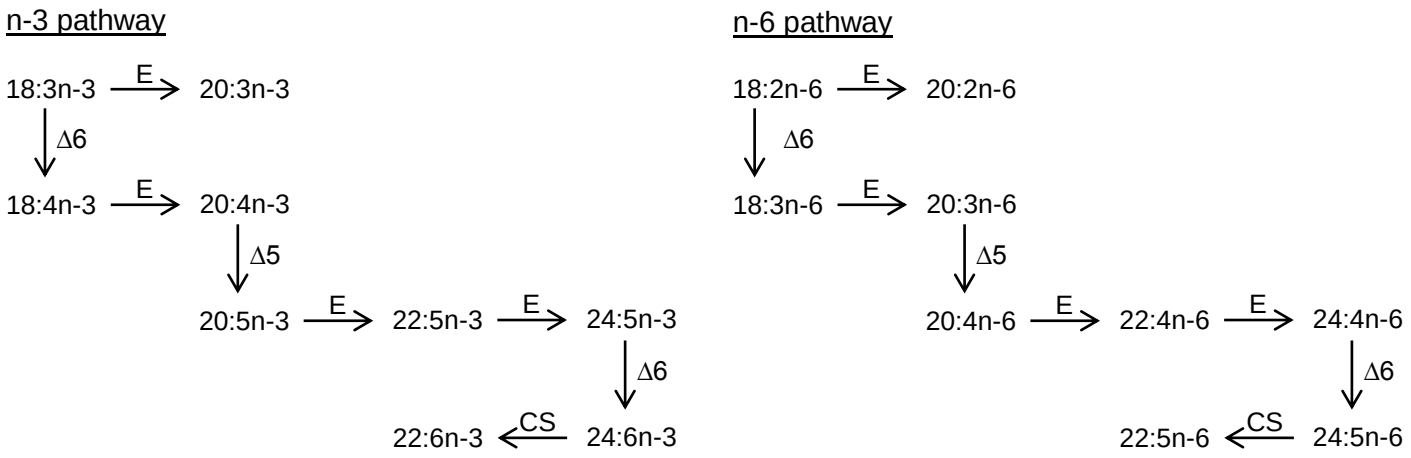

Fig. 3.1. Alpha-linolenic acid (left) and linoleic acid (right) biotransformation pathways in fish hepatocytes. Abbreviation used: Δ5 and Δ6, microsomal fatty acid desaturase enzyme mediated reactions; E, hepatic microsomal elongase mediated reactions; CS, peroxisomal fatty acid chain shortening.

3.2. Material and methods

3.2.1. Cells, culture media and growth conditions

The RTL-W1 cell line derived from the liver of a 4 year old male rainbow trout (Lee et al., 1993) was kindly provided by S. Bony (INRA, France). Cells were routinely grown at 19°C in 75 cm^2 flasks (Easyflasks Nunc, Life Technology, USA) containing 15 mL of Leibovitz's L15 medium supplemented with 5% foetal bovine serum (FBS: PAA Laboratories, France) and 1% antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin). The culture medium was renewed every two days. When 80% confluence was reached, i.e. about once a week, cells were detached with trypsin 0.125% in phosphate-buffered saline supplemented with 0.03% ethylenediamine tetraacetic acid (PBS-EDTA) before being subcultured into a new 75 cm^2 flask. Cell counts and routine viability measurements were made on a Bürker cell in presence of Trypan Blue as an exclusion dye. Cells were regularly checked for the absence of mycoplasma (MycoAlert kit, Promega, The Netherlands).

3.2.2. Experimental design

The experimental sequence used was the following: (i) fatty acid profile manipulation during 7 days by incubating RTL-W1 cells in a medium enriched or not (control) in a specific PUFA from either the n-3 pathway (experimental group 1: ALA, EPA, DHA) or the n-6 pathway (experimental group 2: LA, AA, DPA); (ii) fatty acid profile determination of the control and PUFA-enriched cell batches; (iii) metal challenge (CH_3HgCl or $CdCl_2$) during 24 h and viability assessment via the CellTiter-Blue (CTB) assay (Promega, The Netherlands) and the 5-carboxyfluorescein diacetate, acetoxymethyl ester (5-CFDA,AM) assay (Life Technology, USA). This sequence was entirely repeated three times for each of the two experimental groups studied: n-3 pathway and n-6 pathway.

3.2.3. PUFA enrichment

The PUFAs (Larodan, Sweden) were supplied as complexes with fatty acid free-bovine serum albumin (FAF-BSA) (4:1 mol:mol), as described by Best et al. (2006). When 80% confluence was reached, cells maintained in routine growth conditions were harvested with trypsin 0.0625% in PBS-EDTA and seeded at a density of 20,000 cells/cm^2 in 75 cm^2 flasks containing 15 mL PUFA-enriched media (50 µM of a specific n-3 or n-6 PUFA, 12.5 µM FAF-BSA, 2% FBS, 1% antibiotics) or control medium (no PUFA, 12.5 µM FAF-BSA, 2% FBS, 1% antibiotics). The incubation lasted 7 days during which the enriched or control culture media were renewed twice. At the end of the incubation period, each cell batch was harvested with trypsin 0.0625% in PBS-EDTA and subdivided into two parts of 4,000,000 living cells each: one for the fatty acid profile determination and the other one for the metal challenge.

3.2.4. Fatty acid profile determination

3.2.4.1. Sample preparation

The cell suspension was washed with 5 mL ice-cold FAF-BSA (10 g/L in Leibovitz's L15 medium) and re-suspended in 2 mL ice-cold FAF-BSA (10 g/L in Leibovitz's L15 medium) from which 0.1 mL was used for protein quantification and 2 x 0.8 mL were used for lipid extraction (for each sample, analyses were performed in duplicate). For protein quantification, the cell pellet was washed with L15/ex medium, a restrictive medium that contains the salts, galactose and pyruvate of Leibovitz's L15 medium at the same concentration as Leibovitz's L15 medium (Dayeh et al., 2002). Thereafter, the cell pellet was lysed (lysis buffer: 60 mM Tris, 10 mM EDTA, 1% sodium dodecyl sulphate (w/w) in water). The lysates were kept at -20°C until protein quantification by the Bicinchoninic acid (BCA) Protein Assay (Thermo Scientific Pierce, USA), with BSA as standard.

3.2.4.2. Lipid extraction

Total lipids were extracted with methanol:chloroform:water (2:2:1.8 v:v:v) as described by Bligh and Dyer (1959). A solution composed of 1,2-dipentadecanoyl-sn-glycero-3-phosphatydylcholine (Larodan, Sweden), triheptadecanoin (Larodan, Sweden) and tridecanoic acid (Larodan, Sweden) was used as internal standard.

3.2.4.3. Lipid class separation

Total lipid extracts were separated by solid phase extraction into three lipid fractions, i.e. neutral lipids, free fatty acids and phospholipids, as described by Schneider et al. (2012).

3.2.4.4. Methylation and extraction

Neutral lipid, free fatty acid and phospholipid extracts were methylated as described by Schneider et al. (2012) with minor modifications to ensure sample concentration. Briefly, eluted fractions were evaporated to dryness under a stream of N_2 at 30°C and methylated at 70°C through the addition of 0.5 mL methanol containing KOH (0.1 M) and a 1 h incubation followed by the addition of 0.2 mL of acidified methanol (1.2 M HCl) and a 15 min incubation. The resulting fatty acid methyl esters (FAMEs) were then extracted using 1 mL hexane.

3.2.4.5. Identification and quantification

FAMEs were separated by gas chromatography as described by Schneider et al. (2012), with minor adaptations. The chromatograph (GC Trace-2000, Thermo Quest, Italy) was equipped with an RT2560 capillary column (100 m x 0.25 mm internal diameter, 0.2 µm film thickness: Restek, USA), a GC PAL autosampler (CTC Analytics, Switzerland) and a flame ionisation detector (FID: Thermo Quest, Italy). The carrier gas used was H_2 at constant pressure (200 kPa) and flow rate (2 mL/min). The FID was continuously flowed by H_2 (35 mL/min) and air (350 mL/min) and kept at a constant temperature of 255°C. The temperature program was as follows: an initial temperature of 80°C,

which increased at 25°C/min up to 175°C, a holding temperature of 175°C during 25 min, a new increase at 10°C/min up to 205°C, a holding temperature of 205°C during 4 min, a new increase at 10°C/min up to 215°C, a holding temperature of 215°C during 25 min, a last increase at 10°C/min up to 235°C and a final holding temperature of 235°C during 10 min. The resulting spectra were processed with the ChromQuest software (version 4.2, ThermoFinnigan, Italy). Methyl-undecanoate (Larodan, Sweden) was used as injection standard. Peak identification and quantification were enabled by the use of an external standard, composed of 44 FAMEs (Larodan, Sweden).

3.2.5. Metal challenge and acute metal toxicity assessment

3.2.5.1. Metallic solutions

Three metallic stock solutions were prepared in cell culture grade water: CH_3HgCl (5 mM, CAS 115-09-3, Sigma-Aldrich, USA) and $CdCl_2$ (30 and 100 mM, CAS 7790-78-6, Fisher Chemical, UK). Successive dilutions of these stock solutions in cell culture grade water enabled the constitution of a stock solution set for each metallic compound tested. The concentrations tested ranged from 0 to 100 µM CH_3HgCl and from 0 to 1000 µM $CdCl_2$. These concentration ranges were selected based on preliminary tests. The working solutions were prepared fresh daily by diluting the stock solution set by 100 times in L15/ex medium.

3.2.5.2. Metal challenge

After a 7-day incubation with a specific PUFA, cells were seeded into 96-well black plates (Greiner Bio-One, Belgium), in their respective PUFA-enriched medium, at a cell density of 20,000 living cells per well. Additional wells were left empty from cells (blanks). After a minimum of 10 h incubation under routine growth conditions (time needed to enable cell attachment), the medium was removed and each well was exposed to one concentration of either CH_3HgCl ([0-100] µM) or $CdCl_2$ ([0-1000] µM) in L15/ex medium for 24 h. Five wells with cells and two blanks were exposed to each tested concentration.

3.2.5.3. Viability assays

After 24 h exposure, two cell viability assays were simultaneously performed: CTB and 5-CFDA,AM, to assess the metabolic activity and the membrane integrity of the cells, respectively. CTB consists of a solution of resasurin, which is reduced in metabolically active cells into the fluorescent dye resorufin. It therefore reflects the reductase activity of the cells. 5-CFDA,AM is hydrolysed by unspecific cytosolic esterase into the fluorescent dye carboxyfluorescien. It therefore measures both the esterase activity of the cells and their membrane integrity as only an intact plasma membrane can maintain the cytosolic milieu required to support the esterase activity. The method described by Dayeh et al. (2005) has been slightly adapted. Briefly, the exposure medium was replaced by 120 µL of a reaction mix composed of 16.7% (v:v) CTB and 0.1% (v:v) 5-CFDA,AM in L15/ex medium. Directly after the reaction mix addition, the emitted fluorescence was recorded every 40 s during 26 min using the Fluoroskan Ascent FL (Thermo Electron corporation, USA) spectrofluorometer. The excitation and emission wavelengths were 530 and 590 nm for CTB and 435 and 530 nm for 5-CFDA,AM.

3.2.6. Data analyses

Graphs and statistical analyses were performed using JMP PRO 11.0 software.

3.2.6.1. Fatty acid profile data analysis

For each biological replicate, the average of the duplicates was calculated and used for further analyses. Data in the tables are reported as means (of the three biological replicates) ± standard error of the mean. A Kruskal-Wallis test followed by a Wilcoxon Rank Sum test were used to determine the statistical differences between the respective treatments and the control, with a total alpha set at 0.05. A statistical difference of $p < 0.05$ was considered significant.

3.2.6.2. Viability data analysis

For each well, after correction of the fluorescence intensities by the blank values, the slopes of the kinetics between 0.7 and 20 min were determined and those with a coefficient of determination (r^2) inferior to 0.85 were considered as equal to zero. The slope values were expressed in percentage of the controls (cells; 0 µM metal). The averages of the five wells of the same condition were calculated for each repetition and used for data modelling. Then, for each PUFA and each metal, either a LogLogistic or a Brain & Cousens (in case of a significant hormesis effect) model was fitted to the data from the three independent repetitions (n = 3) (Schabenberger et al., 1999). The EC50s and their confidence interval (at 98.3%) were obtained by estimating the parameters of the models. Differences were considered as being significant ($p < 0.05$) when no overlapping of the confidence intervals (at 98.3%) was observed (Scheffczyk et al., 2014). Inverse predictions enabled the determination of the concentration ranges within which the response (metabolic activity or membrane integrity) was significantly different between PUFA-enriched and control cells.

3.2.6.3. Multivariate analysis

One principal component analysis was performed using, together, the fatty acid profile data (% of the sum of the fatty acids identified in the cell phospholipids, arcsine-root square transformed) and the calculated EC50s (for MeHg and Cd).

3.3. Results

3.3.1. Lipid composition of RTL-W1 cells

The fatty acid composition of phospholipids (Table 3.1), neutral lipids (supplementary data Table 3.A.1) and free fatty acids (supplementary data Table 3.A.2) of the RTL-W1 cells were established after one week incubation in a medium supplemented or not with 50 µM PUFA (ALA, EPA, DHA, LA, AA or DPA).

3.3.1.1. Lipid class separation: importance of the phospholipids

The phospholipids accounted for 63.9 to 75.3% of the total cellular lipids. Free fatty acids and neutral lipids represented 17.5 to 22.4% and 5.0 to 15.1% of the total cellular lipids, respectively. Free fatty acids and neutral lipids classes were essentially composed of the saturated fatty acids (SFAs) 16:0 and 18:0. The only PUFAs found in these two fractions were the supplemented ones.

3.3.1.2. *In vitro* manipulation of the fatty acid profile of RTL-W1 cell phospholipids

The phospholipid composition of the control cells (Table 3.1) globally reflected that of the FBS. Both were rich in monounsaturated fatty acids (MUFAs) and SFAs, mainly 18:1n-9, 16:0 and 18:0, and low in PUFAs. Indeed, FBS was composed of 23.4% PUFAs: 9.1% n-3 (EPA, 22:5n-3 and DHA) and 14.4% n-6 (LA, 20:3n-6 and AA), leading to a n-3/n-6 ratio of 0.63. Control cell phospholipids were composed of 22.1% PUFAs: 9.4% n-3 (EPA, 22:5n-3 and DHA) and 12.7% n-6 (LA, 18:3n-6, 20:3n-6 and AA), leading to a n-3/n-6 ratio of 0.74.

Adding a specific PUFA in the culture medium for 7 days significantly changed the phospholipid composition of the RTL-W1 cells.

Firstly, the total n-3 PUFA content was significantly higher in the phospholipids of the cells incubated with a specific n-3 PUFA ($p < 0.05$), while n-6 PUFAs were significantly more abundant in the phospholipids of the cells incubated with a specific n-6 PUFA ($p < 0.05$). In addition, the phospholipids of the cells enriched in n-3 PUFAs contained significantly less n-6 PUFAs than those of the control cells ($p < 0.05$). This trend was however not significant in the EPA-enriched cells ($p = 0.068$). Similarly, the amount of n-3 PUFAs was significantly lower in the phospholipids of the cells enriched in n-6 PUFAs than in the phospholipids of the control cells ($p < 0.05$). As a result, the n-3/n-6 ratio was significantly higher (by around 10 times) in the n-3 PUFA-enriched cells than in the control cells ($p < 0.05$) and significantly lower (by around 10 to 20 times) in the n-6 PUFA-enriched cells, as compared to the control cells ($p < 0.05$). The

total PUFAs found in the cellular phospholipids significantly increased ($p < 0.05$) in cells supplemented with ALA, EPA, LA and AA while this increase was not significant in DHA- ($p = 0.172$) and DPA-enriched cells ($p = 0.068$). Besides, the total MUFAs found in the phospholipids significantly dropped (between 47 to 80% drop) in each PUFA-treated cell batch ($p < 0.05$), mainly due to a significant drop in 18:1n-9, but without significance in the EPA-enriched cells ($p = 0.068$). By contrast, both the total SFAs and the total phospholipid levels remained similar in each experimental condition.

Secondly, a significant increase of the amount of the specific PUFA added into the cell culture media was observed (from 188.3 up to 327.9 µmol/g protein) in the phospholipids of all enriched cells ($p < 0.05$), allowing the supplemented PUFA to become the main fatty acid within this fraction, accounting for 26 to 49% of the total phospholipids.

Thirdly, the amount of biotransformation products most supposedly derived from the supplemented PUFA increased in all enriched cells, indicating the presence of a bioconversion process via direct elongation and desaturation (see Fig. 3.1 for the biotransformation pathways). The 18:4n-3 and 18:3n-6 levels significantly increased in cells respectively supplemented with ALA and LA ($p < 0.05$), suggesting the occurrence of a Δ6 desaturase activity in the RTL-W1 cells. Direct elongation products of ALA, EPA and AA, i.e. 20:3n-3, 22:5n-3 and 22:4n-6, also significantly increased in cells enriched in these PUFAs ($p < 0.05$). Similarly, traces of 20:2n-6 were found in the LA-enriched cells. However, the amounts of the biotransformation products, resulting from a second step of elongation/desaturation were not different between enriched and control cells, indicating no further elongation and desaturation of the six above-mentioned metabolites.

Fourthly, the cells supplemented with a specific n-6 PUFA were significantly lower in the n-3 PUFAs EPA, 22:5n-3 and DHA, than the control cells ($p < 0.05$). This trend was however not significant for 22:5n-3 in the AA-enriched cells ($p = 0.073$).

Finally, most treatments affected the cellular levels of the n-6 PUFAs LA, 20:3n-6 and AA. Indeed, the amount of LA was lower in cells

enriched in DHA, AA and DPA than in the control cells ($p < 0.05$). This trend was however not significant in AA-enriched cells ($p = 0.073$). In addition, the AA level was lower in cells enriched in ALA, DHA, LA and DPA than in the control cells ($p < 0.05$). Besides, the levels of 20:3n-6 were lowered by a factor of 10.2, 3.6, 11.3 and 9 in cells respectively supplemented with ALA, EPA, DHA and DPA, as compared to the control cells ($p < 0.05$). A similar trend was observed in LA-enriched cells but without significance ($p = 0.068$).

Table 3.1. Fatty acid profile of the RTL-W1 cells phospholipids after a 7 day-supplementation with 50 µM PUFA (µmol/g protein).

Fatty acid	Control	ALA	EPA	DHA	LA	AA	DPA
14:0	9.6 ± 2.9	0.0 ± 0.0 *	2.9 ± 2.9	5.7 ± 2.9	0.0 ± 0.0 *	0.0 ± 0.0 *	2.3 ± 2.3
16:0	196.1 ± 21.6	138.4 ± 12.6	213.8 ± 15.2	143.2 ± 8.5	109.2 ± 13.5	194.0 ± 38.5	175.1 ± 26.7
18:0	104.9 ± 10.1	97.7 ± 9.6	114.1 ± 8.7	65.5 ± 2.5	70.0 ± 7.5	111.4 ± 20.6	93.5 ± 4.5
Total SFA	**310.6 ± 33.3**	**236.1 ± 22.2**	**330.8 ± 21.1**	**214.4 ± 13.6**	**179.2 ± 19.7**	**305.4 ± 58.9**	**270.8 ± 31.7**
16:1n-7	12.8 ± 4.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 1.4	0.0 ± 0.0
18:1n-7	36.1 ± 4.5	15.9 ± 2.2 *	25.6 ± 1.6	11.0 ± 5.6 *	6.1 ± 3.1 *	21.8 ± 3.6	7.0 ± 7.0 *
18:1n-9	276.5 ± 33.7	107.4 ± 14.1 *	148.2 ± 11.4	98.5 ± 8.8 *	57.6 ± 4.7 *	129.5 ± 23.1 *	97.9 ± 25.6 *
Total MUFA	**325.4 ± 42.6**	**123.3 ± 16.2 ***	**173.7 ± 13.0**	**109.5 ± 13.8 ***	**63.7 ± 3.8 ***	**152.7 ± 26.7 ***	**104.9 ± 32.0 ***
ALA 18:3n-3	0.0 ± 0.0	**246.4 ± 29.7 ***	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:3n-3	0.0 ± 0.0	12.0 ± 1.4 *	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
18:4n-3	0.0 ± 0.0	39.8 ± 8.0 *	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:4n-3	0.1 ± 0.1	4.0 ± 4.0	4.0 ± 4.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
EPA 20:5n-3	19.9 ± 2.9	29.6 ± 4.7	**224.3 ± 14.8 ***	21.0 ± 2.0	2.2 ± 2.2 *	0.0 ± 0.0 *	0.0 ± 0.0 *
22:5n-3	24.2 ± 4.1	15.7 ± 1.5	92.4 ± 9.0 *	29.6 ± 2.9	9.7 ± 1.5 *	10.4 ± 4.4	6.7 ± 3.6 *
DHA 22:6n-3	34.6 ± 5.9	20.2 ± 2.0	20.4 ± 1.8	**188.3 ± 19.3 ***	12.8 ± 1.6 *	9.6 ± 4.2 *	8.5 ± 4.7 *
Total n-3 PUFA	**78.8 ± 12.9**	**367.7 ± 48.5 ***	**341 ± 21.5 ***	**238.9 ± 23.9 ***	**24.7 ± 4.5 ***	**20.0 ± 8.6 ***	**15.2 ± 8.3 ***
LA 18:2n-6	31.3 ± 4.8	23.7 ± 3.1	19.2 ± 1.3	13.0 ± 1.1 *	**249.0 ± 30.9 ***	16.9 ± 3.5	9.8 ± 5.0 *
20:2n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.7 ± 2.4	0.0 ± 0.0	0.0 ± 0.0
18:3n-6	2.0 ± 2.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	35.3 ± 3.5 *	1.0 ± 1.0	0.0 ± 0.0

Data are expressed as means ± standard error of mean (n = 3, 4 or 7). Stars indicate significant differences as compared to the control (Wilcoxon Rank Sum Test, total alpha = 0.05, $p < 0.05$). Abbreviation used: ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid; DPA, docosapentaenoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

(continued)

Fatty acid	Control	ALA	EPA	DHA	LA	AA	DPA
20:3n-6	20.3 ± 3.0	2.0 ± 2.0 *	5.6 ± 2.8 *	1.8 ± 1.8 *	11.9 ± 1.2	15 ± 3.3	2.9 ± 2.9 *
AA 20:4n-6	52.6 ± 8.2	26.3 ± 2.9 *	31.9 ± 2.2	14.4 ± 1.1 *	23.1 ± 2.4 *	**326.6 ± 53.5 ***	24.8 ± 2.9 *
22:4n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	52.2 ± 8.1 *	0.0 ± 0.0
DPA 22:5n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	**327.9 ± 83.3 ***
Total n-6 PUFA	**106.1 ± 17.1**	**52.0 ± 5.5 ***	**56.7 ± 3.2**	**29.1 ± 2.4 ***	**324.0 ± 28.3 ***	**411.7 ± 67.4 ***	**365.3 ± 93.2 ***
Total PUFA	**184.9 ± 30.0**	**419.6 ± 53.4 ***	**397.7 ± 23.9 ***	**268.0 ± 25.7**	**348.6 ± 31.2 ***	**431.7 ± 72.9 ***	**380.5 ± 101.4**
Total	**820.8 ± 104.3**	**779 ± 91.2**	**902.2 ± 58.0**	**592 ± 53.0**	**591.6 ± 48.5**	**889.8 ± 158.2**	**756.2 ± 163.5**
n-3/n-6 ratio	**0.74 ± 0.01**	**7.07 ± 0.44 ***	**6.02 ± 0.27 ***	**8.23 ± 0.54 ***	**0.08 ± 0.01 ***	**0.05 ± 0.02 ***	**0.03 ± 0.02 ***

Data are expressed as means ± standard error of mean (n = 3, 4 or 7). Stars indicate significant differences as compared to the control (Wilcoxon Rank Sum Test, total alpha = 0.05, $p < 0.05$). Abbreviation used: ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid; DPA, docosapentaenoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

3.3.2. RTL-W1 cells response to an acute metal challenge

After one week of incubation with a specific PUFA, enriched and control cells were challenged for 24 h with increasing concentrations of MeHg or Cd in a serum- and PUFA-free medium. The cell viability was assessed by measuring simultaneously the metabolic activity (CTB assay, Fig. 3.2 and 3.3) and the membrane integrity (5-CFDA,AM assay, supplementary data Fig. 3.A.1 and 3.A.2) of the cells. The two metallic compounds caused a dose-dependent decline in cell viability, regardless of the PUFA-enrichment or the toxicity test.

3.3.2.1. ALA, EPA, DHA and DPA: four PUFA increasing the cell tolerance to MeHg and/or Cd

The enrichment in ALA, EPA and DPA significantly increased the cell tolerance to MeHg as measured through the metabolic activity. Indeed, the dose-response curves were shifted to the right for cells enriched in these three PUFAs, compared to the control cells (Fig. 3.2a,b and 3.3c). Based on the dose-response models, the metabolic activity of ALA-, EPA- and DPA-enriched cells was significantly higher than that of the control cells for the respective MeHg concentration ranges [0.39-11.47], [0.13-8.83] and [0.24-3.17] µM ($p < 0.05$) (Fig. 3.2a,b and 3.3c). Besides, the calculated MeHg EC50s were respectively 4.0, 4.5 and 3.2 times higher for cells enriched in ALA, EPA and DPA than the MeHg EC50 for the control cells ($p < 0.05$) (Table 3.2).

The enrichment in ALA, EPA, DHA and DPA increased the cell tolerance to Cd assessed through the metabolic activity. Indeed, based on the dose-response models, ALA-, EPA-, DHA- and DPA-enriched cells were significantly more metabolically active than the control cells for the Cd concentration ranges [35.8-555.6], [36.6-331.8], [67.0-191.6] and [0.1-39.6] µM, respectively ($p < 0.05$) (Fig. 3.2d-f and 3.3f). The calculated Cd EC50s were respectively 3.0, 2.9 and 1.9 times higher for cells enriched in ALA, EPA and DHA than for the control cells ($p < 0.05$) (Table 3.2). While the DPA enrichment did not significantly affect the Cd EC50 (Table 3.2), it induced a hormesis effect, i.e. a beneficial effect at

low Cd dose (Fig. 3.3f). The hormesis term of the parametrisation of the Brain & Cousens model (Schabenberger et al., 1999) was significant: 2.30 [0.57-4.88)] (supplementary data Table 3.A.4).

The DPA supplementation significantly increased the cell tolerance to both MeHg and Cd evaluated through measuring the membrane integrity. Indeed, as for the CTB assay, we observed, for the 5-CFDA,AM assay, a shift to the right of the dose-response curves for the DPA-enriched cells compared to the control cells (supplementary data Fig. 3.A.2c,f). Based on the dose-response models, the membrane integrity in the DPA-enriched cells was significantly higher than in the control cells after exposure to MeHg concentrations ranging from 1.41 to 5 µM and to Cd concentrations ranging from 10.8 to 126.3 µM ($p < 0.05$) (supplementary data Fig. 3.A.2c,f). The calculated MeHg and Cd EC50 values increased respectively by 2.0 and 1.5 times in comparison to the controls in case of a MeHg or Cd challenge ($p < 0.05$) (supplementary data Table 3.A.3). The other PUFAs had no impact on the viability decline (in terms of membrane integrity) caused by either MeHg or Cd (supplementary data Fig. 3.A.1a,b,d,e and 3.A.2a,b,d,e).

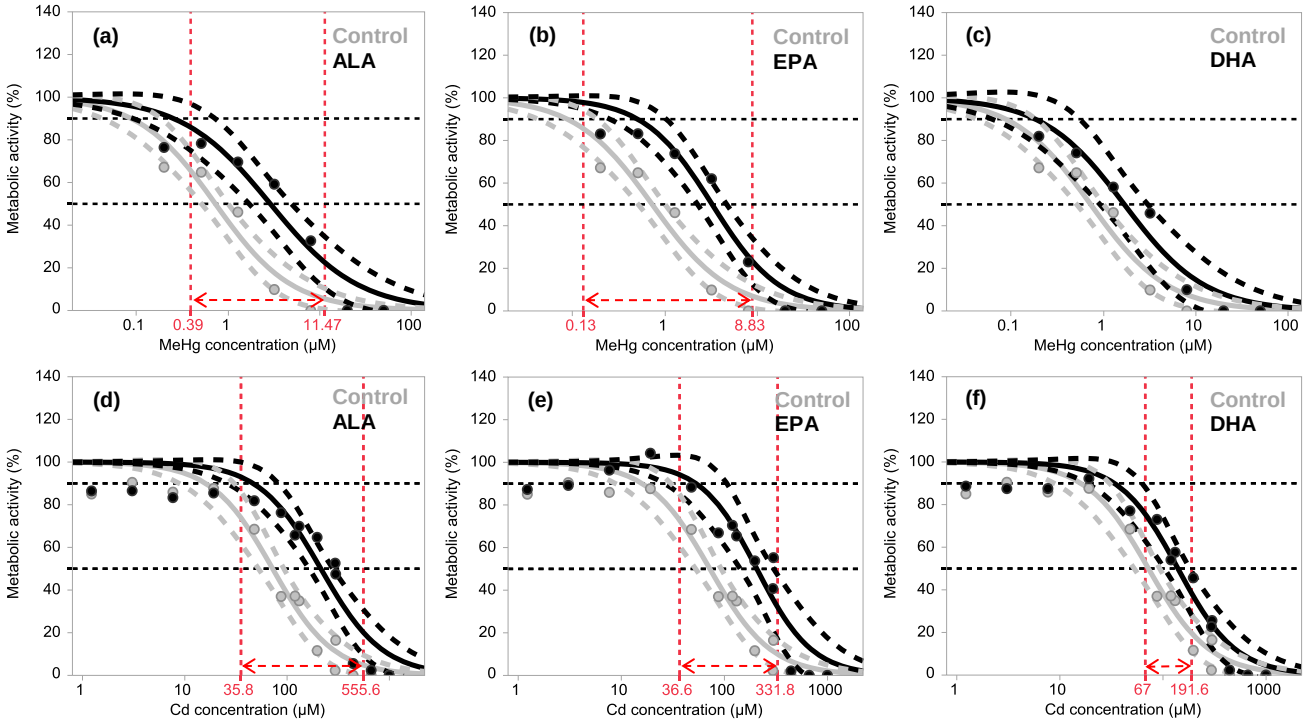

Fig. 3.2. Metabolic activity of the RTL-W1 cells enriched (black) or not (grey) in a specific n-3 PUFA (ALA, EPA or DHA) and challenged with increasing concentrations of MeHg (a–c) or Cd (d–f) during 24 h. Plain curves represent the LogLogistic prediction models; dotted curves represent the confidence intervals at 98.3% for the predictions. Points represent the mean metabolic activities of the cells challenged at specific MeHg or Cd concentrations (n = 3). The dotted arrows delimit the metal concentration ranges withinwhich the predicted metabolic activity of cells enriched with a specific n-3 PUFA is significantly higher than that of the control cells. Abbreviation used: ALA, alpha-linolenicacid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; MeHg, methylmercury; Cd, cadmium.

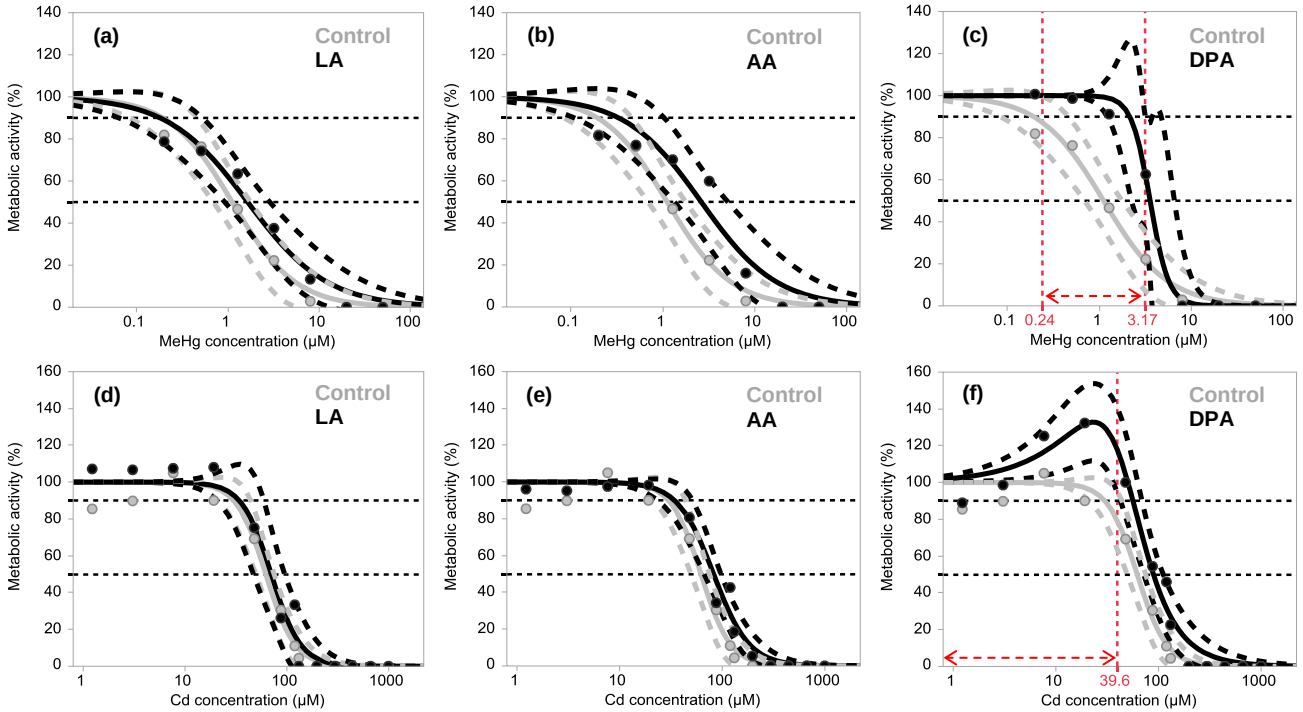

Fig. 3.3. Metabolic activity of the RTL-W1 cells enriched (black) or not (grey) in a specific n-6 PUFA (LA, AA or DPA) and challenged with increasing concentrations of MeHg (a–c) or Cd (d–f) during 24 h. Plain curves represent the LogLogistic prediction models; dotted curves represent the confidence intervals at 98.3% for the predictions. Points represent the mean metabolic activities of the cells challenged at specific MeHg or Cd concentrations (n = 3). The dotted arrows delimit the metal concentration ranges within which the predicted metabolic activity of cells enriched with a specific n-6 PUFA is significantly higher than that of the control cells. Abbreviation used: AA, arachidonic acid; DPA, docosapentaenoic acid; LA, linoleic acid; MeHg, methylmercury; Cd, cadmium.

Table 3.2. EC50s of MeHg and Cd for metabolic activity (as measured by the CTB assay) in RTL-W1 cells enriched in different fatty acids.

PUFA enrichment	MeHg EC50			Cd EC50		
	Estimation (μM)	Confidence interval (μM)	Fold change (compared to the control)	Estimation (μM)	Confidence interval (μM)	Fold change (compared to the control)
Control n-3	0.73	[0.51 - 1.01]	1.0	72.5	[52.3 - 94.6]	1.0
ALA	2.90	[1.66 - 4.79]	4.0	214.7	[161.7 - 275.8]	3.0
EPA	3.25	[2.22 - 4.61]	4.5	207.0	[144.2 - 290.1]	2.9
DHA	1.68	[0.92 - 2.92]	2.3	140.3	[102.5 - 182.3]	1.9
Control n-6	1.11	[0.70 - 1.72]	1.0	62.3	[49.8 - 75.7]	1.0
LA	1.65	[0.92 - 2.79]	1.5	70.6	[49.9 - 95.6]	1.1
AA	2.62	[1.31 - 4.80]	2.4	82.7	[68.2 - 98.0]	1.3
DPA	3.58	[2.50 - 5.14]	3.2	89.7	[72.5 - 110.6]	1.4

The EC50s and their confidence interval (at 98.3%) were obtained by estimating the parameters of the LogLogistic models, which were fitted to the data from the three independent repetitions (n = 3). Abbreviation used: MeHg, methylmercury; EC50, median effect concentration; Cd, cadmium; PUFA, polyunsaturated fatty acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid; DPA, docosapentaenoic acid.

3.3.3. Multivariate analysis

In order to highlight relationships between the phospholipid composition of the RTL-W1 cells and their resistance to MeHg and Cd, we performed a principal component analysis with data from both the fatty acid profile (phospholipid fraction) and the metal challenge (calculated EC50s for MeHg and Cd). The three first principal components (PC) explained 58.9% of the total variance. PC1, PC2 and PC3 explained 24.1, 19.7 and 15.1% of the total variance, respectively (Table 3.3, Fig. 3.4). The three first components clearly separated the PUFA-enriched groups from each other and from the controls (Fig. 3.4). The first axis of the PCA separated cells with a high resistance to both MeHg and Cd from less resistant cells while the second and the third axes separated cells with a higher tolerance to MeHg from those more tolerant to Cd (Fig. 3.4). The higher resistance to both MeHg and Cd was associated with a high phospholipid content in n-3 PUFAs, mainly ALA, 18:4n-3 and 20:3n-3 (PC1) and a lower phospholipid content in n-6 PUFAs, mainly 20:3n-6, LA, 18:3n-6 and 20:2n-6 (PC1, PC2, PC3) (Table 3.3). Besides, some fatty acids were specifically associated with a higher resistance to one metallic compound only. Indeed, a higher phospholipid content in n-3 HUFAs, mainly EPA, 22:5n-3 and DHA, was associated with a higher cellular resistance to Cd (PC2) but not to MeHg (PC3) (Table 3.3). In contrast, a higher phospholipid content in n-6 HUFAs, mainly DPA, 22:4n-6 and AA, was associated with a higher cellular resistance to MeHg (PC3) but not to Cd (PC2) (Table 3.3). Finally, a higher phospholipid content in both MUFAs (mainly 18:1n-9 and 16:1n-7) and SFAs (mainly 16:0 and 14:0) was associated in PC1 with a lower resistance to both MeHg and Cd (Table 3.3). However, the opposite relationship was observed with both MUFAs and SFAs in PC2 and with SFAs in PC3. Indeed, a higher phospholipid content in both MUFAs (e.g. 18:1 n-9) and SFA (e.g. 16:0) was linked to a higher resistance to Cd (PC2) (Table 3.3). In addition, a higher phospholipid content in SFAs (e.g. 16:0) was associated to a higher resistance to MeHg (PC3). These contradictions reduce the plausibility of a preponderant role of the MUFA phospholipid level in modulating the toxicity of MeHg, on the one hand, and of the SFA phospholipid level in modulating the toxicity of both MeHg and Cd, on the other hand.

Table 3.3. Principal component analysis loadings.

	PC1	PC2	PC3
14:0 (1)	-0.46	0.58	0.12
16:0 (2)	-0.49	0.50	-0.56
18:0 (3)	0.00	-0.08	-0.39
16:1n-7 (4)	-0.54	0.30	0.16
18:1n-7 (5)	-0.35	0.45	0.25
18:1n-9 (6)	-0.60	0.60	0.02
ALA (7)	0.81	0.07	0.21
20:3n-3 (8)	0.81	0.07	0.21
18:4n-3 (9)	0.81	0.08	0.21
20:4n-3 (10)	0.39	0.29	0.17
EPA (11)	0.26	0.74	0.22
22:5n-3 (12)	0.05	0.72	0.32
DHA (13)	-0.09	0.48	0.24
LA (14)	-0.14	-0.54	0.76
20:2n-6 (15)	-0.08	-0.56	0.62
18:3n-6 (16)	-0.16	-0.66	0.64
20:3n-6 (17)	-0.72	-0.16	0.31
AA (18)	-0.27	-0.40	-0.41
22:4n-6 (19)	-0.15	-0.44	-0.44
DPA (20)	0.13	-0.24	-0.56
EC50 MeHg	0.70	-0.20	-0.49
EC50 Cd	0.83	0.43	0.09
Part of the total variance explained (%)	24.10	19.70	15.10
Cumulative part of the variance explained (%)	24.10	43.80	58.90

The principal component analysis was performed using, together, the fatty acid profile data (expressed as percentages of the sum of the fatty acids identified in the cell phospholipids, arcsine-root square transformed) and the calculated EC50s (for MeHg and Cd). A loading is the correlation between one variable and one principal component. Numbers in brackets refer to the eigenvectors representation used in the biplot (see Fig. 3.3). Abbreviation used: PC, principal component; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid; DPA, docosapentaenoic acid; EC50, median effect concentration; MeHg, methylmercury; Cd, cadmium.

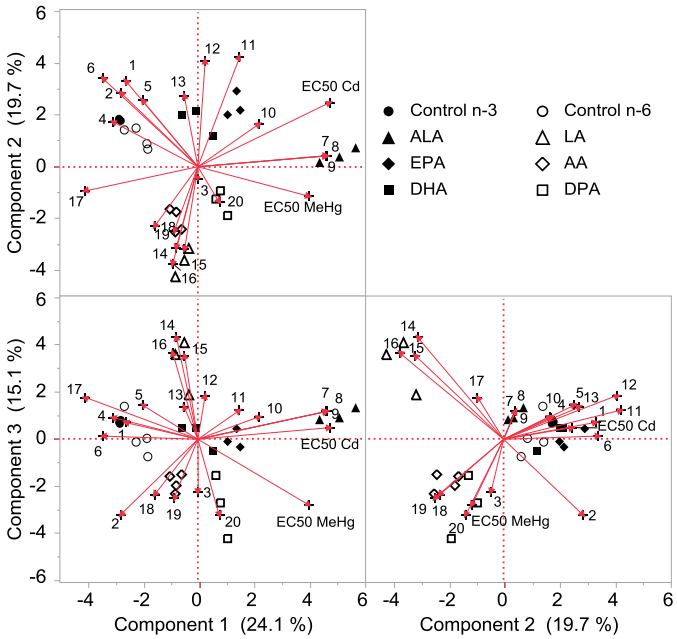

Fig. 3.4. Principal component analysis biplot. The principal component analysis was performed using, together, the fatty acid profile data (expressed as percentages of the sum of the fatty acids identified in the cell phospholipids, arcsine-root square transformed) and the calculated EC50s (for MeHg and Cd). Numbers associated to the eigenvectors refer to a specific fatty acid that composed the cellular phospholipids (see Table 3.3). Abbreviation used: ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid; DPA, docosapentaenoic acid; EC50, median effect concentration; MeHg, methylmercury; Cd, cadmium.

3.4. Discussion

In the present study, we successfully modulated the phospholipid composition of the RTL-W1 cells and highlighted the correlation between that phospholipid composition and cell sensitivity to MeHg and Cd.

The lipid composition of fish tissues is known to globally reflect that of the diet (Bell et al., 2004; Bell et al., 2001; Bell et al., 2006; Caballero et al., 2002; Fonseca-Madrigal et al., 2005; Mourente and Bell, 2006; Petropoulos et al., 2009). Similarly, the phospholipid composition of *in vitro* cultured fish cells globally reflects that of the growth media (Tocher et al., 1988). This property was successfully used in the literature (Gregory et al., 2011; Tocher, 1990; Tocher and Dick, 1990) and in the present study to change the phospholipid composition of fish cells. Cells

differing in their phospholipid composition but from the same origin can thus be obtained by changing their lipid supply.

The PUFA supplementation had no impact either on the proportions of the three cellular lipid classes analysed or on the total lipid content of the cells: cellular lipids were mainly phospholipids and the total phospholipid content was kept constant independently of the PUFA treatment applied. Similar observations were made in the Atlantic salmon cell line (AS) supplemented with 25 µM ALA or LA (Tocher and Dick, 1990). This result indicates that the modification of the phospholipid composition of the RTL-W1 cells resulted from phospholipid turnover rather than from phospholipid accumulation. This turnover was rather efficient as at least 30% of the cellular phospholipid composition was significantly changed after one week of PUFA supplementation. Major changes observed included a high integration of the supplemented PUFA in the cellular phospholipids, the occurrence of direct elongation and desaturation metabolites in the cellular phospholipids as well as a modification of the gross phospholipid composition (MUFA and PUFA levels as well as n-3/n-6 ratio). The high integration of the supplemented PUFA is in accordance with previous literature (Buzzi et al., 1996; Gregory et al., 2011; Tocher, 1990) and corroborates the importance of the lipid composition of the growth media in defining the phospholipid composition of fish cell culture.

Freshwater fish such as rainbow trout are known to be able to synthesize EPA and DHA from ALA (Buzzi et al., 1996; Fonseca-Madrigal et al., 2005; Mourente and Tocher, 1998). However, no significant increase of the EPA and DHA levels was observed after ALA supplementation. Instead, we observed a significant accumulation of its direct desaturation and elongation metabolites (18:4n-3 and 20:3n-3), which were not shown to accumulate *in vivo* (Tocher, 2003). Similarly, the supplementation with EPA led to a significant accumulation of its elongation metabolite (22:5n-3) while no significant increase of DHA was observed. Similar observations were made in cells enriched in the n-6 PUFAs LA and AA as expected since the biotransformation of n-3 and n-6 fatty acids requires the same enzymes (Tocher, 2003). The supplementation with LA was associated with an increase of the phospholipid levels of its desaturation (18:3n-6) and elongation (20:2n-6,

in traces) metabolites without any change in the phospholipid levels of AA and DPA. Likewise, the supplementation with AA led to the accumulation of its elongation metabolite (22:4n-6) while no increase of DPA was observed. The reduction of the ability to synthetize significant amounts of valuable HUFAs such as EPA, DHA and AA from their respective precursors ALA and LA has been already observed after long-term fish cell culture (Gregory et al., 2011; Tocher, 1990; Tocher and Dick, 1990). While this low *in vitro* biotransformation capacity could be disadvantageous when attempting to reproduce *in vivo* conditions, it is interesting as it allows studies of the biological role of the HUFA-precursors ALA, EPA and LA, independently from their indirect role as HUFA-providers.

In the present study, we used the RTL-W1 cell line to assess the impact of individual PUFAs on fish liver cells sensitivity to MeHg and Cd. Our results indicate that enrichment in specific PUFAs was associated with an increase in cell resistance to MeHg (ALA, EPA, DPA) and Cd (ALA, EPA, DHA, DPA). The protective impact of these PUFAs might have occurred through (i) the decrease of the metal cellular uptake due to modifications of membrane lipid composition and/or (ii) the modulation of intracellular parameters increasing the cell resistance to the damages induced by metals.

Modifications of the lipid composition of biological membranes is likely to influence its permeability, fluidity and functions such as ion transport (Arts and Kohler, 2009; Tillman and Cascio, 2003). Since Cd^{2+} uptake occurs through transport pathways of essential metals, its absorption efficiency might be affected by modifications of membrane properties (Klinck and Wood, 2011; McGeer et al., 2011). However, to our knowledge, the influence of the membrane lipid composition on cellular Cd uptake has never been investigated yet.

Changes in membrane properties might also influence both active and passive MeHg transport. In particular, the degree of unsaturation of the membrane modulates its fluidity, which in turn might influence its permeability to pollutants. Indeed, DHA supplementation has been found to decrease MeHg uptake in some mammalian cell lines such as the human embryonic kidney cell line (HEK293) (Nøstbakken et al., 2012) and the rat C6-glial cell line (Kaur et al., 2007) and in primary cultures of

cerebellar neurons and astrocytes from mice (Kaur et al., 2008). However, DHA supplementation had no significant effect on MeHg uptake in the Atlantic salmon kidney cell line (ASK) (Nøstbakken et al., 2012) and in the rat B35-neuronal cell line (Kaur et al., 2007). Similarly, EPA supplementation had no impact on MeHg uptake in both the ASK and HEK293 cell lines. The role of the fatty acid supplementation on cellular uptake of metals remains poorly known. Consequently, further studies are necessary to better understand the role of the membrane lipid composition in MeHg and Cd uptake and to determine whether the protective effect of specific PUFA enrichment against MeHg or Cd toxicity can be attributed to a decrease of the metal uptake.

The toxicity of MeHg and Cd is mainly triggered by the induction of oxidative stress and the alteration of the ionic balance (e.g. calcium), which can lead to apoptosis (Kidd and Batchelar, 2011; Matović et al., 2015; McGeer et al., 2011). The supplementation with n-3 PUFAs (ALA, EPA and DHA) might have increased the RTL-W1 cell ability to cope with these adverse effects. In addition, the fact that each fatty acid treatment exerted similar factors of protection against both metals (Table 3.2) suggests similar mechanisms of protection.

Firstly, the supplementation with n-3 PUFAs could have counteracted the oxidative stress induced by the metals, either directly by decreasing reactive oxygen species production or indirectly by enhancing the cellular antioxidant defences. For example, MeHg and Cd are known to directly increase the mitochondrial production of reactive oxygen species by decreasing the activity of the complex IV and III of the transport electron chain, respectively (Cambier et al., 2009; Wang et al., 2004) while n-3 PUFAs can increase both of them (Hagopian et al., 2010), and thus might counteract the negative impact of these metals. Besides, both MeHg and Cd are generally known to decrease the cellular antioxidant defences by decreasing both the reduced glutathione cellular level and the antioxidant enzyme activities. This results from their high affinity for thiol groups, their affinity for (MeHg) or similarity with (Cd) the cofactors (e.g. manganese, selenium) of some of antioxidant enzymes or their direct interactions with the catalytic sites (Cd) of some antioxidant enzymes (Casalino et al., 2002; Farina et al., 2011; Matović et al., 2015; Wang et al., 2015). The dietary supplementation with ALA (1%) was reported to

counteract the toxic effect of a MeHg contaminated diet (5 mg/kg body weight) by restoring the antioxidant defences and reducing lipid peroxidation in liver, kidneys, brain, plasma and red blood cells of rats (Pal and Ghosh, 2012a, b). The authors suggested that the protective effect of ALA might be due to its bioconversion into EPA, which is known for its antioxidant properties (through enhancing the cellular antioxidant defences) (Palaniswamy et al., 2014; Shakouri Mahmoudabadi and Rahbar, 2014). However, our results show that the biological importance of ALA is not only due to its bioconversion into valuable HUFAs. Indeed, in the present study, not only EPA but also ALA enrichment protected RTL-W1 cells against MeHg and Cd toxicity while no significant bioconversion of ALA into EPA was observed in ALA-enriched cells. Therefore, EPA is unlikely to be responsible for the protective effect of ALA supplementation against MeHg and Cd toxicity. ALA itself and/or its biotransformation products (18:4n-3, 20:3n-3) would rather be at the origin of such protection.

Secondly, the protective effect of enrichment in n-3 PUFAs observed in the present study might be due to a higher resistance of the enriched cells to the apoptosis induced by MeHg and Cd. Indeed, EPA enrichment (300 μM) has been previously shown to significantly reduce the apoptosis induced by 2.5 μM MeHg in the ASK cell line (Nøstbakken et al., 2012). The authors attributed such protection to a lower synthesis of AA-derived eicosanoids in the presence of EPA (Nøstbakken et al., 2012). Indeed, contrary to EPA-derived eicosanoids, AA-derived eicosanoids were reported to increase the calcium intracellular level, which can induce apoptosis (Rizzuto et al., 2003; Tokuda et al., 1992). The replacement of AA by EPA in the membrane phospholipids might favour the production of EPA-derived eicosanoids over the AA-derived ones and decrease apoptosis. Since both MeHg and Cd can induce apoptosis by increasing intracellular calcium level, a decrease of AA-derived eicosanoids might partially explain the protection observed in the RTL-W1 cells supplemented with EPA in the present experiment. However, further investigations are required to test this hypothesis.

Contrary to the other n-3 PUFAs, DHA enrichment had no significant impact on MeHg toxicity in the present experiment. Although DHA is the most studied PUFA, the results of previous studies are contradictory.

Indeed, in some cellular models, DHA exposure increased the damages induced by MeHg. For instance, DHA exposure exacerbated the apoptosis induced by MeHg in the ASK cell line (Nøstbakken et al., 2012), enhanced the production of reactive oxygen species induced by MeHg in the B35-neuronal cell line (Kaur et al., 2007) or increased glutathione depletion due to MeHg exposure in the C6-glial cell line (Kaur et al., 2007) and in primary cultures of cerebellar neurons (Kaur et al., 2008). However, in other models, DHA exposure did not influence GSH depletion induced by MeHg (e.g. in the B35-neuronal cell line (Kaur et al., 2007) and in primary cultures of astrocytes (Kaur et al., 2008)) or even had a protective effect. In primary cultures of cerebellar astrocytes and neurons, DHA exposure decreased the reactive oxygen species production induced by MeHg (Kaur et al., 2008). It is possible that the absence of protection conferred by DHA to MeHg-treated cells could reflect the occurrence of both agonistic and antagonistic actions of this PUFA. These previous results together with our study highlight the need for additional studies to clearly assess the role of DHA supplementation on cellular MeHg sensitivity, particularly in fish.

Compared to the n-3 PUFAs, the enrichment in n-6 PUFAs (LA, AA and DPA) had less impact on the cell tolerance to both MeHg and Cd as only DPA enrichment induced protection. To our knowledge, the influence of only one n-6 PUFA, AA, has previously been investigated on MeHg toxicity (Nøstbakken et al., 2012). In accordance with our results on RTL-W1 cells, AA enrichment did not influence MeHg uptake and apoptosis induced by MeHg in the HEK293 and ASK cell lines (Nøstbakken et al., 2012). The protective effect of DPA against both MeHg and Cd toxicity observed in the present experiment is noteworthy and has, to our knowledge, never been demonstrated before. In particular, DPA was the only tested PUFA that protected the RTL-W1 cells against the membrane integrity damages induced by both MeHg and Cd. The cellular protection induced by DPA might thus have occurred through different mechanisms than the one induced by other tested PUFAs. DPA is a poorly studied PUFA with no clear biological role, which is rarely found in high amounts in organisms, except in some marine algae (Jiang et al., 2004) and fungi (Nakahara et al., 2003). Recent studies have shown that DPA may share similar biological functions with DHA. For instance, DPA supplementation has been recently reported to ameliorate the plasmatic

lipoprotein profile of rats fed a cholesterol-rich diet in a similar extent as a DHA supplementation (Chen et al., 2012). Besides, the DPA level has also been reported to increase in brain and retina of rats in case of n-3 PUFA deficiency, suggesting a compensatory mechanism (Nakahara et al., 2003). However, spatial disorders were observed in rats fed a DHA deficient diet supplemented with DPA as compared to a DHA sufficient diet, suggesting that DPA is not biologically equivalent to DHA (Lim et al., 2005). Further investigations should therefore be performed to better understand the biological functions of this n-6 PUFA and its potential protective role against MeHg and Cd toxicity.

In the present study, a higher tolerance to both MeHg and Cd was associated with a decreased phospholipid content in 20:3n-6, the precursor of AA via Δ5 desaturation. Both 20:3n-6 and AA generate eicosanoids by oxygenation. However, the eicosanoids metabolized from AA are generally considered to have pro-inflammatory properties whereas the eicosanoids produced from 20:3n-6 seem to have anti-inflammatory properties, as those metabolized from EPA (Calder, 2015; Wang et al., 2012). This contrast has increased the interest in 20:3n-6, particularly in oncology. Both anti-cancer (decreasing cell proliferation and increasing apoptosis) and pro-cancer properties (increasing tumour proliferation) were reported for 20:3n-6 in the literature (reviewed by Xu and Qian (2014)). In addition, 20:3n-6 was reported to cause sterility in C. elegans by inducing apoptosis in germ cells (Watts and Browse, 2006). Therefore, the apoptotic potency of 20:3n-6 might partly explain the positive correlation observed, in the present study, between depletion in 20:3n-6 and increase in cell tolerance to MeHg and Cd. However, further investigations, including medium supplementation with 20:3n-6, are needed to test the specific adverse effect of this n-6 PUFA on the RTL-W1 sensitivity to MeHg and Cd.

The present study highlighted a correlation between the phospholipid composition of rainbow trout liver cells and their sensitivity to both MeHg and Cd. At present, *in vivo* effects of dietary lipids on sensitivity to metals are still poorly documented. In one study, fish oil dietary supplementation has been shown to protect rainbow trout against MeHg toxicity (Olsvik et al., 2011) compared to vegetable oil dietary supplementation. The authors attributed this protective impact to the

high level of long chain n-3 HUFAs in fish oil. However, oils also contain other valuable components, such as vitamins and phenolic compounds, the concentration of which largely depends on the oil type and brand. *In vivo* experiments using diets differing only in their lipid composition are therefore still needed to validate the correlations observed in the present *in vitro* study.

In conclusion, the present study highlighted the interest and the pertinence of the RTL-W1 cell model to investigate the biological roles of specific PUFAs separately. We used this model to show that the nature of the PUFA supply modulates fish liver cell tolerance to metallic compounds. In particular, ALA, EPA and DPA enrichment significantly protected the cells against both MeHg and Cd while DHA enrichment significantly protected the cells against Cd but not MeHg. In addition, AA and LA enrichment had no impact on both MeHg and Cd toxicity. Besides, ALA was found to have a protective effect by itself and/or through its direct biotransformation products (18:4n-3, 20:3n-3), independently of its further bioconversion into EPA or DHA. Taken together, these results indicate that the nature of the PUFA supplied is an important factor influencing the tolerance of fish liver cells to both MeHg and Cd toxicity. Further investigations should focus on the mechanisms behind the observed interactions. In addition, *in vivo* experiments with diets differing only by their fatty acid concentrations are required to confirm the results obtained *in vitro* with the RTL-W1 cell line.

3.5. Acknowledgements

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3.6. Supplementary data

Table 3.A.1. Fatty acid profile of the RTL-W1 cells neutral lipids after a 7 day-supplementation with 50 µM PUFA (µmol/g protein).

Fatty acid	Control	ALA	EPA	DHA	LA	AA	DPA
16:0	30.0 ± 5.3	19.9 ± 1.7	28.7 ± 1	19.5 ± 1.8	41.4 ± 21.6	28.9 ± 1.3	36.5 ± 6.8
18:0	25.6 ± 6.8	16.8 ± 1.4	24.5 ± 2.1	15.8 ± 1.7	57.1 ± 34.4	24.7 ± 4.6	33.4 ± 11.4
Total SFA	**55.6 ± 11.7**	**36.7 ± 3.1**	**53.8 ± 2.7**	**35.3 ± 3.5**	**98.5 ± 56.1**	**53.5 ± 4.7**	**69.9 ± 17.1**
16:1n-7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0
Total MUFA	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.3 ± 0.3**	**0.0 ± 0.0**
ALA 18:3n-3	0.0 ± 0.0	16.5 ± 2.9 *	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
EPA 20:5n-3	0.0 ± 0.0	0.0 ± 0.0	6.4 ± 3.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.7
DHA 22:6n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	19.3 ± 3.3	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 1.0
Total n-3 PUFA	**0.0 ± 0.0**	**16.5 ± 2.9**	**6.4 ± 3.3**	**19.3 ± 3.3**	**0.0 ± 0.0**	**0.0 ± 0.0**	**1.7 ± 1.7**
LA 18:2n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	25.4 ± 7.9	0.0 ± 0.0	0.0 ± 0.0
AA 20:4n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	14.2 ± 5.8	0.0 ± 0.0
DPA 22:5n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	114.2 ± 67.2
Total n-6 PUFA	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**	**25.4 ± 7.9**	**14.2 ± 5.8**	**114.2 ± 67.2**
Total PUFA	**0.0 ± 0.0**	**16.5 ± 2.9**	**6.4 ± 3.3**	**19.3 ± 3.3**	**25.4 ± 7.9**	**14.2 ± 5.8**	**115.9 ± 68.8**
Total	**55.6 ± 11.7**	**53.2 ± 6.0**	**60.2 ± 4.7**	**54.6 ± 6.5**	**123.9 ± 58.6**	**68.0 ± 4.5**	**185.8 ± 63.9**
n-3/n-6 ratio					**0.0 ± 0.0**	**0.0 ± 0.0**	**0.01 ± 0.01**

Data are expressed as means ± standard error of mean (n = 3, 4 or 7). Stars indicate significant differences as compared to the control (Wilcoxon Rank Sum Test, total alpha = 0.05, $p < 0.05$). Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 3.A.2. Fatty acid profile of the RTL-W1 cells free fatty acids after a 7 day-supplementation with 50 µM PUFA (µmol/g protein).

Fatty acid	Control	ALA	EPA	DHA	LA	AA	DPA
16:0	130.6 ± 12.1	121.0 ± 9.2	155.9 ± 24.9	105.2 ± 2.6	87.6 ± 24.1	127.3 ± 33.2	134.2 ± 21.3
18:0	95.3 ± 8.2	87.3 ± 6.6	112.0 ± 17.1	76.6 ± 2.5	65.0 ± 16.1	93.0 ± 21.8	102.3 ± 13.8
Total SFA	**225.9 ± 20.2**	**208.4 ± 15.8**	**267.9 ± 42.0**	**181.8 ± 5.0**	**152.6 ± 40.1**	**220.3 ± 55.0**	**236.5 ± 34.8**
Total MUFA	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**
ALA 18:3n-3	0.0 ± 0.0	3.7 ± 3.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
EPA 20:5n-3	0.0 ± 0.0	0.0 ± 0.0	4.6 ± 2.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
DHA 22:6n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.6 ± 2.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total n-3 PUFA	**0.0 ± 0.0**	**3.7 ± 3.7**	**4.6 ± 2.3**	**2.6 ± 2.6**	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**
LA 18:2n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	5.3 ± 2.7	0.0 ± 0.0	0.0 ± 0.0
AA 20:4n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	5.4 ± 3.1	0.0 ± 0.0
DPA 22:5n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.9 ± 2.9
Total n-6 PUFA	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**	**5.3 ± 2.7**	**5.4 ± 3.1**	**2.9 ± 2.9**
Total PUFA	**0.0 ± 0.0**	**3.7 ± 3.7**	**4.6 ± 2.3**	**2.6 ± 2.6**	**5.3 ± 2.7**	**5.4 ± 3.1**	**2.9 ± 2.9**
Total	**225.9 ± 20.2**	**212.1 ± 18.5**	**272.5 ± 40.1**	**184.4 ± 7.6**	**157.9 ± 42.2**	**225.6 ± 54.6**	**239.4 ± 34.5**
n-3/n-6 ratio					**0.00 ± 0.00**	**0.00 ± 0.00**	**0.00 ± 0.00**

Data are expressed as means ± standard error of mean (n = 3, 4 or 7). Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 3.A.3. EC50s of MeHg and Cd for membrane integrity (as measured by the 5-CFDA,AM assay) in RTL-W1 cells enriched in different fatty acids.

PUFA enrichment	MeHg EC50			Cd EC50		
	Estimation (μM)	Confidence interval (μM)	Fold change (compared to the control)	Estimation (μM)	Confidence interval (μM)	Fold change (compared to the control)
Control n-3	6.37	[5.09 - 7.93]	1.0	205.5	[173.4 - 243.0]	1.0
ALA	7.23	[5.67 - 8.87]	1.1	190.0	[170.4 - 211.5]	0.9
EPA	5.94	[4.62 - 7.49]	0.9	191.8	[161.3 - 228.3]	0.9
DHA	5.23	[3.91 - 6.92]	0.8	205.2	[179.9 - 233.7]	1.0
Control n-6	3.80	[3.07 - 4.66]	1.0	76.5	[68.2 - 85.4]	1.0
LA	4.87	[3.35 - 6.90]	1.3	85.0	[60.1 - 114.0]	1.1
AA	5.31	[3.64 - 7.47]	1.4	80.3	[64.2 - 98.5]	1.0
DPA	7.52	[5.05 - 10.79]	2.0	113.2	[92.5 - 137.3]	1.5

The EC50s and their confidence interval (at 98.3%) were obtained by estimating the parameters of the LogLogistic or Brain & Cousens models, which were fitted to the data from the three independent repetitions (n = 3 or 4). Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; Cd, cadmium; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EC50, median effect concentration; EPA, eicosapentaenoic acid; LA, linoleic acid; MeHg, methylmercury; PUFA, polyunsaturated fatty acid.

Table 3.A.4. Parameters of the LogLogistic and Brain & Cousens models fitted to the cytotoxicity data.

Metal and viability assay	PUFA enrichment	Model	EC50 (µM)	Relative slope around EC50 (b) (no unit)	Hormesis term (f) (no unit)
MeHg CTB	Control n-3	LogLogistic	0.73	1.04	-
	ALA	LogLogistic	2.9	0.9	-
	EPA	LogLogistic	3.25	1.18	-
	DHA	LogLogistic	1.68	0.99	-
	Control n-6	LogLogistic	1.11	1.25	-
	LA	LogLogistic	1.65	0.99	-
	AA	LogLogistic	2.62	1.06	-
	DPA	LogLogistic	3.58	4.3	-
MeHg 5-CFDA, AM	Control n-3	LogLogistic	6.37	2.16	-
	ALA	LogLogistic	7.23	2.98	-
	EPA	LogLogistic	5.94	2.04	-
	DHA	LogLogistic	5.23	2.04	-
	Control n-6	LogLogistic	3.8	1.74	-
	LA	LogLogistic	4.87	1.4	-
	AA	LogLogistic	5.31	1.52	-
	DPA	LogLogistic	7.52	2.67	-
Cd CTB	Control n-3	LogLogistic	72.54	1.46	-
	ALA	LogLogistic	214.75	1.44	-
	EPA	LogLogistic	207.01	1.62	-
	DHA	LogLogistic	140.25	1.55	-
	Control n-6	LogLogistic	62.31	3.02	-
	LA	LogLogistic	70.63	3.03	-
	AA	LogLogistic	82.67	2.51	-
	DPA	Brain & Cousens	89.68	2.59	2.3
Cd 5-CFDA,AM	Control n-3	LogLogistic	205.46	1.25	-
	ALA	LogLogistic	189.97	1.14	-
	EPA	LogLogistic	191.84	1.48	-
	DHA	LogLogistic	205.21	1.45	-
	Control n-6	LogLogistic	76.54	1.44	-
	LA	LogLogistic	84.95	1.72	-
	AA	LogLogistic	80.34	1.14	-
	DPA	Brain & Cousens	113.16	1.94	1.26

Model	Function
LogLogistic	$f(x) = \frac{100}{1 + exp(b \times ln\left(\frac{x}{EC50}\right))}$
Brain & Cousens	$f(x) = \frac{100 + f \times x}{1 + (1 + \frac{f \times EC50}{50}) \times exp(b \times ln\left(\frac{x}{EC50}\right))}$

Abbreviations used: EC50, median effect concentration; AA, arachidonic acid; ALA, alpha-linolenic acid; Cd, cadmium; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LA linoleic acid; MeHg, methylmercury ; PUFA, polyunsaturated fatty acid.

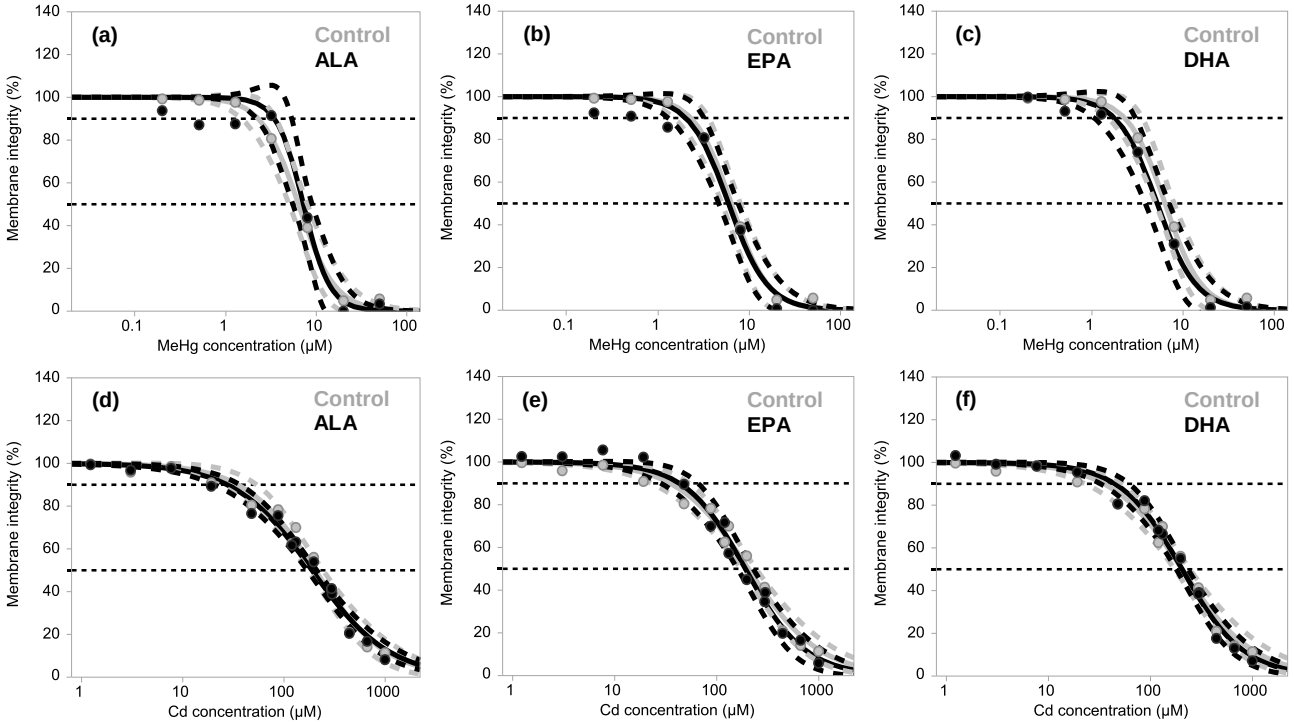

Fig. 3.A.1. Membrane integrity of the RTL-W1 cells enriched (black) or not (grey) in a specific n-3 PUFA (ALA, EPA or DHA) and challenged with increasing concentrations of MeHg (a–c) or Cd (d–f) during 24 h. Plain curves represent the LogLogistic prediction models; dotted curves represent the confidence intervals at 98.3% for the predictions. Points represent the mean membrane integrities of the cells challenged at specific MeHg or Cd concentrations (n = 3). Abbreviations used: ALA, alpha-linolenic acid; Cd, cadmium; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MeHg, methylmercury.

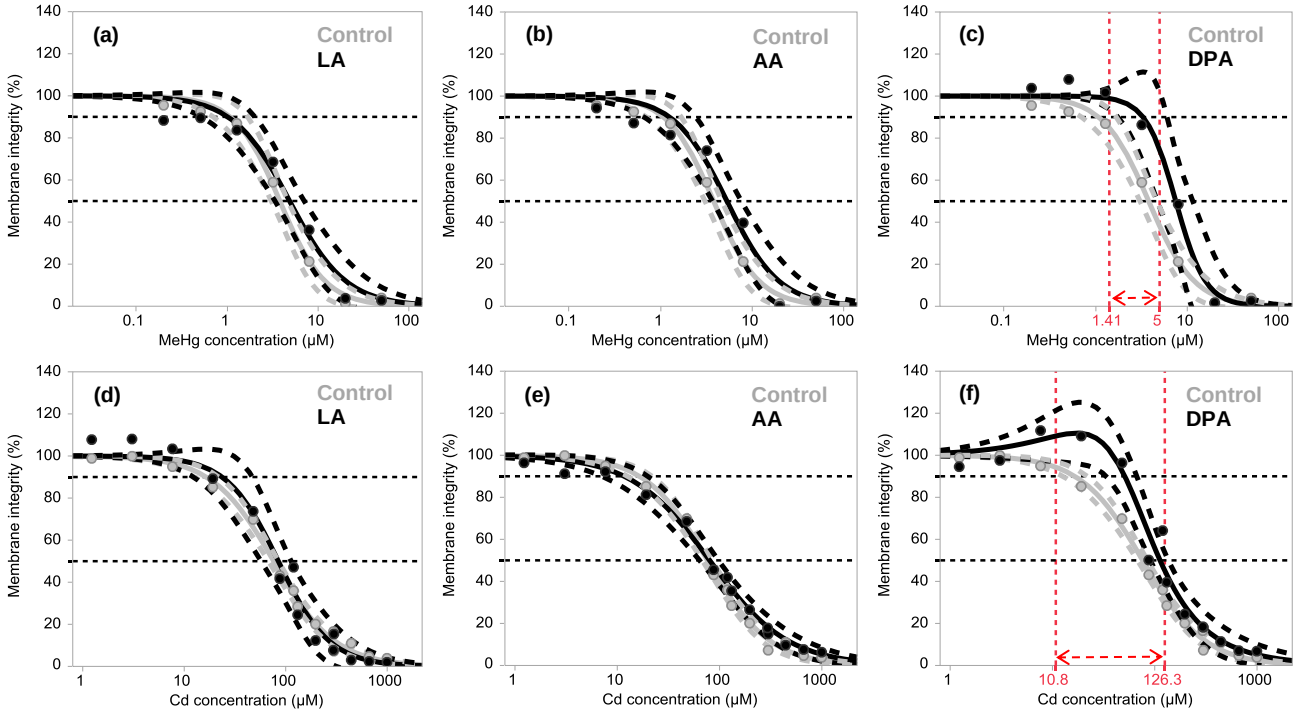

Fig. 3.A.2. Membrane integrity of the RTL-W1 cells enriched (black) or not (grey) in a specific n-6 PUFA (LA, AA or DPA) and challenged with increasing concentrations of MeHg (a–c) or Cd (d–f) during 24 h. Plain curves represent the LogLogistic or Brain & Cousens prediction models; dotted curves represent the confidence intervals at 98.3% for the predictions. Points represent the mean membrane integrities of the cells challenged at specific MeHg or Cd concentrations (n = 3 or 4). The dotted arrows delimit the metal concentration ranges within which the predicted membrane integrity of cells enriched with a specific n-6 PUFA is significantly higher than that of the control cells. Abbreviations used: AA, arachidonic acid; Cd, cadmium; DPA, docosapentaenoic acid; LA, linoleic acid; MeHg, methylmercury.

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Chapter 4

Exploring the interactions between polyunsaturated fatty acids and cadmium in rainbow trout liver cells: a genetic and proteomic study

Foreword

In the previous chapter, we showed that rainbow trout liver cells with different fatty acid profiles, obtained through exposure to media enriched in specific polyunsaturated fatty acids (PUFAs) during one week, had different sensitivities to an acute exposure to methylmercury (MeHg) and cadmium (Cd). In particular, enrichment in alpha-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) and docosapentaenoic acid (DPA, 22:5n-6)) protected RTL-W1 cells against both MeHg and Cd while enrichment in docosahexaenoic acid (DHA 22:6n-3) conferred protection against Cd only and enrichment in linoleic acid (LA, 18:2n-6) and arachidonic acid (AA, 20:4n-6) had no impact. Based on these results we selected one metallic compound (Cd) and four specific PUFAs (ALA, EPA, LA and AA) for further *in vitro* mechanistic investigations. Cd was chosen over MeHg because a wider range of PUFAs tested in the previous chapter conferred protection against Cd. The four selected PUFAs included two protective n-3 PUFAs (ALA and EPA) and their unprotective n-6 counterparts (LA and AA). We tested herin whether specific PUFAs protect RTL-W1 cells by decreasing cellular Cd burden and/or enhancing molecular defences. We also assessed whether Cd influences RTL-W1 cell fatty acid homeostasis and metabolism. The present results were published in Aquatic Toxicology.

Exploring the interactions between polyunsaturated fatty acids and cadmium in rainbow trout liver cells: a genetic and proteomic study

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Abstract

Polyunsaturated fatty acids (PUFAs) have key biological roles in fish cells. We recently showed that the phospholipid composition of rainbow trout liver cells (RTL-W1 cell line) modulates their tolerance to an acute Cd challenge. Here, we investigated (i) the extent to which PUFAs and Cd impact fatty acid homeostasis and metabolism in these cells and (ii) possible mechanisms by which specific PUFAs may confer cytoprotection against Cd. First, RTL-W1 cells were cultivated for one week in growth media spiked with 50 μmol L^{-1} of either alpha-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3), linoleic acid (LA, 18:2n-6) or arachidonic acid (AA, 20:4n-6) in order to modulate their fatty acid profile. Then, the cells were challenged with Cd (0, 50 or 100 μmol L^{-1}) for 24 h prior to assaying viability, fatty acid profile, intracellular Cd content, proteomic landscape and expression levels of genes involved in fatty acid metabolism, synthesis of PUFA-derived signalling molecules and stress response. We observed that the fatty acid supply and, to a lesser extent, the exposure to Cd influenced cellular fatty acid homeostasis and metabolism. The cellular fatty acid composition of fish liver cells modulated their tolerance to an acute Cd challenge. Enrichments in ALA, EPA, and, to a lesser extent, AA conferred cytoprotection while enrichment in LA had no impact on cell viability. The present study ruled out the possibility that cytoprotection reflects a decreased Cd burden. Our results rather suggest that the PUFA-derived cytoprotection against Cd occurs through a reduction of the oxidative stress induced by Cd and a differential induction of the eicosanoid cascade, with a possible role of peroxiredoxin and glutaredoxin (antioxidant enzymes) as well as cytosolic phospholipase A2 (enzyme initiating the eicosanoid cascade).

Key words (5): cadmium; polyunsaturated fatty acids; RTL-W1 cells; gene expression; proteomics

4.1. Introduction

Cadmium (Cd) is an ubiquitous heavy metal that is toxic to human and wildlife. In fish, liver and kidney are the primary organs of accumulation under acute and chronic Cd exposure, respectively (Chowdhury et al., 2003). Cytotoxicity of Cd is mainly driven by (i) disruption of ion homeostasis, (ii) modulation of protein structure and properties and (iii) oxidative stress generation. Indeed, Cd binds to sulfhydryl, carboxyl and phosphate groups in proteins and competes with essential metals (e.g. zinc, calcium, manganese and selenium), leading to enzyme dysfunction, decreased antioxidant defence, loss of redox balance and ultimately apoptosis (Casalino et al., 2002; Matović et al., 2015; McGeer et al., 2011; Moulis, 2010; Wang et al., 2015). Research indicates that these adverse effects of Cd can be alleviated by nutrition (e.g. supplementation with essential metals, vitamins, phenolic compounds and vegetable oils) (Amamou et al., 2015; Choi et al., 2002; Eriyamremu et al., 2008; Fox, 1979; Jamwal et al., 2016; Linhartova et al., 2016; Linhartova and Sampels, 2015; Peraza et al., 1998).

Lipids, and their constitutive fatty acids, are important components of fish diets, with vital cellular roles. They (i) constitute an important energy source, (ii) directly participate in the maintenance of cell structure and membrane fluidity, (iii) are precursors of signalling molecules and (iv) act as nuclear receptor ligands (Tocher, 2003). Pollutants, such as Cd, may interfere with these functions. For example, Cd has been reported to modulate cellular fatty acid metabolism, with consequences for tissue and cellular fatty acid composition (Chen et al., 2013; Fadhlaoui and Couture, 2016; Fadhlaoui et al., 2018; Konar et al., 2010; Koskinen et al., 2004; Madejczyk et al., 2015). On the other hand, using an *in vitro* model (rainbow trout liver cell line RTL-W1), we recently showed that cells of which the phospholipids had been enriched in alpha-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) or docosahexaenoic acid (DHA, 22:6n-3) were significantly more tolerant to an acute Cd challenge (while enrichment in linoleic acid (LA, 18:2n-6) or arachidonic acid (AA, 20:4n-6) had no impact (Ferain et al., 2016). In line with this result, it has been reported that a mixture of EPA and DHA protected human hepatocellular cells (Hep G2 cell line) against acute Cd challenges (24 or 48 h) (Linhartova and Sampels, 2015). Studies with individual PUFAs are

required to illuminate the mechanisms underlying this protective effect. Actually, numerous sites of interactions between polyunsaturated fatty acids (PUFAs) and Cd may explain the observed cytoprotection. First, by modifying the physicochemical properties of the plasma membrane (e.g. fluidity, permeability) (Arts and Kohler, 2009; Kremmyda et al., 2011), PUFAs may influence Cd uptake and/or excretion and thereby alter cytotoxicity. Second, PUFAs, as precursors of signalling molecules and ligands of nuclear receptors (Kremmyda et al., 2011; Tocher, 2003), may also promote activation of cellular defences. Importantly though, PUFA-rich biological membranes are prone to oxidative stress (i.e., lipid peroxidation). This could increase risks of cytotoxic effects in fish cells exposed to pro-oxidant metals such as Cd.

The present study therefore aimed at investigating (i) the extent to which PUFAs and Cd impact fatty acid homeostasis and metabolism in fish liver cells and (ii) possible mechanisms by which specific PUFAs may confer cytoprotection against Cd. To this end, RTL-W1 cells were first differentially enriched in a specific PUFA (ALA, EPA, LA or AA) prior to an acute Cd challenge (24 h). Assays included cellular viability, Cd burden, fatty acid composition, proteome landscape and expression of genes involved in fatty acid metabolism, PUFA-derived signalling and stress response.

4.2. Material and methods

4.2.1. Experimental design

The experimental sequence used was the following: (i) fatty acid profile manipulation by incubating RTL-W1 cells for one week in a control or PUFA-enriched (ALA, EPA, LA or AA) medium; (ii) Cd challenge during 24 h and (iii) endpoints assessment: cytotoxicity (n = 6), intracellular cadmium concentration (n = 4), fatty acid profile (n = 3), targeted gene expression (n = 4) and untargeted proteomic analysis (n = 3). The entire sequence (i, ii and iii) was repeated independently up to 6 times (n), depending on the endpoint measured.

4.2.2. Cell culture and PUFA enrichment

The RTL-W1 cell line was kindly provided by S. Bony (INRA, France). Cells were routinely grown at 19°C in 175 cm^2 flasks (Cellstar, Greiner Bio-One, Belgium) containing 25 mL of Leibovitz's L15 medium supplemented with 5% foetal bovine serum (FBS; PAA Laboratories, France) and 1% antibiotics (100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin) as detailed earlier (Ferain et al., 2016). Cells from passage 54 to 64 were used in the present study. Cell counts and routine viability measurements were made on disposable hemocytometers (C-chip Neubauer improved, NanoEnTek, USA) using Trypan Blue as exclusion dye.

PUFA enrichment was carried out as previously described (Ferain et al., 2016). Briefly, when 80% confluence was reached, RTL-W1 cells maintained in routine growth conditions were harvested with trypsin 0.0625% in a phosphate buffered solution containing 0.03% ethylenediamine tetraacetic acid (PBS-EDTA) and seeded at a density of 2 x 10^4 living cells cm^{-2} in 25 mL of growth medium in 175 cm^2 flasks. Growth medium consisted in Leibovitz's L15 medium supplemented with 2% FBS, 1% antibiotics. For the PUFA-enriched conditions, growth medium was spiked with individual PUFAs (either ALA, EPA, LA or AA, 50 µmol L^{-1}). Prior to this, PUFAs (Larodan, Sweden) were complexed with fatty acid free-bovine serum albumin (FAF-BSA) at a molar ratio of 4 to 1 (PUFAs to FAF-BSA), as described by Best et al. (2006). Control cells were supplied with a FAF-BSA level (12.5 µmol L^{-1}) similar to that of PUFA-enriched cells. PUFA enrichment lasted one week during which the growth media were renewed twice.

4.2.3. Cd challenge

Stock solutions of CdCl2 (0, 5 and 10 mmol L^{-1}, CAS 7790-78-6, Fisher Chemical, UK) were prepared in cell culture grade water and kept at 4°C in the dark until use. The contaminated media were prepared fresh daily by diluting stock solutions 100 times with L15/ex medium, a restrictive medium containing salts, galactose and pyruvate at the same concentration as the Leibovitz's L15 medium (Dayeh et al., 2002). After one week of PUFA enrichment (see section 2.2.), the growth media were

replaced by 25 mL of contaminated media (0, 50 or 100 µmol L^{-1} Cd). These contamination levels were chosen within the range in which ALA and EPA enrichment conferred cytroprotection against Cd (Ferain et al., 2016). Actual contamination levels were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES) (see section 4.2.5.). After 24 h of Cd exposure, RTL-W1 cells were sampled for downstream analyses. Besides, 24 h cytotoxicity induced by the two doses of Cd tested was assessed using both the CellTiter-Blue (CTB) (Promega, The Netherlands) and the 5-carboxyfluorescein diacetate, acetoxymethyl ester (5-CFDA,AM), (Life Technology, USA) assays, as previously explained (Ferain et al., 2016).

4.2.4. Cell sampling

After 24 h of Cd exposure, RTL-W1 cells were washed with 10 mL of PBS-EDTA, detached from the flask with trypsin (0.0625% in PBS-EDTA), washed once with ice-cold FAF-BSA (10 g L^{-1} in Leibovitz's L15 medium) and once with L15/ex medium. From each cell suspension, 4 x 10^6 cells were directly used for fatty acid profile analysis, while the remaining cells were subdivided into aliquots of 1-2 x 10^6 cells each. The aliquots were then centrifuged (400 g, 5 min, 19°C) and cell pellets were flash-frozen in liquid N_2 and kept at -80°C until downstream analyses (intracellular Cd concentration, gene expression, proteomic analysis).

4.2.5. Cd quantification

Cd quantification was performed by ICP-AES and, for the non-exposed cells only, by graphite furnace atomic absorption spectrometry (GFAAS). Frozen cell pellets, containing 1 x 10^6 cells, were resuspended in Tris Buffer (0.05 mol L^{-1}, pH 6.8). From this suspension, one aliquot was used for Cd quantification while another one was used for protein quantification (Bradford, 1976). The former was first digested with 100 µL HNO_3 (65%) at 80°C for 3 h and then diluted to 2.5 mL with deionized water. ICP-AES analyses were run on an iCap 7200 duo spectrophotometer (Thermo Scientific, UK) with argon as plasma gas. Wavelengths for Cd detection were 228.802 nm and 226.502 nm, with the torch in the axial position. GFAAS analyses were run on an ICE3500 spectrophotometer (Thermo Scientific, UK) with argon. A matrix modifier

($MgNO_3$, 5 µL, 4 g L^{-1}) was added to the samples (15 µL). The temperature program was as follows: (i) drying at 110°C for 35 s, 130°C for 10 s, (ii) pyrolysis at 450°C for 20 s, (iii) atomisation at 1500°C for 3 s and (iv) cleaning at 2500°C for 3 s. During the atomisation step, Cd atomic absorption was measured at 228.802 nm, with a Zeeman background correction. For both ICP-AES and GFAAS analyses, Cd concentration was calculated thanks to a calibration curve made from certified standards (Fluka, Germany). Besides, the accuracy of the whole process was controlled both by the standard addition method (recovery 93 ± 2 %) and by the analysis of reference materials (TM-28.4 and TMDA-70.2, Environment Canada, Canada) every 25 samples (recovery 99 ± 1 % in ICP-AES and 96 ± 3 % in GFAAS). For each experimental condition from each biological replicate, quantification was performed on two independent frozen cell pellets. Data from these technical duplicates were averaged. The Cd concentration in contaminated media was also determined by ICP-AES. Exact contamination levels were 48.7 ± 1.0 and 98.0 ± 2.1 µmol L^{-1}.

4.2.6. Cellular fatty acid profile determination

After centrifugation (800 g, 6 min, 19°C), the freshly sampled 4 x 10^6 cells were resuspended in 2 mL of L15/ex medium from which 0.1 mL was used for protein quantification and 2 x 0.8 mL was used for lipid extraction (analyses were performed in duplicate). The 0.1 mL aliquots were centrifuged (550 g, 5 min, 4°C). The isolated cell pellet was lysed (ice-cold lysis buffer: 60 mmol L^{-1} Tris, 10 mmol L^{-1} EDTA, 1% sodium dodecyl sulphate (w/w) in water) and stored at -20°C until protein quantification using the Bicinchoninic acid (BCA) Protein Assay (Thermo Scientific Pierce, USA), with BSA as standard.

Cellular total lipids were first extracted with methanol:chloroform:water (2:2:1.8, v:v:v) (Bligh and Dyer, 1959), then separated into neutral lipids, free fatty acids and phospholipids (Schneider et al., 2012) and finally methylated (Ferain et al., 2016). The resulting fatty acid methyl ester (FAMEs) from the neutral lipids and phospholipid fractions were extracted (Ferain et al., 2016) and separated by gas chromatography (Ferain et al., 2018). The chromatograph (GC Trace-1310, Thermo Fisher Scientific, Italy) was equipped with an

RT2560 capillary column (100 m x 0.25 mm internal diameter, 0.2 μm film thickness: Restek, USA), a TriPlus TP100 autosampler (Thermo Fisher Scientific, Italy) and a flame ionisation detector (FID: Thermo Quest, Italy). The carrier gas used was H_2 at constant pressure (200 kPa) and flow rate (2 mL min^{-1}). The FID was continuously flowed by H_2 (35 mL min^{-1}), air (350 mL/min) and N_2 (40 mL min^{-1}) and kept at a constant temperature of 255°C. The temperature program was as follows: an initial temperature of 80°C, which increased at 25°C min^{-1} up to 175°C, a holding temperature of 175°C during 25 min, a new increase at 10°C min^{-1} up to 200°C, a holding temperature of 200°C during 20 min, a new increase at 10°C min^{-1} up to 220°C, a holding temperature of 220°C during 5 min, a last increase at 10°C min^{-1} up to 235°C and a final holding temperature of 235°C during 15 min. FAMEs were finally identified and quantified by comparing the obtained chromatograms (retention times and peak areas) with that of a standard containing 44 FAMEs (Larodan, Sweden) at known concentrations. The chromatogram management software used was ChromQuest (version 5.0, ThermoFinnigan, Italy). Data from the technical duplicates were averaged.

4.2.7. Gene expression

Total RNA was extracted from 1 x 10^6 frozen cells using the Aurum Total RNA Mini Kit (Bio-Rad Laboratories, USA) following manufacturer's Spin Protocol. The DNAse I treatment was always included. RNA quality and quantity was assessed spectrophotometrically on a Nanodrop ND-1000 (Isogen, Life Sciences, The Netherlands) using the optical densities (OD) measured at 260, 280 and 230 nm (OD260, OD260/280 and OD260/230). Total RNA (1 μg) was then reverse transcribed with the Iscript cDNA Synthesis Kit (Bio-Rad Laboratories, USA) according to manufacturer's instructions. cDNA was diluted 3 times in nuclease free water and kept at -20°C until quantification of the expression level of targeted genes (Table 4.1). Quantitative polymerase chain reactions (qPCR) were run on a Step One Plus thermocycler (Applied Biosystems, Life Technologies, USA). Samples were run in technical duplicates. Each reaction contained cDNA (1 μL), primers and SYBR Select Master Mix I (Applied Biosystems, Life Technologies, USA) in a final volume of 20 μL. The temperature programme was: (i) 95°C for 2 min, (ii) 95°C for 15 s, (iii) gene-specific annealing-extension temperature (see Table 4.1) for 1

min. Steps (ii, iii) were repeated 40 times. The programme ended with a melt curve analysis. Primer pairs for 17 target genes and 2 housekeeping genes (Table 4.1) were either obtained from literature or designed using transcript sequence information available at the European Nucleotide Archive (for identifiers, see Marancik et al. (2014)). The targeted genes 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*), fatty acid elongases 2 and 5 (*ELOVL2* and *ELOVL5*)), β-oxidation (carnitine palmitoyl transferase 1 (*CPT1*)), synthesis of PUFA-derived signalling molecules (cytosolic phospholipase A2 (*cPLA2*), cyclooxygenase 2 (*COX2*), arachidonate 5-lipoxygenase (*ALOX5*)) and stress response (metallothioneins a and b (*MTa* and *MTb*), heat shock proteins 70a and 70b (*HSP70a* and *HSP70b*), apoptosis regulator BCL2-Like1 (*BCLx*), tumor protein 53 (*p53*), calreticulin (*CALR*)) (Fig. 4.1). The orphan cytochrome P450 20A1 gene (*CYP20A1*) was also studied. Each primer pair used was experimentally validated: (i) the linearity of the amplification was assessed, (ii) optimum primer concentrations were determined and (iii) the identity of the amplified sequence was confirmed by sequencing (Beckman Coulter Genomics, UK). Relative expression analysis was performed with the $E^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). For each biological replicate, Ct values of technical duplicates were averaged prior to relative expression calculation. The geometric mean of *tubulin* and *18S* Ct values was used as housekeeping value (Vandesompele et al., 2002). Other genes and gene combinations tested as housekeepers were proved unreliable (i.e., lower stability of Ct values across treatments; data not shown). Control cells that were not exposed to Cd were used as reference condition, for relative expression of data. An arbitrary expression value of 1 was given to each biological replicate of this condition. Note that for *MTa*, the reference condition for relative expression of data was control cells exposed to 50 μmol L^{-1} Cd as no detectable signal of amplification could be obtained for this gene in the absence of Cd challenge. The *MTa* expression level of the latter experimental condition was set at 1 for each biological replicate.

Table 4.1. Sequence, amplicon size, annealing temperature and optimal concentration in master mix for the primers of the genes investigated.

Gene	Sequence 5'→3'	Size (bp)	Annealing T (°C)	Concentration (nmol L^{-1})	Reference
Tubulin *housekeeping gene 1*	F: GGTCAATGTGGAAATCAGATCG R: TCTCTCAAGCTGCAGATCG	111	60	300	*Present study*
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. 2018*
CPT1 *carnitine palmitoyl transferase 1a*	F: CTCCTGGAAGAAGAGGTTCATACG R: ACTCACCACCACCACCAAAAAC	95	60	100	*Ferain et al. 2018*
FADS2 *fatty acid desaturase 2*	F: AGGGTGCCTCTGCTAACTGG R: TGGTGTTGGTGATGGTAGGG	175	60	200	*Kamalam et al. 2013*
ELOVL5 *fatty acid elongase 5*	F: CCAAGTACATGAGACACAGAC R: CACACAGCAGACACCATCTC	115	60	100	*Ferain et al. 2018*
cPLA2 *cytosolic phospholipase A2*	F: GAGATGTCGTTAGAGGTGTG R: GTCTCTGCGTGTCTGTCTGA	94	60	200	*Ferain et al. 2018*
COX2 *cyclooxygenase 2*	F: CATCGGACAAGAACCCTTGA R: CTTCATTGGAGAGGCTGGTGTGC	73	60	100	*Chettri et al. 2011*
ALOX5 *arachidonate 5-lipoxygenase*	F: CGGCAGAGCACGGTTGCCAAAG R: CCACTCACTCCATCTGTAGGTT	104	58	200	*Baron et al. 2005*

Transcript sequences used for primer design were gleaned from the literature or designed (*"Present study"*) based on transcript sequences annotated by *Marancik et al. 2014.* (see *"Reference"* column). Abbreviations used: bp, base pairs; F, forward; R, reverse; T, temperature.

(continued)

Gene	Sequence 5'→3'	Size (bp)	Annealing T (°C)	Concentration (nmol L^{-1})	Reference
HSP70a *heat shock protein 70a*	F: CAGCATTGAGATTGACTCC R: GTCAGAACACATCTCCTC	86	60	300	*Ferain et al. 2018*
HSP70b *heat shock protein 70b*	F: CAGCATTGAGATTGACTCT R: GTCGGAACACATTTCCTC	71	60	200	*Ferain et al. 2018*
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. 2018*
CALR *calreticulin*	F: GTTCGGTCCAGACATCTGTG R: GTGTACTCATCATCCTTGC	115	60	300	*Present study*
CYP20A1 *cytochrome P450 20A1*	F: GCTGTCACTCAACTCGCTCTG R: CTGATGGAGCTCTTCTCCATG	137	60	300	*Ferain et al 2018*

Transcript sequences used for primer design were gleaned from the literature or designed (*"Present study"*) based on transcript sequences annotated by *Marancik et al. 2014.* (see *"Reference"* column). Abbreviations used: bp, base pairs; F, forward; R, reverse; T, temperature.

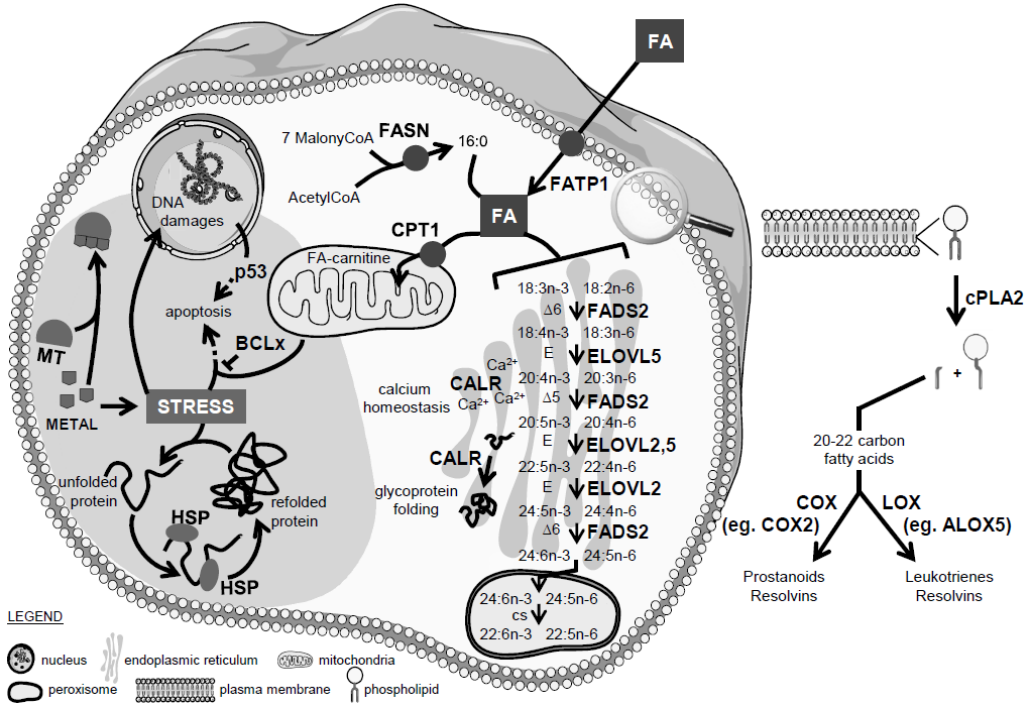

Fig. 4.1. Roles of the proteins encoded by the genes investigated. The illustration (adapted from Ferain et al. (2018)) has been done using the "cell biology" package from Servier Medical Art. Abbreviations used: Δ5, Δ5 desaturation; Δ6, Δ6 desaturation; ALOX5, arachidonate 5-lipoxygenase; BCLx, apoptosis regulator BCL2-Like1; CALR, calreticulin; COX2, cyclooxygenase 2, cPLA2, cytosolic phospholipase A2; CPT1, carnitine palmitoyl transferase 1; cs, peroxisomal chain shortening; E, elongation; ELOVL2, fatty acid elongase 2; ELOVL5, fatty acid elongase 5; FA, fatty acid; FADS2, fatty acid desaturase 2; FASN, fatty acid synthase; FATP1, fatty acid transport protein 1; HSP, heat shock protein; MT, metallothionein; p53, tumor protein 53.

4.2.8. Proteomic analysis

A subset of 8 experimental conditions was selected for proteomic analysis (CT, ALA-, EPA- and AA-enriched cells, exposed to either 0 or 50 µmol L^{-1} Cd). Sample analysis was carried out as described by Villers et al. (2017), with minor adaptations. Briefly, frozen cell pellets (1 x 10^6 cells) were resuspended in tetraethylammonium bromide buffer (TEAB, 0.5 mol L^{-1}, pH 8.0, Thermo Scientific, USA) containing a protease inhibitor (Halt Protease and Phosphatase Inhibitor Cocktail, Thermo Scientific, USA), homogenized (15 s, Vortex), sonicated (30 s) and centrifuged (90720 g, 30 min, 4°C, Optima ultra-speed centrifuge equipped with a TLA-55 rotor, Beckman Coulter, USA). Supernatants containing soluble proteins were isolated. Proteins were quantified with the BCA Protein Assay, using BSA as standard. Soluble proteins (20 µg) were digested and labelled for iTRAQ (8-plex) as described by Villers et al. (2017). Samples (8) from the same biological replicate were then pooled and labelled peptides were separated by reversed phase chromatography on a C18 PepMap100 pre-column and analytical column (LC Packings, The Netherlands) for 180 min at a flow rate of 300 nL min^{-1} using a linear gradient from 8% acetonitrile in water/0.1% trifluoroacetic acid to 76% acetonitrile in water/0.085% trifluoroacetic acid. The eluted peptides were mixed with α-cyano-4-hydroxycinnamic acid matrix (2 mg mL^{-1} in 70% acetonitrile, 0.1% trifluoroacetic acid) and spotted directly onto a MALDI target using a Probot system (LC Packings The Netherlands) (Villers et al., 2017). The spotted plate was analysed on an 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Life Technologies, USA) and MS, MS/MS spectra were obtained and processed as described by Villers et al. (2017). LC-MS/MS data were confronted to the trout peptide database (http://www.genoscope.cns.fr/trout/data/). The same search options as in Villers et al. (2017) were applied in the analysis method. Results were further processed by the Pro Group™ Algorithm to determine the minimal set of justifiable identified proteins (Villers et al., 2017). Protein identification was confirmed using the Mascot 2.2.04 program (Matrix Science, USA) using the same database as for ProteinPilot (SCIEX, USA). The following variable modifications were allowed: C-carbamidomethyl, K-acetylation, methionine oxidation, dioxidation and trypsin. Proteins

were finally annotated based on Oncrorhynchus_genome database, and Oncorhynchus_peptide database (both available at the genoscope trout genome resources - URL cited above).

4.2.9. Statistical analyses

Graphs and statistical analyses were performed with the JMP PRO 12.0 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).

The fact that metabolic activity, membrane integrity and intracellular Cd concentration of the non-exposed cells as well as gene expression levels of the reference condition had no variance restricted the use of classical statistical tests. This limitation was overcome by performing Student's t-tests (Dagnelie, 2006) to determine (i) whether metabolic activity and membrane integrity were significantly different from 100%, (ii) whether intracellular Cd concentrations were different from 0 and (iii) whether relative gene expression levels were different from 1. In any other case, two one-way ANOVAs were performed to assess impacts of the PUFA enrichment strategy and the Cd exposure level. For each PUFA enrichment strategy separately, a first one-way ANOVA followed by a post-hoc Tukey test was performed to assess impact of the Cd exposure level on membrane integrity, metabolic activity (both arsin-rootsquare transformed), Cd intracellular concentrations (no data transformation required) and gene expressions (Log transformed). For each Cd exposure level separately, the impact of the PUFA enrichment strategy was assessed by a second 1-way ANOVA followed by a post-hoc Dunnett's test, with control cells as reference condition.

Statistical analyses of the proteomic data were performed by combining the p-values computed by ProteinPilot, using weighed z-scores (Zaykin, 2011), as described by Villers et al. (2017).

4.3. Results

4.3.1. PUFA enrichment modulates the phospholipid composition of the RTL-W1 cells

The fatty acid profile of RTL-W1 cell phospholipids was modified by changing the PUFA composition of the culture medium (Table 4.2).

Phospholipids of cells cultivated for one week in a medium enriched in a specific PUFA were significantly richer in the supplemented PUFA and some of its biotransformation products than control cells (see Fig. 4.1 for the biotransformation pathway). Particularly, ALA, 18:4n-3, 20:3n-3 and 20:4n-3 were found in the phospholipids of cells grown in a medium enriched in ALA but not in those of control cells. ALA-enriched cells contained also about 4-fold more EPA in their phospholipids than control cells. Phospholipids of cells grown in an EPA-rich medium were about 19-fold richer in EPA than those of control cells. Phospholipids of EPA-enriched cells also contained a significantly higher amount of 22:5n-3 than control cells. Adding LA to the culture medium significantly increased the cell phospholipid content in LA (by about 15-fold), 18:3n-6 and 20:3n-6, without affecting AA phospholipid level. The cell phospholipid richness in AA increased by about 9-fold following AA addition to the growth medium. AA-enriched cells also contained 22:4n-6 in their phospholipids while control cells were deprived of it. As a consequence of these individual changes, the phospholipids of RTL-W1 cells grown in a medium enriched in a specific n-3 PUFA (vs a n-6 PUFA) were characterized by a significantly higher (vs a lower) n-3/n-6 ratio than control cells. The PUFA enrichment strategies applied enabled to obtain RTL-W1 cells with n-3/n-6 ratios encompassing a 200-fold range. Finally, phospholipids of cells cultivated in media enriched in a specific PUFA were significantly richer in PUFAs (about 3.5- to 4.5-fold) and poorer in MUFAs (about 2- to 3.5-fold). The decrease of the total MUFA level observed under PUFA enrichment was mainly due to a lower 18:1n-9 level. The SFA content and the total amount of fatty acids

identified in cell phospholipids were not changed upon PUFA enrichment.

4.3.2. PUFA enrichment modulates the neutral lipid composition of RTL-W1 cells

Cellular neutral lipid levels were about 10 times lower than phospholipid levels irrespective of the PUFA enrichment strategy (Tables 4.2, 4.3). Neutral lipids of control cells were composed of SFAs, MUFAs but were deprived of PUFAs (Table 4.3). Adding a specific PUFA to the growth medium had no impact on SFA level in cellular neutral lipids but significantly decreased MUFA level (by about 3- to 4.5-fold), especially 18:1n-9, as it did in cellular phospholipids. Higher level of the specific PUFA added to the culture medium was recovered in neutral lipids of PUFA-enriched RTL-W1 cells, as well as direct desaturation products for ALA- and LA-enriched cells and elongation products for EPA- and AA-enriched cells (Table 4.3).

4.3.3. Cd has limited impact on the phospholipid composition of RTL-W1 cells

A 24-h exposure to Cd (50 or 100 µmol L^{-1}) had no significant impact on the fatty acid profile of RTL-W1 cell phospholipids. A few trends were however noticed in control, LA- and AA-enriched cells (Table 4.4). The phospholipid level of 22:6n-3 slightly increased under Cd exposure (100 µmol L^{-1}, 24 h) in control cells (+22% as compared to non-exposed control cells) while it decreased (100 µmol L^{-1}, 24 h) in AA-enriched cells (-44% as compared to non-exposed AA-enriched cells). These opposite variations resulted in a significantly lower 22:6n-3 level in phospholipids of AA-enriched cells challenged with the highest Cd dose tested (100 µmol L^{-1}) as compared to control cells challenged with the same Cd level (-67%) (Table 4.4). Besides, the 20:3n-6 phospholipid level slightly decreased in LA-enriched cells under Cd exposure (-16 to -24%) while it remained stable in control cells. As a result, the significant difference of 20:3n-6 phospholipid level between LA-enriched and control cells disappeared under Cd challenge (Table 4.4).

Table 4.2. Fatty acid composition of RTL-W1 cell phospholipids after one week culture in a growth medium supplemented or not (control) in a specific PUFA (50 µmol L^{-1} ALA, EPA, LA or AA) followed by 24 h in a medium deprived of fatty acid (µmol g^{-1} protein).

Fatty acids	Control	ALA	EPA	LA	AA
14:0	12.7 ± 3.3	6.0 ± 1.4	10.1 ± 0.9	7.1 ± 0.8	9.2 ± 0.9
16:0	206.6 ± 72.6	154.6 ± 13.9	193.2 ± 10.7	180.1 ± 34.0	175.8 ± 21.4
18:0	129.3 ± 39.8	135.4 ± 6.4	121.4 ± 8.4	132.2 ± 20.1	109.7 ± 18.2
Total SFA	**348.5 ± 115.6**	**296.1 ± 18.4**	**324.7 ± 18.5**	**319.4 ± 54.9**	**294.8 ± 40.0**
16:1n-7	11.6 ± 3.5	0.0 ± 0.0 (*)	6.4 ± 0.3	0.0 ± 0.0 *	5.5 ± 0.3
18:1n-7	27.5 ± 6.9	13.2 ± 0.8 *	20.8 ± 1.4	13.2 ± 0.9 (*)	17.0 ± 1.0
18:1n-9	232.4 ± 42.9	87.6 ± 5.2 *	110.5 ± 6.8 *	67.3 ± 6.2 *	91.5 ± 5.4 *
Total MUFA	**271.5 ± 53.0**	**100.8 ± 6.0 ***	**137.7 ± 8.4 ***	**80.5 ± 7.1 ***	**114.1 ± 6.7 ***
ALA 18:3n-3	0.0 ± 0.0	**164.2 ± 2.1 ***	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
18:4n-3	0.0 ± 0.0	52.4 ± 6.5 *	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:3n-3	0.0 ± 0.0	12.9 ± 1.0 *	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:4n-3	0.0 ± 0.0	18.4 ± 0.9 *	11.7 ± 2.1 *	0.0 ± 0.0	0.0 ± 0.0
EPA 20:5n-3	8.2 ± 1.1	36.1 ± 4.2 *	**153.5 ± 18.0 ***	3.9 ± 0.7 (*)	0.0 ± 0.0 *
22:5n-3	9.4 ± 1.3	8.0 ± 0.6	90.7 ± 10.3 *	7.2 ± 1.2	7.5 ± 0.7
22:6n-3	14.6 ± 2.4	10.4 ± 0.8	13.0 ± 1.3	10.0 ± 2.0	10.6 ± 3.5
Total n-3 PUFA	**32.2 ± 4.2**	**302.3 ± 10.7 ***	**268.9 ± 31.0 ***	**21.1 ± 2.5**	**18.0 ± 4.0 (*)**

Data are expressed as means ± standard error of mean (n = 3). Stars indicate significant differences between control and PUFA-enriched conditions ($p < 0.05$) while stars into brackets indicate similar trends but without significance ($0.05 < p < 0.1$). Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

(continued)

Fatty acids	Control	ALA	EPA	LA	AA
LA 18:2n-6	19.3 ± 3.9	11.8 ± 2.8	11.8 ± 0.9	**282.3 ± 45.8 ***	9.6 ± 1.0
18:3n-6	4.1 ± 0.8	0.0 ± 0.0 *	0.0 ± 0.0 *	47.6 ± 2.4 *	4.7 ± 0.3
20:2n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	12.6 ± 2.2 *	0.0 ± 0.0
20:3n-6	12.8 ± 2.0	0.0 ± 0.0 *	6.0 ± 0.7 *	26.4 ± 2.4 *	12.9 ± 0.6
AA 20:4n-6	20.6 ± 3.4	11.0 ± 1.0 *	16.6 ± 1.2	20.5 ± 1.8	**184.9 ± 18.6 ***
22:4n-6	0.0 ± 0.0	0.0 ± 0.0	5.3 ± 0.7 *	0.0 ± 0.0	71.7 ± 4.1 *
Total n-6 PUFA	**56.8 ± 10.1**	**22.9 ± 2.7 ***	**39.7 ± 3.5**	**389.3 ± 54.6 ***	**283.9 ± 23.7 ***
Total PUFA	**89.0 ± 14.2**	**325.1 ± 12.9 ***	**308.6 ± 34.6 ***	**410.4 ± 57.1 ***	**301.9 ± 25.5 ***
Total	**709.0 ± 179.7**	**722.0 ± 27.4**	**771.1 ± 57.9**	**810.3 ± 119.1**	**710.7 ± 70.7**
n-3/n-6 ratio	**0.58 ± 0.04**	**13.52 ± 1.31 ***	**6.74 ± 0.17 ***	**0.06 ± 0.01 ***	**0.06 ± 0.01 ***

Data are expressed as means ± standard error of mean (n = 3). Stars indicate significant differences between control and PUFA-enriched conditions ($p < 0.05$) while stars into brackets indicate similar trends but without significance ($0.05 < p < 0.1$). Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 4.3. Fatty acid composition of RTL-W1 cell neutral lipids after one week culture in a growth medium supplemented or not (control) in a specific PUFA (50 µmol L^{-1} ALA, EPA, LA or AA) followed by 24 h in a medium deprived of fatty acid (µmol g^{-1} protein).

Fatty acid	Control	ALA	EPA	LA	AA
16:0	24.7 ± 6.1	24.0 ± 3.5	21.6 ± 4.3	29.9 ± 7.9	21.2 ± 1.6
18:0	33.2 ± 4.1	31.9 ± 7.7	26.4 ± 6.9	34.6 ± 14.3	28.9 ± 5.0
Total SFA	**57.9 ± 10.2**	**55.9 ± 11.2**	**48.0 ± 11.2**	**64.4 ± 22.2**	**50.2 ± 6.4**
18:1n-9	16.8 ± 0.9	4.6 ± 0.4 *	5.3 ± 0.6 *	4.7 ± 1.2 *	3.5 ± 0.5 *
Total MUFA	**16.8 ± 0.9**	**4.6 ± 0.4 ***	**5.3 ± 0.6 ***	**4.7 ± 1.2 ***	**3.5 ± 0.5 ***
ALA 18:3n-3	0.0 ± 0.0	**10.7 ± 1.3 ***	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
18:4n-3	0.0 ± 0.0	5.4 ± 0.8 *	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
EPA 20:5n-3	0.0 ± 0.0	0.0 ± 0.0	**9.2 ± 1.5 ***	0.0 ± 0.0	0.0 ± 0.0
22:5n-3	0.0 ± 0.0	0.0 ± 0.0	5.5 ± 0.8 *	0.0 ± 0.0	0.0 ± 0.0
Total PUFA n-3	**0.0 ± 0.0**	**16.2 ± 1.8 ***	**14.6 ± 2.2 ***	**0.0 ± 0.0**	**0.0 ± 0.0**
LA 18:2n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	**19.9 ± 7.2 (*)**	0.0 ± 0.0
18:3n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.6 ± 0.5 *	0.0 ± 0.0
AA 20:4n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	**6.0 ± 0.5 ***
22:4n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.6 ± 0.0 *
Total PUFA n-6	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**	**24.5 ± 7.6 (*)**	**8.6 ± 0.5 ***
Total PUFA	**0.0 ± 0.0**	**16.2 ± 1.8 ***	**14.6 ± 2.2 ***	**24.5 ± 7.6 (*)**	**8.6 ± 0.5 ***
Total	**74.7 ± 11.0**	**76.7 ± 10.1**	**67.9 ± 13.5**	**93.6 ± 31.0**	**62.3 ± 7.0**

Data are expressed as means ± standard error of mean (n = 3). Stars indicate significant differences between control and PUFA-enriched conditions ($p < 0.05$) while stars into brackets indicate similar trends but without significance ($0.05 < p < 0.1$). Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 4.4. Fatty acid composition of RTL-W1 cell phospholipids after one week culture in a growth medium supplemented or not (control) in a specific PUFA (50 µmol L^{-1} LA or AA) and a subsequent challenge with Cd (0, 50 or 100 µmol L^{-1}) during 24 h in a medium deprived of fatty acid (µmol g−1 protein).

	0 µmol L^{-1} Cd			50 µmol L^{-1} Cd		
	Control	LA	AA	Control	LA	AA
14:0	12.7 ± 3.3	7.1 ± 0.8	9.2 ± 0.9	9.8 ± 0.5	6.0 ± 0.7 *	7.4 ± 0.3
16:0	206.6 ± 72.6	180.1 ± 34.0	175.8 ± 21.4	165.5 ± 17.9	158.0 ± 28.8	148.2 ± 21.7
18:0	129.3 ± 39.8	132.2 ± 20.1	109.7 ± 18.2	120.4 ± 12.3	123.3 ± 19.3	99.5 ± 18.9
Total SFA	**348.5 ± 115.6**	**319.4 ± 54.9**	**294.8 ± 40.0**	**295.7 ± 30.1**	**287.3 ± 48.7**	**255.2 ± 40.4**
16:1n-7	11.6 ± 3.5	0.0 ± 0.0 *	5.5 ± 0.3	9.5 ± 1.1	0.0 ± 0.0 *	4.6 ± 0.5 (*)
18:1n-7	27.5 ± 6.9	13.2 ± 0.9 (*)	17.0 ± 1.0	24.7 ± 2.2	10.7 ± 1.3 *	14.4 ± 1.3 (*)
18:1n-9	232.4 ± 42.9	67.3 ± 6.2 *	91.5 ± 5.4 *	216.1 ± 18.0	59.9 ± 7.0 *	76.9 ± 6.9 *
Total MUFA	**271.5 ± 53.0**	**80.5 ± 7.1 ***	**114.1 ± 6.7 ***	**250.3 ± 20.5**	**70.6 ± 8.3 ***	**95.9 ± 8.7 ***
ALA 18:3n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
18:4n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:3n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:4n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
EPA 20:5n-3	8.2 ± 1.1	3.9 ± 0.7 (*)	0.0 ± 0.0 *	9.4 ± 0.6	4.1 ± 1.3 (*)	0.0 ± 0.0 *
22:5n-3	9.4 ± 1.3	7.2 ± 1.2	7.5 ± 0.7	9.0 ± 0.9	6.0 ± 1.1	5.7 ± 0.5
22:6n-3	14.6 ± 2.4	10.0 ± 2.0	10.6 ± 3.5	13.6 ± 1.6	8.8 ± 1.4	5.4 ± 0.4
Total n-3 PUFA	**32.2 ± 4.2**	**21.1 ± 2.5**	**18.0 ± 4.0 (*)**	**32.0 ± 3.1**	**18.8 ± 3.7**	**11.1 ± 0.8 ***

Data are expressed as means ± standard error of mean (n = 3). For each level of Cd exposure, stars indicate significant differences between control and PUFA-enriched conditions (1-way ANOVA with a post-hoc Dunnett's test with control cells as reference condition, $p < 0.05$) while stars into brackets indicate similar trends but without significance ($0.05 < p < 0.1$). Abbreviations used: AA, arachidonic acid; Cd, cadmium; LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

(continued)

	0 µmol L⁻¹ Cd			50 µmol L⁻¹ Cd		
	Control	LA	AA	Control	LA	AA
LA 18:2n-6	19.3 ± 3.9	**282.3 ± 45.8 ***	9.6 ± 1.0	18.4 ± 2.5	**239.2 ± 46.9 ***	7.8 ± 0.8 *
18:3n-6	4.1 ± 0.8	47.6 ± 2.4 *	4.7 ± 0.3	4.1 ± 0.3	41.1 ± 4.0 *	4.1 ± 0.3
20:2n-6	0.0 ± 0.0	12.6 ± 2.2 *	0.0 ± 0.0	0.0 ± 0.0	10.3 ± 2.1 *	0.0 ± 0.0
20:3n-6	12.8 ± 2.0	26.4 ± 2.4 *	12.9 ± 0.6	12.4 ± 1.0	20.0 ± 2.5	10.5 ± 0.7
AA 20:4n-6	20.6 ± 3.4	20.5 ± 1.8	**184.9 ± 18.6 ***	20.2 ± 2.0	16.9 ± 2.1	**155.4 ± 15.4 ***
22:4n-6	0.0 ± 0.0	0.0 ± 0.0	71.7 ± 4.1 *	0.0 ± 0.0	0.0 ± 0.0	56.0 ± 3.2 *
Total n-6 PUFA	**56.8 ± 10.1**	**389.3 ± 54.6 ***	**283.9 ± 23.7 ***	**55.1 ± 5.6**	**327.6 ± 57.5 ***	**233.8 ± 19.3 ***
Total PUFA	**89.0 ± 14.2**	**410.4 ± 57.1 ***	**301.9 ± 25.5 ***	**87.1 ± 8.6**	**346.4 ± 61.3 ***	**244.9 ± 20.2 ***
Total	**709.0 ± 179.7**	**810.3 ± 119.1**	**710.7 ± 70.7**	**633.2 ± 55.7**	**704.3 ± 113.7**	**596.0 ± 65.9**
n-3/n-6 ratio	**0.58 ± 0.04**	**0.06 ± 0.01 ***	**0.06 ± 0.01 ***	**0.58 ± 0.00**	**0.06 ± 0.00 ***	**0.05 ± 0.00 ***

	100 µmol L⁻¹ Cd		
	Control	LA	AA
14:0	10.7 ± 0.4	6.9 ± 0.3	8.1 ± 0.7
16:0	191.9 ± 8.0	180.3 ± 17.7	166.4 ± 17.3
18:0	141.4 ± 4.7	140.7 ± 14.2	111.6 ± 10.3
Total SFA	**344.0 ± 11.7**	**327.9 ± 31.7**	**286.1 ± 28.0**

Data are expressed as means ± standard error of mean (n = 3). For each level of Cd exposure, stars indicate significant differences between control and PUFA-enriched conditions (1-way ANOVA with a post-hoc Dunnett's test with control cells as reference condition, $p < 0.05$) while stars into brackets indicate similar trends but without significance ($0.05 < p < 0.1$). Abbreviations used: AA, arachidonic acid; Cd, cadmium; LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

(continued)

	100 µM Cd		
	Control	LA	AA
16:1n-7	10.5 ± 1.1	0.0 ± 0.0 *	5.6 ± 0.6
18:1n-7	28.6 ± 3.4	12.7 ± 0.5 *	16.7 ± 1.8
18:1n-9	248.2 ± 15.1	72.7 ± 5.2 *	93.3 ± 9.8 *
Total MUFA	**287.2 ± 19.4**	**85.4 ± 5.7 ***	**115.6 ± 12.1 ***
ALA 18:3n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
18:4n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:3n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:4n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
EPA 20:5n-3	10.4 ± 0.5	5.3 ± 0.4 (*)	0.0 ± 0.0 *
22:5n-3	10.2 ± 0.5	6.6 ± 0.6	6.4 ± 0.8
22:6n-3	17.9 ± 3.1	9.9 ± 0.9	5.9 ± 0.5 *
Total n-3 PUFA	**38.5 ± 3.8**	**21.7 ± 1.9**	**12.3 ± 1.2 ***
LA 18:2n-6	20.9 ± 1.8	**272.5 ± 29.7 ***	9.5 ± 1.3
18:3n-6	4.6 ± 0.7	48.9 ± 2.5 *	5.2 ± 0.3
20:2n-6	0.0 ± 0.0	11.3 ± 0.9 *	0.0 ± 0.0
20:3n-6	14.0 ± 0.7	22.2 ± 0.9	11.6 ± 1.0
AA 20:4n-6	23.0 ± 1.3	19.2 ± 1.3	**179.3 ± 23.5 ***

Data are expressed as means ± standard error of mean (n = 3). For each level of Cd exposure, stars indicate significant differences between control and PUFA-enriched conditions (1-way ANOVA with a post-hoc Dunnett's test with control cells as reference condition, $p < 0.05$) while stars into brackets indicate similar trends but without significance ($0.05 < p < 0.1$). Abbreviations used: AA, arachidonic acid; Cd, cadmium; LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

(continued)

	100 µM Cd		
	Control	LA	AA
22:4n-6	0.0 ± 0.0	0.0 ± 0.0	62.1 ± 4.2 *
Total n-6 PUFA	**62.6 ± 4.2**	**374.1 ± 34.8 ***	**267.8 ± 29.0 ***
Total PUFA	**101.1 ± 7.7**	**395.8 ± 36.6 ***	**280.1 ± 30.2 ***
Total	**732.3 ± 36.4**	**809.2 ± 65.6**	**681.8 ± 69.6**
n-3/n-6 ratio	**0.62 ± 0.03**	**0.06 ± 0.00 ***	**0.05 ± 0.00 ***

Data are expressed as means ± standard error of mean (n = 3). For each level of Cd exposure, stars indicate significant differences between control and PUFA-enriched conditions (1-way ANOVA with a post-hoc Dunnett's test with control cells as reference condition, $p < 0.05$) while stars into brackets indicate similar trends but without significance ($0.05 < p < 0.1$). Abbreviations used: AA, arachidonic acid; Cd, cadmium; LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

4.3.4. Cd increases the neutral lipid content in n-6 PUFAs of AA-enriched cells

A 24-h exposure to Cd (50 or 100 µmol L^{-1}) had no significant impact on neutral lipid composition in control, ALA-, EPA- and LA-enriched cells (data not shown). The same phenomenon was observed in AA-enriched cells submitted to 50 µmol L^{-1} of Cd. On the other hand, a 2- to 3-fold increase of the absolute levels of AA, 22:4n-6, n-6 PUFAs and PUFAs in the neutral lipids was noticed in AA-enriched cells submitted to 100 µmol L^{-1} of Cd (Table 4.5).

Table 4.5. Fatty acid composition of RTL-W1 cell neutral lipids after one week culture in a growth medium supplemented in AA (50 µmol L^{-1}) and a subsequent challenge with Cd (0, 50 or 100 µmol L^{-1}) during 24 h in a medium deprived of fatty acid (µmol g^{-1} protein).

Fatty acid	0 µmol L^{-1} Cd	50 µmol L^{-1} Cd	100 µmol L^{-1} Cd
16:0	21.2 ± 1.6	22.9 ± 7.3	14.8 ± 1.1
18:0	28.9 ± 5.0	28.5 ± 8.7	21.3 ± 4.9
Total SFA	**50.2 ± 6.4**	**51.4 ± 15.9**	**36.1 ± 6.0**
18:1n-9	3.5 ± 0.5	6.0 ± 3.4	4.3 ± 0.5
Total MUFA	**3.5 ± 0.5**	**6.0 ± 3.4**	**4.3 ± 0.5**
Total PUFA n-3	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**
AA 20:4n-6	**6.0 ± 0.5** $^{(a)}$	**7.4 ± 1.6** $^{(ab)}$	**16.3 ± 4.6** $^{(b)}$
22:4n-6	2.6 ± 0.0 ^{a}	2.6 ± 0.3 ^{a}	5.0 ± 0.8 ^{b}
Total PUFA n-6	**8.6 ± 0.5** ^{a}	**10.0 ± 1.8** $^{ab(a)}$	**21.3 ± 5.4** ^{b}
Total PUFA	**8.6 ± 0.5** ^{a}	**10.0 ± 1.8** $^{ab(a)}$	**21.3 ± 5.4** ^{b}
Total	**62.3 ± 7.0**	**67.4 ± 20.9**	**61.7 ± 11.6**

Data are expressed as means ± standard error of mean (n = 3). Letters indicate significant impact of the Cd exposure level, with levels not connected to the same letter being significantly different from each other, ($p < 0.05$) while letters into brackets indicate similar trends but without significance ($0.05 < p < 0.1$). Abbreviations used: AA, arachidonic acid; Cd, cadmium; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

4.3.5. PUFA enrichment modulates cell tolerance to Cd

Cd decreased both RTL-W1 cell viability proxies, that are metabolic activity and membrane integrity (Fig. 4.2). None of the PUFA enrichments modified the apparent drop in membrane integrity caused by Cd. By contrast, ALA, EPA and AA enrichments significantly modulated cell metabolic activity under Cd challenge (Fig. 4.2). ALA- and EPA-enriched cells challenged with Cd (50 or 100 µmol L^{-1}) were 1.6- to 1.9-fold more metabolically active than control cells exposed to the same Cd dose. A similar significant cytoprotection was induced by AA enrichment at the lowest Cd dose tested (50 µmol L^{-1}) while no impact of AA enrichment on cellular metabolic activity was observed at 100 µmol L^{-1} Cd. On the other hand, LA enrichment had no impact on any of the cell viability indicators whatever the Cd concentration (Fig. 4.2).

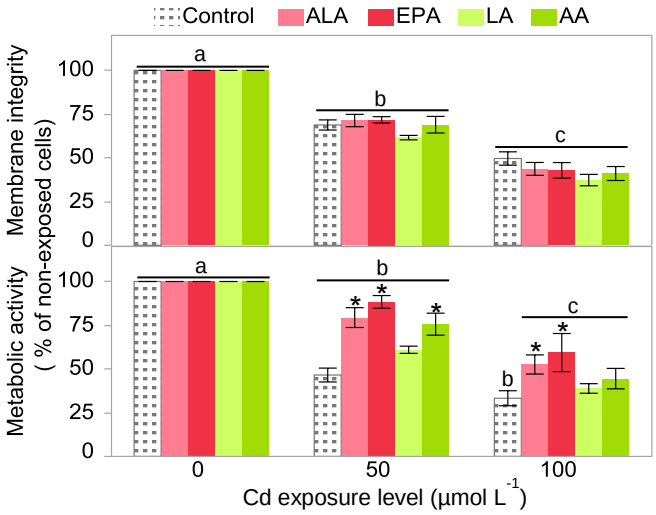

Fig. 4.2. Membrane integrity (up) and metabolic activity (down) of RTL-W1 cells after one week culture in a growth medium supplemented or not (control) in a specific PUFA (50 µmol L^{-1} ALA, EPA, LA or AA) and a subsequent challenge with Cd (0, 50 or 100 µmol L^{-1}) during 24 h in a medium deprived of fatty acid. Data are expressed as means ± standard error of mean (n = 6). For each level of Cd exposure, stars indicate significant differences between control and PUFA-enriched conditions ($p < 0.05$). For each PUFA enrichment strategy, letters indicate significant impact of the Cd exposure level, with levels not connected to the same letter being significantly different from each other ($p < 0.05$). Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; Cd, cadmium; EPA, eicosapentaenoic acid; LA, linoleic acid, PUFA, polyunsaturated fatty acid.

4.3.6. PUFA has no impact on Cd burden

RTL-W1 dose-dependently accumulated Cd, irrespective of the PUFA enrichment strategy (Fig. 4.3). The total Cd burden was not significantly different between PUFA-enriched cells and control cells (Fig. 4.3).

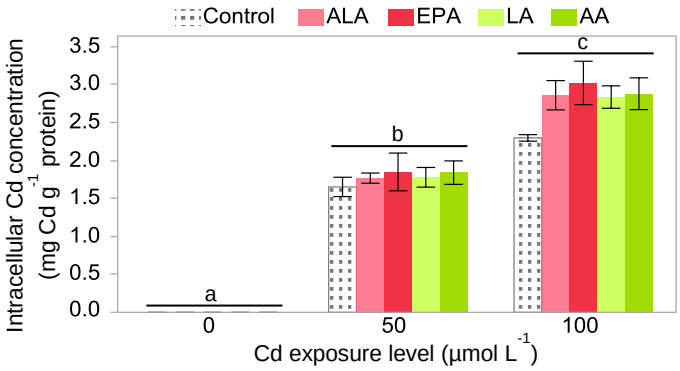

Fig. 4.3. Intracellular Cd concentration in RTL-W1 cells after one week culture in a growth medium supplemented or not (control) in a specific PUFA (50 µmol L^{-1} ALA, EPA, LA or AA) and a subsequent challenge with Cd (0, 50 or 100 µmol L^{-1}) during 24 h in a medium deprived of fatty acid. Data are expressed as means ± standard error of mean (n = 4). For each PUFA enrichment strategy, letters indicate significant impact of the Cd exposure level ($p < 0.05$). For more details, refer to the legend of Fig. 4.2 PUFA enrichments had no significant impact on the intracellular Cd concentration. Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; Cd, cadmium; EPA, eicosapentaenoic acid; LA, linoleic acid, polyunsaturated fatty acid.

4.3.7. PUFA and Cd modulate the expression of genes and the proteomic landscape

Raw data for proteomic analyses are available in supplelmentary data Table 4.A.1.

4.3.7.1. Fatty acid metabolism

The expression level of the *ELOVL2* gene was below quantification limit for all experimental conditions (not shown). In the absence of Cd challenge, PUFA enrichments significantly increased *FATP1* and *CPT1* gene expression levels, while having no significant impact on the expression level of the other fatty acid metabolism genes analysed (*FASN*, *FADS2* and *ELOVL5*) (Fig. 4.4). At the protein expression level,

ALA, EPA and AA enrichments significantly downregulated the fatty acid binding protein 7 (FABP7), a protein involved in fatty acid uptake, transport and metabolism. This effect was unaffected by Cd exposure (proteomic analyses, Table 4.6).

Cd had no significant impact on *FATP1*, *CPT1* and *ELOVL5* gene expression (Fig. 4.4). Cd tended to decrease the *FASN* gene expression in PUFA-enriched cells (especially in ALA- and LA-enriched cells), while the *FASN* gene expression remained constant under Cd exposure in control cells (Fig. 4.4). As a result, a trend for lower *FASN* gene expression was observed in PUFA-enriched cells (especially ALA-, EPA- and LA-enriched cells) exposed to 100 μmol L^{-1} Cd as compared to control cells exposed to the same Cd dose (Fig. 4.4). Besides, Cd tended to downregulate the *FADS2* gene in PUFA-enriched cells (especially in EPA-enriched cells) (Fig. 4.4).

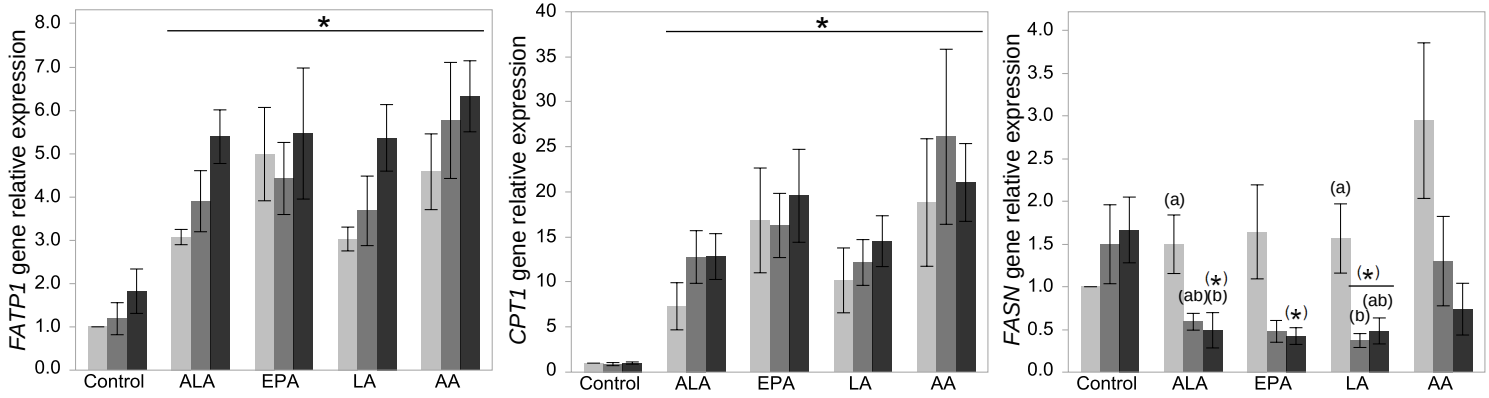

Fig. 4.3. Relative expression of genes involved in lipid metabolism in RTL-W1 cells after one week culture in a growth medium supplemented or not (control) in a specific PUFA (50 µmol L^{-1} ALA, EPA, LA or AA) and a subsequent challenge with Cd (0 µmol L^{-1} in light grey, 50 µmol L^{-1} in grey or 100 µmol L^{-1} in dark grey) during 24 h in a medium deprived of fatty acid. Data are expressed as means ± standard error of mean (n = 4). For each level of Cd exposure, stars indicate significant differences between control and PUFA-enriched conditions ($p < 0.05$), while stars intro brackets indicate similar trends but without significance ($0.05 < p < 0.1$). For each PUFA enrichment strategy, letters indicate significant impact of the Cd dose, with levels not connected to the same letter being significantly different from each other ($p < 0.05$), while letters intro brackets indicate similar trends but without significance ($0.05 < p < 0.1$). Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; Cd, cadmium; CPT1, carnitine palmitoyl transferase 1; 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; PUFA, polyunsaturated fatty acid.

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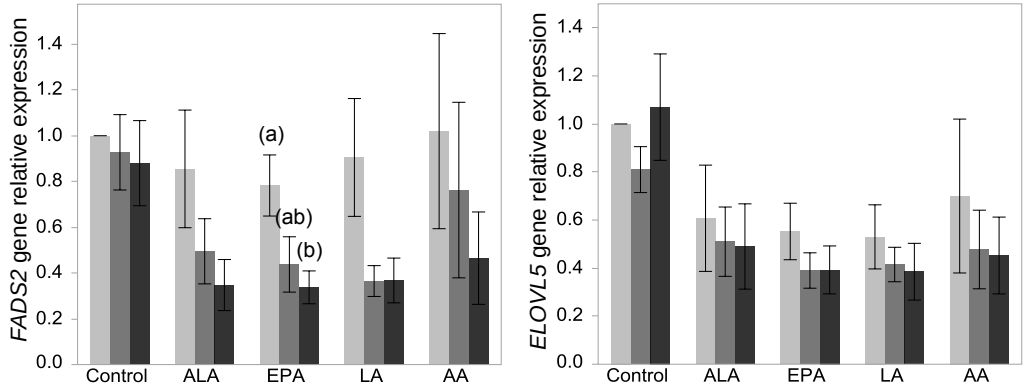

Fig. 4.3. Relative expression of genes involved in lipid metabolism in RTL-W1 cells after one week culture in a growth medium supplemented or not (control) in a specific PUFA (50 µmol L^{-1} ALA, EPA, LA or AA) and a subsequent challenge with Cd (0 µmol L^{-1} in light grey, 50 µmol L^{-1} in grey or 100 µmol L^{-1} in dark grey) during 24 h in a medium deprived of fatty acid. Data are expressed as means ± standard error of mean (n = 4). For each level of Cd exposure, stars indicate significant differences between control and PUFA-enriched conditions ($p < 0.05$), while stars intro brackets indicate similar trends but without significance ($0.05 < p < 0.1$). For each PUFA enrichment strategy, letters indicate significant impact of the Cd dose, with levels not connected to the same letter being significantly different from each other ($p < 0.05$), while letters intro brackets indicate similar trends but without significance ($0.05 < p < 0.1$). Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; Cd, cadmium; CPT1, carnitine palmitoyl transferase 1; 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; PUFA, polyunsaturated fatty acid.

Table 4.6. Differentially expressed proteins in RTL-W1 cells after one week culture in a growth medium supplemented or not (control) in a specific PUFA (50 µmol L^{-1} ALA, EPA or AA) and a subsequent challenge with Cd (0 or 50 µmol L^{-1}) during 24 h in a medium deprived of fatty acid.

Proteins	PUFA impact in absence of Cd			PUFA impact in presence of Cd			Cd impact			
	ALA0/ CT0	EPA0/ CT0	AA0/ CT0	ALA50/ CT50	EPA50/ CT50	AA50/ CT50	CT50/ CT0	ALA50/ ALA0	EPA50/ EPA0	AA50/ AA0
Glucose metabolism										
Pyruvate kinase	1.08	1.14	1.27 *	0.98	1.03	1.09	1.00	0.91	0.95	0.90
Fatty acid metabolism										
Fatty acid binding protein 7	0.57 *	0.52 *	0.59 *	0.62 *	0.53 *	0.64 *	1.08	1.05	0.87	0.96
Stress response										
Annexin-A1	0.93	1.00	1.05	1.01	1.03	0.96	0.73 (*)	0.86	0.82	0.67 (*)
Glutaredoxin-1	0.82	0.69 (*)	0.80	0.77	0.92	0.93	1.11	0.91	1.06	1.11
Heat shock protein 30	0.66	0.76	0.67	1.33	1.09	1.31	4.07 (*)	5.16	3.26	5.00
Heat shock protein 70-1	1.00	0.88	1.25	1.03	0.94	0.91	3.73 *	1.48 *	1.35 *	1.32 *
Heat shock protein 70-2	0.82	1.09	1.11	1.08	1.04	0.95	5.92 *	4.99	3.82	3.81
Peroxiredoxin-1	1.19	1.03	1.07	1.39 *	0.97	1.26 (*)	0.90	1.09	0.89	1.12
Protein S100-A1	0.56 (*)	0.66	0.62 (*)	0.90	1.37	1.32	1.11	1.00	1.30	1.16

Data are expressed as mean ratios (n = 3). Stars indicate significant upregulation (green) or downregulation (red) by PUFAs or Cd (i.e. ratios significantly different from 1, weighed z-scores, $p < 0.05$) while stars into brackets indicate similar trends but without significance ($0.05 < p < 0.1$). Desciption (Oncorhynchus_peptide database): Annexin-A1, GSONMP00063408001; Cytokeratin-18, GSONMP00010164001; Fatty acid binding protein 7, GSONMP00072181001; Glutaredoxin-1, GSONMP00066197001; Heat shock protein 30, GSONMP00081507001; Heat shock protein 70-1, GSONMP00064818001; Heat shock protein 70-2, GSONMP00020100001; Intermediate filament protein ON3, GSONMP00040665001; Myosin-9, GSONMP00017263001; Peroxiredoxin-1, GSONMP00041410001; Protein S100-A1, GSONMP00014770001; ; Pyruvate kinase, GSONMP00052888001;. Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; Cd, cadmium; CT, control; EPA, eicosapentaenoic acid; LA, linoleic acid, PUFA(s), polyunsaturated fatty acid(s).

(continued)

Proteins	PUFA impact in absence of Cd			PUFA impact in presence of Cd			Cd impact			
	ALA0/ CT0	EPA0/ CT0	AA0/ CT0	ALA50/ CT50	EPA50/ CT50	AA50/ CT50	CT50/ CT0	ALA50/ ALA0	EPA50/ EPA0	AA50/ AA0
Cytoskeleton										
Cytokeratin-18	1.37 *	1.32	1.38 *	0.93	0.89	0.98	1.20	0.97	0.92	1.14
Intermediate filament protein ON3	1.26	1.29	1.39 *	1.01	1.00	1.07	1.16	0.96	0.94	0.96
Myosin-9	0.88 (*)	0.83 *	0.78 *	0.92	0.92	0.90	1.03	1.04	0.96	1.03

Data are expressed as mean ratios (n = 3). Stars indicate significant upregulation (green) or downregulation (red) by PUFAs or Cd (i.e. ratios significantly different from 1, weighed z-scores, $p < 0.05$) while stars into brackets indicate similar trends but without significance ($0.05 < p < 0.1$). Desciption (Oncorhynchus_peptide database): Annexin-A1, GSONMP00063408001; Cytokeratin-18, GSONMP00010164001; Fatty acid binding protein 7, GSONMP00072181001; Glutaredoxin-1, GSONMP00066197001; Heat shock protein 30, GSONMP00081507001; Heat shock protein 70-1, GSONMP00064818001; Heat shock protein 70-2, GSONMP00020100001; Intermediate filament protein ON3, GSONMP00040665001; Myosin-9, GSONMP00017263001; Peroxiredoxin-1, GSONMP00041410001; Protein S100-A1, GSONMP00014770001; ; Pyruvate kinase, GSONMP00052888001;. Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; Cd, cadmium; CT, control; EPA, eicosapentaenoic acid; LA, linoleic acid, PUFA(s), polyunsaturated fatty acid(s).

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4.3.7.2. PUFA derived signalling

In the absence of Cd challenge, EPA enrichment slightly, yet significantly, upregulated the *cPLA2* gene expression (1.4-fold) while the other PUFA enrichments had no impact (Fig. 4.5). The expression level the *COX2* gene was significantly lower (4-fold) in AA-enriched cells than in control cells. A similar trend, but of lower magnitude, was observed in EPA-enriched cells. ALA and LA enrichments had no impact on the *COX2* gene expression level (Fig. 4.5). AA enrichment tended to decrease *ALOX5* gene expression level while the other PUFA enrichments had no impact (Fig. 4.5).

Cd significantly upregulated the *cPLA2* gene expression in control cells (4.5-fold increase at the highest dose tested) (Fig. 4.5). The *cPLA2* gene expression level was significantly lower in ALA-, EPA- and AA-enriched cells exposed to 100 µmol L^{-1} Cd than in control cells challenged with the same Cd dose (Fig. 4.5). The *COX2* gene expression was significantly decreased by Cd in ALA- and LA-enriched cells (Fig. 4.5). Similar downregulations, not significant though, were observed in EPA-enriched cells (at both Cd doses) and in control cells (at the lowest Cd dose tested only) while Cd had no impact on the *COX2* gene expression level in AA-enriched cells (Fig. 4.5). Finally, Cd had no impact on the *ALOX5* gene expression level, regardless of the PUFA enrichment strategy (Fig. 4.5).

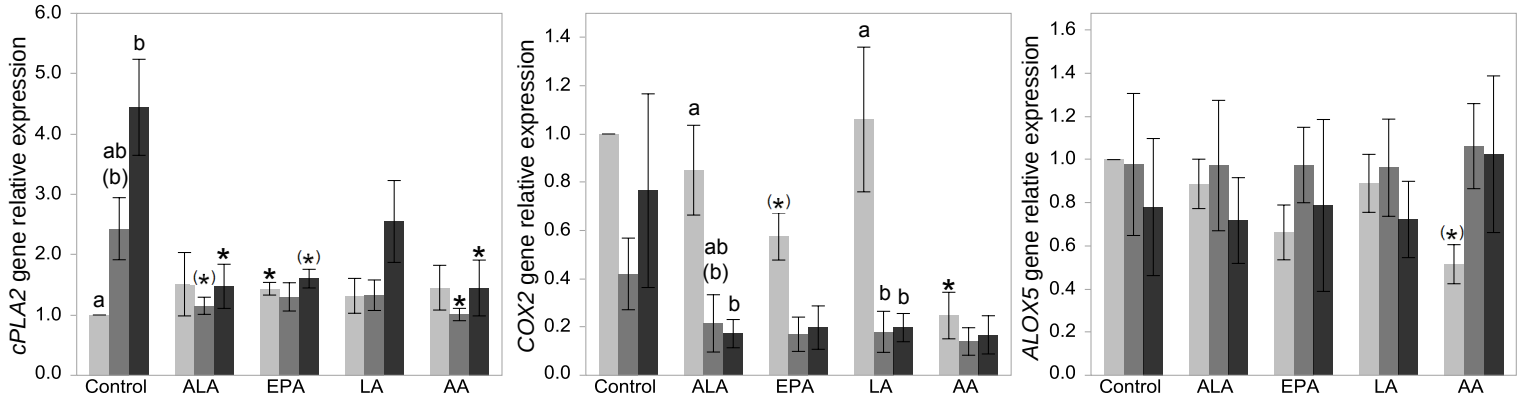

Fig. 4.5. Relative expression of genes involved in PUFA-derived signalling in RTL-W1 cells after one week culture in a growth medium supplemented or not (control) in a specific PUFA (50 µM ALA, EPA, LA or AA) and a subsequent challenge with Cd (0 µmol L^{-1} in light grey, 50 µmol L^{-1} in grey or 100 µmol L^{-1} in dark grey) during 24 h in a medium deprived of fatty acid. Stars and letters indicate significant impacts of PUFA enrichment and Cd exposure, respectively. For more details, refer to the legend of Fig. 4.4. Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; ALOX5, arachidonate 5-lipoxygenase; Cd, cadmium; cPLA2, cytosolic phospholipase A2; COX2, cyclooxygenase 2, EPA, eicosapentaenoic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acid.

4.3.7.3. Stress response

The expression level of the *HSP70b* gene was below quantification limit for all treatments (not shown). In the absence of Cd challenge, RTL-W1 cells did not express the *MTa* gene at detectable levels while they markedly expressed the *MTb* isoform (Fig. 4.6). The basal expression level of the latter gene was not influenced by PUFA enrichment strategies (Fig. 4.6). PUFA enrichments did not impact the gene expression of *HSP70a*, *p53* or *CALR* (Fig. 4.6). ALA, LA and AA enrichments tended to downregulate the *BCLx* gene (Fig. 4.6). At the protein expression level, EPA enrichment tended to downregulate glutaredoxin-1. ALA and AA enrichments tended to downregulate S100-A1, a protein involved in many cellular functions, including calcium homeostasis, cytoskeleton dynamics and the inflammatory response. The observed downregulation of glutaredoxin-1 and protein S100-A1 disappeared after Cd exposure (50 µmol L^{-1}) (proteomic analyses, Table 4.6).

Cd significantly induced *MTa*, *HSP70a* and *MTb* gene expression levels while it decreased that of *BCLx* and had no significant impact on *p53* and *CALR* gene expression levels (Fig. 4.6). The upregulation of the *MTb* gene was less pronounced in PUFA-enriched cells and decreased with the Cd exposure level (Fig. 4.6). As a result, *MTb* gene expression level was significantly lower (or tended to be so) in ALA-, EPA- and AA-enriched cells exposed to 100 µmol L^{-1} Cd than in control cells exposed to the same Cd level (Fig. 4.6). The *MTa* gene expression followed a similar pattern, with a significantly lower expression level in AA-enriched cells exposed to 100 µmol L^{-1} Cd than in control cells (Fig. 4.6). At the protein expression level, Cd strongly upregulated several HSPs, namely HSP30 and two isoforms of HSP70. However, due to a high variability of response, the upregulation of one of the HSP70 isoforms was only significant in control cells, and the upregulation of HSP30 was not significant (only a trend was seen in control cells) (proteomic analyses, Table 4.6). Besides, ALA-enriched cells showed higher levels of an isoform of the antioxidant enzyme peroxiredoxin (predicted: peroxiredoxin-1, blast NCBI) when challenged with Cd (50 µmol L^{-1}), while no impact was observed in the absence of Cd (proteomic analyses,

Table 4.6). A similar trend, but without significance, was observed in AA-enriched cells, while EPA enrichment had no impact on the production of this protein. Finally, in control and AA-enriched cells, Cd (50 μmol L^{-1}) significantly downregulated annexin-A1, a protein involved in trafficking and organisation of vesicles, exocytosis and endocytosis processes as well as in calcium ion channel formation (proteomic analyses, Table 4.6).

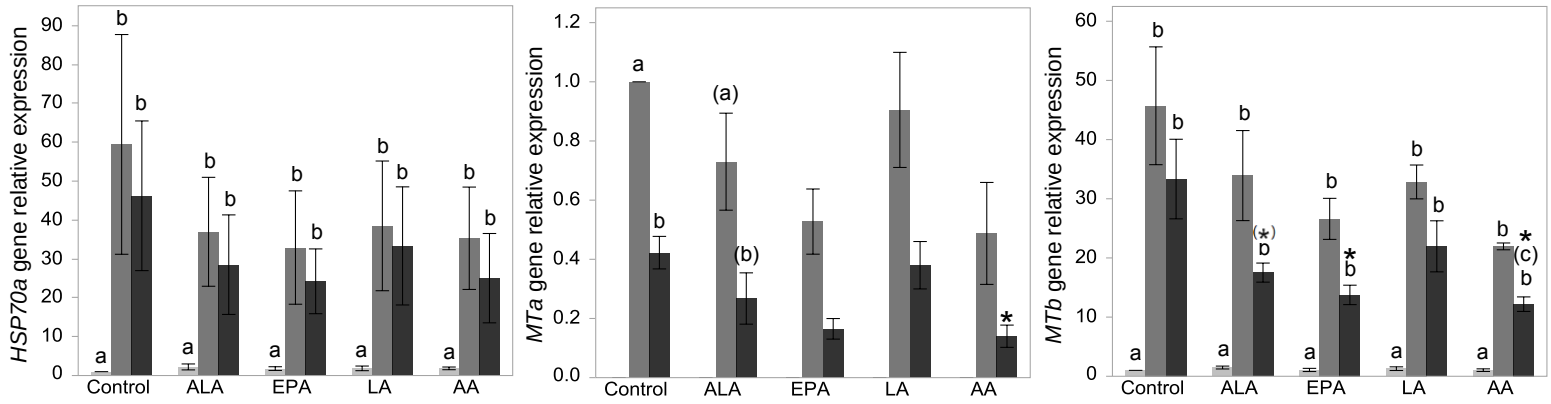

Fig. 4.6. Relative expression of genes involved in stress response in RTL-W1 cells after one week culture in a growth medium supplemented or not (control) in a specific PUFA (50 µM ALA, EPA, LA or AA) and a subsequent challenge with Cd (0 µmol L^{-1} in light grey, 50 µmol L^{-1} in grey or 100 µmol L^{-1} in dark grey) during 24 h in a medium deprived of fatty acid. Stars and letters indicate significant impacts of PUFA enrichment and Cd exposure, respectively. For more details, refer to the legend of Fig. 4.4. Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; BCLx, apoptosis regulator BCL2-Like1; CALR, calreticulin; EPA, eicosapentaenoic acid; HSP70a, heat shock protein 70a; LA, linoleic acid; MTa, metallothionein a; MTb, metallothionein b; p53, tumor protein 53; PUFA, polyunsaturated fatty acid.

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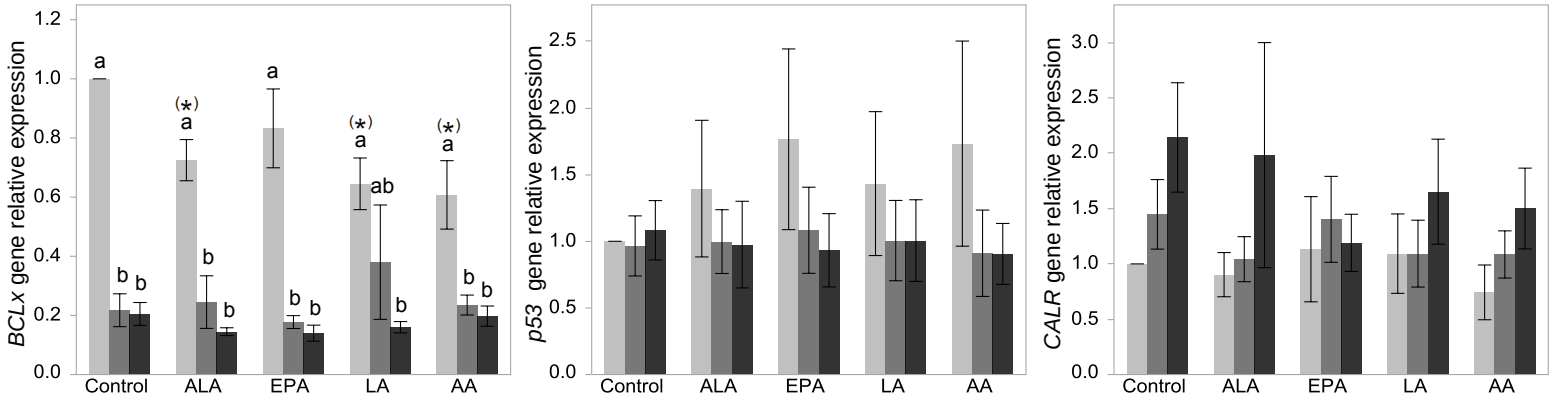

Fig. 4.6. Relative expression of genes involved in stress response in RTL-W1 cells after one week culture in a growth medium supplemented or not (control) in a specific PUFA (50 µM ALA, EPA, LA or AA) and a subsequent challenge with Cd (0 µmol L^{-1} in light grey, 50 µmol L^{-1} in grey or 100 µmol L^{-1} in dark grey) during 24 h in a medium deprived of fatty acid. Stars and letters indicate significant impacts of PUFA enrichment and Cd exposure, respectively. For more details, refer to the legend of Fig. 4.4. Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; BCLx, apoptosis regulator BCL2-Like1; CALR, calreticulin; EPA, eicosapentaenoic acid; HSP70a, heat shock protein 70a; LA, linoleic acid; MTa, metallothionein a; MTb, metallothionein b; p53, tumor protein 53; PUFA, polyunsaturated fatty acid.

4.3.7.4. CYP20A1

In absence of Cd challenge, EPA and AA enrichments significantly increased the expression level of the *CYP20A1* gene, an orphan of the CYP family (Fig. 4.7).

Both doses of Cd repressed the *CYP20A1* gene expression level in EPA-enriched cells, whereas no significant impact of Cd on the *CYP20A1* expression level could be detected in control cells and cells enriched in other PUFAs (Fig. 4.7).

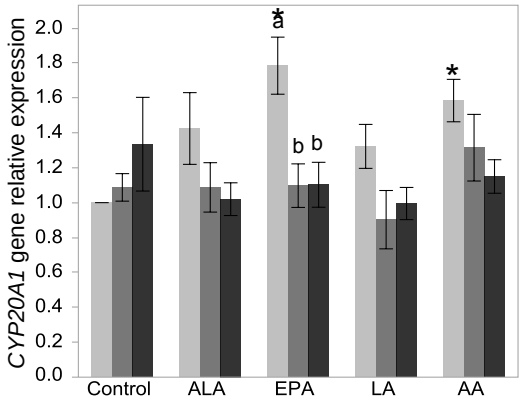

Fig. 4.7. Relative expression of *CYP20A1* gene in RTL-W1 cells after one week culture in a growth media supplemented or not (control) in a specific PUFA (50 µmol L^{-1} ALA, EPA, LA or AA) and a subsequent challenge with Cd (0 µmol L^{-1} in light grey, 50 µmol L^{-1} in grey or 100 µmol L^{-1} in dark grey) during 24 h in a medium deprived of fatty acid. Stars and letters indicate significant impacts of PUFA enrichment and Cd exposure, respectively. For more details, refer to the legend of Fig. 4.4. Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; Cd, cadmium; CYP20A1, cytochrome P450 20A1; EPA, eicosapentaenoic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acid.

4.3.7.5. Cytoskeleton

At the protein expression level, PUFA enrichments influenced the abundance of some cytoskeletal intermediate filaments. In absence of Cd challenge, cytokeratin-18 level was significantly higher in ALA- and AA-enriched cells than in control cells. Besides, AA-enriched cells contained a significantly higher amount of intermediate filament protein ON3 than control cells. Finally, ALA, AA and EPA enrichments slightly reduced myosin-9 level (significant for AA and EPA enrichments). These PUFA-

derived regulations disappeared after Cd exposure (50 µmol L^{-1}) (proteomic analyses, Table 4.6).

4.3.7.6. Glucose metabolism

In absence of Cd challenge, AA enrichment significantly upregulated pyruvate kinase expression (proteomic analyses, Table 4.6), a protein involved in glucose metabolism, while ALA and EPA enrichment had no impact. This upregulation was not observed under Cd exposure (50 µmol L^{-1}).

4.4. Discussion

The present study aimed at investigating the interactions between PUFAs and Cd at the molecular level. Globally, our results indicate that PUFAs strongly influenced fatty acid homeostasis and metabolism while Cd moderately impacted these parameters. They further indicated that some PUFAs modulated the sensitivity of rainbow trout liver cells to Cd. This protective effect could not be ascribed to a differential Cd accumulation. Rather, it is suggested that modulation of intracellular parameters was responsible for the increased tolerance to Cd.

4.4.1. Impact of PUFA on fatty acid homeostasis and metabolism

As previously observed (Ferain et al., 2016), RTL-W1 cells supplemented with single PUFAs incorporated these compounds as well as some biotransformation products mainly in their phospholipids, while keeping the total cellular fatty acid content stable. This indicates that the fatty acid profile modulation occurred either through the replacement of some fatty acids (mainly 18:1n-9) in pre-existing lipids or thanks to the lipid turnover process rather than through supplementary lipid accumulation, as previously pointed out (Ferain et al., 2016). As a result, RTL-W1 cells grown in a culture medium supplemented with a specific PUFA were not only richer in PUFAs but also lower in MUFAs than the control cells, as previously described (Ferain et al., 2016). The major incorporation of the absorbed PUFAs in

the cellular phospholipids emphasises once more the preponderant role of PUFAs as structural components of biological membranes (Tocher, 2003).

Fatty acids are absorbed by cells through either a passive mechanism or by transmembrane proteins, such as FATP1 (Frohnert and Bernlohr, 2000; Zhou et al., 2010). The upregulation of *FATP1* gene detected with the PUFA treatments may be a cellular response aiming at increasing the efficiency of the PUFA uptake, which may have been driven either by the high PUFA concentration in the growth medium or by an increased mitochondrial β-oxidation rate. Indeed, it has been suggested that the metabolic demand for fatty acids and not the substrate availability is the driving force for cellular fatty acid uptake (Mashek and Coleman, 2006). The upregulation of *CPT1* gene supports this view since the enzyme encoded by this gene catalyses the complexation of activated fatty acids with carnitine, a crucial step for their translocation into mitochondria for β-oxidation (Fig. 4.1) (Leaver et al., 2008). The intracellular transport of free fatty acids is facilitated by proteins called fatty acid binding proteins as they bind the hydrophobic fatty acids (Leaver et al., 2008). Contrary to expectations, PUFA treatments decreased the level of FABP7, a fatty acid binding protein isoform. Other isoforms may thus preponderantly ensure cytosolic transport of fatty acids in RTL-W1 cells.

Rainbow trout, like other salmonids, have high fatty acid biotransformation abilities and can efficiently produce DHA and AA from 18 carbon precursors (ALA and LA) (Tocher, 2003). These biotransformation steps (Fig. 4.1), mainly occurring in the liver, are catalysed by elongases and desaturases. These enzymes are encoded by the *ELOVL2*, *ELOVL5* and *FADS2* genes (Leaver et al., 2008). In line with previous reports (Ferain et al., 2016; Mellery et al., 2017), RTL-W1 cells were unable to produce DHA from ALA or from EPA, and AA from LA. Instead, an accumulation of intermediary elongation and desaturation (Δ5 and Δ6) products immediately downstream of the given PUFAs in the biotransformation pathway was observed, as previously described (Ferain et al., 2016; Mellery et al., 2017). The absence of *ELOVL2* expression in RTL-W1 cells under the present experimental conditions

might explain their inability to generate DHA, as previously suggested (Ferain et al., 2018).

4.4.2. Impact of Cd on fatty acid homeostasis and metabolism in RTL-W1 cells

The fatty acid profile of fish tissue is influenced by environmental factors, including pollution by metals (Fadhlaoui and Couture, 2016; Filimonova et al., 2016; Konar et al., 2010). Nevertheless, very few works addressed the specific impact of Cd on the fatty acid composition of cellular lipid classes. A previous study on rat hepatocytes showed that a sub-lethal dose of Cd increased the relative parts of 16:0 and 18:0 while decreasing that of AA in neutral lipids (Kudo and Waku, 1996). In the present study, Cd neither impacted on the fatty acid composition of cellular lipids (neutral lipids and phospholipids), nor the expression of genes involved in fatty acid metabolism in control cells. On the other hand, Cd modulated these parameters in PUFA-enriched cells. In particular Cd significantly increased (2.5-fold) the incorporation of AA in neutral lipids of AA-enriched cells at the expense of other fatty acids (mainly SFAs). Since Cd exposure was carried out in a fatty acid free medium and the n-6 PUFA level remained constant in phospholipids under Cd exposure, n-6 PUFAs incorporated into neutral lipids in response to Cd exposure likely originated from the cellular free fatty acid pool. Further investigation is, however, needed to validate this hypothesis as the free fatty acid fraction was not analysed in the present experiment. Besides, in the present study, DHA phospholipid level was significantly lower in AA-enriched cells than in control cells after exposure to the highest dose of Cd, while there was no difference in absence of Cd challenge. This resulted from two opposite trends (not significant), i.e. an increase (vs a decrease) of DHA level following Cd exposure in control-cells (vs in AA-enriched cells). The biological relevance of these changes remains unclear.

Previous studies have shown that Cd can modulate the expression of genes involved in fatty acid metabolism. However, the reported effects are highly variable and depend on the exposure characteristics (concentration, duration) (Koskinen et al., 2004), on the tissue targeted (Chen et al., 2013; Fadhlaoui et al., 2018), on environmental conditions

(water temperature during fish growth) (Fadhlaoui et al., 2018). For instance, a Cd challenge of high intensity (0.5 mg L^{-1}, 96 h) downregulated genes related to biosynthesis of fatty acids in rainbow trout while a Cd challenge of a lower intensity (0.05 or 0.025 mg L^{-1}, 24 h) upregulated these genes (Koskinen et al., 2004). In the present study, Cd tended to downregulate *FASN* (about 3- to 4-fold, not significant) and *FADS2* (about 2- to 2.5-fold, not significant) in PUFA-enriched cells while Cd did not affect these genes in control cells. Downregulations of these genes by Cd were also reported in primary cultures of Atlantic salmon hepatocytes (Olsvik et al., 2016) and in liver of rats (Madejczyk et al., 2015). All together, these data suggest that Cd might negatively affect salmonid ability to synthesize EPA and DHA, thereby decreasing the nutritional value of its flesh. Further studies are needed to identify the critical conditions in which Cd really alters PUFA biosynthesis in fish.

4.4.3. Impact of PUFA on Cd sensitivity

At both Cd doses tested, ALA- and EPA-enriched cells were more metabolically active than the control cells, while LA enrichment had no impact. This cytoprotection exerted by ALA and EPA confirmed our previous observation with the same cell line (Ferain et al., 2016). Similarly, n-3 PUFAs (EPA and DHA mix) increased cell tolerance to 24 h and 48 h Cd challenge in Hep G2 cells (Linhartova and Sampels, 2015). Still, in contrast to our previous observations, AA-enrichment also increased cell tolerance to 50 µmol L^{-1} Cd but not to 100 µmol L^{-1} Cd, in the present study. This divergence could most probably reflect differences in the experimental procedures. Indeed, the protective effect of PUFAs toward Cd toxicity was herein evaluated at two discrete Cd concentrations, while it was assessed by EC50 calculation through modelling toxicity data in our previous study (Ferain et al., 2016).

In the present study, we explore two hypotheses that may explain the cytoprotection exerted by specific PUFAs. PUFAs might (i) decrease Cd cellular uptake and/or (ii) modulate intracellular parameters increasing the cell tolerance to the damages induced by Cd (Ferain et al., 2016).

4.4.3.1. Cd burden was not influenced by PUFA enrichment

Depending on their number of carbons and unsaturation level, PUFAs confer different physicochemical properties to the biological membranes (fluidity, permeability), which can affect both active and passive transports of solutes (Arts and Kohler, 2009; Tillman and Cascio, 2003). However, in the present study, cytoprotection conferred by ALA, EPA and AA was not associated with a decrease in intracellular Cd concentration. Interestingly, n-3 PUFAs (EPA and DHA mixture) increased Hep G2 cells tolerance to Cd (5 µmol L^{-1}, 24 h) while they significantly increased Cd intracellular burden (Linhartova and Sampels, 2015). Overall, these results highlight that PUFAs do not protect cells against Cd toxicity by decreasing Cd burden. Nevertheless, one cannot exclude that PUFA enrichments could influence Cd intracellular distribution, thereby modulating Cd toxicity. In cells, Cd can indeed be found in five separate pools: (i) metallothionein-like proteins, (ii) metal-rich granules, (iii) cellular debris (membrane), (iv) heat sensitive proteins (e.g. enzymes) and (v) organelles (e.g. nucleus, mitochondria) (Wang and Rainbow, 2006). The two latter pools represent the metal sensitive fraction, while the two former ones represent the detoxification fraction (Wallace et al., 2003; Wang and Rainbow, 2006). Whether individual PUFAs may modulate the repartition of Cd between these two fractions and thereby affect the fate of cells (survival or death) is currently unknown but worth investigating.

4.4.3.2. PUFAs modulated proteomic and genetic responses to Cd

MTs are cysteine-rich proteins that scavenge metal ions and reactive oxygen species (Fig. 4.1). They can thus reduce cellular availability of toxic metals such as Cd. In accordance with previous studies in mammals (Cartularo et al., 2015; Go et al., 2014) and fish (Chen et al., 2014; Qiang et al., 2017; Walker et al., 2008), Cd strongly induced the expression of two isoforms of *MT*, i.e. *MTa* and *MTb*, in RTL-W1 cells. Interestingly, cytoprotection conferred by ALA, EPA and AA seemed to go along with a lower induction of *MT* genes. Since Cd intracellular concentrations were not influenced by PUFA enrichment,

such a decrease in *MT* expression is likely not linked to a decrease in Cd level but rather might indicate a reduction of intracellular levels of free metal ions or ROS by other mechanisms such as subcellular Cd partitioning.

Cd is known to induce oxidative stress in liver cells (Jamwal et al., 2016; Linhartova et al., 2016; Matović et al., 2015; Wang et al., 2014). Interestingly, the supplementation with a mixture of EPA and DHA protected Hep G2 cells against the Cd-induced oxidative damages to lipids and proteins (Linhartova et al., 2016). This effect was associated with a lower level of superoxide dismutase activity (Linhartova et al., 2016). This suggests that PUFA-derived cytoprotection could, at least partly, be explained by a reduction of the oxidative stress induced by Cd. Likewise, in the present study, interactive effects between cytoprotective PUFAs (EPA, ALA and AA) and the levels of antioxidant enzymes were observed either in the absence (EPA) or in the presence (ALA and AA) of Cd. On the one hand, the lower level of the antioxidant enzyme glutaredoxin-1 in EPA-enriched cells in the absence of Cd challenge as compared to control cells, pleads for lower basal ROS production in cells enriched in this PUFA. This could result from a direct impact of EPA on the mitochondrial electron transport chain, i.e. decrease of electron leakage and hydrogen peroxide generation through modulations of the activities of the mitochondrial complexes I, III and IV, as previously demonstrated in mice (Hagopian et al., 2010). On the other hand, we observed that the ALA-enriched cells had significantly higher protein levels of peroxiredoxin-1 under Cd stress than control cells, while the basal level of this antioxidant enzyme was similar in the absence of Cd challenge. A similar trend was observed in AA-enriched cells. Since peroxiredoxin-1 catalyses the reduction of hydrogen peroxide, peroxynitrite and organic hydroperoxides (Wood et al., 2003), the higher level of this antioxidant enzyme may have conferred a higher detoxification ability against Cd-induced ROS production in ALA- and AA-enriched cells. All together, these results reinforce the view that PUFAs are likely to increase cell tolerance and response to oxidative stress. This may counteract to some extent Cd toxicity.

Oxidative stress induced by Cd can damage lipids, DNA and proteins. HSPs play an important role in cell response to metal toxicity

as these chaperone proteins refold damaged proteins and target the highly-damaged ones for degradation (Deane and Woo, 2011) (Fig. 4.1). In agreement with the literature (Deane and Woo, 2011; Lu et al., 2011; Mills and Gallagher, 2017), results from the present study highlight a strong induction of several HSPs at both transcriptomic and proteomic levels in RTL-W1 cells exposed to Cd. PUFA enrichments had no impact on this parameter, suggesting that the PUFA-derived cytoprotection observed in the present experiment is not linked to a differential expression of HSPs. As it is the case for proteins, cells possess mechanisms to repair DNA damages inflicted by oxidative stress. However, Cd has been reported to affect DNA repair mechanisms. Specific mechanisms are not well understood but the implication of p53, a transcription factor involved in the regulation of DNA repair and cell cycle arrest, has been suggested (Antoniali et al., 2015). In the present study, though, neither Cd nor PUFA enrichments influenced *p53* gene expression.

Cd is known to alter ion homeostasis, especially calcium homeostasis, which can lead to increased ROS production, mitochondrial membrane depolarisation and apoptosis (McGeer et al., 2011; Thévenod and Lee, 2013; Wang et al., 2014). In the present study, the level of annexin-A1, a multifunctional protein involved in calcium ion channel formation, was reduced by Cd exposure in control and AA-enriched cells. On the other hand, no impact of Cd could be detected on S100-A1, a calcium-binding protein, or on CALR, a calcium-dependent chaperone located in the endoplasmic reticulum (Fig. 4.1). However, in the absence of Cd challenge, ALA and AA enrichments influenced the level of S100-A1. Further research is needed to determine whether a reduced basal level of S100-A1 could contribute to the lower sensitivity to Cd observed in ALA- and AA-enriched cells.

As mentioned above, Cd toxicity is partly due to its capacity to trigger cell apoptosis through different mechanisms. Among these, the increase in ROS production, the rise in free cytosolic calcium or the modulation of anti- and pro-apoptotic members of BCL2 family (Morcillo et al., 2016; Olszowski et al., 2015; Thévenod and Lee, 2013; Wang et al., 2014). Here, Cd exposure strongly decreased the expression level of *BCLx* gene, an anti-apoptotic member of the BCL2 family (Fig.

4.1). However, none of the PUFA enrichments that protected RTL-W1 cells against Cd prevented *BCLx* gene decreased expression. This is in agreement with the previous report stating that EPA (300 μmol L^{-1}) had no impact on *BCLx* gene expression in ASK cells exposed to methylmercury (2.5 μmol L^{-1}, 48 h) although it reduced cell apoptosis (Nøstbakken et al., 2012). The reduction of metal-driven apoptosis by PUFA enrichments may result from other direct or indirect mechanisms such as a direct modulation of other members of the BCL2 family (e.g. pro-apoptotic Bax) or a differential modulation of calcium intracellular level by AA- or EPA-derived eicosanoids. Further studies are needed to better understand the mechanisms behind PUFA protective effect on metal-induced apoptosis.

Fatty acids with 20 and 22 carbons (such as AA, EPA, 20:3n-6 and DHA) are precursors of eicosanoids, protectins and resolvins (Kremmyda et al., 2011). These PUFA-derived bioactive molecules are modulators of several processes such as inflammation, ion transport and cellular redox status (Kremmyda et al., 2011). PUFAs are first released from the cellular membrane through the action of phospholipases (e.g. cPLA2), then oxidized into bioactive prostanoids, leukotrienes, protectins and resolvins by cyclooxygenases (e.g. COX2) and lipoxygenases (e.g. ALOX5) (Kremmyda et al., 2011) (Fig. 4.1). Cd has been shown to induce the eicosanoid cascade through notably upregulation of the *cPLA2* gene (Madejczyk et al., 2015; Miyahara et al., 2001). Likewise, Cd upregulated the *cPLA2* gene in control and LA-enriched cells in the present study (4.5- and 2-fold increase, significant in control cells). Together with the lower level annexin-A1, an inhibitor of cPLA2 (Parente and Solito, 2004), observed in control cells exposed to Cd, this upregulation of the *cPLA2* gene pleads for a high induction of the eicosanoid cascade by Cd in control cells. Interestingly, the *cPLA2* gene expression remained constant under Cd challenge in the PUFA-enriched cells that were more resistant to Cd, i.e. ALA, EPA- and AA-enriched cells. This suggests that PUFA-derived cytoprotection mechanism against Cd might include a repression of the eicosanoid cascade. This repression may, for instance, have been driven through the reduction of the cellular ROS level or the inhibition of the NF-κB signalling pathway by PUFA (Hagopian et al., 2010; Kremmyda et al.,

2011; Marion-Letellier et al., 2015), as both were shown to mediate *cPLA2* gene induction (Lin et al., 2016; Lin et al., 2011).

Metals, including Cd, have been reported to modulate the expression of various *CYPs* involved in cellular defence (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 PUFAs and decreased by methylmercury both in zebrafish larvae and RTL-W1 cells, suggesting CYP20A1 could represent a molecular target of interactive effects between PUFAs and some heavy metals (Ferain et al., 2018; Lemaire et al., 2016). In the present study, we observed that Cd significantly downregulated *CYP20A1*, but in EPA-enriched cells only. This suggests that the orphan *CYP20A1* may be involved in EPA-derived cytoprotection against Cd. Besides, in the absence of Cd challenge, EPA and AA enrichments significantly upregulated *CYP20A1* and downregulated *COX2* gene expression. These observations suggest that the orphan *CYP20A1* gene might have role in the eicosanoid cascade, as previously highlighted (Ferain et al., 2018).

Cd has been shown to cause abnormalities in the cytoskeleton architecture (Nawaz et al., 2005; Vergilio and De Melo, 2013) and influence the expression of structural constituents of the cytoskeleton (Dorts et al., 2012; Koskinen et al., 2004). However, the proteomic data of the present experiment revealed no such impact of Cd. In contrast, ALA, EPA and AA enrichments influenced the amount of different proteins of the cytoskeleton (cytokeratin-18, the intermediate filament protein ON3 and myosin-9). Still, the observed differences between PUFA-enriched cells and control cells disappeared after Cd exposure. The initial differences in the basal levels of some components of the cytoskeleton might have contributed to the PUFA-derived cytoprotection.

Stress response has a non-negligible energetic cost, which can be further increased by Cd since this metal has been shown to impair ATP production in rainbow trout (Adiele et al., 2012). A higher energy provision at the start of Cd exposure is thus likely to increase the ability of cells to cope with metal stress, as suggested by our observation on AA-enriched cells. Indeed, in absence of Cd, AA-enriched cells had a higher level of pyruvate kinase than control cells. Since this enzyme

catalyses the tenth step of glycolysis (production of pyruvate and ATP from phosphoenolpyruvate and ADP) (Nelson et al., 2008), its upregulation may have provided AA-enriched cells with a high basal energy level, which may have contributed to the higher tolerance of AA-enriched cells towards Cd exposure.

4.5. Conclusion

The present study highlighted molecular interactions between PUFAs and Cd in fish liver cells. On the one hand, the fatty acid supply and, to a lesser extent, Cd influenced cellular fatty acid homeostasis and metabolism. On the other hand, the cellular fatty acid composition of fish liver cells modulated their tolerance to an acute Cd challenge. Enrichments in ALA, EPA, and, to a lesser extent, AA conferred cytoprotection while enrichment in LA had no impact on cell viability. Although the mechanisms of cytoprotection still remain to be fully understood, the present study ruled out the possibility that cytoprotection could result from a decreased Cd accumulation. It rather suggests that the PUFA-derived cytoprotection against Cd may occur through a reduction of the oxidative stress induced by Cd and a differential induction of the eicosanoid cascade. However, it remains difficult to discriminate between the direct and indirect mechanisms by which PUFAs modulate Cd toxicity. Other mechanisms such as modulations of the subcellular partitioning of Cd and of the cellular energy provision, as well as a reduction of the apoptosis triggered by Cd might also be involved. Further studies are needed to address these possibilities.

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4.7. Supplementary data

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Chapter 5

Body lipid composition modulates acute cadmium toxicity in *Daphnia magna* adults and juveniles

Foreword

In the previous chapters, we showed that specific PUFAs such as eicosapentaenoic acid (EPA, 20:5n-3) can protect rainbow trout liver cells (RTL-W1 cell line) against acute Cd toxicity. Cytoprotection did not seem to result from a lower Cd burden but would rather occur through a reduction of the oxidative stress induced by Cd and a differential induction of the eicosanoid cascade. This interaction has never been investigated *in vivo* in aquatic organism. Still, EPA was reported to modulate *Daphnia spp.* ability to cope with other environmental stressors, such as temperature or infection. EPA awas also reported to influence daphnid reproductive success and to be transferred to the progeny. In the present chapter, we thus tested our hypothesis that dietary EPA would protect adult daphnids and the juveniles they released against acute Cd toxicity. The present results were published in Chemosphere.

Body lipid composition modulates acute cadmium toxicity in *Daphnia magna* adults and juveniles

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Abstract

Long chain polyunsaturated fatty acids (LC-PUFAs) such as eicosapentaenoic acid (EPA, 20:5n-3) affect zooplankton fitness and ability to cope with environmental stressors. However, the impact of LC-PUFAs on zooplankton sensitivity to chemical stressors is unknown. Here, we aimed to document the interaction between EPA and cadmium (Cd), as model chemical stressor, in *Daphnia magna*. A life-history experiment was performed in which daphnid neonates were raised into adulthood on three diets of different lipid composition: (i) algae mix; (ii) algae mix supplemented with control liposomes; (iii) algae mix supplemented with liposomes containing EPA. Juveniles (3rd, 4th and 5th brood) released by daphnids during this life-history experiment were sampled, challenged with Cd during 48 h and their immobility was assessed. At the end of this life-history experiment, another immobilisation test was performed with adults from each treatment. Daphnids absorbed, incorporated and transferred ingested EPA to their offspring. Liposome feeding increased adult tolerance to Cd. The presence of EPA in liposomes did not increase adult tolerance to Cd. Offspring's tolerance to Cd was influenced by the brood number and the maternal diet. It was positively correlated with the PUFA level in body neutral lipids, especially alpha-linolenic acid (ALA, 18:3n-3) and negatively correlated with the saturated fatty acid level in body neutral lipids, especially stearic acid (18:0). Overall, these results emphasize the importance of dietary lipids and maternal transfer of body lipids in *D. magna* sensitivity to Cd and highlight the need to take into account these parameters in ecotoxicological studies and risk assessment.

Key words (5): eicosapentaenoic acid, liposome, cadmium, *Daphnia magna*, acute toxicity

5.1. Introduction

Cadmium (Cd) is a highly toxic heavy metal that has been extensively extracted from the Earth crust and released in the environment as a consequence of its direct or indirect (waste-product) use in various industrial applications (Pan et al., 2010). At the individual level, Cd impairs growth, reproduction and survival of aquatic organisms (McGeer et al., 2011). Cd toxicity is commonly linked to its potency to (i) alter ion homeostasis, (ii) bind sulfhydryl groups and modulate the properties of many proteins and enzymes and (iii) generate oxidative stress (Barata et al., 2005b; Connon et al., 2008; McGeer et al., 2011; Matovic et al., 2015).

Lipids, and their constitutive fatty acids, have important biological roles in aquatic organisms as they (i) provide a significant part of the energy required for somatic growth, locomotion and reproduction, (ii) constitute the biological membranes and maintain them in an appropriate structure for a good functioning (iii) are precursors of signalling molecules (e.g. the eicosanoids) (Tocher, 2003; Heckmann et al., 2008; Parrish, 2009). Some fatty acids can be synthesized *de novo* in zooplankton and fish but others cannot and must therefore be assimilated from their diet and are called essential fatty acids (Parrish, 2009). Linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3), two essential fatty acids, are exclusively synthesized by primary producers and some heterotrophic protists (Desvilettes and Bec, 2009; Parrish, 2009). These two fatty acids can be further desaturated and elongated by heterotrophic organisms to produce the valuable long chain polyunsaturated fatty acids (LC-PUFAs): arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Bell and Tocher, 2009). However, these biotransformation steps being of poor efficiency in zooplankton (Farkas et al., 1981; von Elert, 2002; Martin-Creuzburg et al., 2010), the LC-PUFA content of these organisms is highly dependent on the lipid composition of their food, i.e. the primary producers (Brett et al., 2009). The fatty acid profile of these primary producers has been shown to vary a lot among taxa (Brett et al., 2009; Lang et al., 2011) and is modulated by environmental conditions, such as temperature (e.g. Hixson and Arts (2016)) and water pollution (e.g. Chia et al. (2013)).

Zooplankton has a key role in aquatic food webs as it transfers biomass and macronutrients, such as lipids, from the primary producers to higher trophic levels. Among the freshwater zooplankton, the large water flea, *Daphnia magna*, can be found in many lakes and ponds of temperate regions and is commonly used as model species in ecotoxicological studies as it (i) has a short generation time, (ii) can easily be handled and maintained in controlled laboratory conditions and (iii) has a parthenogenetic reproduction cycle, which ensures genetic stability of the clones. As other filter feeders, *D. magna* does not select its food based on its nutrient richness but rather based on its size (DeMott, 1986). The natural diet of *D. magna* is thus composed of a wide variety of microorganisms and particulate suspended matter with size ranging from 0.6 to 40 µm (Geller and Müller, 1981). This property can be used in the laboratory to study the impact of a wide variety of food sources on the organism fitness. Particularly, the biological roles of specific fatty acids can be studied through feeding *Daphnia spp*. with liposomes loaded with specific fatty acids. For example, using that technique, it has been shown that EPA and AA influence *Daphnia spp*. somatic growth and/or reproductive success (Ravet et al., 2003; Martin-Creuzburg et al., 2009, 2010, 2012; Sperfeld and Wacker, 2012; Schlotz et al., 2013, 2014) and modulate their ability to cope with environmental stressors, such as temperature (Martin-Creuzburg et al., 2012), predation (Brzezinski and von Elert, 2015) or infection (Schlotz et al., 2013, 2014). Several mechanisms have been suggested to explain these interactions. It has first been suggested that, in ectotherms with poor fatty acid biotransformation efficiencies, such as *D. magna*, limitations in dietary LC-PUFAs could prevent the organisms from performing the homeoviscous adaptation required to maintain membrane fluidity in case of cold temperature shocks, which can happen either during winter or during diel vertical migrations (Martin-Creuzburg et al., 2012; Brzezinski and von Elert, 2015). Besides, it has also been suggested that dietary fatty acids with 20 carbons could modulate the immune response to parasite infection through influencing the eicosanoid cascade induction (Schlotz et al., 2013, 2014).

Following a similar reasoning, we hypothesized that dietary LC-PUFAs could also influence the ability of aquatic organisms to cope with other environmental stressors, such as chemical pollutants. Indeed, the

membrane unsaturation level could modulate chemical uptake, excretion, cellular/subcellular partitioning and/or body burden, which, in turn, could modulate the organism general sensitivity to the chemical. In addition, lipid signalling could enhance the organism defences against chemical toxicity. However, such interactions have been poorly investigated. Still, there is evidence from *in vitro* studies that specific fatty acids can modulate the sensitivity to heavy metals such as Cd. For example, ALA, EPA and DHA given either alone (Ferain et al., 2016) or in combination (Linhartova and Sampels, 2015) to rainbow trout liver cells or human hepatocellular cells have been shown to increase the cellular tolerance to an acute Cd challenge, while supplementation with either LA or AA did not have any impact (Ferain et al., 2016). However, the influence of specific fatty acids on sensitivity to Cd has, to our knowledge, never been investigated *in vivo*. We therefore aimed at investigating this interaction using *D. magna* as experimental model and EPA as targeted fatty acid.

We hypothesized that EPA would protect *D. magna* against an acute Cd challenge. To test this hypothesis, we carried out a life history experiment with *D. magna Straus* raised into adulthood on diets specifically enriched in EPA, using the liposome supplementation technique. Adult daphnids from each dietary treatment as well as the offspring they have released during the life-history experiment (3rd to 5th brood) were sampled and challenged with Cd. After 48 h of exposure, the immobilisation of the daphnids was assessed as toxicity endpoint. *D. magna* are known to have a fatty acid composition that globally reflects that of their diet. In addition, they preferentially drain PUFA to their progeny and have a better fitness under EPA feeding. We therefore expected that 21-day old daphnids fed EPA and their juvenile progeny would contain a higher EPA body level and be more tolerant to an acute Cd challenge than those fed no EPA and their progeny, respectively.

5.2. Material and methods

5.2.1. Experimental design

A life history experiment was first carried out with *D. magna* raised into adulthood (21 days old) on three diets differing in their lipid

composition: (i) algae mix; (ii) algae mix supplemented with control liposomes (CT-liposomes); (iii) algae mix supplemented with liposomes containing EPA (EPA-liposomes). The algae mix was supplied at saturating level to ensure differences in quality of the diet only. Juveniles released by these daphnids during the life-history experiment were sampled. Those belonging to the 3rd, 4th and 5th brood were used for running a standardized acute immobilisation test, using Cd as chemical stressor. At the end of the life-history experiment, a similar acute immobilisation test was performed with the 21-day old daphnids from each treatment. In addition, the fatty acid profile of two lipid classes (phospholipids and neutral lipids) was monitored in individuals at the start of each acute test performed. Finally, the sensitivity of juveniles to Cd was linked to fatty acid profile of their neutral lipids and phospholipids using two principal component analyses.

5.2.2. *D. magna* maintenance

The *D. magna Straus* K6 clone used in the present study was sampled in a pond in Kallo (Antwerp, Belgium) in 1983. It is routinely maintained in aerated carbon filtered tap water (Ghent, Belgium) supplemented with Na_2SeO_3 (2.2 mg/L; Prolabo, France) and vitamins, i.e. thiamin (B1, 0.75 mg/L; Fluka, Germany), biotin (B8, 75 mg/L; Sigma-Aldrich, USA) and cyanocobalamin (B12, 100 mg/L; Sigma-Aldrich, USA), at a density of 50 animals/L, at 20°C and under a light cycle of 12 h:12 h light:darkness. Daphnids from this stock are daily fed an algae mix composed of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* at a 3 to 1 cell number ratio. This algae mix is given at concentration of (i) 2-2.5 mg carbon/L from day 0 to day 8 and (i) 4-5 mg carbon/L from day 9 to day 21 of their life. The fatty acid profile of the algae mix used has been determined. It contains PUFAs from both the n-3 (ALA and 18:4n-3) and n-6 (LA and 18:3n-6) families but is devoid of LC-PUFAs, such as EPA, DHA and AA.

5.2.3. Liposome preparation and characterisation

The liposome suspensions were produced as described by Martin-Creuzburg et al. (2009), with minor adaptations. Briefly, stock solutions of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC; Lipoid, Germany)

and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG; Lipoid Germany) were prepared in ethanol (6.7 g/L), subdivided into vials and kept at -20°C. Prior use, each vial was homogenized and sonicated (ultrasonic bath, 20°C, 3 min). CT-liposomes stock suspensions were prepared from 315.3 mg POPC (in ethanol), 135.1 mg POPG (in ethanol). For EPA-liposomes stock suspensions, 150.0 mg EPA (Larodan, Sweden) was added. The resulting suspensions were evaporated to dryness using a rotary evaporator (25°C, 45 mbar), resuspended in 50 mL buffer (20 mM NaH_2PO_4, 20 mM Na_2HPO_4, 150 mM NaCl, pH 7.0), incubated on a rotary shaker during 30 min (25°C, 100 revolutions/min) and sonicated (ultrasonic bath, 25°C, 10 min) before being washed from remaining free fatty acids. For doing so, liposome suspensions were centrifuged (35,000 g, 90 min, 4°C, L7-65 ultra-speed centrifuge equipped with a 55.2 Ti fixed angle rotor Beckman Coulter, USA) and the resulting liposome pellets were resuspended in fresh buffer (40 mL). The obtained liposome suspensions were then subdivided into aliquots, flushed with N_2 and kept at -20°C until use either for feeding daphnids or for liposome characterisation (size and lipid composition). Prior any use, thawed liposome suspensions were homogenized and sonicated (ultrasonic bath, 20°C, 2 min). Liposome size assessment was performed on three independent aliquots of each liposome suspension with a Mastersizer S long bed (version 2.15, Malvern Instruments Ltd, UK) equipped with a 300RF lens, as previously described (Kasinos et al., 2014). A refractive index of 1.33, 1.5295 and 0.10000 was set for the continuous phase (i.e. water), the real and the imaginary dispersed phase (i.e. liposomes), respectively (Evens et al., 2011). Particle size distribution was performed using the polydisperse model of the Malvern Mastersizer software (UK) and volumetric diameters (D[4,3]) were calculated. D[4,3] were 10.6 ± 0.1 and 13.0 ± 0.1 mm, for CT- and EPA-liposome suspensions, respectively. The method for fatty acid composition of the liposomes assessment is detailed in section 5.2.5.

5.2.4. Life-history experiment

Neonate daphnids from the stock culture (3rd brood, born within 24 h) were raised on three diets differing in their lipid content during 21 days: (i) algae mix; (ii) algae mix supplemented with CT-liposomes; (iii) algae mix supplemented with EPA-liposomes. Each treatment consisted

in 3 aquaria of 2 L containing 100 individuals each. Daphnids were fed daily and water was renewed three times a week. The composition of both the algae mix and the water used for the life-history experiment were similar as for the stock culture. The algae mix was initially fed at 2 mg carbon/L per day, then increased to 4 mg carbon/L per day and 6 mg carbon/L per day from day 8 and 15, respectively, in order to keep its level saturated throughout the life-history experiment and thus avoid differences due to the quantities fed and ensure differences in quality of the diet only. EPA-liposome supplementation was increased proportionally to the algae mix, starting with 1.5 mg EPA per daphnid and per day, then increased at 3.0 and 4.5 mg EPA per daphnid and per day from day 8 and day 15, respectively (Table 5.1). EPA-liposomes also contained palmitic acid (16:0) and oleic acid (18:1n-9) as these two fatty acids constituted the POPC and POPG used in liposome preparation. The amount of CT-liposomes given was therefore calculated to be equivalent to the level of 16:0 and 18:1n-9 given through EPA-liposome supplementation (Table 5.1), in order to ensure differences in EPA level only, between these two diets.

Table 5.1. Amounts of fatty acids daily fed through liposome supplementation throughout a life-history experiment comparing three diets differing in their lipid composition (mg/daphnid/day).

Day	Fatty acid	Algae	CT-liposome	EPA-liposome
1-7	EPA 20:5n-3	-	0.0 ± 0.0	**1.5 ± 0.0**
	16:0	-	1.7 ± 0.0	1.7 ± 0.0
	18:1n-9	-	1.8 ± 0.0	1.8 ± 0.0
8-14	EPA 20:5n-3	-	0.0 ± 0.0	**3.0 ± 0.0**
	16:0	-	3.3 ± 0.1	3.4 ± 0.0
	18:1n-9	-	3.6 ± 0.1	3.6 ± 0.0
15-20	EPA 20:5n-3	-	0.0 ± 0.0	**4.5 ± 0.1**
	16:0	-	5.0 ± 0.1	5.0 ± 0.1
	18:1n-9	-	5.4 ± 0.1	5.4 ± 0.1

Data are expressed as means ± standard error of mean (n = 2). Abbreviations used: CT, control; EPA, eicosapentaenoic acid.

The life-history experiment was carried out at 20°C and under a 16 h:8 h light:dark cycle, in order to ensure comparability of the physical conditions between the present experiment and the literature about the impact of dietary LC-PUFAs on effects of environmental stressors to

Daphnia spp. that is currently available (Martin-Creuzburg et al., 2012; Schlotz et al., 2013; Schlotz et al., 2014; Brzezinski and von Elert, 2015). Water chemistry was recorded (pH, dissolved organic carbon (DOC), dissolved metal concentrations) at every renewal (old and new water) and was constant throughout the life-history experiment (supplementary data Table 5.A.1). Juveniles of the 3rd, 4th and 5th brood released by daphnids during the life-history experiment were sampled at day 15, 18 and 21, respectively, and were used for running an acute immobilisation test, using Cd as chemical stressor. At the end of the life-history experiment, the 21-day old daphnids from each dietary treatment were also sampled and used for running a similar immobilisation test. In addition, daphnids were sampled for lipid analyses at the start of each acute test performed.

5.2.5. Lipid analyses

5.2.5.1. Sample preparation

Daphnids were first transferred in clean medium deprived from food during 24 h in order to empty their gut content before being sampled, freeze-dried and stored at -80°C until lipid analyses. A visual check on a few individuals indicated that the Daphnia guts were cleared within 24 h.

5.2.5.2. Lipid extraction, separation and quantification

Total lipids from liposome suspensions (n = 2) and freeze-dried daphnids (n = 3) were extracted with methanol:chloroform:water (2:2:1.8, v:v:v) as described by Bligh and Dyer (1959). Daphnid samples were disrupted through homogenisation in presence of ceramic beads (MagNaLyser Green Beads, Roche Applied Biosystem, Germany) for 30 s at a speed of 6000 g (MagNaLyser Instrument Roche Applied Biosystem, Germany) prior total lipid extraction. Total lipid extracts from liposome were then methylated as previously described (Ferain et al., 2016) while those from daphnids were separated by solid phase extraction into three lipid fractions, i.e. neutral lipids, free fatty acids and phospholipids, as described by Schneider et al. (2012) prior methylation. Fatty acid methyl esters (FAMEs) of the neutral lipid and

phospholipid fractions were then extracted with hexane and separated by gas chromatography, as detailed by Ferain et al. (2016) with minor adaptations. The gas chromatograph (GC Trace 1310, Thermo Fisher Scientific, Italy) was equipped with an autosampler TriPlus TP100 (Thermo Fisher Scientific, Italy), a RT2560 capillary column (biscyanopropyl polysiloxane, 100 m x 0.25 mm internal diameter, 0.2 mm film thickness; Restek, USA) and a flame ionisation detector (FID, Thermo Quest, Italy). The carrier gas was H_2 (200 kPa, 2 mL/min). The FID was kept at 255°C and flowed by H_2 (35 mL/min), air (350 mL/min) and N_2 (40 mL/min). The temperature program was as follows: (1) initial temperature set at 80°C; (2) 25°C/min increase up to 175°C; (3) 25 min at 175°C; (4), 10°C/min increase up to 200°C; (5) 20 min at 200°C; (6) 10°C/min increase up to 220°C; (7) 5 min at 220°C; (8) 10°C/min increase up to 235°C; (9) 15 min at 235°C; (10) 20°C/min decrease down to initial temperature (80°C). Chromatogram processing was performed with the ChromQuest software (version 5.0, ThermoFinnigan, Italy). FAME identification and quantification were made by comparing the retention times and peak areas, respectively, with an external standard made from known concentrations of 44 FAMEs (Larodan, Sweden). An internal standard composed of 1,2-dipentadecanoylsn-glycero-3-phosphatydylcholine, triheptadecanoin and tridecanoic acid (Larodan, Sweden) and an injection standard (methylundecanoate; Larodan, Sweden) were added during the extraction step and before the injection, respectively, in order to control the quality of the whole process.

5.2.6. Acute Cd challenge

Juveniles from three successive broods (3rd to 5th brood, born within 24 h) released by daphnids from each dietary treatment during the life-history experiment were sampled and used for running a standardized acute immobilisation test (OECD guidelines, test 202), using Cd as chemical stressor. Twenty-one-day old daphnids from each treatment were also sampled at the end of the life-history experiment and used for running an additional immobilisation test (OECD-guidelines, test 202, with minor adaptations). Briefly, 3 replicates of 10 individuals each were exposed to different Cd concentrations ranging from 58.7 to 974.6 mg Cd/L for juvenile tests and from 260.0 to 1122.1

mg Cd/L for adults (dissolved concentrations measured by inductively coupled plasma atomic emission spectrometer, ICP-AES, Thermo Fisher Scientific, Italy). Exposure media were prepared by spiking the culture media (similar composition as for life-history experiment) with $CdCl_2$ (CAS 7790-78-6; Prolabo, France). Test vessels (polypropylene cups) contained 25 mL and 200 mL exposure media for juvenile and adult tests, respectively. Acute tests were carried out at 20°C, pH 8.4 ± 0.0, hardness 178.9 ± 2.1 mg/L $CaCO_3$, DOC 1.9 ± 0.1 mg/L, under a light cycle of 16 h:8 h light:darkness (supplementary data Table 5.A.1). Daphnids were not fed during the immobilisation test. In any case, immobility was monitored after 48 h of exposure.

5.2.7. Data analyses

Graphs and statistical analyses were performed using the JMP PRO 12.0 software. For all statistical tests, results were considered significant at the level $\alpha = 0.05$. A Bonferroni-Sidak correction was applied to correct this α level whenever relevant, i.e. in any case of multiple testing ($\alpha = 1 - (1 - 0.05)^{(1 / \text{comparison number})}$) (Sidak, 1967).

5.2.7.1. Fatty acid profile data analyses

Data are expressed in mmol/g of freeze-dried daphnid or in % of the sum of the fatty acids identified in the fraction analysed. For data expressed in %, an arcsine-root square transformation was applied to the data. The ANOVA's conditions of application, i.e. normality of the residuals and homogeneity of the variance, were checked with appropriate tests (Shapiro-WilkWtest, skewness and kurtosis calculations, Levene test and Bartlett test). For each sampling time, i.e. before each acute test performed, a 1-way ANOVA followed by a post-hoc Tukey test (with α set at 0.025, due to Bonferroni-Sidak correction) was performed to assess the impact of the diet on the fatty acid composition of each lipid class analysed. For each diet, a 1-way ANOVA followed by a post-hoc Tukey test (with α set at 0.025, due to Bonferroni-Sidak correction) was performed to assess the impact of the groups of daphnids, i.e. 3^{rd}, 4^{th}, 5^{th} brood juveniles or 21-day old adults, on the fatty acid composition of each lipid class analysed.

5.2.7.2. Immobility data analyses

For each dietary treatment (3) and for each acute test performed (4), a LogLogistic model was fitted to the immobility data (% survival). The median effect concentrations (EC50s) and their standard errors were obtained by estimating the parameters of these models. For each acute test performed, Wheeler ratio tests (Wheeler et al., 2006) (with α set at 0.0170, due to Bonferroni-Sidak correction) were performed to highlight significant differences of 48 h-EC50s between daphnids from the 3 dietary treatments. For each dietary treatment, Wheeler ratio tests (with α set at 0.0085, due to Bonferroni-Sidak correction) were also performed to highlight significant differences of 48 h-EC50s between the 4 groups of daphnids (3^{rd}, 4^{th}, 5^{th} brood juveniles and 21-day old adults).

5.2.7.3. Multivariate analyses

The sensitivity of juveniles to Cd (calculated 48 h-EC50s) was linked to the fatty acid profile of either their neutral lipids or their phospholipids (% of the sum of the fatty acids identified in the fraction, arcsine-root square transformed) using two principal component analyses. Each principal component analysis was performed on the correlation matrix in order to give the same weight to each variable (Husson et al., 2017).

5.3. Results

5.3.1. Fatty acid composition and Cd tolerance of 21-day old daphnids

5.3.1.1. EPA accumulation

In absence of supplemented EPA in the diet, 21-day old daphnids contained small amounts of EPA in their phospholipids (less than 1% of the total identified fatty acids in the fraction) while none could be detected in their neutral lipids (Fig. 5.1).

Under EPA-liposome feeding, increased EPA levels in both lipid fractions, but especially in phospholipids, were observed (Fig. 5.1). The EPA level was thus 7.5-10.0 times higher in phospholipids of daphnids fed the EPA-liposomes than in those of daphnids fed the two other diets ($p < 0.05$).

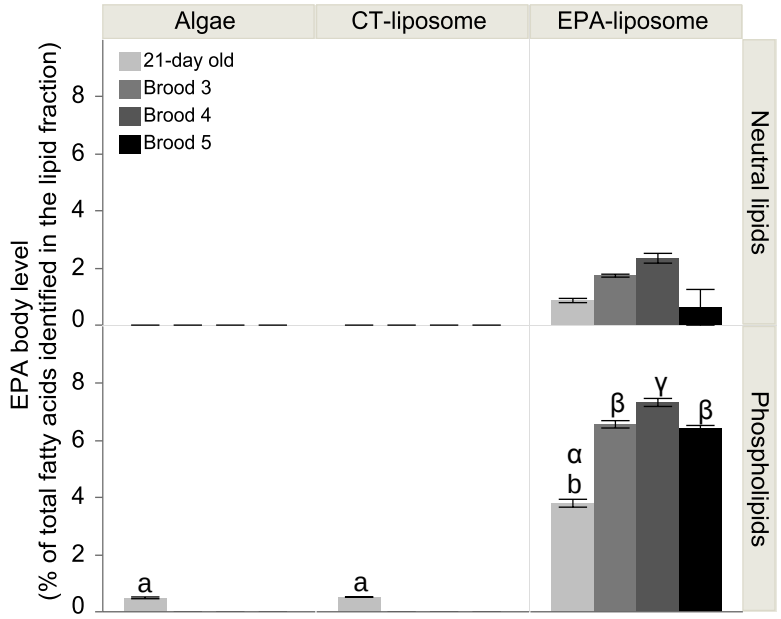

Fig. 5.1. EPA level in phospholipids and neutral lipids of 21-day old daphnids and of the 3rd, 4th and 5th brood juveniles they have released during a life-history experiment comparing three diets differing in their lipid composition. The three diets were an algae mix (Algae), an algae mix supplemented with control liposomes (CT-liposome) and an algae mix supplemented with liposomes containing EPA (EPA-liposome). Bars represent means, error bars represent standard error of mean (n = 3). For each group of individuals separately, letters indicate significant impact of the diet, with levels not connected to the same letter being significantly different from each other (ANOVA followed by a post-hoc Tukey test, $p < 0.025$ due to Bonferroni-Sidak correction). For each dietary treatment separately, Greek letters indicate significant impact of the group of individuals, with levels not connected to the same Greek letter being significantly different from each other (ANOVA followed by a post-hoc Tukey test, $p < 0.025$ due to Bonferroni-Sidak correction). Abbreviations used: CT, control; EPA; eicosapentaenoic acid.

5.3.1.2. Modulation of the fatty acid profile of phospholipids by the diet

Neutral lipids were more abundant (1.3-1.6 fold) than phospholipids in 21-day old daphnids, whatever the diet they had been

fed throughout the experiment. Aside from the EPA accumulation, the diet had no significant impact on the fatty acid profile of their neutral lipids (supplementary data Table 5.A.2). In contrast, the fatty acid composition of phospholipids was significantly affected by the diet (Table 5.2). The levels of 16:0 and 18:1n-9 were significantly higher ($p < 0.05$), or tended to be higher ($p < 0.1$) in phospholipids of daphnids fed both types of liposomes than in those fed no liposome. In addition, the n-3/n-6 ratio of phospholipids was 1.3 fold higher in daphnids fed EPA-liposomes as compared to those fed the two other diets.

Table 5.2. Phospholipid fatty acid composition of 21-day old daphnids at the end of a life-history experiment comparing three diets differing in their lipid composition (mmol/g of freeze-dried daphnid).

Fatty acid	Algae	CT-liposome	EPA-liposome
12:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
14:0	0.8 ± 0.1	0.8 ± 0.0	0.8 ± 0.0
16:0	18.2 ± 1.0 $^{a\ (a)}$	22.6 ± 0.2 $^{b\ (b)}$	21.5 ± 0.8 $^{ab\ (b)}$
18:0	5.0 ± 0.3 $^{ab\ (b)}$	6.2 ± 0.5 $^{a\ (a)}$	4.9 ± 0.0 $^{b\ (b)}$
Total SFA	**24.0 ± 1.2** ^{a}	**29.6 ± 0.6** ^{b}	**27.2 ± 0.9** ^{ab}
16:1n-7	1.0 ± 0.0 ^{a}	1.7 ± 0.1 ^{b}	1.4 ± 0.1 ^{b}
18:1n-7	3.3 ± 0.3	3.8 ± 0.2	4.0 ± 0.3
18:1n-9	13.0 ± 0.8 ^{a}	17.5 ± 1.3 ^{b}	17.2 ± 0.7 ^{b}
Total MUFA	**17.4 ± 1.1** $^{a\ (a)}$	**23.0 ± 1.6** $^{ab\ (b)}$	**22.6 ± 1.1** $^{b\ (b)}$
18:3n-3	9.1 ± 0.7	10.1 ± 0.7	9.6 ± 0.7
18:4n-3	1.2 ± 0.0	1.3 ± 0.1	1.3 ± 0.1
EPA 20:5n-3	0.3 ± 0.0 ^{a}	0.4 ± 0.0 ^{a}	**3.0 ± 0.2** ^{b}
Total n-3 PUFA	**10.6 ± 0.8**	**11.8 ± 0.9**	**13.8 ± 1.0**
18:2n-6	13.0 ± 1.1	14 ± 0.7	13.1 ± 0.6
18:3n-6	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
20:4n-6	0.5 ± 0.1	0.7 ± 0.0	0.5 ± 0.0
Total n-6 PUFA	**13.8 ± 1.2**	**15.1 ± 0.8**	**14.1 ± 0.6**
Total PUFA	**24.4 ± 2.0**	**27.0 ± 1.7**	**27.9 ± 1.6**
Total	**65.8 ± 4.2**	**79.5 ± 2.6**	**77.7 ± 3.4**
n-3/n-6 ratio	**0.77 ± 0.01** ^{a}	**0.78 ± 0.02** ^{a}	**0.98 ± 0.03** ^{b}

Data are expressed as means ± standard error of mean (n = 3). Letters indicate significant impact of the diet, with levels not connected to the same letter being significantly different from each other (ANOVA followed by a post-hoc Tukey test, $p < 0.05$) while letters into brackets indicate similar trends but without significance (ANOVA followed by a post-hoc Tuckey test, $0.05 < p < 0.1$). Abbreviations used: CT, control; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

5.3.1.3. Higher tolerance to Cd in 21-day old daphnids fed both types of liposome

Twenty-one-day old daphnids fed both types of liposomes were significantly more tolerant to an acute Cd challenge as compared to those fed no liposomes (Table 5.3). Indeed, the calculated 48 h-EC50s were 13.8% and 20.8% higher for daphnids fed the CT- and EPA-liposomes as compared to that of daphnids fed no liposome ($p < 0.017$). However, there was no significant difference between the 48 h-EC50s that were calculated for daphnids fed the EPA- and CT-liposomes ($p > 0.05$).

Table 5.3. Cd sensitivity of 21-days old daphnids and their 3rd, 4th and 5th brood juveniles (released during a life-history experiment comparing three diets differing in their lipid composition) following 48 h of exposure to increasing concentrations of the metal.

Individual	Diet	Cd 48 h - EC50 (µg/L)
21-day old adults	Algae	302.5 ± 8.7 $^{a,\alpha}$
	CT-liposome	344.2 ± 13.6 $^{b,\alpha}$
	EPA-liposome	365.4 ± 5.6 $^{b,\alpha}$
3rd brood juveniles	Algae	452.6 ± 8.0 $^{a,\beta}$
	CT-liposome	497.9 ± 10.5 $^{b,\beta}$
	EPA-liposome	455.7 ± 10.4 $^{a,\beta}$
4th brood juveniles	Algae	509.8 ± 14.9 $^{\gamma}$
	CT-liposome	465.7 ± 13.9 $^{\beta}$
	EPA-liposome	489.1 ± 16.2 $^{\beta}$
5th brood juveniles	Algae	354.3 ± 13.0 $^{\delta}$
	CT-liposome	356.3 ± 10.8 $^{\alpha}$
	EPA-liposome	370.0 ± 10.6 $^{\alpha}$

Data are expressed as means ± standard error of mean (n = 3). For each group of individuals separately, letters indicate significant impact of the diet, with levels not connected to the same letter being significantly different from each other (Wheeler ratio test, $p < 0.0170$ due to Bonferroni-Sidak correction). For each dietary treatment separately, Greek letters indicate significant impact of the group of individuals, with levels not connected to the same Greek letter being significantly different from each other (Wheeler ratio test, $p < 0.0085$ due to Bonferroni-Sidak correction). Abbreviations used: Cd, cadmium; CT, control; EC50, median effect concentration; EPA, eicosapentaenoic acid.

5.3.2. Fatty acid composition and Cd tolerance of the juvenile progeny

5.3.2.1. Maternal transfer of EPA

EPA was recovered in neutral lipids and phospholipids of juveniles released by daphnids fed the EPA-liposomes, where it accounted for about 0.6-2.3% and 5.4-7.3% of the total fatty acids identified in the fraction, respectively, while juveniles released by daphnids fed the two other diets were deprived of EPA (Fig. 5.1). In addition, phospholipids of juveniles released by daphnids fed the EPA-liposomes were relatively richer in EPA than those of their mothers, with maximal EPA phospholipid level observed in 4th brood juveniles (Fig. 5.1).

5.3.2.2. Complex variations of the fatty acid profile in juvenile daphnids

The fatty acid composition of neutral lipids and phospholipids of juveniles was influenced by both the diet received by their mothers and the brood to which they belong, leading to complex variations. Detailed fatty acid profiles of neutral lipids and phospholipids are given as supplementary material (supplementary data Tables 5.A.3 and 5.A.4). Fatty acid profile data were summarized and linked to the tolerance to Cd using multivariate analyses (see below).

5.3.2.3. Complex variations of the tolerance to Cd in juvenile daphnids

Dose response curves, calculated 48 h-EC50s and parameters of the LogLogistic models are shown in Fig. 5.2, Table 5.3 and supplementary data Table 5.A.5, respectively. Third brood juveniles released by daphnids fed the CT-liposomes were significantly more tolerant to an acute exposure to Cd than those released by daphnids fed no liposomes (significantly higher 48 h-EC50, Table 5.3). In contrast, the tolerance of 4th and 5th brood juveniles to an acute Cd challenge was not significantly affected by the diet received by their mothers (Table 5.3). The sensitivity to Cd varied among the successive broods released by

daphnids from the same dietary treatment, with the 5th brood showing the highest sensitivity (significantly lowest 48 h-EC50, Table 5.3). In addition, 3rd and 4th brood juveniles were more tolerant to Cd than their mothers (significantly higher 48 h-EC50, Table 5.3). Similarly, 5th brood juveniles released by daphnids fed no liposomes were more tolerant to an acute Cd challenge than their mothers (significantly higher 48 h-EC50, Table 5.3), while those released by daphnids fed the two other diets were equally sensitive to Cd as their mothers (similar 48 h-EC50, Table 5.3).

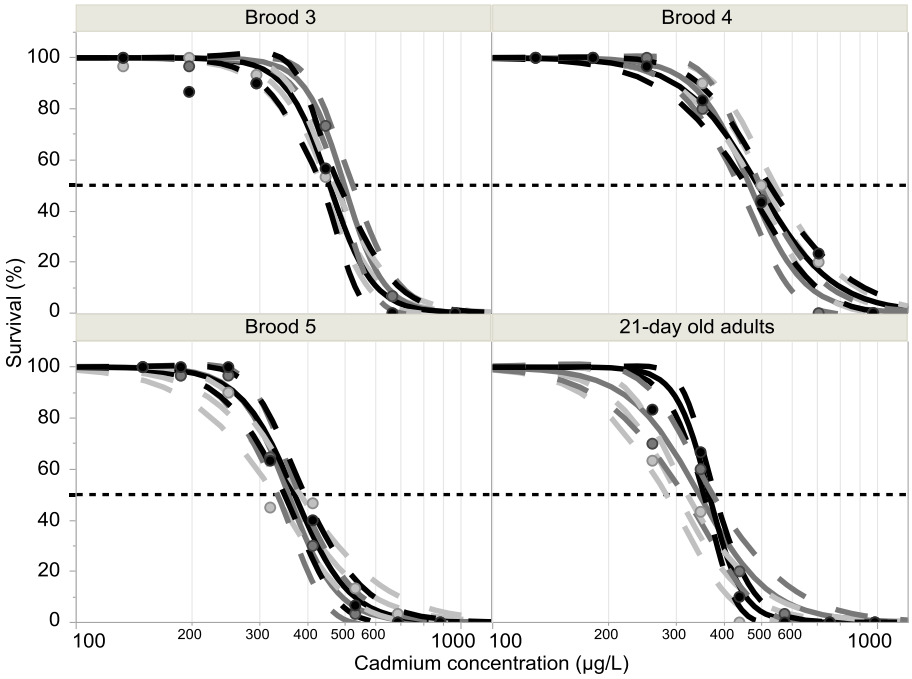

Fig. 5.2. Viability of 21-day old daphnids and of their 3rd, 4th and 5th brood juveniles (released during a life-history experiment comparing three diets differing in their lipid composition) following 48 h of exposure to increasing Cd concentrations. The three diets were an algae mix (Algae), an algae mix supplemented with control liposomes (CT-liposome), and an algae mix supplemented with liposomes containing EPA (EPA-liposome). Plain curves represent the LogLogistic prediction models. Dotted curves represent the confidence intervals at 95% for the predictions. Points represent the mean viabilities of daphnids challenged at specific Cd concentrations (n = 3).

5.3.2.4. Link between fatty acid composition of neutral lipids, brood number and sensitivity to Cd in juveniles

A first principal component analysis was performed using the fatty acid composition of juvenile neutral lipids and the calculated 48 h-EC50s (Fig. 5.3a, Table 5.4). The two first principal components (PC) explained 67.1% of the total variance, with PC1 and PC2 explaining 44.5% and 22.6% of the total variance, respectively (Fig. 5.3a, Table 5.4). The addition of a third component did not add pertinent information to differentiate among the experimental groups. PC1 separates juveniles according to their brood number (Fig. 5.3a). Given the PC1 loadings, it suggests that 5th brood juveniles were characterized by a higher neutral lipid level in 16:0 and 18:0, a lower neutral lipid level in 14:0, ALA, 18:4n-3, LA and 18:3n-6 and a higher sensitivity to Cd than 3rd and 4th brood juveniles, regardless of the diet their mothers were fed throughout their lifespan (Table 5.4). Linear regressions highlight the negative correlations between the tolerance to Cd (48 h-EC50) and the neutral lipid content in 18:0 ($r = -0.85$) and total saturated fatty acids (SFAs; $r = -0.85$), and positive correlations between the tolerance to Cd and the neutral lipid content in ALA ($r = 0.82$), total n-3 PUFAs ($r = 0.80$), total PUFAs ($r = 0.78$) and n-3/n-6 ratio ($r = 0.76$) (supplementary data Fig. 5.A.1).

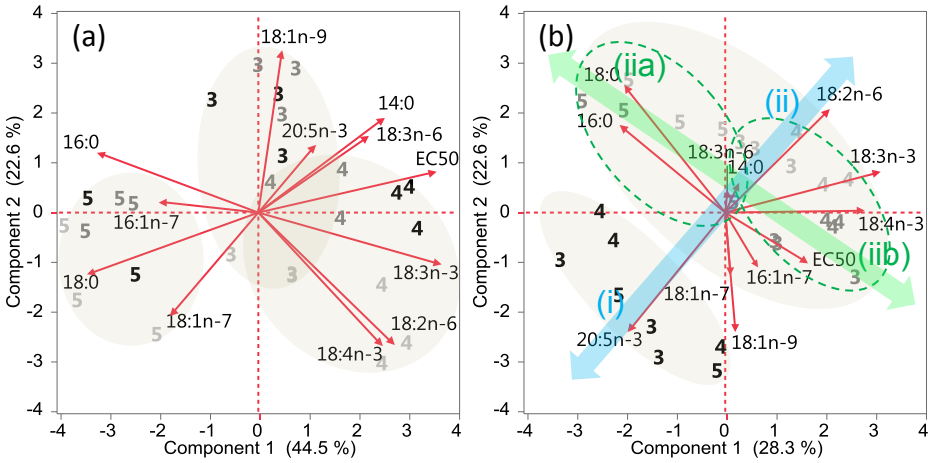

Fig. 5.3. Principal component analysis biplots based on fatty acid composition and Cd tolerance of 3rd, 4th and 5th brood juveniles released by daphnids during a life-history experiment comparing three diets differing in their lipid composition and a subsequent 48 h of Cd exposure. Two principal component analyses were performed using, together, the fatty acid profile data across broods and dietary treatments (expressed as percentages of the sum of the fatty acids identified (a) in the neutral lipids or (b) phospholipids of juvenile daphnids, arcsine-root square transformed) and the calculated Cd 48 h-EC50s. Legend for the scores: numbers (3, 4, 5) refer to the brood numbers, colours refer to the maternal diet: an algae mix (Algae) in light grey, an algae mix supplemented with control liposome (CT-liposome) in dark grey and an algae mix supplemented with liposomes containing EPA (EPA-liposome) in black. Blue and green arrows, shaded areas, dotted circles as well as the terms (i), (ii), (iia) and (iib) highlight the group separations by the principal component analyses. Abbreviations used: Cd, cadmium; CT, control; EC50, median effect concentration, EPA; eicosapentaenoic acid.

Table 5.4. Principal component analysis loadings based on fatty acid composition and Cd tolerance of 3rd, 4th and 5th brood juveniles released by daphnids during a life-history experiment comparing three diets differing in their lipid composition and a subsequent 48 h of Cd exposure.

	Neutral lipids		Phospholipids	
	PC1	PC2	PC1	PC2
14:0	0.65	0.49	0.07	0.16
16:0	-0.83	0.31	-0.64	0.53
18:0	-0.87	-0.32	-0.61	0.77
16:1n-7	-0.50	0.05	0.19	-0.32
18:1n-7	-0.45	-0.53	0.03	-0.37
18:1n-9	0.12	0.83	0.06	-0.72
18:3n-3	0.94	-0.27	0.94	0.25
18:4n-3	0.63	-0.68	0.84	0.01
EPA 20:5n-3	0.29	0.34	-0.59	-0.72
18:2n-6	0.70	-0.68	0.63	0.62
18:3n-6	0.56	0.39	0.01	0.12
EC50	0.92	0.21	0.49	-0.30
Part of the total variance explained (%)	44.5	22.6	28.3	22.6
Cumulative part of the variance explained (%)	44.5	67.1	28.3	50.9

Two principal component analyses were performed using, together, the fatty acid profile data across brood and dietary treatments (expressed as percentages of the sum of the fatty acids identified in the neutral lipids or phospholipids of juvenile daphnids, arcsine-root square transformed) and the calculated 48 h-EC50s. A loading is the correlation between one variable and one principal component. Abbreviations used: PC, principal component; EPA, eicosapentaenoic acid; EC50, median effect concentration.

5.3.2.5. Link between fatty acid composition of phospholipids, maternal diet and sensitivity to Cd in juveniles

A second principal component analysis was performed using the fatty acid composition of juvenile phospholipids and the calculated 48 h-EC50s (Fig. 5.3b, Table 5.4). The two first PCs explained 50.9% of the total variance, with PC1 and PC2 explaining respectively 28.3% and 22.6% of the total variance (Fig. 5.3b, Table 5.4). The addition of a third component did not add pertinent information to differentiate among the experimental groups. The PC1/PC2 biplot separates juveniles released by daphnids fed the EPA-liposomes (group (i)) from juveniles released by daphnids fed the two other diets (group (ii)) (Fig. 5.3b, shaded

areas). This subdivision is along an axis depicted by a blue arrow in Fig. 5.3b. The loadings of these components suggest that phospholipids of juveniles released by daphnids fed the EPA-liposomes were richer in EPA, lower in LA and, to a lower extent, lower in ALA than the phospholipids of juveniles released by daphnids fed the two other diets, regardless of their brood number (Fig. 5.3b, Table 5.4). The PC1/PC2 biplot further subdivides group (ii) in two subgroups: one including 5th brood juveniles (group (iia)) and another one clustering 3rd and 4th brood juveniles (group (iib)) (Fig. 5.3b, dotted circles). The subdivision is along an axis depicted by a green arrow in Fig. 5.3b. Given the loadings of PC1 and PC2, it suggests that 5th brood juveniles released by daphnids fed either the CT-liposomes or no liposomes were characterized by a higher phospholipid level in 16:0, 18:0, a lower phospholipid level in ALA, 18:4n-3 and a higher sensitivity to Cd than 3rd and 4th brood juveniles released by daphnids fed the same diet (Fig. 5.3b, Table 5.4).

5.4. Discussion

In the present study, we aimed at investigating both direct and maternal impact of dietary EPA on acute Cd toxicity to *D. magna*. Using the liposome supplementation technique, we successfully enriched daphnids and their juvenile progeny in EPA. Contrary to expectations, EPA did not protect daphnids against an acute Cd challenge. Instead, we observed that liposomes, but not EPA itself, increased adult daphnid tolerance to the metal, while the sensitivity of juveniles to Cd was correlated to the fatty acid composition of their neutral body lipids. Below, we discuss these main findings in some more detail.

5.4.1. Plasticity of the body fatty acid composition in daphnids

Daphnids have naturally low ability for fatty acid bioconversion. Various authors have reported either poor or no ability of *Daphnia spp*. for EPA synthesis from dietary ALA (Farkas et al., 1981; von Elert, 2002; Martin-Creuzburg et al., 2010; Koussoroplis et al., 2013). In the present experiment, small but quantifiable amounts of EPA were detected in

phospholipids of 21-day old daphnids in absence of dietary EPA. The strain of daphnid used was thus able to bioconvert dietary ALA into EPA. However, even under dietary EPA supplementation, no further bioconversion of EPA into DHA was observed here, suggesting the inability of *D. magna* to synthesize DHA, as previously reported in *Daphnia spp*. (Farkas et al., 1981; von Elert, 2002; Schlechtriem et al., 2006; Martin-Creuzburg et al., 2010). Similarly, daphnids were able to bioconvert dietary LA into AA, as expected since the same enzymes (elongases and desaturases) are required for the bioconversion of n-3 and n-6 fatty acids (Tocher, 2003).

Diet has been reported to have a pronounced impact on the fatty acid composition of *Daphnia spp*. (von Elert, 2002; Becker and Boersma, 2005; Brett et al., 2009), with neutral lipids being overall more affected than phospholipids (Brett et al., 2009). However, in the present experiment, diet had a higher impact on fatty acid composition of phospholipids. Phospholipids of daphnids fed both types of liposomes were richer in palmitic (16:0) and oleic (18:1n-9) acids, the two fatty acids that constituted the backbone of the phospholipids used for liposome preparation. Also, a higher phospholipid content in EPA was observed under dietary EPA supplementation. Daphnids thus ingested and absorbed liposomes and incorporated the given fatty acids in their body, mainly in phospholipids. The predominant incorporation of both dietary and synthesized EPA in phospholipids may emphasize the importance of that LC-PUFA as structural component of biological membranes.

D. magna significantly transferred the ingested EPA to their offspring. It was previously shown that daphnids preferentially drain their constituting PUFAs, including EPA, in their eggs, which can be richer in these fatty acids than their mothers (Becker and Boersma, 2005; Wacker and Martin-Creuzburg, 2007; Sperfeld and Wacker, 2012). Similarly, in the present experiment, EPA accounted for about 7% of the total fatty acids identified in the phospholipids in juveniles while it accounted for about 4% of that fraction in their mothers. This significant EPA transfer to the progeny may highlight a preponderant role of this LC-PUFA in reproduction and development, as previously suggested (Martin-Creuzburg et al., 2009; Sperfeld and Wacker, 2012,

2015). However, in absence of dietary EPA, adults did not transfer the synthesized EPA to their offspring, suggesting that there may be a threshold for maternal transfer of EPA.

Daphnids sequentially accumulate and mobilize lipids along the reproduction cycle. Lipids are known to accumulate in the fat cells, principally located ventrally along the gut, until their mobilisation at the start of the yolk formation (Tessier and Goulden, 1982; Smirnov, 2017). Successive broods may thus be of different lipid composition depending on the fatty acid composition of the fat body when mobilisation is initiated. In the present experiment we observed complex variations of the fatty acid profiles of both phospholipids and neutral lipids in juveniles, with the fatty acid composition of the phospholipids being predominantly affected by the diet while that of neutral lipids was predominantly affected by the brood number (see principal component analyses). While the impact of dietary fatty acids on reproduction efficiency (size, number of juveniles) and total fatty acid profile of eggs has been investigated (Ravet et al., 2003; Wacker and Martin-Creuzburg, 2007; Martin-Creuzburg et al., 2010; Sperfeld and Wacker, 2015), the present experiment is, to our knowledge, the first showing differential impacts of dietary lipids and brood number on the fatty acid composition of lipid classes in juvenile daphnids. Fatty acids having important biological roles, this complex plasticity of fatty acid profile in juveniles might explain variability of biological response in *D. magna* neonates.

5.4.2. Impact of dietary lipids and maternal transfer of body lipids on daphnid sensitivity to Cd

EPA did not confer protection against Cd toxicity in both adult and juvenile daphnids, under the present experimental conditions. By contrast, EPA increased by 4.5 times the Cd 24 h-EC50 (CellTiter-Blue assay, assessing metabolic activity) in the rainbow trout liver cell line RTL-W1 (Ferain et al., 2016). This emphasizes the need for studies with more experimental models and for cautiousness when comparing data obtained from models of different complexity. Besides, differences in the

extent to which the fatty acid profile could have been experimentally modified might explain the different results between the two studies. Indeed, EPA incorporation and biotransformation were more efficient in RTL-W1 cells (Ferain et al., 2016) than in *D. magna*. For instance, the phospholipid richness in EPA was increased from 2.4% to 25.0% *in vitro* (Ferain et al., 2016), while EPA constituted maximum 7.3% of body phospholipids in the present *in vivo* experiment. Besides, RTL-W1 cells elongated EPA into 22:5n-3 and incorporated this metabolite in their cellular phospholipids (Ferain et al., 2016) while no 22:5n-3 was detected in *D. magna*, in the present experiment. EPA and/or 22:5n-3 phospholipid levels might be important modulators of Cd toxicity. Differences in these levels might thus explain the contrasting results.

In adults, dietary supplementation with both types of phospholipid-based liposomes increased daphnid tolerance to Cd. However, EPA did not confer additional protection. Phospholipids are important nutritional factors in fish and crustaceans. Increased performance and reproductive success under phospholipid feeding has been reported in fish and crustaceans (Coutteau et al., 1997; Tocher et al., 2008). While the role of phospholipids in *Daphnia spp*. nutrition has, to our knowledge, not been determined yet, it was recently shown that the beneficial impact of EPA on *D. magna* fitness was potentiated when it was given in the form of a phospholipid instead of a free fatty acid, as we did (Denoux et al., 2017). Although the mechanisms remain to be fully understood, authors suggest that the uptake of EPA by enterocytes might be increased when it is administrated in the form of a phospholipid (Denoux et al., 2017). Together with the present results, this suggests an important role of phospholipids in *D. magna* nutrition. In addition, the types of the fatty acids that constitute the phospholipids may be important. Here, liposomes contained palmitic (16:0) and oleic (18:1n-9) acids, two fatty acids that were significantly incorporated into body phospholipids and that might have conferred a higher Cd tolerance on daphnids. Besides, the protective impact of liposome feeding observed here may be linked to a higher phosphorous dietary level under liposome supplementation. However, it has been previously reported that phosphorous increased Cd toxicity in *D. magna* (Zhang et al., 2012). A final explanation of the protection conferred by liposome feeding may be the increased choline dietary level (given in the form of

phosphatidylcholine), which is known to be an important nutrient for both fish and crustacean health since it is not easily synthesized (Gouillou-Coustans and Guillaume, 2001). Choline is involved in cell structure maintenance (phosphatidylcholine) and in neurotransmission (acetylcholine), two biological functions that have been shown to be dysregulated under Cd contamination (McGeer et al., 2011; Khan et al., 2016). Data so far are thus insufficient to explain the liposome derived protection against Cd toxicity observed here. Further investigations are required for unravelling mechanisms of interactions.

In juveniles, the sensitivity to Cd was mainly related to the brood number, with the 5th brood juveniles showing the highest sensitivity to an acute Cd challenge, regardless of the diet. In addition, a higher sensitivity to Cd across all broods and diets was associated with a lower neutral lipid content in PUFAs, especially ALA, a lower n-3/n-6 ratio and a higher neutral lipid content in SFAs, especially 18:0 (see principal component analyses). In other words, these results suggest that neonates with neutral lipids characterized by a higher level of PUFAs, especially ALA, a higher n-3/n-6 ratio, and a lower level of SFAs, especially 18:0, are more tolerant to Cd. The protective effect of ALA against acute Cd toxicity has previously been demonstrated in RTL-W1 cells (Ferain et al., 2016). However, the interaction highlighted here is somewhat different as the sensitivity of juvenile *D. magna* is correlated to the fatty acid profile of the neutral lipids instead of that of the phospholipids. Neutral lipids are used as energy storage (Tessier and Goulden, 1982). Under metal stress, the energy demand can be increased (Sokolova et al., 2012). Whether fatty acids are differentially mobilized depending on both their degree of unsaturation and the position of the unsaturation under such events in *D. magna* neonates, as described in vertebrate (Raclot, 2003; Mustonen et al., 2007; Louis et al., 2015), and whether this would have an impact on daphnid ability to cope with a rise in energy demand is currently unknown and would require further investigation.

5.4.3. Higher sensitivity of the adult daphnids to Cd, as compared to most of their progeny

Larger and older organisms are often reported to be more tolerant to pollutants (Enserink et al.,1990; Hutchinson et al.,1998). However, in the present experiments, adult daphnids were more sensitive to an acute Cd exposure than their 3rd and 4th brood progeny. Only a few authors investigated the impact of *D. magna* age on Cd acute toxicity. Data so far are contradictory as older daphnids showed either higher (Traudt et al., 2017a; b), lower (Stuhlbacher et al., 1993) or equal (Nebeker et al., 1986) sensitivity to an acute Cd challenge. The higher sensitivity of adult daphnids observed in the present experiment is counterintuitive and may not be explained by differences in water chemistry, as pH and DOC were similar across experiments. It might rather be related to the decrease in antioxidant defences that occurs during the aging process of *D. magna* (Barata et al., 2005a). Nevertheless, other mechanisms are probably also involved as adult sensitivity to Cd was either similar to or only slightly higher than their 5th brood progeny. Although moulting of juvenile and adult daphnids was not monitored during the experiments, the observed differences of sensitivity to Cd between adults and the 3rd and 4th brood juveniles could potentially also be explained in part by differences in the timing and fraction of moulting between these groups during the 48-h exposure period, since daphnids are known to be more sensitive to aqueous metal exposure during moulting (Lee and Jr., 1979). It would finally be interesting to see if the difference in toxicity between adults and 3rd and 4th brood juveniles could be explained by differences in Cd body burden or Cd toxicokinetics and/or toxicodynamics. Toxicokinetic-toxicodynamic (TK-TD) models, as developed by Tan and Wang (2012) for *Daphnia spp*. exposed to metals (Cd, mercury, zinc), are known to be good predictors of toxicity. However, as evidenced by the authors, the toxicodynamic parameters of such models may depend on the developmental stadium of the daphnids (Tan and Wang, 2012), which currently restricts their use.

5.5. Conclusion

In conclusion, the present experiment highlighted the importance of dietary lipids and maternal transfer of body lipids on *D. magna* sensitivity to Cd. Diet modulated the fatty acid composition of daphnids and their juvenile progeny, with notably higher EPA levels in phospholipids of EPA-liposome fed mothers and their juvenile progeny. *D. magna* fed both types of liposomes (i.e. CT- and EPA-liposomes) during 21 days were more tolerant to an acute Cd challenge, while EPA itself had no impact on Cd sensitivity in either adults or juveniles. In juveniles, the tolerance to Cd acute toxicity was influenced by the brood number and positively correlated to the neutral lipid content in PUFAs, especially ALA, and negatively correlated to the neutral lipid content in SFAs (e.g. 18:0). Additional experiments testing the effect of dietary supplementation with other n-3 PUFAs, such as ALA, or SFAs, such as 18:0, on *D. magna* sensitivity to Cd across generations are needed to validate the correlations observed here. In addition, mechanisms of interactions should be further investigated. Finally, the impact of the brood number on Cd acute toxicity observed in the present experiment might explain inconsistencies between different ecotoxicological studies and emphasizes the need to take this into account in risk assessment.

5.6. Acknowledgements

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5.7. Supplementary data

Table 5.A.1 Water chemistry throughout a life-history experiment comparing three diets differing in their lipid composition and a subsequent 48 h of Cd exposure.

Medium [1]			Na [2]	Mg [2]	K [2]	Ca [2]	P [3]
new			21.9 ± 0.6	6.4 ± 0.2	2.9 ± 0.1	62.5 ± 1.2	*bql* [5]
Day	Medium [1]	Diet	Na [2]	Mg [2]	K [2]	Ca [2]	P [3]
3	old	Algae	20.8 ± 0.1	6.9 ± 0.0	2.9 ± 0.0	64.9 ± 0.1	*bql* [5]
3	old	CT	21.4 ± 0.1	6.9 ± 0.0	2.9 ± 0.0	64.3 ± 0.1	118.3 ± 2.9
3	old	EPA	21.5 ± 0.0	6.9 ± 0.0	2.9 ± 0.0	65.1 ± 0.1	122.3 ± 2.3
6	old	Algae	21.4 ± 0.1	6.1 ± 0.0	2.8 ± 0.0	58.9 ± 0.1	*bql* [5]
6	old	CT	22.5 ± 0.1	6.2 ± 0.0	2.8 ± 0.0	59.1 ± 0.2	182.4 ± 16.7
6	old	EPA	22.4 ± 0.0	6.1 ± 0.0	2.8 ± 0.0	58.9 ± 0.1	182.4 ± 5.3
8	old	Algae	21.9 ± 0.0	6.2 ± 0.0	2.9 ± 0.0	60.1 ± 0.1	5.6 ± 5.6
8	old	CT	22.6 ± 0.1	6.2 ± 0.0	2.9 ± 0.0	60.0 ± 0.2	196.1 ± 1.5
8	old	EPA	22.7 ± 0.1	6.2 ± 0.0	2.9 ± 0.0	60.6 ± 0.2	201.7 ± 2.5
10	old	Algae	21.8 ± 0.0	6.3 ± 0.0	2.8 ± 0.0	62.3 ± 0	17.4 ± 0.9
10	old	CT	23.5 ± 0.0	6.3 ± 0.0	2.9 ± 0.0	62.9 ± 0.1	373.6 ± 4.2
10	old	EPA	23.6 ± 0.1	6.3 ± 0.0	2.9 ± 0.0	62.8 ± 0.1	381.1 ± 2.7
13	old	Algae	22.2 ± 0.1	6.5 ± 0.0	2.9 ± 0.0	63.0 ± 0.2	*bql* [5]
13	old	CT	24.6 ± 0.1	6.5 ± 0.0	2.9 ± 0.0	63.1 ± 0.2	406.3 ± 11.0
13	old	EPA	24.8 ± 0.0	6.5 ± 0.0	2.9 ± 0.0	63.6 ± 0.1	436.9 ± 26.2
15	old	Algae	23.8 ± 0.0	6.6 ± 0.0	3.1 ± 0.0	63.7 ± 0.1	*bql* [5]
15	old	CT	25.2 ± 0.1	6.6 ± 0.0	3.1 ± 0.0	63.6 ± 0.2	174.9 ± 11.7
15	old	EPA	25.3 ± 0.1	6.7 ± 0.0	3.1 ± 0.0	63.8 ± 0.2	240.8 ± 33.7
17	old	Algae	23.6 ± 0.0	6.7 ± 0.0	3.2 ± 0.0	63.4 ± 0.1	*bql* [5]
17	old	CT	25.8 ± 0.0	6.5 ± 0.0	3.0 ± 0.0	63.4 ± 0.0	384.2 ± 11.3
17	old	EPA	26.1 ± 0.1	6.7 ± 0.0	3.1 ± 0.0	64.1 ± 0.2	453.0 ± 22.9
20	old	Algae	24.7 ± 0.1	6.7 ± 0.0	3.4 ± 0.0	64.4 ± 0.2	24.0 ± 0.9
20	old	CT	28.0 ± 0.1	6.6 ± 0.0	3.3 ± 0.0	64.2 ± 0.3	627.4 ± 19.9
20	old	EPA	26.8 ± 0.0	6.3 ± 0.0	3.1 ± 0.0	62.0 ± 0.1	690.8 ± 19.5
Acute test			Na [2]	Mg [2]	K [2]	Ca [2]	P [3]
3rd brood			23.6 ± 0.1	6.3 ± 0.0	3.1 ± 0.0	62.9 ± 0.3	*bql* [5]
4th brood			23.4 ± 0.0	6.3 ± 0.0	3.0 ± 0.0	61.9 ± 0.1	*bql* [5]
5th brood			23.5 ± 0.0	6.1 ± 0.0	3.0 ± 0.0	60.6 ± 0.1	*bql* [5]
21-day old adults			23.4 ± 0.1	6.1 ± 0.0	3.0 ± 0.0	59.7 ± 0.2	*bql* [5]

[1] type of medium analysed (new or old)
[2] mg/L
[3] µg/L
[4] mg/L $CaCO_3$
[5] below limits of quantification, i.e. below 15.0 µg/L, 0.6 µg/L and 1.0 µg/L for P, Mn and Cd, respectively.
[6] 7-8 discrete Cd doses ranging from 58.7 to 974.6 µg Cd/L for juvenile tests and from 260.0 to. 1122.1 µg Cd/L for adults. In any case, Cd concentration of the blank condition was bql (i.e. below 1.0 µg Cd/L)
Data are expressed as means ± standard error of mean (n = 3). Other metals analysed but with concentrations below limit of quantification: Fe, Cu, Ni, Pb, Co, Mo, Sb (i.e. below 2.4 µg/L, 5.0 µg/L, 4.0 µg/L, 15.0 µg/L, 2.0 µg/L, 3.3 µg/L and 28.1 µg/L, respectively. Abbreviations used: bql, below quantification limit; CT, algae mix based diet supplemented with control liposomes; DOC, dissolved organic carbon; EPA, algae mix based diet supplemented with liposomes containing eicosapentaenoic acid

(continued)

Medium [1]			Zn [3]	Al [3]	Ba [3]	B [3]
new			10.7 ± 1	21.7 ± 2.8	13.8 ± 0.7	36.7 ± 6
Day	Medium [1]	Diet	Zn [3]	Al [3]	Ba [3]	B [3]
3	old	Algae	10.5 ± 0.5	19.7 ± 0.4	15.2 ± 0.1	41.6 ± 0.2
3	old	CT	10.1 ± 0.1	19.6 ± 0.4	15.1 ± 0.0	41.5 ± 0.2
3	old	EPA	10.1 ± 0.0	20.8 ± 0.7	15.0 ± 0.0	41.3 ± 0.2
6	old	Algae	9.5 ± 1.1	42.6 ± 15.7	12.9 ± 0.1	29.6 ± 0.1
6	old	CT	7.6 ± 0.2	27.7 ± 0.7	12.9 ± 0.1	30.3 ± 0.3
6	old	EPA	7.4 ± 0.1	27.3 ± 0.3	12.9 ± 0.0	29.7 ± 0.2
8	old	Algae	9.1 ± 0.1	28.8 ± 0.3	13.2 ± 0.0	30.3 ± 0.2
8	old	CT	9.0 ± 0.2	29.2 ± 0.7	13.3 ± 0.1	30.6 ± 0.1
8	old	EPA	8.9 ± 0.1	29.2 ± 0.2	13.3 ± 0.1	30.5 ± 0.2
10	old	Algae	9.4 ± 0.2	24.1 ± 0.4	12.6 ± 0.1	24.3 ± 0.2
10	old	CT	9.3 ± 0.3	24.0 ± 0.5	12.5 ± 0.0	24.1 ± 0.1
10	old	EPA	9.4 ± 0.2	24.5 ± 0.4	12.7 ± 0.1	24.2 ± 0.2
13	old	Algae	8.4 ± 0.1	25.1 ± 0.2	13.0 ± 0.0	25.1 ± 0.1
13	old	CT	8.7 ± 0.2	23.8 ± 0.4	12.6 ± 0.1	25.3 ± 0.3
13	old	EPA	9.7 ± 1.1	23.5 ± 0.3	12.5 ± 0.1	25.7 ± 0.2
15	old	Algae	11.5 ± 0.4	17.1 ± 1.0	15.2 ± 0.1	49.3 ± 0.3
15	old	CT	12.1 ± 0.1	16.2 ± 1.2	14.8 ± 0.0	48.7 ± 0.3
15	old	EPA	13.8 ± 1.0	15.3 ± 0.2	14.6 ± 0.1	49.1 ± 0.5
17	old	Algae	9.9 ± 0.3	17.4 ± 1.0	15.2 ± 0.1	49.0 ± 0.1
17	old	CT	9.5 ± 0.2	14.9 ± 0.5	14.8 ± 0.0	49.0 ± 0.3
17	old	EPA	10.0 ± 0.4	15.8 ± 0.9	14.9 ± 0.1	49.0 ± 0.2
20	old	Algae	8.2 ± 0.1	24.5 ± 0.5	14.9 ± 0.0	33.0 ± 0.4
20	old	CT	7.7 ± 0.0	23.9 ± 0.5	14.6 ± 0.1	33.0 ± 0.3
20	old	EPA	9.0 ± 0.5	23.8 ± 0.6	14.1 ± 0.1	32.4 ± 0.2
Acute test			Zn [3]	Al [3]	Ba [3]	B [3]
3rd brood			11.2 ± 0.5	25.3 ± 0.4	14.4 ± 0.0	32.0 ± 0.2
4th brood			11.2 ± 0.1	25.8 ± 0.2	14.3 ± 0.1	31.8 ± 0.1
5th brood			11.4 ± 0.2	23.8 ± 0.1	13.7 ± 0.0	30.0 ± 0.1
21-day old adults			12.1 ± 0.2	25.4 ± 0.2	13.8 ± 0.0	30.6 ± 0.1

[1] type of medium analysed (new or old)
[2] mg/L
[3] µg/L
[4] mg/L $CaCO_3$
[5] below limits of quantification, i.e. below 15.0 µg/L, 0.6 µg/L and 1.0 µg/L for P, Mn and Cd, respectively.
[6] 7-8 discrete Cd doses ranging from 58.7 to 974.6 µg Cd/L for juvenile tests and from 260.0 to. 1122.1 µg Cd/L for adults. In any case, Cd concentration of the blank condition was bql (i.e. below 1.0 µg Cd/L)
Data are expressed as means ± standard error of mean (n = 3). Other metals analysed but with concentrations below limit of quantification: Fe, Cu, Ni, Pb, Co, Mo, Sb (i.e. below 2.4 µg/L, 5.0 µg/L, 4.0 µg/L, 15.0 µg/L, 2.0 µg/L, 3.3 µg/L and 28.1 µg/L, respectively. Abbreviations used: bql, below quantification limit; CT, algae mix based diet supplemented with control liposomes; DOC, dissolved organic carbon; EPA, algae mix based diet supplemented with liposomes containing eicosapentaenoic acid

(continued)

Medium [1]			Mn [3]	Cd [3]	Hardness [4]	DOC [2]	pH
new			0.5 ± 0.4	*bql* [5]	182.9 ± 3.6	2.0 ± 0.0	8.4 ± 0.0
Day	Medium [1]	Diet	Mn [3]	Cd [3]	Hardness [4]	DOC [2]	pH
3	old	Algae	1.2 ± 0.6	*bql* [5]	191.1 ± 0.4	2.6 ± 0.2	8.5 ± 0.0
3	old	CT	1.4 ± 0.0	*bql* [5]	189.5 ± 0.4	2.2 ± 0.1	8.5 ± 0.0
3	old	EPA	1.4 ± 0.0	*bql* [5]	191.4 ± 0.2	2.4 ± 0.1	8.4 ± 0.0
6	old	Algae	0.9 ± 0.9	*bql* [5]	172.8 ± 0.3	3.0 ± 0.2	8.4 ± 0.1
6	old	CT	1.3 ± 0.1	*bql* [5]	173.3 ± 0.6	2.6 ± 0.1	8.3 ± 0.0
6	old	EPA	1.2 ± 0.1	*bql* [5]	172.7 ± 0.3	2.6 ± 0.1	8.3 ± 0.0
8	old	Algae	3.2 ± 0.0	*bql* [5]	176.3 ± 0.2	2.6 ± 0.1	8.2 ± 0.0
8	old	CT	1.9 ± 0.1	*bql* [5]	175.9 ± 0.6	2.6 ± 0.1	8.2 ± 0.0
8	old	EPA	1.9 ± 0.1	*bql* [5]	177.2 ± 0.6	2.4 ± 0.1	8.2 ± 0.0
10	old	Algae	6.3 ± 0.2	*bql* [5]	181.9 ± 0.1	3.1 ± 0.2	8.3 ± 0.0
10	old	CT	5.4 ± 0.3	*bql* [5]	183.5 ± 0.2	2.8 ± 0.1	8.2 ± 0.0
10	old	EPA	5.8 ± 0.2	*bql* [5]	183.2 ± 0.2	2.7 ± 0.2	8.2 ± 0.0
13	old	Algae	9.4 ± 0.8	*bql* [5]	184.3 ± 0.5	4.6 ± 0.1	8.4 ± 0.0
13	old	CT	12.5 ± 0.5	*bql* [5]	184.6 ± 0.6	5.1 ± 0.1	8.3 ± 0.0
13	old	EPA	16.6 ± 0.3	*bql* [5]	186.1 ± 0.4	5.0 ± 0.2	8.2 ± 0.0
15	old	Algae	1.5 ± 0.1	*bql* [5]	186.8 ± 0.3	3.7 ± 0.1	8.6 ± 0.0
15	old	CT	5.3 ± 1.3	*bql* [5]	186.4 ± 0.5	3.5 ± 0.0	8.4 ± 0.0
15	old	EPA	7.6 ± 0.6	*bql* [5]	187.3 ± 0.6	3.6 ± 0.0	8.3 ± 0.1
17	old	Algae	4.1 ± 0.2	*bql* [5]	186.3 ± 0.2	3.3 ± 0.1	8.4 ± 0.0
17	old	CT	3.2 ± 0.1	*bql* [5]	185.7 ± 0.1	4.5 ± 0.5	8.4 ± 0.0
17	old	EPA	3.2 ± 0.8	*bql* [5]	187.9 ± 0.7	3.6 ± 0.1	8.3 ± 0.0
20	old	Algae	1.6 ± 0.1	*bql* [5]	188.7 ± 0.6	5.0 ± 0.3	8.1 ± 0.0
20	old	CT	1.6 ± 0.4	*bql* [5]	187.9 ± 0.8	4.7 ± 0.1	8.1 ± 0.0
20	old	EPA	1.9 ± 0.3	*bql* [5]	181.2 ± 0.3	4.9 ± 0.2	7.9 ± 0.0
Acute test			Mn [3]	Cd [3]	Hardness [4]	DOC [2]	pH
3rd brood			*bql* [5]	gradient [6]	183.7 ± 0.8	2.1 ± 0.1	8.3 ± 0.0
4th brood			*bql* [5]	gradient [6]	180.7 ± 0.3	2.1 ± 0.1	8.5 ± 0.0
5th brood			*bql* [5]	gradient [6]	176.9 ± 0.3	1.8 ± 0.2	8.4 ± 0.0
21-day old adults			*bql* [5]	gradient [6]	174.4 ± 0.7	1.8 ± 0.2	8.4 ± 0.0

[1] type of medium analysed (new or old)
[2] mg/L
[3] µg/L
[4] mg/L $CaCO_3$
[5] below limits of quantification, i.e. below 15.0 µg/L, 0.6 µg/L and 1.0 µg/L for P, Mn and Cd, respectively.
[6] 7-8 discrete Cd doses ranging from 58.7 to 974.6 µg Cd/L for juvenile tests and from 260.0 to. 1122.1 µg Cd/L for adults. In any case, Cd concentration of the blank condition was bql (i.e. below 1.0 µg Cd/L)
Data are expressed as means ± standard error of mean (n = 3). Other metals analysed but with concentrations below limit of quantification: Fe, Cu, Ni, Pb, Co, Mo, Sb (i.e. below 2.4 µg/L, 5.0 µg/L, 4.0 µg/L, 15.0 µg/L, 2.0 µg/L, 3.3 µg/L and 28.1 µg/L, respectively. Abbreviations used: bql, below quantification limit; CT, algae mix based diet supplemented with control liposomes; DOC, dissolved organic carbon; EPA, algae mix based diet supplemented with liposomes containing eicosapentaenoic acid

Table 5.A.2 Neutral lipid fatty acid composition of 21-day old daphnids at the end of a life-history experiment comparing three diets differing in their lipid composition (μmol/g of freeze-dried daphnid).

Fatty acid	Algae	CT-liposome	EPA-liposome
12:0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0
14:0	6.5 ± 0.6	6.6 ± 0.9	7.7 ± 0.8
16:0	29.1 ± 3.1	31.4 ± 4.3	39.2 ± 2.9
18:0	4.6 ± 0.0	4.7 ± 0.4	3.8 ± 0.3
Total SFA	**40.8 ± 3.7**	**43.2 ± 4.8**	**51.3 ± 4.1**
16:1n-7	5.5 ± 0.6	6.3 ± 0.8	6.2 ± 0.7
18:1n-7	3.7 ± 0.3	3.4 ± 0.3	4.3 ± 0.5
18:1n-9	16.5 ± 1.6	18.1 ± 1.8	20.6 ± 0.6
Total MUFA	**25.6 ± 2.5**	**27.7 ± 2.9**	**31.1 ± 1.8**
18:3n-3	19.0 ± 2.3	16.8 ± 2.3	19.4 ± 0.6
18:4n-3	3.7 ± 0.4	3.3 ± 0.5	3.9 ± 0.2
EPA 20:5n-3	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	**1.1 ± 0.1** [b]
Total n-3 PUFA	**22.7 ± 2.7**	**20.1 ± 2.8**	**24.4 ± 0.5**
18:2n-6	13.1 ± 1.3	12 ± 1.8	14.6 ± 0.2
18:3n-6	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
20:4n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total n-6 PUFA	**14.0 ± 1.4**	**12.9 ± 2**	**15.6 ± 0.1**
Total PUFA	**36.7 ± 4.1**	**33.0 ± 4.8**	**40.0 ± 0.6**
Total	**103.1 ± 10.0**	**104.0 ± 12.5**	**122.4 ± 6.3**
n-3/n-6 ratio	**1.61 ± 0.05**	**1.56 ± 0.02**	**1.56 ± 0.02**

Data are expressed as means ± standard error of mean (n = 3). Letters indicate significant impact of the diet, with levels not connected to the same letter being significantly different from each other (ANOVA followed by a post-hoc Tukey test, $p < 0.05$) Abbreviations used: CT, control; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 5.A.3 Neutral lipid fatty acid composition of 3rd, 4th and 5th brood juveniles released by daphnids during a life-history experiment comparing three diets differing in their lipid composition (% of total fatty acids identified in neutral lipids).

Fatty acid	3rd brood			4th brood		
	Algae	CT-liposome	EPA-liposome	Algae	CT-liposome	EPA-liposome
14:0	5.8 ± 0.3 $^{\alpha}$	6.0 ± 0.1 $^{\alpha}$	5.7 ± 0.1 $^{\alpha}$	5.0 ± 0.1 $^{\alpha\beta}$	5.4 ± 0.1 $^{\alpha}$	5.3 ± 0.1 $^{\alpha}$
16:0	28.0 ± 1.0	29.7 ± 0.3 $^{\alpha}$	30.0 ± 0.9	26.7 ± 1.0	29.5 ± 0.2 $^{\alpha}$	27.0 ± 0.5
18:0	12.8 ± 0.5 $^{a,\alpha}$	9.1 ± 0.6 ^{b}	10.6 ± 0.8 $^{ab,\alpha}$	9.6 ± 0.8 $^{\alpha}$	10.0 ± 1.5	8.2 ± 0.3 $^{\alpha}$
Total SFA	**46.6 ± 1.3** $^{\alpha}$	**44.7 ± 0.9**	**46.3 ± 1.6** $^{\alpha\beta}$	**41.4 ± 1.0** $^{\alpha}$	**44.9 ± 1.6**	**40.5 ± 0.7** $^{\alpha}$
16:1n-7	4.1 ± 0.1 $^{a,\alpha}$	3.2 ± 0.1 $^{b,\alpha}$	3.2 ± 0.2 $^{b,\alpha}$	2.3 ± 0.2 $^{\beta}$	2.0 ± 0.2 $^{\beta}$	1.9 ± 0.1 $^{\beta}$
18:1n-7	2.7 ± 0.1	2.4 ± 0.0 $^{\alpha\beta}$	2.4 ± 0.1 $^{\alpha}$	2.9 ± 0.3	2.2 ± 0.0 $^{\alpha}$	2.5 ± 0.1 $^{\alpha}$
18:1n-9	10.4 ± 0.4 ^{a}	18.0 ± 0.5 $^{b,\alpha}$	15.0 ± 0.3 ^{c}	10.3 ± 0.4 ^{a}	14.4 ± 0.1 $^{b,\beta}$	13.5 ± 0.4 ^{b}
Total MUFA	**17.2 ± 0.5** ^{a}	**23.5 ± 0.4** $^{b,\alpha}$	**20.5 ± 0.6** ^{c}	**15.5 ± 0.5** ^{a}	**18.6 ± 0.2** $^{b,\beta}$	**17.9 ± 0.3** ^{b}
18:3n-3	22.4 ± 0.5 $^{\alpha}$	19.8 ± 0.7 $^{\alpha\beta}$	19.3 ± 0.8 $^{\alpha}$	27.4 ± 0.4 $^{a,\beta}$	23.2 ± 1.0 $^{b,\alpha}$	24.4 ± 0.4 $^{ab,\beta}$
18:4n-3	3.2 ± 0.1	2.6 ± 0.1	2.7 ± 0.1	3.8 ± 0.1	3.2 ± 0.2	3.6 ± 0.2
EPA 20:5n-3	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	**1.7 ± 0.1** ^{b}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	**2.3 ± 0.2** ^{b}
Total n-3 PUFA	**25.6 ± 0.6** $^{\alpha}$	**22.4 ± 0.7** $^{\alpha\beta}$	**23.8 ± 1.0** $^{\alpha}$	**31.1 ± 0.5** $^{a,\beta}$	**26.5 ± 1.2** $^{b,\alpha}$	**30.3 ± 0.5** $^{ab,\beta}$
18:2n-6	10.6 ± 0.3 $^{a,\alpha\beta}$	8.9 ± 0.2 $^{b,\alpha}$	9.1 ± 0.2 $^{b,\alpha}$	11.7 ± 0.4 $^{a,\alpha}$	9.8 ± 0.2 $^{b,\beta}$	10.5 ± 0.0 $^{ab,\beta}$
18:3n-6	0.0 ± 0.0	0.5 ± 0.2	0.2 ± 0.2 $^{\alpha\beta}$	0.3 ± 0.3	0.3 ± 0.3	0.9 ± 0.1 $^{\alpha}$
Total n-6 PUFA	**10.6 ± 0.3** $^{a,\alpha\beta}$	**9.3 ± 0.2** $^{b,\alpha\beta}$	**9.3 ± 0.3** $^{b,\alpha}$	**12.0 ± 0.1** $^{a,\alpha}$	**10.1 ± 0.3** $^{b,\alpha}$	**11.4 ± 0.1** $^{a,\beta}$
Total PUFA	**36.2 ± 0.8** $^{\alpha}$	**31.7 ± 0.7** $^{\alpha\beta}$	**33.2 ± 1.2** $^{\alpha}$	**43.1 ± 0.5** $^{a,\beta}$	**36.5 ± 1.4** $^{b,\alpha}$	**41.7 ± 0.6** $^{a,\beta}$
n-3/n-6 ratio	**2.41 ± 0.04** $^{\alpha}$	**2.41 ± 0.09**	**2.55 ± 0.07** $^{\alpha}$	**2.60 ± 0.05** $^{\alpha}$	**2.63 ± 0.08**	**2.65 ± 0.01** $^{\alpha}$

Data are expressed as means ± standard error of mean (n = 3). For each brood separately, letters indicate significant impact of the maternal diet, with levels not connected to the same letter being significantly different from each other (ANOVA followed by a post-hoc Tukey test, $p < 0.025$ due to Bonferroni-Sidak correction). For each dietary treatment separately, Greek letters indicate significant impact of the brood number, with levels not connected to the same Greek letter being significantly different from each other (ANOVA followed by a post-hoc Tukey test, $p < 0.025$ due to Bonferroni-Sidak correction). Abbreviations used: CT, control; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

(continued)

Fatty acid	5th brood		
	Algae	CT-liposome	EPA-liposome
14:00	4.0 ± 0.4 $^{\beta}$	4.2 ± 0.3 $^{\beta}$	4.3 ± 0.3 $^{\beta}$
16:00	32.2 ± 1.8	31.5 ± 0.3 $^{\beta}$	30.6 ± 1.8
18:00	19.1 ± 1.0 $^{\beta}$	16.2 ± 2.6	15.0 ± 1.0 $^{\beta}$
Total SFA	**55.4 ± 2.4 $^{\beta}$**	**51.9 ± 2.0**	**49.9 ± 2.5 $^{\beta}$**
16:1n-7	2.7 ± 0.1 $^{\beta}$	3.5 ± 0.3 $^{\alpha}$	3.6 ± 0.2 $^{\alpha}$
18:1n-7	3.2 ± 0.1	2.7 ± 0.2 $^{\beta}$	4.2 ± 0.7 $^{\beta}$
18:1n-9	10.0 ± 0.5 ^{a}	13.5 ± 0.6 $^{b,\beta}$	13.9 ± 0.7 ^{b}
Total MUFA	**15.9 ± 0.7**	**19.7 ± 1.1 $^{\beta}$**	**21.7 ± 1.6**
18:3n-3	16.3 ± 0.9 $^{\gamma}$	16.7 ± 1.1 $^{\beta}$	15.8 ± 0.9 $^{\alpha}$
18:4n-3	3.1 ± 0.2	2.8 ± 0.1	2.9 ± 0.2
EPA 20:5n-3	0.0 ± 0.0	0.0 ± 0.0	**0.6 ± 0.6**
Total n-3 PUFA	**19.4 ± 1.1 $^{\gamma}$**	**19.5 ± 1.2 $^{\beta}$**	**19.3 ± 0.5 $^{\gamma}$**
18:2n-6	9.3 ± 0.6 $^{\beta}$	8.9 ± 0.2 $^{\alpha\beta}$	9.1 ± 0.4 $^{\alpha}$
18:3n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0 $^{\beta}$
Total n-6 PUFA	**9.3 ± 0.6 $^{\beta}$**	**8.9 ± 0.2 $^{\beta}$**	**9.1 ± 0.4 $^{\alpha}$**
Total PUFA	**28.7 ± 1.7 $^{\gamma}$**	**28.4 ± 1.0 $^{\beta}$**	**28.4 ± 0.9 $^{\alpha}$**
n-3/n-6 ratio	**2.10 ± 0.08 $^{\beta}$**	**2.20 ± 0.18**	**2.14 ± 0.05 $^{\beta}$**

Data are expressed as means ± standard error of mean (n = 3). For each brood separately, letters indicate significant impact of the maternal diet, with levels not connected to the same letter being significantly different from each other (ANOVA followed by a post-hoc Tukey test, $p < 0.025$ due to Bonferroni-Sidak correction). For each dietary treatment separately, Greek letters indicate significant impact of the brood number, with levels not connected to the same Greek letter being significantly different from each other (ANOVA followed by a post-hoc Tukey test, $p < 0.025$ due to Bonferroni-Sidak correction). Abbreviations used: CT, control; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 5.A.4 Phospholipid fatty acid composition of 3rd, 4th and 5th brood juveniles released by daphnids during a life-history experiment comparing three diets differing in their lipid composition (% of total fatty acids identified in phospholipids).

Fatty acid	3rd brood			4th brood		
	Algae	CT-liposome	EPA-liposome	Algae	CT-liposome	EPA-liposome
14:0	0.6 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
16:0	31.5 ± 0.4	31.4 ± 0.3	32.0 ± 0.8	31.3 ± 0.3	30.8 ± 0.1	32.7 ± 1.1
18:0	14.3 ± 0.4 $^{\alpha\beta}$	12.4 ± 0.5 $^{\alpha\beta}$	13.4 ± 0.7	13.2 ± 0.4 $^{\alpha}$	12.2 ± 0.1 $^{\alpha}$	13.5 ± 1.3
Total SFA	**46.4 ± 0.5**	**43.7 ± 0.8**	**45.3 ± 1.5**	**44.5 ± 0.7**	**43.0 ± 0.1**	**46.1 ± 2.3**
16:1n-7	1.4 ± 0.1	1.2 ± 0.0	1.5 ± 0.4 $^{\alpha}$	0.3 ± 0.3	0.2 ± 0.2	0.0 ± 0.0 $^{\beta}$
18:1n-7	4.3 ± 0.0	3.7 ± 0.1	4.4 ± 0.2	4.3 ± 0.0	3.9 ± 0.0	3.9 ± 0.4
18:1n-9	11.0 ± 0.2 ^{a}	16.0 ± 0.1 $^{b,\alpha}$	14.4 ± 0.3 ^{c}	11.6 ± 0.3 ^{a}	15.0 ± 0.2 $^{b,\alpha\beta}$	13.7 ± 0.8 ^{ab}
Total MUFA	**16.7 ± 0.2 ^{a}**	**20.9 ± 0.2 $^{b,\alpha}$**	**20.2 ± 0.9 ^{b}**	**16.2 ± 0.4**	**19.1 ± 0.0 $^{\alpha\beta}$**	**17.6 ± 1.1**
18:3n-3	18.0 ± 0.3 $^{a,\alpha}$	17.8 ± 0.5 $^{a,\alpha}$	14.0 ± 0.3 ^{b}	19.0 ± 0.3 $^{a,\alpha}$	18.8 ± 0.0 $^{a,\alpha}$	14.7 ± 0.7 ^{b}
18:4n-3	1.6 ± 0.1	1.7 ± 0.1 $^{\alpha\beta}$	1.4 ± 0.0	1.7 ± 0.0 ^{ab}	1.8 ± 0.0 $^{a,\alpha}$	1.6 ± 0.0 ^{b}
EPA 20:5n-3	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	**6.6 ± 0.1 $^{b,\alpha}$**	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	**7.3 ± 0.1 $^{b,\beta}$**
Total n-3 PUFA	**19.6 ± 0.4 $^{a,\alpha}$**	**19.5 ± 0.6 $^{a,\alpha}$**	**22.0 ± 0.4 ^{b}**	**20.7 ± 0.3 $^{a,\alpha}$**	**20.6 ± 0.0 $^{a,\alpha}$**	**23.7 ± 0.5 ^{b}**
18:2n-6	17.2 ± 0.3 ^{a}	15.9 ± 0.0 ^{b}	12.4 ± 0.2 ^{c}	18.5 ± 0.3 ^{a}	17.3 ± 0.0 ^{a}	12.6 ± 0.7 ^{b}
18:3n-6	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
Total n-6 PUFA	**17.3 ± 0.1 ^{a}**	**15.9 ± 0.0 $^{b,\alpha}$**	**12.4 ± 0.2 ^{c}**	**18.7 ± 0.1 ^{a}**	**17.3 ± 0 $^{a,\beta}$**	**12.6 ± 0.7 ^{b}**
Total PUFA	**36.9 ± 0.5 $^{\alpha\beta}$**	**35.3 ± 0.6 $^{\alpha\beta}$**	**34.5 ± 0.6**	**39.3 ± 0.4 $^{\alpha}$**	**37.9 ± 0.1 $^{\alpha}$**	**36.3 ± 1.2**
n-3/n-6 ratio	**1.13 ± 0.01 $^{a,\alpha}$**	**1.23 ± 0.03 $^{a,\alpha}$**	**1.77 ± 0.02 $^{b,\alpha}$**	**1.11 ± 0.01 $^{a,\alpha}$**	**1.19 ± 0.00 $^{a,\alpha}$**	**1.88 ± 0.06 $^{b,\alpha}$**

Data are expressed as means ± standard error of mean (n = 3). For each brood separately, letters indicate significant impact of the maternal diet, with levels not connected to the same letter being significantly different from each other (ANOVA followed by a post-hoc Tukey test, $p < 0.025$ due to Bonferroni-Sidak correction). For each dietary treatment separately, Greek letters indicate significant impact of the brood number, with levels not connected to the same Greek letter being significantly different from each other (ANOVA followed by a post-hoc Tukey test, $p < 0.025$ due to Bonferroni-Sidak correction). Abbreviations used: CT, control; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

(continued)

Fatty acid	5th brood		
	Algae	CT-liposome	EPA-liposome
14:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
16:0	32.5 ± 0.7	32.5 ± 0.8	30.0 ± 0.9
18:0	15.9 ± 0.5 $^{\beta}$	15.8 ± 1.1 $^{\beta}$	12.4 ± 1.3
Total SFA	**48.4 ± 1.1**	**48.4 ± 1.9**	**42.4 ± 2.2**
16:1n-7	0.3 ± 0.3	0.3 ± 0.3	0.5 ± 0.5 $^{\alpha\beta}$
18:1n-7	4.3 ± 0.1 ^{a}	3.8 ± 0.1 ^{b}	5.0 ± 0.1 ^{c}
18:1n-9	11.8 ± 0.2 ^{a}	14.1 ± 0.5 $^{b,\beta}$	15.4 ± 0.4 ^{b}
Total MUFA	**16.3 ± 0.4** ^{a}	**18.2 ± 0.9** $^{ab,\beta}$	**20.9 ± 1.1** ^{b}
18:3n-3	15.3 ± 0.4 $^{\beta}$	15.2 ± 0.5 $^{\beta}$	13.6 ± 0.3
18:4n-3	1.6 ± 0.0	1.4 ± 0.1 $^{\beta}$	1.5 ± 0.1
EPA 20:5n-3	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	**6.4 ± 0.1** $^{b,\alpha}$
Total n-3 PUFA	**16.8 ± 0.4** $^{a,\beta}$	**16.7 ± 0.6** $^{a,\beta}$	**21.5 ± 0.5** ^{b}
18:2n-6	18.4 ± 0.4 ^{a}	16.6 ± 0.5 ^{ab}	14.9 ± 0.4 ^{b}
18:3n-6	0.0 ± 0.0	0.2 ± 0.2	0.3 ± 0.3
Total n-6 PUFA	**18.4 ± 0.4** ^{a}	**16.8 ± 0.4** $^{ab,\alpha\beta}$	**15.2 ± 0.7** ^{b}
Total PUFA	**35.2 ± 0.8** $^{\beta}$	**33.5 ± 1.0** $^{\beta}$	**36.7 ± 1.2**
n-3/n-6 ratio	**0.91 ± 0.00** $^{a,\beta}$	**0.99 ± 0.01** $^{b,\beta}$	**1.42 ± 0.03** $^{c,\beta}$

Data are expressed as means ± standard error of mean (n = 3). For each brood separately, letters indicate significant impact of the maternal diet, with levels not connected to the same letter being significantly different from each other (ANOVA followed by a post-hoc Tukey test, $p < 0.025$ due to Bonferroni-Sidak correction). For each dietary treatment separately, Greek letters indicate significant impact of the brood number, with levels not connected to the same Greek letter being significantly different from each other (ANOVA followed by a post-hoc Tukey test, $p < 0.025$ due to Bonferroni-Sidak correction). Abbreviations used: CT, control; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 5.A.5 Parameters of the LogLogistic models fitted to the cytotoxicity data.

Individual	Diet	EC50 (µg/L)	Relative slope around EC50 (b) (no unit)
21-day old adults	Algae	302.5	5.0
	CT-liposome	344.2	4.7
	EPA-liposome	365.4	10.0
3rd brood juveniles	Algae	452.6	6.6
	CT-liposome	497.9	8.5
	EPA-liposome	455.7	7.7
4th brood juveniles	Algae	509.8	5.1
	CT-liposome	465.7	6.1
	EPA-liposome	489.1	4.4
5th brood juveniles	Algae	354.3	4.2
	CT-liposome	356.3	6.9
	EPA-liposome	370.0	6.1
Prediction function:		$f(x) = \frac{100}{1 + exp(b \times ln\left(\frac{x}{EC50}\right))}$	

Abbreviations used: CT, control; EC50, median effect concentration; EPA, eicosapentaenoic acid.

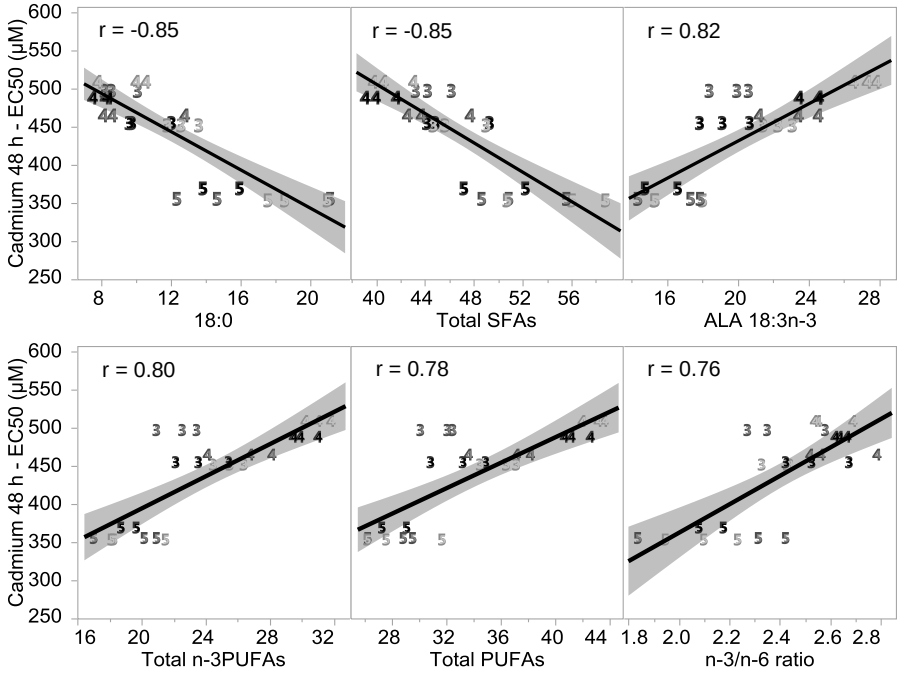

Fig. 5.A.1. Link between Cd sensitivity and body neutral lipid composition of 3rd, 4th and 5th brood juveniles released by daphnids during a life-history experiment comparing three diets differing in their lipid composition. Plain curves represent the linear regressions of the calculated Cd 48 h-EC50s as a function of the relative neutral lipids abundance of (a) 18:0, (b) total SFAs, (c) ALA, (d) total n-3 PUFAs, (e) total PUFAs or (f) n-3/n-6 ratio in juvenile daphnids across broods and dietary treatments. Shaded areas represent the confidence interval at 95% for the regressions. Numbers (3, 4, 5) refer to the brood numbers. Colours refer to the maternal diet: an algae mix in light grey, an algae mix supplemented with control liposomes (CT-liposome) in dark grey, an algae mix supplemented with liposomes containing EPA (EPA-liposome) in black). Abbreviations used: ALA, alpha-linolenic acid; Cd, cadmium; CT, control; EC50, median effect concentration, EPA; eicosapentaenoic acid, PUFA, polyunsaturated fatty acid: r, Pearson's correlation; SFA saturated fatty acid.

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Chapter 6

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

Foreword

In the previous chapter, we showed that, contrary to our expactations, dietary eicosapentaenoic acid (EPA, 20:5n-3) neither protected adult daphnids nor the juveniles they released against acute cadmium (Cd) toxicity while it was the case *in vitro* using the RTL-W1 cell line as model (chapters 3-4). Still, offspring's fatty acid profile and tolerance to Cd was influenced by the brood number and the maternal diet. Multivariate analyses pointed out a potential protection by alpha-linolenic acid (ALA, 18:3n-3) against Cd toxicity in juveniles, which corroborates our *in vitro* results (chapters 3-4). These divergences highlight the model-dependance of results and suggest taking caution while extrapolating conclusions from one model to another. In the present chapter, we thus studied the interactions between dietary lipids and Cd in a second *in vivo* model, i.e. rainbow trout juveniles, with a focus on the liver. The present results were published in Aquatic Toxicology.

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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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.

Key words (4): Polyunsaturated fatty acids, cadmium, rainbow trout liver, gene expression

6.1. 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 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. 6.1 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).

6.2. Material and methods

6.2.1. 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. 6.A.1). 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. 6.A.1). 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. 6.A.1). In addition, the hepatic fatty acid profile was assessed at the beginning of the experiment (day 0).

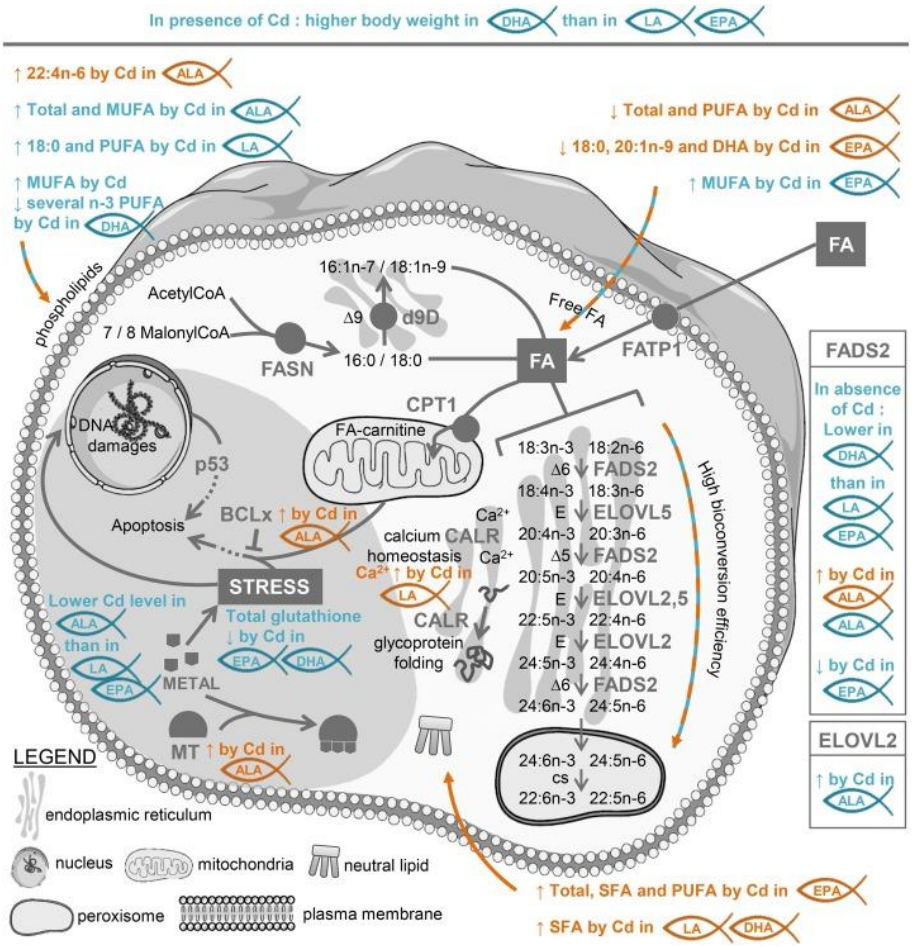

Fig. 6.1 Roles of the proteins encoded by the genes investigated and main interactive results between Cd and dietary lipids observed in the liver of rainbow trout juveniles after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 or 0.3 µg/L) (day 29, 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; FA, fatty acid; FADS2, fatty acid desaturase 2; FASN, fatty acid synthase; FATP1, fatty acid transport protein 1; LA, linoleic 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.

6.2.2. 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 6.1 and 6.2, 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).

Table 6.1 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).

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 (E161-g) 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 Vit E levels in the premix compensated the different Vit 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).

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

Table 6.2 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).

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.

6.2.3. 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 6.2.7.).

6.2.4. 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).

6.2.5. 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.

6.2.6. 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.

6.2.7. 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 315.887 nm, 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.

6.2.8. 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 6.3) were obtained from the litterature. 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 6.3) for 40 cycles.

A melt curve analysis always followed the 40 cycles. The targeted genes (Fig. 6.1) 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., 2018a,b), 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).

Table 6.3 Sequence, amplicon size, annealing temperature and optimal concentration in master mix for the primers of the genes investigated.

Gene	Sequence 5'→3'	Size (bp)	Annealing T (°C)	Concentration (nmol/L)	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*

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

(continued)

Gene	Sequence 5'→3'	Size (bp)	Annealing T (°C)	Concentration (nmol L^{-1})	Reference
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 literature (see *"Reference"* column). Abbreviations used: bp, base pairs ; F, forward; R, reverse; T, temperature.

6.2.9. 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.

6.3. Results

6.3.1. Growth and HSI

At day 29, fish weights were similar (Table 6.4). 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 6.4). 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 6.4). Cd had no significant impact on fish weight (Table 6.4, day 29 and day 70). Neither the diet nor the exposure to Cd had any significant impact on the HSI (Table 6.4, day 29 and day 70).

Table 6.4 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).

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.

6.3.2. Hepatic fatty acid composition

6.3.2.1. 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).

6.3.2.2. 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. 6.2-6.6). We thus chose to develop here under the results for fish unexposed to Cd only.

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. 6.2F) or on their content in SFA (Fig. 6.2A) and PUFA (Fig. 6.2E) 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. 6.2B, 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. 6.2B, day 70). The

MUFA content of neutral lipids and free fatty acids was not affected by the diet throughout the experiment (Fig. 6.2B, day 29 and day 70).

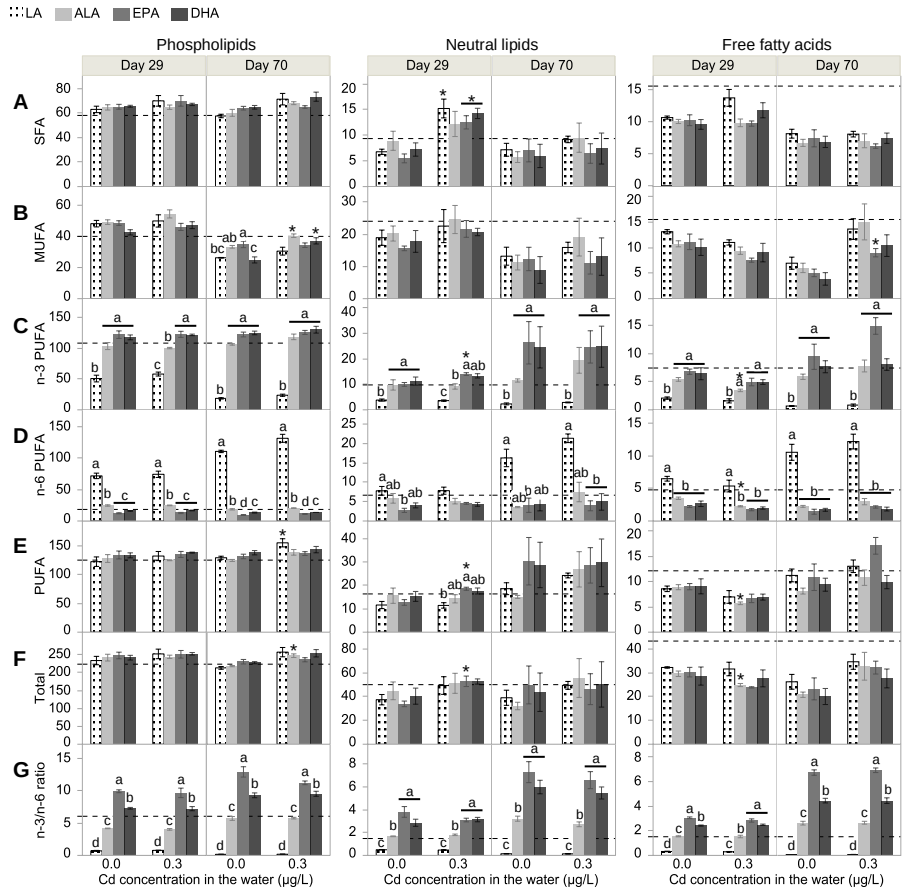

Fig. 6.2. Levels of lipid classes, i.e. SFA (A), MUFA (B), n-3 PUFA (C), n-6 PUFA (D), PUFA (E), Total fatty acids (F) (µmol/g freeze dried liver) and n-3/n-6 ratio (G) in rainbow trout juvenile hepatic phospholipids, neutral lipids and free fatty acids after 4 weeks of feeding diets enriched in specific 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.

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. 6.2D), lower in n-3 PUFAs (x0.1-0.5, Fig. 6.2C) and exhibited a lower n-3/n-6 ratio (x0.01-0.30, Fig. 6.2G) 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. 6.2D). 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. 6.3B-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. 6.4I). 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 decreased by 49%, 52% and 63% between the start of the experiment and day 29, and decreased again by 65%, 47% and 76% between day 29 and day 70 (Fig. 6.4I). 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.

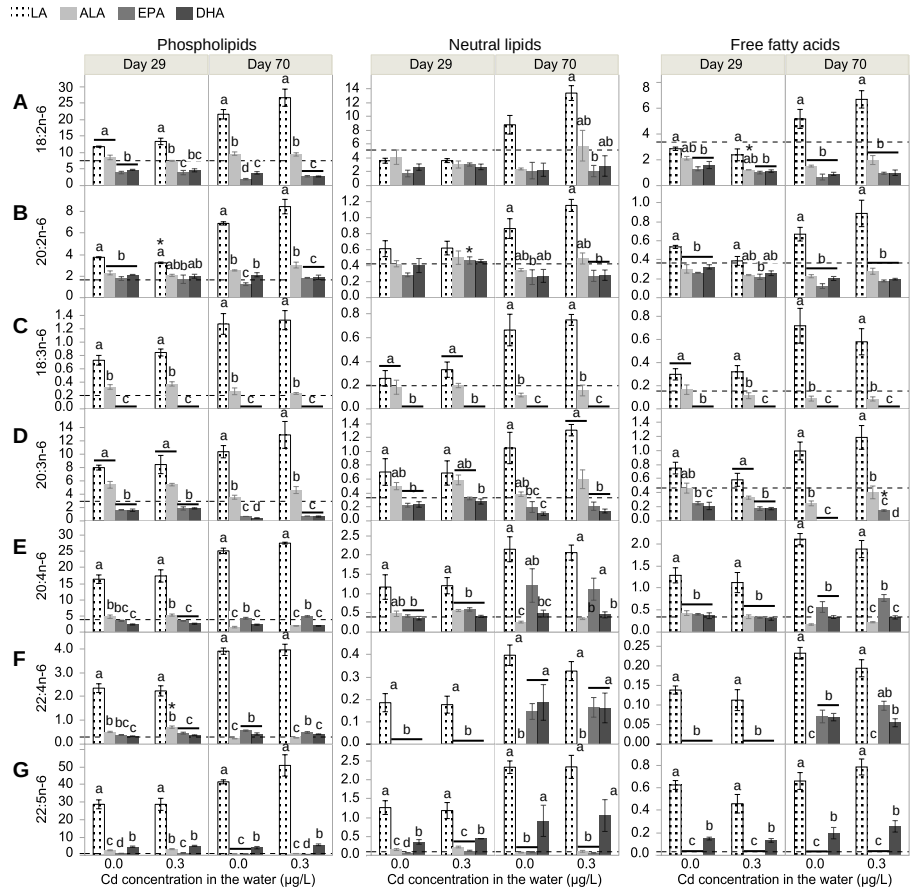

Fig. 6.3. Levels of individual n-6 PUFAs, i.e. 18:2n-6 (A), 20:2n-6 (B), 18:3n-6 (C), 20:3n-6 (D), 20:4n-6 (E), 22:4n-6 (F), 22:5n-6 (G), (µmol/g freeze dried liver) in rainbow trout juvenile hepatic phospholipids, neutral lipids and free fatty acids after 4 weeks of feeding diets enriched in specific 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. 6.2. Abbreviations used: ALA, alpha-linolenic acid; Cd, cadmium; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acid.

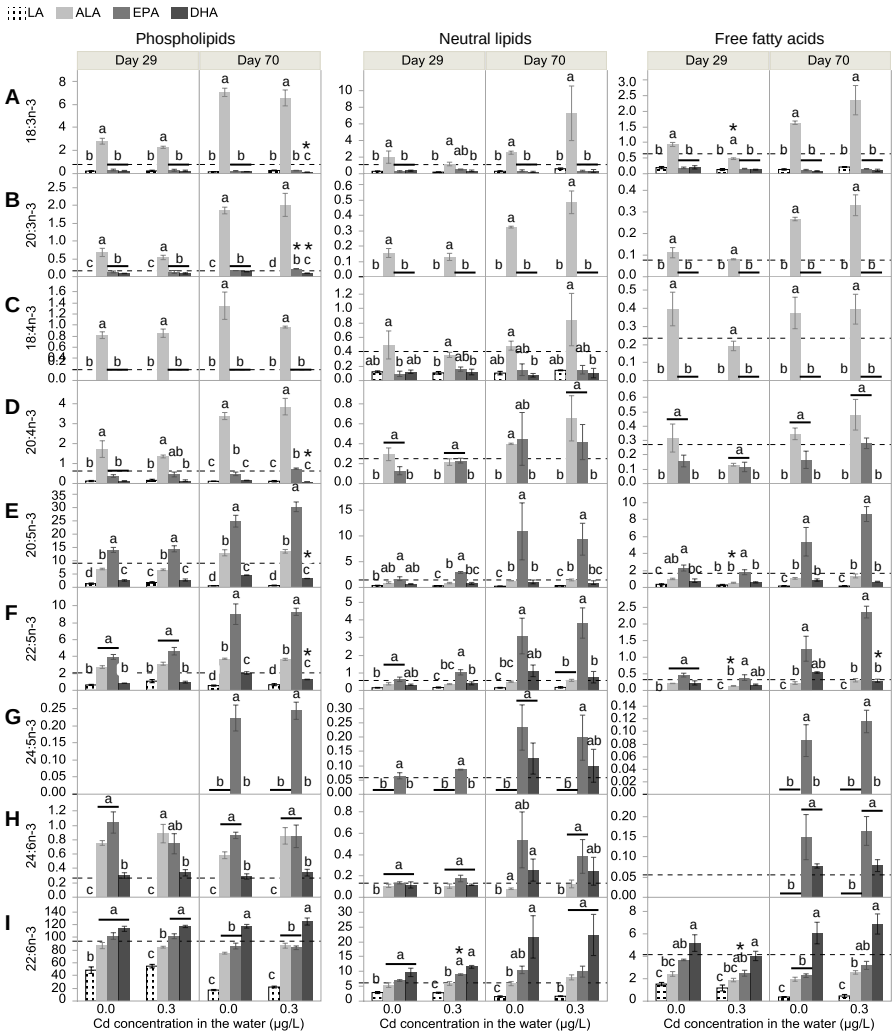

Fig. 6.4. Levels of individual n-3 PUFAs, i.e. 18:3n-3 (A), 20:3n-3 (B), 18:4n-3 (C), 20:4n-3 (D), 20:5n-3 (E), 22:5n-3 (F), 24:5n-3 (G), 24:6n-3 (H), 22:6n-3 (I), (µmol/g freeze dried liver) in rainbow trout juvenile hepatic phospholipids, neutral lipids and free fatty acids after 4 weeks of feeding 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. 6.2. Abbreviations used: ALA, alpha-linolenic acid; Cd, cadmium; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acid.

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. 6.2D, day 29 and day 70) and the n-3/n-6 ratio in every lipid classes (Fig. 6.2G, 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. 6.2C, 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. 6.2D, 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. 6.2D). 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. 6.2G, 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. 6.2G, day 29 and day 70.

The hepatic lipids of fish fed the ALA-enriched diet were richer in ALA (x4.6-39.3, Fig. 6.4A) and its direct elongation and desaturation products (20:3n-3 and 18:4n-3, Fig. 6.4B-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. 6.4C). 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. 6.4C). 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. 6.4D, 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. 6.4E, 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. 6.4E). 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. 6.4F). 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. 6.4G). 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. 6.4H, day 29 and day 70). The neutral lipid level of 24:6n-3 was similar among fish fed the n-3 enriched diets (Fig. 6.4H, 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. 6.4H). At day 29, the hepatic DHA level in phospholipids and neutral lipids did not differ between fish fed the n-3-enriched diets (Fig. 6.4I). 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. 6.4I, 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. 6.4I). 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. 6.4I).

6.3.2.3. Cd impacts on hepatic lipids

Phospholipids

The level of 20:2n-6 decreased by 14% (Fig. 6.3B, day 29) and the level of 22:4n-6 increased by 39% (Fig. 6.3F, day 29) in hepatic phospholipids of fish exposed to Cd for 24-h that were 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. 6.2F, day 70). This increase mainly reflected a rise in the levels of MUFAs (Fig. 6.2B, day 70), among which predominantly 18:1n-9 (Fig. 5C, day 70) and 20:1n-9 were increased (Fig. 6.5D, day 70). Besides, Cd significantly increased by 49% the total MUFA level recovered in hepatic phospholipids of fish fed the DHA-enriched diet (Fig. 6.2B, day 70), mainly 18:1n-7, 18:1n-9 and 20:1n-9 (Fig. 6.5B-D, day 70), without affecting significantly the total phospholipid level (Fig. 6.2F, day 70). In addition, Cd significantly decreased the hepatic phospholipid levels of ALA (-31%, Fig. 6.4A, day 70), 20:3n-3 (-33%, Fig. 6.4B, day 70), 20:4n-3 (-38%, Fig. 6.4D, day 70), EPA (-28%, Fig. 6.4E, day 70), 22:5n-3 (-37%, Fig. 6.4F, 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 6.4B, day 70). Finally, Cd significantly increased the total PUFA (+20%, Fig. 6.2E, day 70) and the 18:0 (+24%, Fig. 6.6D, day 70) levels in phospholipids of fish fed the LA-enriched diet.

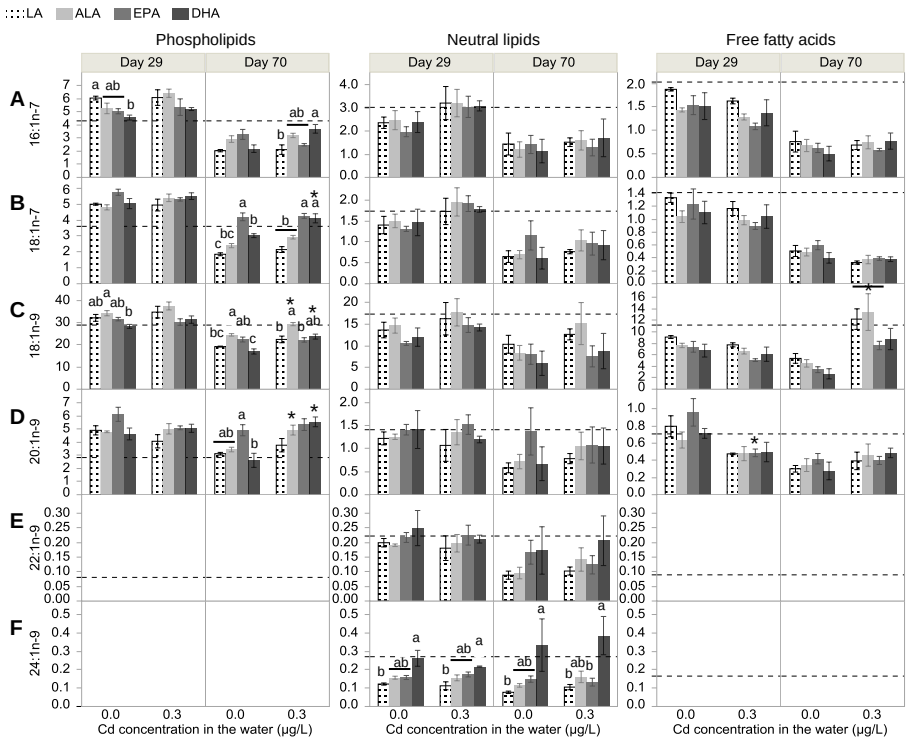

Fig. 6.5. Levels of individual MUFAs, i.e. 16:1n-7 (A), 18:1n-7 (B), 18:1n-9 (C), 20:1n-9 (D), 22:1n-9 (E), 24:1n-9 (F), (µmol/g freeze dried liver) in rainbow trout juvenile hepatic phospholipids, neutral lipids and free fatty acids after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, 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. 6.2. 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.

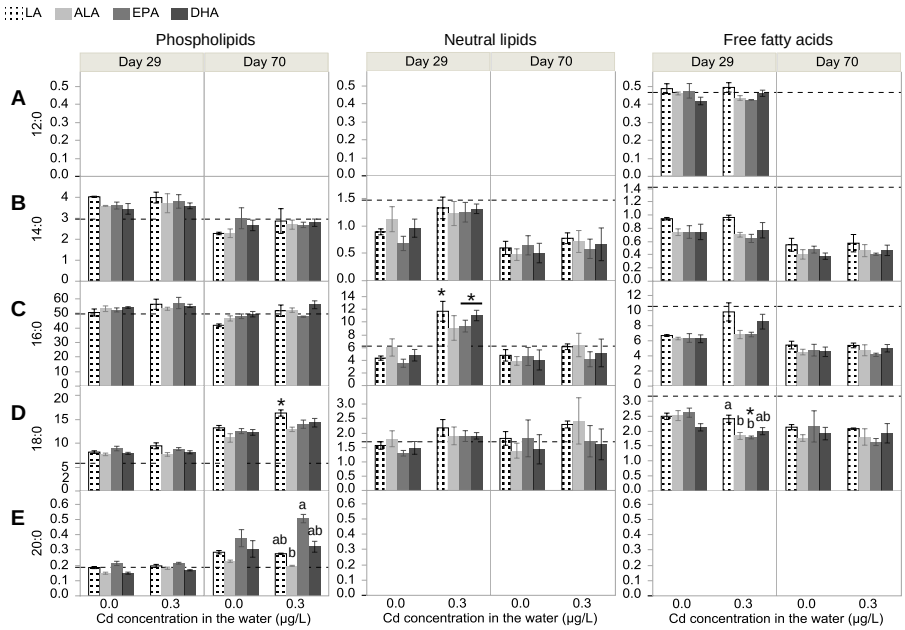

Fig. 6.6. Levels of individual SFAs, i.e. 12:0 (A), 14:0 (B), 16:0 (C), 18:0 (D), 20:0 (E), (µmol/g freeze dried liver) in rainbow trout juvenile hepatic phospholipids, neutral lipids and free fatty acids after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (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. 6.2. 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.

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. 6.2A, day 29). This increase was mainly due to enrichment in 16:0 (Fig. 6.6C, 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. 6.2E, day 29). The increase in PUFAs was essentially due to a rise of n-3 PUFAs (+41%, Fig. 6.2C, day 29), among which mainly DHA (+29%, Fig. 6.4I, day 29), but also, to a lesser extent, to an increase in 20:2n-6 (+63%, Fig. 6.3B, 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. 6.2F, day 29).

These effects were not maintained upon long-term Cd exposure (Fig. 6.2-6.4 and 6.6, day 70).

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. 6.2F, day 29). This decrease mainly reflected a decline in both the n-3 (-36%, Fig. 6.2C, day 29) and n-6 (-35%, Fig. 6.2D, day 29) PUFA levels, including ALA (-49%, Fig. 6.4A, day 29), EPA (-45%, Fig. 6.4E, day 29), 22:5n-3 (-36%, Fig. 6.4F, day 29) and LA (-42%, Fig. 6.3A, day 29). In fish fed the EPA-enriched diet, the 24-h Cd exposure induced a decline in 18:0 (-32%, Fig. 6.6D, day 29), 20:1n-9 (-49%, Fig. 6.5D, day 29) and DHA (-32%, Fig. 6.4I, day 29) in the free fatty acid fraction. These effects were not maintained upon long-term Cd exposure (Fig. 6.2-6.6, 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. 6.2B, day 70). This increase mainly reflected a rise in 18:1n-9 (x2.2, Fig. 6.5C, 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. 6.5C, day 70).

6.3.3. Hepatic total glutathione level

The diet had no impact on the total hepatic glutathione level (Fig. 6.7A, 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. 6.7A, day 29). Cd had no impact on the total hepatic glutathione level in fish fed the LA- and ALA-enriched diets (Fig. 6.7A, day 29 and day 70).

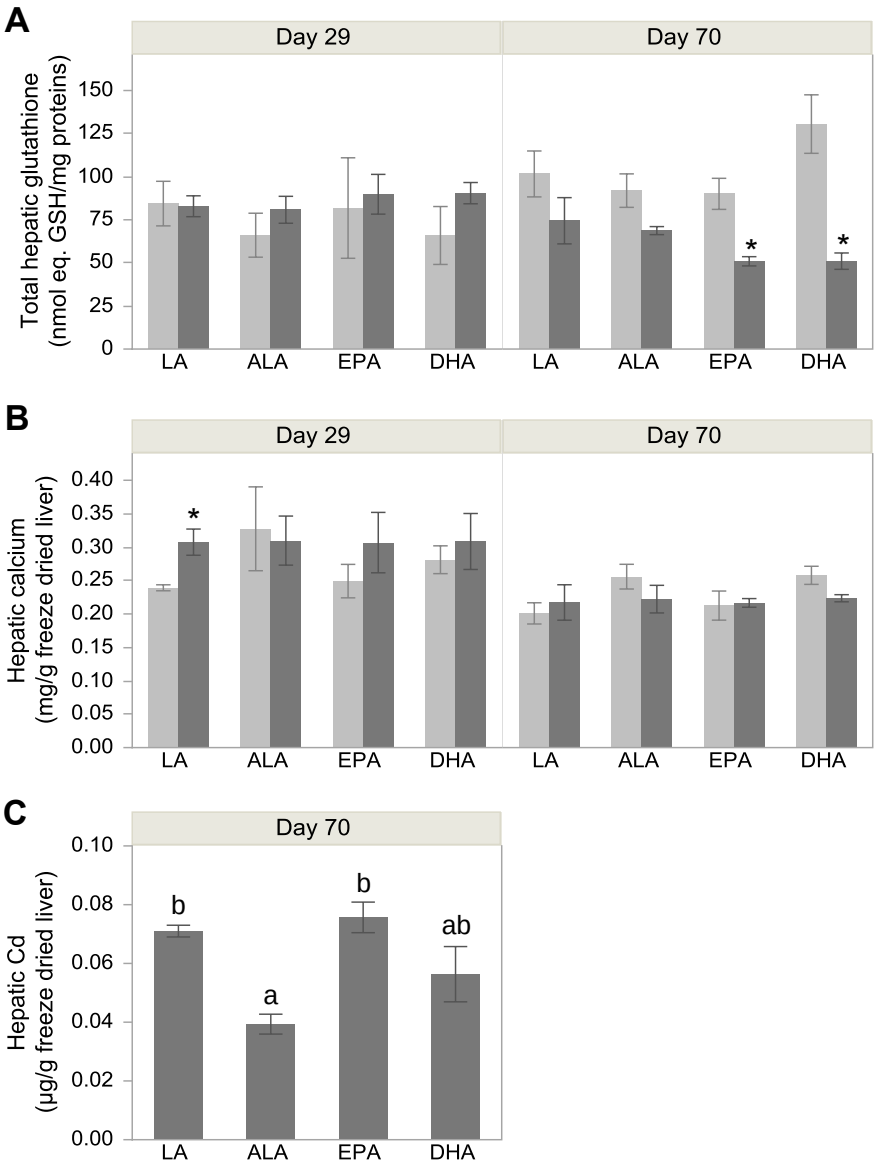

Fig. 6.7. Total hepatic glutathione level (A), hepatic calcium level (B), hepatic Cd level (C) in juvenile rainbow trout after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 µg/L in light grey or 0.3 µg/L in dark grey) (day 29) and after 10 weeks 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.

6.3.4. Hepatic calcium level

The diet had no impact on the hepatic calcium level (Fig. 6.7B, 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. 6.7B, day 29). This effect disappeared upon long-term Cd exposure (Fig. 6.7B, day 70).

6.3.5. 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. 6.7C, 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. 6.7C, day 70).

6.3.6. Hepatic gene expression pattern

6.3.6.1. 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. 6.8 and Supplementary data Fig. 6.A.2). 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. 6.8, day 70). These differences were no longer observed upon long-term Cd exposure (Fig. 6.8, 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. 6.8, 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. 6.8, day 70). Due to a higher variability of the data, this difference was significant upon short-term Cd exposure (supplementary data Fig. 6.A.2, day 29).

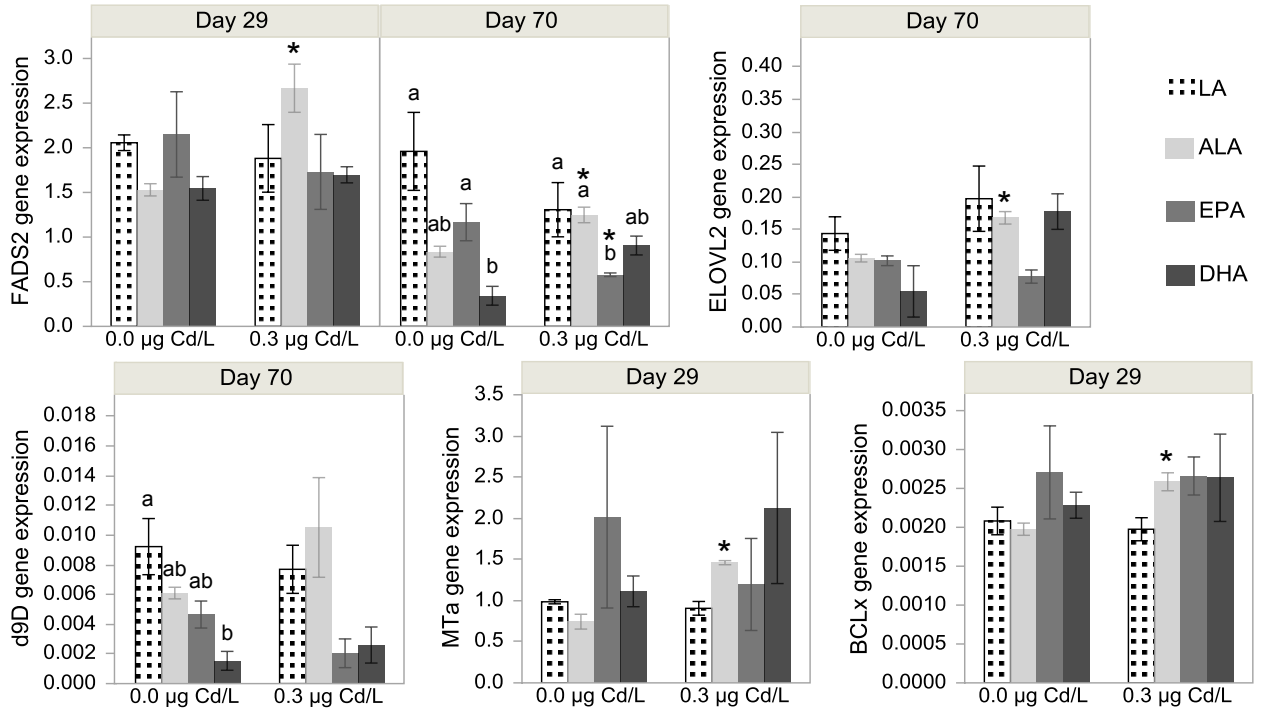

Fig. 6.8. Relative expression of genes involved in fatty acid metabolism or in stress response in juvenile rainbow trout liver after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 or 0.3 µg/L) (day 29) and/or after 10 weeks of feeding and 6 weeks 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.

Cd had no impact on the hepatic *FATP1*, *CPT1*, *FASN*, *d9D* and *ELOVL5* gene expressions at both 29 and 70 days (Fig. 6.8 and supplementary data Fig. 6.A.2). The 24-h exposure to Cd significantly upregulated hepatic *FADS2* gene expression (x1.7) in fish fed the ALA-enriched diet only (Fig. 6.8, day 29). This upregulation was maintained upon long-term Cd exposure (x1.5) (Fig. 6.8, 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. 6.8, 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. 6.8 and supplementary data Fig. 6.A.2).

6.3.6.2. Stress response

The diet had no impact on hepatic *MTa*, *MTb*, *CALR*, *BCLx* and *p53* gene expressions (Fig. 6.8 and Supplementary data Fig. 6.A.3, day 29). The 24-h Cd exposure significantly upregulated hepatic *MTa* (x2.0) and *BCLx* (x1.3) gene expressions in fish fed the ALA-enriched diet only (Fig. 6.8, day 29). Cd had no impact on hepatic *MTb*, *CALR* and *p53* gene expressions (supplementary data Fig. 6.A.3, day 29).

6.3.6.3. CYP20A1

Neither the diet nor the Cd exposure impacted *CYP20A1* gene expression (supplementary data Fig. 6.A.3, day 29 and day 70).

6.4. Discussion

The present study aimed at investigating to what extent (i) dietary lipids and Cd influence the fatty acid homeostasis and metabolism and (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. 6.1.

6.4.1. 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-PUFA 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.

6.4.2. Cd impact on fatty acid homeostasis and metabolism

6.4.2.1. 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. However, further investigations are required to test this hypothesis.

6.4.2.2. 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 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, respectively (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 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.

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).

6.4.3. Dietary lipid impact on biological responses to Cd

Body weight was not influenced by Cd exposure in the present experiment. In accordance with 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 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 RTL-W1 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.

6.5. 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, 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. In future studies focus should be placed on genomic, proteomic and metabolomic analyses to provide a deeper understanding of the specific mechanisms of individual PUFA enrichments towards environmentally realistic Cd stress.

6.6. Acknowledgements

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6.7. Supplementary data

Total hepatic glutathione level determination – Detailed procedure

Aliquots stored for total glutathione analyses were first thawed on ice and centrifuged (5000 g, 5 min, 4°C). Samples were kept on ice throughout the manipulations and analysed in triplicates. Supernatant (50 µL) was spiked with internal standard (glutathione ethyl ester, GEE) then diluted to 250 µL in SSA/HCl, a solution containing 1.3% sulphosalicylic acid (SSA) and 8 mM HCl. Then, 100 µL of this suspension was neutralised by the addition of $NaHCO_3$ (in excess). After centrifugation (5000 g, 5 min, 4°C), the isolated supernatant (100 µL) was reduced by the addition of 100 µL of DL-dithiothreitol (DTT, 25 mM) and 50 µL of TRIS buffer (100 mM, pH 8.5). After a 30 min-incubation at 4°C, samples were acidified by the addition of metaphosphoric acid (MPA, 6%, 800 µL). Derivatization with ortho-phtalaldehyde (OPA) was performed by mixing 100 µL of the resulting suspension with 100 µL of OPA reagent and a subsequent 5 min-incubation at room temperature. The OPA reagent was prepared fresh daily from 50 mg of OPA dissolved in 500 µL of methanol then diluted to

10 mL in sodium tetraborate buffer (100 mM, pH 9.0). Derivatized samples were finally neutralized by addition of 800 µL of sodium phosphate buffer (500 mM, pH 7.0) before high performance liquid chromatography (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 the mobile phase at a constant rate of 0.7 mL/min. For the first 8 min of run, the mobile phase consisted of 100% sodium acetate (50 mM, pH 6.0)/acetonitrile (92/8, v/v). Then, a linear gradient was applied to obtain in 5 min a mobile phase consisting of 70% sodium acetate (50 mM, pH 6.0)/acetonitrile (92/8, v/v) and 30% acetonitrile, which was kept during 8 min. Then, a second linear gradient was applied to return in 3 min to the initial mobile phase. The system was finally conditioned during 7 min before the next injection. Samples were kept at 4°C until injection (20 µL). After 6 min and 20 min of run, respectively, the fluorescence emitted by GSH-OPA and GEE-OPA adducts was detected at 420 nm following excitation at 340 nm. Each run lasted 31 min. 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.

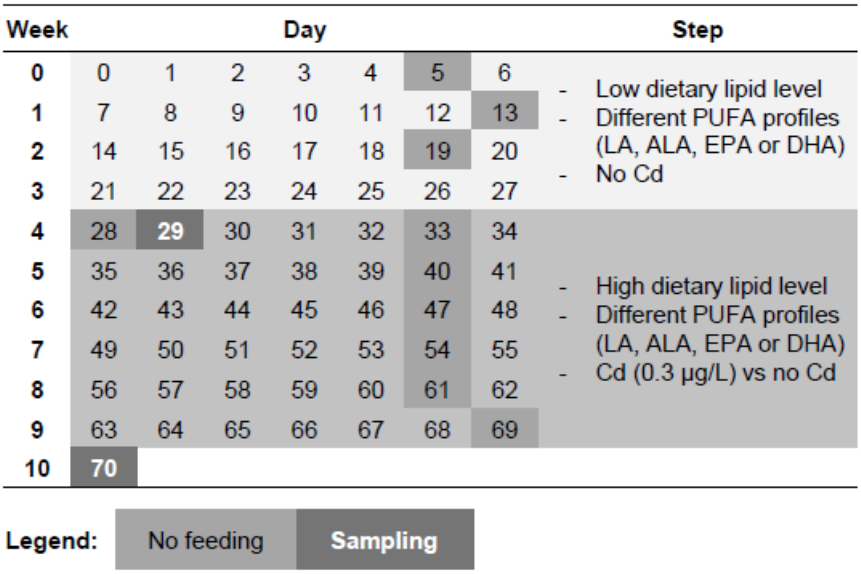


Fig. 6.A.1. Experimental design. Abbreviations used: ALA, alpha-linolenic acid; Cd, cadmium, DHA, docosahexaenoic acid, EPA, eicosapentaenoic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acid.

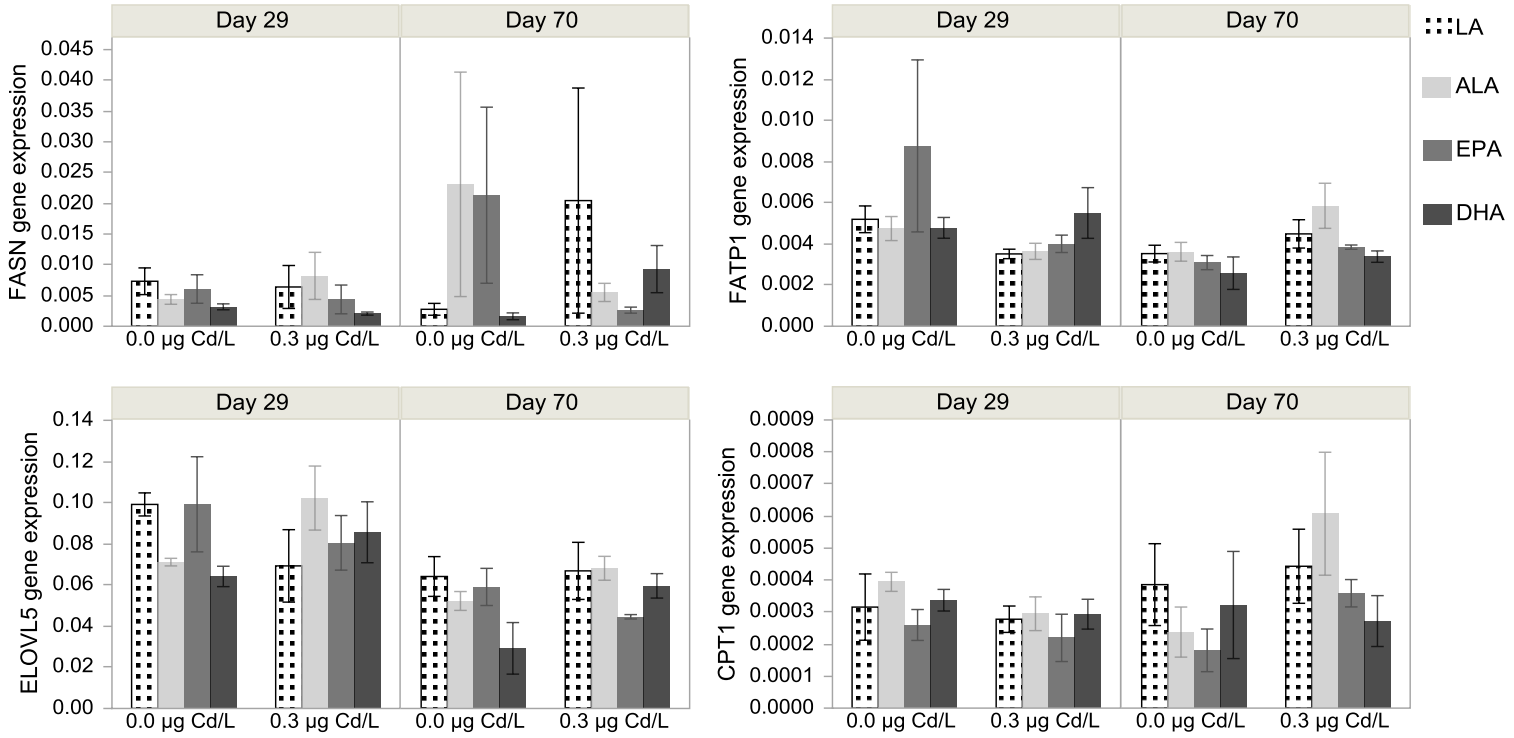
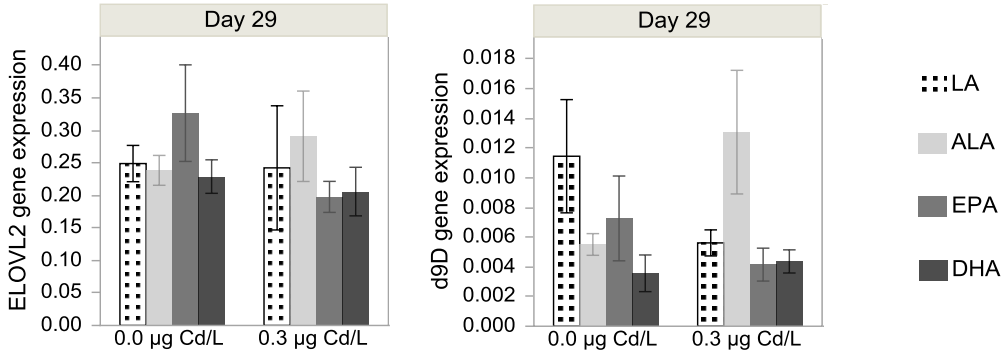


Fig. 6.A.2. Relative expression of genes involved in fatty acid metabolism in juvenile rainbow trout liver after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 or 0.3 µg/L) (day 29) and/or after 10 weeks of feeding and 6 weeks of Cd exposure (day 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.

(continued)



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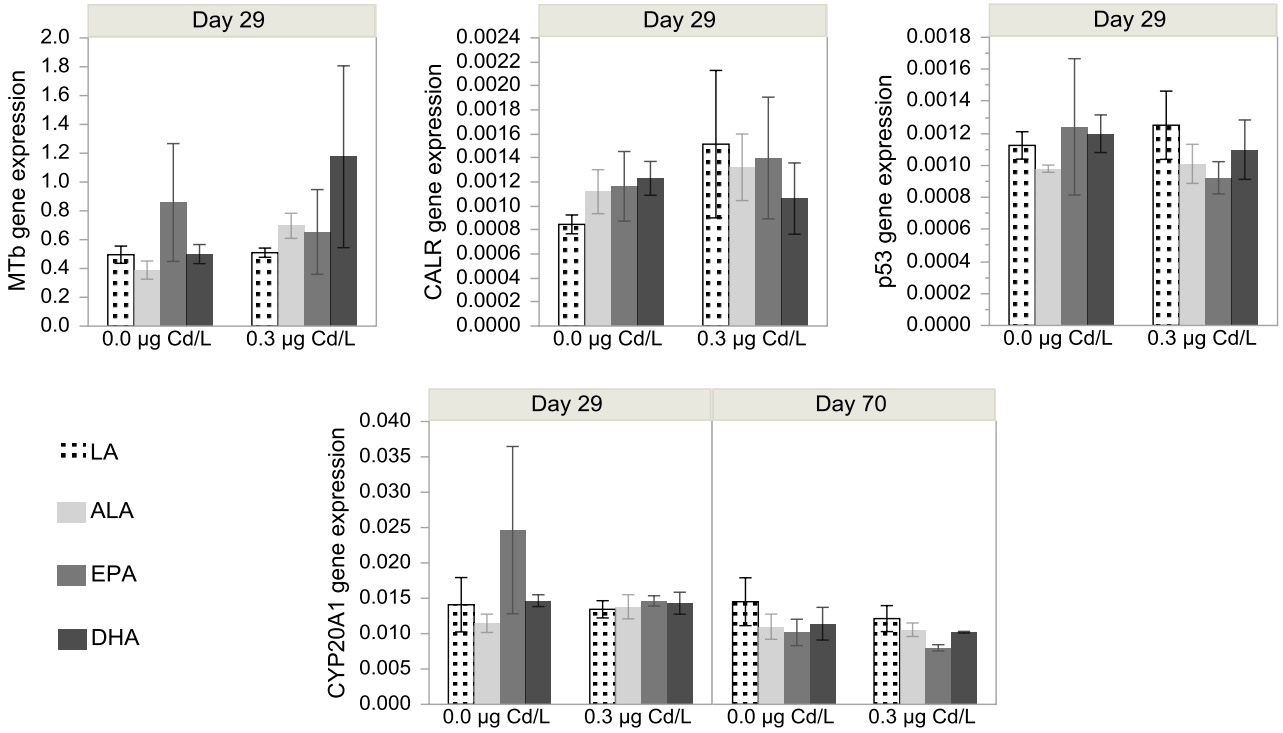

Fig. 6.A.3. Relative expression of genes involved in stress response and of CYP20A1 gene in juvenile rainbow trout liver after 4 weeks of feeding diets enriched in specific PUFAs (LA, ALA, EPA or DHA) and 24 h of Cd exposure (0.0 or 0.3 µg/L) (day 29) and/or after 10 weeks of feeding and 6 weeks of Cd 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.

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Chapter 7

General discussion and perspectives

The aim of this work was to characterize the interactions between the nature of dietary lipids and the biological responses to chemical stressors, especially cadmium (Cd), in aquatic biota. More particularly we focussed our study on investigating if and how (i) dietary lipids and Cd affect fatty acid homeostasis and metabolism, (ii) the individual/tissue/cell fatty acid profile modulates the sensitivity to Cd, in aquatic organisms. To address these questions, we used an *in vitro* approach with a rainbow trout liver cell line (RTL-W1, Lee et al. (1993)) for screening and mechanistic investigations and two *in vivo* models, belonging to two different trophic levels, i.e. the large water flea (*Daphnia magna*, a planktonic crustacean) and the rainbow trout (*Oncorhynchus mykiss*, a fish).

The major outcomes derived from this research project, as well as some perspectives, are given in the next sections.

7.1. Dietary lipids modulate fatty acid homeostasis and metabolism

OUTCOME 1 – The cellular, tissular and individual fatty acid profile of the selected aquatic exoperimental models was successfully modulated.

Whatever the experimental model used (*in vitro* with the RTL-W1 cell line or *in vivo* with juvenile rainbow trout or the large water flea), our experimental strategy included, as first step, growth in culture media or with diets specifically enriched in one targeted polyunsaturated fatty acid (PUFA) to generate cell batches or individuals with different fatty acid profiles, that were, in a second step,

challenged (or not) with Cd. Targeted PUFAs were alpha-linolenic acid (ALA, 18:3n-3) (Chapters 3, 4, 6), eicosapentaenoic acid (EPA, 20:5n-3) (Chapters 3-6) and docosahexaenoic acid (DHA, 22:6n-3) (Chapters 3, 6) and their n–6 counterparts linoleic acid (LA, 18:2n-6) (Chapters 3, 4, 6), arachidonic acid (AA, 20:4n-6) (Chapters 3-4) and docosapentaenoic acid (DPA, 22:5n-6) (Chapter 3) (see Fig. 7.1 for the biotransformation pathways). Techniques used for fatty acid profile modification were (i) RTL-W1 cell culture during one week in media spiked (or not) with a targeted PUFA complexed with fatty acid free-bovine serum albumin (FAF-BSA) (Chapters 3-4), (ii) feeding 3rd brood daphnid neonates (< 24 h old) a common algae based diet devoid of long chain (LC)-PUFAs, supplemented (or not) with liposomes loaded (or not) with a LC-PUFA until their 5th brood progeny was released (Chapter 5) and (iii) feeding juvenile rainbow trout during 10 weeks with isolipidic diets specifically enriched in one targeted PUFA, through inclusion of a specific oil in the aquafeed (Chapter 6). Fatty acid profile modification was efficiently archived using these techniques. In each experimental model, the supplemented PUFAs were absorbed and incorporated predominantly in phospholipids, emphasizing the preponderant role of PUFAs as structural component of biological membranes (Chapters 3-6) (Tocher et al., 2003). Our *in vitro* results (Chapters 3-4) suggest that cellular fatty acid profile modification occurred either through the replacement of some fatty acids (mainly 18:1n-9) in pre-existing lipids or thanks to the lipid turnover process rather than through supplementary lipid accumulation, since the total cellular lipid concentration was not affected by the PUFA supplementation. Our *in vivo* results with trout fed isolipidic diets specifically enriched in one targeted PUFA corroborate this suggested mechanism of fatty acid profile modification (Chapter 6). In Chapter 5, daphnid not only integrated the supplemented LC-PUFA (EPA) in their somatic tissues, but also transferred it to and concentrated it in their progeny, whose phospholipids were twice richer in EPA than those of their mothers. This drain of PUFA was previously documented in the literature, and emphasizes the preponderant role of LC-PUFAs in reproduction and development of daphnids (Becker and Boersma, 2005; Martin-Creuzburg et al., 2009; Sperfeld and Wacker, 2012, 2015; Wacker and Martin-Creuzburg, 2007). However, we showed that, in absence of dietary EPA, adult daphnids were able to synthetize EPA from dietary ALA (Fig. 7.1) but they did not transfer this

EPA to their progeny, suggesting that there may be a quantitative threshold for maternal transfer of EPA (Chapter 5). Besides, in addition to the EPA maternal transfer, the whole fatty acid profile of the progeny (3rd to 5th brood) was found to be highly dynamic, with, overall, a major impact of the maternal diet on the phospholipid profile of the progeny and a major impact of the brood number on the neutral lipid profile of the progeny (Chapter 5). The dynamic observed may reflect the successive cycles of lipid accumulation in the fat body followed by lipid mobilisation to the ovaries at the start of the yolk formation (Smirnov, 2017; Tessier and Goulden, 1982), with, as consequence, the release of successive broods with different fatty acid compositions depending on the fatty acid composition of the fat body at the start of lipid mobilisation. This complex plasticity of fatty acid profile in juvenile daphnids might partly explain variability of biological responses in neonates, and would be worth taking into account in future experimental research.

OUTCOME 2 – PUFAs impacted fatty acid transporters *in vitro* but not *in vivo*.

Fish cells have high predisposition to absorb and use fatty acids as energy source (Tocher, 2003). Accordingly, supplementation of RTL-W1 cell growth media with any of the tested PUFAs induced an upregulation of the genes coding for fatty acid transport protein 1 (FATP1), a transmembrane protein facilitating cellular fatty acid uptake (Frohnert and Bernlohr, 2000; Nelson et al., 2008; Zhou et al., 2010), and carnitine palmitoyl-CoA transferase 1 (CPT1), an enzyme catalysing the formation of acyl-carnitine from activated fatty acids and carnitine, which is required for their translocation to the mitochondrial matrix where β-oxidation takes place (National Research Council, 2011; Nelson et al., 2008; Tocher, 2003) (Chapter 4). The expression of these genes was however not impacted by feeding strategies tested *in vivo* (Chapter 6). Such divergences most likely ensue from the fact that isolipidic diets were provided to the fish *in vivo* while growth media supplemented with PUFAs were richer in lipids than the control growth medium, supplemented with FAF-BSA only. Our results contradict the suggestion that the metabolic demand for fatty acids rather than the substrate availability is the driving force for cellular fatty acid uptake (Mashek and

Coleman, 2006). In cytosol, fatty acids are bound to fatty acid binding proteins (FABP) (Tocher, 2003). Contrary to expectations, PUFA treatments decreased the RTL-W1 cell level of one fatty acid binding protein isoform (FABP7), suggesting that other isoforms may thus preponderantly ensure cytosolic transport of PUFAs in RTL-W1 cells (Chapter 4).

OUTCOME 3 – The three selected aquatic experimental models had different fatty acid bioconversion abilities.

Salmonids, such as rainbow trout, have a naturally high ability for fatty acid bioconversion (Tocher, 2003). In Chapters 3-4, we observed that RTL-W1 cells were able to bioconvert the given PUFAs, although to a lower extent than observed *in vivo*, in Chapter 6, and in literature (Fonseca-Madrigal et al., 2005; Glencross, 2009; Mellery et al., 2017; Mourente and Tocher, 1998). Indeed, while DHA and AA, two major biotransformation products of the n-3 and n-6 PUFA pathways respectively, were efficiently synthetized in rainbow trout liver *in vivo* (Chapter 6), RTL-W1 cells showed no production of these LC-PUFAs from their respective precursors ALA (and EPA) and LA (Chapters 3-4) (Fig. 7.1). Instead, an accumulation of intermediary metabolites resulting from elongation and/or desaturation (Δ5 and Δ6) of the given precursors was observed (Chapters 3-4) (Fig. 7.1). The observed elongation and desaturation abilities of RTL-W1 cells were confirmed by the quantifiable expression of the genes coding for elongase (the elongation of very long chain fatty acids 5, ELOVL5) and desaturase (fatty acid desaturase 2, FADS2) (Chapter 4). The inability of RTL-W1 cells to produce DHA from 22:5n-3 could result from either a non-functional *ELOVL2* gene or an inactivation of the gene product (enzyme) catalysing the elongation of 22:5n-3 into 24:5n-3 (Gregory and James, 2014), i.e. the first and limiting step for DHA production from EPA. The absence of *ELOVL2* gene expression in RTL-W1 cells pleads for the former option (Chapter 4). While this low *in vitro* biotransformation capacity could be disadvantageous when attempting to reproduce *in vivo* conditions, it can also be interesting as it allows comparing the biological role of the LC-PUFA-precursors (e.g. ALA and LA), independently from their indirect role as LC-PUFA-providers. Although high, the *in vivo* bioconversion ability of rainbow trout can be

overwhelmed. Indeed a drop of n-3 LC-PUFAs (especially DHA) is often observed in the tissues of fish fed plant based diets (lacking n-3 LC-PUFAs) as compared to fish fed fish-oil based diets. Tissue fatty acid profile generally reflects that of the diet (Bell et al., 2004; Bell et al., 2006; Bell et al., 2001; Caballero et al., 2002; Fonseca-Madrigal et al., 2005; Francis et al., 2014; Mourente and Bell, 2006; Petropoulos et al., 2009). In accordance, liver of juvenile rainbow trout fed diets enriched in n-6 PUFAs (or n-3 PUFAs) were richer in n-6 (or n-3 PUFAs) than those fed diets enriched in n-3 PUFAs (or n-6 PUFAs) and had a n-3/n-6 ratio that followed the same decreasing order than the diet (EPA- > DHA- > ALA- > LA-enrichments) (Chapter 6). DHA hepatic level in rainbow trout fed a diet lacking or deficient in n-3 PUFAs (LA-enriched diet, obtained through inclusion of sunflower oil in the diet) gradually dropped throughout the 10-week feeding trial, but proportionally less in the phospholipid and neutral lipid fractions than in the free fatty acid fraction. Fish fed the LA-enriched diet also highly bioconverted LA into AA and DPA and incorporated them in hepatic phospholipids (Fig. 7.1). The retention of DHA in hepatic phospholipids, the synthesis AA and DPA from LA and their incorporation in hepatic phospholipids were probably strategies to maintain a sufficient unsaturation level of biological membranes under dietary n-3 (LC)-PUFA deficiency. The inclusion of the n-3 LC-PUFA-precursor ALA in the trout diet (ALA-enriched diet, obtained through inclusion of linseed oil in the diet) enabled to maintain high hepatic DHA level through an efficient bioconversion pathway (Fig. 7.1). Indeed, the hepatic level of DHA was up to 5 times higher in fish fed the ALA-enriched diet than in fish fed the LA-enriched diet. Although at the end of the 10-week feeding trial, fish fed the ALA-enriched diet had a lower hepatic DHA level than fish fed the DHA-enriched diet (obtained through inclusion of DHA-rich cod liver oil in the diet), they maintained their hepatic DHA level as high as fish fed the EPA-enriched diet (obtained through inclusion of EPA-rich cod liver oil in the diet) (Chapter 6). The increased efficiency of fatty acid bioconversion has been explained by combination of increased substrate level, decreased LC-PUFA level, increased desaturate and elongase activity and gene expression (Fonseca-Madrigal et al., 2005; Gregory et al., 2016; Leaver et al., 2008a; Leaver et al., 2008b; Mellery et al., 2017; Minghetti et al., 2011; Thanuthong et al., 2011; Tocher, 2015; Zheng et al., 2009). Particularly, dietary DHA has been shown to

downregulate hepatic *FADS2* and *ELOVL2* genes in juvenile rainbow trout (Gregory et al., 2016). In accordance, hepatic *FADS2* gene expression was higher in fish fed LA- and EPA-enriched diets as compared to those fed the DHA-enriched diet (Chapter 6). However, hepatic *FADS2* gene expression was similar in ALA- and DHA- enriched diets, and diet had no impact on *ELOVL2* gene expression (Chapter 6). The high bioconversion of ALA into DHA observed in liver of fish fed the ALA-enriched diet was thus not explained by an upregulation of gene expression (Chapter 6).

In contrast to salmonids, daphnids have naturally low ability for fatty acid bioconversion. Either poor or no ability of *Daphnia spp*. for EPA (and AA) synthesis from dietary ALA (and LA) are reported in literature (Farkas et al., 1981; Koussoroplis et al., 2013; Martin-Creuzburg et al., 2010; von Elert, 2002; Wacker and Martin-Creuzburg, 2007). In Chapter 5, small but quantifiable amounts of EPA (and AA) were detected in phospholipids of 21-day old daphnids in absence of dietary EPA (and AA) and in presence of dietary ALA (and LA). But, even under dietary EPA supplementation (through liposome feeding), no further bioconversion of EPA into DHA was observed, as previously reported (Farkas et al., 1981; Martin-Creuzburg et al., 2010; Schlechtriem et al., 2006; von Elert, 2002). The strain of daphnid used as experimental model had thus a limited but existing ability to bioconvert dietary ALA (and LA) into EPA (and AA) but not into DHA (Fig. 7.1).

The three experimental models used are complementary and enable investigation and comparison of biological roles of individual PUFAs in systems with low (the large water flea), intermediate (RTL-W1 cell line) and high (rainbow trout juveniles) fatty acid bioconversion abilities (Fig. 7.1). Besides, the short lifespan and the high plasticity of the progeny fatty acid profile of the large water flea make it a suitable model for investigation of biological role of maternal transfer of dietary and body lipids.

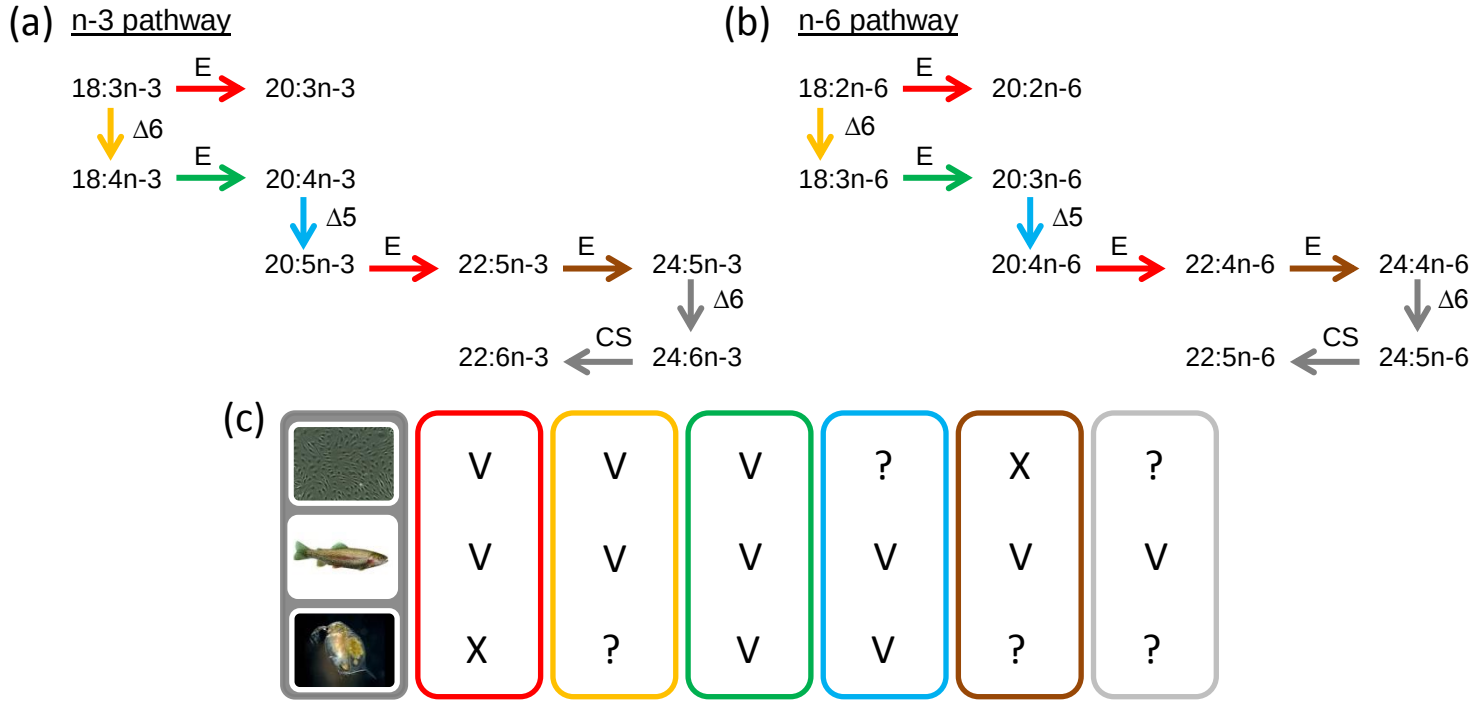

Fig. 7.1. Suggested (a) alpha-linolenic acid and (b) linoleic acid biotransformation pathways in experimental models. The colour of the arrow in biotransformation pathways (a, b) refers to (c) the occurence of the reaction observed in the experimental models. Abbreviations used: Δ5 and Δ6, microsomal fatty acid desaturase enzyme mediated reactions; ?, not proved by present data; CS, peroxisomal fatty acid chain shortening; E, hepatic microsomal elongase mediated reaction; V, reaction observed; X, no reaction observed.

7.2. Cd influences fatty acid homeostasis and metabolism

Cd impacts on fatty acid homeostasis and metabolism were assessed both *in vitro* (Chapter 4) and *in vivo* (Chapter 6), using rainbow trout as organism model. The general strategy was similar to what is described in section 7.1., i.e. a first experimental period of fatty acid profile modification in absence of Cd challenge followed by a short term (24 h, Chapters 4, 6) or a long term (6 weeks, Chapter 6) Cd challenge at either high (100 µM, Chapter 4), medium (50 µM, Chapter 4) or low (0.3 µg/L, Chapter 6) Cd levels. Impacts observed were dynamic and depended on the PUFA supply, Cd challenge duration and concentration (Chapters 4, 6).

7.2.1. Short-term effects

OUTCOME 4 – Short-term Cd exposure influenced the fatty acid profile of the phospholipids *in vitro* and of both the neutral lipids and the free fatty acids *in vivo*. Observed effects depended on the PUFA supply and were overall not explained by genetic variations.

In vitro, Cd (100 µM, 24 h) increased (2-3x) the incorporation of n-6 PUFAs (AA and 22:4n-6) at the expense of saturated fatty acids (SFA), in neutral lipids of AA-enriched cells, only (Chapter 4). We suggested these n-6 PUFAs were derived from the cellular free fatty acid pool as the phospholipid composition of AA-enriched cells was not impacted by Cd and the metal challenge was performed in a fatty acid free medium. This hypothesis still needs *in vitro* confirmation as we did not analyse free fatty acids from these cells. *In vivo*, Cd (0.3 µg/L, 24 h) decreased the hepatic levels of several free fatty acids in fish fed the ALA- and EPA-enriched diets, only, and in a PUFA-supply dependent manner (Chapter 6). In fish fed the ALA-enriched diet, Cd decreased total free fatty acid levels, among which especially PUFAs (LA, ALA, EPA, 22:5n-3) (Chapter 6). As the levels of these PUFAs were not impacted by Cd in other fatty acid fractions, we suggested a loss by oxidation (oxidative stress generated by Cd or β-oxidation to yield the required energy for

initiating detoxification responses) and/or by increased bioconversion. The two oxidation markers analysed (total glutathione level for the oxidative stress and *CPT1* gene expression for β-oxidation) were, however, not impacted by Cd in fish fed the ALA-enriched diet (Chapter 6). Still, other markers would be worth testing (e.g. other molecular or enzymatic antioxidants, other enzymes from the β-oxidation pathway such as CPT2 at both protein and genetic levels). On the other hand, the hepatic *FADS2* gene upregulation and the increase of the phospholipid 22:4n-6 level in fish fed the ALA-enriched diet both plead for at least partial implication of increased bioconversion in the loss of hepatic free PUFA observed in fish fed this diet after 24 h exposure to an environmentally realistic Cd level (Chapter 6). However, no impact was observed on expression of genes coding for elongases (*ELOVL2* and *ELOVL5*) (Chapter 6). In fish fed the EPA-enriched diet, Cd decreased the levels of some free SFA (18:0), monounsaturated fatty acids (MUFA, 20:1n-9) and PUFA (DHA) without affecting total free fatty acid level. This Cd-induced loss of specific free fatty acids went along with an increase of the total neutral lipid level, among which, mainly SFA (16:0), and PUFA (DHA), but not MUFA (Chapter 6). Besides, the SFA deposition in neutral lipids exceeded their disappearance from the free fatty acid fraction (Chapter 6). We therefore suggested Cd induced fatty acid storage (SFA, PUFA), coupled with increased SFA uptake from the bloodstream or with *de novo* synthesis while increasing catabolism of MUFA for energy generation through β-oxidation. However, no impact was observed on hepatic fatty acid synthase (*FASN)*, *FATP1* and *CPT1* gene expressions in fish fed the EPA-enriched diet (Chapter 6), as previously observed *in vitro* (Chapter 4), suggesting that Cd impact would either be exerted on other targeted genes or at the protein level. Similarly to what was observed in fish fed the EPA-enriched diet, Cd (0.3 µg/L, 24 h) increased SFA level in hepatic neutral lipids of fish fed the LA- and DHA-enriched diets. But, in contrast to what was observed in fish fed the EPA-enriched diet, Cd did not affect the levels of total neutral lipid or free fatty acid in fish fed the LA- and DHA-enriched diets (Chapter 6). We therefore suggested that in fish fed the LA- and DHA-enriched diets, incorporation of SFA in neutral lipids induced by Cd was balanced by a higher SFA uptake or *de novo* synthesis and/or a lower use of SFA as energy source, resulting in a constant free SFA level. However, again, modulations of fatty acid profile observed were not

mirrored by genetic variations (Chapter 6). Overall, an untargeted omic approach would help unravelling molecular mechanisms of effect.

7.2.2. Longt-term effects

Except for the upregulation of hepatic *FADS2* gene in fish fed the ALA-enriched diet, none of the Cd effects observed after 24 h exposure were maintained upon 6 weeks of Cd exposure (Chapter 6). Other effects and interactions were still observed.

OUTCOME 5 – Long-term Cd exposure had opposite effects on hepatic *FADS2* gene expression in rainbow trout fed ALA- and EPA enriched diets, without any impact on their hepatic PUFA composition. Long-term Cd exposure influenced the free 18:1n-9 level and the phospholipid synthesis/turnover in a PUFA supply dependent manner.

Cd (0.3 µg/L, 6 weeks) upregulated hepatic *FADS2* and *ELOVL2* genes in fish fed the ALA-enriched diet, downregulated hepatic *FADS2* gene in fish fed the EPA-enriched diet, without any impact on hepatic PUFA levels in fish fed these two diets (except small, but significant, increase of phospholipid 20:3n-3 and free 20:3n-6 levels in fish fed the EPA-enriched diet) (Chapter 6). These interactions highlight the complexity of biological responses to Cd and the importance of taking the characteristic of the challenge (duration, concentration) and the nature of the diet (fatty acid composition) into account when comparing studies. The inconsistence between effects on hepatic desaturase/elongase gene expressions and PUFA levels in fish fed these diets could indicate the existence of antagonistic effects on these enzymes at post-translational level and/or on other pathways. For example, upregulation of desaturase and elongase gene expression observed in fish fed the ALA-enriched diet (Chapter 6) might be balanced by the oxidation of the generated PUFAs, leading to unchanged hepatic PUFA levels. On the other hand, downregulation of desaturase gene expression observed in fish fed the EPA-enriched diet (Chapter 6) might be balanced by increased uptake from the bloodstream leading to unchanged or even slightly increased hepatic PUFA levels. In accordance, the increase of total free MUFA level, among which

especially 18:1n-9, without any impact on MUFA levels of other lipid fractions, induced by Cd in fish fed the EPA-enriched diet pleads for higher fatty acid uptake under long term Cd exposure in fish fed this diet (Chapter 6). However, the fatty acid uptake marker analysed (*FATP1* gene expression) was not impacted by Cd in fish fed this diet (Chapter 6). Note that fatty acid uptake also occurs through passive diffusion or through other fatty acid transporters (e.g. CD36) (Frohnert and Bernlohr, 2000; Nelson et al., 2008; Zhou et al., 2010), which were not assessed in the present research project. The observed higher free 18:1n-9 could alternatively reflect a higher *de novo* synthesis. However, Cd had no impact on *FASN* and Δ9 desaturase (*d9D)* gene expressions in fish fed the EPA-enriched diet (Chapter 6), suggesting either no impact on *de novo* synthesis, or an impact at protein level rather than genetic level. Free 18:1n-9 level was also increased by Cd in the liver of fish fed the LA- and ALA-enriched diet without any impact on gene expression (Chapter 6).

Cd (0.3 µg/L, 6 weeks) modulated phospholipid content and composition in a PUFA-supply dependent manner (Chapter 6). Indeed, Cd (0.3 µg/L, 6 weeks) increased total hepatic phospholipid level and MUFA level in hepatic phospholipid of fish fed the ALA-enriched diet. It increased 18:0 and PUFA levels in hepatic phospholipids of fish fed the LA-enriched diet, without, however, any impact on total phospholipid. It finally increased MUFA and decreased n-3 PUFA levels in hepatic phospholipids of fish fed the DHA-enriched diet, without, again, any impact on the total phospholipid level (Chapter 6). Based on these results, we suggested Cd induced phospholipid synthesis and turnover in fish fed these three diets, probably in a strategy to replace phospholipids that were damaged by oxidative stress induced by Cd. Phospholipid synthesis and turnover seemed to be particularly efficient in fish fed the ALA-enriched diet as it lead to hepatic phospholipid accumulation. The loss of n-3 PUFAs (ALA, 20:3n-3, 20:4n-3, EPA and 22:5n-3) in hepatic phospholipid and the related decrease of the unsaturation level of hepatic phospholipids, induced by Cd in fish fed the DHA-enriched diet might reflect uncontrolled oxidative chain reactions in the liver of fish fed this diet with possible consequences on hepatic membranes functionality and integrity (Chapter 6).

7.3. Dietary lipids modulate biological responses to Cd

Lipids, and their constitutive fatty acids, are important nutrients. They are crucial energetic resources, play a key role in cell structure maintenance, are precursors of signalling molecules (e.g. eicosanoids) and act as nuclear receptor ligands (Tocher, 2003). It therefore appears plausible that lipids could play key roles in organismal, tissue and cellular response to pollutants such as Cd. We first aimed at validating the existence of such interaction between cellular (Chapters 3, 4), individual (Chapters 5, 6) fatty acid profile and sensitivity to Cd. We then investigated mechanisms behind the observed interactions (Chapters 4, 6). The general experimental strategy was similar to what is described in previous sections, i.e. a first experimental period of fatty acid profile modification in absence of Cd challenge followed by a short term (24 h in Chapters 4, 6 or 48 h in Chapter 5) or a long term (6 weeks, Chapter 6) Cd challenge.

7.3.1. The fatty acid profile modulates cellular and individual sensitivity to Cd

OUTCOME 6 – PUFA supply influenced Cd toxicity. Results diverged between the selected aquatic experimental models, with EPA being protective *in vitro* but not *in vivo* and ALA being protective both *in vitro* and *in vivo.*

The phospholipid composition of rainbow trout liver cells is an important parameter influencing their sensitivity to Cd. Indeed, RTL-W1 cells with phospholipids enriched in ALA, EPA or DHA were significantly more tolerant to an acute Cd stress (1.9-3.0 times higher 24 h median effect concentration (EC50) value than non-enriched cells) while an enrichment in their n-6 counterparts, i.e. LA, AA and DPA, had no impact on Cd EC50 (Chapter 3). Although DPA-enrichment did not significantly affect the Cd EC50, it induced a hormesis effect, i.e. a beneficial effect at low Cd dose (Chapter 3). Cytoprotection exerted by ALA- and EPA-enrichments against Cd challenge of a medium (50 µM, 24 h,) or a high (100 µM, 24 h) intensity was confirmed in Chapter 4, with metabolic

activity of ALA- and EPA-enriched cells being 1.6-1.9 times higher than that of unenriched cells. Note that DHA-enrichment was not included in the experimental design of mechanistic investigations performed in Chapter 4. This would be worth investigating in future research. In contrast to expectations, AA-enrichment also increased cell tolerance to 50 µM Cd only (Chapter 4). This divergence with Chapter 3, most probably reflects that two discrete Cd concentrations were tested in Chapter 4, while EC50 data in Chapter 3 were calculated based on modelling data (i.e., dose-response analyses and curve fitting implying extrapolation). As shown in section 7.1., PUFA-enrichment strategies not only enriched the cells with the targeted PUFAs but rather modified the whole cellular fatty acid profile. Using a principal component analysis (PCA) approach, we identified fatty acids that may preponderantly have influenced cellular sensitivity to Cd (Chapter 3). Indeed, the higher resistance to Cd was associated with a higher phospholipid content in n-3 PUFAs (ALA, 18:4n-3, 20:3n-3, EPA, 22:5n-3 and DHA) and a lower phospholipid content in n-6 PUFAs (20:3n-6, LA, 18:3n-6 and 20:2n-6). The absence of negative impact of AA and DPA on Cd sensitivity that came from this PCA (Chapter 3), together with the AA-enrichment derived protection against one discrete Cd concentration (50 µM, Chapter 4) and the hormesis effect induced by DPA-enrichment (Chapter 3) highlight the importance of better defining biological role of individual PUFAs instead of focussing on effects of n-3 PUFAs vs n-6 PUFAs.

In contrast to the *in vitro* observations (Chapters 3-4), EPA did not protect adult nor neonate daphnids against Cd (Chapter 5). Indeed, while feeding *D. magna* with an algae based diet supplemented with liposomes during 21 days increased their tolerance to an acute Cd challenge (higher 48 h-EC50), loading liposomes with EPA did not confer additional protection against Cd to either adults or their progeny (Chapter 5). Aside from differences of biological organisation levels, we suggested that the divergence might also result from differences in the extent to which the fatty acid profile could have been experimentally modified (lower EPA accumulation, absence of EPA elongation in *Daphnia* model). In neonate daphnids, the tolerance to Cd acute toxicity was influenced by the brood number and positively correlated to the neutral lipid content in PUFA, especially ALA, the neutral lipid n-3/n-6

ratio, and negatively correlated to the neutral lipid content in SFA, especially 18:0. This corroborates the cytoprotective effect of ALA against acute Cd toxicity (Chapter 3).

7.3.2. Suggested mechanisms underlying PUFA-derived protection against Cd toxicity

Two main mechanisms underlying protection by individual PUFA were explored first *in vitro* (Chapters 4), then and *in vivo* (Chapter 6).

We first hypothesized that ALA-, EPA-, DHA- and, to a lower extent, AA-enrichments could increase tolerance to Cd by decreasing Cd burden, through a reduced uptake and/or an increased efflux of metal. We alternatively hypothesized that individual PUFAs could act to improve the cellular (Chapter 4) or individual (Chapter 6) capacity to cope with toxic effects of Cd, among others through the expression of defensive genes.

OUTCOME 7 – ALA-enrichment decreased Cd hepatic burden *in vivo* but not *in vitro.*

Our first mechanistic hypothesis was ruled out *in vitro* (Chapter 4), as the total RTL-W1 cell Cd burden was not influenced by PUFA-enrichments. However, long term (6 weeks) *in vivo* exposure to an environmentally realistic Cd concentration (0.3 µg/L) yielded to a different conclusion, as juvenile rainbow trout fed the ALA-enriched diet accumulated twice less Cd in their liver than fish fed the LA- or EPA-enriched diets (Chapter 6). Response patterns vary thus between short- and long-term exposure to high and low Cd concentration, *in vitro* and *in vivo*, respectively. Cd being known to predominantly accumulate in the liver and in the kidneys during short-term and long-term Cd exposure respectively (Chowdhury et al., 2003), we suggested the lower Cd hepatic level in fish fed the ALA-enriched diet might reflect a lower long-term hepatic uptake rate and/or a higher/earlier metal transfer to the kidneys. Mechanisms still need to be unravelled but PUFAs are known to modulate physicochemical properties of biological membranes and embedded proteins (Arts and Kohler, 2009; Tillman and Cascio, 2003), potentially influencing uptake/efflux of Cd. Besides, the genetic

upregulation of one metallothionein isoform (*MTa*) induced by short-term (24 h) Cd exposure in fish fed the ALA-enriched diet only while Cd hepatic burden was still below quantification limit might indicate early hepatic preparation for Cd efflux to the kidney as part of the Cd is excreted from the liver as MT-Cd complex (Chapter 6) (Chowdhury et al., 2003; McGeer et al., 2011). Finally, one cannot exclude that PUFA-enrichments could also influence Cd intracellular distribution, thereby modulating Cd toxicity. These aspects of Cd toxicokinetic/toxicodynamic modelling were not measured in the present research project and would be worth investigating in fish fed diets enriched in specific PUFAs.

OUTCOME 8 – Activation of the first line (MT and glutathione) and the second line (general antioxidant- and stress-defence systems) of Cd detoxification were modulated by PUFA both *in vitro* and *in vivo*. ALA-derived protection against Cd seemed to be, at least partly, triggered by an efficient detoxification response both *in vitro* and *in vivo*.

The first line of cellular Cd detoxification is mediated by MT, a low molecular weight protein with a high cysteine content, and glutathione, the major non-protein thiol in cells, that both act as metal and ROS scavengers. Cd having a high affinity for thiol groups, it is rapidly chelated by MT and glutathione as soon as it enters the cells. This chelation system decreases the cellular level of free Cd ion and thus Cd toxicity. The thiol groups of MT and glutathione can also scavenge ROS generated during normal aerobic process and the excess of ROS generated during (Cd) stress, thus preventing oxidative damage. Chelation of Cd by MT and glutathione can thus be beneficial for cells as it decreases the bioavailability of the toxic form of the metal but also detrimental to cells if not coupled with recruitment of the general antioxidant- and stress-defence systems (i.e. the second line of cellular detoxification) as it decreases the total antioxidant cellular capacity (Cuypers et al., 2010; Đukić-Ćosić et al., 2020; Klaassen et al., 1999; McGeer et al., 2011; Thévenod and Lee, 2013; Zalups and Ahmad, 2003). Both lines of defense were activated *in vitro* (Chapter 4) and *in vivo* (Chapter 6). Interestingly these activations were modulated by the PUFA-enrichments.

In accordance with literature (Chen et al., 2014; Walker et al., 2008), Cd (50-100 µM, 24 h) upregulated two isoforms of the *MT* gene (*MTa* and *MTb) in vitro* (Chapter 4). Interestingly, ALA-, EPA-, and AA-derived cytoprotection against Cd (100 µM, 24 h) went along with a lower induction of *MTb* gene expression by Cd (Chapter 4). A similar effect was observed with *MTa* gene expression, for the AA-enrichment only (Chapter 4). As fatty acid treatment had no impact on cellular Cd burden, we suggested this lower *MT* gene induction observed in cells that were more tolerant to Cd might be the consequence of a lower cellular free Cd level through differential subcellular metal partitioning and/or a lower cellular ROS level, through either reduction of ROS generation or stimulation of cellular antioxidant systems, as free Cd and ROS can both enhance *MT* gene expression (Klaassen et al., 1999; Kling and Olsson, 2000; McGeer et al., 2011; Tan et al., 2016; Thévenod and Lee, 2013; Wan et al., 2009). As mentioned above, subcellular distribution of Cd in cells differentially enriched in PUFAs has not been assessed yet and this parameter would be worth testing in futur research. We neither measured cellular ROS levels but proteomic analyses showed different amounts of antioxidant enzymes (glutaredoxin-1 and peroxiredoxin-1) in cells enriched in protective PUFAs as compared to non-enriched cells, either in the absence (EPA-enrichment) or in the presence (ALA- and AA-enrichments) of Cd (Chapter 4). These results suggest that the differential induction of the antioxidant system could be one of the mechanisms behind PUFA-derived cytoprotection, as suggested in human hepatocellular cells (Hep G2 cell line) (Linhartova et al., 2016). More particularly, the lower glutaredoxin-1 level (enzyme catalysing the reduction of protein disulphides and glutathione-protein mixed disulphides) of EPA-enriched cells in the absence of Cd as compared to non-enriched cells might indicate a lower basal ROS level under EPA-enrichment (Chapter 4). This could result from direct impact of EPA on complex I, III and IV of the mitochondrial electron transport chain reducing net hydrogen peroxide production, as demonstrated in mice (Hagopian et al., 2010). Cd has been reported to exert to opposite effect on the complex III of the mitochondrial electron transport chain, stimulating thereby the production of ROS (Wang et al., 2004). Electron transport chain III complex conformation and functionality under differential PUFA-enrichment strategies and Cd stress have not been assessed and would

be worth investigating to validate this possible mechanism of EPA-derived cytoprotection. Besides, the level of peroxiredoxin-1, an antioxidant enzyme catalysing the reduction of hydrogen peroxide, peroxynitritre and organic hydroperoxides, was higher in ALA-enriched cells challenged with Cd (50 µM, 24 h) as compared to non-enriched cells. This might indicate a faster or more efficient antioxidant response in ALA-enriched cells as compared to non-enriched cells. A similar trend was observed in AA-enriched cells (Chapter 4).

In vivo, Cd (0.3 µg/L, 24 h, Chapter 6) upregulated the hepatic *BCLx* gene, which codes for an anti-apoptotic member of the BCL2 family and the hepatic *MTa* (but not *MTb* genes), in fish fed the ALA-enriched diet only, while the hepatic Cd level was still below the quantification limit. We suggested these very early upregulation of *MTa and BCLx* genes could be the consequence of a more efficient short-term hepatic Cd uptake and/or, again, a faster detoxification response in fish fed the ALA-enriched diet, as compared to fish fed the other diets. Whether the upregulation of *BCLx* gene was balanced by an upregulation of apoptotic members of the BCL2 family (e.g. Bax) was not assessed in the present research project and would be worth investigating. Given its role as ROS scavenger (Klaassen et al., 1999), the genetic early upregulation of *MTa* by Cd could also have enhanced the total hepatic antioxidant capacity of fish fed ALA-enriched diet. In contrast, the lower total glutathione level observed in fish fed the DHA- and EPA-enriched diets after long-term Cd exposure (0.3 µg/L, 6 weeks, Chapter 6) as compared to fish fed the same diet but not exposed to Cd might reflect a lower total antioxidant capacity of fish fed these two diets and potentially higher susceptibility to the oxidative stress induced by long term Cd exposure. In that way, the decrease in n-3 PUFAs levels observed in hepatic phospholipids of fish fed the DHA after long term Cd exposure might reflect oxidative damage to biological membranes. The Cd-induced decrease in total glutathione level could result from impairment of the glutathione synthesis or from enhancement of glutathione degradation (Đukić-Ćosić et al., 2020; Jamwal et al., 2016; Ramakrishnan et al., 2017). These specific impacts were not assessed in the present research project and would be worth investigating.

OUTCOME 9 – The energy provision and storage systems might, at least partly, have played a role in the differential responses to Cd among the PUFA treatments observed both *in vitro* and *in vivo.*

Activation of cellular defences has a non-negligible energetic cost, which can be further increased by Cd since this metal has been shown to impair ATP production (Adiele et al., 2012). Any cellular mechanism that increases energy production and storage might increase cellular ability to cope with a subsequent Cd stress. In that way, the higher level of pyruvate kinase that catalyses the tenth step of glycolysis (production of pyruvate and ATP from phosphoenolpyruvate and ADP) (Nelson et al., 2008) observed in AA-enriched cells as compared to non-enriched cells might have provided AA-enriched cells with a higher basal energy level and ability to cope with the acute Cd challenge applied thereafter (50 µM, 24 h, Chapter 4). Neutral lipids constitute an important energy storage that is mobilized when energy demand excesses the energy provision (Nelson et al., 2008; Tessier and Goulden, 1982; Tocher, 2003); which can happen during Cd stress (Adiele et al., 2012; Berntssen and Lundebye, 2001; Sokolova et al., 2012). Interestingly, we showed that neonate daphnid with neutral lipids characterized by a higher PUFA (especially ALA) level, a higher n-3/n-6 ratio and a lower SFA (especially 18:0) level were more tolerant to Cd (Chapter 5). Differential mobilisation of fatty acids and use as energy source under Cd stress has not been assessed yet and would be worth investigating in future research to unravel mechanisms of protection. Prolonged lipid mobilisation to yield energy for cellular detoxification under prolonged Cd stress might result in impaired growth and reproduction. While long-term environmentally realistic Cd stress (0.3 µg/L, 6 weeks) had no direct impact on body weight, interactions between PUFA-enrichment strategy and Cd contamination on fish weight was highlighted (Chapter 6). Indeed, fish fed the EPA-enriched diet were lighter than fish fed the DHA-enriched diet after long-term Cd exposure while no impact was observed in absence of Cd challenge (Chapter 6). This contradiction between short term EPA-derived cytoprotection (Chapters 3, 4) and long-term higher sensitivity to Cd (Chapter 6) might reflect the energetic cost of activation and maintenance of detoxification responses. If the stress is prolonged, energy stocks might be weakened and the detoxification system might be overwhelmed. The lower total

glutathione level observed in fish fed the EPA-enriched diet under long-term Cd exposure as compared to fish fed the same diet but unexposed to Cd might be an example of such a decline (Chapter 6).

OUTCOME 10 – A differential induction of the eicosanoid cascade might, at least partly, explain PUFA-derived *in vitro* protection against Cd.

Eicosanoids and resolvins are important PUFA-derived signalling molecules playing roles in biological processes such as inflammation, ion transport and cellular redox balance. Their production starts with the liberation of PUFAs (with 20 or 22 carbons) from the phospholipid membranes; step catalysed by phospholipases, mainly the cytosolic phospholipase A2 (cPLA2). The released precursors are then oxidized to generate prostanoids, leukotrienes or resolvins; steps catalysed by cyclooxygenases (COX), such as COX2, and lipoxygenases, such as arachidonate 5-lipoxygenase (ALOX5) (Kremmyda et al., 2011). (Kremmyda et al., 2011). In line with the literature (Madejczyk et al., 2015; Miyahara et al., 2001), Cd (100 µM, 24 h, Chapter 4) upregulated *cPLA2* gene expression in non-enriched cells. Interestingly, a similar trend was observed in LA-enriched cells, while no *cPLA2* induction was observed in cells enriched in cytoprotective PUFAs (ALA, EPA and AA). This suggests that mechanisms of PUFA-derived cytoprotection against Cd might include a repression of the eicosanoid cascade. This repression may, for instance, have been driven by the reduction of the cellular ROS level or the inhibition of the NF-κB signalling pathway by PUFA as both were shown to mediate *cPLA2* gene induction (Hagopian et al., 2010; Kremmyda et al., 2011; Lin et al., 2016; Lin et al., 2011; Marion-Letellier et al., 2015). Proteomic data also showed that non-enriched cells and AA-enriched cells exposed to Cd (50 µM, 24 h) had lower levels of Annexin A1, a cPLA2 inhibitor (Parente and Solito, 2004), than cells unexposed to Cd (Chapter 4). Together with the genetic results, this suggests a potential induction of the eicosanoid cascade by Cd in non-enriched cells.

OUTCOME 11 – Cytochrome P450 (CYP) 20A1 might be involved in the eicosanoid cascade and in the EPA-derived protection against Cd *in vitro*, while no impact was observed under the *in vivo* experimental conditions.

The CYP family plays an important role in oxidative metabolism and detoxification of xenobiotics. Some vertebrate CYPs isoforms have unidentified functions and are termed orphans. CYP20A1 is one of those orphans (Stark and Guengerich, 2007; Stark et al., 2008). Cd has been reported to modulate the gene expression of various CYPs (Chen et al., 2019; Madejczyk et al., 2015; Olsvik et al., 2016; Reynders et al., 2006). In Chapter 4, we showed that Cd (50-100 µM, 24 h) downregulated the expression of *CYP20A1* gene, in EPA-enriched cells only, suggesting that the orphan CYP20A1 might be involved in EPA-derived cytoprotection against Cd. On the other hand, in absence of Cd, EPA- and AA-enrichments upregulated *CYP20A1* gene expression and downregulated *COX2* gene expression (Chapter 4), suggesting that *CYP20A1* might be involved in the eicosanoid cascade, as previously suggested (Ferain et al., 2018). However, these effects were not observed under the *in vivo* experimental conditions tested in Chapter 6. Additional research is needed to deorphanize *CYP20A1* gene.

OUTCOME 12 – Cytoprotective PUFAs modulated the abundance of some cytoskeletal intermediate filaments, in absence of Cd only.

Cd has been reported to cause abnormalities in cytoskeleton architecture and to modulate the expression of components of the cytoskeleton (Nawaz et al., 2005; Vergilio and De Melo, 2013). Proteomic data revealed no impact of Cd (50 µM, 24 h, Chapter 4) on cytoskeleton components. Although, cytoprotective PUFAs (ALA, EPA and AA) influenced the amount of specific proteins of the cytoskeleton (cytokeratin-18, the intermediate filament protein ON3 and myosin-9), in absence of Cd challenge only (Chapter 4). Whether the different basal levels of these cytoskeleton components have helped cells to cope with the Cd challenged applied has not been investigated and would be worth exploring.

OUTCOME 13 – Cd modulated the hepatic calcium burden *in vivo* and the abundance of specific proteins involved in calcium homeostasis *in vitro* in a PUFA-supply dependent manner.

Cd impairs the homeostasis of essential metals, among which calcium (Biagioli et al., 2008; Choong et al., 2014; McGeer et al., 2011; Niyogi et al., 2004; Niyogi et al., 2015). In Chapter 6, we showed that Cd (0.03 µg/L, 24 h) increased hepatic calcium level in fish fed the LA-enriched diet only. This effect was not maintained upon long term (6 weeks) exposure, suggesting a potential adaptation to the Cd stress. Subcellular calcium partitioning was not assessed and would be worth investigating to characterise the fate and possible consequences of this transient rise in hepatic calcium level. For example, if coupled with blockage of plasma membrane, endoplasmic reticulum or sarcoplasmic reticulum calcium pumps as previously reported under Cd contamination, the rise of total hepatic calcium level could reflect a rise of free cytosolic calcium level, which can lead to apoptosis (Biagioli et al., 2008; Choong et al., 2014; Thevenod and Lee, 2013; Wang et al., 2014). The apoptosis marker analysed, *BCLx* gene expression, was not impacted by Cd in fish fed the LA-enriched diet (Chapter 6). This contrasts with our *in vitro* results, where Cd (50-500 µM, 24 h, Chapter 4) decreased *BCLx* gene expression (50-500 µM, 24 h, Chapter 4) in LA-enriched cells, but also in any other RTL-W1 cell batch tested. However this sole marker is not a sufficient piece of evidence to conclude about the cellular apoptotic fate. Additional research should focus on these aspects. Other interactions between Cd, PUFAs and calcium homeostasis actors were observed *in vitro*, without, however, a clear relationship to toxicity (Chapter 4). For example, Cd (50 µM, 24 h) decreased the level of Annexin A1, a multifunctional protein involved in ion calcium channel formation, in non-enriched and AA-enriched cells only. Cells enriched in specific cytoprotective PUFAs (ALA, AA but not EPA), had a lower basal level of S100-A1 protein, a calcium binding protein. The exact meaning of these interactions has not been solved and would require further investigation.

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The multi-model approach adopted in the present project enabled to highlight molecular interactions beween Cd and PUFA. Cd and the fatty acid supply influence fatty acid metabolism and homeostasis. The fatty acid supply modulates cell, tissue and individual response to Cd. Interplay is highly dynamic. Effects depend on the experimental conditions (PUFA supply, contamination level, duration of exposure) and on the model used (*in vitro* vs *in vivo*, brood number), pointing out the need for multi-model and multi-factorial experimental designs. Still, in any model tested in the present research project, ALA conferred protection against Cd. This beneficial effect of ALA itself independently of its further bioconversion into the well-known valuable LC-PUFAs EPA and DHA is worth investigating in deeper details. Our results finally emphasize the need to take into account feed composition in ecotoxicological studies and risk assesment.

7.4. Perspectives

The present research project highlighted impacts of both lipids and Cd on fatty acid homeostasis and metabolism. It also showed that the nature of the individual/tissue/cell fatty acid profile influences the biological responses to Cd contamination. We suggested, based on our data, potential mechanistic tracks which open the doors to future research in the fields of nutrition and ecotoxicology. Several calls to actions were already put forward in this chapter (see sections 7.1.-7.3.). This last section is thus a non-exhaustive compilation of open questions underlying this research project and analytic suggestions.

In Chapter 3, we showed that DPA induced a hormesis effect, i.e. a beneficial effect at low Cd dose. This n-6 LC-PUFA is understudied and has no clear biological role. It has been suggested to share similar functions with DHA without being its equivalent. We did not include these two LC-PUFAs in the experimental design of *in vitro* mechanistic investigations (Chapter 4). It should be interesting to do it in future research to better understand common and different properties of these two LC-PUFAs.

In Chapter 4, we suggested protective effect of specific PUFAs might be linked to a reduction of the oxidative stress induced by Cd and a differential induction of the eicosanoid cascade, with a possible role of peroxiredoxin-1 and glutaredoxin-1 (antioxidant enzymes) as well as cytosolic phospholipase A2 (enzyme initiating the eicosanoid cascade). We also suggested the complex III of the electron transport chain as potential site of interaction between EPA and Cd. Further research should investigate these possible mechanisms of interaction in deeper details to validate or infirm our hypotheses.

In Chapters 4 and 6, we analysed total cell and tissue Cd burdens to assess whether PUFA-derived protection against Cd was linked to differential Cd influx/efflux. However, Cd repartition between the metal sensitive fraction (heat sensitive proteins, organelles) and the detoxified fraction (heat stable proteins, metal-rich granules) rather than total Cd burden defines the toxicity level. As subcellular partitioning is highly dynamic, organ dependant and does not follow a priority order, it would be important to analytically measure repartition between these pools. In the same line, subcellular repartition of calcium rather than total calcium level (as assessed in Chapter 6) would have been an interesting endpoint. These measurements would probably give important mechanistic clues for defining cellular sites of interactions.

In Chapters 4 and 6, we showed that Cd impacts on fatty acid profiles were generally not reflected by impacts on expression levels of the selected genes. Overall an untargeted transcriptomic approach might have enabled better identify genetic targets.

In Chapter 5, we showed that the fatty acid profile of the daphnid's 3rd-5th brood progeny was highly dynamic with, overall a major impact of the maternal diet on the phospholipid profile of the progeny and a major impact of the brood number on the neutral lipid profile of the progeny. This complex plasticity of fatty acid profile in juvenile daphnids would be worth exploring in deeper details. We analysed 3 successive broods (3rd-5th), what about the next ones? We tested 3 different diets (algae based diet supplemented (or not) with liposomes loaded (or not) with free EPA), what about other diets (ex: liposomes loaded with other free PUFAs or liposomes made with EPA-phospholipid instead of loaded with free EPA)? In general, would the

choice of a specific brood number have influenced results of other research with the daphnid model?

In Chapter 5, we observed a protective impact of liposome supplementation against Cd acute toxicity in adult daphnid while loading EPA in the liposomes did not give further protection. We suggested this liposome-derived protection might indicate positive impact of dietary phospholipids in general or of one component of the given phospholipids, i.e. 16:0, 18:0, phosphorous or choline. Further investigations are required to refine this list.

Across the chapters we observed complex interactions between Cd and dietary lipids. Impacts depended on the PUFAs supplied, exposure characteristics (concentration, duration) and the model used. Similar interactions would be worth investigating using other experimental models both *in vitro* and *in vivo*. In chapters 3 and 4, we used classical monolayer cultures of the RTL-W1 cell line for running our *in vitro* tests. It has been shown that RTL-W1 cells regain structural and functional characteristics of hepatocytes when cultured in a three-dimensional context with notably a higher organelle concentration and a higher basal and induced *CYP1A* gene expression (Lammel et al., 2019; Malhão et al., 2013). Spheroid RTL-W1 cells may thus represent a more physiologically relevant *in vitro* experimental system that benefits from all advantages of continuous liver cell lines over the primary culture (easier to handel, lower intrinsic genetic variability, less expensive), while overcoming their main shortcoming (i.e. limited metabolic capabilities). However, experimental cautions should be taken to overcome the major disasdvantages of the speroids over the monolayer culture that are a higher variability in the local exposure level and chemical availability to cells from the external vs internal layers of the spheroid. This variability can be further increased if the size of the spheroid is not fully controlled and reproductible (Lammel et al., 2019). Besides, we focussed our research project on rainbow trout and *Daphnia magna.* Other well-characterized models would be worth investigating such as zebrafish (*Danio rerio*) for which a liver cell line has also been developed (ZFL cell line) allowing combination of both *in vitro* and *in vivo* approaches. The richness of the present multi-model approach would be further increased.

7.5. References

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