UNIVERSITAT DE BARCELONA

Application of omic approaches on the mechanisms of pollutants using *Daphnia magna* as model species

Inmaculada Fuertes Rodríguez

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PhD Thesis

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Application of **omic approaches** on the characterization of novel toxicity mechanisms of pollutants using *Daphnia magna* as model species

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Application of omic approaches on the characterization of novel toxicity mechanisms of pollutants using *Daphnia magna* as model species

Thesis presented by

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HACEN CONSTAR,

Que la presente memoria doctoral, titulada "Application of omic approaches on the characterization of novel toxicity mechanisms of pollutants using *Daphnia magna* as model species" ha sido realizada bajo su dirección y que todos los resultados presentados en ella son fruto de la investigación realizada por la citada doctoranda en el Departamento de Química Ambiental del Instituto de Diagnóstico Ambiental y Estudios del Agua adscrito al Consejo Superior de Investigaciones Científicas.

Y para que así conste expiden el presente certificado.

Barcelona, noviembre de 2020

Dr. Carlos Barata

Dr. Demetrio Raldúa

A mi familia,

"La cultura del consumo, cultura del desvínculo,
nos adiestra para creer que las cosas ocurren porque sí."
Eduardo Galeano

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Resumen

La toxicología ambiental está experimentando un cambio de paradigma debido a la preocupante nueva realidad medioambiental. Hoy en día, gestionar los efectos más sutiles y crónicos de los compuestos químicos, ya sean individualizados o en mezclas, es una necesidad imperiosa, especialmente en concentraciones bajas y relevantes para el medio ambiente. No menos importante es ocuparse de los contaminantes emergentes (EC), cuyos efectos nocivos en los ecosistemas y sus mecanismos de toxicidad aún se desconocen. Por lo tanto, deben elaborarse nuevas estrategias con mayor relevancia ambiental para evaluar la toxicidad de los contaminantes, lo que requiere la aplicación de enfoques integradores que combinen herramientas de distintas disciplinas. Las tecnologías ómicas permiten medidas holísticas de efectos producidos a bajos niveles de organización biológica en plataformas de alto rendimiento, y proporcionan datos mecanicistas que pueden resultar esenciales para el desarrollo y la aplicación de estrategias de ensayo más eficientes y eficaces. En general, el objetivo de esta tesis ha sido demostrar la importancia de integrar enfoques toxicológicos ómicos con ensayos toxicológicos convencionales a fin de obtener información significativa que ayude a desentrañar cualquier nuevo mecanismo de toxicidad desencadenado por ECs en el medio acuático utilizando *Daphnia magna (D. magna)* como especie modelo.

Los contaminantes estudiados en esta tesis incluyeron aquellos sospechosos de ser disruptores de lípidos (compuestos disruptores endocrinos, EDC) y ECs que se sabe que afectan al sistema nervioso central (es decir, fármacos neuroactivos y otros productos químicos). Se han desarrollado diferentes enfoques integradores para evaluar la toxicidad de estos compuestos vinculando los efectos sobre la reproducción y el comportamiento (respuestas individuales del organismo) con los cambios en la expresión de los genes y su posterior alteración metabolómica (y por tanto lipidómica) en *D. magna*. A lo largo de esta tesis se ha abordado la capacidad de los EDCs y de los fármacos neuroactivos de afectar a la reproducción y perturbar la homeostasis lipídica, así como a las vías de señalización molecular que modulan esta perturbación. En el capítulo 2, se realizó un análisis transcriptómico mediante microarrays de hembras adultas de *D. magna* expuestas a algunos EDCs durante su etapa reproductiva y se estudiaron los efectos producidos en su lipidoma mediante un análisis lipidómico utilizando UHPLC-TOF MS. Se identificaron mecanismos transcripcionales comunes descritos con categorías funcionales relacionadas con la

energía, la muda y la reproducción, así como diferentes categorías funcionales de lípidos. Los resultados obtenidos permitieron vincular los efectos reproductivos con cambios en los perfiles de lípidos, así como con una alterada transferencia de lípidos de las hembras de *D. magna* a sus huevos. En el capítulo 3 se estudiaron los efectos lipidómicos de productos farmacéuticos neuroactivos en concentraciones ambientalmente relevantes y los mecanismos moleculares asociados a ellos. La hipótesis de que la serotonina puede participar en la regulación de la dinámica de los lípidos y las respuestas de la fecundidad en *D. magna* se confirmó mediante el análisis del lipidoma de clones con el gen triptófano hidrolasa silenciado. Por último, en el capítulo 4 se desarrolló un enfoque metabolómico dirigido para analizar neurotransmisores en *D. magna* y se empleó en el estudio de los efectos de fármacos neuroactivos que afectaban a su comportamiento cognitivo. Los resultados metabólicos se vincularon a la alteración transcripcional asociada estudiada a través del RNAseq, probando la idoneidad de estos organismos para estudios de neurotoxicidad ambiental.

En términos generales, los resultados obtenidos a lo largo de esta tesis permitieron vincular las vías de señalización transcriptómica con efectos metabolómicos (perfiles lipidómicos y de neurotransmisores) y con respuestas apicales (reproducción y comportamiento).

Summary

Environmental toxicology is undergoing a paradigm shift due to the new concerning environmental reality. Nowadays, to manage subtler and chronic effects of chemicals, either single or mixtures, is an imperative need, especially at low and environmentally relevant concentrations. Not least important is to deal with emerging contaminants (ECs), whose harmful effects in ecosystems and toxicity mechanisms are still unknown. Therefore, new strategies for assessing the toxicity of pollutants with greater environmental relevance must be developed, what requires the application of integrative approaches combining tools from different disciplines. Omic technologies allow the holistic measurement of effects at low levels of biological organization in high throughput platforms, and provide mechanistic data which may become essential in the development and application of more efficient and effective testing strategies. Overall, this thesis aimed to prove the importance of integrating omic and conventional toxicological approaches in order to obtain significant information that helps to unravel any new toxicity mechanism triggered by ECs on the aquatic environment using *Daphnia magna* (*D. magna*) as model species.

The ECs studied included suspected lipid disruptors (endocrine disrupting compounds, EDCs) and ECs that are known to affect the central nervous system (i.e. neuroactive pharmaceuticals and other chemicals). Different integrative approaches have been developed to assess the toxicity of these compounds by linking effects on reproduction and behavior (individual organism responses) with gene expression changes and its subsequent metabolomic (and thus lipidomic) disruption in *D. magna*. The ability of EDCs and neuroactive pharmaceuticals to affect reproduction and disrupt lipid homeostasis, as well as the molecular signaling pathways that modulate this disruption, has been addressed throughout the thesis. Within the chapter 2, microarray transcriptomic analysis of *D. magna* adult females exposed to some EDCs during reproduction was performed, together with the effects on their lipidome by a lipidomic analysis using UHPLC-TOF MS. Common transcriptional mechanisms were identified as energy-related categories, molting and reproduction, and different lipid functional categories. The obtained results allowed to link reproductive effects with changes in lipid profiles and disrupted transference of lipids to eggs in *D. magna* females. Lipidomic effects of neuroactive pharmaceuticals at environmental concentrations and the driven molecular mechanisms behind them were studied in chapter 3. The hypothesis that serotonin may be involved in regulating lipid dynamics and fecundity responses in *D. magna* was confirmed by the analysis of the lipidome of genetically

tryptophan hydrolase gene knockout clones. Finally, in chapter 4 a targeted metabolomic approached was developed to analyze neurotransmitters in *D. magna* samples and employed in the study of the effects of neuroactive pharmaceuticals that affected *Daphnias'* cognitive behavior. Metabolomic results were linked to the associated transcriptional disruption studied through RNAseq, probing the suitability of these organisms for environmental neurotoxicity studies.

Overall, the results obtained throughout this thesis allowed to link transcriptomic signaling pathways with metabolomic effects (lipidomic and neurotransmitter profiles) and with apical responses (reproduction and behavior).

Abbreviations and acronyms

20E	20-hydroxyecdysone
3-MT	3-methoxytyramine
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
6OH	6-hydroxydopamine

A

AAAD	Aromatic Amino Acid Decarboxylase
ACAT	Acyl-Coenzyme A: Cholesterol-O-Acyltransferase
ADHD	Attention Deficit Hyperactivity Disorder
ANOVA	Analysis of Variance
AO	Adverse Outcome
AOP	Adverse Outcome pathway
APO	Apomorphine
ASD	Autism Spectrum Disorder
ASTA-R2	Allatostatin A Receptor 2
ASTM	American Society for Testing and Materials
AUC	Area Under the Curve
AZ	Amplicon Size

B

BHT	Butylated Hydroxytoluene
BL	Body Length
BPA	Bisphenol A
BPAH	Bisphenol-A High
BPAL	Bisphenol-A Low
BWA	Burrows-Wheeler Alignment

C

CA	Concentration Addition
CAFF	Caffeine
CaMKII	Calcium/Calmodulin-Dependent Protein Kinase II
CAS	Chemical Abstract Service
CBZ	Carbamazepine
CBZH	Carbamazepine High
CBZL	Carbamazepine Low
Cda9	Chitin Deacetylase-like 9
Cdk5	Cyclin-Dependent Kinase 5
Cdk7	Cyclin-Dependent Kinase 7

cDNA Complementary DNA
CE Capillary Electrophoresis
CEs Cholesteryl Esters
CHH Crustacean Hyperglycemic Hormone
CHL Cholesterol
Cht3 Chitinase 3
CIM Cimetidine
CNS Central Nervous System

D

DEG Differentially Expressed Gene
DEP Differentially Expressed Probes
DESI Desorption Electrospray Ionization
DG Diacylglycerol
DIMS Direct Infusion Mass Spectrometry
DIPH Diphenhydramine
DNA Deoxyribonucleic Acid
DO Dissolved Oxygen
DPMRA Daphnia Photomotor Response Assay
DSP4 N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
DVOC DanioVision Observation Chamber
DVTCU DanioVision Temperature Control Unit
DZP Diazepam
DZPH Diazepam High
DZPL Diazepam Low

E

EC Emerging Contaminant
ECM Extracellular Matrix
EcR Ecdysone Receptor
EDC Endocrine Disrupting Compound
EI Electronic Impact
EPR Enhanced Photomotor Response
ER Endoplasmic Reticulum
ERA Environmental Risk Assessment
ESI Electrospray Ionization
EU European Union

F

F Slope
FA Fatty Acid
FC Fold-Change
FDR False Discovery Rate
FIA Flow Injection Analysis

FTICR Fourier-Transform Ion Cyclotron Resonance
FWHM Full Width at Half Maximum
FX Fluoxetine
FxH Fluoxetine High
FxL Fluoxetine Low

G

GABA γ-Aminobutyric Acid
GalT1 1,4-Galactosyltransferase
GC Gas Chromatography
Gish Gilgamesh
GO Gene Ontology
GSH Glutathione

H

H Habituation
HILIC Hydrophilic Interaction Chromatography
HPLC High-Performance Liquid Chromatography
HPTLC High-Performance Thin Layer Chromatography
HRMS High Resolution Mass Spectrometry

I

IDL Instrumental Detection Limit
ILCNC International Lipid Classification and Nomenclature Committee
IMI Imidacloprid
Inos Myo-Inositol-1-Phosphate Synthase
Ins1,4,5P_3 Inositol 1,4,5-Triphosphate
IP_3 Inositol 1,4,5-trisphosphate
Ire1 Inositol-Requiring Enzyme-1
IS Internal Standard
ISI Interstimulus Selected Interval
ISO International Organization for Standardization
IT IS Integrated Taxonomic Information System

J

JH Juvenile Hormone

K

KE Key Event
KEGG Kyoto Encyclopedia of Genes and Genomes
KER Key Event Relationship

L

LBS Ligation-Based Synthesis
LC Liquid Chromatography
L-DOPA 3,4-dihydroxyfenilalanina
LION Lipid Ontology

LoE Lines of Evidence
LOEC Lowest Observed Effect Concentration
Loess Locally Weighted Linear Regression
LOOCV Leave-One-Out Cross-Validation
LPC Lysophosphatidylcholine
LPE Lysohosphatidylethanolamine
LPG Lysophosphatidylglycerol
LPI Lysohosphatidylinositol
LPS Lysohosphatidylserine
LRP1 Low Density Lipoprotein Receptor Protein 1
LTQ-Orbitrap Linear Ion Trap-Orbitrap

M

mAChR Muscarinic Acetylcholine Receptor
MALDI Matrix-Assisted Laser Desorption Ionization
MDL Method Detection Limit
ME Matrix Effect
MEC Mecamylamine
MEM Memantine
MET Methoprene tolerant
MF Methyl Farnesoate
MFH Methyl Farnesoate High
MFL Methyl Farnesoate Low
MfR Methyl farnesoate Receptor
MG Monoacylglycerol
MIE Molecular Initiating Event
MQL Method Quantification Limit
MRM Multiple Reaction Monitoring
mRNA Messenger RNA
MS Mass Spectrometer
MTBE Methyl-Tert-Butyl Ether

N

nAChR Nicotinic Acetylcholine Receptor
NGS Next-Generation Sequencing
NIC Nicotine
NMDA N-Methyl-D-aspartate
NMR Nuclear Magnetic Resonance
NPLC Normal Phase Liquid Chromatography
NR Nuclear Receptor
NSAID Non-Steroidal Anti-inflammatory Drug
NT Neurotransmitter

O

OECD Organization for Economic Co-operation and Development
OPLS-DA Orthogonal Partial Least Square-Discriminant Analysis
OZ Offspring Size

P

PA Phosphatidic Acid
PAM Partition Around Medoids
PBS Phosphate-Buffered Saline
PC Phosphatidylcholine
PCA Principal Component Analysis
PCB Polychlorinated Biphenyls
PC-O Plasmanyl-Phosphatidylcholine
PC-P Plasmenyl-Phosphatidylcholine
PCPA Para-Chloro-DL-Phenylalanine
PCR Polymerase Chain Reaction
PE Phosphatidylethanolamine
PE Paired-End
PE-O Plasmanyl-Phosphatidylethanolamine
PE-P Plasmenyl-Phosphatidylethanolamine
PG Phosphatidylglycerol
PI Phosphatidylinositol
PI3K Phosphatidylionositol-3-Kinase
Pi3K21B Phosphatidylinositol 3-Kinase
PI-PLC PI-specific Phospholipases C
PKA Cyclic Adenosine Monophosphate-Activated Protein Kinase A
Pkcdelta Protein Kinase C Delta
PLA2 Phospholipase A2
PLS Partial Least Square
PLS-DA Partial Least Square-Discriminant Analysis
PP Pyriproxyfen
PPAR Peroxisome Proliferator Activated Receptor
PPH Pyriproxyfen High
PPL Pyriproxyfen Low
PPRE Peroxisome Proliferators Response Element
Pr Propranolol
PrH Propranolol High
PrL Propranolol Low
PS Phosphatidylserine
PUFA Poly-Unsaturated Fatty Acid

Q

QqQ Triple Quadrupole

qRT-PCR Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Q-TOF Quadrupole Time-Of-Flight

QTrap Quadrupole Ion Trap

R

RA Response Addition

REACH Registration, Evaluation and Authorization of Chemicals

RIN RNA Integrity Number

rl Rolled Kinase

RNA Ribonucleic Acid

RNAseq RNA Sequencing

Rok Rho Kinase

RPLC Reverse Phase Liquid Chromatography

RSD Relative Standard Deviation

Rt Retention time

RXR Retinoic X Receptor

S

S6k S6 Kinase

SAK Sak Kinase

SBS Sequencing By Synthesis

SCO Scopolamine

SD Standard Deviation

SDS-PAGE Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SE Standard Error

SIMS Secondary Ion Mass Spectrometry

SM Sphingomyelin

SPE Solid-Phase Extraction

SR Selectivity Ratio

SRC Steroid Receptor Coactivator

SREBP Sterol Regulatory Element-Binding Protein

SRM Single Reaction Monitoring

SSRI Selective Serotonin Reuptake Inhibitor

T

TBT Tributyltin

TG Triacylglycerol

TLC Thin Layer Chromatography

TMM Trimmed Means of M-Values

TNT 2,4,6-Trinitrotoluene

TOF Time-Of-Flight

TRH Tryptophan Hydrolase

U

UHPLC	Ultra-High Performance Liquid Chromatography
US	United States
US-EPA	United States Environmental Protection Agency
USP	Ultraspiracle

V

VIP	Variable Importance in Projection

W

WFD	Water Framework Directive

Objectives and thesis outline

Objectives

In recent decades, environmental toxicology is undergoing a paradigm shift due to the new concerning environmental reality. Nowadays, to manage subtler and chronic effects of chemicals, either single or mixtures, is an imperative need, especially at low and environmentally relevant concentrations. Not least important is to deal with emerging contaminants (ECs), whose harmful effects in ecosystems and toxicity mechanisms are still unknown. In order to address this problem and to develop strategies for evaluating the toxicity of pollutants with greater environmental relevance, it is necessary to build up integrative approaches combining tools from different disciplines. Unconventional testing of toxicity is needed, which represents the major task of modern environmental toxicology and of this thesis. Technology advances to measure effects at lower levels of biological organization in high throughput platforms (i.e. omic approaches) have potential to contribute to the development and application of more efficient and effective testing strategies.

Within this thesis, different integrative approaches have been carried out to assess ECs toxicity by linking effects at different levels of biological organization, i.e. reproduction and behavior as organism responses, gene expression (transcriptomics) and the subsequent metabolomic disruption (metabolomics and lipidomics). We focused mainly on the analysis of novel specific sublethal effects of ECs, specifically those classified as endocrine disruptors (chapter 2) and pharmaceuticals or neuroactive compounds (chapter 3 and 4), using the aquatic model organisms *Daphnia magna (D. magna)*.

In this context, the general objectives of this thesis are:

- To develop and apply high throughput transcriptomic and metabolomic techniques using *D. magna* as a model organism.
- To conduct integrative approaches at different levels of biological organization in order to unravel novel toxicity mechanisms produced by emerging contaminants.
- To study the effects of endocrine disruptors and neuroactive chemicals in *D. magna.*

In order to meet these main objectives, this thesis faces the following specific objectives:

- To study the ability of endocrine disrupting compounds (bisphenol A, methyl farnesoate, pyriproxyfen and tributyltin), many of them known as obesogens, to disrupt *D. magna* lipid homeostasis and their associated lipid molecular processes relating them with reproductive effects (Scientific articles I and II).
- To study the molecular mechanisms by which neuroactive pharmaceuticals (carbamazepine, diazepam, fluoxetine and propranolol) affect reproduction and lipid homeostasis in *D. magna* at environmentally relevant concentrations (Scientific articles III and IV).
- To test the hypothesis of the involvement of serotonin in lipid dynamics and reproduction (Scientific article IV).
- To develop and validate a targeted metabolomic strategy for the analysis of neurotransmitters in *D. magna* matrices (Scientific article V).
- To develop an integrative method to evaluate neurotoxicity using *D. magna* as model species, linking behavioral with transcriptional and metabolomic responses (Scientific article V and VI).

Thesis outline

This thesis is divided into five chapters:

Chapter 1: General introduction

An introduction of the aspects around which this thesis is articulated is presented. This chapter covers from more general aspects, such as the environmental crisis, emerging contaminants description and the model organism used throughout this thesis, as well as the different omic techniques (transcriptomics, metabolomics and lipidomics) and analytical approaches applied.

This section also includes a book chapter that was published as a result of the knowledge acquired throughout this thesis regarding transcriptomic studies with RNAseq:

- Fuertes, I., Vila-Costa, M., Asselman, J., Piña, B., Barata, C., 2020. Data Processing for RNA/DNA Sequencing, in: Comprehensive Chemometrics. Elsevier, pp. 507–514. https://doi.org/10.1016/B978-0-12-409547-2.14595-0

Chapter 2: Lipidomic and transcriptomic changes induced by compounds enhancing accumulation of storage lipids in *Daphnia magna.*

The study of the effects of endocrine disrupting compounds in *D. magna* molecular processes, particularly those associated with lipid homeostasis and fecundity consequences, is presented. First there is a short introduction to the topic and to the studied pollutants. Then, two publications where the results obtained have been reported are presented, and finally a cross-sectional discussion of the results obtained in both publications is addressed.

- Scientific article I:

  Fuertes, I., Jordão, R., Piña, B., Barata, C., 2019. Time-dependent transcriptomic responses of *Daphnia magna* exposed to metabolic disruptors that enhanced storage lipid accumulation. Environ. Pollut. 249, 99-108. https://doi.org/10.1016/j.envpol.2019.02.102

- Scientific article II.

  Fuertes, I., Jordão, R., Casas, F., Barata, C., 2018. Allocation of glycerolipids and glycerophospholipids from adults to eggs in *Daphnia magna*: Perturbations by compounds that enhance lipid droplet accumulation. Environ. Pollut. 242. https://doi.org/10.1016/j.envpol.2018.07.102

Chapter 3: Effects of environmental relevant concentrations of neuroactive pharmaceuticals on transcriptomic and lipidomic *Daphnia magna* responses.

An integrative study of the effects of low environmental relevant concentrations of human prescribed neuroactive pharmaceuticals in *D. magna* molecular processes, lipid homeostasis and fecundity effects is presented. First there is a short introduction to the topic and to the studied pollutants. Then, two published works enclosing the results are gathered, and finally a cross-sectional discussion of both articles is reported.

- Scientific article III:

  Fuertes, I., Campos, B., Rivetti, C., Pinã, B., Barata, C., 2019. Effects of Single and Combined Low Concentrations of Neuroactive Drugs on *Daphnia magna* Reproduction and Transcriptomic Responses. Environ. Sci. Technol. 53. https://doi.org/10.1021/acs.est.9b03228

- Scientific article IV:

  Fuertes, I., Piña, B., Barata, C., 2020. Changes in lipid profiles in *Daphnia magna* individuals exposed to low environmental levels of neuroactive pharmaceuticals. Sci. Total Environ. 139029. https://doi.org/10.1016/j.scitotenv.2020.139029

Chapter 4: *Daphnia magna* as a model organism for neurotoxicity studies.

The development and validation of a targeted metabolomic methodology for neurotransmitter analysis is presented. Furthermore, an integrative strategy to evaluate neurotoxicity using *D. magna* as model species linking behavioral, transcriptional and metabolomic responses is shown. First there is a short introduction to the topic and to the studied pollutants and measured neurotransmitters. Then, two scientific articles with the obtained results are reported, and finally a cross-sectional discussion of both publications is presented.

- Scientific article V:

  Fuertes, I., Barata, C., 2021. Characterization of neurotransmitters and related metabolites in *Daphnia magna* juveniles deficient in serotonin and exposed to neuroactive chemicals that affect its behavior: A targeted LC-MS/MS method. Chemosphere 263, 127814. https://doi.org/10.1016/j.chemosphere.2020.127814

- Scientific article VI:

  Fuertes, I., Piña, B., Barata, C., De Schampheleare, K., Asselman, J., 2021. Effects of behavior-disrupting neuroactive chemicals in *Daphnia magna* cephalic transcriptome. To be submitted to Environmental Science and Technology.

Chapter 5: Conclusions

This chapter includes the main conclusions of this thesis.

Chapter 1

General introduction

"Future generations are unlikely to condone our lack of prudent concern for the integrity of the natural world that supports all life."
Rachel Carson, *Silent Spring* (1962)

1.1. The social value of science facing an environmental crisis

It is important not to lose sight of what is indispensable. Science is and must continue being at the service of society and its needs. This aspect, sometimes forgotten, is especially relevant when we talk about environmental sciences due to the fact that the human being is an inseparable element of the environment in which he cohabits. Because of this, Humans produce changes in the environment for which we must take responsibility. This necessary care about the ecosystem where we live, as well as the demand to respond to social needs and to the advances, improvements or solutions that society is asking for, is what science offers solutions to. Environmental chemistry and toxicology are articulated around this motivation, as is the present thesis.

One of the greatest challenges that society, and thus science, is facing in the 21st century is that related to the environmental crisis. Humanity is constantly growing. According to the United Nations, the world's population is expected to increase by two billion people in the next 30 years, from 7.7 billion today to 9.7 billion in 2050 (United Nations, 2019). This growth, linked to our absolute dependence on chemical products, is putting environmental health at risk. The number of new substances and products entering our lives is increasing and the pace of their introduction is continuously accelerating. Every day, we surround ourselves with a multitude of chemicals used in industrial and agricultural applications, and pharmaceuticals and personal care products, many of which may be threatening the environment. Nevertheless, tools to assess their safety are barely increasing. There is a growing gap between assessment capacities and assessment needs (Hartung, 2011).

The emergence of environmental awareness should probably be attributed to Rachel Carson for her 1962 book "Silent Spring" (Paull, 2013). Since then, and during the last decades, social concern about the anthropogenic effect on the environment and its possible remediation has increased. In response to this, the scientific community has deeply focused on assessing the toxicity of widely distributed chemicals and their potential damage to the environment. Specific guidelines have been developed for the reduction of the use of compounds of particular concern, based on which many governments have legislated their use and developed environmental protection strategies. However, there are many chemical compounds whose presence in the environment has been reported but for which there is still no regulation. According to the Network of reference laboratories, research centers and related

organizations for monitoring of emerging environmental substances (NORMAN), "emerging substances" can be defined as substances that have been detected in the environment, but which are currently not included in routine monitoring programs at European Union (EU) level and whose fate, behavior and ecotoxicological effects are not well understood (NORMAN, 2016). According to the latest NORMAN list (February 2016), there are still 1,036 substances under this name. Despite some of them are not new chemical compounds and have been present in the environment for decades, their occurrence and potential effects are not currently elucidated.

1.2. Environmental toxicology and regulatory frameworks

Due to the increase in the amount of chemical compounds to which the environment is exposed, the need to possess tools to evaluate and detect the effects related to these pollutants arises. The study of these adverse effects is the main subject of modern environmental toxicology. In particular, environmental toxicology is defined as a multidisciplinary science (integrated by ecology, toxicology and environmental chemistry) that investigates the effect of pollutants at organismal, organ, tissue, cell, organelle and biochemical reaction levels (Tolleson, 2018). Based on this, the central mission of this discipline is to understand, describe and explain how and by what mechanism chemical compounds affect the environment, in order to develop measures to prevent these adverse effects. Thus, this discipline is a fundamental part of the environmental risk assessment (ERA). ERA was firstly introduced in 1980s, and since then it is compulsory in the EU to set up a comprehensive ERA for all new commercialized chemicals. It determines the nature and likelihood of harmful effects occurring to organisms such as humans, animals, plants, or microbes, due to their exposure to stressors like chemicals. ERA can be used in a retrospective or a prospective way, in order to estimate adverse effects after exposure has occurred or conversely to predict adverse effects based on estimated exposure. Thus, it is a useful tool for both estimating amounts of chemicals in environmental resources that are associated with minimal harm and guide environmental management decisions about chemicals (Society of Environmental Toxicology and Chemistry (SETAC), 2018).

In 2007, the Registration, Evaluation and Authorization of Chemicals (REACH) came into force in the European Union, which regulates and restricts the production and use of chemicals to minimize, from a protection point of view, the environmental impacts of chemicals throughout their whole life cycle (European Commission, 2006). According to this European regulatory framework, standardized toxicity tests should be used for chemical safety assessment (European Chemicals Agency, 2011).

1.3. The aquatic ecosystem

The aquatic ecosystem is one of the natural systems most affected by pollution, being the ultimate sink for many chemicals and their degradation products (Hampel et al., 2015). The discharge of pollutants into the aquatic environment is the outcome of countless anthropogenic activities, damaging the quality of the environment by rendering water bodies unsuitable and threatening the health of the living beings (Bashir et al., 2020). Chemical compounds can reach aquatic ecosystems through wastewater due to inefficient disposal in purification treatments but also through industrial and agricultural effluents, direct dumping, atmospheric deposition and runoff, among others. The list of contaminants that have been reported in the aquatic environment is extensive and includes radioactive elements, metals, industrial solvents and volatile organic compounds, agrochemicals, household products, fuel combustion, nanoparticles, personal care products, microplastics, antibiotics as well as a huge variety of prescription and nonprescription drugs and pharmaceuticals of human and veterinary medicine (Hampel et al., 2015). Despite the development of water treatment technologies, the increased emissions of these compounds and the introduction of compounds specially designed to be persistent and active such as pharmaceuticals, make chemical pollution a major threat to aquatic ecosystems (Hampel et al., 2015; Hughes et al., 2013; Larsson, 2014). Therefore, and linked to the invaluable connection between water and life, maintaining the quality of aquatic ecosystems represents one of the most important challenges for society in the 21st century.

Aware of this problem, specific regulatory frameworks have been established for the aquatic ecosystem. In 2000, in the EU came into force the Water Framework Directive (WFD) - EU Directive 2000/60/EC (European Commission, 2000), constituting one of the most important EU pieces of environmental legislation concerning the protection, enhancement and restoration of water bodies to date. It was a pioneering legislation that changed the paradigm of water management by shifting from an anthropocentric perspective of water of previous EU regulations (defining it as a resource for direct exploitation by humankind) to an ecocentric perspective, where water is seen as an ecosystem holder (Santos et al., 2021). WFD defines standard approaches and methodologies to assess the status of water bodies based on different lines of evidence (LoE), with especial emphasis on ecological data (biological communities) (Santos et al., 2021). It implicitly relies on a good knowledge of the ecosystem functioning under specific environmental conditions, an ambitious assumption considering the complexity and heterogeneity of aquatic ecosystems (Martinez-Haro et al., 2015), and taking into account the large amount of emerging

pollutants that are dumped into the environment every day. For this reason, the identification of ecological risks of chemical compounds to aquatic organisms is essential when establishing efficient regulations and legislations to protect water bodies.

1.4. New challenges and paradigm shift of environmental toxicology

In the past, high local levels of a limited number of chemical compounds were detected in the environment causing acute and chronic effects. However, the reality of environmental health today coupled with the development of analytical techniques that can detect more contaminants in lower concentrations (Richardson and Ternes, 2018) means that environmental toxicology issue has shifted to managing subtler, chronic effects of mixtures of chemicals at lower concentrations (Schmitt-Jansen et al., 2008). In order to address this problem and develop strategies for evaluating the toxicity of pollutants with greater environmental relevance, it is necessary to expand on integrative approaches combining tools from different disciplines. Traditionally, environmental toxicology has putatively evaluated harmful effects employing individual level assays focused on apical endpoints like mortality, reproduction and individual growth in animals exposed to high doses of pollutants (Bal-Price and Meek, 2017; Chevalier et al., 2015). In fact, this type of endpoints has been traditionally used in most standardized tests by organizations like Organization for Economic Co-operation and Development (OECD) and United States Environmental Protection Agency (US-EPA), without providing information regarding to toxic mechanisms and being maybe not sensitive enough to detect effects at low environmentally relevant concentrations and longer chronic exposure times. In order to understand possible harmful effects and yield deeper insights than the standard toxicity testing, mechanism-based approaches are necessary. Therefore, regulatory environmental toxicology is currently undergoing a transformation from a descriptive analysis of animal experiments to a pathway-based paradigm of cellular and molecular mechanisms (Hartung, 2011). Technology advances to measure effects at lower levels of biological organization in high throughput platforms that have potential to contribute to the development and application of mechanistic data in designing more efficient and effective testing strategies (Bal-Price and Meek, 2017). These strategies may include *in silico* models, *in vitro* assays, and short-term *in vivo* tests with molecular and/or biochemical endpoints (including “omics”) indicative of perturbation of biological pathways. In order to translate this information into apical responses applicable to risk assessment, the

adverse outcome pathway (AOP) framework was developed (Ankley and Edwards, 2018).

It was in 2007 that the National Academy of Sciences from the United States proposed in its report “Toxicity Testing for the 21st Century: a vision and a strategy”, that chemical safety assessment could be improved by shifting to an approach based on an explicit delineation of biological pathways (National Research Council, 2007). In 2012, the OECD initiated a program that aimed to evolve the concept of AOP, which was first introduced by Ankley et al., (2010). The AOP framework reflects an evolution of prior pathway-based concepts, most notably mechanism or mode of action, for assembling and depicting toxicological data across biological levels of organization (Ankley and Edwards, 2018). The objective of AOPs is to explain in a structured way causal relationships linking initial perturbation of a biological system after chemical interaction with molecular biological targets (molecular initiating event, MIE) to an adverse outcome (AO) through a sequential series of key events (KE) that reflect changes at different biological levels (Sachana, 2019). A general scheme if this AOP framework is represented in Figure 1.1. In other words, it aims to describe a full cascade of biological events (KE) after a molecular initiating event (MIE) to lead into an observable adverse effect (AO). MIE is the first KE that captures the interaction between a chemical and the biomolecules within an organism (e.g., receptor-ligand interaction, DNA binding, etc.), and it is the trigger for the following steps in the pathway. Between the MIE and the AO, there are a series of KEs that represent essential and empirically observable steps that need to take place across different levels of biological or physiological levels (i.e., cellular, tissue, organ). A KE can be the altered activity of an enzyme, upregulation or downregulation of a gene transcription, increased or decreased levels of a protein, of a metabolite, histopathological fingerprints in a tissue, functional changes of an organ, etc., and so a defined biological perturbation can progress and eventually culminate with the manifestation of a specific AO in either individuals or populations. The AO is a specialized key event (KE) that represents the final step of an AOP, which should be relevant to regulatory decision-making in chemical safety (i.e., corresponding to an apical endpoint or measurements that are done in a test guideline study such as developmental neurotoxicity, reproductive toxicity, etc.). Between each KE, key event relationships (KERs) are established, that are the causal and predictive linkage between one key event to another, describing the likelihood and conditions by which a particular KE trigger the next one (Bal-Price and Meek, 2017; Sachana, 2019). An important property of AOPs is that they are chemically agnostic, capturing response-response relationships that

result from a given perturbation of a MIE that could be caused by any stressor (Ankley and Edwards, 2018). AOPs have received substantial attention as an organizing framework for toxicologically-relevant biological information. The OECD defined best practices for development and assessment of AOPs (OECD, 2017, 2016), using this framework as a critical tool supporting the mutual acceptance of toxicological data by diverse regulatory authorities (Ankley and Edwards, 2018).

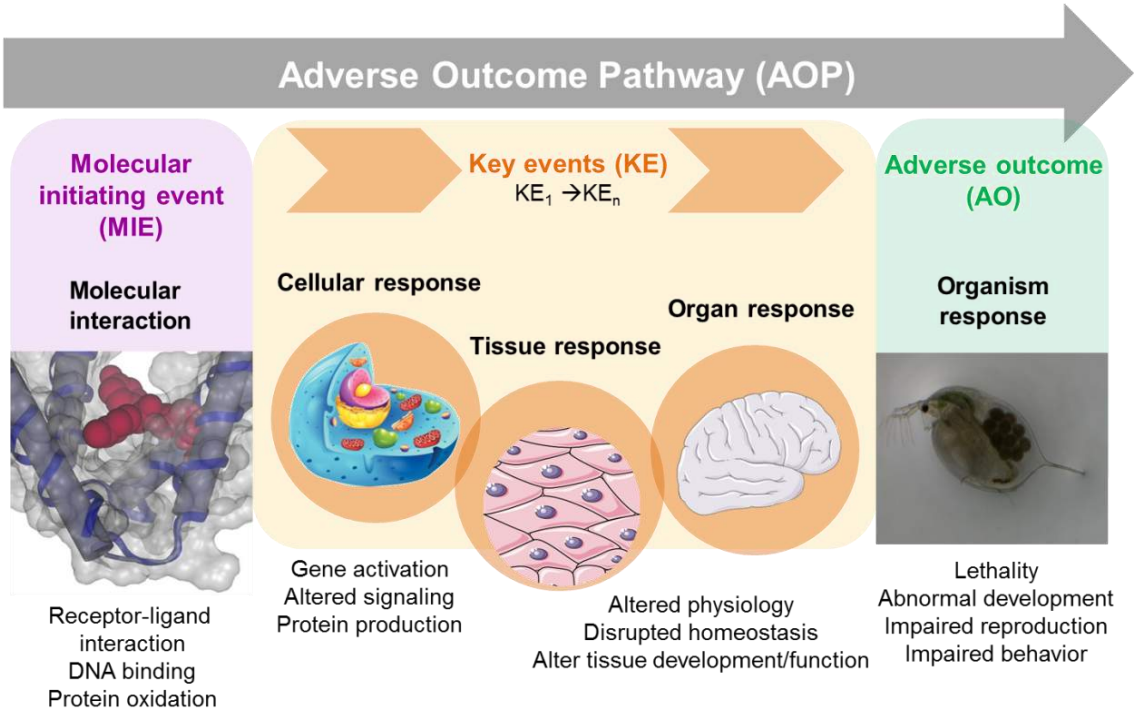

Figure 1.1. General scheme of an adverse outcome pathway (AOP) framework, based on Ankley and Edwards (2018); OECD (2017) and Sachana (2019).

A detailed mechanistic knowledge of observed effects would facilitate the development of alternative testing methods as well as help to prioritize higher tiered toxicity testing (Wittwehr et al., 2017). In the same manner, for the correct development of AOP, it is necessary to have mechanistic techniques and to understand KE biological processes. This would allow scientists to better extrapolate the results and make inferences about the resulting toxic potential, so that regulatory measures can be established for the pollutants causing these toxic effects and potential damage to the environment.

1.5. Emerging contaminants and associated new mechanism of toxicity

Emerging substances or emerging compounds (ECs) are environmental pollutants that have been widely investigated only in the last two decades and include, among others, both naturally occurring and anthropogenic chemicals such as pharmaceuticals and personal care products and their metabolites, illicit drugs, nanomaterials, flame retardants, plasticizers, food additives, polar pesticides, algal toxins and antibiotic resistance genes (Noguera-Oviedo and Aga, 2016; Richardson and Kimura, 2017; Ternes et al., 2015).

As aforementioned, while these contaminants are called “emerging”, most of them have been present in the environment for many years or even decades (Richardson and Kimura, 2017). The reason for this denomination may be due to either they are new compounds for which no adequate toxicological data is available, or they are already known compounds whose toxic effects have been insufficiently characterized or that have not been previously detected (Sauvé and Desrosiers, 2014). This deficiency in the environmental detection of compounds was due to the lack of highly sensitive and powerful analytical instrumentation, that allowed identification and trace quantification of unknown contaminants in complex environmental matrices (Noguera-Oviedo and Aga, 2016). The improvement of analytical methods within the last decades (mainly high efficiency chromatographic separations coupled to high-resolution mass spectrometers) has pushed the levels of detection in many denominated ECs up to the part per trillion (ng/L) or even parts per quadrillion (pg/L) range, allowing the monitoring of harmful chemical compounds that were already known and present but remained undetected in environmental matrices due to their low concentration (Richardson and Ternes, 2018; Tang et al., 2019). Because of this, there are a growing number of chemical compounds whose presence in the environment is now known, and whose toxicity and harmful effects are currently being studied and reported. Reliable analytical and toxicity assessment methods are the basis of either the management or the elimination of ECs (Tang et al., 2019). Although most of these emerging pollutants show low acute toxicity, their potential sublethal effects and toxicological mode of action is unknown or only known in humans (Claessens et al., 2013; Cristale et al., 2013; Gibs et al., 2007; Hutchinson et al., 2013; Noguera-Oviedo and Aga, 2016). Unconventional testing of effects and toxicity are needed (Noguera-Oviedo and Aga, 2016), which represents the major task of modern environmental toxicology, and therefore of this thesis.

Wastewater effluents are one of the major sources for many of these emerging contaminants due to their use in products like pharmaceuticals, detergents, fabric coatings, foam cushions, lotions, sunscreens, cosmetics, hair products, foods and beverages and food packaging. After household use, these chemicals are released in wastewater, and because many of them are incompletely removed in wastewater treatment plants (Bueno et al., 2012; Fuertes et al., 2017) they enter our rivers and drinking water supplies. Issues surrounding these emerging contaminants include widespread occurrence, bioaccumulation, persistence and toxicity. Furthermore, these compounds are continuously being introduced into the environment, so they do not even have to be persistent to compensate their transformation or removal rate. Many of these pollutants do not exert acute toxicity like some of the traditional contaminants, but exert their effects in more subtle ways; e.g. through endocrine disruption or impairing specific responses as reproduction or behavioral performance (Richardson and Kimura, 2017).

Between some of the safety concerns that have arisen are endocrine disruption, and neurotoxicities (Hartung, 2011). Hereupon, within this perspective, in this thesis we focused mainly on the analysis of novel specific sublethal effects of ECs, specifically those classified as endocrine disruptors (in particular lipid disruptors or obesogens) and pharmaceuticals or neuroactive pharmaceuticals.

1.5.1. Endocrine disrupting compounds as lipid disruptors

Endocrine disrupting compounds (EDCs) are defined as exogenous chemicals that can interfere with any aspect of the endocrine system (Barouki, 2018; Janesick and Blumberg, 2016), by mimicking, blocking and/or altering hormone roles and metabolism. Although more than 1,300 chemicals have been identified to potentially interfere with hormonal metabolism, very few have been screened for their capacity to cause endocrine effects *in vivo* (Capitão et al., 2017). EDCs have a variety of applications, as synthetic hormones (e.g. ethynilestradiol), plastics (e.g. bisphenol A and phthalates), pesticides and fungicides (e.g. organitins and chlorpyrifos), flame retardants (e.g. perfluoroalkyl compounds), solvents (e.g. polychlorinated biphenyls (PCBs) and dioxins), pharmaceuticals (e.g. thiazolidinediones, atypical anti-psychotics, antihistamines, antidepressants) and personal care products (e.g. triclosan) (Barouki, 2018; Capitão et al., 2017). Some of these compounds can be bioaccumulated and biomagnificated through the food-chain, being persistent in the environment (Capitão et al., 2017). Endocrine disruption can provoke effects as estrogenicity, androgenicity, obesity, lipid and adipogenesis dysregulation, thyroid dysregulation, steroid-related

pathways alteration, infertility, developmental problems and several metabolic disorders (Papalou et al., 2019), and thus alterations in reproduction, behavior and neural system (Brander, 2013).

Among all the possible alterations produced by EDCs, those related with lipid metabolism are of particular interest (Maradonna and Carnevali, 2018). In 2002, Baillie-Hamilton suggested for the first time that the current obesity epidemic could be associated to the exponential increase of the production of chemical products, establishing a link between obesity and the exposure to environmental pollutants, including some pesticides, solvents, plastics, flame retardants and heavy metals (Baillie-Hamilton, 2002). However, it was not until 2006 when Grün and Blumberg introduced the term “obesogen” (Grün and Blumberg, 2006), defining it as a group of EDCs that inappropriately regulate and promote lipid accumulation and adipogenesis (Grün and Blumberg, 2009, 2007). Adipose tissue is a true endocrine organ and therefore highly susceptible to disturbance by EDCs (Janesick and Blumberg, 2016). This “obesogen” or lipid disruptor subset of EDCs have the potential to interact with hormone receptors and neuroendocrine signaling, leading to a disruption of the lipid homeostasis (balance between capture, transport, storage, biosynthesis, metabolism and lipid catabolism) on organisms (Barata et al., 2004; Haeba et al., 2008; LeBlanc, 2007; Wang et al., 2011; Wang and LeBlanc, 2009). Due to the relationship between lipid metabolism deregulation with several important human diseases, the mode of action of these compounds is now under strong scrutiny (Capitão et al., 2017; Castro and Santos, 2014; de Cock and van de Bor, 2014; Grün and Blumberg, 2006; Machado Santos et al., 2012).

However, little is known about effects of EDCs in non-vertebrates (Haeba et al., 2008). The concern over endocrine disruption in aquatic invertebrates has been highlighted by two well-known examples: tributyltin inducing reproductive damage and population declines in mollusks (Hutchinson, 2002), and ethinylestradiol alterating the fecundity and sex ratio of fish (Runnalls et al., 2015; Soares et al., 2009). Studies on EDCs conducted in small crustaceans such as copepods and *Daphnia* often assume that these chemicals only produce effects on developmental rate, growth and reproduction by disrupting pathways that control maturation and/or associated with reproduction. Nevertheless, other toxic effects such as stage or sex related differences in sensitivity (i.e. survival) to EDCs or reduced energy resources acquisition may also affect the above mentioned life history traits (Barata et al., 2004). In fact, linked to the fact that EDCs can interact with endocrine receptors, affect hormonal levels and deregulate their metabolism, often reported effects of certain EDCs in *Daphnia* include

alterations in testosterone metabolism (Baldwin and LeBlanc, 1994), perturbations in the moult cycle (Zou and Fingerman, 1997), growth delay (Leblanc and Mclachlan, 1999), developmental abnormalities (Olmstead and LeBlanc, 2000) or modulations of fecundity (Bryan et al., 1986). The intracellular lipid flux and the adipocyte proliferation and differentiation are controlled by gene networks that are regulated by a number of master transcriptional regulators (Grün and Blumberg, 2009). In vertebrates, the mode of action of some EDCs that show obesogenic effects is known (Grün et al., 2006; Grün and Blumberg, 2006; Tingaud-Sequeira et al., 2011). Equally, is known that in arthropods, some effects of EDCs seem to be mediated by steroid or ecdysteroid regulated processes acting via receptors and transcription factors in a way similar to vertebrates (Baldwin and LeBlanc, 1994; Haeba et al., 2008). Although an increasing number of studies report changes in lipid metabolism and related gene pathways in invertebrates after exposure to ECDs (Jordão et al., 2016, 2015; Sengupta et al., 2017; Seyoum et al., 2020), it is still necessary to complement ecotoxicological studies with genomic and metabolomic technologies (i.e. omic technologies) in order to unravel the mode of action of EDCs that help us to understand the complexity of the effects and consequences of the exposure to these compounds, particularly in invertebrates within the aquatic environment.

a. Transcriptional factors controlling endocrine signaling and lipid homeostasis

Nuclear receptors (NRs) are a large family of proteins responsible for the control of diverse processes, from sexual characteristics and behavior to different metabolic pathways (e.g. oxidative metabolism or lipid imbalance) in vertebrates or moulting and reproduction in invertebrates. NRs are activated or antagonized by their binding to natural molecules (ligands), and both act as homo- or heterodimers that bind to specific DNA elements in order to promote transcriptional activation of target genes (Castro and Santos, 2014; Johnson and O'Malley, 2012). Chemical compounds can interfere in this interaction between a NR and its natural ligand (Wang et al., 2007). Ligand binding typically occurs at rather low concentrations in the nanomolar or micromolar range, which makes them prime targets of ECDs (Castro and Santos, 2014). Classically, EDCs have been documented to act by their ability to bind (in an agonist or antagonist mode of action) NRs related with the endocrine system, such as androgen receptors, estrogen and estrogen-related receptors, aryl hydrocarbon receptor, pregnane X receptors, constitutive androstane receptors, glucocorticoid receptors, retinoid X receptors, thyroid hormone receptors, peroxisome proliferator-activated receptors or farnesoid X receptors (Capitão et al., 2017; Lauretta et al., 2019). However, other EDCs' modes of action have also been described, such as

interference with the biosynthesis, transport and/or elimination of natural hormones, direct disruption of enzymatic activity along a metabolic pathway or deregulation of the NRs transcription (Capitão et al., 2017; Lauretta et al., 2019; Roig et al., 2013; Yang et al., 2015).

In particular, effects and key events produced by obesogenic EDCs has been mostly related in vertebrates with the activation of the retinoid X receptor (RXR) and the peroxisome proliferator activated receptors (PPARs), key regulators in several processes related with lipid metabolism and homeostasis (Lempradl et al., 2015; Yang et al., 2015). PPARs are crucial in the regulation of fat storage and fatty acid (FA) β-oxidation, and each of its three isoforms described in mammals (α, β, γ) has a specific function: PPARα regulates enzymes involved in the up-take, esterification and β-oxidation of FAs, PPARβ regulates FA oxidation specifically in the muscles and PPARγ regulates and is essential for adipocyte differentiation and survival, lipogenesis and regulation of insulin sensitivity, contributing to energy storage (Capitão et al., 2017; Lefterova et al., 2014; Lempradl et al., 2015). PPARs isoforms form heterodimers with retinoic X receptor (RXR) isoforms (α, β, γ), whose main natural ligand is the 9-cis-acid retinoic. PPAR/RXR heterodimers have been reported to affect different target genes related to ketogenesis (e.g. hydroxymethylglutaryl-CoA synthase), lipid transport (e.g. lipoproteins), lipogenesis (e.g. fatty acid desaturases or stearoyl-CoA desaturase), cholesterol metabolism (e.g. liver X receptor alpha or cholesterol monooxygenases), FA transport (e.g. fatty acid binding protein, lipoprotein lipase, acyl-CoA synthetase or low density lipoprotein receptor) and oxidation (e.g. acetyl-CoA acyltransferase, acyl-CoA oxidase or acyl-CoA dehydrogenase), and thus disrupting lipid metabolism, as well as target genes related to adipocyte differentiation (e.g. fatty acid binding protein or adiponectin) or gluconeogenesis (e.g. glycerol kinase or phosphoenolpyruvate carboxykinase) (Desvergne et al., 2006; Desvergne and Wahli, 1999; Feige et al., 2006; Hauner, 2002; Mandard et al., 2004; Savage, 2005; Semple, 2006; Steinberg et al., 2008; Takada and Kato, 2005).

The master nuclear receptor heterodimer related to adipogenesis is formed between PPARγ and RXRα, that are involved in the regulation of food intake, metabolic efficiency and energy storage (Grün and Blumberg, 2007; Lefterova et al., 2014; Machado Santos et al., 2012; Wafer et al., 2017; Yang et al., 2015). This PPARγ/RXRα heterodimer binds to peroxisome proliferators response elements (PPREs) in several target genes to regulate their transcription (Berger and Moller, 2002; Lefterova et al., 2014; Yang et al., 2015; Zhang et al., 2015), as e.g. apolipoprotein transcription, that encapsulate neutral lipids as triglycerides or cholesterol esters and transport lipid

molecules through the plasma (Cindrova-Davies et al., 2017; Dahabreh and Medh, 2012). Deregulation of apolipoprotein transcription has been related to obesity in some species (Oka et al., 2010). A general overview of PPARγ/RXRα heterodimer formation is shown in Figure 1.2.

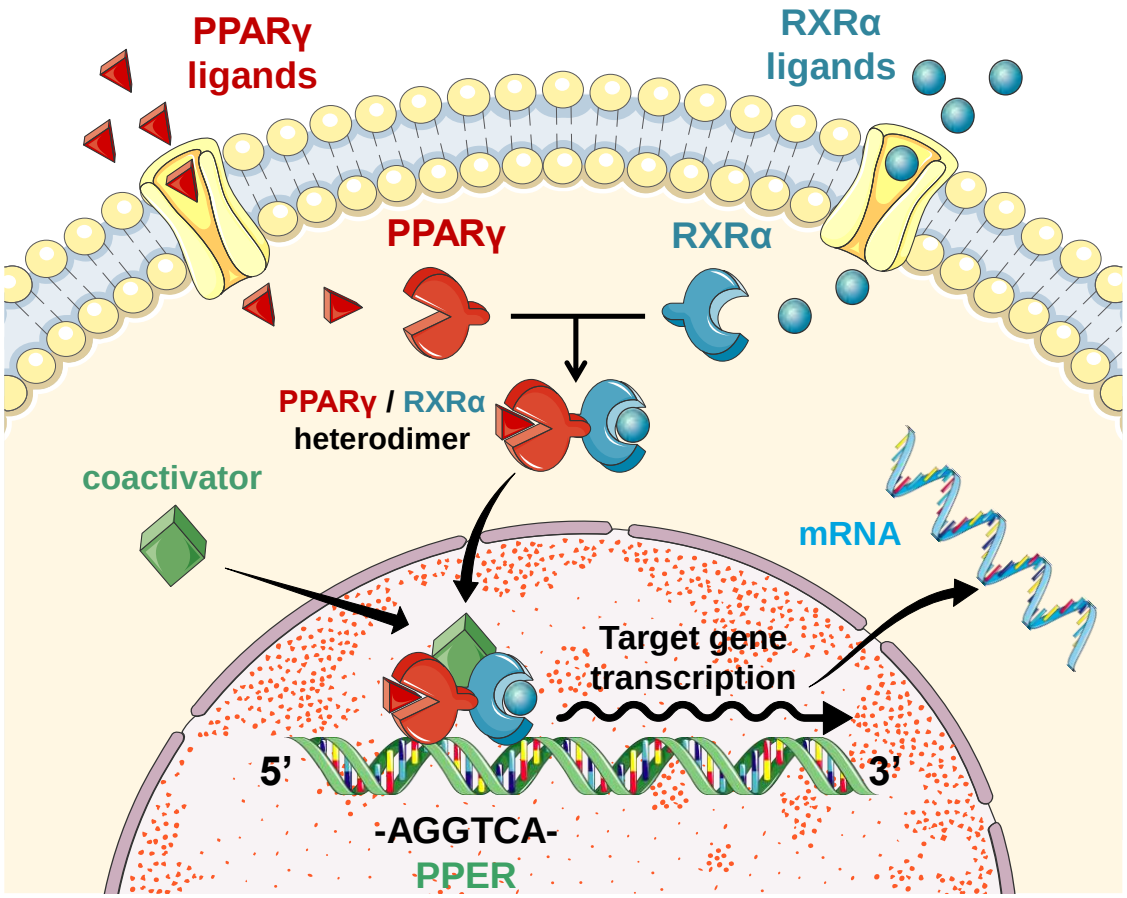

Figure 1.2. Schematic diagram of the formation of the PPARγ/RXRα heterodimer, from the cellular cytosol to the nucleus, where it binds to peroxisome proliferators response elements (PPREs) in several target genes to regulate their transcription in concert with nuclear coactivators (based on Choudhuri et al., (2018), Sainis et al., (2008) and Wagner et al.,(2010)).

b. Endocrine signaling in invertebrates

Invertebrates represent more than 95% of the known animal species and they have key roles in ecosystem functions. However, most research on NR-mediated disruption has focused on vertebrate models, significantly hindering our understanding on the wider biological and phylogenetic impact of EDCs and emerging contaminants (Castro and Santos, 2014). Very few studies have linked NRs to endocrine perturbation in invertebrate phyla (Gesto et al., 2013), most likely related to the lack of knowledge of invertebrate endocrinology and genomic NR collection (Castro and Santos, 2014).

Although many NRs remain largely uncharacterized in invertebrates (e.g. PPAR), RXR is perhaps the best characterized example of NR functions (Castro and Santos, 2014), originally called ultraspiracle (USP) in insects (Machado Santos et al., 2012). Ecdysteroids and juvenile hormones (JHs) are two major insect hormone families famous for their roles in development, moulting, reproduction and metamorphosis, that act via nuclear receptors, thereby regulating the transcription of several response genes (Lenaerts et al., 2019). In arthropods, ecdysteroid hormones regulate these varieties of activities by binding to a heterodimeric complex of nuclear receptors, the ecdysone receptor (EcR) and RXR (André et al., 2014; Lenaerts et al., 2019). The ecdysteroid 20-hydroxyecdysone (20E) is the natural ligand of EcR, synthetized from cholesterol through a series of oxidation and hydroxylation steps. Ecdysteroids are particularly well characterized with respect to their role in regulating the moulting process (ecdysis) and in different aspects of female reproductive physiology (Lenaerts et al., 2019; Mykles, 2011). JH are acyclic sesquiterpenoid hormones that regulate important physiological and development processes among arthropods, including metamorphosis, moulting, growth, reproduction and sex determination (Miyakawa et al., 2013). JH acts via the methoprene tolerant (MET) receptor (Jindra et al., 2015; Konopova and Jindra, 2008; Lenaerts et al., 2019). In crustaceans, JH lost the epoxide group becoming methyl farnesoate (MF) (Mu and Leblanc, 2004). MF has many regulatory functions such as reproductive maturation in decapods, where it increases production of vitellogenin and eggs, hence stimulatiing gonadal maturation. It has been reported that high levels of MF in reproductive *Daphnias* result in the production of male offspring (LeBlanc, 2007). Although it is still unclear how RXR may interact with the JH signalling pathway in arthropods, some studies have identified a putative methyl farnesoate receptor (MfR) complex in *Daphnia pulex* and *Daphnia magna* (Kakaley et al., 2017; LeBlanc et al., 2013; Miyakawa et al., 2013), that include MET transcription factor and the steroid receptor coactivator (SRC), involved in the reception and signal transduction of MF. It is not known if the putative MfR complex can dimerize with RXR but one of its components (SRC) induces structural changes in agonist bound nuclear receptors like RXR (Johnson and O'Malley, 2012).

1.5.2. Pharmaceuticals as neuroactive compounds

Pharmaceutical products are a wide variety of organic compounds used in the prevention and treatment of human and animal diseases (Bottoni et al., 2010). They represent an important group of ECs because of their widespread presence in environmental waters, contamination of drinking water and their potential to produce

adverse impacts on ecosystems and humans (Richardson and Kimura, 2017). In the last century, as a result of the rapid medical advances, a still increasing number of new medications and treatments have been developed, thus resulting in an increased consumption of drugs and their consequent release into wastewaters. Nowadays, more than 3,000 different pharmaceuticals are available on the market, including analgesics, antibiotics, neuro-active compounds (e.g. antidepressants) or lipid regulators, among others (Bottoni et al., 2010; Fent et al., 2006). During the last decades there has also been an increase in the occurrence of psychiatric disorders, thus triggering a particular increase in the use of neuroactive pharmaceuticals, i.e. antidepressants, anxiolytics and sedatives (Calisto and Esteves, 2009). These compounds are excreted through feces and urine as a mixture of unmetabolized pharmaceuticals and their metabolites. Altogether, they can reach water bodies through domestic, urban and hospital wastewater, effluents from sewage treatment plants, aquaculture, and livestock farming (Bottoni et al., 2010; Hughes et al., 2013), and also industrial discharges from drug manufacturing (Larsson, 2014). At present, the presence of antibiotics, steroids, blood lipid regulators, estrogens, painkillers, anti-inflammatories, antiseptics, antihypertensive drugs, antiepileptics, antidepressants, antineoplastic agents and other substances in surface water bodies is well-documented (Bottoni et al., 2010). As a consequence of their extensive application, together with their tendency of persistence and accumulation, pharmaceuticals can reach water concentrations of ng/L to µg/L.

Despite representing a major group of environmental contaminants over the past decade, relatively little is known about their occurrence and consequences on freshwaters compared to other pollutants, one of the reasons for which they are considered ECs (Bottoni et al., 2010; Fabbri, 2015; Fent et al., 2006; Hughes et al., 2013; Richardson and Kimura, 2017). These molecules are designed to produce a therapeutic effect on the body, usually active at very low concentrations, they can pass through biological membranes and persist in the body long enough to avoid being inactivated before having an effect (Bottoni et al., 2010). Thus, experimental evidence indicates that pharmaceuticals may cause adverse effects on the environment, such as morphological, metabolic and sex alterations on aquatic species at environmental relevant concentracions, and induction of antibiotic resistance in aquatic pathogenic microorganisms (Bottoni et al., 2010). Several countries have reported the present of recalcitrant pharmaceuticals in water after wastewater treatment. Triclosan, ibuprofen, carbamazepine and clofibric acid are some ot the compounds detected in higher concentrations, up to high ng/L levels (Hughes et al., 2013; Richardson and Kimura, 2017). While many pharmaceuticals have the potential to adversely impact

ecosystems, most of the reported environmental concentrations are well below the lowest observed effect concentrations (LOECs). However, some pharmaceuticals (e.g. diclofenac, clofibric acid, salicylic acid, propranolol, fluoxetine, and carbamazepine) can be found in the environment at levels close to the LOECs (Richardson and Ternes, 2014, 2011). Furthremore, due to their ocurrence levels at the ng/L range, the risk of acute toxicity is thought to be negligible. However, there are substantial knowledge gaps in terms of chronic effect, long-term exposure of non-target aquatic organisms and the effects on ecosystem functioning (Hughes et al., 2013). In rivers, concentrations within the range known to cause acute or chronic toxicity in aquatic systems have been reported for antibiotics, painkillers, antidepressants, anxiolytics and antidepressants, highlighting the need for the adoption of more realistic ecotoxicological experiments (Hughes et al., 2013). Their highly specific biological activity (i.e. neuroactive pharmaceuticals) on the neuroendocrine system makes them an important group of pharmaceuticals for evaluating ecotoxicological effects in aquatic non-target organisms (Fong and Ford, 2014; van der Ven et al., 2006). As a matter of fact, several authors have already reported effects on invertebrates at very low concentrations (Calisto and Esteves, 2009; Campos et al., 2012; Fent et al., 2006; Ford and Fong, 2016; Rivetti et al., 2016).

It is likely that pharmaceutical consumption will increase in coming years, particularly in developing countries and those with aging human demographics. (Daughton, 2003; Hughes et al., 2013). Regrettably, pharmaceutical compounds currently receive minimal consideration by regulators and policy makers (Farré et al., 2008). For this reason, more research is needed to shed light on the harmful effects of these emerging contaminants on the environment, and on water ecosystems in particular as one of the major environmental compartments affected by their continued discharge.

1.6. *Daphnia* as environmental toxicity model organism

Invertebrates include approximately the 95% of all know animal species and are ecologically indispensable in the trophic transfer of nutrients and carbon. Furthermore, many invertebrate species also can serve as biological sentinels of environmental insult. Identification and characterization of the effects of ECs on invertebrate species may prove useful in recognizing and characterizing them before they are elicited in vertebrate populations, including humans.

Daphnia magna (*D. magna*), a freshwater micro-crustacean, is advocated as one of the best model organisms for environmental toxicology studies in freshwater ecosystems and was used as test organism throughout this thesis. The taxonomy classification of *Daphnia magna* is shown in Table 1.1.

Table 1.1. Taxonomy classification of *D. Magna* Strauss, retrieved (01/10/20) from the Integrated Taxonomic Information System (ITIS) on-line database (http://www.itis.gov).

Kingdom	Animalia
Subkingdom	Bilateria
Infrakindom	Protostomia
Superphylium	Ecdysozoa
Phylum	Arthropoda
Subphylum	Crustacea
Class	Branchiopoda
Order	Diplostraca
Suborder	Cladocera
Family	Daphniidae
Genus	*Daphnia*
Species	*Daphnia Magna* Straus

D. magna is a brackish and freshwater organism, found in lakes and ponds with low fish predation pressure all around the world. It plays a key ecologycal rool in the trophic chain, being one of the most important primary consumers and fundamental to control algae blooms, while is also important as a food source for both invertebrates and vertebrates predators (De Gelas and De Meester, 2005; Lampert, 2011). *Daphnia* species are non-selective filter feeders, mainly grazing on algae, but able to retain and ingest without selection all the suspended particles that can be withheld by their filtering apparatus (Gillis et al., 2005). Due to this, as filter feeders, these small crustaceans are exposed to numerous environmental insults to which they respond via numerous mechanisms.

Like all crustaceans, *D. magna* has an exoskeleton, consisting of a dimerous chitinous carapace. This exoskeleyon is non-elastic, as a consequence of which growth is only possible by regular renewals of it, a phenomenon known as moulting (Ebert et al., 1991). *D. magna* has two pairs of antennas: the first are known to function as sensory organs and the second are modified for swimming. Due to the position and

movement of this second antenna, *Daphnia* locomotion activity is mainly restricted to vertical movements, presenting its characteristic movement of hop-and-sink. In the head region, there are the composed eye, the brain, connected to the naupliar eye and the gut opening (mouth) (Ebert et al., 1991). The anatomy and morphology of *D. magna* is shown in Figure 1.3.

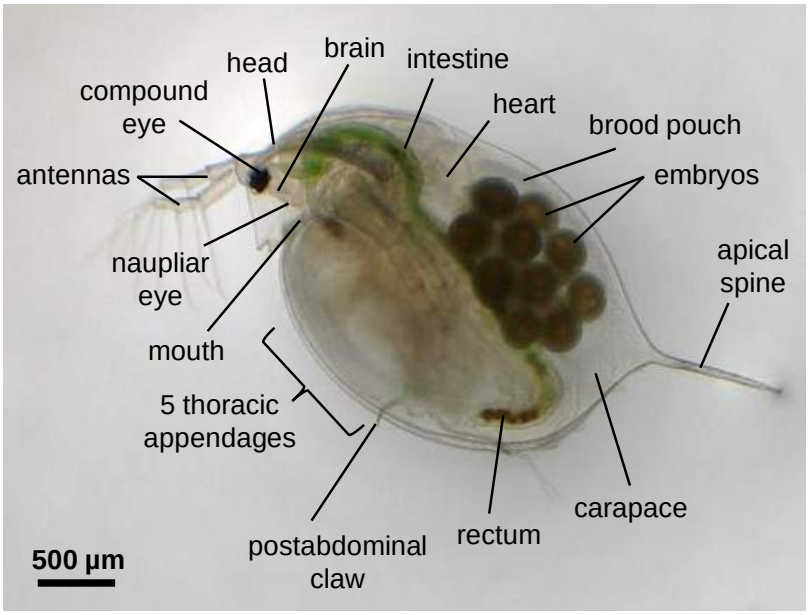

Figure 1.3. Photography of an adult *D. magna* used as model organism throughout this thesis, with annotations of its functional anatomy.

This species is internationally recognized as an indicator of environmental health and fitness and, consequently, as an important and widely used bioassay of aquatic toxicology. In particular, *Daphnia* is recognized as a human health model organism by the US National Institutes of Health (Colbourne et al., 2011) and it is formally endorsed in testing bioassays by major international institutions such as OECD and International Organization for Standardization (ISO). During the last decades, *Daphnia* has been successfully used as a model organism in a broad range of applications, from ecology and evolution (Lampert, 2011; Stollewerk, 2010) to different disciplines of toxicology (Colbourne et al., 2011; J. R. Shaw et al., 2008). In addition to its use in traditional toxicological assays, it has been used in an increasingly number of studies where different "omic" techniques have been applied. The close relationship of crustaceans and insects in both molecular and morphological traits makes *Daphnia* a valuable outgroup for comparative genomic studies (Le et al., 2016). The genome of *D. pulex* was recently published (Colbourne et al., 2011) and the transcriptome of its close relative *D. magna* was fully assembled in 2016 (Orsini et al.,

2016). As a result, it has also been suggested as a primary model species for genomics and transcriptomics studies (Colbourne et al., 2011; Ebert, 2011; Orsini et al., 2016, 2011). In addition to this, it has been used as a model organism in e.g. metabolomic (Nagato et al., 2016; Sengupta et al., 2016; Taylor et al., 2018) or proteomic studies (Otte et al., 2019; Trotter et al., 2015). In this perspective, the possibility of combining modern "omic" tools together with well-documented traditional and standardized toxicological approaches, as well as community and ecosystem impacts, makes *Daphnia* the ideal species for integrative investigation of mechanisms of action and development of AOPs that underlie responses to environmental changes (Miner et al., 2012; J. R. Shaw et al., 2008).

A peculiar characteristic of *Daphnia* is its unusual life cycle (Figure 1.4), which has been studied for more than 150 years (Lubbock, 1857), and makes it an easy to use species in environmental science (Colbourne et al., 2011). *Daphnia* reproduces through cyclical parthenogenesis, and it is able to produce two different types of eggs in response to surrounding environmental cues. In presence of optimal environmental and living conditions *Daphnia* reproduces through cyclical parthenogenesis, producing diploid eggs. During this phase, the population is composed exclusively by females, that produce genetically identical offspring (Hebert and Ward, 1972). Nevertheless, changes in the environmental conditions (e.g. food limitation, high population densities, short photoperiod or desiccation) can activate sexual reproduction, leading to the production of haploid sexual eggs and thus both male and female offspring (Brown and Banta, 1935; Hobaek and Larsson, 1990; Kleiven et al., 1992; Stuart et al., 1932). Likewise, sex determination is also environmentally driven and males showing sexual dimorphism are able to be produced in response to suitable environmental prompts indicating fast adaption to external stimuli (Kleiven et al., 1992; Weider and Pijanowska, 1993). The produced haploid eggs are fertilized by the male during mating to form diapausing resting eggs contained in an ephippium. These resting eggs can lay dormant in the sediment for prolonged periods of time, and hatch when environmental conditions improve (Orsini et al., 2013; Schwartz and Hebert, 1987).

D. magna life cycle is ideally suited for experiments due to its short reproduction time when compared to most eukaryotic model species: under optimal laboratory conditions (at temperature of 20 °C, daily photoperiod of approximately 12 h and abundant food) the reproductive maturity is reached within six to ten days and a reproductive batch occurs every three days hereafter (Ebert, 1991). In addition, due to the asexual clonal reproduction, the genetic background of *Daphnia* can be kept constant and genotypes can be maintained completely and continuously, providing an

exceptional opportunity to study responses to environmental stimuli in a defined and constant genetic background with unlimited replication (Le et al., 2016). On the other hand, clonal lines with different genetic background can also be maintained to create experimental populations with controlled genetic variation and studying the genetic architecture underlying phenotypic variation in natural populations (Orsini et al., 2012).

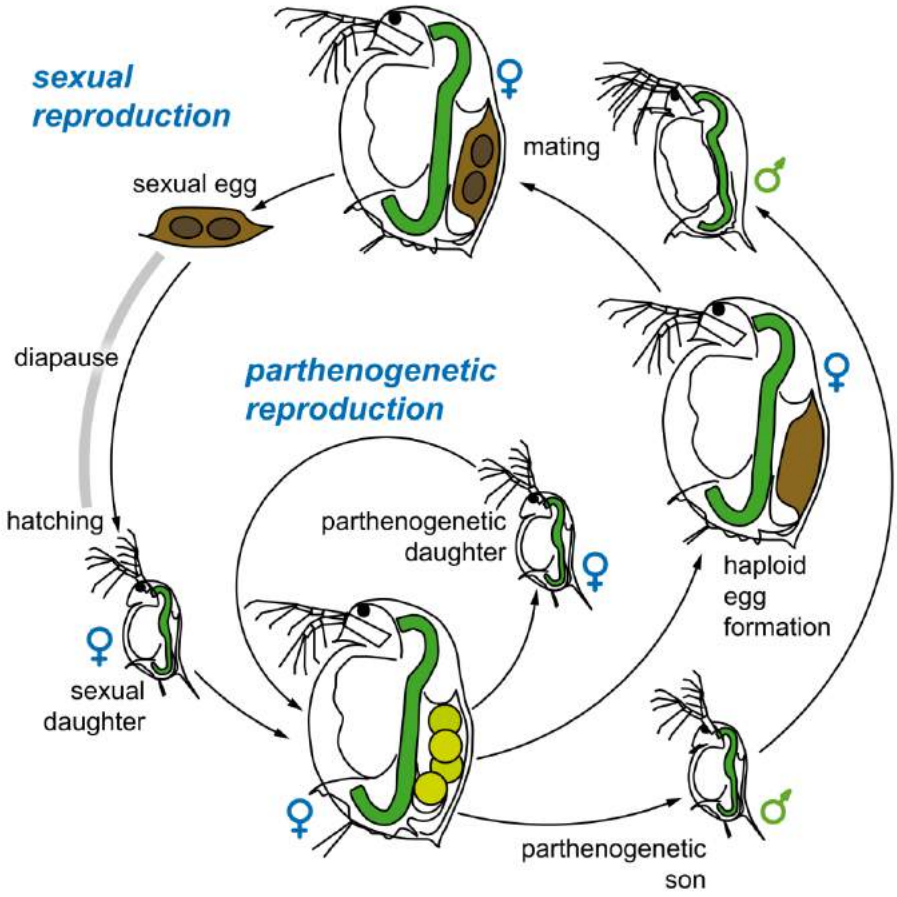

Figure 1.4. Life cycle of *Daphnia,* showing alternating asexual (phartenogenic) and sexual cycle, from Ebert (2005).

Thanks to recent advances in gene manipulation techniques (i.e. CRISPR/Cas9), it is possible to genetically manipulate organisms as *Daphnia*, generating transgenic organisms (Nakanishi et al., 2014). In this way, specific genes linked to mechanisms of action of certain contaminants can be silenced, and thus elucidate their mechanisms of action. Within this thesis, modified CRISPR/Cas9 *D. magna* with mutations in the tryptophan hydrolase (TRH) gene enzyme (rate limit enzyme in serotonin synthesis) were also used for some experiments (Rivetti et al., 2018).

1.6.1. Lipid dynamics in *Daphnia*

In *Daphnia*, lipids represent between 16 to 41 % of their total dry mass (Goulden and Place, 1990). Lipid dynamics vary during the cladocerans moult and reproductive cycle, which is regulated by the EcR and JH signalling pathway. During an intermoult or/and two reproductive events, an individual *Daphnia* take up lipids mostly from food building up its lipid content. At the end of the intermoult and/or reproductive period these lipids are used for moulting or transferred into ovary and then into the next clutch of eggs. Thus, in juveniles or in adults the total content of lipids decreases dramatically after moulting or reproduction. Age-related changes in some lipid species, such as triacylglycerides, diacylglycerides, phosphatidylcholines, phosphatidylethanolamines, ceramides and sphingomyelins, have been recently reported, relating it to this moult and reproductive cycle (Constantinou et al., 2020). In cladocerans, storage lipids are primarily triacylglycerides located in scattered spherical droplets throughout the animal's hemocoel (Sterner et al., 1992). In adition, recent studies have also reported that the content of particular lipids in its food clearly affects resource allocation in *D. magna*, and suggests that cholesterol is important for somatic growth, while poly-unsaturated fatty acids (PUFAs) are primarily needed for reproduction (Sengupta et al., 2017; Wacker and Martin-Creuzburg, 2007). As stated above (section 1.5.1, subsection b), these lipid dynamics are regulated by three receptors: MfR, EcR and RXR (Jordão et al., 2016). These lipid changes, as well as the associated changes in genetic expression, mean that for correct experimental results adequate control of the sampling timepoint within an assay is essential.

1.7. Integrated approaches at different levels of biological organization

The use of integrative approaches covering a wide range of endpoints can aid in a comprehensive understanding of the effects of toxicants, also supporting risk assessment and helping in the perspective of developing AOPs of ECs (Fabbri, 2015; Simões et al., 2018). In order to achieve full comprehensive assessment at different organization levels, e.g. cellular, tissue, organ or organism response, the use of a wide selection of methods and skills is required, that allow us to unravel new potential modes of action of ECs (Ankley et al., 2010; Brennan et al., 2006; Campos et al., 2016). The integration of these different methods give us the possibility to identify KEs and effects and then use this information to design novel tools for ERA (Ankley et al., 2010).

As already stated earlier, *D. magna* was used as model organism throughout the progress of this thesis. Using this model, an integrative approach was carried out to assess ECs toxicity by linking effects at different levels of biological organization, i.e. reproduction and behavior as organism responses, gene expression and the subsequent metabolomic disruption. Figure 1.5 represent the different methods applied throughout this work.

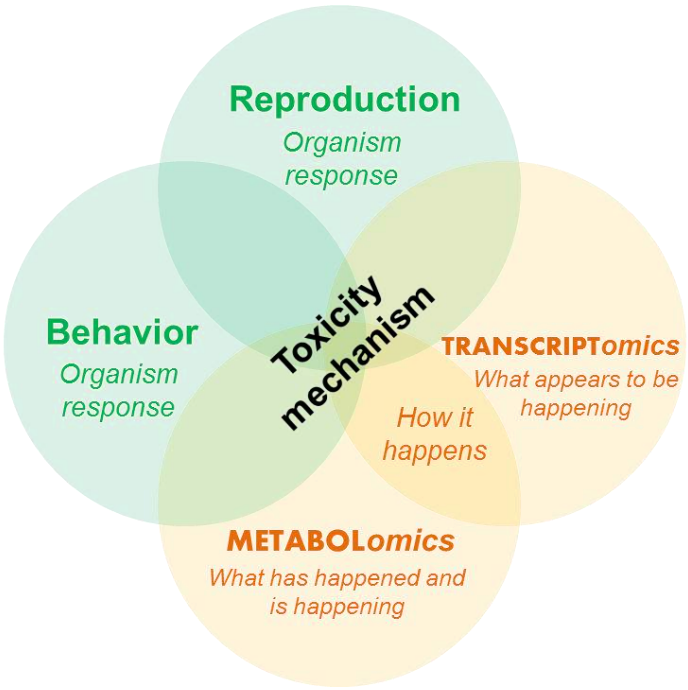

Figure 1.5. Graphical abstract of the different toxicological approaches carried out within this thesis in order to unravel mechanism of action of different emerging contaminants.

In the current environmental situation, where it is necessary to apply methods able to monitor sublethal effects, the evaluation of effects on the reproduction and behavior of *D. magna* represents a very useful tool. *D. magna* reproduction is one of the responses considered to evaluate chronic effects of contaminants by OECD (21 days reproduction test, according to OECD guideline 211) (OECD, 2012) and represent a sensitive method to detect toxicity. Effects of endocrine disruptors or pharmaceuticals on reproduction of *D. magna* have been extensively used in previous studies (Campos et al., 2012; Dietrich et al., 2010; Dzialowski et al., 2006; Rivetti et al., 2016; Sengupta et al., 2016). Behavioral assays can be considered another powerful and effective method to screen for sublethal toxicity. Behavioral tests are increasingly being applied in *D. magna*, particularly to assess the effects of pharmaceuticals and neuroactive compounds (McCoole et al., 2011; McWilliam and Baird, 2002; Ren et al., 2015; Rivetti

et al., 2016; Simão et al., 2019). Behavior is both an assay of fitness and an adaptive (although maladaptive at times) response to environmental stimuli (Peterson et al., 2017). Behavioral assays can be powerful endpoints for measuring contaminant effects due to the fact that behavior integrates both the internal physiological state of an animal and its response to an external stimuli (Legradi et al., 2018). There is extensive research indicating that behavior is an indicator of multiple levels of biological outcomes, is among the most sensitive indicators of impact of exposure and is considered an early warning tool (Hellou, 2011; Peterson et al., 2017; Scott and Sloman, 2004; Weis et al., 2001), and thus it could be used as an alternative or complement to the current acute standard test for the predictive evaluation of toxic effects in the aquatic environment (Chevalier et al., 2015). Nevertheless, even with the emergence of behavioral environmental toxicology, little integrative work using its application has been published (Peterson et al., 2017). There is therefore a need to conduct studies that integrate and develop behavioral testing as a tool for environmental toxicology.

Within this integrative perspective, the development of high-throughput “omic” technologies could be considered as a milestone in environmental toxicology, being excellent approaches to study different KEs and thus to understand the reason for an adverse outcome in an organism (Piña et al., 2018). The aplication of these techniques (e.g. transcriptomics and metabolomics) to evaluate toxicological effects is becoming increasingly important and is helpful for risk assessment (Simões et al., 2018). Their implementation make possible to understand what is happening (or has happened) after the exposure to a contaminant that produces a response in the organism (i.e. impaired normal reproduction or behavior) and furthermore how it has happened. In particular, using transcriptomics and metabolomics tools, it is possible to establish links between the regulation of genes involved in responses to enviornmental stressors and the metabolic disrupted homeostasis that triggered that expression, which subsequently led to a response in the organism. These approaches, able to be synthesized in AOP framework, can have an immediate impact on regulatory decisions (Ankley et al., 2010).

1.8. The omic era

The suffix ”*-omics*“ is used frequently to describe something big, and here refers to a field of study in life sciences that focuses on large-scale data/information to understand life summed up in “omes” (Yadav, 2007). In this way, omics is a broad scientific field that comprises a large variety of disciplines aiming to the study of the

abundance and the structural characterization of different families of molecules within organisms (Bedia et al., 2018). Since the beginning of the Human Genome Project, and the consequent availability of the entire human genome, there was an exponential growth of the application of these omic disciplines, entering in the post-genic biology era. Figure 1.6 ilustrates this growth with the number of scientific publications related to omics since then. This new perspective of biological systems, in terms of how to explore the function of genes, will represent a new and powerful multidisciplinary approach that will allow, with a better predictive capacity, the identification of biological markers.

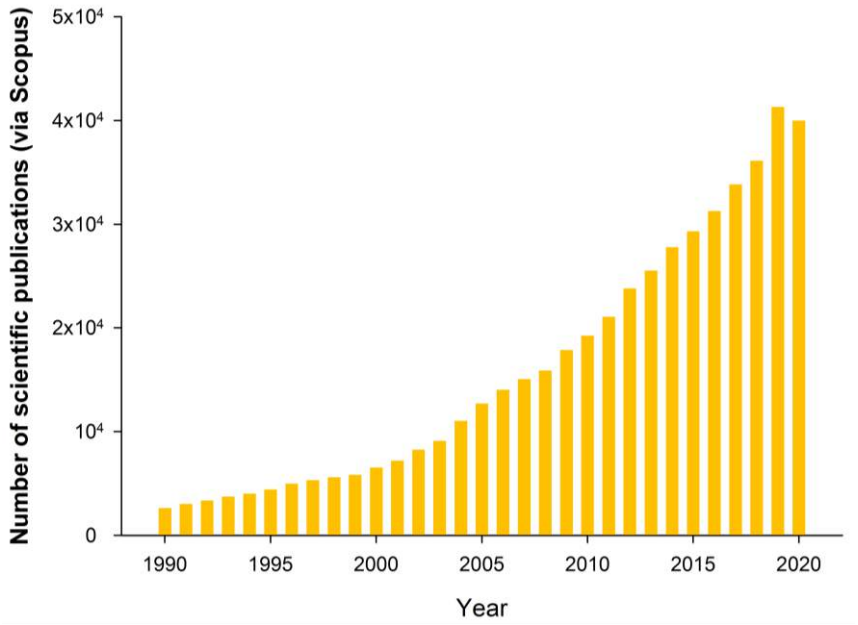

Figure 1.6. Number of scientific publications within the last 30 years (via Scopus, accessed 25/09/20) containing the term "omic" in the title, the keywords or the abstract.

Omic sciences adopt a holistic view of the molecules that make up a cell, tissue or organism (Horgan and Kenny, 2011). Within the so called omics, different disciplines can be distinguished, which as a whole define the omic cascade (Figure 1.7). Among the omic sciencies are included: genomics, for the study of variations in DNA sequences; transcriptomics, for the characterization of gene expression using the measurement of messenger RNA (mRNA); proteomics, for the measurement of the levels and expression of proteins; and metabolomics, for the global evaluation of the metabolites present in a given biological system. The range of metabolites is extensive, including many metabolite families with different physical and chemical properties, that it can be further subdivided according to the type of metabolite that is specifically analyzed, such as carbohydrates (glycomics), nucleic acids (nucleotidomics), amino

acids (aminoacidomics) or lipids (lipidomics) (Piña et al., 2018). These omic sciences, in order, constitute the omic cascade, from genetic coding to the development of a determined phenotype. In order to achieve a full comprehension in an *in toto* approach, more categories have been progressively developed, like interactomics, that describes the complete set of molecular interactions (usually protein-protein) (Iida and Takemoto, 2018) or fluxomics, that determines the metabolic rates of all the different reactions that take place in a particular biological system (Gonzalez and Pierron, 2015). The use of these sciences allows us to obtain a large amount of genes, gene products and metabolites that are generated simultaneously, and allows us to generate a global impression of how organizations and cells respond to e.g. contaminants or stressors (Jason R. Snape et al., 2004). The integration and interaction of the different components of the omic cascade have given rise to the concept “systems biology”, that understand more thoroughly a complex system considering it as a whole, with a holistic view of the biological and biochemical mechanisms present in a living organism (Horgan and Kenny, 2011; Westerhoff and Palsson, 2004).

These technologies have been promoted by their wide range of applications and much potential. Omic sciences have generated important insights to increase our understanding in fields as diverse as medical sciences, pharmacology, physiology and environmental sciences (Horgan and Kenny, 2011; Piña et al., 2018). The revolution in these sciences has allowed us to obtain data from the molecular changes that occur in cells, tissues and the body, without knowing how they are subject to stress or external stimuli. In most omic studies, the main aim is the discovery of highly reliable variables whose values are significantly different in two populations being labelled “control” and “case”, e.g. in the environmental field, omics evaluates the alterations that model organisms might suffer after exposure to environmental stressors (Bedia, 2018).

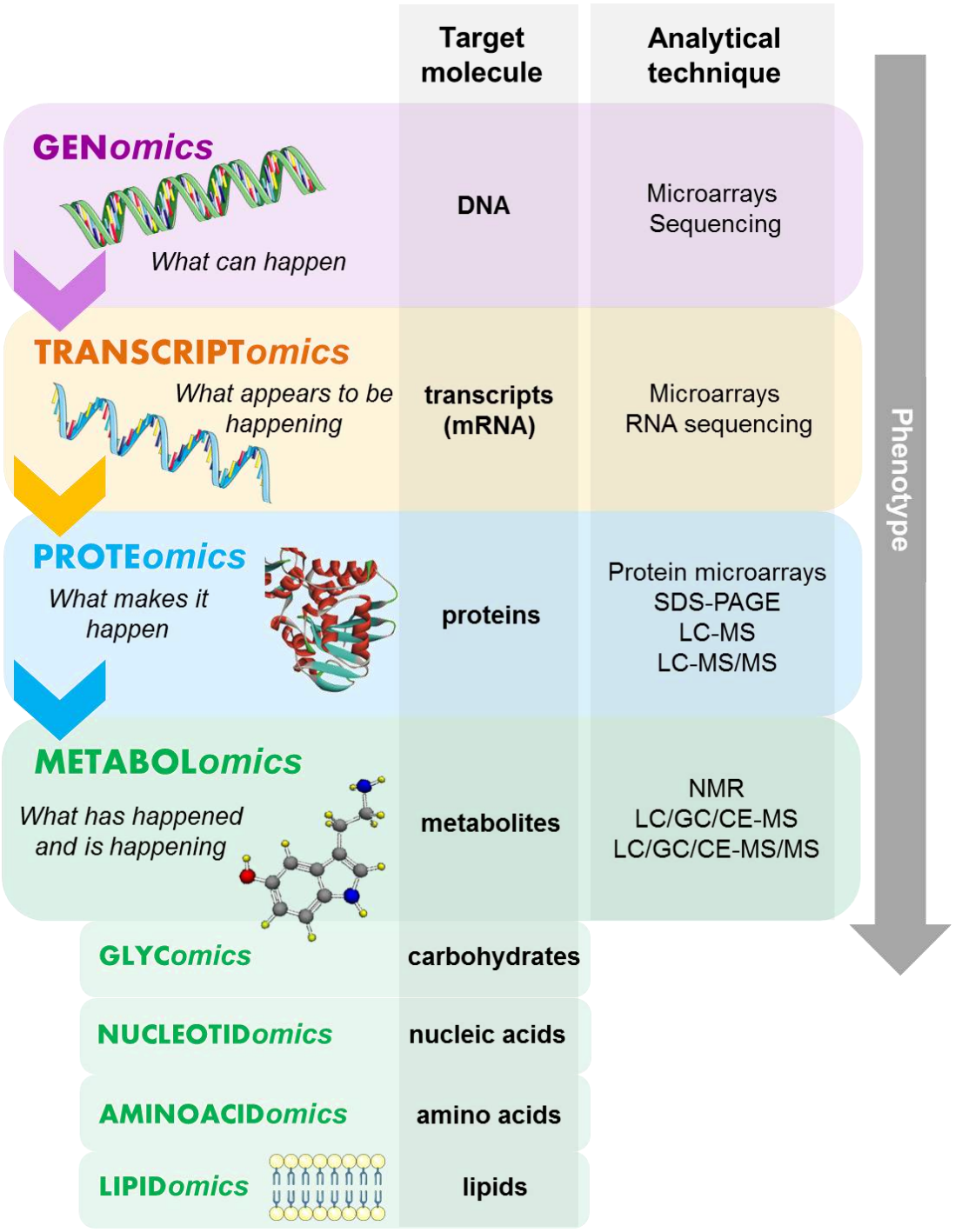

Figure 1.7. Different omic technologies represented as an information cascade in order to determine a phenotype, with their target analyzed molecules and examples of the main analytical techniques used in each one. SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; LC: liquid chromatography; GC: gas chromatography; CE: capillary electrophoresis; MS: mass spectrometry; NMR: nuclear magnetic resonance.

Advantages in omic technologies have been also important in environmental toxicology, where they have allowed to significantly improve our understanding of environmental stress responses at individual level (Zhang et al., 2018). Omics allow to connect molecular events and adverse outcomes (AO) with biological levels of

organization, improving the knowledge and undertanding of the potential risks of environmental contaminants over the health of ecosystems and thus relevant for risk assessment schemes (Simmons et al., 2015; Simões et al., 2018). Systems toxicology, as systems biology, describe the toxicological interactions within a living system and thus involves the analysis of interactions of a large network of biological molecules and macromolecules and their perturbations following a given exposure to a contaminant (Choudhuri et al., 2018). The term toxicogenomics was firstly introduced in 2004 and refers to the integration of genomic-based science, i.e. omic sciences, into toxicological and risk assessment analyses by using transcriptomic, proteomic and metabolomic data (Kim et al., 2015; Jason R Snape et al., 2004). This integrative approach has been fundamental in our current comprehension of the effects of environmental hazards, the characterization of multiple cell targets for a single contaminant, or the development of new and more precise methodologies for monitoring toxic effects both in humans and wildlife (Piña et al., 2018).

In comparison with traditional toxicological techniques, that deals with extensive observations of phenotypic endpoints *in vivo* and complementing *in vitro* models (Kinaret et al., 2020), these new techniques provide more detailed molecular information which is more appropriate for the identification and quantification of molecular markers sensitive to environmental stressors (Van Aggelen et al., 2010). Many traditional toxicity assays have the disadvantage that, although being informative about a toxic effect, they cannot allow to understand the mechanisms that is causing that toxicity. Responses in an organism results from molecular responses. Therefore, when exposed to a contaminant, changes in molecular level (e.g. gene or protein expression; or metabolic disruption) should happen first, and thus be a more sensitive indicator of toxicity (Le et al., 2016). With the deterioration of environmental health and the increasing ocurrence of emerging contaminants and more complex pollution scenarios, the conventional toxicity tests are not sufficient to evaluate total environmental risk, so that omic techniques have covered that need for more robust methodological approaches to assess the hazard of toxicants to aquatic organisms (Le et al., 2016), and on a large scale, adverse effects to the ecosystem (Daniel L. Villeneuve et al., 2012). Thus, it is clear that a single omic approach is not enough to characterize the complexity of ecosystems, and a higher comprehension of harmful effects after exposure to contaminants is achieved when the information from different techniques is combined (Choudhuri et al., 2018). However, the data obtained by means of omic sciences should not be considered independently, but rather as complementary to traditional techniques that study biological processes.

In all omic approaches, the analysis of the different families of compounds involved in this variety of biological levels is possible because of the recent instrumental revolution, which has made possible to develop highly efficient analytical techniques (Bedia, 2018; Piña et al., 2018). Depending on the experimental question addressed for which the omic technique is used, an analytical platform (some of the most applied per omic technique are listed in Figure 1.7) and an analytical approach (i.e. targeted, untargeted or suspected screenning) most be defined.

Targeted studies focus on the detection, analysis and quantification of a reduced set of known preselected biological molecules previously characterized (usually in the order of tens of molecules) (Bedia, 2018; Griffiths et al., 2010). Usually, these selected analyzed molecules belong to a specific metabolic pathway or to the same family of biological compounds, aiming to corroborate a preconceived hypothesis by means of determining their relative concentrations or abundances (Yang and Han, 2016). Targeted approaches have the advantage that the extraction procedure and instruments used in the analytical workflow are optimized and calibrated to detect specific molecules, increasing the sensitivity and precision of the measurements. Furthermore, the obtained data is relatively easier to analyze when comparing different samples. However, only a limited part of the sample data is examined, hence, there is potentially relevant information in the sample that is left unexplored. This aspect is covered in the untargeted analysis of samples, that attempt the investigation of all the data obtained in the instrumental analysis of the sample allowing a more comprehensive evaluation of the considered biological system, without any a priori hypothesis of the potential molecules involved in the process studied. Nevertheless, this analytical approach implies increased complexity in the data analysis requiring a highly-extensive computacional processing (Bedia, 2018). Halfway between this two approaches is the suspected screening analysis or semi-targeted. In this case, the data is obtained in an untargeted analysis, but it is processed as in a targeted approach, where only the information of known preselected biological molecules is extracted. This strategy is usually performed in some metabolomic studies (Che et al., 2018).

1.9. Analytical technologies in omic sciences

In the following section, each of the peculiarities of the analytical omic techniques applied throughout this thesis will be explained.

1.9.1. Transcriptomics

The increasing occurrence of ECs dictates the need for a better understanding of the molecular changes occurring in exposed biological systems (Kinaret et al., 2020). During the last decades, advances in DNA sequencing and genome characterization have made possible the development of high throughput molecular based technologies, whose potential for ERA and developing modelling approaches for predicting AO has been highlighted (Connon et al., 2012; Garcia-Reyero and Perkins, 2011; Kinaret et al., 2020; Milan et al., 2015; Daniel L Villeneuve et al., 2012). Gene expression (i.e. transcription) can be altered as a direct (e.g. the binding of a steroid hormone or an analogue to a specific steroid hormone receptor) and/or indirect interaction of a contaminant exposure, which results in changes (activation or repression) of target or related genes (Piña et al., 2018). In fact, gene expression is expected to be stress-specific and to respond quickly after stressor exposures (López-Maury et al., 2008; Orsini et al., 2018), having the potential of providing early detection of environmental stress (being gene transcription the first affected step). Therefore, it has become of crucial importance to determine changes in the gene expression of an organism to understand ECs mode of action and identify KE (Altenburger et al., 2012; Piña et al., 2007). Transcriptomics enables this exploration of organism responses to environmental stressors by observing the molecular alterations in more detail (Kinaret et al., 2020). It consists of the detection and quantification of mRNA expression levels of specific genes encoded by the genome (i.e.transcriptome). In contrast to the genome, the transcriptome of a cell is a more dynamic layer due to the continuous specific transcription processes that reflect the activity of cells and its response to external stimuli (Bedia, 2018). A major mechanism underlying almost all the responses to environmental changes is mediated by a shift in the transcription of genes. Thus, in the environmental toxicology context, the comparison of the transcriptome under different stimuli or environmental conditions allows the identification of the genes that are differentyally expressed as response to that environmental stress factors. It is important to note that different mechanisms of toxicity generate specific patterns of gene expression and that these patterns can provide molecular biomarkers specific for each mechanism or mode of action of ECs or other environmental stressors (Merrick and Bruno, 2004; Piña et al., 2018; Piña and Barata, 2011). Due to this reason, transcriptomics is becoming a key discipline for the assessment of environmental impacts of ECs, highlighting toxicant specific gene expression paterns and signalling pathways.

Nowadays, due to the improvement of analytical technologies, transcriptomics is the layer in systems biology with the best analytical coverage (Bedia, 2018). Currently, the most widely used techniques in transcriptomics are real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR), microarrays and RNA sequencing (RNAseq), that has revolutionized the way how we were able to address genomic processes. Whereas qRT-PCR analyses allow the monitoring of few genes at the time with high resolution in a quantitative way, microarrays and RNAseq offer opportunity for the screening of thousands of genes at once, providing a more complete picture of transcription effects (Martyniuk and Simmons, 2016; Wang et al., 2009). Figure 1.8 ilustrates the experimental workflow in which these techniques have been applied throughout this thesis, and which will be discussed in detail below.

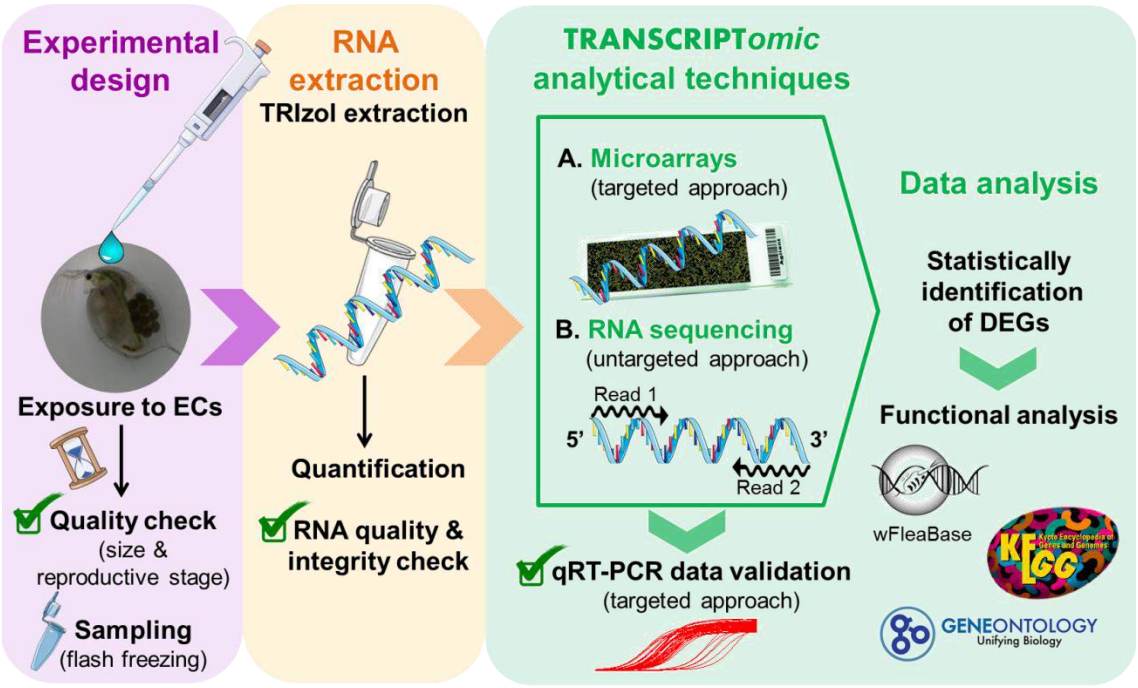

Figure 1.8. Experimental workflow of transcriptomics experiments applied throughout this thesis. In the figure, the different phases of a transcriptomic study are simplified: experimental design considerations, extraction procedure, applied analytical techniques and data analysis workflow with used databases and bioinformatics tools. DEGs: differentially expressed genes; ECs: emerging contaminants.

a. Microarrays

Microarrays consist in a small rigid support, that hosts thousands of complementary DNAs (cDNAs) or oligonucleotides spotted sequences called probes, which are printed or attached in known and fixed locations. Therefore, although the analytical coverage is very high, it represents a targeted transcriptomic approach.

cDNA microarrays are usually created by spotting amplified cDNA fragments in a high-density pattern onto a solid surface such as a glass slide, meanwhile probes for oligonucleotides arrays are either spotted or synthesized directly onto a glass or silicon surface using various technologies including photolithography or ink-jets (Gonzalo and Sánchez, 2018; Venkatasubbarao, 2004). Hybridization between these cDNA fragments or probes and complementary target sequences labelled with a fluorescent dye is the principle behind microarrays technique. Total RNA from the biological sample of interest for which the array was designed is extracted. Target sequences are obtained by reverse transcription of this RNA, using nucleotide derivatives that are either fluorescent or that can be subsequently attached to fluorescent molecules.

Two different approaches to detect differentially expressed genes (DEGs) between samples can be applied in a microarray: one- or two-color technology. In one-color approach, one microarray is used per sample, and a sigle fluorescent label is applied, meaning that different fltuorescent images are compared to obtain differentially expressed targetes. In two-color experiments, a reference RNA sample is needed, and both reference and experimental samples are labelled separately with different fluorescent dyes, then hybridized to a single microarray, and scanned to generate fluorescent images from the two specific wavelengths of each dye (Gonzalo and Sánchez, 2018). While the one-color approach requires very consistent manufacturing to minimize array-to-array variation, the two-color approach benefits from the direct comparison between samples on a single array. However, comparing more than two samples becomes complicated, being necessary either the use of a common reference sample and/or a hybridization strategy that combines multiple different pairs of samples. Nevertheless, both approaches have been frequently applied and yield comparable results (Oberthuer et al., 2010; Patterson et al., 2006). Within this thesis, oligonucleotide (probes) one-color microarrays have been applied.

The conditions in which hybridization and subsequent washings are carried out, promote the formation of only strongly paired strands (between sample and probes), or in other words, those with a high number of complementary base pairs, avoiding non-specific interactions. The total fluorescent signal emitted by each spot depends on the amount of target sequence binding to the probes present on that spot, which determine the level of expression on the related target gene, identifying those genes whose expression change due to the condition under study. Although microarray technology has a very high throughput interrogating thousands of genes simultaneously, there are possible sources of variability that make necessary the application of statistical tools in

experimental design and in data analysis in order to obtain high quality results (Gonzalo and Sánchez, 2018; Kammenga et al., 2007).

Despite its success and reliable results, microarrays present some limitations. On the one hand, microarrays measure gene expression indirectly, based on dye fluorescence. This is known to be linearly related to gene expression only in a range of concentrations, but not for small or high values, where it can be undetectable (low) or saturated (high). On the other hand, it is very difficult to avoid some degree of cross-hybridization (i.e. the sequence bound to a given probe may not belong to the gene it was intended to belong but is somehow very similar). Finally, a microarray is a targeted approach, and therefore it can only detect sequences present on the slide, and thus it cannot detect any new or previously unknown sequence (Gonzalo and Sánchez, 2018). This one is probable its main limitation, also linked to the incomplete knowledge of the entire genome or incomplete annotations of many existing species for proper microarray design (Piña and Barata, 2011). These limitations are overcome by next-generation sequencing (NGS) (i.e. RNAseq), which has meant that in recent years some studies have suggested that the time of microarrays is over. Although this may seem a reasonable evolution, specially when NGS becomes more affordable, the fact is that nowadays microarrays are the option of choice for many studies where the flexibility of NGS may be not necessary (Gonzalo and Sánchez, 2018). Indeed microarray studies have been widely apply in *Daphnia* environmental toxicology (Campos et al., 2019, 2018, 2013; De Schamphelaere et al., 2008; Dom et al., 2012; Garcia-Reyero et al., 2012; Giraudo et al., 2017, 2015; Gomes et al., 2018; Poynton et al., 2012; Soetaert et al., 2007; Vandenbrouck et al., 2009).

b. RNA sequencing

Although microarrays are cheaper than sequencing techniques, they represent a targeted approach based on a priori knowledge of the genetic content of the sample. In contrast, sequencing allows detection of changes without a previous knowledge and it has a broader dynamic range of detection and quantification (Bumgarner, 2013; Manzoni et al., 2018; Wang et al., 2009). RNAseq is an untargeted approach that requires no pre-knowledge of the sample, performing absolute count of the transcripts within a sample allowing higher precision, and being able to identify and quantify also rare transcripts, alternative splicing or mutations, without any previous knowledge. Furthermore, it also requires lower amount of RNA than microarrays, presenting a much higher dynamic range (Lowe et al., 2017).

Despite the fact that RNAseq is a relatively new methodology, it has rapidly evolved in the last years and thus nowadays is widely used in environmental toxicology studies. It is therefore not surprising that many studies have applied this technique in recent years to observe the effects of environmental contaminants and stressors in *Daphnia* (Jeong et al., 2018a; Li et al., 2021; Orsini et al., 2018, 2016b; Schmidt et al., 2017; Toyota et al., 2019).

The knowledge acquired throughout the realization of this thesis allowed the publication of a book chapter addressing the issue of RNAseq (Fuertes et al., 2020). Therefore, to further explain this subsection, the referred published chapter is attached bellow. Among the different sequencing techniques explained in the referred article, in this thesis paired-end RNAseq was applied. At the same sequencing depth, the paired-end RNAseq increase the sensitivity and specificity of the detection and mapping of the reads in comparison with the single-end sequencing. Therefore, paired-end sequencing is a more efficient strategy for characterizing and quantifying transcriptome (Fang and Cui, 2011), and specially in the case of *D. magna*, due to the fact that has many repetitive regions in its genome and is a species that is not still well sequenced (Colbourne et al., 2005). More details on this aspect can be found in the following published chapter.

Data Processing for RNA/DNA Sequencing

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4.21 Data Processing for RNA/DNA Sequencing

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4.21.1 The Sequencing Era

Genomics and transcriptomics are essential to understand the functional elements of the genome, explore the molecular mechanisms of cells and tissues, or the organism patterns affected by development, disease, or different environmental factors.[1] There are mainly two current techniques used in the last decades in these fields, having made it possible to convert them in key disciplines in biological sciences. DNA and RNA microarrays, where DNA or RNA samples are hybridized with a set of predefined oligonucleotide probes corresponding to regions of the genome or transcriptome of interest,[2] and sequencing methods, based on the fragmentation of genetic material from a sample into pieces that are then sequenced.[3,4] Although microarrays are cheaper than sequencing techniques, they are based on a priori knowledge of the genetic content of the sample, looking to target predefined genome or transcriptome regions, they have high background signal owning to cross-hybridization and have a limited dynamic range of detection and quantification of lowly and highly expressed genes; in contrast, sequencing allows detection of changes without a previous knowledge and it has a broader dynamic range of detection and quantification.[1–3]

Despite the fact that DNA and RNA-sequencing (DNA-seq and RNA-seq) are relatively new methodologies, they have rapidly evolved in the last several years, improving to be high throughput and to require low input amounts. Since the development of the Sanger chain termination method in 1977,[5] going through the first capillary electrophoresis-based first-generation sequencing instruments that were determinant for the National Institutes of Health and Celera Human Genome Projects,[6] sequencing technologies have been under development. Over the last decades, after the launch of 454 and Illumina sequencing, massively parallel sequencing has increased its capacity whiles dropping its costs, thus changing the way in which genetic research is carried out.[7]

Here we will focus on the two most challenging case scenarios explaining the existing pipelines and constraints in DNA/RNA sequencing of microbial communities and on the functional analysis of transcriptomic changes in the crustacean *Daphnia magna*.

4.21.2 Sequencing Technologies

There are two major approximations carried out by sequencing techniques: short-read and long-read sequencing (Sanger, PacBio, Minion). The former provide lower-cost and higher-accuracy data useful for population-level studies or clinical variant research, while the latter provides read lengths more useful for de novo assembly applications and full-length isoform sequencing.[7] Short-read sequencing can be performed by ligation (LBS or by synthesis SBS). LBS is carried out in presence of a probe sequence bound to a fluorophore that hybridizes to the sample fragment and then is ligated to an adjacent oligonucleotide, identifying the base by the emission spectrum of the fluorophore. In SBS sequencing, a polymerase is used and the incorporation of a nucleotide into an elongating strand is identified.[7] Related to long-read sequencing, there are mainly two different technologies, single-molecule real-time sequencing approaches and synthetic approaches that use existing short-read technologies to construct long reads in silico.[7]

4.21.3 Single-Read and Paired-End Sequencing

The development of paired-end (PE) sequencing was a major advance in sequencing technologies.[8] Single-read sequencing involves sequencing just for one end of the fragment to be sequenced, whereas in paired-end sequencing both ends of the fragment are

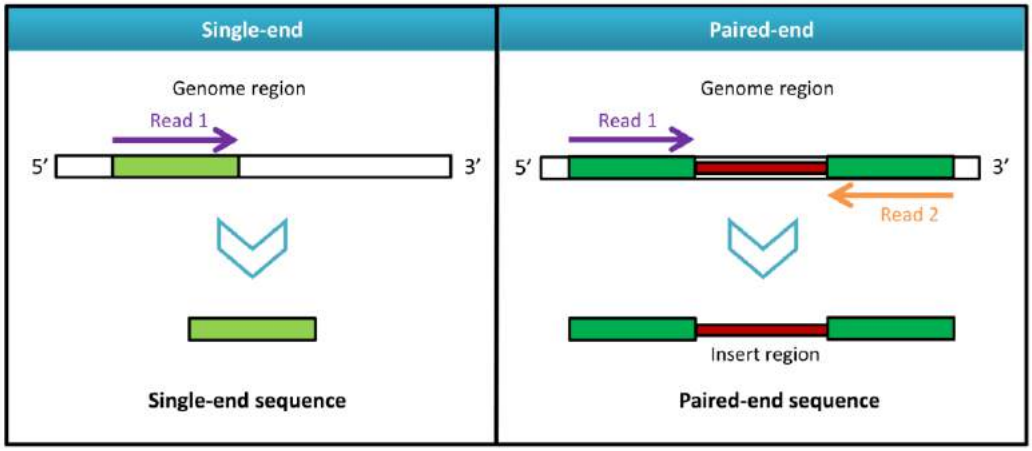

Fig. 1 Single-read and paired-end sequencing techniques. Single-end sequence just from one end of the DNA, whereas paired-end sequencing enables both ends to be sequenced, where the distance between each paired read (insert region) is known.

sequenced (Fig. 1). This technology produces twice the number of reads for the same time and library preparation effort, and also allows a more accurate read alignment of the forward and reverse reads, providing elevated coverage.[9] Since the approximate distance between pairs is known, alignment algorithms use this information to map the reads over repetitive regions in a more accurate way, especially across repetitive regions of the genome that are difficult to sequence,[9] as occurs, for example, in the case of sequencing studies with the environmentally aquatic model crustacean species *Daphnia magna*.[10] The genome of the previous mentioned species has a lot of repeated regions and is a species that is not still well sequenced.[10] When using single-read sequencing the same read can be mapped in many different places to the reference genome or transcriptome. On the other hand, having PE, two different reads of the same region have to be mapped with a known-distance gap between them, increasing the specificity and yield when aligning back to the reference genome or transcriptome, producing highly quality sequence data.[11] PE sequencing also allows removal of PCR duplicates produced during PCR amplification and the detection of insertion-deletion variants. Although metaomics is fundamentally different from genomics of sequenced model organisms in that there are reference genomes only for some species, PE sequencing is nevertheless also the preferred for these approaches, considering that paired reads are more powerful both in annotation of raw reads and assembly.

4.21.4 Basics of Sequencing Data Generation

The basic steps performed during a standard DNA or RNA sequencing experiment are shown in Fig. 2. After getting DNA/RNA isolated, pure and integer from the samples of interest, the sequencing library is prepared by breaking DNA or cDNA into smaller pieces by enzymatic, chemical or physical fragmentation. Then, each sequencing technology follows a specific strategy to sequence the reads. For instance, one of the most popular methodologies for SBS, Illumina sequencing, ligates adapters to DNA fragments that are subsequently PCR amplified and purified. The obtained library is loaded into a flow cell or reaction chamber, and fragments are captured by surface-bound oligos complementary to the library adapters. Each fragment is then amplified through bridge amplification generating clonal clusters, being the templates ready to be sequence.[12] Different sequencing technologies can be used based on different methods of detection of nucleotide sequence, including Ion Torrent PGM and several Illumina systems. All of them, however, after a basic clean-up, provide information on a base-by-base sequencing, which further requires a deep data analysis procedure and an alignment process to a reference genome or transcriptome. Following this, many different analyses are possible depending on the final research question and based in the obtained differences between the reference and the sequenced reads.

4.21.5 DNA and RNA-seq Data Analysis

The pipeline shown in Fig. 3 can be divided in at least three blocks in which, the raw output of the DNA/RNA-seq machine has to be converted in readable sets of DNA/RNA sequences, pasting together (i.e., assembling) those belonging to a same transcript and

Fig. 2 DNA-seq or RNA-seq experimental workflow: from experimental design to data generation and analysis. We included a specific step (clustering) for one of the most widely use methods (illumine).

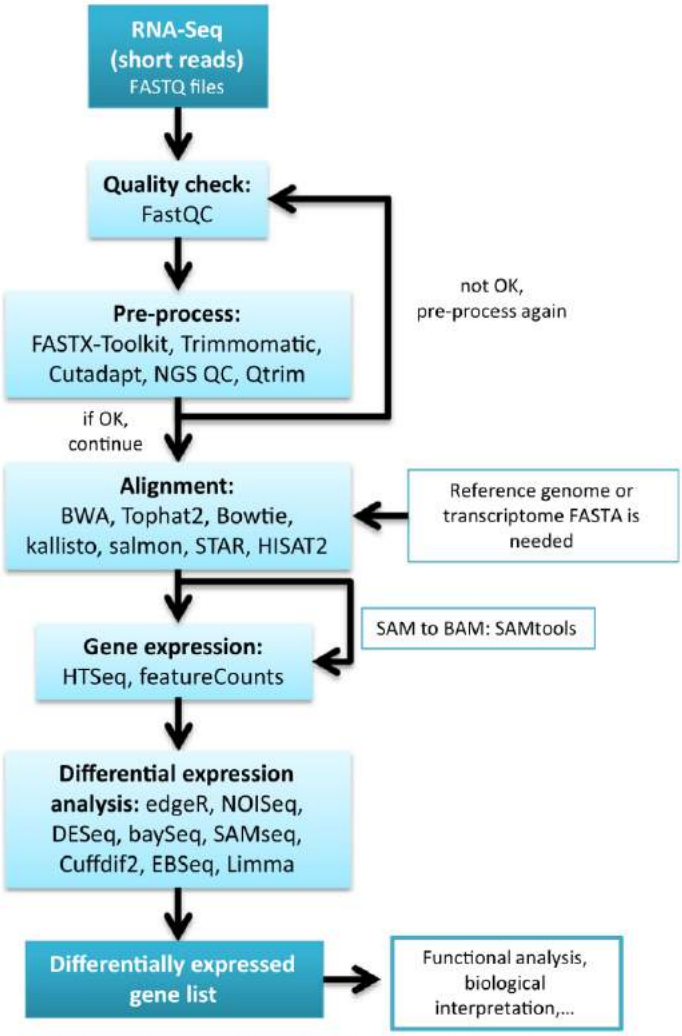

Fig. 3 General bioinformatics pipeline showing typical steps followed by RNA-seq analysis and some of the existing bioinformatics tools for each task.

calculating the number of hits for each one of these transcriptional units. The first or pre-procession step aims to improve the quality of sequencing data. Following sequencing reaction, DNA/RNA sequencing technologies originate massive quantity of output data in ".fastq" format. The ".fastq" is a text format containing a set of read sequences (FASTA data) and base calling information encoded in ASCII characters. These characters contain additional information as quality score values (Phred score) indicating the probabilities of an erroneous base call.[13] The initial step in both bioinformatic pipelines is checking of the quality of the reads using FastQC tool (Java program available at http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), the results of which can be summarized with MultiQC (https://multiqc.info/). FastQC generates a HTML file with graphical illustrations providing different quality analysis, from basic statistical results, until per base sequence quality, per sequence quality scores, per sequence GC content, sequence length distribution, overrepresented sequences or adapter content, among others. Depending on the outcome of FastQC/MultiQC, steps to delete problems from sequences are undertaken. Most commonly, this involves identifying and removing any non-biological sequences, in particular primers and "adapters," sequences introduced by the sequencing method, and bases of low quality, "trimming". Programs used for these purposes include Cutadapt (https://github.com/marcelm/cutadapt), Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic), Sickle (https://github.com/najoshi/sickle), QTrim or NGS QC Toolkit.[14–17] After a first trimming step, a new quality check analysis with FastQC tool is recommended in order to determine if the trimming process has been successful and the resulting high quality reads can follow the downstream analysis.

The second step in the DNA/RNA-seq analysis is the annotation of the sequences, what it means to determine the function and taxonomy (in metagenomics) or the gene (single species transcriptomic analyses) of the reads. This means that in metagenomics the functional analysis step (see the section below), at least in part, is conducted here in order to identify bacterial taxons, whereas in single species transcriptomic functional genomics is performed after gene transcriptomic analysis. Two different strategies for gene-annotation are followed (a pipeline summary is in Fig. 3): (1) read-based annotation, in which each read is annotated individually

(either as single-read or paired-read sequencing); (2) assembly-based annotation, in which all reads are assembled together to reconstruct contigs from the original samples, and then proceed with gene annotation.

Contigs from assembly are often binned to form so-called metagenome-assembled genomes or MAGs, i.e., candidate genomes from populations in the samples, but this is not a requirement. In the assembly-based annotation pipeline, assembly of metagenomic is performed using e.g., MEGAHIT.[18] Gene annotation, i.e., assignment of names to sequence reads or genes, is usually performed by alignment to an annotation database like NCBI RefSeq. For this purpose there are alignment programs available, that are orders of magnitude faster than BLAST (Altschul).[19] Examples include the Diamond aligner[20] which aligns sequences only to amino acid databases but has a blastx that can be used for nucleotide query sequences. Another fast aligner is LAST,[21] which can perform alignments against both amino acid and nucleotide databases. The resulting alignments need to be post-processed to arrive at taxonomic and functional classifications for the sequences. This can be done e.g., with MEGAN[22] which will classify sequences using the SEED, InterPro2GO and eggNOG. There are many other options for classification, several of which rely on using the profile search tool HMMER[23] with a set of profiles typically downloaded from an annotation database like Pfam[24] or eggnog.[25] In assembly-based annotation, contigs are quantified by mapping reads back to contigs using e.g., Bowtie 2[25] and SAMtools.[17]

As shown in Fig. 3, additional aligners have been developed and are commonly used for RNA sequencing studies. Various approaches are possible[14]: spliced read aligners as TopHat,[26] STAR[27] or HISAT,[28] that applies the exon-first methodology; unsigned read aligners like Burrows-Wheeler alignment tool (BWA)[29] or Bowtie,[30] that apply the Burrows-Wheeler transform; pseudo-aligner kallisto[31] or quasi-mapping approaches like in salmon software.[32]

Existing problems for alignment are the lack of information about the complete genome, the poor gene annotation and the existence of many duplicated genes and uncharacterized proteins. This is the case for the species *Daphnia magna*, in which about 80% of short RNA reads can be aligned[33] and the most common case for microorganisms which are mostly unculturable.[34]

Resulting data is then exported to statistical programs to extract patterns and tendencies of the samples and identify those genes that are significantly differently present in the samples (see below).

The quantification of the differentially expressed genes or transcripts between selected biological conditions is at the basis of the use DNA but mostly RNA-seq as a transcriptome analysis tool, as it assumes that the higher the number of hits for a given RNA, the higher its representation in the total transcriptome. This assumption is far less obvious than it seems, as its fulfillment requires a comparable rate of sequence reading for all RNA species in all samples, which is not trivial. It is not unusual that different total RNA, even prepared in parallel and complying with all quality control standards, may result in significantly different total number of readings. Part of this heterogeneity usually comes from different rates of compliance in sequence quality checks among samples, which result in variable numbers of usable sequences from a same number of total reads. Thus, the first step in the processing of the annotated reads (i.e., hits or contings) is the homogenization of the different datasets corresponding to each sample in a given experiment, to ensure that any observed variation in the representation of any RNA/DNA species truly corresponds to physiological/taxon changes and not to sample heterogeneity. The usual procedure is to treat numerically the data set to make all samples as similar in the overall profile as possible. The hypothesis underlying this principle is that most of transcripts, proteins or metabolites (taxons for metagenomics) would not be affected by the parameter we are studying in any given experiment (condition, toxicant, stage of development, etc.). For relatively small datasets, a simple median/mean centering is normally enough, but large transcriptomic/metagenomic datasets require more sophisticated tools, like quantile normalization to make the distributions the same across samples or local weighted regression (Loess) to account for non-linear bias.[35] At this point, the structure of the data is mostly relevant. As transcriptomic/metagenomic data may vary by several orders of magnitude among different genes, using logarithmic transformation helps reducing the overall dispersion of values.

The normalization steps are essentially directed to minimize "false positives," that is, minimizing the differences between samples not directly related to the experiment itself. The next step is the determination of the molecular species whose representation changed between experiments, that is, transcripts whose abundance was affected by a give substance, genetic condition or environmental agent, the so-called de-regulated genes, or DEGs. Many different tools are available, which apply different normalization methods, read count distribution assumptions or differential expression test. Some of the most common ones are edgeR,[36] NOISeq,[37] DESeq,[38] baySeq,[39] SAMseq,[40] Cuffdif,[26] EBSeq[41] and Limma.[35] In many of them, the applied normalization method is Trimmed Mean of M values (TMM), which has been reported as the recommended one after comparison between different normalization methods.[42] The TMM measure is computed multiple times selecting one sample as a reference and the others as test samples, under the assumption that most genes are not differentially expressed. For each sample, TMM is estimated as the weighted mean of log ratios against the reference, after excluding the ones that are most expressed throughout samples and the genes with the largest log-ratios. Following the DE hypothesis, this measure should result close to 1. If the latter is not the case, the estimated value provides a correction factor to be used in conjunction with library sizes in order to concur with standards. In order to so and obtain normalized read counts, these counts are re-scaled by the mean of the normalized library sizes.

For relatively small datasets, univariate Student's *t*-tests (two conditions) or ANOVA (more than two conditions) should be enough to identify the changed molecular species, but for the larger datasets, with more than 1000 to 2000 datapoints, multivariant approaches, like rMANOVA,[43] ASCA[44] or ANOVA-PLS[45] are better suited to determine the contribution of the different molecular species to the total variation of the samples. In ASCA, the original data are split into orthogonal data blocks that can be attributed to a specific factor of interest. It combines the advantages of SCA and ANOVA. SCA analyses data by summarizing and projecting these in a new space. This new space is defined by so-called simultaneous components, which are linear combinations of the original axis (i.e., genes) and that meet some specific mathematical demands. These demands are defined in such a way that the first

simultaneous component describes the largest amount of variance possible. Consecutive simultaneous components are orthogonal to all the previous ones and each one describes less variation than the previous simultaneous component. Using the simultaneous components, it is usually possible to describe the data more condensed than originally. This means that a dimension reduction can be performed which facilitates a visual inspection of the data. The aim of ANOVA is to separate the sources of variation and to assign them to specific factors. This is done by splitting the variations into orthogonal and independent parts using a linear model matching a specific experimental design that include one or more factors and their interaction. The model uniquely separates the total data into orthogonal (independent) data blocks that represent the known factors from the design. The remaining part of the data equals the difference between the sum of the known data blocks and the total data. That part is called the residual part and contains sources of variation that cannot be attributed to a known factor. These sources of variation can originate from unknown factors such as instrumental drift, batch effects, sample work up errors, or measurement errors. In the present data set, the residual part will largely consist of biological variability.

Similar as with ASCA, ANOVA-PLS is the combination of variance decomposition to extract different effects and a subsequent statistical analysis. In this case this analysis is regression with PLS, which has been described extensively and, more recently, for genomic data.[46] It is a data modeling technique that is used to determine the relationship between a multivariate data set and a univariate phenotype. Depending on the fact if the phenotype is discrete (e.g., a treatment) or continuous (e.g., a concentration, phenotypic response), this becomes a classification or a regression analysis, respectively. PLS is able to analyze large numbers of genes in small sample sizes by reducing the dimensionality of the data. The dimension reduction is achieved by constructing latent components (PLS factors), in such a way that these components have maximal covariance with the outcome variable whereas the latent components themselves are uncorrelated. Note that the optimal number of PLS factors is a model meta-parameter that needs to be estimated independently from the regression performance.

For ASCA, each data block is analyzed separately by SCA to interpret the different effects. ANOVA-PLS slightly differs in the sense that different *combinations* of effects are used to determine the relation between the data types rather than single effects. The advantage of analyzing selected combinations of effects is that certain effects are highlighted or excluded, compared to the total data, which enables a specific zoom into the data. An additional statistical reason to use effect combinations instead of single effects is that the rank of single effects is too low to build a reliable regression model. This originates from the ANOVA principles where effects are represented by corresponding group means instead of individual values.

ASCA assumes that none of the genes/transcripts are correlated and that they all have the same variance. Because of these assumptions, ASCA may relate the wrong genes to a factor. This reduces the power of the method and hampers interpretation. In the regularized-MANOVA (rMANOVA) models the optimal weight is determined in a data-driven fashion. Compared to ASCA, this method assumes that genes can correlate, leading to a more realistic view of the data. Compared to MANOVA, the model is also applicable when the number of samples is (much) smaller than the number of genes.

At the end, the result should reflect the initial hypothesis that most genes/transcripts are not affected by the experiment: a reasonable result would be that 80–90% of genes do not vary significantly among samples. The level of confidence required to define the significance of this variability is difficult to set a priori. Most omic datasets include less than five biological replicates, and this makes the estimation of any p value a mere guesswork. In addition, they usually present a large number of molecular entries (genes, proteins, metabolites), so a correction for multiple testing is mandatory; the relatively mild FDR (false discovery ratio) with a $p \leq 0.05$ or 0.01 cut off is probably the most used of the available methods.[47] Some packages, like *limma* or *EdgeR*, both in the R environment and meant for transcriptomic datasets, propose the use of Bayesian methods to estimate the gene-specific biological variation, even for experiments with minimal levels of biological replication.[36,48]

Profile analysis is perhaps the most sensitive step on determining the reliability of the previous normalization and selection steps. A direct prediction of this is that biological replicates for each experimental condition should be more similar between them than to any other replicate from other conditions. Clustering methods test exactly this hypothesis. The easiest and more interpretable of these methods are heatmaps with hierarchical clustering, which visually display the relative proximity of the different samples. Principal Component Analysis, or PCA, is another practical way to test whether or not the variation of the selected genes contributes to separate the different experimental conditions. These methods also provide a hint of the behavior of the different genes/transcripts across the experimental conditions. At this point, a second working hypothesis is that most genes/transcripts will change in a coordinate fashion. This means that the sense and, in some cases, the extent of the concentration changes of molecules physiologically or metabolically related should be similar, and, therefore, we should be able to define a limited number of specific patterns of variations. In a well-conducted experiment, one should expect to find two, three or, at most, four of these patterns. Neither hierarchical clustering nor PCA are optimal tools to define which genes, or gene transcripts belong to each pattern; K-means, K-median or K-medoids clustering provide more univocal results.[49,50] The determination of the number of clusters is again tentative, although some algorithms, like PAM (Partitioning Around Medoids), provide an indicative value.[49] The use of partial least squared (PLS) procedures per se, discriminant (PLS-DA) or orthogonal discriminant analysis (OPLS-DA), combined with variable important in projection (VIP) or selectivity ratio (SR) scores are also good alternatives to select genes whose transcriptional levels or abundance covary with a particular factor or phenotype.[51–54] Nevertheless, the use of PLS related clustering methods in RNA or DNA-seq data is still scarce compared with the use of K-means, K-median or K-medoids clustering.[55]

After applying all these steps, a complete list of differentially expressed genes (DEGs) is obtained, with which is possible to carry out functional analysis and biological interpretation of the results.

4.21.6 Functional Analysis

The next step in the process of extracting information from omic data is to correlate the list of molecular entities identified by the previous steps with specific biological functions (note that in metagenomics this task is performed in the annotation step). This is a very important step of the whole process, as the mere enumeration of genes, proteins or metabolites, even combined with the information about their behavior under a series of environmental conditions, has very little predictive or explanatory capacity about what is happening to a particular biological system under those conditions. Having a list of DEGs is of little use if we cannot correlate these variations to physiological responses and, ultimately, to macroscopic phenotypes. This "bottom up" approach is complemented with a "top down" one, consisting in identifying the molecular initial event, or MIE, which triggered the observed changes in DEG transcription. This process is called "functional annotation," and it incorporates all the current knowledge about the role of genes either as agents of biological effects or as their consequences. This information has been collected from decades of biochemical, physiological, genetic, and medical investigations.[56] Differences between biological taxa are most relevant: functional information for model species is far more developed than the same information for non-model ones, although there is an active field of research to correlate functions across species, especially those phylogenetically close. For obvious reasons, mammals, and in particular, humans, are the species for which better and more accurate functional information is available, but the gap with other models is closing very fast, mainly because of the current tendency of implementing non-mammalian (and even non-animal) models for research in medicine, pharmacology, and toxicology.[57]

Most of the relevant functional information about genes, proteins, and metabolites is deposited in vast public databases, usually providing the necessary taxonomic information. Perhaps the older one, and likely one of the most completes, is the Gene Ontology Database (http://www.geneontology.org/), with more than 6.6 million annotations corresponding to more than 100 species, both Prokaryotes and Eukaryotes. From these annotation, about 1.5 million correspond to three species (not surprisingly, *H. sapiens*, *M. musculus* and *R. norvegicus*), but there are other eleven species with more than 20,000 annotations each: two non-mammalian vertebrates (*G. gallus* and *D. rerio*), the fruit fly *D. melanogaster*, the nematode *C. elegans*, the filamentous fungus *A. oryzae*, three yeasts (*S. cerevisiae*, *S. pombe*, and *C. albicans*), the slime mold *D. discoideum*, the thale cress *A. thaliana*, and the bacterium *E. coli*. These annotations refer exclusively to genes, and they are organized in hierarchical ("parent-child") GO categories, from the more general ones (like "metabolic process" or "cytoplasm") to very specific ones ("cholestenol delta-isomerase activity" or "telomeric loop formation"), including information about subcellular localization, metabolic activity, or roles in cell division or development, among many others. Most important, each one of these GO categories is identified by a key number (e.g., "GO:0005737," "cytoplasm"), which greatly facilitates the process of functional annotation of omic results. This process is then reduced to confronting the list of genes identified in the previous steps (normally, some hundreds of genes) with the 6 million plus entries of the GO database. It can be done "in house," as the GO databases are publicly available, but there are several web pages that allow to do the process on-line (e.g., the AmiGO! database, http://amigo1.geneontology.org/cgi-bin/amigo/term_enrichment, or the DAVID database, https://david.ncifcrf.gov). The algorithms embedded in these webpages perform the so-called "Term enrichment," that is, to associate each of the genes of the input list to one or more GO terms and to calculate for each of these terms whether or not they are overrepresented in the input list relative to a random gene list of the same length. With this information, one can define what biological processes were affected by a given experimental situation under a biologically relevant point of view.

The functional analysis of proteomic data takes advantage of the intimate correlation between gene and protein sequences, and of the ability to recognize functional protein domains from amino acid sequences. This is particularly important when dealing with non-model species, as very often it is much easier to recognize protein domains in peptide sequences from apparently divergent species than to identify homologous or orthologous genes even among closely related ones. Protein-specific sequence databases, like SWISS-Prot (www.uniprot.org/), facilitate this domain/homology search; other databases, like INTERPRO (https://www.ebi.ac.uk/interpro/), PIR (https://pir.georgetown.edu), or SMART (http://smart.embl-heidelberg.de), which also has a genomic section, are instrumental in associating these functional domains to specific genetic functions. At this point, it is important to note that these databases are also included in DAVID searches; therefore, with a single search one can obtain both genetic annotation and protein function. It also means that lists from proteomics and transcriptomic data can be functionally annotated together.

As previously mentioned, the lack of information about the complete genome and a poor gene annotation severely limit functional analysis. For example, only about 40% of *D. magna* genes have homologous in the closest model species (*Drosophila* sp.).[58] Further developments on the biology and genome annotation of other evolutionary close species (Decapods, other insects, etc.) will indubitably improve this situation. Conversely, the comprehension of the functional roles of homologous genes in different taxa (fungi, protostomata, deuterostomata, etc.) will increase notably our understanding of their role in model species (including humans). It will also improve our capacity to predict physiological responses to both internal and external inputs, including mutations, medical drugs, and pollutants, with large implications in many key fields of knowledge, as Medicine, Pharmacy, Toxicology, and Biotechnology.

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c. qRT-PCR as high throughput data validation technique

The polymerase chain reaction (PCR) was developed by Kary Mullis in 1980s (Saiki et al., 1988) and represents the basis of transcriptomics. PCR is a transcriptomic targeted approach that is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary (cDNA) to the template strand, needing a primer (i.e. short single-stranded oligonucleotide probe complementary to a certain region of the target cDNA) to which it can add the first nucleotide and iniciate the replication and elongation processes (amplification). At the end of the PCR reaction, that specific sequence will have been copied billions of times (i.e. amplicons). Real-time quantitative reverse transcription PCR (qRT-PCR) enables reliable detection and measurement of products generated during each cycle of PCR process (Heid et al., 1996). qRT-PCR uses a fluorophore, either DNA sequence non-specific fluorescent dyes that intercalate with any double-stranded DNA (e.g. SYBR Green) or fluorescent reporters attached to specific primers (e.g. TaqMan probes), to monitor in real time the amplification process in each termal cycle (Zhang et al., 2011). This fluorescence is directly related to the amount of cDNA molecules accumulated, that exponentially increases in each thermal cycle (Higuchi et al., 1992).

qRT-PCR is characterized by a wide dynamic range, low quantification limits and the least biased results when compared to other transcriptomic methods (e.g. microarrays or RNAseq (Dallas et al., 2005; Wang et al., 2009). It is specifically suitable when analyzing a restricted number of genes whose sequence is well known. Due to its accuracy and robustness this technique is widely used in order to generate corroborative data and validate results from high throughput transcriptomic techniques. Indeed, the need to conduct corroborative studies has now become official editorial policy for several journals (Rockett and Hellmann, 2004). Although Northern blotting has also been used for this issue (Kammenga et al., 2007), qRT-PCR has been reported to be able to quantify smalller samples (Roth, 2002) and thus as a more suitable validation method of both microarray (Kammenga et al., 2007; Rockett and Hellmann, 2004) and RNAseq (Fang and Cui, 2011; Rajkumar et al., 2015) transcriptomic techniques.

d. *D. magna* genomic and functional available information

Although *Daphnia*'s ecology has been extensive studied, still little is known about the molecular basis of its responses to environmental stressors. Nevertheless, genomic sequencing projects and databases are being developed to advance understanding gene-environment interactions. *Daphnia* has slowly matured into a

versatile genomic model thanks to the work of the *Daphnia* Genomics Consortium, an international network of researchers whose joint efforts are committed to establish *Daphnia* as a premiere model system (Shaw et al., 2008; Stollewerk, 2010). This lead to the publication of the first *Daphnia* species genome for the related species *D. pulex* (Colbourne et al., 2011) and later the fully assembled transcriptome of *D. magna* (Orsini et al., 2016). Thanks to this, wFleaBase was possible to be developed, a database with the necessary infrastructure to curate, archive and share genetic, molecular and functional genomic data for the microcrustacean *Daphnia* (Colbourne et al., 2005). As a result, *Daphnia* is considered a model species for genomics and transcriptomics studies (Colbourne et al., 2011; Ebert, 2011; Orsini et al., 2016, 2011). Recently, the genome of *D. magna* has also been characterized (Lee et al., 2019) and new resources for a better understanding of gene expression patterns after environmental perturbations are being developed, as the *Daphnia* stressor database, that performed a stressor based annotation strategy for a better understanding and interpretation of the functional role of the understudied or uncharacterized *Daphnia* genes (Lee et al., 2019). In fact, the poor gene functional annotation of *Daphnia*'s genome is still one of the biggest limitations to correctly interpret genomic and transcriptomic responses in this genus includding *D. magna*. There is still a large porcentage of genome regions coding for uncharacterized or hypothetical proteins, significantly divergent from many of the available arthropod model species (Lee et al., 2019). In fact, only about 40% of *D. magna* genes have homologous in the closest model species (*Drosophila melanogaster*) (Campos et al., 2018).

Thanks to the development of these genomic projects and databases, it is possible to correlate genes identified to be affected by environmental stressors with specific biological functions (i.e.functional annotation). The keystone of this process is the development of bioinformatic platforms and databases as the Gene Ontology (GO) Database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa, 2000), which make possible to link gene transcription with metabolic and signalling pathways. The GO Database (http://www.geneontology.org/) has more than 6.6 million annotations corresponding to more than 100 species, although 1.5 million from them correspond to only three species (not surprisingly, *Homo sapiens, Mus musculus* and *Rattus norvegicus*). Other 11 species are represented by more than 20.000 annotations each, among which is the fruit fly *Drosophila melanogaster*. This makes feasible to have the possibility of carrying out a large part of the functional analysis of *D. magna* by relating its genes to *Drosophila* ortologs. GO annotations are organized in hierarchical GO categories, from the more general ones (e.g. metabolic process or

cytoplasm) to very specific ones. They include information about subcellular localization, metabolic activity, or roles in cell division or development, among others. KEGG (http://www.genome.jp/kegg/) is another bioinformatics resource that allows to understand the different functions of biological systems at molecular level. In fact, KEGG is integrated by smaller databases, classified according to whether they offer system information (BRITE, MODULE and PATHWAY), genomic information (GENES, GENOME and ORTHOLOGY), chemical information (COMPOUND, ENZYME, GLYCAN, LIGAND and REACTION) and biomedical information (DISEASE, DRUG, ENVIRON and MEDICUS). The PATHWAY database is especially useful for visualizing molecular data sets in maps and thus facilitating the biological interpretation of the transcriptomic (and other omics) data, with the advantage of allowing to position genes in specific places within a molecular pathway. Genes modify their expresion in order to maintain cell homeostasis in a compatible way with the external impact, which implies that genes within the same pathway or under the same control mechanism would change their transcription in a coordinated manner. Thus, through functional analysis of DEGs, it is possible to better understand the pathways and functions affected by exposure to environmental stressors and to characterize specific gene expression patterns after toxicant exposure, to select possible new molecular biomarkers and to try to understand their possible mechanisms of action (Piña and Barata, 2011).

1.9.2. Metabolomics

Metabolomics is the systematic study of all chemical processes concerning metabolites, providing characteristic chemical fingerprints that specific cellular processes yield, by means of the study of their small-molecule metabolite profiles (typically with a molecular mass lower than 1,500 Da). It represents the down stream endpoint of the omic cascade, being metabolites the end products of gene and protein expression and therefore most closely correlate with phenotype (Manchester and Anand, 2017). Metabolomics has demonstrated its potential in many different areas, such as in environmental studies (Lankadurai et al., 2013), clinical toxicology (Robertson et al., 2011), nutrition and food sciences (Wishart, 2008) and disease diagnosis (Shao and Le, 2019). Environmental metabolomics is a relatively recent subdiscipline based on the application of metabolomics to characterise the interactions of living organisms with their environment (Bundy et al., 2009; Viant and Sommer, 2013). Thus, metabolomics is able to reflect metabolic biological system responses derived from environmental stressor exposures. Therefore, the study of the metabolic responses of organisms to stressors will improve the understanding of the molecular

pathways that control different physiological processes and it may assist regulatory policy and decision-making processes in chemical risk assessment (Piña et al., 2018).

Compared to genomic, transcriptomic and proteomic studies, metabolomics has some limitations, due to the enormous physicochemical diversity of metabolites, broad ranges of concentrations, and the large and yet undetermined size of the metabolome (Godzien et al., 2018). Metabolomics aims to measure thousands of molecules (metabolome) with disparate physical propertines (e.g. carbohydrates, nucleic acids, amino acids, amines or lipids, among others), so it presents a significant analytical challenge. For this reason, as previously highlighted in section 1.8 and Figure 1.7, comprehensive metabolomic technology platforms typically take the strategy of dividing the metabolome into subsets of metabolites, and thus the metabolome is measured as a patchwork of results from different analytical methods (Clish, 2015). For this purpose, metabolomics benefits from the development of analytical technologies. The measurement of extracted metabolites within a biological sample can be performed via direct analysis or by separation prior to the detection step, which uses either chromatographic (liquid (LC) or gas (GC) chromatography) or electrophoretic (capillary electrophoresis (CE)) principles. The subsequent detection of metabolites is predominantly based on nuclear magnetic resonance (NMR) and mass spectrometry (MS) (Bedia, 2018; Godzien et al., 2018).

The first metabolomics approaches generally used NMR-based methods (Manchester and Anand, 2017). NMR approaches typically give a broad view of metabolism changes and are useful for identifying unknowns due to its ability to elucidate chemical structures (Wishart, 2016). NMR is a quantitative technique recommended for its high reproducibility, high selectivity, non-destructive nature and simplicity of sample preparation (Bedia, 2018; de Raad et al., 2016). It is based on the energy absorption and reemission of the atom nuclei due to changes in an external magnetic field. Metabolite identification is based on the pattern of the obtained spectral peaks, that are as a reused to indirectly measure the quantity of the metabolite present in the sample. Hydrogen is the most frequentlytargeted nucleus (hydrogen-1 NMR) in metabolomic NMR-based studies, although other less frequently used atoms likecarbon (carbon-13 NMR) and phosphorous (phosphorus-31 NMR) are also targeted (Bedia, 2018). NMR metabolomic approaches have also been performed for lipidomic analysis (Melvin et al., 2019) and widely applied in the field of environmental toxicology using *Daphnia* as a model species. Nagato et al.,(2016) determined changes in amino acids, glucose and lactate after exposure to diazinon, malathion and bisphenol A (Nagato et al., 2016) applying hydrogen-1 NMR metabolomics. Kovacevic et al. have

several studies using this technique to determine disruption in *Daphnia* metabolome after exposure to sublethal concentrations of drugs (Kovacevic et al., 2016), organophosphates (Kovacevic et al., 2018a), perfluorates (Kovacevic et al., 2018b), or other ECs in the presence of organc matter (Kovacevic et al., 2019). There are many more studies have applied this technique to determine the metabolome of D*aphnia* in recent years (Falanga et al., 2018; Jeong et al., 2018b; Kariuki et al., 2017; Wagner et al., 2018), even in *in vivo* studies with whole *Daphnia* organism (Ghosh Biswas et al., 2020).

Although the use of NMR has a long tradition in metabolomics, its sensitivity is lower than that from MS-based techniques (Piña et al., 2018). MS has more recently dominated the metabolomics field due to its high sensitivity and high resolution (Godzien et al., 2018), that achieved the detection and quantification of several hundred small-molecules in a single analysis (Bedia, 2018). Although direct-infusion mass spectrometry (DIMS) has been successfully applied in metabolomics (Kai et al., 2019, 2017), having the great potential of providing more concise raw data than chromatography-based methods without limiting the throughput, the chromatographic step is beneficial to comprehensive analysis. DIMS methodology has also been sucessfully applied for the assessment of some specific metabolites and metabolomic biomarkers in *Daphnia* species (Grintzalis et al., 2019; Taylor et al., 2018). Nevertheless, despite the advantages of DIMS and the shorter analysis time required, especially beneficial in the case of large amounts of samples, matrix effects are inevitable because all the components of the sample are infused together without separation, which reduces the sensitivity and capability for metabolite identification and produces ionic supression (Bedia, 2018). This is especially disadvantageous when dealing with biological matrices due to their huge complexity. Therefore, in metabolomic studies it is often necessary to separate the metabolites of interest prior to MS acquisition. The most applied separation techniques in metabolomic studies coupled to MS are capillary electrophoresis (CE), gas chromatography (GC) and liquid chromatography (LC), each of which has some advantages and disadvantages. Among them, LC-MS is the one most applied due to its capability to detect the widest range of metabolites with a huge chemical diversity, generating information of thousands of molecules with specific mass/charge ratio (m/z) values in a given retention time (Rt). GC-MS has been applied in multiple studies, also determining *Daphnia*'s metabolites (Garreta-Lara et al., 2018, 2016; Vandenbrouck et al., 2010) and PUFAs (Martin-Creuzburg et al., 2010). However, GC is only suitable for the analysis of small and volatile metabolites (e.g. fatty acids, sugars or organic acids), and requires an

additional sample derivatization step. In contrast, capillary electrophoresis (CE) and liquid chromatography (LC) allow direct analysis of non-volatile metabolites. Capillary electrophoresis is especially suitable for the analysis of polar or ionic compounds according to their ionic mobility (Bedia, 2018; Ramautar et al., 2011) and LC allows the separation of a wide range of metabolites, regardless of their hydrophilic or hydrophobic nature (Engskog et al., 2016), using either reverse phase liquid chromatography (RPLC) or hydrophilic interaction chromatography (HILIC) (Manchester and Anand, 2017).

Regarding MS analysis, ionization is a critical step in metabolomics. The most frequently applied ionization methods in metabolite detection are electrospray ionization (ESI) and electronic impact (EI) (Godzien et al., 2018). Whereas ESI is a soft ionization preferred for LC-MS, EI is a hard ionization method that causes fragmentation of metabolites, and is the method of choice of GC-MS analyses (Bedia, 2018). Reviewing the type of MS most commonly applied in metabolomics, there are nowadays a huge diversity of analysers with different resolution and sensitivies, both in sigle (MS) or tandem (MS/MS) configurations. Some of the most used in single MS approaches are time-of-flight (TOF), fourier-transform ion cyclotron resonance (FTICR) and Orbitrap, due to their high resolution and thus commonly applied in untargeted approaches. In the other hand, tandem MS/MS quadrupole ion trap (QTrap), triple quadrupole (QqQ), quadrupole-TOF (Q-TOF), and linear ion trap-Orbitrap (LTQ-Orbitrap) are some of the most applied, and commonly implemented for targeted studies (Bedia, 2018; Gowda and Djukovic, 2014). Alternatively, tissue sections or whole organisms can be directly analyzed by techniques such as matrix-assisted laser desorption ionization (MALDI), secondary ion MS (SIMS) and desorption electrospray ionization (DESI) (Goto-Inoue et al., 2011; Wiseman et al., 2008), which allows *in situ* analysis of the metabolites in order to visualize its distribution within the biological sample.

Figure 1.9 ilustrates the experimental workflow of the two different metabolomic approaches that have been applied throughout this thesis, whose peculiarities will be discussed in detail below.

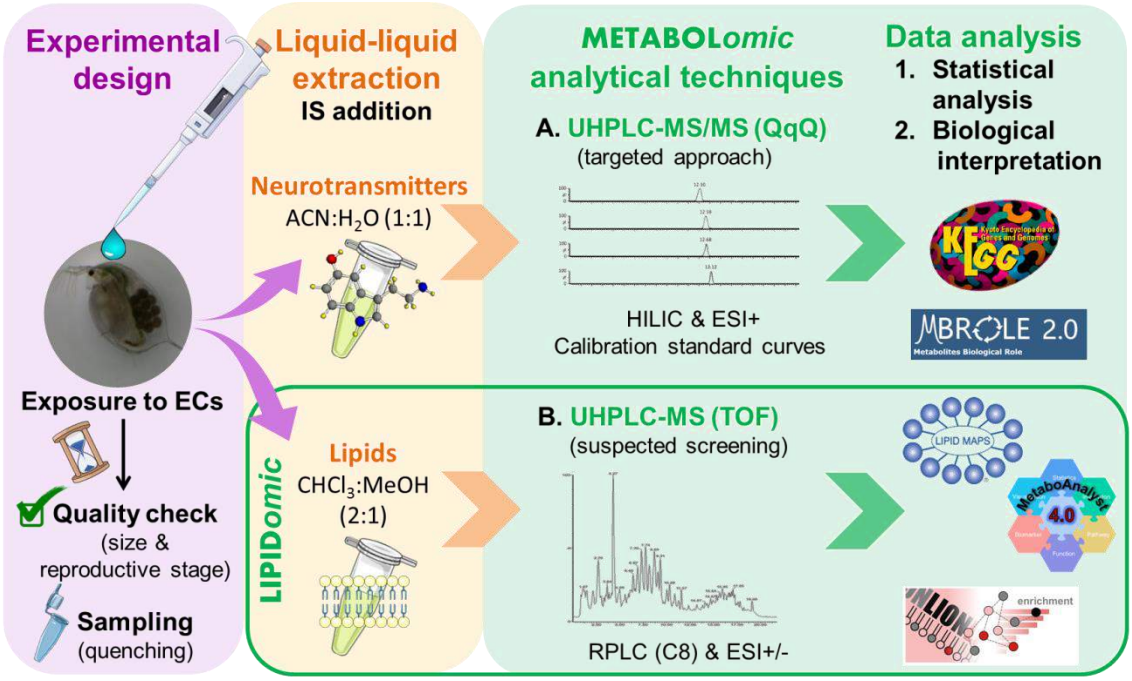

Figure 1.9. Experimental workflow of metabolomics experiments applied throughout this thesis. The different phases of a metabolomic study are simplified: experimental design considerations, extraction procedure, applied analytical techniques and data analysis with used databases and bioinformatics tools. ECs: emerging contaminants; ESI: electrospray ionization; HILIC: hydrophilic interaction chromatography; HPLC: high-performance liquid chromatography; IS: internal standard; MS: mass spectrometer; QqQ: triple quadrupole; TOF: time-of-flight; UHPLC: ultra-high performance liquid chromatography; RPLC: reverse phase liquid chromatography.

a. Liquid chromatography (LC) metabolite separation

LC has become the reference technique for the separation and characterization of a large number of molecules for many different applications (Swartz, 2005). As stated before, this separation technique is particularly suitable for metabolomic studies as it allows the separation of a wide range of structurally diverse metabolites, regardless of their hydrophilic or hydrophobic nature (Engskog et al., 2016). Furthermore, it facilitates the separation of isobaric and isomeric specimens that would not be possible to differenciate with DIMS, and greatly guarantees the quality of quantity results because each chromatographic pic is in 2D space with a characteristic Rt against a specific m/z (Jurowski et al., 2017; Yang and Han, 2016).

Over the years, various modes of separation of molecules associated with LC have been developed acoording to the stationary phase and the type of interaction with the target analyzed molecules (i.e. metabolites). Linked to technological advances, one of the trends in LC has been the reduction of the chromatographic columns particle

size in order to increase the chromatographic separation, which produces a considerable increase of the column pressure. Therefore, LC traditional systems (i.e. high-performance liquid chromatography (HPLC)) had to evolved in parallel with this diameter particle size decrease, developing ultra-high performance liquid chromatography (UHPLC) systems, that supports higher working pressures (higher than 1,000 bars), and has better efficiency, speed and sensitivity compared to HPLC systems (Swartz, 2005). This system typically used, particularly in metabolomics applications, either reverse phase liquid chromatography (RPLC) or hydrophilic interaction chromatography (HILIC) columns (Manchester and Anand, 2017).

Reverse phase columns, such as the C8 and C18, have been the most popular stationary phases for many years because of their versatility in the analysis of a wide range of compounds. However, in the field of metabolomics, RPLC columns present a limitation due to the low retention of the polar and hydrophilic compounds. Therefore, it has been proposed the use of other modes of separation, such HILIC to overcome this disadvantage of reversed phase columns without applying derivatization (Hemström and Irgum, 2006; Park et al., 2013; Tufi et al., 2015). Furthermore, due to its selectivity, HILIC provides greater freedom from matrix effects compared to RPLC (Van Eeckhaut et al., 2009).

HILIC was introduced in 1990 (Alpert, 1990), although its first application in the metabolomics field was in 2002 (Tolstikov and Fiehn, 2002). Since then, it has been an increasing number of metabolomic studies applying this separation technique (Gallart-Ayala et al., 2018; H.-J. Lee et al., 2019; Tang et al., 2016; Tufi et al., 2015), also applied to *Daphnia* matrices (Rivetti et al., 2019). HILIC chromatography can be considered a variant of normal phase liquid chromatography (NPLC) considering that both methodologies use polar stationary phases and that the retention increases with the polarity of the analytes (Greco and Letzel, 2013), but HILIC uses mobile phases similar to RPLC, i.e. aqueous-organic mobile phases rich in organic solvents (usually acetonitrile). Polar target analytes are soluble in this water fraction, and due to the high organic content of mobile phases (that provides lower viscosity), HILIC allows an increase sensitivity of MS improving the efficiency of the ionization (Jandera, 2011). The HILIC separation mode is based on the partitioning of the analytes between the mobile phase and a hydrophilic layer that is on the stationary polar phase (Buszewski and Noga, 2012). Various stationary phases can be used in the HILIC mode for separations of metabolites, as bare silica gel, or silica-based modified with polar functional groups (e.g. amino-, amido-, carbamate-, diol-, polyol-, zwitterionic sulfobetaine or poly(2-sulphoethyl aspartamide)), but also ion exchangers or

zwitterionic materials showing combined HILIC-ion interaction retention mechanism (Jandera, 2011).

In the particular case of the methodology applied in this thesis (represented in Figure 1.9), both modes of separations have been applied, either C8 column (RPLC) for lipid separation (i.e. lipidomic studies) in chapter 2 and 3, or HILIC column for neurotransmitter separation in chapter 4.

b. Mass spectrometry (MS) based metabolomic approaches

Two different approached based on MS have been applied within this thesis, a targeted approach using tandem MS/MS with a triple quadrupole (QqQ) and a suspected screnning or semi-targeted approach with a time-of-flight (TOF) mass analyzer. ESI was used as an ionization source in either way. ESI is the ultimate source of ionization for metabolomic studies, due to its compatibility with moderately polar molecules and thus suitable for the analysis of many metabolites. After LC, samples are pumped through a metal capillary (at 3 to 5 kV) and nebulised at the tip to form a fine spray of charged droplets. These droplets are rapidly evaporated by heat and dry nitrogen, and the residual electrical charge is transferred to the analytes. Then, the ionized analytes are transferred into the high vacumm of the specific mass spectrometer, i.e. QqQ and TOF. The ion source and subsequent ion optics can be operated to detect positive or negative ions, and switching between these two modes within an analytical run can be performed (Pitt, 2009).

As stated in section 1.8, MS-based metodologies can be performed according to the omic approach of interest, i.e. targeted, untargeted, or suspected screening. In the present thesis, a targeted study was carried out in order to analyze different neurotransmitters in *Daphnia* biological samples using a QqQ as MS (chapter 4), meanwhile suspected screening or semi-targeted approaches were performed for lipidomic studies using a TOF MS (chapter 2 and 3). For this reason, the peculiarities associated with these two MS techniques are explained below.

- Tandem MS-based targeted studies

Targeted studies focus on the detection, analysis and quantification of a reduced set of known preselected and previously characterized biological molecules (Bedia, 2018; Griffiths et al., 2010). In this strategy, MS-based methods are optimized to accurately detect that predetermined set of targeted compounds to the detriment of a general coverage of the metabolome. With this approach, relationships and novel

associations between the analyzed metabolites may be illuminated in the context of specific physiological states.

When utilizing targeted metabolomics sample preparation can be optimized, reducing the dominance of not of interest high-abundance molecules in the analyses and analytical artifacts, since all analyzed species are clearly defined. Standards of the target metabolites are also analyzed, which are used for the development and the optimization of the analytical method with the corresponding generation of its calibration curve, which enables a quantitative analysis. Internal standards (ISs) spiked into each sample can be used to normalize concentrations of metabolites across sample sets and batches (Roberts et al., 2012). Furthermore, as ISs are also affected by the extraction, they allow to minimize the variability of the extraction and of the instrument measure and matrix effects and interferences of the sample. Quadrupole analyzer consist of a set of four parallel metal rods that trasmit a narrow band of m/z values by combination of constant and varying voltages, resulting in a mass spectrum. A particularly useful mass spectrometer configuration is obtained by placing a collision cell between two quadrupole mass analysers, what is call QqQ (Pitt, 2009). A metabolite precursor is ionized at the ESI source and then resolved and isolated in the first quadrupole (Q1). The second quadrupole (Q2) functions as a collision cell with an inert gas (such as nitrogen or argon) where the target ion is fragmented. Finally, the ions are accelerated into the third quadrupole (Q3), which also acts as a mass filter, selecting for a particular *m/z* of a fragment ion, which is then introduced to the detector. Only analytes with this precursor/product ion (transition) combination will be detected. This targeted strategy allows the quantification in the same analysis of many target metabolites with the acquisition by MRM (Multiple Reaction Monitoring) of the specific signals. MRM on a QqQ mass spectrometer has long been used for targeted metabolite quantification, and this technique is regarded as the gold standard for quantitation of small molecules and metabolites (Zhou and Yin, 2016). Since individual metabolites have specific precursor/product ions (transition), the identity of the metabolite can be ensured, especially when combined with a known chromatographic retention time (Roberts et al., 2012). This tandem MS/MS offers increased selectivity and sensitivity over single MS by selection of a compound-specific precursor ion in the first mass spectrometer, fragmentation of the precursor ion in a collision cell and subsequent selection of the specific fragment or product ions in a second mass spectrometer (van der Gugten, 2020). These kind of approach has been used in several *Daphnia* and other species metabolomic studies e.g. for the determination of neurotransmitters (Rivetti et al., 2019; Tufi et al., 2015) or amino acids, nucleotides and

carbohydrates in order to characterized endocrine disruptor effects (Jeong and Simpson, 2020) or reproduction stage metabolomic differences (Jeong and Simpson, 2019).

In the above mentioned targeted analytical strategies, given the previously known nature of the analyzed metabolites, the data processing tends to be straightforward. Generally, the quantitation of the peaks can be performed in the vendor-specific software (e.g. MassLynx (Waters)). After metabolite peak identification according on its mass spectrum and Rt, this software enables the integration of the appropriate peak, normalization to the internal standard and calibration to a standard curve (Lamichhane et al., 2018).

- Time-of-flight (TOF)-based suspected screening analysis

As aforementioned, suspected screening, semi-targeted or large scale targeted approach is an strategy that is halfway between targeted and untargeted apporaches. This methodology is usually performed in some metabolomic studies (Che et al., 2018). While targeted assays are usually applied to validate and translate the novel discoveries of a hypothesis-generating study, both untargeted and semi-targeted approaches are applied in hypothesis-generating studies. Data is obtained in an untargeted analysis, but it is processed as in a targeted approach, where only the information of known preselected biological molecules is extracted. Contrary to targeted strategies, no reference standard per each individual metabolite is used in this kind of approaches. Instead, metabolite identification is performed according to intrinsic characteristics of the analytes, such as the m/z precission, the isotopic pattern or Rt. These semi-targeted metabolomic approaches typically used high resolution mass spectrometry (HRMS) platform, were full scan-based untargeted analysis is applied to extract target ions from MS and to generate targeted ion lists. However, actual quantification of MS is performed on the peak areas of precursor ions and no fragmentation is involved, and interference is therefore more likely which results in data of lower reliability (Zhou and Yin, 2016). For this reason, currently there is a tendency to preferably use semi-targeted or untargeted MS as Q-TOF or QTrap, where HRMS is applied but also make possible the fragmentation of the molecule into an additional quadrupole, which allows the unique identification of the metabolites. During this thesis, however, there was no access to this instrumentation, and therefore the analyses were carried out by means of a TOF, which is also widely used in metabolomics (and thus lipidomics) studies (Pitt, 2009). Nevertheless, since it is not possible to carry out a fragmentation with this analyzer, it is not possible to know the total structure of the

specific lipid, but only the category or family to which it belongs and the number of carbons and unsaturations of its hydrocarbon chains can de determined.

The time-of-flight (TOF) MS operates by accelerating ions through a high voltage. The velocity of the ions, and hence the time taken to travel down a flight tube from the ionization source to reach the detector, depends on their m/z values (Marshall and Hendrickson, 2008). Therefore, ions with higher charge and lower mass will arrive at the detector faster than the lower charge and higher mass ions. The development of orthogonal acceleration technology has considerably improved both the resolution and the mass accuracy of these analyzers (Guilhaus et al., 2000). This technology consists in making the ions pass through a reflector that inverts the direction of its flight in order to compensate the differences in the kinetic energy of the ions coming from the ionization source. Thus, if a pack of ions of a given m/z has different kinetic energies, the reflector will decrease the dispersion of the time of flight of those ions (García-Reyes et al., 2018). TOF analyzer can acquire spectra extremely quickly with high sensitivity and high mass accuracy (Pitt, 2009), with errors of less than 1-5 ng/µL working at high resolution (up to 11,500 FWHM (full width at half maximum) at m/z 556) (Gorrochategui et al., 2014a) and providing a high acquisition rate (20-500 spectra). Moreover, the TOF is a relatively simple and economical analyzer. All these characteristics make it a very suitable MS analyzer for this kind of metabolomic approaches. This type of TOF-based metabolomic methods has been applied in environmental studies, determining the metabolic profiling of biological samples exposed to environmental stressors (Ortiz-Villanueva et al., 2015). It has been widely applied for lipidomic environmental studies, as in this thesis (Gorrochategui et al., 2014a, 2014b; Guercia et al., 2017; Marqueño et al., 2019), and even also in *Daphnia* matrices (Jordão et al., 2015).

For suspected screening metabolomics analysis, relative quantification or semi-quantification is often preferred. This is due to the fact that achieving absolute simultaneous quantification of large numbers of metabolites is expensive because of the need of standards and internal standards (the isotopic labeled standard or an analogue of the analyte) for every compound, that in many cases are not even commercially available. Relative quantification is thus a common choice for large-scale targeted metabolomics analysis (Zhou and Yin, 2016). In the particular case of lipidomic studies, this is normally performed with the addition of an internal standard per lipid family. Thus, lipid identification is based on the accurate m/z measurement of the monoisotopic peak, its relative retention time (combining the retention time of the internal standard used for each lipid family with the retention time of the individual lipid

of interest, and taking into account the changes in the retention time due to the number of carbons and of unsaturations), and the correct isotopic distribution of the main adduct of each lipid.

- Obtained information and biological interpretation

Figure 1.10 summarizes the information that is obtained through each of the analytical phases carried out for the metabolomics studies developed in this thesis (i.e. LC and MS), which have already been discussed above.

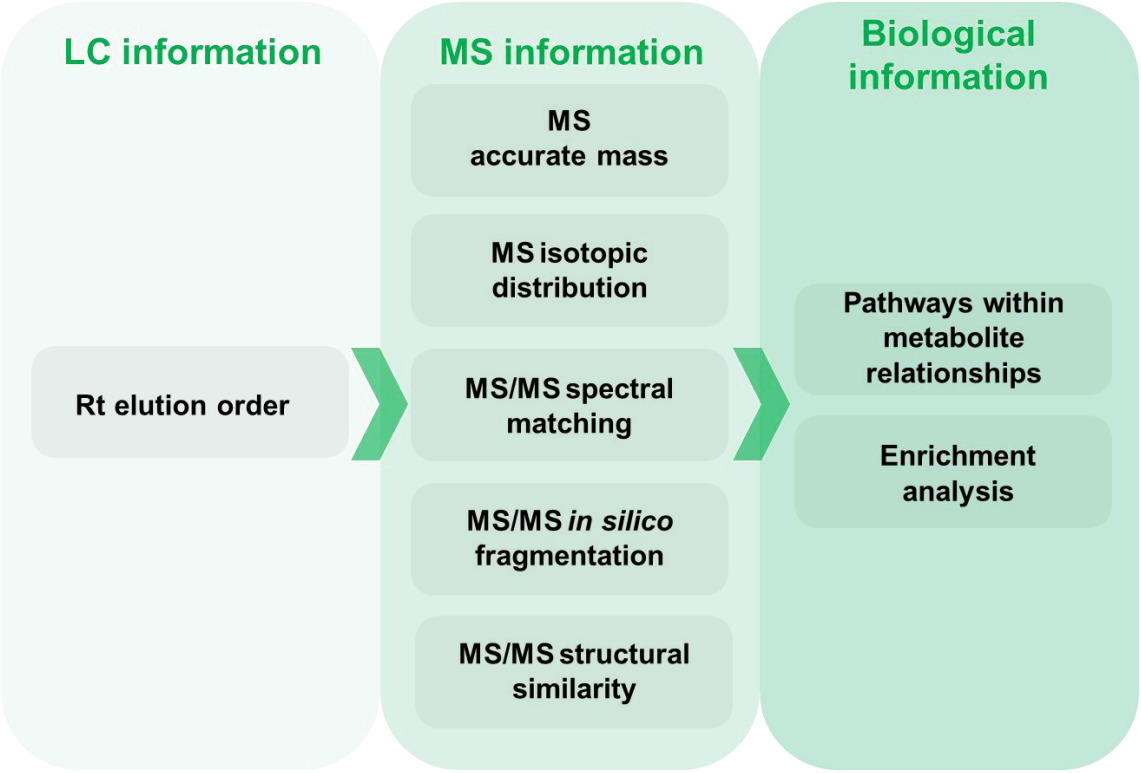

Figure 1.10. Information obtained at different levels of a metabolomic study (adapted from Godzien et al. (2018). LC: liquid chromatography; MS: mass spectrometry; MS/MS: tandem mass spectrometry; Rt: retention time.

Nowadays, there are multiple metabolite online datases with pracical analytical information, accurate mass and expected adduct information or mass spectra (MS and MS/MS) information, such as METLIN (https://metlin.scripps.edu/) (Guijas et al., 2018), HMDB (https://hmdb.ca/) (Wishart et al., 2007), or mzCloud (https://www.mzcloud.org/), and specific for lipids as Lipid Maps (https://www.lipidmaps.org/) (Fahy et al., 2007), Lipid Library (https://lipidlibrary.aocs.org/), Cyberlipid (http://cyberlipid.gerli.com/), LipidBank (http://lipidbank.jp/) (Watanabe et al., 2000) or LipidBlast

(https://fiehnlab.ucdavis.edu/projects/lipidblast) (Kind et al., 2013). These databases have facilitated the broad usage of metabolite identification as well as method construction for metabolomics analysis (Zhou and Yin, 2016).

Figure 1.9 shows the resources that have been applied in this thesis for metabolomic data analysis and interpretation. MetaboAnalyst (https://www.metaboanalyst.ca) is a web-based tool suite for comprehensive metabolomic data analysis, interpretation, and integration with other omics data. In addition to providing a variety of data processing and normalization procedures, MetaboAnalyst supports a wide array of functions for statistical, functional, as well as data visualization tasks (Chong et al., 2019). Regarding to the biological interpretation of metabolomic results, KEGG (already mentioned in transcriptomics section) (http://www.genome.jp/kegg/) is also a valuable bioinformatics resource for the biological interpretation of metabolomics. Nevertheless, the interpretation of affected metabolic pathways with metabolomic data is not as straightforward as with transcriptomic data, especially for lipidomics due to the fact that within each lipid family there is a large number of lipids and to the existence of interconnections between metabolic pathways.

Pathway enrichment analysis is an useful strategy to gain mechanistic insight into metabolites generated from metabolomic experiments. This method identifies biological and metabolic pathways that are enriched in a metabolite list more than would be expected by chance (Reimand et al., 2019). There are several enrichment resources (Chagoyen and Pazos, 2013), as e.g. the already mentioned MetaboAnalyst, MetExplore (http://www.metexplore.fr/) (Cottret et al., 2010) or MetScape (http://metscape.ncibi.org/) (Karnovsky et al., 2012). Among the available options, in this thesis were used MBrole (http://csbg.cnb.csic.es/mbrole2/) (López-Ibáñez et al., 2016) for neurotransmitter metabolomic analysis and Lipid Ontology (LION) enrichment analysis web application for lipidomic studies (Molenaar et al., 2019).

1.9.3. Lipidomics

As it was pointed out when dealing with the different omic sciences, lipidomics emerged as a sub-discipline of metabolomics, but in some aspects is considered as an independent branch due to the high number of lipid species and its high biological relevance. Furthermore, as with other types of biomolecules, lipid structural, physical and chemical properties are so different to other metabolites that requires different considerations from other metabolomic disciplines, including the extraction and analytical procedures.

In a similar way to other omic sciences, lipidomics has been defined as the complete characterization of lipid species (lipidome) and their biological roles with respect to expression of proteins involved in lipid metabolism and function, including gene regulation (Spener et al., 2003), aiming to obtain a lipid profile which provides information on the composition and abundance of lipid species in a biological sample. Traditionally, lipidomics has been mainly applied in the field of human medicine and clinical sciences, but the versatility and utility of lipidomic applications has been increasingly recognized in environmental toxicology in recent years (Aristizabal-Henao et al., 2020). This is mainly due to the fact that lipids are highly abundant and ubiquitous across all organisms and lipid profiles often change drastically in response to external stimuli (Koelmel et al., 2020).

Some of the peculiarities regarding to lipidomics studies have already been explained within the metabolomics section. Nevertheless, due to the particular nature of lipids and the important weight of these studies within this thesis, in this section some characteristics of lipid extraction and separation, complementary methods applied for their analysis as well as the peculiarities of lipid categories are further explained.

a. Lipid families and functions

Etymologically, the word lipid comes from the Greek word "lípos", which means "fat". Lipid are key biomolecules within organisms, since they are essential for energy supply and homeostasis, reproduction, organ physiology and numerous aspects of cellular biology (Lee et al., 2003), and thus they reflect environmental changes and effects of environmental stressors (Lee et al., 2018). The interest in studying lipids in environmental toxicology became especially relevant with the emergence of potential endocrine disruptors or other ECs suspected of being able to affect the maintenance of lipid homeostasis or disrupt signaling pathways related with lipids and lipid metabolism. Lipid metabolism is crucial for survival and propagation of widely distributed aquatic invertebrates that are ubiquitously exposed to environmental factors such as ECs. Nutritional status is a crucial factor in maintaining nominal biological processes against environmental changes in aquatic invertebrates to ensure homeostasis (Lee et al., 2018). Pollutants alter storage and can disrupt lipid metabolism and homeostasis in somatic organs (Parrish, 2013). Lipids can also act as solvent and absorption carrier for organic contaminants and thus can be drivers of pollutant bioaccumulation (Laender et al., 2010). Therefore, understanding lipid metabolism in the presence of

environmental stressors is important in regard to aquatic invertebrates (Lee et al., 2018).

Lipids are a diverse type of hydrophobic or amphiphilic small metabolites. Lipid families or categories are composed of a broad spectrum of individual species that differ in the nature of their hydrophobic and hydrophilic moieties so that the complexity of the lipidome may exceed that of the proteome (Dowhan and Bogdanov, 2008). The International Lipid Classification and Nomenclature Committee (ILCNC) (Fahy et al., 2011) defined eight major categories of lipids, represented in Figure 1.11, based on their chemically functional backbones and biochemical principles. This classification is spearheaded by the Lipid Maps consortium (Fahy et al., 2009), whose database contains nowadays more than 44,000 individual lipid species. Among these categories, the ones studied within this thesis are mainly glycerolipids and glycerophospholipids, and some types of sphingolipids and sterol lipids. These lipid categories can be further subdivided in sub-categories, some of which are further detailed in Figure 1.12. Fatty acids (FA) are important constituents of a large number of more complex lipids. FA are formed by a carboxyl group (-COOH) and an aliphatic or linear hydrocarbon chain, that can present different unsaturation degree. The most common FAs in living organisms are of even chain, especially those containing 16 and 18 carbons. Glycerolipids are lipids that contain a glycerol group. The most common ones can incorporate one, two or three FAs to their structure, being respectively monoacylglycerol (MG), diacylglycerol (DG) and triacylglycerol (TG), as represented in Figure 1.12 (Fahy et al., 2009). Glycerophospholipids are lipids that contain also a glycerol, but with a phosphate group at the sn-3 position. The positions sn-1 and sn-2 contain two FAs. If the structure does not contain one of the two acyl groups, they are defined as lysophospholipids. The different subclasses of GPs are differentiated by the polar functional group linked to the phosphate, being: choline (PC), ethanolamine (PE), serine (PS), glycerol (PG), inositol (PI) or without group as the phosphatidic acid (PA) (Figure 1.12). In addition, in the sn-1 position there may be an ester or vinyl ester bond, being either plasmanyl- (O-) or plasmenyl- or plasmalogen (P-), respectively. Sphingolipids have a long chain sphingoid base, which can include different substituents, although the ones analyzed within this thesis are sphingomyelins (SMs) that contain an amide-related FA and a phosphocholine group. The sterol lipids include cholesterol and its derivatives. Within this thesis, the analyzed ones have been cholesteryl esters (CEs), which have an ester bond between the carboxylate group of a FA and the hydroxyl group of cholesterol.

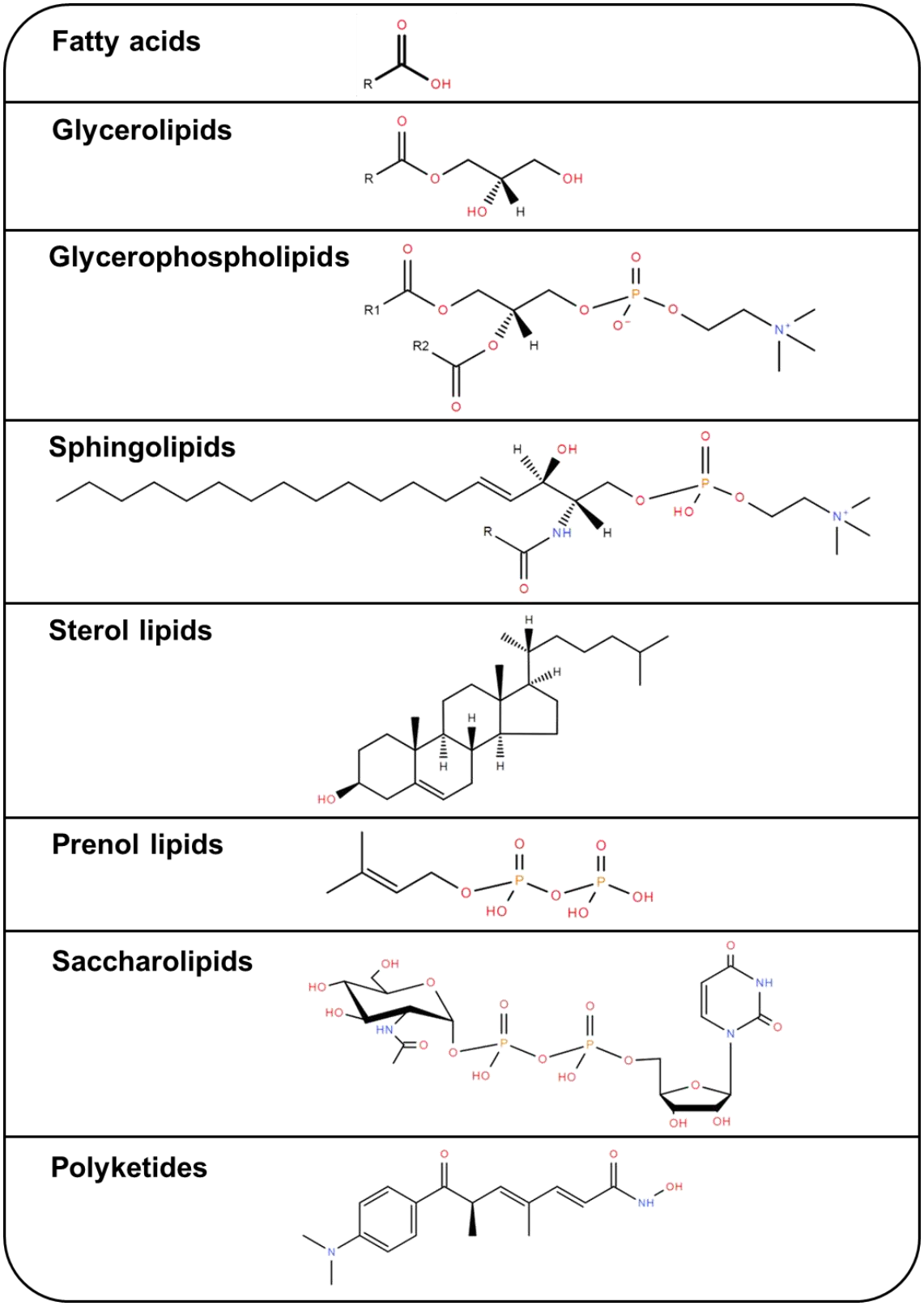

Figure 1.11. Representative structures of the 8 lipid categories defined by the International Lipid Classification and Nomenclature Committee (ILCNC) (Fahy et al., 2011). Example structures were obtained from LIPID MAPS (www.lipidmaps.org). R, R1 and R2 correspond to the fatty acid hydrocarbon chains.

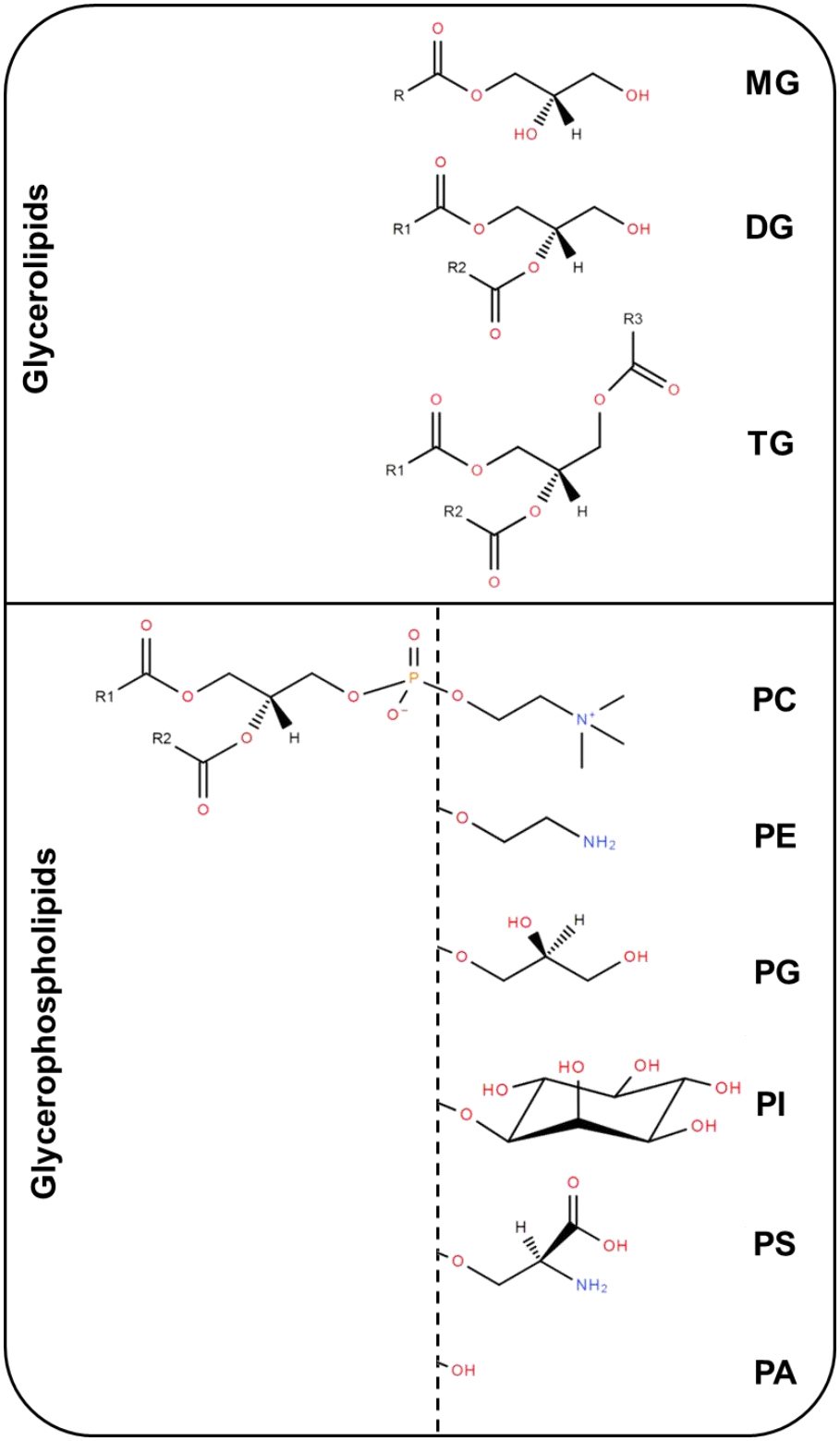

Figure 1.12. Representative structure of the subcategories of glycerolipids and glycerophospholipids defined by the International Lipid Classification and Nomenclature Committee (Fahy et al., 2011). Example structures obtained from LIPID MAPS (www.lipidmaps.org). R1, R2 and R3 correspond to the fatty acid hydrocarbon chains. MG: monoacylglycerol, DG: diacylglycerol; TG: triacylglycerol; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PS: phosphatidylserine; PA: phosphatidic acid. Corresponding lysophospholipids from represented glycerophospholipids would be structurally similar but without one acyl group.

Table 1.2. Lipid categories and subcategories with their main described biological functions.

Lipid categories	Biological function
Fatty acids (FA)	• Components of other lipid families, they undergo several metabolic reactions such as oxidation, scission or polymerization (Qiu et al., 2020). • Energy production (via oxidation) or lipid synthesis (via esterification) (de Carvalho and Caramujo, 2018). • Signaling molecules and second messengers (Itoh et al., 2003).
Glycerolipids MG: monoacylglycerols DG: diacylglycerols TG: triacylglycerols	• MG is an intermediate in the degradation of DG and TG and of the catabolism of some phospholipids (Lee et al., 2020). • Energy storage (mainly TG, that is also involved in thermal insulation) (Aguilera-Méndez et al., 2013). • DG has signaling function with protein kinase C (Hodgkin et al., 1998).
Glycerophospholipids PC: phosphatidylcholines PE:phosphatidylethanolamines PG: phosphatidylglycerols PI: phosphatidylinositols PS: phosphatidylserines PA: phosphatidic acids	• Membrane lipids, key components of the lipid bilayers (especially PC and PE) (van Meer et al., 2008). • Involved in metabolism and signaling (Hishikawa et al., 2014). • PS is involved in calcium binding in bone growth (Merolli and Santin, 2009) • PI is related to protein anchorage and is precursor for other signaling molecules (Clarke, 2003) • PG produces the activation of protein kinase C family and is precursor of cardiolipins (Morita and Terada, 2015). • Phospholipases hydrolyze them to generate lysophospholipids (Wilton, 2008).
Sphingolipids Ceramides Sphingomyelins (SM) Glycosphingolipids	• Implicated in numerous intra and extracellular signaling processes (Bartke and Hannun, 2009). • Components of the lipid bilayers (van Meer et al., 2008). • Form lipid microdomains or rafts, which function as hubs for effective signal transduction and protein sorting (Bartke and Hannun, 2009).
Sterol lipids Cholesterol and derivatives Steroids Secosteroids Bile acids and derivatives	• Components of the lipid bilayers (van Meer et al., 2008). • Hormones and signaling molecules (Wollam and Antebi, 2011).
Prenol lipids Isoprenoids Quinones and hydroquinones Polyprenols	• Antioxidants and precursors of vitamins (Stephenson et al., 2017).
Saccharolipids Acylaminosugars Acyltrehaloses Acyltrehalose glycans	• Form structures that are compatible with membrane bilayers (Fahy et al., 2005). • Cell membrane lipids in bacteria (Fahy et al., 2005).
Polyketides Acetogenins Macrolides and lactones Ansamycins Aflatoxins Cytochalasins Flavonoids	• Some are linked with non-ribosomally synthesized peptides to form hybrid scaffolds (Fahy et al., 2005). • Used as antimicrobial, antiparasitic and anticancer agents (Fahy et al., 2005; Stephenson et al., 2017).

Lipids are a class of molecules that displays a wide diversity in both structure and biological function. A primary role of lipids (especially glycerophospholipids) in cellular function is in the formation of the permeability barrier of cells and subcellular organelles in form of a lipid bilayer. The external layer is mainly formed by PC and sphingolipids and the internal layer by PS and PE, while cholesterol molecules are incorporated in a random way within the two layers (Pande, 2000). Poly-unsaturated fatty acids (PUFAs) are constituents of a large variety of glycerophospholipids and provide several important properties to the cellular membranes as fluidity and flexibility (Rolim et al., 2015). Moreover, lipids are the main form of energy storage within the cell, mainly in TG as lipid droplets, generating ATP energy by the β-oxidation of their FA (Tocher, 2003). Nevertheless, lipids are not only energy depots and structure builders in the cell, but they also play active roles in membrane functions and can act as messenger molecules (Dowhan and Bogdanov, 2008). Further details regarding to their biological functions and processes in which they are involved are summarized in Table 1.2.

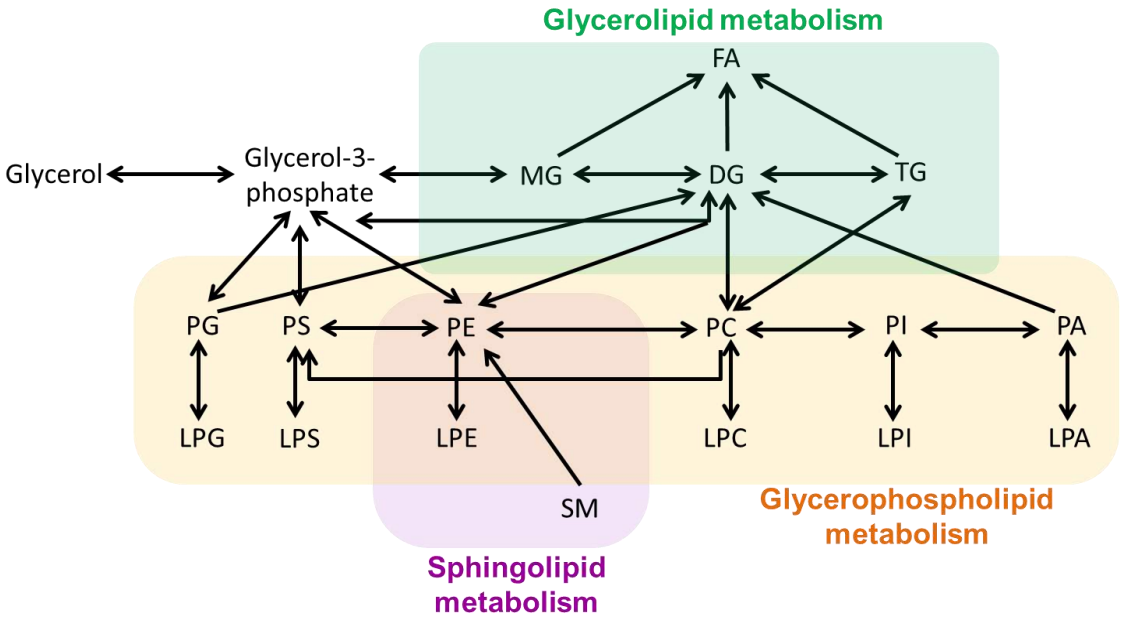

Figure 1.13. Simplified pathway that represents some lipid metabolic processes relating different lipid categories. The information represented was obtained from KEGG pathway database.

The homeostasis of these lipids (balance between capture, transport, storage, biosynthesis, metabolism and lipid catabolism) (Tocher, 2003) is regulated by different transcription factors, already explained in section 1.5.1. A simplified pathway

representing metabolic processes that relate different lipid categories is represented in Figure 1.13. As it can be seen in the figure, which only represents some metabolic processes that relate some lipid categories and sub-categories without the intermediate reactions and subsequent metabolic cascades, the interpretation of lipidomic results is often complicated due to the wide number of relationships and interconnections among them.

b. Peculiarities of the extraction and analytical separation of lipids

There are different types of lipid extraction depending on the lipid categories to be analyzed. The already mentioned database Lipid Maps collects the most suitable extraction methods for each lipid category. In general, a liquid-liquid extraction is performed, focused on the non-polar fraction.

Most lipidomic studies aim to analyze the maximum possible lipid categories in order to obtain a representative profile of the lipidome. Therefore both non-polar lipids (glycerolipids and sterols) and more polar lipids (glycerophospholipids and sphingolipids) must be extracted at once. To be able to carry out this, it is important to incorporate solvents of different polarities within the extraction procedure in order to solubilize as many lipid species as possible. In addition, it must be taken into account that removing as much interference (i.e. other metabolites) as possible is the most convenient way to be able to carry out a correct instrumental analysis afterwards. Thus, the extraction with organic non-polar solvents provides low levels of polar metabolites, proteins and salts, reducing the complexity of the samples and the subsequent MS-based instrumental analysis.

The chloroform-methanol-water extractions of either Folch (Folch et al., 1957) or Bligh and Dyer (Bligh and Dyer, 1959) have been traditionally recognized as the best lipid extraction methods. More recently, other methods that use methyl-tert-butyl ether (MTBE) (Abbott et al., 2013) or ethanol (Satomi et al., 2017) as extraction solvents have also been reported as suitable procedures for lipidomics. The Folch method consists in a chloroform:methanol 2:1 (v/v) lipid extraction, that then is mixed with 0.2 times its volume of water phase. To obtain an optimum efficiency, the mixture of solvents should be 20 times the volume of the sample (e.g. for 500 µL of sample, 10 mL of solvents). Bligh and Dyer is a similar extraction procedure based in the previous one, but the solvent system used less amounts of chloroform and methanol for the primary extraction step, with a final proportion of chloroform:methanol:water 2:2:1.8 (v/v/v). Both methods provide very good lipid recoveries, where methanol helps in the separation of lipids from the proteins within the sample, and chloroform generates two

well-differentiated phases that facilitate obtaining the lipophilic fraction of the extract. In addition, generally antioxidant agents are added previous to the extraction to prevent lipids from oxidation during the extraction process (e.g. butylated hydroxytoluene (BHT) or hydroxybutylanisole).

Regarding to the chromatographic separation for subsequent MS lipidomic analysis, both RPLC (Avela and Sirén, 2020) and HILIC (Sandra and Sandra, 2013) approaches have been proposed. Nevertheless, while HILIC separates lipids according to their head group polarity, and thus by lipid categories or families, the RPLC separates them based on general polarity/hydrophobicity, the length of its FAs, as well as its degree of unsaturation. However, nonpolar lipids (e.g. CEs and TGs) and lipids with only one hydroxyl group (e.g. ceramides, DGs, MGs and cholesterol) are often barely retained with HILIC columns. In contrast, PA and PS lipid species are known to have broad or barely detectable peaks in RPLC, and thus, proper methods for the detection of these lipid categories should been specifically approached with HILIC (Avela and Sirén, 2020). For this reason, RPLC is more common among lipidomic studies, where separation is based on the interaction of a non-polar stationary phase with non-polar analytes (Avela and Sirén, 2020). This chromatographic separation method was the one applied in this work with a C8 column.

c. Complementary lipid analysis techniques

The studies in which *Daphnia*'s lipidome is determined in the present thesis are mostly based on LC-MS lipidomic strategies. However, as a preliminary screening test and to validate and contrast the results obtained by this technique, two other methods were applied.

Nile red fluorescence staining assay was performed in order to determine the accumulation of storage lipids into lipid droplets (i.e. TGs). Nile red is a lipophilic stain that is intensely fluorescent in a lipid-rich environment. This method, suitable for toxicological studies (Tingaud-Sequeira et al., 2011), has been previously applied for the determination of lipid droplets in *Daphnia* samples (Jordão et al., 2016, 2015), showing a close relationship with whole organism levels of TGs.

Thin layer chromatography (TLC) was used for qualitatively validate lipidomic MS-based analysis. TLC is a widely used, fast, highly reproducible, and relatively inexpensive method of separation of complex mixtures and provides a robust and reliable qualitative measure of the existing lipid groups within a given sample (Olsen and Henderson, 1989). This method allows the separation of different lipid families (but

not individual lipid species) based on their partitioning between the mobile and the stationary phases (usually a silica coated glass plate), which depends on the lipid hydrophobicity. Each lipid family is represented by a spot in the silica plate, whose relative amount can be calculated by densitometry (Touchstone, 1995). Over the years, the traditional TLC introduced some improvements, such as a smaller silica particle size, a thinner plate and/or double elution. This approach, with a double plate elution with polar and non-polar solvents, has been previously applied for aquatic organisms lipidome determination (Martínez et al., 2020; Navarro and Villanueva, 2000; Reis et al., 2017; Valverde et al., 2012). Although being a low cost and time methodology, this technique show higher matrix effects than MS-based lipidomic approaches, as well as minor sensibility and accuracy. Nevertheless, it represents an effective method to validate high throughput lipidomic data.

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Chapter 2

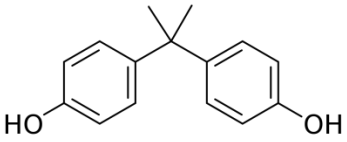
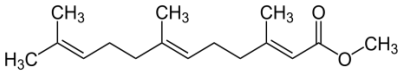
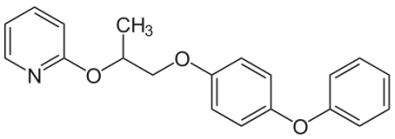
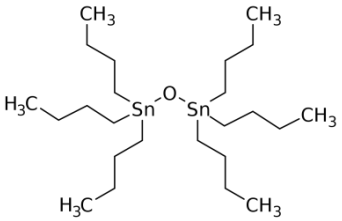
Lipidomic and transcriptomic changes induced by compounds enhancing accumulation of storage lipids in *Daphnia magna*

2.1. Introduction

As highlighted in Chapter 1, endocrine disrupting compounds (EDCs) are ECs of environmental interest and concern. Between all the possible alterations produced by EDCs, those related with lipid metabolism are of particular interest in environmental toxicology studies (Maradonna and Carnevali, 2018). Thus, in 2006 Grün and Blumberg introduced the term “obesogen” (Grün and Blumberg, 2006), defining a group of EDCs that inappropriately regulate and promote lipid accumulation and adipogenesis (Grün and Blumberg, 2009, 2007). This “obesogen” subset of EDCs have the potential to interact with hormone receptors and neuroendocrine signaling, leading to a disruption of the lipid homeostasis on organisms (Barata et al., 2004; Haeba et al., 2008; LeBlanc, 2007; Wang et al., 2011; Wang and LeBlanc, 2009). This compounds are nowadays under strong scrutiny due to the relationship between lipid metabolism deregulation with several important human diseases (Capitão et al., 2017; Castro and Santos, 2014; de Cock and van de Bor, 2014; Grün and Blumberg, 2006; Machado Santos et al., 2012) but relatively few is known about the effects of EDCs in aquatic and non-vertebrates species (Haeba et al., 2008). Furthermore, little is known the mechanisms by which ECs disrupt storage lipid accumulation in invertebrates, probably due to our lack of knowledge about invertebrate lipid metabolism and regulation (Jordão et al., 2015; Machado Santos et al., 2012).

Although an increasing number of studies report changes in lipid metabolism and related gene pathways in invertebrates after exposure to ECDs (Jordão et al., 2015, 2016; Sengupta et al., 2017; Seyoum et al., 2020), it is still necessary to complement ecotoxicology studies with genomic (i.e. transcriptomics) and metabolomic technologies (i.e. lipidomics) to unravel the mode of action of EDCs that help us to understand the complexity of the effects and consequences of the exposure to these compounds. In order to shed some light on this purpose, the effects of some reported EDCs in lipidomic and transcriptomic *D. magna* responses were explored. Tested chemical compounds, with their structures and described modes of action, are summarized in Table 2.1.

Table 2.1. Endocrine disrupting compounds (EDCs) tested within this chapter. Their structures, applications and described mode of action are detailed.

EDC	Structure	Applications	Mode of action
Bisphenol A (BPA)		Plasticizers	Bind to the estrogen receptor (ER) and interact with other targets in mammalian cells (Bašić et al., 2012; Wetherill et al., 2007)
Methyl farnesoate (MF)		Crustacean natural hormone analogous to the juvenile hormone (JH)	Roles in development, moulting, reproduction and metamorphosis via nuclear receptors (MfR) (Lenaerts et al., 2019).
Pyriproxyfen (PP)		Insecticide	Juvenile hormone analog and an insect growth regulator (Ginjupalli and Baldwin, 2013)
Tributyltin (TBT)		Biocides and antifouling applications	Activation of RXR and PPARγ (Bašić et al., 2012)

Bisphenol A (BPA) is a common industrial chemical component in many consumer products. It is a monomer used in the production of polycarbonate and epoxy resins and as a non-polymer additive in plastics, which has been reported to have endocrine disrupting properties (Mu et al., 2005). BPA has become ubiquitous in the environment as a result of its high production, consumption and subsequent environmental release (Corrales et al., 2015). It is frequently detected in wastewater discharged from municipal and industrial sources in reported concentrations up to 17 mg/L (Corrales et al., 2015) without being able to achieve its removal after sewage treatment processes (Santhi et al., 2012). As a result, it has been measured in surface waters in concentrations up to 56 µL/L (Corrales et al., 2015). Both pyriproxyfen (PP) and methyl farnesoate (MF) are juvenoid compounds. PP is an insecticidal juvenile hormone (JH) analog that perturbs insect and tick development. PP also alters phartenogenic reproduction in non-target cladoceran species as it induces male

production that can lead to a decrease in fecundity, a reduction in population density and subsequent ecological effects (Ginjupalli and Baldwin, 2013). Due to its low solubility, high partition coefficients and hydrophobicity, and because it is applied outdoors to agricultural crops, the potential for PP to reach surface waters and sediments from runoff or spray drift is a major concern (Sullivan and Goh, 2008). This pesticide is measured in surface waters, as e.g. in Ebro river water, at concentrations up to 38 ng/L (Ccanccapa et al., 2016). MF is a crustacean natural hormone analogous to the JH, and thus comparing the effect of PP and MF can help to unravel their mechanisms of action. Tributyltin (TBT) is a biocide compound used because of its antifouling properties, and traditionally applied as antifouling coating ship paints. Even the legal restrictions in its production and usage, TBT has been still measured in surface waters in concentrations up to 5 µg/L (Gianguzza, 1997; Takahashi, 2009).

Some effects of these compounds have been reported in *Daphnia*. There is reported evidence that the studied juvenoids (MF and PP) reduced the clutch size, leading to the production of all-male broods (Olmstead and LeBlanc, 2003; Wang et al., 2005). MF and PP juvenoids, TBT and BPA have been previously reported to increase the accumulation of lipid droplets in the crustacean *D. magna* (Jordão et al., 2016b; Zaffagini and Zeni, 2009). Recently, a study of the effects of TBT on *Daphnia*'s lipidome during a reproductive cycle reported that this compound impaired the allocation of TGs to eggs, promoting their accumulation into lipid droplets in the adult females after reproduction (Jordão et al., 2015). Accumulation of TGs after exposure to TBT and BPA has been also reported in other aquatic species as zebrafish (Lyssimachou et al., 2015; Martínez et al., 2020). Furthermore, all the tested compounds have been described to act through *Daphnia*'s RXR, EcR and MfR (Jordão et al., 2015, 2016a, 2016b). Nevertheless, little is known about how juvenoids and BPA affect the lipid dynamics in adult *Daphnias* and the allocation of lipids to its eggs. There are relatively few studies addressing the molecular pathways that regulate lipid metabolism in *Daphnia* and their modulation after chemical exposure (Koussoroplis et al., 2017; Schlotz et al., 2016, 2012; Schwarzenberger and Fink, 2018; Windisch and Fink, 2019, 2018). However, there are no studies that have addressed the molecular signaling pathways behind the reported lipid disruptive effects of all the previous mentioned ECs in *D. magna*.

It is important to highlight that although many studies focus on transcriptomic responses at single time points, this may lead to inconclusive results and incomplete understanding of the underlying molecular and thus lipidomic mechanism (Asselman et al., 2019). This is especially important to take into account when performing

transcriptomic and lipidomic studies. On the one hand, as already explain in chapter 1, lipid dynamics varying during the cladocerans moult and reproductive cycle. It has been previously reported that fat accumulation into lipid droplets follows a temporal pattern in *Daphnia* females, increasing dramatically during the first period of their reproductive and moulting cycle to be invested into eggs (Goulden and Place, 1993; Jordão et al., 2015). On the other hand, RNA molecules have a short lifetime, what indicates that the time-point of measurement is crucial (Asselman et al., 2019). The lack of gene transcription at a specific time point means either than the RNA is not yet produced or that it has been already translated into functional proteins. Therefore, the effects of the referred EDCs in *Daphnia* have been studied at different time points.

2.2. Experimental section and results

Within this chapter, an integrative approach is presented linking EDCs effects at different levels of biological organization, as organism response (i.e. reproduction), gene expression (transcriptomics) and the subsequent lipid metabolism disruption (lipidomics). This is presented divided into two published scientific articles:

- Scientific article I:

  Fuertes, I., Jordão, R., Piña, B., Barata, C., 2019. Time-dependent transcriptomic responses of *Daphnia magna* exposed to metabolic disruptors that enhanced storage lipid accumulation. Environ. Pollut. 249. https://doi.org/10.1016/j.envpol.2019.02.102

The main objective of this work was to study the molecular mechanisms by which BPA, PP and TBT increase the accumulation of storage lipids in *D. magna* adult females during reproduction by microarray transcriptomic analysis of the exposed animals. Previous to transcriptomic analysis, Nile red staining assay was assessed as a screening strategy in order to select the time points and the concentrations at which the greatest changes in lipid droplets occurred. These time points were the ones selected for further transcriptomic analyses. Effects on fecundity were also examined. High throughput microarray results were further validated by qRT-PCR.

- Scientific article II:

  Fuertes, I., Jordão, R., Casas, F., Barata, C., 2018. Allocation of glycerolipids and glycerophospholipids from adults to eggs in *Daphnia magna*: Perturbations by compounds that enhance lipid droplet accumulation. Environ. Pollut. 242. https://doi.org/10.1016/j.envpol.2018.07.102

The aim of this article was to assess the effects of BPA and the two juvenoids (MF and PP) at different dosses on *D. magna* females' lipidome during their first reproductive cycle and in their eggs. Accordingly, the TG profile of the algae used to feed the

experimental *D. magna* individuals was also assessed in order to test how this organism incorporates TGs from its diet. This lipid determination was assessed by suspected screening lipidomic analysis using UHPLC-TOF MS. Lipidomic analyses were further validated by TLC. Furthermore, lipid droplet determination by Nile red staining assay and effects of these compounds on the fecundity of *D. magna* were also determined.

2.2.1. Scientific article I

Time-dependent transcriptomic responses of *Daphnia magna* exposed to metabolic disruptors that enhanced storage lipid accumulation.

Fuertes, I., Jordão, R., Piña, B., Barata, C., 2019. Environ. Pollut. 249, 99-108.

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Time-dependent transcriptomic responses of *Daphnia magna* exposed to metabolic disruptors that enhanced storage lipid accumulation.☆

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ABSTRACT

The analysis of lipid disruption in invertebrates is limited by our poor knowledge of their lipidomes and of the associated metabolic pathways. For example, the mechanism by which exposure of the crustacean *Daphnia magna* to tributyltin, juvenoids, or bisphenol A increase the accumulation of storage lipids into lipid droplets is largely unknown/presently unclear. Here we analyze transcriptome changes subsequent to this lipid accumulation effect induced by either the pesticide pyriproxyfen (a juvenoid agonist), the plasticizer bisphenol A, or the antifouling agent tributyltin. Changes in the whole transcriptome were assessed after 8 and 24 h of exposure, the period showing the greatest variation in storage lipid accumulation. The three compounds affected similarly to a total of 1388 genes (965 overexpressed and 423 underexpressed transcripts), but only after 24 h of exposure. In addition, 225 transcripts became upregulated in samples exposed to tributyltin for both 8 h and 24 h. Using *D. melanogaster* functional annotation, we determined that upregulated genes were enriched in members of KEGG modules implicated in fatty acid, glycerophospholipid, and glycerolipid metabolic pathways, as well as in genes related to membrane constituents and to chitin and cuticle metabolic pathways. Conversely, downregulated genes appeared mainly related to visual perception and to oocyte development signaling pathways. Many tributyltin specifically upregulated genes were related to neuro-active ligand receptor interaction signaling pathways. These changes were consistent with the phetotypic effects reported in this and in previous studies that exposure of *D. magna* to the tested compounds increased lipid accumulation and reduced egg quantity and quality.

1. Introduction

A large variety of anthropogenic chemicals, including phthalates, bisphenol A (BPA), alkylphenols and organotin pesticides can promote fat accumulation and cause obesity in humans and in other vertebrates (Grün and Blumberg, 2009). Recent results showed that ecdysteriods, juvenoids, tributyltin and bisphenol A promoted the accumulation of storage lipids such as triacylglycerols and cholesterol into lipid droplets in the environmentally-relevant microcrustacean *Daphnia magna*, which constitutes an invertebrate version of the lipidic disruption observed in vertebrates. (Fuertes et al., 2018; Jordão et al., 2016a; Jordão et al., 2015; Jordão et al., 2016c).

Exogenous chemicals may alter fat accumulation in vertebrates by multiple mechanisms. The so-called chemical obesogens promote adipocyte differentiation and storage lipid accumulation by disrupting signaling pathways regulated by the peroxisome proliferator-activated receptor (PPARγ) in combination with its heterodimeric partner the retinoid X receptor (RXR) (Grün and Blumberg, 2009). Obesogens can also increase the accumulation of fat by disrupting sex steroids, by affecting the hypothalamic-pituitary-adrenal axis, or by altering metabolic signaling pathways under the regulation of thyroid and/or glucocorticoid hormones (Grün and Blumberg, 2006, 2009). Conversely, very little is known about the mechanisms by which anthropogenic chemicals disrupt storage lipid accumulation in invertebrates, probably due to our lack of knowledge about invertebrate lipid metabolism pathways and about their regulation (Jordão et al., 2015; Santos et al., 2012).

Many of the reported vertebrate obesogens disrupt transcription

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factors that also exists in invertebrates, like RXR and HR96 (Karimullina et al., 2012; Wang and LeBlanc, 2009), although there is evidence that other mechanisms may also apply. For example, in the model insect species *Drosophila melanogaster* the natural ligand of the ecdysteriod receptor, EcR, ecdysone, controls lipid metabolism and facilitates oocyte production interacting with lipogenic transcription factors and the central nervous system (Sieber and Spradling, 2015). In *Daphnia magna*, it has been proposed that the disruption of lipid homeostasis by obesogens like tributyltin, bisphenol A, and juvenoids may be mediated by their interaction with a regulatory complex that includes both the EcR and the methyl farnesoate receptor, the latter identified as the crustacean juvenile hormone receptor (Fuertes et al., 2018; Jordão et al., 2016a; Jordão et al., 2015; Jordão et al., 2016c). In vertebrates and invertebrates, storage lipids are accumulated mainly in lipid droplets, which are cell organelles that store neutral lipids such as cholesterol and triacylglycerols and have the necessary enzymatic machinery to regulate storage lipid deposition and mobilization (Kühnlein, 2012; Pol et al., 2014). Interestingly, lipid droplet-based storage fat metabolism is quite similar in mammals, yeast, and *Drosophila,* and it requires the coordination of fatty acid metabolism, *de novo* synthesis and catabolism of neutral lipids, activation of the glyceropohospholipid metabolism, and the involvement of modulatory associated proteins (Kühnlein, 2012; Pol et al., 2014).

Reported information on lipid metabolism in *Daphnia* species has been mostly focused on the allocation and metabolism of dietary lipids (fatty acids, glycerophospholipids, glycerolipids) and their role in development, growth and reproduction (Goulden and Place, 1990; Goulden and Place, 1993; Tessier et al., 1983; Wacker and Martin-Creuzburg, 2007). There are relatively few studies addressing the molecular pathways that regulate lipid metabolism in *Daphnia*, their modulation by chemicals and individual level consequences (Koussoroplis et al., 2017; Schlotz et al., 2016; Schlotz et al., 2012; Schwarzenberger and Fink, 2018). Exceptions include the deregulation of eicosanoid signaling pathways by nonsteroidal anti-inflammatory drugs (NSAID) (Heckmann et al., 2008), and the reduction of the metabolism of sphingomyelins into ceramides or sphingosine by specific toxicants and fatty acids (Sengupta et al., 2017), resulting both in detrimental effects on maturation and fecundity. The two previously mentioned studies proposed as key initiating molecular events for the observed effects, either the inhibition of COX by NSAID (Heckmann et al., 2008), or the activation/inhibition of the receptor HR96 by fatty acids, atrazine or triclosan (Sengupta et al., 2017). Changes in glycerophospholipids and related gene metabolic pathways have also been reported in *D. magna* exposed to TNT (2,4,6-Trinitrotoluene) and to different flame retardants (Scanlan et al., 2015; Stanley et al., 2013). There are, however, no studies on the effect of these toxicants in the genetic regulation of changes in storage lipids.

The main objective of this work is to study the molecular mechanisms by which tributyltin, pyriproxyfen and bisphenol A increase the accumulation of storage lipids in *D. magna* adult females during reproduction by analyzing the transcriptomes of the exposed animals. Physiological storage fat accumulation into lipid droplets follows a temporal pattern in *Daphnia* females, increasing dramatically during the first period of their reproductive/moulting cycle to be invested into eggs, which are released during the molting occurring at the end of the cycle (Goulden and Place, 1993; Jordão et al., 2015). To cope with this cyclic variation on lipid content, the disruptive effect of chemicals in storage lipid metabolism was studied considering two time points, one at the onset of the natural lipid accumulation and a second one just when storage lipid accumulation is the greatest. We used a new custom microarray that includes probes from the full transcriptome of *D. magna* and that has 50% of its genes functionally annotated (Campos et al., 2018). *D. magna* differentially transcribed genes were compared to *Drosophila* homologous ones of known function and related to effects observed on storage lipid accumulation and reproduction, thus providing information about the gene pathways associated to these processes.

2. Material and methods

2.1. Studied compounds and chemicals

Studied compounds included the antifouling tributyltin chloride (TBT) (CAS Number 1461-22-9), the juvenoid pesticide pyriproxyfen (PP, CAS 95737-68-1), the plasticizer bisphenol A (BPA, CAS 80-05-7). These compounds, and the chemical Nile red (CAS Number 7385-67-3) were obtained from Sigma Aldrich. All other chemicals were analytical grade and were obtained from Merck.

2.2. Experimental animals

Parthenogenetic cultures of a single clone of *D. magna* (clone F) were used in all experiments. Photoperiod was set to 14 h light: 10 h dark cycle, and temperature at 20 ± 1 °C. Individual cultures were maintained in 100 ml of ASTM hard synthetic water at high food ration levels (5×10^5 cells/ml of *Chlorella vulgaris*, respectively), following Barata and Baird (1998).

2.3. Experimental procedures

Experiments follow previous procedures (Jordão et al., 2015; Jordão et al., 2016c) and aimed to determine temporal transcriptomic changes during exposure to contaminants known to promote the accumulation of storage lipids in lipid droplets across an entire adolescent intermolt cycle. Concentrations were selected to cause high accumulation of lipid droplets and included 1 μg/L for tributyltin and pyriproxyfen and 10 mg/L for bisphenol A (Jordão et al., 2016c), hereafter referred, respectively, to TBT, PP and BPA. Experiments started with <4–8 h old newborn individuals obtained from females reared individually at high food ration levels. Newborns were reared in groups of 5 in 200 ml of ASTM hard water at high food ration conditions until about 4–8 h before molting for the third time. At this point experimental exposures were initiated. Acetone at a concentration of 20 μL/L was used as a chemical carrier, which was also added to controls. The test medium was renewed every 24 h. Experiments included three samplings: 8 h (just after the third molt), 24 h and just after the forth molt (48 h). At each sampling, four replicates of 5 individuals were collected and processed for lipid droplet quantification using Nile red and another 4 replicate of 5 individuals were preserved in RNA latter, snap frozen in liquid N_2 and preserved at −80 °C for transcriptomic studies. At the 48 h sampling point, females were debrooded following Jordão et al. (2015) and eggs were counted.

2.4. Nile Red determination

Lipid droplet accumulation was determined fluorometrically using Nile red following previous procedures (Jordão et al., 2015).

2.5. Transcriptomic analyses

Transcriptomic analyses were restricted to 8 and 24 h samples since the greatest changes in lipid droplets occurred between these periods (Table 1). RNA extraction, labelling and microarray analyses were performed as described (Campos et al., 2018), complete protocols are given as supplementary material (SI). Raw microarray data from this study have been deposited at the Gene Expression

Table 1
Lipid droplet accumulation (% relative to the 8 h control) and fecundity (egg number) (Mean ± SE, N = 20) in *D. magna* females exposed to the studied chemicals. Lipid droplet accumulation is expressed as relative fluorescence relative to control treatments at 8 h. Different letters means significant (P < 0.05) differences following ANOVA and Tukey's post-hoc multi-comparison tests.

	8 h	24 h	48 h	Fecundity
Control	0 ± 0.1 a	41.8 ± 0.8 b	35.7 ± 1.0 b	9.1 ± 0.2 a
BPA	7.3 ± 0.3 a	86.7 ± 1.9 d	94.9 ± 1.8 d	9.1 ± 0.3 a
PP	1.4 ± 0.1 a	62.3 ± 1.2 c	57.7 ± 1.1 c	3.7 ± 0.1 c
TBT	0.32 ± 0.0 a	152.67 ± 4.0 e	145.02 ± 5.0 e	7.8 ± 0.2 b

Omnibus Web site (www.ncbi.nlm.nih.gov/geo/) with accession number GSE119329.

Gene model annotation: For an optimal interpretation of transcriptional changes it is fundamental to have a functional annotation of all genes. About 50% of the 41317 probes included in the microarray corresponded to genes functionally annotated in Gene Ontology (GO) and/or Kyoto Encyclopaedia of Genes and Genomes (KEGG) databases. This annotation was performed using Blast2Go Suit and the BLASTX alignment algorithm (Conesa et al., 2005). Search was performed using as target the three main databases of interest to our platform: Drosophila, RefSEQ and SwissprotKB. The three annotation sets were finally merged, resulting in a high value annotation set. Further details can be found in Campos et al. (2018).

2.6. Data analysis

Fecundity and Nile Red fluorescence: Fecundity differences across treatments was analyzed using one way ANOVA, whereas Nile red fluorescence responses across exposure treatments and time periods by two way ANOVA (Zar, 1996). Within each time period chemical treatment differences were compared to the respective controls by post-hoc Dunnett's tests. Prior to analysis we confirmed that our data meet ANOVA assumption of data normality and variance homoscedasticity (Zar, 1996).

Gene Expression Analysis: Raw microarray data were analyzed using Gene Spring GX v13.0 software (Agilent, USA). Fluorescence data was normalized using quantile normalization and baseline transformation to the median of all samples. The quantile 95 of the added 3500 negative probes was calculated and this value was assumed as being the fluorescence background noise value of each sample.

The transcriptomic patterns of *D. magna* individuals vary dramatically within a single reproductive or moulting cycle due to the many processes involved such as the destruction and synthesis of new carapace, the accumulation and mobilization of energy reserves and the production of a new clutch of eggs (Campos et al., 2018). This means that a two-way ANOVA comparing two different reproductive/moulting stages across exposure treatment would identify mostly moulting/reproduction/energetic metabolic pathways masking those changes truly related to chemical treatments that promote lipid storage. Accordingly, fluorescence data were centered relative to the respective time control and log2 transformed. Significant changes in treated and control samples were analyzed using one-way ANOVA (analysis of variance) in R (v. 1.0.136, R Core Team), considering each one of the time x treatment conditions (including controls) as a class. Probes showing significant variations among the classes ($p \leq 0.05$, 1964 probes belonging to 1613 genes in total) were selected as DEGs for further analysis.

Hierarchical clustering and PAM (partition around medoids) clustering analysis were performed using the packages *gplots*, *fpc*, and *cluster* in R. Significant differences between scaled values of all probes included in each cluster were assessed by one-way ANOVA followed by post hoc Tukey's tests ($p < 0.05$) in R (*foreign* and *agricolae* packages); further graphs, including heatmaps, were performed with the *gplots* package, also in R.

2.6.1. Functional gene analysis

Functional analysis of selected genes was performed using DAVID Bioinformatic Resources 6.8 (Huang et al., 2009). Gene enrichment analysis was estimated in DAVID using gene homologous to *Drosophila melanogaster* and the microarray set up as background gene set. Enrichment significance was set at $P < 0.05$ after being corrected for multiple testing using the Benjamini-Hochberg and a false discovery rate of 5%. The gene function of de-regulated lipid related genes was further studied by annotating them into KEGG and into lipid droplet-based storage fat metabolism pathways (Kühnlein, 2012; Pol et al., 2014). The designed array contained about 60% of its genes functional annotated into KEGG lipid metabolic pathways having a coverage ranging from 29% for steroid biosynthesis to 91.4% for fatty acid degradation (Table S1, Supplementary Material).

2.6.2. Validation of microarray results by qPCR

Microarray results were validated with real-time quantitative polymerase chain reaction (qPCR). We selected 15 differentially expressed genes, 14 of them implicated in lipid metabolic pathways (CG5966, LIP4 or Mag, Pect, Dma/PLA2, CG6567, CG31522,CG4500, DESAT1, CG3635, CG4380, NLaz, α4GT1, INOS, SL), plus Hb, which is highly transcribed upon exposure to juvenoids (Jordão et al., 2015). An additional gene (HR96) was also included in the qPCR analysis despite not being de-regulated significantly due to its key regulatory role in *Daphnia* lipid homeostasis (Karimullina et al., 2012; Sengupta et al., 2016). The gene G3PDH (glyceraldehyde 3-phosphate dehydrogenase) was used as an internal control (house-keeping) (Campos et al., 2013) as its mRNA levels did not changed across samples. Primers for each one of these genes were designed with Primer Express® Software v3.0.1 (Thermofisher, USA); their sequences are provided in Table S2, Supplementary material. Further details of the specific names and signaling pathways of the above mentioned genes are depicted in Table S2, Supplementary material. Amplification efficiencies were ≥90% for all tested genes as described by Pfaffl et al. (2002). qPCR was performed according to manufacturer's protocols using four experimental replicates per treatment and time.

Relative mRNA abundances were calculated from the second derivative maximum of their respective amplification curves (Cp, calculated from technical triplicates). To minimize errors on RNA quantification among different samples, Cp values for target genes (Cptg) were normalized by Cp values for G3PDH for each sample. Changes in mRNA abundance in samples from different treatment were calculated by the ΔΔCp method (Pfaffl, 2001), using corrected Cp values from treated and non-treated samples.

3. Results

3.1. Effects on lipid droplets and fecundity

Accumulation of lipids into lipid droplets, measured as Nile Red fluorescence, increased significantly ($P < 0.05$, Time, $F_{2,217} = 497.4$) from 8 h to 24 h and level out afterwards (Table 1). Females treated with the studied chemicals significantly accumulated more lipid droplets than controls (Treatment effect, $F_{3,217} = 95.4$, $p < 0.05$). Most relevant changes in lipid droplet accumulation among and within treatments occurred between the 8- and 24-h periods (interaction, $F_{6,217} = 24.6$). TBT was the compound promoting the greatest accumulation of lipid droplet

followed by BPA and PP (Table 1). Fecundity of females exposed to BPA was similar to those of controls, and that of females exposed to TBT and PP were significantly lower ($p < 0.05$, $F_{3,\ 76} = 191.4$) (Table 1).

3.2. Determination and classification of deregulated probes and genes

ANOVA identified 1964 probes belonging to 1613 unique genes differentially transcribed in at least one of the experimental subsets (Control, TBT, BPA, or PP) relative to the rest. Normalized transcription values of differentially transcribed probes (DEP) and related genes (DEG) are shown in File S1, supplementary material. Normalized microarray transcription patterns of 15 DEG were significantly ($P < 0.05$) correlated with those measured by qPCR (Fig. S1, supplementary material). Despite of not being de-regulated significantly in the micro-array, the qPCR transcription patterns of the HR96 gene was significantly ($P < 0.05$, $F_{3.11} = 4.4$.) down-regulated in 24 h samples upon exposure to the three studied chemicals (Fig. S2, supplementary material). Hierarchical clustering showed that the selected DEPs correctly clustered the different biological replicates (Fig. 1). PAM clustering defined three clusters: A, B, and C, including 1199, 279 and 486 probes, respectively, corresponding to 965, 225 and 423 genes (Fig. S3 from supplementary material shows the distribution of DEPs). Cluster A and C included genes whose mRNA levels increased and decreased, respectively, in *D. magna* individuals exposed to any of the three selected contaminants at 24 h. Cluster B included only genes with high transcription levels after exposure to TBT for both 8 and 24 h (Fig. 2).

3.3. Functional characterization

From the 1613 deregulated genes, 606 were functionally annotated to the *D. melanoster* genome (Supplementary File S2), from which 46 of them belonged to lipid metabolic pathways (Table S3, supplementary material). All the 606 genes annotated in *D. melanogaster* genome were used for the functional enriched analysis. Up to 190 gene signaling pathways were obtained by DAVID functional analysis after Benjamini-Hochberg correction (complete results in Supplementary File S3). Fig. 3 includes main specifically enriched GO, KEGG and lipid-related UP Keywords categories. Cluster A (up regulated genes after 24 h of exposure) showed the greatest number of enriched functional terms, 10 of them related to lipid metabolism. Cluster C (down regulated genes after 24 h of exposure) was enriched in several functional categories related to visual perception, oocyte development and filopodium assembly. Finally, Cluster B, which grouped genes up-regulated upon TBT treatment, showed a similar functional profile as Cluster A, with specific enrichment in neuroactive ligand-receptor interaction and metallocarboxypeptidase activity functional classes (Fig. 3).

Twenty three out of the 46 lipid metabolic genes included in Table S3 (supplementary material) were annotated into KEGG lipid-related and signaling pathways. We added three more genes not initially annotated in KEGG: HR96, whose qPCR transcripts varied significantly across treatments (see Fig. S2, Supplementary Material) and Dma/PLA2, Dma/LPGAT, de-regulated *D. magna* genes that encoded, respectively, a phospholipase A2 and acyl-CoA:lysophosphatidylglycerol acyltransferase (Table S3, Supplementary Material). The most enriched lipid-related functional classes were fatty acid, phosphoinositol, glycerophospholipid, and glycerolipid metabolic pathways. There were also 5 genes involved in sphingolipid- (CDase - ceramidase), terpenoid- (CG33671 - Mevalonate kinase), steroid biosynthesis (Sc2 - 3-oxo-5-alpha-steroid 4-dehydrogenase, LIP4 - sterol esterase) and ketone-metabolic pathways (CG3699 - reductase). Transcription patterns of most of those genes belonged to cluster A as their mRNA levels increased in treated organisms after 24 h of exposure (see Fig. S4, S5 and Supplementary Material for further details). There were, however, three lipogenic genes up regulated only after TBT treatments (CG6472 - TG lipase, CG13282 - TG lipase, CDase), and nine genes down regulated in 24 h samples (Cluster C). The latter group

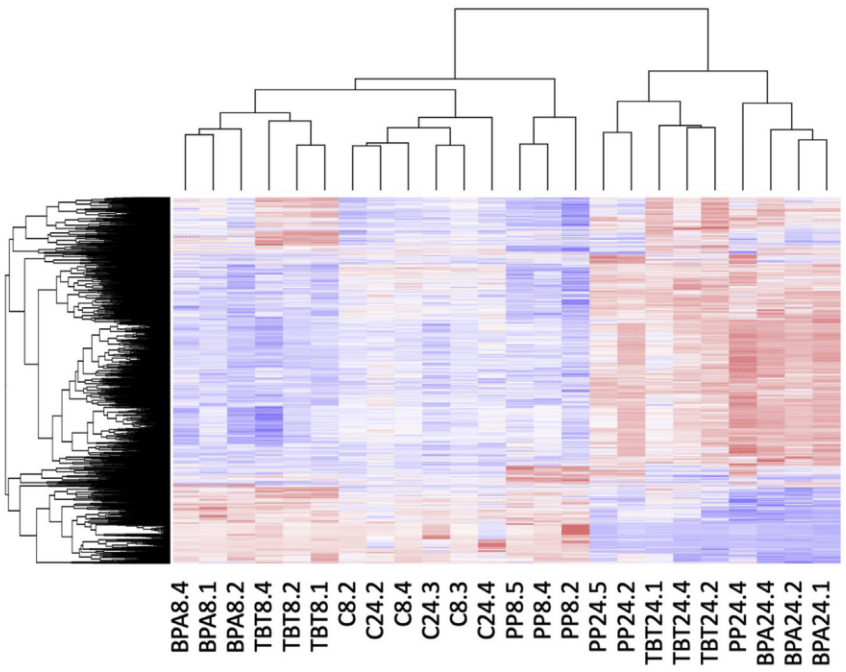

Fig. 1. Hierarchical clustering of the normalized fluorescence values of the 1965 differentially transcribed probes (DEPs) at 8 and 24 h following exposures to BPA, PP and TBT in *D. magna* females for all samples. Colours represent overexpressed (red) and underexpressed (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

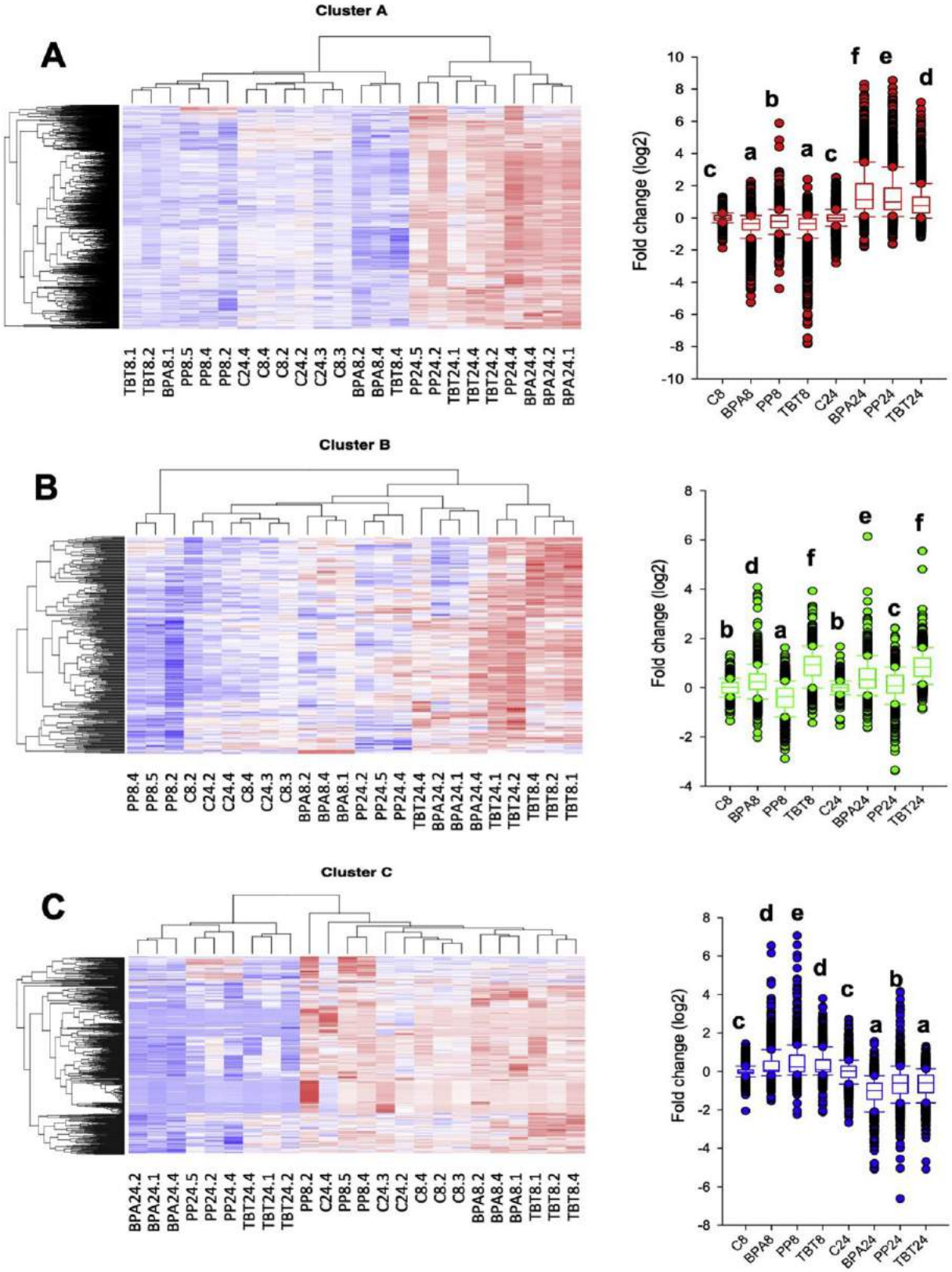

Fig. 2. Transcription patterns of differentially expressed probes (PEGs) belonging to the three clusters identified by the medoid cluster analysis. The total number of probes in cluster A, B and C were 1199, 279 and 486 respectively. Left panels (A–C) show hierarchical clustered heatmaps; colours indicate overexpressed (red) or under-expressed (blue) PEGs relative to the controls. Right panel show box-plots of the distribution of the corresponding transcription values. Boxes indicate ranges from the first to the third quartile, the thick lane indicates the media, the whiskers the 95% confidence interval and filled symbols outliers. Letters in the boxplots represent significantly different sets of values (ANOVA + Tukey's). Data have been scaled to its time control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

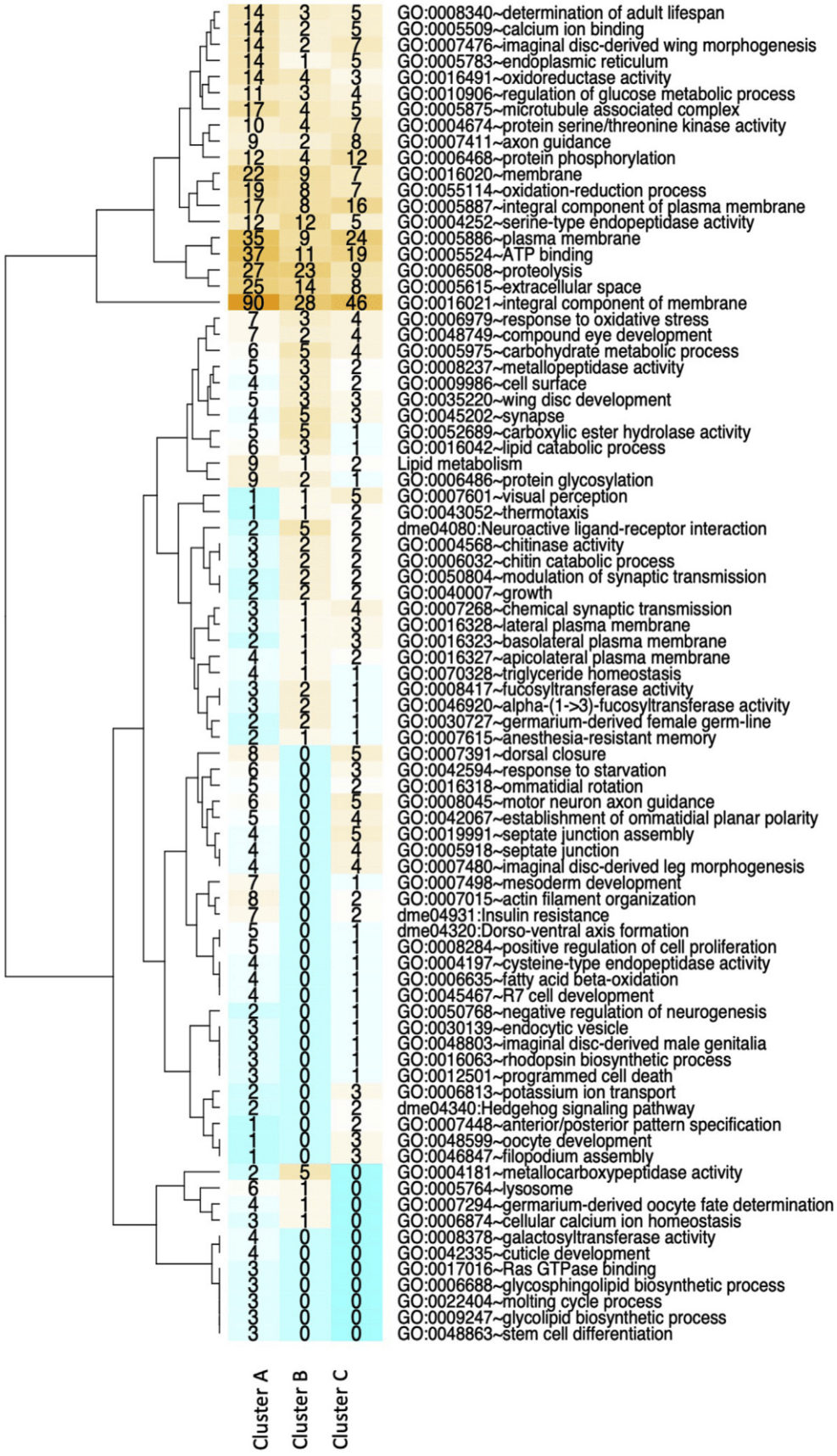

Fig. 3. Distribution of genes belonging to GO, KEGG or UP_KEYWORDS functional categories, calculated by DAVID. Only categories with significant enrichment in at least one of the treatments are shown. Colours correspond to the relative importance of each cluster in the total distribution of the genes belonging to each category. Orange and cyan correspond to genes over- or under-represented in each particular cluster; the actual number of hits for each cluster and categories is indicated with black numbers. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

included five nuclear receptors (LPR1, MGL, Teq, UPS or RXR, HR96).

In Fig. 4, thirteen of the genes reported in Table S3 are schematically allocated within the lipid droplet-based storage fat metabolism adapted from *Drosophila*, mammals and yeast. More than half of the reported *Drosophila* genes encoding for lipogenic, lipolitic and associated proteins (de Paula et al., 2018; Kühnlein, 2012) were de-regulated in the present study (13 out 23). From these 13 genes, ten were up-regulated and three were down-regulated in individuals exposed to the three studied compounds at 24 h. Non de-regulated genes within the above mentioned pathways are depicted in italics in Fig. 4 and included in Table S4.

4. Discussion

Accumulation of lipid droplets and storage lipids in *D. magna* upon exposure to exogenous substances has been related to the well-known obesogenic effect of the same compounds in vertebrates (Fuertes et al., 2018; Jordão et al., 2016b; Jordão et al., 2015). However, the evolutionary divergence between arthropods and fish or mammals casts reasonable doubts on whether or not both phenomena may occur by ortholog mechanisms. This is for instance the case of juvenoids, which are key arthropod hormones that control maturation, reproduction and sex in *Daphnia* and also promote its accumulation of storage lipid into lipid droplets (Fuertes et al., 2018; Jordão et al., 2016b; Jordão et al., 2016c). To explore this point, we performed a genome wide transcriptome analysis comparing gene expression of fed control and exposed animals, using three substances that induce accumulation of lipid droplets in *Daphnia*, and using the same time window in which this droplet accumulation occurs at its maximal extend (8–24 h). Given our very limited direct knowledge of *Daphnia* metabolic pathways, all functional inferences of the observed effects were made using the corresponding *Drosophila* homologs. In total, our analysis identified 606 DEGs with predictable functions based in their *Drosophila* counterparts, most of them (349) up-regulated by the three studied contaminants after 24 h of exposure. An additional 101 were specifically up-regulated by TBT across the two studied exposure periods. This similarity in the transcriptomic signature of the exposure to three compounds with very little or no structural similarities, and only related because of their common ability to promote lipid droplet accumulation, suggest a common mechanism of action. In addition, only 11 out of the 606 DEGs identified in our experiments genes appeared also as de-regulated in starved *Drosophila* (Grönke et al., 2005): a putative glycerol-3-phosphate O-acyltransferase (CG59669), a kinase (PKA-C3) and juvenile responsible protein (pAbp), as up-regulated genes, and eight down-regulated genes related to protease activity (Grönke et al., 2005). This suggest that the changes observed in *Daphnia* were related to the action of the tested chemicals rather than to feeding alterations.

The largest functional group of DEGs (46 genes, 8.1% of the de-regulated genes annotated into *D. melanogaster*) encodes lipid metabolic and/or lipid related proteins, thirty-six of them showing up-regulation upon exposure to the studied chemicals. Thirteen of these lipid-related gene/enzymes were annotated into the five pathways involved in lipid droplet biogenesis and metabolism in *Drosophila*, yeast and vertebrates (Kühnlein, 2012; Pol et al., 2014): fatty acid activation by acyl-CoA synthetases (CG4500), *de novo* synthesis of neural lipids (Dma/LPGAT, CG6567, CG4729, CG6847, CG5966, Thiolase, CG8552, LP4 or Mag), the Lands (Dma/PLA2, CdsA) and Kennedy (Pect) pathways of glycerophospholipid metabolism, and modulatory associated proteins (Lds) (Fig. 4). Kühnlein (2012) described with detail the *Drosphila* genes involved

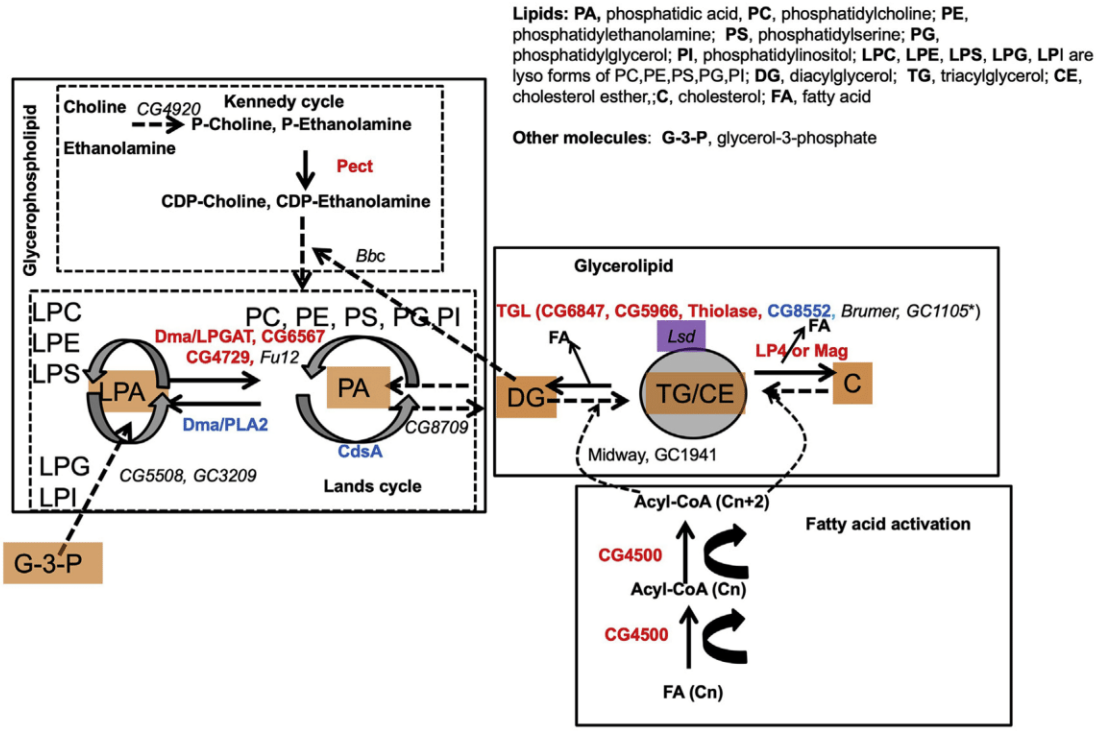

Fig. 4. Schematic representation of the full set of *Drosophila* genes homologous to those of *D. magna* involved in the Lipid droplet-based storage fat metabolism: the fatty acid activation by acyl-CoA, *de novo* synthesis of neutral lipids (orange boxes), glycerophospholipid synthesis (Kennedy, Lands cycle) and associated proteins (purple box, adapted from Kühnlein, 2012, de Paula et al., 2018; Pol et al., 2014). Red and blue gene names indicate transcription patterns up and down-regulated at 24 h in exposed individuals to the three chemicals, respectively. Italic gene names are those not de-regulated. Dotted and continuous arrow lines indicated the specific pathways that were, respectively, not de-regulated and de-regulated. Further details of specific genes and KEGG pathways are depicted in Tables S3 and S4 Supplementary Information and details of gene transcription patterns are in Fig. S4, Supplementary Information. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in lipid droplet-based storage fat metabolism, which were homologous to 22 genes present in our microarray (12 of those genes were upregulated). More recently, de Paula et al. (2018) characterized a *Drosophila* Acyl-CoA Synthetase, a gene involved in fatty acid activation and obesity, which was also de-regulated in our study (GC4500). This means that our results point out that the three tested compounds were able to de-regulate 56% of all the *Daphnia* genes that can be related to lipid droplet-based storage fat metabolism, based on their sequence similarity with *Drosophila* genes.

Four additional DEGs were annotated as related to the inositol phosphate metabolic KEGG pathway (Balakrishnan et al., 2015; Bloomquist et al., 1988; Park et al., 2000): the phosphatidylinositol 3-kinase (Pi3K21B), which catalyses phosphorylation reactions of phosphatidylinositol (PI) into polyphosphoinositides (i.e. PI4,5$P_{2)}$, many of which have messenger functions (Abel et al., 2001; Balakrishnan et al., 2015); phosphatidylinositol phospholipase C (norpA) and 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase (sl) that hydrolysed polyphosphoinositide PI4,5P_2 to the second messengers inositol 1,4,5-trisphosphate (Ins1,4,5P_3) and diacylglycerol (DG) (Bloomquist et al., 1988); and myo-inositol-1-phosphate synthase (Inos) that produces *1L*-myo-inositol-1-phosphate (Inositol 3P) from *D*-glucose 6-phosphate (Park et al., 2000). In addition to these four functional modules, other identified DEGs encoded enzymes from the steroid (Sc2), terpenoid (CG33671), sphingolipid (CDase) and glycosphingolipid biosynthesis (α4GT1,4GalNAcTB, Brn, C1GalTA), all of them also related to lipid metabolism (see Table S2 for further material).

Of particular interest is a group of regulatory genes that were down-regulated upon exposure to the studied compounds (Figs. S5 and SI): LRP1, involved in Notch signaling (Meng et al., 2010), Mgl involved in endocytic processes (De et al., 2014), Teq, which regulated insulin signaling (Huang et al., 2015), UPS, also known as the retinoic X receptor in Daphnia (RXR) and the nuclear HR96 receptor. The *Daphnia* RXR heterodimerizes, with the ecdysteriod receptor and probably with the juvenile hormone receptor, a regulatory complex that modulates storage lipid metabolism (Jordão et al., 2016a). There is also ample information showing that HR96 signaling pathways can be activated and de-activated by fatty acids and certain contaminants and that this receptor regulates lipid homeostasis in *Daphnia* (Karimullina et al., 2012; Sengupta et al., 2016). Ligand-dependent downregulation of transcription factors, as well as the degradation of the protein itself, are well-known mechanisms to regulate the activity of signal transduction, and the RXR receptor is one example of such regulatory mechanism (Osburn et al., 2001). There is reported information that TBT and probably juvenoids act as a ligand of RXR in *Daphnia* (Jordão et al., 2016a; Wang and LeBlanc, 2009; Wang et al., 2007) and that some fatty acids and pyriproxyfen do so for *Daphnia* HR96 (Karimullina et al., 2012). Therefore, the observed reduction of transcription factors implicated in lipid homeostasis is consistent with the general activation of the lipid metabolism by the tested compounds.

Additional de-regulated signaling pathways included genes (32) involved in the breakdown and modification of sugars. These include three gene/enzymes involved in carbohydrate metabolism: the amylase proximal Amy-p, the NADP-malic enzyme Men-b, and the alpha-glucosidase αGcs2 (Chng et al., 2014; Geer et al., 1980; Hickey and Benkel, 1982). There were also chitinase enzymes, like chitinase 3 (Cht3) and chitin deacetylase-like 9 (Cda9) (Funkhouser and Aronson, 2007; Petkau et al., 2012), and glycosyltransferases like the 1,4- β galactosyltransferase GalT1 (Kim et al., 2011).

Eleven of the up-regulated genes were involved in two significant insulin receptor signaling related pathways (Fig. 3), which include several genes encoding for proteins from insulin (phosphatidylinositol-3-kinase (PI3K), LDL receptor protein 1 (LRP1), ribosomal protein S6 kinase (S6k)) (Boucher et al., 2010), as well as elements from other receptors that can be modulated by insulin such as the Notch, Wnt and SREBP receptor signaling (He et al., 2007; Hsu and Drummond-Barbosa, 2009; Jones et al., 2009).

Up-regulated genes also include 18 regulatory kinases or kinase subunits, many of them involved in cell/tissue differentiation, morphogenesis and development (Basson, 2012): protein kinase C delta (Pkcdelta), rolled (rl), Rho kinase (Rok), Cyclin-dependent kinase 5 and 7 (Cdk5,7), Sak kinase (SAK), Inositol-requiring enzyme-1(Ire1), Calcium/calmodulin-dependent protein kinase II (CaMKII), Gilgamesh (gish). Kinases also included the cAMP-activated protein kinase A (PKA) that promotes glycogen and triacylglycerol catabolism (Londos et al., 1999).

Down regulated genes included kinases and binding proteins involved in oogenesis (MOS, ROK and PABP) (Clouse et al., 2008; Ivanovska et al., 2004; Verdier et al., 2006) and several genes involved in metabolic processes related to eye pigments and photoreceptors and ultimately to visual perception (Fig. 3) (transporter and adaptor proteins (CG9317, AVA), homeo-box (B–H1), the protein tyrosine kinase (SEV) and phosphoinositide phospholipase C (NORPA) (Chaturvedi et al., 2016; Higashijima et al., 1992; Kim et al., 2006; Ni et al., 2017; Roignant et al., 2006). All these data suggest that a relevant part of the action of the three tested chemicals involves signaling pathways related to energy metabolism (carbohydrate, insulin), molting, development, reproduction and life-span functions (Fig. 3).

Crustacean's growth and reproduction are regulated by the ecdysteroid and juvenile hormone receptor signaling pathways (LeBlanc, 2007). Ecdysteriods exert their effects through the interaction with the ecdysteroid receptor (EcR), known to heterodimerize with RXR (LeBlanc, 2007; Rewitz and Gilbert, 2008; Wang and LeBlanc, 2009). TBT, which is an agonist of RXR together with methyl farnesoate-the crustacean juvenile hormone, and other juvenoids like pyriproxyfen enhanced the ecdysteroid-dependent activation of the EcR: RXR heterodimer (Wang et al., 2011; Wang and LeBlanc, 2009). This means that agonists of the RXR and of the methyl farnesoate receptors, TBT and pyriproxyfen, respectively, are likely to affect moulting, growth and reproduction. Indeed, there is evidence that at higher concentrations, TBT and pyriproxyfen, inhibit moulting and promote embryo arrest, which is directly linked with an anti-ecdysteriod effect (Mu and LeBlanc, 2004; Wang et al., 2011). On the other hand, there is proof indicating that BPA enhances juvenoid activity, decreasing the enzymatic degradation of methyl farnesoate (Mu et al., 2005). Little is known about the role of insulin in *Daphnia*, but nevertheless, in *Drosophila* it is related with growth (Boucher et al., 2010; Kaplan et al., 2008). Therefore, the above mentioned studies provide common mechanism of action for the three studies chemicals. In fact, in *Daphnia* or phylogenetically related species other studies have reported similar transcriptomic/proteomic responses. In the marine crustacean copepod *Eurytemora affinis,* pyriproxyfen also affects the transcription of gene signaling pathways related with reproduction, moulting and development, and up-regulated insulin growth factor (Legrand et al., 2016). In the amphipod *Gammarus fossarum*, pyriproxyfen induced 32 proteins, many of them involved in energetic metabolism (Trapp et al., 2018). Transcriptomic responses of *D. magna* neonates exposed to BPA at 10 mg/L for two days also revealed de-regulation of many signaling pathways related with molting, development and energetic metabolic processes (Jeong et al., 2013). TBT not only can alter carbohydrate and lipid metabolism in crustaceans interacting with the RXR and the crustacean hyperglycemic hormone (CHH) signaling, but also can disrupt methyl farnesoate and ecdysteroid signaling, thereby altering growth and reproduction (Vogt et al., 2018).

Exposure to TBT triggered expression of a specific subset of 101

genes after only 8 h of exposure. These TBT-specific DEGs included several genes annotated to the neuro-active ligand receptor interaction signaling KEGG functional module (dme: 04080) (Fig. 3) and included four receptors involved in neurotransmitter and neuropeptide signaling: The serotonin receptor 5HT1B, the gamma-aminobutyric acid receptor RDL, the G-protein coupled receptor TKR86C, the Allatostatin A receptor (ASTA-R2) (Gasque et al., 2013; Johnson et al., 2003; Larsen et al., 2001; Towers and Sattelle, 2002). Other DEGs also specifically up regulated by TBT were identified as serine type endopeptidases, known to regulate the activities of the above mentioned receptors (Audsley and Weaver, 2004; Huang et al., 1993; Tong et al., 2008). This link between neurotransmitter regulation and lipid droplet accumulation may be related with the observed energy metabolic effects of SSRIs in *Daphnia* (Campos et al., 2013; Campos et al., 2012) and provides a (molecular) phenotypic evidence of the neurohormonal regulation of lipid accumulation and metabolism in Daphnia. The fact that these functional modules were clearly underrepresented in the DEGs associated to BPA or PP exposure suggests that these compounds act downstream the signaling cascade from neurological stimuli to lipid droplet formation.

In summary, the study of whole-genome transcription changes associated to enhanced accumulation of storage lipids in *D. magna* identified energy (carbohydrate, insulin), molting, and reproduction as the functional categories preferentially affected by the three treatments. Key master receptors involved in lipid homeostasis, such as HR96 and RXR, were down-regulated by the treatments, probably as a feedback regulatory mechanism. These nuclear receptors have been involved in the regulation of different DEGs, particularly of those involved in lipid, glycerolipid and fatty acid metabolic pathways. Finally, TBT may interact with the neurohormonal control of lipid homeostasis, although this mechanism needs further investigation. Our results provide a comprehensive view of the molecular mechanisms explaining chemical disruptive effects in storage lipid accumulation in *D. magna*.

Competing interests

The authors have declared that no competing interests exist.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.02.102.

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Supplementary Material: scientific article I

Time-dependent transcriptomic responses of *Daphnia magna* exposed to metabolic disruptors that enhanced storage lipid accumulation.

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METHODS

RNA Extraction. Total RNA was isolated from samples using Trizol reagent (Invitrogen, USA) and following manufacturer protocols with slight modifications. After RNA isolation, DNAse treatment was performed according to manufacturer protocols, followed by a double phenol-chloroform and another chloroform extraction for further purification. RNA was precipitated using sodium acetate and 100% ethanol, being re-suspended in RNAse free water, and lastly, quantified and quality checked in a NanoDrop D-1000 Spectrophotometer (NanoDrop Technologies, USA). Samples presenting a ratio 230/260-260/280 between 1.9-2.1 were selected. RNA integrity was checked using a Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Only the samples showing RIN values above 9 were used for microarray analysis.

Microarrays. A 8 x 60 K Agilent array containing the full set of the 41,317 gene models (Orsini et al., 2016) representing the full transcriptome of *Daphnia magna* was used. This platform was designed from a previous 4 x 180 K one that contained four probes per gene model and which was tested across seven life-stages (Campos et al., 2018). The 8 x 60 K new platform included 39,000 probes belonging to unique genes scoring the max fluorescence signal in at least five stages and the two best probes having the highest signal for the remaining 2,317 genes, which showed a less consistent signaling pattern across life-stages. Further e-array based quality controls were added, resulting in a microarray with 50,000 probes, as well as an extra 3,500 negative probes computer generated. This was then printed on a 8 x 60 K format (Agilent 079797design; GPL23826).

A total of three replicates per treatment and sampling point were used. One µg of total RNA was used for all hybridizations. cDNA synthesis, labeling, amplification and hybridizations were performed following the manufacturer's kits and protocols (Quick Amp labeling kit; Agilent, Palo Alto, CA). The Agilent one-color Microarray

Based Gene Expression Analysis v6.5 was used for microarray hybridizations according to the manufacturer's recommendations. Microarray images were generated by an Agilent high-resolution C microarray scanner. Data was resolved from microarray images using Agilent Feature Extraction software v10.7. Raw microarray data from this study have been deposited at the Gene Expression Omnibus Web site (www.ncbi.nlm.nih.gov/geo/) with accession number GSE119329.

FIGURES

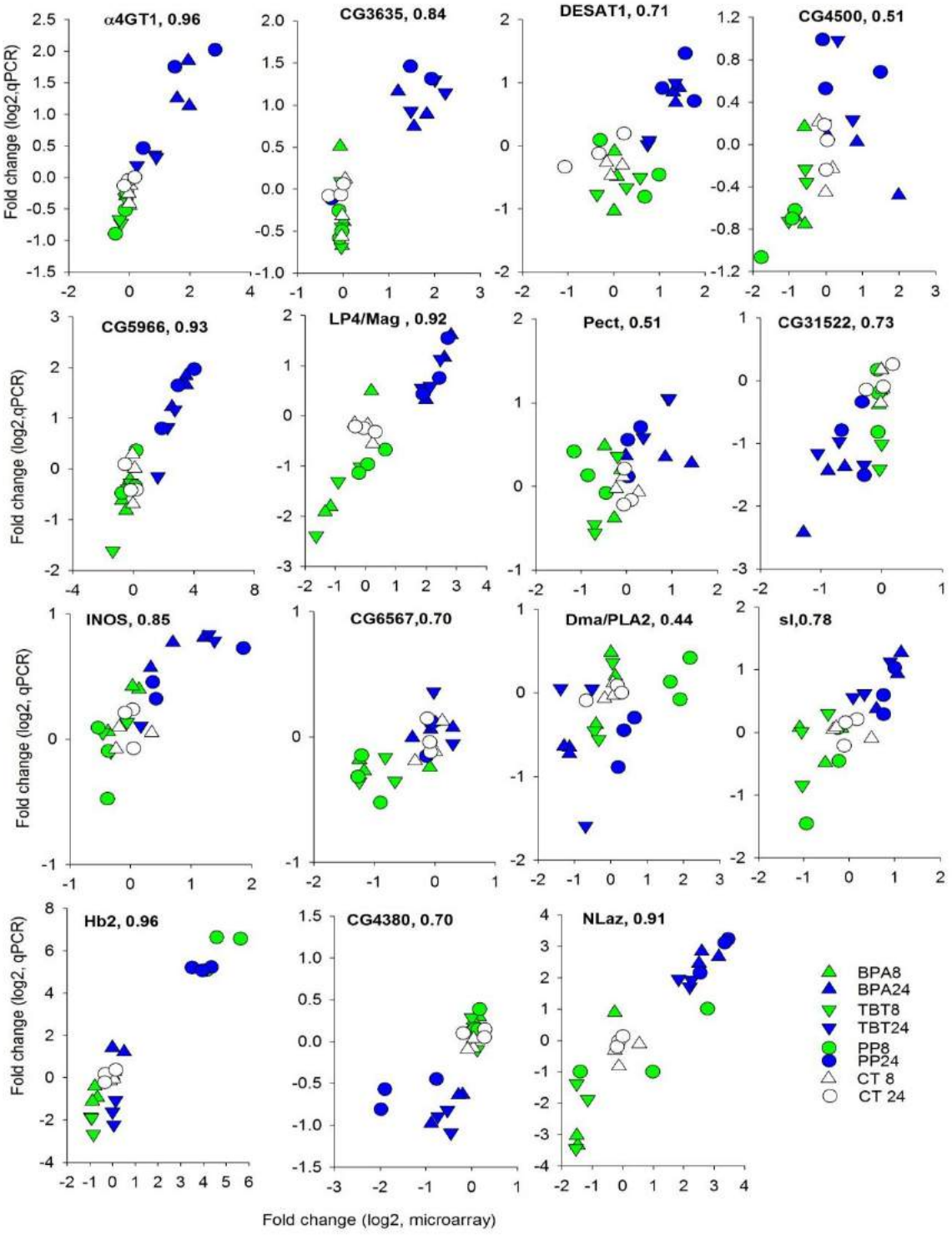

Figure S1. qPCR validation of microarray transcriptomic results for 15 selected de-regulated genes. Results are reported as log 2 normalized transcriptomic responses relative to the respective control time. Each symbol is a single observation. Numbers following gene name are Pearson correlation coefficients. All correlations were significant $P<0.05$.

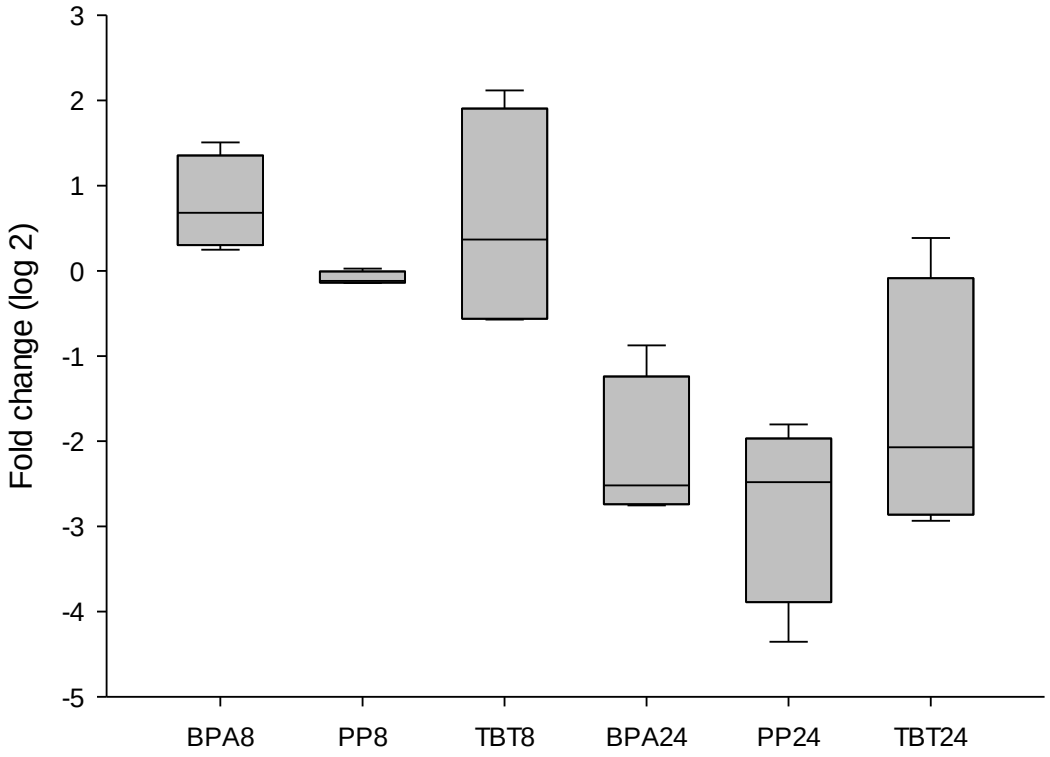

Figure S2. Box plots of HR96 qRT-PCR transcription patterns across treatments and time points. Data have been scaled to its time control.

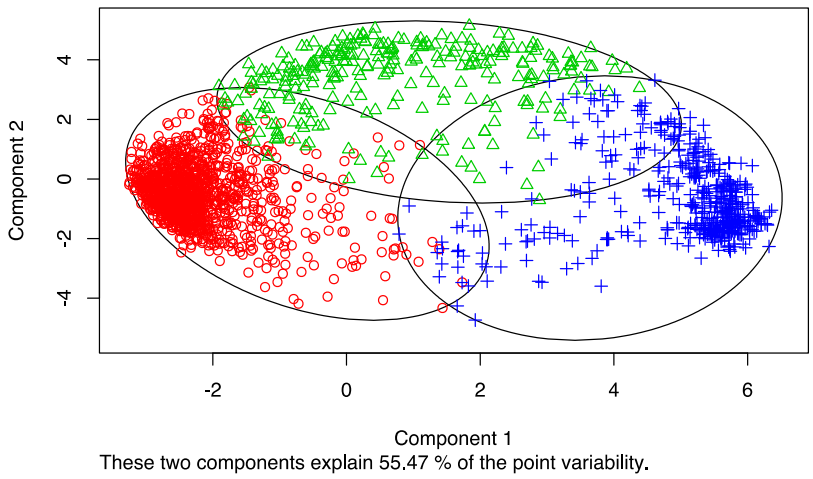

Figure S3. First two components of the medoid cluster analysis. Red, green and blue symbols represent genes belonging, respectively, to clusters A, B and C.

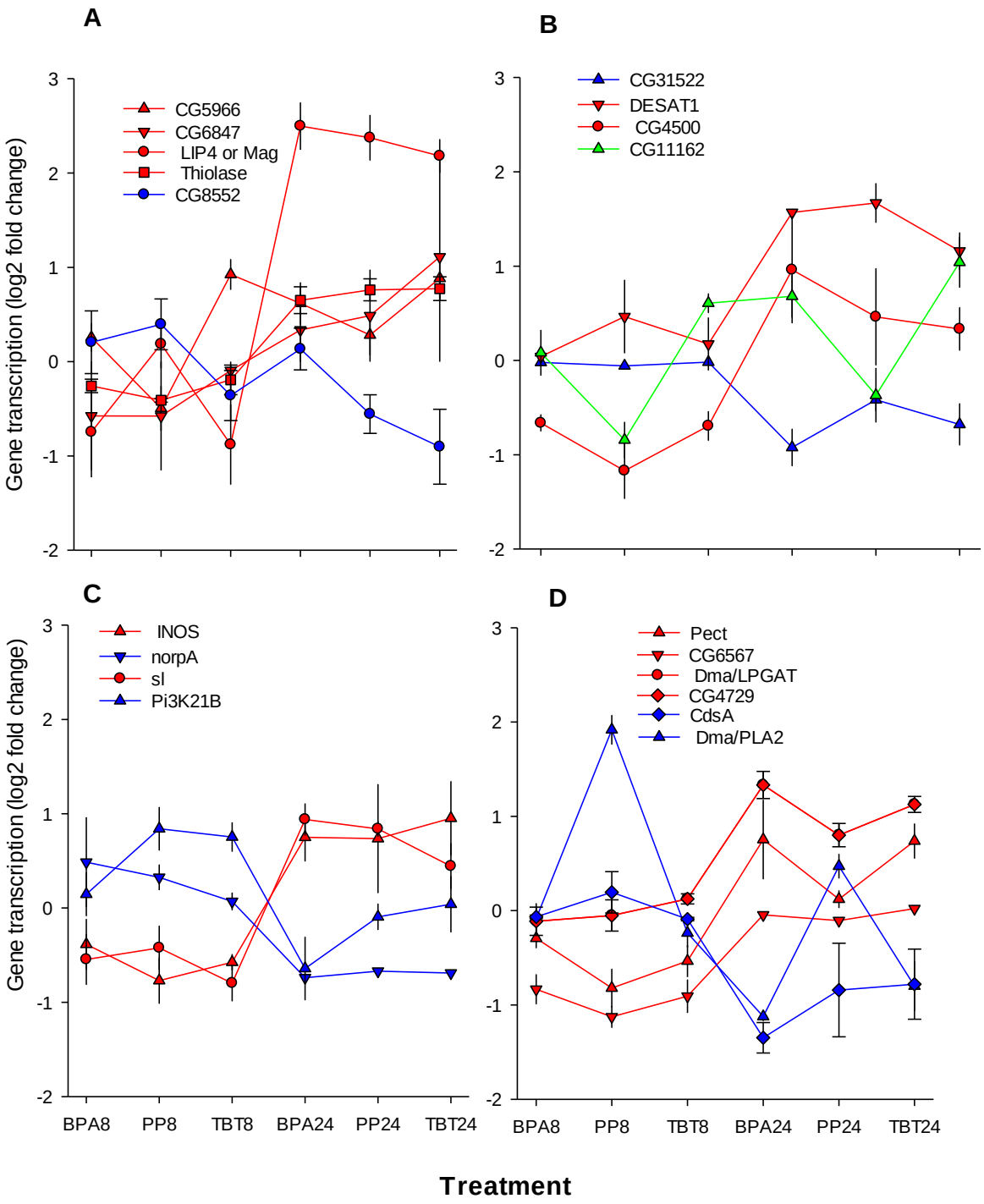

Figure S4. Transcription patterns across treatments and time points (Mean SE, N=3) of differentially transcribed genes (DEGs) belonging to the glycerolipid (A), fatty acid (B) , phosphoinositol (C) and glycerophospholipid (D) KEGG signaling pathways. Data has been scaled to its time control. Genes belonging to clusters A, B and C are depicted, respectively, in red, green and blue.

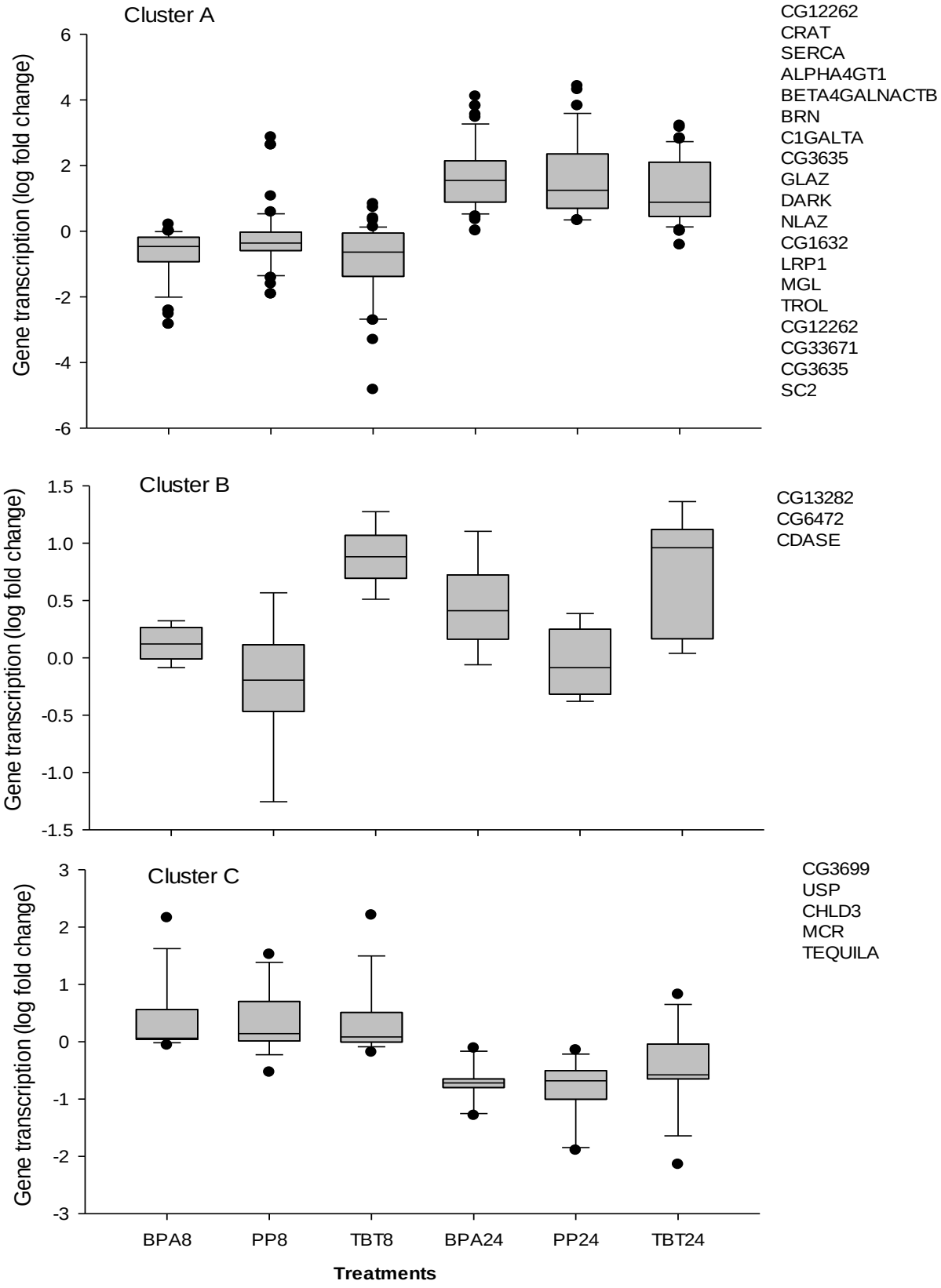

Figure S5. Box plots of transcription patterns across treatments and time points of selected differentially transcribed genes (DEGs) belonging to clusters A, B and C and to KEGG or DAVID functional categories related to lipid metabolic signaling pathways. Data has been scaled to its time control.

TABLES

Table S1. Total number of genes and those present in the microarray and deregulated (DEGs) belonging to the major KEGG pathways of lipid metabolism. The inositol phosphate metabolism is also included. The coverage (Cv) of the microarray respect to the total and that of the DEGs are also reported.

Code	Name	Total	Array		DEGs	
		Genes	Genes	Cv (%)	Genes	Cv (%)
	Lipid metabolism					
ec00564	Glycerophospholipid metabolism	110	73	66.4	5	6.8
ec00561	Glycerolipid metabolism	86	58	67.4	15	25.9
ec01212	Fatty acid metabolism	79	50	63.3	18	36.0
ec00071	Fatty acid degradation	58	53	91.4	16	30.2
ec00590	Arachidonic acid metabolism	44	31	70.5	3	9.7
ec00600	Sphingolipid metabolism	41	26	63.4	3	11.5
ec00061	Fatty acid biosynthesis	33	16	48.5	3	18.8
ec01040	Biosynthesis of unsaturated fatty acids	32	10	31.3	2	20.0
ec00072	Synthesis and degradation of ketone bodies	32	10	31.3	1	10.0
ec00100	Steroid biosynthesis	31	9	29.0	3	33.3
ec00565	Ether lipid metabolism	30	22	73.3	3	13.6
ec00062	Fatty acid elongation	25	14	56.0	5	35.7
ec00592	alpha-Linolenic acid metabolism	25	16	64.0	8	50.0
ec00591	Linoleic acid metabolism	25	14	56.0	3	21.4
	Carbohydrate metabolism					
ec00562	Inositol phosphate metabolism	74	45	60.8	9	20.0

Table S2. Primer pairs designed from existing sequences used for amplification of selected *D. magna* partial gene sequences.

Official gene symbol	Name/function	Acc. Number	Forward	Reverse	Amplicon size (bp)
ALPHA4GT1	α1,4-galactosyltransferase1	KZS12650.1	GCACCGACTCATCGACGAA	CGTGCCACATGTAATCGTCC	71
CG31522	Fatty acid elongase	KZS21395	ACTGAGCCAGCCGAAACG	CAGCCTTCAAACTACGCGGA	101
CG3635	Putative lipase 3	KZS16640.1	CTGGCTTCTGACTCAACTTGGA	GGAAATCTCCGTGGTTGTAAGTTT	71
CG4500	Fatty-acid-CoA ligase	KZS06643	CAATACGACACGAGAACGCCTA	CGGCATCAGTTCATTCTCTTGAT	71
CG5966	Triacylglycerol lipase	KZS12532	GTTGACCAATGATACGGTGACG	GATTTGCTAGATCCCAGTTGCTTT	81
CG6567	Lysophospholipase	KZS14236	AATGGGAGTTTCCGTGTTGC	GGTGTTGTGGCTCTGTCTTGC	81
DESAT1	Desaturase	KZS17645.1	GTTTGCAAGGGCCGTATTCA	ACGGGAAACGATAAGGAAATTTC	71
G3PDH	HK glyceraldehyde-3-phosphate dehydrogenase	AJ292555	GACCATTACGCTGCTGAATACG	CCTTTGCTGACGCCGATAGG	100
HB2	Hemoglobin 2	AB021136	CCCAGGTTCTTTTCCGCCTTC	CGGATTGAGGAACATCGGC	81
HR96	Hormone receptor-like in 96 (but characterized)	JAM35624	CTTCCGTTAATGGTGCCAGG	ATTGTCACCCGTACGCAC	
INOS	Myo-inositol-1-phosphate synthase	KZS15705	CGTGACGTGCAGTAATCGTAATC	GACTAAATTCCAAGTTGACAGCCC	81
LIP4	Gastric triacylglycerol lipase	KZS21232.1	TGTTCAAGTAACTCGAGAGGAAACG	CTTCTTGCTTTCTACCATTAAAACACA	71
NLAZ	Neural Lazarillo	KZS14940.1	TCTATAGACACCATAAAAGTTTGGCAA	CACTTTCCCACTTTAAACTAAAACGA	71
Pect	Phosphocholine/ethanolamine cytidylyltransferase	KZS13222	ATGAGCAGGCAGTTGCAGCT	TGCCTGTATCATTTTGGGCC	81
PLA2	Phospholipase A2	KZS08312.	TGCTCGTCGTCGTTCTTCG	TCCCTGTCGTTGTTGGCTG	81
RXR	Retinoid X Receptor	DQ530508	GTGTCGAGTGCAAGGACGAG	CCCATTCAACCAACTGGAAAA	100
sl	Small wing; 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase	KZS14804	TCGACCAGCCTATACCACAGC	CGCCTGTGTTTTGGTAGGATG	71

Table S3. Deregulated *D. magna* genes homologous to *D. melanogaster* ones and involved in lipid and lipid-related metabolic signaling pathways.

Official gene symbol	Drosophila Accession Number /Dapma7 geneID	Gene Name/Description	KEGG/GO
Brn	AAF45918	Brainiac, Beta-1,3-galactosyltransferase	GlySL
C1GalTA	NP_609258	Core 1 Galactosyltransferase A	GlyL
CDase	NP_651797	Ceramidase	SL
CG10849	AAF47807	Very-long-chain enoyl-CoA reductase	SD
CG11162	AAF48301.1	Fatty acid hydroxylase	FA
CG12262	NP_648149	Acyl-CoA dehydrogenase	FAβOx
CG13282	AAF53578	Triacylglycerol lipase	LC
CG1632	NP_572467	Low-density lipoprotein	LDL
CG31522	NP_730841	Fatty acid elongase	FA
CG33671	NP_001027412	Mevalonate kinase	TB
CG3635	NP_610138	Putative lipase 3	LC
CG3699	NP_569875	Acyl-CoA dehydrogenase	FAβOx
CG4380 (RXR)	NP_476781	Retinoid X receptor	TG
CG4500	NP_609696	Fatty-acid-CoA ligase	FA
CG5966	NP_001284922	Triacylglycerol lipase	GL
CG6472	NP_611166	Triacylglycerol lipase	LC
CG6567	AAF54564	Lysophospholipase	GPL
CG6847	NP_573259	Triacylglycerol lipase	GL, GPL
ChLD3	NP_609806	ChLD3-Low density lipoprotein	LDL
CRAT	NP_649650	Carnitine O-Acetyl-Transferase	FaβOx
Dark	NP_725637	Death-associated APAF1-related killer	TG
DESAT1	NP_652731	Desaturase	TG
Dma/LPGAT	Dapma7bEVm006286	Acyl-CoA:lysophosphatidylglycerol acyltransferase	GPL
Dma/PLA2	Dapma7bEVm011274	Phospholipase A2	GPL
Glaz	AAF58418	Glial Lazarillo	LC
HR96	NP_524493	Hormone receptor-like in 96 (but characterized)	TG
Inos	NP_477405	Myo-inositol-1-phosphate synthase	IP, TB
LIP4, Mag	NP_001188785, NP_649229	Magro (Gastric triacylglycerol lipase), Sterol esterase	GL, SB
LRP1	NP_788284	LDL receptor protein 1	LDL
Mcr	AAF52601	Macroglobulin complement-related	LDL
MGL	NP_001096924	Megalin	LDL
NLaz	NP_001259867	Neural Lazarillo	TG, LC
norpA	NP_525069	No receptor potencial A, Phosphatidylinositol phospholipase C activity	IP
Pect	NP_723790	Phosphocholine/ethanolamine cytidylyltransferase	GPL
Pi3K21B	NP_001259815	Phosphatidylinositol 3-kinase	IP
Sc2	AAF47807	3-oxo-5-alpha-steroid 4-dehydrogenase	SB
SERCA	AAF47102	Sarco/endoplasmic reticulum Ca(2+)-ATPase	FAβOx
Sl	NP_476726	Small wing; 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase	IP

Teq	NP_001163393	Tequila-Low density Lipoprotein	LDL
Thiolase	AAF47083	Acetyl-CoA -acyltransferase	FA, TB
TROL	NP_001245496	Terribly reduced optic lobes-Low density Lipoprotein	LDL
α4GT1	NP_608737	α1,4-galactosyltransferase 1	GlySL
β4GalNAcTB	AAF56843	β1,4-N-acetylgalactosaminyltransferase B	GlySL
CG4729	AAF49473	Acylglycerol-3-phosphate O-acyltransferase 4	GLP
CdsA	AAF50483	CDP-diacylglycerol synthase	GLP
CG8552	NP_609185	Triglyceride lipase	GL

FA, fatty acid metabolism (ec0061, ec00062, ec00071, ec00592, ec01040); FAβOx, fatty acid beta-oxidation (GO:0006635); GL, Glycerolipid metabolism (ec00561); GLP, Glycerophospholipid metabolism (ec00564); GlySL, glycosphingolipid biosynthetic process (GO:0006688); IP, Inositol phosphate metabolism (ec00562); LC, lipid catabolic process (GO:0016042); LDL, Low-density lipoprotein (LDL) receptor class A (IPR002172); SB, Steroid biosynthesis (ec00100); SD, Steroid degradation (ec00984); SL, Sphingolipid metabolism (ec00600); TB, Terpenoid backbone biosynthesis (ec00900); TG, triglyceride homeostasis (GO:0070328). Bold gene names belong to de novo synthesis pathway of neutral lipids according to Pol et al. (2014).

Table S4. Non-deregulated genes present in the microarray belonging to the lipid droplet-based storage fat metabolism in *Drosophila sensu* Pol et al. (2014).

Official *Drosophila* gene symbol	Dapma7 geneID	Probe	Gene description
CG4920	Dapma7bEVm004832	CUST_1748_PI429715507	Ethanolamine kinase
Bbc	Dapma7bEVm000746	CUST_73232_PI429715507	CDP-ethanolamine phosphotransferase
CG5508	Dapma7bEVm001798	CUST_133998_PI429715507	glycerol-3- phosphate acyltransferase 1 or 2 (GPAT1 or 2)
CG3209	Dapma7bEVm015513	CUST_156291_PI429715507	glycerol-3- phosphate acyltransferase 3 (GPAT 3)
Fu12/CG3812	Dapma7bEVm005324	CUST_27420_PI429715507	1-acyl-sn-glycerol-3-phosphate O – acyltransferases (AGPAT 1,2)
CG4729/CG4753	Dapma7bEVm006286	CUST_64377_PI429715507	1-acyl-sn-glycerol-3-phosphate O – acyltransferases (AGPAT 3,4)
CG8709	Dapma7bEVm000171	CUST_132216_PI429715507	Mg 2+ -dependent PA phosphatase (lipin)
midway	Dapma7bEVm001874	CUST_139290_PI429715507	diacylglycerol O –acyltransferase (DGAT1)
CG1941/GC1942/GC1946	Dapma7bEVm004344	CUST_133645_PI429715507	Di or mono- acylglycerol O –acyltransferases (DGAT or MGAT)
Brummer	Dapma7bEVm003238	CUST_1139_PI429715507	Brummer lipase
CG11055	Dapma7bEVm005082	CUST_45471_PI429715507	Hormone-sensitive lipase
Lsd-1, Lsd-2	Dapma7bEVm009909	CUST_143643_PI429715507	Perilipin family of Lipid droplet associated proteins

2.2.2. Scientific article II

Allocation of glycerolipids and glycerophospholipids from adults to eggs in *Daphnia magna:* Perturbations by compounds that enhance lipid droplet accumulation.

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Allocation of glycerolipids and glycerophospholipids from adults to eggs in *Daphnia magna*: Perturbations by compounds that enhance lipid droplet accumulation☆

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ABSTRACT

Analysis of the disruptive effects of chemicals on lipids in invertebrates is limited by our poor knowledge of the lipid metabolic pathways and the complete lipidome. Recent studies shown that juvenoids and bisphenol A disrupted the dynamics of lipid droplets in the crustacean *Daphnia magna*. This study used ultra-high performance liquid chromatography/time-of-flight mass spectrometry (UHPLC/TOFMS) to study how juvenoids (pyriproxyfen and methyl farnesoate) and bisphenol A disrupt the dynamics of glycerophospholipids and glycerolipids in *Daphnia* adults and their allocation to eggs. Lipidomic analysis identified 234 individual lipids corresponding to three classes of glycerolipids, seven of glycerophospholipids, and one of sphingolipids, of which 194 changed according to the chemical treatments and time. Adult females in the control and bisphenol A treatment groups had low levels of triacylglycerols but high levels of glycerophospholipids, whereas those in the juvenoid treatment groups had high levels of triacylglycerols and low levels of glycerophospholipids. The opposite trend was observed for the lipid contents in the eggs produced. Because the juvenoids reduced reproduction dramatically, the females allocated less triacylglycerols to their eggs than the controls did. Interestingly, females exposed to bisphenol A allocated less triacylglycerols to their eggs despite producing a similar number of eggs as that of the controls. Thin-layer chromatography analyses confirmed the UHPLC/TOFMS results and allowed qualitative determination of cholesterol, which was also accumulated in females exposed to the juvenoids.

1. Introduction

Lipids are important structural and energetic cell components which are involved in many organismic vital functions, such as development, growth, and reproduction. Deregulation of the lipids and their related metabolic pathways often results in detrimental health problems. One such problem is obesity and its associated illnesses, such as coronary artery diseases, diabetes, and hypertension (Grün and Blumberg, 2006; Sharma and Staels, 2007). Fat accumulation, and hence obesity, can be promoted upon an organism's exposure to chemicals, such as phthalates, bisphenol A, alkylphenols, and organotin pesticides (Grün and Blumberg, 2009).

The molecular mechanisms by which some chemicals called obesogens promote fat accumulation are well known in mammals and other vertebrates. Several obesogens can alter adipocyte differentiation and lipid metabolism, disrupting the signalling pathways of two receptors that regulate them; namely, peroxisome proliferator-activated receptor gamma and its heterodimeric partner retinoid X receptor (RXR) (Grün and Blumberg, 2009). Obesogens can also promote the accumulation of fat by disrupting sex steroids, the hypothalamic-pituitary-adrenal axis, and thyroid and glucocorticoid hormones (Grün and Blumberg, 2006, 2009). Therefore, the accumulation of lipids can be deregulated by multiple mechanisms.

Compounds such as juvenoids (methyl farnesoate and pyriproxyfen), tributyltin, and bisphenol A have been shown to increase the accumulation of lipid droplets in the crustacean *Daphnia*

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magna, whereas others like nonylphenol, fluoxetine, fenarimol, and testosterone have the opposite effect (Jordão et al., 2016b). Indeed, the accumulation of storage lipids into lipid droplets in *D. magna* has been mechanistically linked to three master receptors that regulate growth and reproduction in arthropods; namely, the ecdysteroid receptor and its heterodimeric partner RXR, and the methyl farnesoate receptor (homologous to the juvenile hormone receptor in insects) (Jordão et al., 2016a). Consequently, it is not surprising to find that most of the tested compounds that disrupted storage lipid accumulation in *D. magna* also altered the growth and reproductive performance of the crustacean.

Ecdysteroids and juvenoids regulate moulting, growth, and reproduction in *D. magna* and modulate the quantity and fate of glycerolipids, mainly of triacylglycerols (TGs) located in lipid droplets (Tessier and Goulden, 1982; Zaffagini and Zeni, 2009). During the intermoult period, reproductive *D. magna* females reared on plenty of algae as food accumulate large quantities of fatty acids into TGs, which are stored in lipid droplets and allocated to the moult and egg formation (Goulden and Place, 1990; Tessier and Goulden, 1982). There is reported evidence that *Daphnia* species bioaccumulate highly polyunsaturated fatty acids from their diet and allocate them to the eggs (Sengupta et al., 2016).

Recently, a study on the effects of tributyltin on the whole lipid profile of *Daphnia* during a reproductive cycle reported that this compound impaired the allocation of TGs to eggs, promoting their accumulation into lipid droplets in the adult females after reproduction (Jordão et al., 2015). Eggs containing less TGs developed into smaller offspring that matured later and reproduced less than those produced by non-exposed females (Jordão et al., 2015). Another study, focused on phospholipids, found that triclosan reduced the metabolism of sphingomyelin (SM) into ceramides or sphingosine (important signalling compounds), thereby promoting SM accumulation in the adults and neonates (Sengupta et al., 2017). Such effects were associated with delayed development in the neonates and delayed maturation and reduced fecundity in the adults.

In *D. magna*, juvenoids are known to dramatically affect reproduction by decreasing the number of offspring and promoting the production of males. There is reported evidence that the studied juvenoids (methyl farnesoate and pyriproxyfen) reduced the clutch size, leading to the production of all-male broods (Olmstead and Leblanc, 2002, 2003; Wang et al., 2005). Reported studies have indicated that bisphenol A at 2.5–10 mg/L is embryotoxic and hence reduces viable offspring production (Jeong et al., 2013; Mu et al., 2005). Nevertheless, little is known about how these compounds affect the lipid dynamics in adults and the allocation of lipids to the eggs.

The aim of this study was to assess the effects of bisphenol A and two juvenoids (methyl farnesoate and pyriproxyfen) on the lipid profiles of adolescent *D. magna* females during their first reproductive cycle and of their eggs. During the adolescent instar, primiparous *Daphnia* females provision the first clutch of eggs, which are released to the brood pouch after moulting. A previous study found that all three chemical compounds as well as tributyltin increased the accumulation of lipid droplets in adolescent females (Jordão et al., 2016b). In the case of tributyltin, the lipid droplets accumulated were of TGs that were not allocated to the eggs. Since the exposure to juvenoids dramatically decreased offspring production, it is plausible to test the hypothesis that TGs not invested into eggs are accumulated in the adult females.

It is well known that in *Daphnia* species, the fatty acids, which are major components of TGs and other lipids, resemble those of algae (Brett et al., 2006; Goulden and Place, 1990; Wacker and Martin-Creuzburg, 2007). Accordingly, the TG profile of the algae used to feed the experimental *D. magna* individuals was also assessed. This allowed us to test whether *D. magna* adults and eggs differentially accumulate TGs that have fatty acids with greater degrees of unsaturation than those in algae. The lipid profiles in the algae, and *Daphnia* adults and eggs were assessed by means of lipidomic analysis, using ultra-high performance liquid chromatography/time-of-flight mass spectrometry (UHPLC/TOFMS) (Gorrochategui et al., 2014). Furthermore, the relative abundance of the major lipid groups quantified by UHPLC/TOFMS were qualitatively validated by thin-layer chromatography (TLC), which is a widely used, fast, highly reproducible, and relatively inexpensive method of separating complex mixtures and provides a robust and reliable qualitative measure of the existing lipid groups within a given sample (Olsen and Henderson, 1989).

2. Materials and methods

2.1. Studied compounds and chemicals

The chemical compounds studied were the crustacean juvenile hormone methyl farnesoate (CAS 10485-70-8); the juvenoid pesticide pyriproxyfen (CAS 95737-68-1), and the plasticiser bisphenol A (CAS 80-05-7). All the compounds were obtained from Sigma-Aldrich (St. Louis, MO, USA) except methyl farnesoate, which was supplied by Echelon Bioscience (Salt Lake City, UT, USA). Nile red (CAS No. 7385-67-3) and 2,6-di-tert-butyl-4-methylphenol (BHT, CAS No. 128-37-0) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipid standards were from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany).

2.2. Experimental animals

Parthenogenetic cultures of a single clone of *D. magna* (clone F) were used in all the experiments. The photoperiod was set to a 14-h light: 10-h dark cycle and the temperature to $20 \pm 1\,^{\circ}C$. Individual cultures were maintained in 150-mL glass vessels filled with 100 mL of ASTM hard synthetic water at high food ration levels (5×10^5 cells/mL of *Chlorella vulgaris*), following the method described by Barata and Baird (1998).

2.3. Experimental procedures

All the experiments were carried out according to previously described procedures (Jordão et al., 2015, 2016b) and aimed to determine the effects of high and low concentrations of the studied chemicals on the lipid dynamics across an entire adolescent intermoult cycle. The concentrations, selected to cause the lowest and highest detectable accumulation of lipid droplets (Jordão et al., 2016a, 2016b), were as follows: 50 and 250 μg/L for methyl farnesoate, 0.5 and 3 μg/L for pyriproxyfen, and 1 and 10 mg/L for bisphenol A (hereafter referred to as MFL, MFH, PPL, PPH, BPA, and BPAH, respectively). Experiments were started with 4–8-h-old newborn individuals obtained from females reared individually on high food ration levels. The newborns were reared in groups of five in 100 mL of ASTM hard water at high food ration conditions until about 4–8 h before moulting for the third time. At this point, the exposure experiments were initiated, where groups of five juveniles each were exposed to 500 mL of the test medium. Acetone at a concentration of 20 μL/L was used as the chemical carrier, and was also used for the controls. The test medium was renewed every 24 h. Three samplings were obtained: at 0 h (just after the third moult and being exposed for about 4-8 h), 24 h, and 48 h (just after the fourth moult). At each sampling, three replicates of five individuals were collected and processed for total lipid measurements. At the 48-h sampling point, females were de-brooded

according to the method described by Jordão et al. (2015). The obtained eggs and de-brooded females were then collected, counted, and used for lipid analyses. At 48 h, accumulated lipid droplets in the post-spawning females were also measured in an additional group of ten animals, using the Nile red assay. Samples of the *C. vulgaris* alga used for lipidomic analyses were cultured under conditions identical to those used to feed the experimental animals, and were then freeze dried following previously described procedures (Barata and Baird, 1998). Triplicate algal samples were used for the lipidomic analyses. The physicochemical water quality and test concentrations were monitored in the fresh and old test solutions. Further information is provided in the Supplementary Materials.

2.4. Nile red determination

The accumulation of storage lipids into lipid droplets was quantified using the Nile red fluorescence assay according to previously described procedures (Jordão et al., 2015).

2.5. Lipidomic analysis

Lipidomic analysis of three replicates from each treatment and sampling period was performed following the procedures described by Jordão et al. (2015) with minor modifications. Each replicate consisted of a pool of 5 *D. magna* adults, 40 eggs, or 50 mg dry weight of algae that were homogenised in 500 μL of phosphate-buffered saline (pH 7.40) with 0.01% BHT as an antioxidant. Homogenates of the *Daphnia* adults were split in two portions: one for UHPLC/TOFMS and the other for TLC analyses. Lipid extraction for UHPLC/TOFMS was performed using similar extraction conditions as described by Olsen and Henderson (1989) with minor modifications, which are explained in the Supplementary Materials.

Lipidomic analysis was performed with an Acquity UHPLC system (Waters Corp., Milford, MA, USA) coupled to a Waters/LCT Premier XE TOF analyser operated in positive and negative electrospray ionisation modes. The analytical separation was performed with an Acquity UPLC BEH C8 column (1.7 mm particle size, 10 × 2.1 mm; Waters Ireland, Dublin, Ireland) at 30 °C and a flow rate of 0.3 mL/min. The chromatographic conditions and MS parameters used have been previously reported (Gorrochategui et al., 2014) and are fully explained in the Supplementary Materials.

The lipids identified fell into eight classes of glycerophospholipids (phosphocholine, PC; lysophosphatidylcholine, LPC; phosphatidylethanolamine, PE; lysophosphatidylethanolamine, LPE; phosphatidylglycerol, PG; lysophosphatidylglycerol, LPG; and phosphatidylserine, PS), three of glycerolipids (monoacylglycerol, MG; diacylglycerol, DG; and TG), and one of sphingolipids (SM). These lipids were annotated as <lipid subclass> <total fatty acyl chain length>: <total number of unsaturated bonds>, except for SM, which was annotated as <total fatty acyl chain length>: <total number of unsaturated bonds in the acyl chain>.

Daphnia lipid extraction for TLC analysis was performed using the methods described by Folch et al. (1957). The TLC analysis was performed according to the method described by Olsen and Henderson (1989). Detailed descriptions of these methods are provided in the Supplementary Materials.

2.6. Data analysis

The effects of treatment and sampling period (time) on the lipidomic profiles of the major lipid groups were analysed by two-way analysis of variance (ANOVA). The treatment effects on the lipid profiles of the eggs and algae, fecundity, and Nile red values were determined by one-way ANOVA. Prior to analyses, all data were log transformed to achieve normality and variance homoscedasticity. Significance levels were set at $p < 0.05$ unless indicated otherwise. Tests were performed with IBM-SPSS Statistics version 23 software. Changes in individual lipids across the treatments and sampling periods in the females and eggs were further analysed using MetaboAnalyst version 4.0. Data were autoscaled, and partial least squares discriminant analysis (PLS-DA) was carried out to identify clusters of individual lipids similarly affected by the studied chemicals. Taking into account the variable importance in projection (VIP) score values obtained from the PLS-DA, individual lipids with a VIP score higher than 1 were submitted to hierarchical clustering analysis (represented as a heatmap) using autoscaled feature standardisation, Pearson's distance measure, and Ward's clustering algorithm.

3. Results

3.1. Effects of the chemicals on fecundity as well as on lipid droplets

The fecundity of females exposed to bisphenol A was similar to that of the controls, but it was significantly reduced more than 50% ($p < 0.05$, $F_{6, 113} = 99.5$) in those exposed to the juvenoids (Table 1). Moreover, females treated with methyl farnesoate, pyriproxyfen, or bisphenol A accumulated significantly more lipid droplets (measured as Nile red fluorescence) than the controls did, in a concentration-dependent manner ($p < 0.05$, $F_{6, 38} = 23.5$), reaching values as high as 52% (Table 1).

3.2. UHPLC/TOFMS vs. TLC

The use of UHPLC/TOFMS allowed us to identify and quantify 234 individual lipids whose calculated and measured mass, elemental composition, mass accuracy error, double-bond equivalent, and retention time were obtained from formula determination tools (elemental composition search) of MassLynx software and are reported in Supplemental Materials, Table S2. These comprised 60 TGs, 50 PCs, 41 DGs, 25 PEs, 13 PSs, 12 LPCs, 11 PGs, 8 SMs, 6 LPEs, 4 LPGs, and 4 MGs. TLC analysis allowed us to separate eight different spots corresponding to specific lipid groups, which were (in order of polarity) TG, cholesterol (CHL), PE, PG, phosphatidylinositol, PS, PC, and SM. An additional spot situated between CHL and PE corresponded to pigments with traces of DG and MG, and when there were free fatty acids, a stain between TG and CHL would be observed (for further details see Supplementary Materials, Figure S1).

Fig. 1A illustrates an example of the relative contribution of six of the major lipid groups present in adult control samples, as quantified by UHPLC/TOFMS and TLC. The results indicated that TLC

Table 1
Quantitative assessment of lipid droplets in *Daphnia magna* across treatments after moulting and fecundity (number of eggs per brood). Lipid droplets were quantified as Nile red fluorescence relative to that of the control treatments. The sample size was 15–20 individuals for fecundity and 5 for lipid droplets. Different letters indicate significant ($p < 0.05$) differences among treatments following ANOVA.

Treatment	Lipid droplet	Fecundity
	Mean ± SE	Mean ± SE
Control	0.0 ± 2.5 a	7.5 ± 0.2 a
MFL	24.7 ± 2.7 b	3.8 ± 0.3 b
MFH	51.6 ± 3.1 c	1.4 ± 0.3 c
PPL	18.5 ± 4.7 ab	4.9 ± 0.2 b
PPH	49.1 ± 3.5 c	3.9 ± 0.1 b
BPAL	25.1 ± 4.6 b	7.0 ± 0.2 a
BPAH	52.6 ± 11.5 c	6.6 ± 0.2 a

underestimated the TGs and overestimated the PCs, but provided equivalent abundance values for PE, PG, PS, and SM when compared with the UHPLC/TOFMS results. Nevertheless, the abundance levels of TG, PC, PG, and PS across the 21 studied treatments obtained by UHPLC/TOFMS and TLC were significantly ($p < 0.05$) correlated (Fig. 1B). The quantities of PG and SM were too low to get accurate measurements by TLC, and hence, good correlations against the UHPLC/TOFMS estimates could not be determined (Fig. 1B).

3.3. Effects of the chemicals on the lipid dynamics

Changes in the lipid contents of *D. magna* juveniles, either unexposed (control) or exposed to the studied chemicals during the adolescent instar, are indicated in Fig. 2 (supporting statistics are provided in the Supplemental Materials, Table S3). The sampling period significantly affected ($p < 0.05$) the levels of 10 out of 12 studied lipid classes within and across the studied concentrations (Time, Time × Treatment effects, from Supplemental Materials, Table S3). In most treatments, the levels of DG, TG, PC, PE, PG, PS, and CHL in the adolescent females increased at the beginning of the instar (0–24 h) and levelled out afterwards (24–48 h). The exceptions were those females exposed to juvenoids, whose lipid levels increased across the entire instar. The TG levels showed the greatest changes across the 0–48-h samples, increasing up to 3- and 5-folds in the control and juvenoid-exposed females, respectively. The maximum levels of TGs in females exposed to juvenoids were from 1.6- to 4-fold greater than those of control females, whereas those of females exposed to bisphenol A were of similar magnitude to the controls. Note, however, that the TLC results indicated that females exposed to bisphenol A accumulated more TGs than those from the control treatments (inset graph of TG, $F_{9, 15} = 12.5$; $p < 0.001$). The levels of LPC and LPE were lower in females exposed to MFH and showed similar patterns, increasing across the entire instar in most treatments except upon exposure to bisphenol A, which had the highest values at 0 h and decreased afterwards. The levels of LPG were greater in the females exposed to juvenoids. The MG graph is not shown because the measured lipid levels were not significantly affected.

In the egg samples, PG, LPG, and PS were not detected and CHL was not measured by TLC. The levels of the lipid classes allocated to reproduction (number of eggs resealed) generally showed a similar pattern as that of fecundity (Fig. 2). The levels of DG, TG, PC, and PE allocated to reproduction were lowest in the females exposed to juvenoids. Females exposed to BPAH invested less TGs to the eggs.

PLS-DA was performed to explain the variation of individual lipids across treatments and sampling periods. In adult females, the two components obtained from the PLS-DA explained 63% of the total variance, and identified 93 out of 234 lipids having VIP scores greater than 1. These lipids clustered into two groups that separated the juvenoid samples from the rest (Fig. 3A). Females exposed to juvenoids were enriched in DGs and TGs, and depleted in PCs, LPCs, and PEs. The two selected components of the PLS-DA performed on individual lipids measured in the eggs explained 68% of the total variance, and identified 69 out of 132 lipids having VIP scores greater than 1. These lipids were grouped into two clusters and also separated the juvenoids from the other treatments (Fig. 3B). Eggs produced by juvenoid-exposed females had less DGs and TGs and more PEs, LPCs, and LPEs. The VIP score values from each individual lipid represented in Fig. 3 are shown in Supplementary Materials, Table S4.

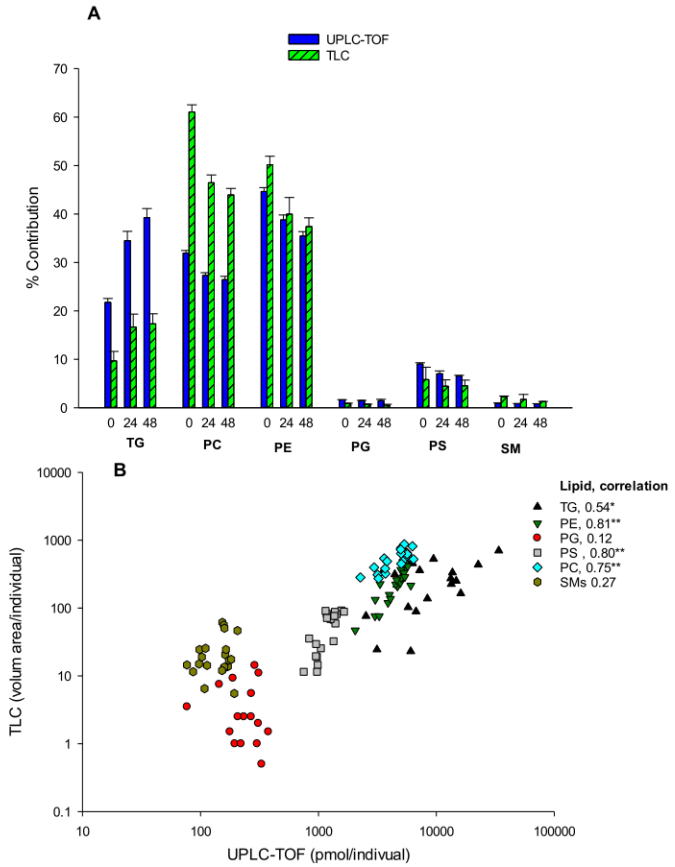

Fig. 1. Relative contribution of major lipid groups measured by TLC and by UHPLC/TOFMS in control females at different times (A), and bi-plots of the measured levels of major lipid groups by TLC and UHPLC-TOF across samples (B). The correlation values of those bi-plots are also shown. *$0.01 < p < 0.05$; **$0.01 < p < 0.001$. Error bars in graph A are standard errors (N=3).

3.4. Effects of the algal diet on the TG profile of adults and eggs

In the *C. vulgaris* samples, only TGs were analysed. The relative abundance of individual TGs in the de-brooded adults was closely related to that of the algal species given as food, although most - females had enriched levels of less unsaturated TGs (Fig. 4A). Females exposed to juvenoids were the exception, since their TG profiles showed similar levels of unsaturation than those of the algae, especially when exposed to the highest levels of methyl farnesoate. The eggs were slightly more enriched in poly-unsaturated TGs (having 7–8 degrees of unsaturation) than the adult females (dotted grey line) and showed little changes across the treatments (Fig. 4B).

4. Discussion

The results presented herein indicated that the studied juvenoid compounds (methyl farnesoate and pyriproxyfen) perturbed the lipid profiles that are crucial in reproduction and development, enhancing the accumulation of glycerolipids into lipid droplets in the reproductively active *D. magna* females. Bisphenol A had minor effects on the whole-organism lipid profiles, but enhanced the accumulation of TGs into lipid droplets, preventing their allocation to the eggs. These results are in line with those of previous studies, which found that juvenoids dramatically reduced fecundity (Olmstead and Leblanc, 2002, 2003; Wang et al., 2005) and both juvenoids and bisphenol A promoted the accumulation of storage lipids into lipid droplets (Jordão et al., 2015, 2016b).

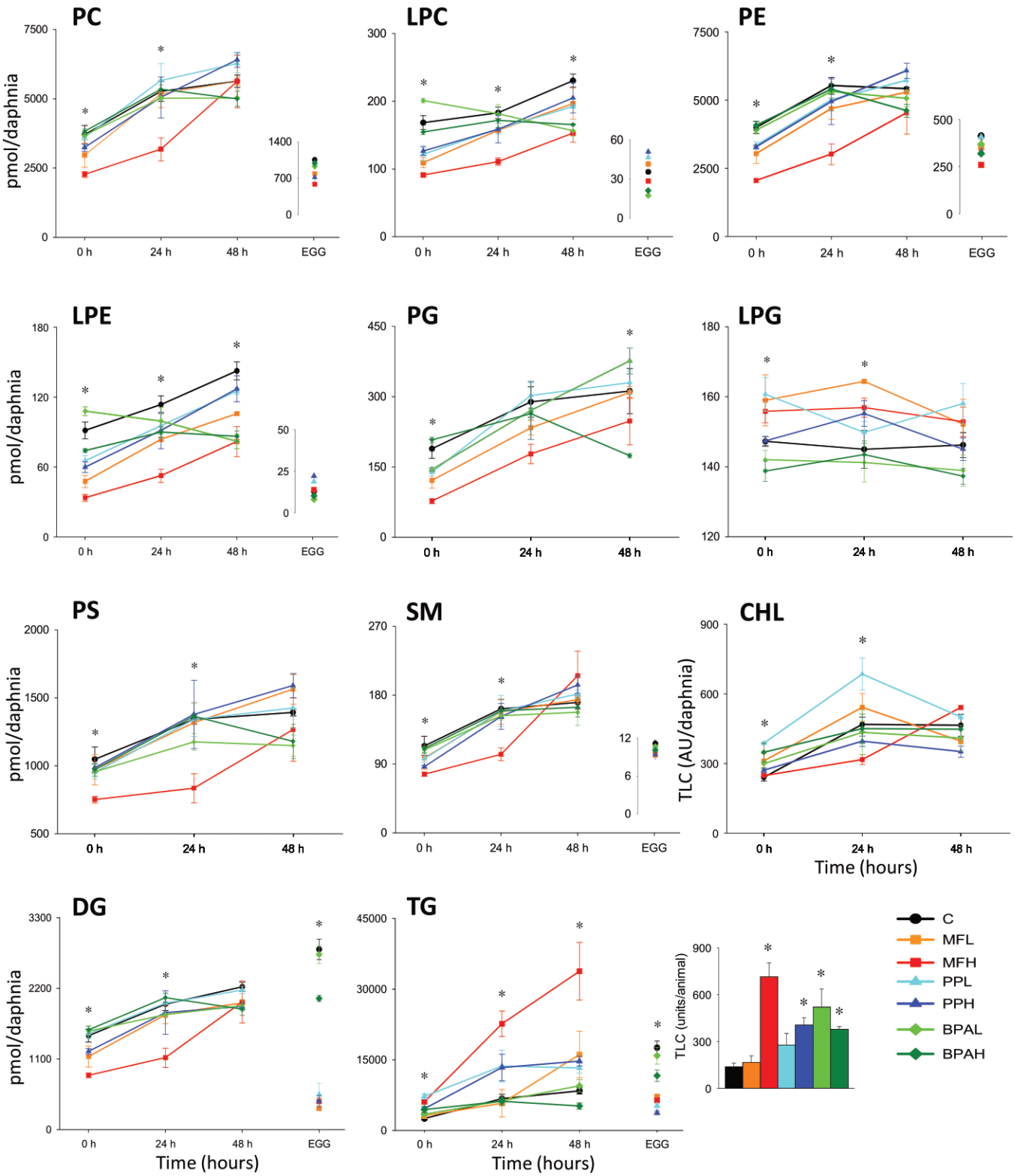

Fig. 2. Lipidomic profiles of major lipid classes (mean ± SE, N = 3) following control (C), and low and high concentrations of methyl farnesoate (MFL, MFH), pyriproxyfen (PPL, PPH), and bisphenol A (BPAL, BPAH) treatments along the adolescent instar in females at 0 and 24 h, in de-brooded females just after the fourth moult (48 h), and in the eggs. Each represented lipid group corresponds to the sum of the measured individual lipids. Graphs include seven glycerophospholipids (phosphocholine, PC; lysophosphatidylcholine, LPC; phosphatidylethanolamine, PE; lysophosphatidylethanolamine, LPE; phosphatidylglycerol, PG; lysophosphatidylglycerol, LPG; and phosphatidylserine, PS); two glycerolipids (diacylglycerols, DG; and triacylglycerols, TG); one sphingolipid (sphingomyelin, SM); and cholesterol (CHL). * indicates significant ($p < 0.05$) differences among treatments within a given sampling period following ANOVA. In the TG graph, the inset graph depicts TGs measured by TLC in 48-h females.

4.1. Lipid dynamics associated with lipid droplets

Lipid droplets are not only a passive reservoir of lipids but also cell organelles, serving a central role in fat and energy metabolism (Olofsson et al., 2009). A lipid droplet includes a central core of glycerolipids (TGs) and CHL esters surrounded by a monolayer of phospholipids and CHL (Pol et al., 2014). Whereas little is known about the metabolism of lipids inside lipid droplets in *Daphnia*, there is ample information of such in *Drosophila*. Much like *Drosophila*, *Daphnia* lipogenesis also occurs mainly in the fat cells (Arrese and Soulages, 2010; Tessier and Goulden, 1982; Zaffagini and Zeni, 2009).

The lipidomic analysis results showed that in most treatments, most lipid classes accumulated linearly during the first phase of the intermoult interval (0–24 h) and then levelled out or even decreased in de-brooded females (48 h) (Fig. 3). The observed complex lipid dynamics of *Daphnia* individuals reflected the cyclic changes in storage lipids (TG, DG, and CHL), glycerophospholipids (PC, PE, PG, and PS), and sphingolipids (SM) during the reproduction cycle (Goulden and Place, 1990; Sengupta et al., 2016; Tessier et al., 1983). Reproductively active *Daphnia* females accumulate large quantities of storage lipids and glycerophospholipids ingested from food into lipid droplets and cell membranes, respectively. High amounts of storage lipids and glycerophospholipids are allocated to the eggs, and glycerophospholipids are additionally used in the formation of the new adult carapace. Recently, it was reported that *D. magna* females transfer high amounts of sphingolipids to their offspring to favour their correct development (Sengupta et al., 2016). Thus, our results are in line with the reported findings.

Furthermore, the observed accumulation of lipid droplets in females after release of the eggs (48 h) also reflected the residual amount of TGs present in those females. In females exposed to the juvenoids and bisphenol A for 48 h, the levels of TG measured by UHPLC/TOFMS or/and TLC were greater than those of the controls (Fig. 2) and likewise the amount of lipid droplets (Table 1).

4.2. Effects of the juvenoids on the lipid profiles of adults and eggs

The lipidomic analysis results showed major effects of both methyl farnesoate and pyriproxyfen on TGs and differential effects on DGs and glycerophospholipids. The excess of TGs in females exposed to MFH, coupled with the low levels of DG, PC, PE, LPC, and LPE, may indicate that an important amount of glycerophospholipids and DGs were used to synthesize TGs, probably by the glycerophosphate pathway (Arrese and Soulages, 2010; Pol et al., 2014). Alternatively, the observed enhanced levels of TGs, but not of DGs or PCs and PEs, in females exposed to the other juvenoid treatments probably means that TGs were also synthesized directly from the acylation of fatty acids (Arrese and Soulages, 2010; Pol et al., 2014).

Fecundity (Table 1) and the amounts of TGs and other glycerophospholipids invested in reproduction (Fig. 2) were the lowest in females exposed to juvenoids. These females also produced fewer eggs, and thus only a small amount of the accumulated TGs and glycerophospholipids were invested into egg production. Juvenoids are known to dramatically reduce fecundity and promote male offspring in *Daphnia*, two responses closely related to coping with adverse living conditions (LeBlanc and Medlock, 2015; Olmstead and LeBlanc, 2003). The observed increase in lipid reserves in exposed females may be a part of this coping mechanism, since the accumulation of high amounts of TGs after reproduction can also be advantageous for survival under adverse conditions.

The lipidomic profile of females exposed to bisphenol A only differed from the rest of the treatments in the levels of LPC and LPE. These two lipids showed the opposite response pattern, having the greatest concentrations in 0-h samples and decreasing afterwards (Fig. 2). LPC and LPE accumulation can be related to a chemical-mediated inhibition of lysophospholipases, which would further metabolise them into the bioactive lipid lysophosphatidic acid. The above-mentioned inhibitory effects have been reported for aromatic phosphate compounds (Jiang et al., 2011) but not for bisphenol A. Further research should be conducted to elucidate the mechanisms by which bisphenol A increases the accumulation of lysophospholipids shortly after exposure.

Despite producing a similar number of eggs to that from the controls (Table 1), females exposed to bisphenol A invested less TGs into reproduction (Fig. 2). Eggs having less TGs develop into smaller offspring that are less fit than the larger ones (Gliwicz and Guisande, 1992; Tessier and Consolatti, 1989, 1991; Wacker and Martin-Creuzburg, 2007). The impairment effects of bisphenol A on the allocation of TGs to eggs were similar to those reported for tributyltin, which also enhanced the accumulation of TGs in *Daphnia* (Jordão et al., 2015). Since the tributyltin effects have been mechanistically linked to the RXR (Jordão et al., 2015), both compounds could act throughout similar mechanisms of action; for example, disrupting the RXR signalling pathway. In fact, there is evidence that this is one the mechanism by which bisphenol A causes obesity in mammals (Boucher et al., 2014; Li et al., 2008).

4.3. Effects of the algal diet on the TG levels of adults and eggs

The profiles of TGs in the *Daphnia* adults and eggs and in the algal species showed that *D. magna* incorporates TGs mostly from the diet (Fig. 4). Cellular TGs are the reservoirs of fatty acids, and previous studies have indicated that the fatty acid composition in adults and eggs of *Daphnia* species resembled that of algae (Brett et al., 2006; Goulden and Place, 1990; Wacker and Martin-Creuzburg, 2007), which agrees with our study.

Fatty acids in TGs vary in carbon-chain lengths and degree of unsaturation. In our analysis, we observed that *D. magna* adults preferentially accumulated TG 48:5, 50:5–6, 52:6–7, and 54:6–7, whereas eggs accumulated TGs with higher degrees of unsaturation.

The essential fatty acids, linoleic acid and linolenic acid, contributed about 20–30% of the total fatty acids in *D. magna* fed with the alga *C. vulgaris* (Barata et al., 2005). *Daphnia* has a limited capacity to convert C18 fatty acids into longer ones, such as arachidonic acid and eicosapentaenoic acid, which are less abundant at 1–2% (Barata et al., 2005), but are also essential for growth and reproduction (Ginjupalli et al., 2015; Goulden and Place, 1990). *Daphnia* allocates high amounts of long-chain polyunsaturated fatty acids to eggs (Wacker and Martin-Creuzburg, 2007), which may explain why the eggs were enriched in TGs with 9 degrees of unsaturation. Indeed, reported results have shown that glycerolipids (i.e., TGs) of *D. magna* eggs contain similar levels of arachidonic and eicosapentaenoic acids than those present in the diet (Putman et al., 2014). Therefore, those TGs in adults and eggs having more than 50 carbons and 9 degrees of unsaturation may contain the essential fatty acids (i.e., linoleic, linolenic, arachidonic, and eicosapentaenoic acids).

Adult females exposed to MFH, and to a lower extent, those exposed to MFL and PPH, had distinctive TG profiles characterised by enriched levels of TGs with 48 carbons and a more homogeneous distribution of those having 3–9 degrees of unsaturation (Fig. 4A). For TGs with 52 carbons, their distributions in females exposed to MFH and in algae were similar (Fig. 4A). These results indicated that the juvenoids not only increased the levels of TGs in adults, but also changed their composition to favour the accumulation of all TGs present in the food and enhance those with 48 carbons. This can also be a strategy to cope with unfavourable conditions, since low

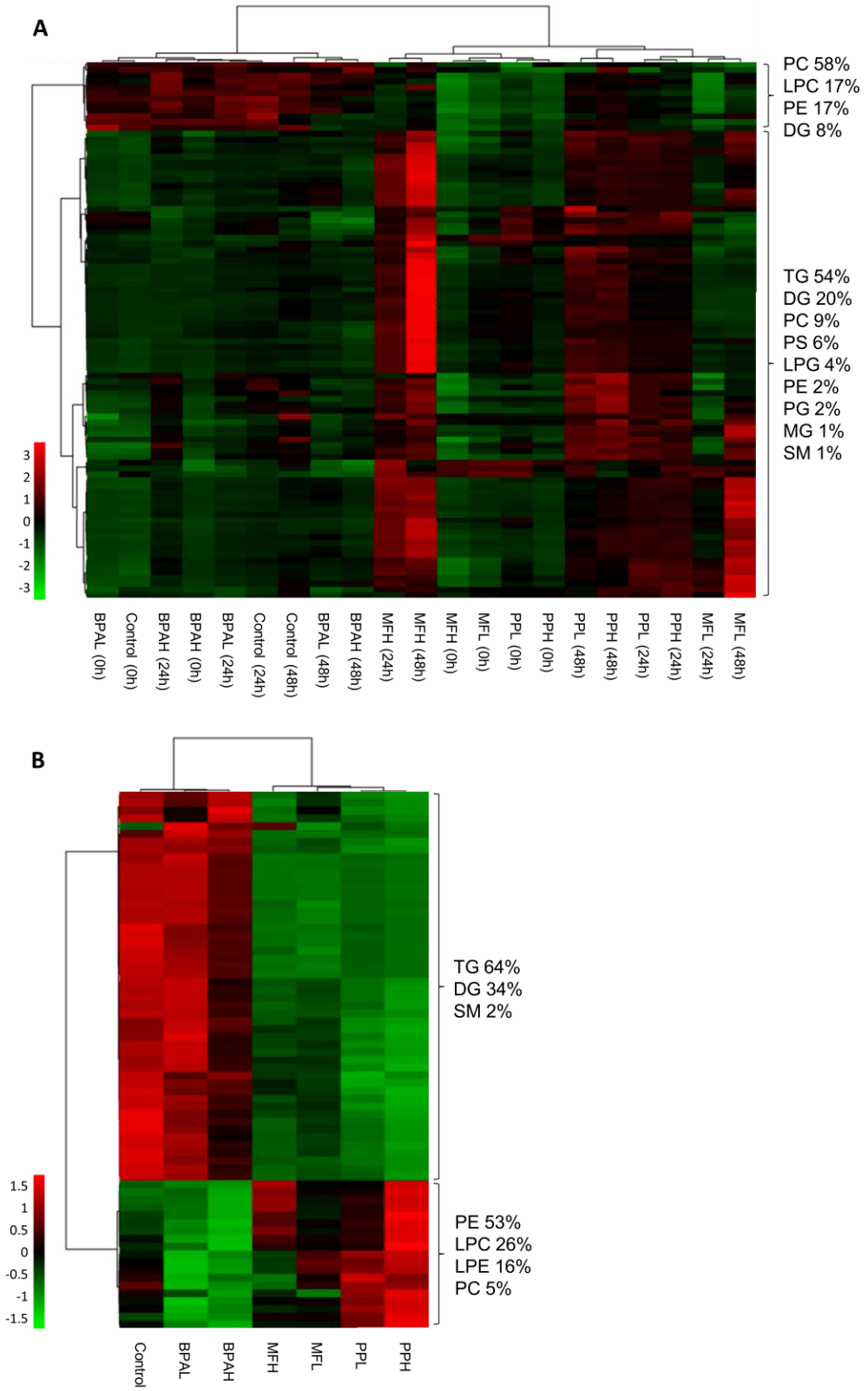

Fig. 3. Heatmap of the clustering results (Pearson's distance measure, and Ward's clustering algorithm) obtained using individual lipids with VIP scores greater than 1 after PLS-DA analysis, from females (A) and eggs (B). Group averages of three replicates per condition are shown.

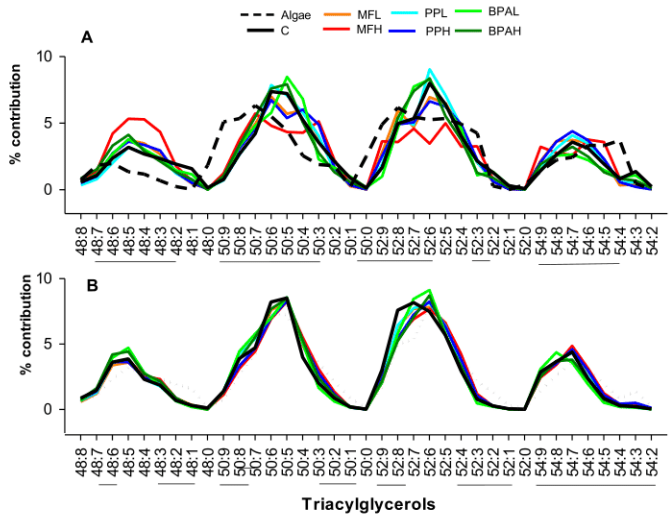

Fig. 4. Relative profiles of triacylglycerols (TGs) in females exposed to the selected chemicals for 48 h and the algae used as a food source (A) and in eggs (B). In graph B, the dotted line corresponds to 48-h control adult females. In each graph, TGs showing significant ($p < 0.05$) differences following ANOVA across treatments are underlined.

unsaturated fatty acids, which are likely to be present in low unsaturated TGs, are preferentially used as a source of energy in crustaceans (Brett et al., 2006), whereas highly unsaturated ones are used as a source of fatty acids for growth and reproduction (Ginjupalli et al., 2015).

5. Conclusion

In summary, the observed lipidomic profiles in *D. magna* adult individuals confirm previous results indicating that during a reproductive cycle, the levels of glycerolipids and glycerophospholipids increase to be invested into reproduction and the formation of the new carapace. The composition of *D. magna* TGs differed only slightly from that of the algae, and the TGs of eggs contained more unsaturated fatty acids. This indicates that *D. magna* incorporates fatty acids from the diet into its TGs and invests more polyunsaturated fatty acids to the eggs. The juvenoid compounds promoted the accumulation of TGs both directly from the acylation of fatty acids obtained from the diet, and indirectly from the metabolism of glycerophospholipids. The juvenoids reduced fecundity and consequently the investment of glycerolipids and glycerophospholipids towards reproduction. Consequently, females exposed to the juvenoids accumulated large amounts of glycerolipids into lipid droplets after reproduction. Bisphenol A impaired the transfer of TGs to the eggs in a similar way as was reported for tributyltin (Jordão et al., 2015); thus, the excess TGs not invested into reproduction accumulated as lipid droplets in the females.

Conflicts of interest

The authors have declared that no competing interests exist.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.07.102.

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Supplementary Material: scientific article II

Allocation of glycerolipids and glycerophospholipids from adults to eggs in *Daphnia magna:* Perturbations by compounds that enhance lipid droplet accumulation.

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Environ. Pollut. 242.

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METHODS

Lipidomic analyses. Lipidomic analyses were performed following Jordão et al., (2016b) with minor modifications, with three replicates from each treatment and sampling period. Each replicate consisted of a pool of five *D. magna*, 40 eggs or 50 mg d.w of algae that were homogenized in 500 µL of phosphate-buffered saline (PBS), pH 7.4, with 0.01% BHT as an antioxidant. Adult homogenates were split in two: one used for UHPLC-TOF and the other for TLC analyses. Lipid extraction for UHPLC-TOF was performed with similar extraction conditions as described by Christie (Christie, 1985) with minor modifications. Briefly, 100 µL of the homogenized sample was mixed with chloroform:methanol (2:1, v/v). Internal standards (200 pmol) were also added (described in Supplemental Material, Table S1). Samples were shaken with a vortex and extracted in an ultrasonic bath. Afterwards, samples were heated at 48°C overnight and dried under N_2. Lipid extracts were reconstituted in 150 µL methanol and centrifuged at 10,000 g for 10 min. The supernatant was then transferred to a new micro vial and analyzed.

Lipidomic instrumental analyses were performed with an Acquity UHPLC system (Waters, USA) coupled to a Waters/LCT Premier XE time-of-flight (TOF) mass analyzer operated in positive and negative electro spray ionization (ESI) mode. The analytical separation was performed with an Acquity UPLC BEH C8 column (1.7 mm particle size, 10×2.1 mm, Waters, Ireland) at 30°C and a flow rate of 0.3 mL/min. Chromatographic conditions and MS parameters have been previously reported (Gorrochategui et al., 2014). Full-scan spectra from 50 to 1,800 Da were acquired, and individual spectra were summed to produce data points each of 0.2 sec. Mass accuracy at a resolving power of 10,000 and reproducibility were maintained by using an independent reference spray (Lock Spray Waters). Mobile phases used were A,

methanol:2 mM ammonium formiate:0.2% formic acid and B, water:2 mM ammonium formiate:0.2% formic acid.

Glycerolipids (MG, DG, TG) and some glycerophospholipids (PC, LPC, LPG, SM) were detected under ESI (+) as their ammonium and hydrogen adducts, respectively, while PE, LPE, PS and PG were detected under ESI (-) as their hydrogen adducts. Identification and relative quantification of lipids was carried out using the ion chromatogram obtained for each compound using 0.05 Da windows. Positive identification of lipids was based on the accurate mass measurement, with a minimum mass error (<5 mg/L) respect to the measured m/z ratio of the monoisotopic peak considering possible adducts, its relative retention time and correct isotopic distribution. The LipidMaps database and MassLynx software (Waters, USA) were used to identify individual lipids from specific exact masses. Relative quantification was done by comparison of peak areas in extracted ion chromatograms between expected lipids and its corresponding internal standards.

A total of 234 individual lipids were identified and quantified by UHPLC-TOF. These included eight classes of glycerophospholipids (phosphocholine, PC; lysophosphatidylcholine, LPC; phosphatidylethanolamine ,PE; lysophosphatidylethanolamine, LPE; phosphatidylglycerol, PG; lysophosphatidylglycerol, LPG; phosphatidylserine, PS), three of glycerolipids (monoacylglycerols, MG; diacylglycerols, DG; triacylglycerols, TG) and one of sphingolipids (sphingomyelins, SM). All were annotated as <lipid subclass> <total fatty acyl chain length>:<total number of unsaturated bonds>, except SM, that were annotated as <total fatty acyl chain length>:<total number of unsaturated bonds in the acyl chain>.

Daphnia lipid extraction for TLC analysis was performed following Folch et al., (1957). Briefly, 300 µL of the homogenized sample was mixed with 1 mL of chloroform:methanol (2:1, v/v) and 250 µL of potassium chloride (0.88%). Samples were centrifuged 10 min at 2500 rpm before phase separation, where the aqueous phase was discarded. The extraction was carried out twice. The organic fraction was dried under N_2. Lipid extracts were reconstituted in 500 µL chloroform:methanol (2:1, v/v) and dried under N_2 again. The dry lipid samples were left overnight in a desiccator and the amount of dry lipid weight was determined. Samples were reconstituted with chloroform:methanol (2:1, v/v) to the same concentration. 1.5 µL of each sample were placed in pre-coated HPTLC silica gel plates from Merck (Darmstadt, Germany). Thin layer chromatography lipid analysis was performed as describe by Olsen and

Henderson, (1989). Separated lipid classes were detected by spraying the plate with Fewster‘s die (3% cupric acetate in 8 % phosphoric acid). Quantitation was performed on a Kodak Gel Logic 200 imaging system and data obtained with freeware GelAnalyzer 2010, from which volume area corresponding to each lipid chromatographic spot was reported.

Chemical analyses. Duplicated water samples of freshly prepared and old (48 h) test solutions were collected at the beginning and end of the tests to determine that measure oxygen levels and pH were within the limit established by OECD guidelines (OECD, 1981) and to assess the stability of the tested compounds. Dissolved oxygen concentration (DO) was measured using an oxygen electrode model 1302 (Strathkelvin Instruments, Glasgow). pH was measured using an epoxy-body combination electrode, coupled to a Crison micro pH 2001 meter and calibrated with standard pH buffer solutions (Sigma, Madrid, Spain). In all test oxygen levels were within 92% of saturation and pH values varied between 7.5 and 8.1.

Duplicated water samples of low and high concentrations of each studied compounds were used to assess the stability of the compounds during exposures, which were confirmed using liquid chromatography-tandem mass spectrometry following the methods of Gómez et al., (2006) and using the Mass Spectrometry services of the IDAEA, CSIC (Barcelona). Detailed analytical methods and results are reported in Jordão et al., (2016b). In the previous study it was found that most studied compounds were stable in water except MF that at 48 h had concentrations 30% lower than nominal ones.

For the sake of clarity, results are referred to nominal vales.

TABLES

Table S1. Internal standards purchased from Avanti Polar Lipids.

Lipid class	Lipid name	Formula	Ionization
Glycerolipids	MG 17:0	C20H40O4	[M+NH4]+
	DG 1,3-17:0 D5	C37H67D5O5	[M+NH4]+
	TG 1,2,3-17:0	C54H104O6	[M+NH4]+
Glycerophospholipids	PC 16:0 D31-18:1	C42H51D31NO8P	[M+H]+
	LPC 17:0	C25H52NO7P	[M+H]+
	PE 16:0 D31-18:1	C39H45D31NO8P	[M-H]-
	LPE 17:1	C22H44NO7P	[M-H]-
	PS 16:0 D31-18:1	C40H44D31NO10PNa	[M-H]-
	PG 16:0 D31-18:1	C40H45D31O10PNa	[M-H]-
	LPG 17:1	C23H44O9PNa	[M+H]+
Sphingolipids	SM 12:0	C35H71N2O6P	[M+H]+

Table S2. Elemental composition of glycerophospholipids, glycerolipids and sphingolipids species found in *D. magna* lipid samples, calculated by mass accuracy within error of 5 ppm, with atom constraints and with -0.5 ≤ DBE ≤ 50.0 (DBE, double-bond equivalent). Elemental composition of neutral glycerolipids refer to their ammonium adducts detected under ESI (+), and elemental composition of glycerophospholipids and sphingolipids refer to their hydrogen adducts detected under ESI (+) for PC, LPC, LPG and SM, and under ESI (-) for PE, LPE, PS and PG.

This table is reported within the annexes section, annex I.

Table S3. Two way ANOVA results (Fisher's quotient: F, degrees of freedom: df) comparing the response of lipid classes across treatments along the adolescent instar including debrooded females just after releasing the first clutch of eggs. Results for eggs are also reported.

	Female						**Eggs**	
	time		treatment		interaction		treatment	
Lipid	F 2,41	p	F 6,41	p	F 12,41	p	F 6,14	p
TG	56.2	<0.001	14.6	<0.001	2.6	<0.001	28.0	<0.001
DG	32.0	<0.001	5.1	<0.001	1.8	0.072	100.9	<0.001
PC	77.7	<0.001	7.1	<0.001	2.4	<0.001	13.4	<0.001
LPC	34.7	<0.001	17.4	<0.001	5.1	<0.001	40.8	<0.001
PE	63.4	<0.001	13.6	<0.001	2.4	<0.001	17.0	<0.001
LPE	49.9	<0.001	22.3	<0.001	5.4	<0.001	122.3	<0.001
SM	94.3	<0.001	2.3	<0.001	2.7	<0.001	2.4	0.086
CHL	0.2	0.825	1.9	0.123	3.7	<0.001		
MG	0.1	0.951	1.5	0.204	1.3	0.236		
PS	28.0	<0.001	4.9	<0.001	1.1	0.359		
PG	70.6	<0.001	8.7	<0.001	5.5	<0.001		
LPG	1.1	0.328	9.2	<0.001	0.7	0.701		

Table S4. Individual lipids shown in Figure 4, obtained after PLS-DA analysis of all individual lipids in MetaboAnalyst. Only lipids with a VIP score higher than 1 are depicted. Lipids are given in the table in the same order that is represented in Figure 4.

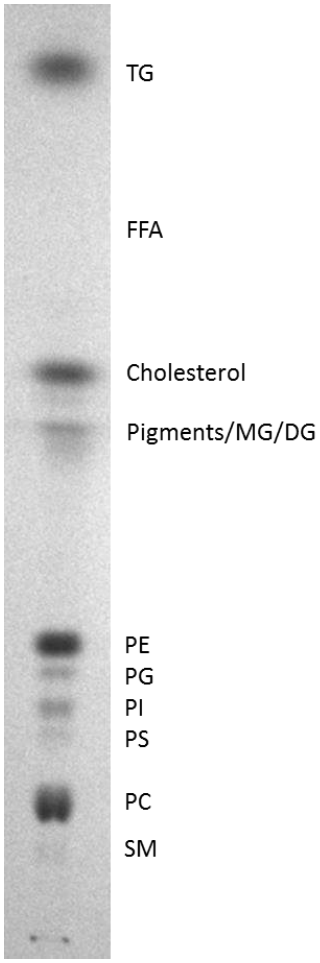
Exposed *Daphnias*		**Eggs**	
Lipid	VIP score	Lipid	VIP score
LPC 14:1	1.6225	TG 48:1	1.1156
PC 28:2	1.2753	TG 54:1	1.0681
PC 30:2	1.3170	TG 50:0	1.0048
PC 40:10	1.1737	TG 52:0	1.0003
DG 42:11	1.0211	SM16:0	1.0726
PC 28:1	1.6032	DG 32:1	1.2646
PE 30:1	1.5335	TG 50:1	1.2663
PC 30:1	1.4532	TG 52:2	1.2454
PE 32:1	1.3676	DG 36:6	1.2080
PC 30:0	1.3157	DG 34:0	1.1772
PC 28:0	1.3861	DG 34:5	1.1973
LPC 14:0	1.2775	DG 34:4	1.1804
DG 36:2	1.6900	DG 36:2	1.2073
DG 34:3	1.2653	DG 32:3	1.1955
DG 36:3	1.3137	DG 34:3	1.1731
DG 36:6	1.1137	DG 34:1	1.2136
TG 52:3	1.5713	DG 36:4	1.1820
TG 44:0	1.3523	DG 32:5	1.0498
TG 46:0	1.5405	DG 32:0	1.1107
TG 52:2	1.5836	DG 32:2	1.1008
TG 50:1	1.3739	DG 34:2	1.1281
TG 42:0	1.0726	DG 32:4	1.1266
TG 46:1	1.0281	DG 36:5	1.1635
TG 50:2	1.1368	DG 34:6	1.1614
TG 48:1	1.1335	TG 48:6	1.1793
DG 38:3	2.0530	TG 52:6	1.2109
MG 20:0	2.0348	TG 48:5	1.1934
DG 34:0	1.6255	TG 50:7	1.2174
DG 36:0	1.7156	TG 48:7	1.2036
DG 38:2	1.7096	TG 48:2	1.2955
TG 60:5	1.6058	TG 50:2	1.2858
TG 60:4	1.1058	TG 50:4	1.2584
DG 40:0	1.5646	TG 48:3	1.2470
DG 38:1	1.3873	TG 48:4	1.2193
DG 36:1	1.1605	TG 50:3	1.2622
TG 56:4	1.4283	TG 52:4	1.2179
TG 56:3	1.4255	TG 54:4	1.1290
TG 54:1	1.3250	TG 52:3	1.2206
TG 56:2	1.3214	TG 54:5	1.1219
TG 54:0	1.2703	TG 52:5	1.1877

TG 58:3	1.4472
TG 58:1	1.4226
TG 56:1	1.4446
TG 56:0	1.4634
TG 58:2	1.3766
TG 52:0	1.4075
TG 48:0	1.3424
TG 50:0	1.2560
TG 54:4	1.7447
TG 52:1	1.3956
TG 56:5	1.5870
TG 54:2	1.5190
TG 54:3	1.7263
TG 58:4	1.554
SM 24:0	1.0810
PS 36:2	1.2500
PC 38:3	1.2373
PG 36:6	1.1130
PS 38:2	2.0319
PS 40:2	1.5487
PS 38:1	1.3328
PC 32:5	1.1886
PS 38:3	2.1621
PC 36:5	1.7117
PC 36:6	1.5113
PG 44:10	1.0024
PC 34:6	1.6324
PC 34:5	1.6308
PC 36:4	1.4514
LPG 20:1	1.3629
LPG 18:1	1.6119
LPG 14:1	1.5438
TG 48:6	1.2483
TG 46:5	1.0581
TG 48:4	1.0879
TG 46:3	1.0632
TG 50:5	1.0710
TG 50:6	1.2284
TG 52:6	1.1164
TG 54:5	1.4805
TG 54:6	1.4625
TG 52:4	1.4505
TG 50:3	1.2933
TG 52:5	1.3605
TG 50:4	1.3130
TG 48:3	1.1869
TG 54:6	1.1810
TG 54:7	1.0988
TG 52:7	1.0722
TG 54:9	1.0393
TG 54:8	1.0774
TG 50:5	1.1517
TG 50:6	1.0682
TG 48:8	1.1428
TG 50:9	1.1398
TG 50:8	1.1268
PE 36:4	1.0783
PE 34:2	1.1189
PE 34:1	1.1309
PE 36:3	1.1159
PE 34:3	1.1695
PE 36:5	1.2787
PE 36:2	1.1896
PC 38:6	1.2597
PE 36:6	1.2032
LPC 18:3	1.3726
LPC 18:2	1.3647
LPC 16:0	1.3278
LPC 16:1	1.0905
LPC 18:1	1.0736
PE 34:4	1.0184
LPE 18:2	1.3839
LPE 18:3	1.3228
LPE 16:0	1.3451
PE 32:2	1.2144
PE 32:2	1.2144

DG 34:4	1.0451
DG 32:3	1.2805
DG 30:3	1.2253
DG 34:5	1.3863
DG 32:4	1.0978
PE 38:8	1.8218
PE 38:7	1.2907

FIGURES

Figure S1. Image of a TLC lane, in which the identification of each spot corresponding to a different lipid class is indicated.



2.3. Further discussion and final remarks

Despite the large number of reported results pointing the harmful effects of EDCs, there is still a great demand for the development of frameworks that can integrate mechanistic and toxicological observations (Maradonna and Carnevali, 2018). It has been already stated that EDCs have been reported to interfere with the endocrine (or hormonal) system inappropriately, stimulating adipogenesis as well as perturbing lipid metabolism and energy balance (Maradonna and Carnevali, 2018; Street et al., 2018). Because of this, within this chapter an integrative approach has been presented linking EDCs reproductive effects with gene expression (transcriptomics) and the subsequent lipid metabolism disruption (lipidomics) in *D. magna*.

In the scientific article I, gene transcription analyses of *D. magna* samples exposed to BPA, PP and TBT were performed. These compounds were previously tested to increase the accumulation of storage lipids in *D. magna* lipid droplets by Nile red staining assays (Jordão et al., 2016b). Times at which the greatest changes in lipid droplets of unexposed animals occurred (8 h and 24 h of exposure) were selected for transcriptomic analysis.

Effects on the gene expression of these EDCs were assessed by means of microarray transcriptomic analysis. Microarrays, although being a high throughput technique, are targeted transcriptomic approaches since only the gene transcription regarding to the genes which are complementary to the spotted probes is determined. Within this study, a custom-made microarray previously developed in my research group was used. This microarray platform was designed from the complete set of gene models representing the whole transcribed genome of *D. magna* (Campos et al., 2018). This custom-made microarray, as well as the genome of *D. magna*, has only the 50% of its genes functionally annotated. For this reason, the obtained DEGs were compared to *Drosophila melanogaster* homologous ones of known function and related to effects observed on storage lipid accumulation and reproduction.

BPA, PP and TBT seemed to affect similarly a total of 1,388 genes: 965 over-represented (cluster A) and 423 under-represented DEGs (cluster C) after 24 h of exposure. This DEGs similarity after the exposure to three compounds with no structural similarities and only related because of their common ability to promote lipid droplet accumulation, suggested a common mechanism. In addition, TBT showed 225 specific DEGs over-expressed after both 8 h and 24 h (cluster B). Functional analysis

of DEGs using *Drosophila* functional annotation identified different KEGG modules enriched for each group of genes. Cluster A was the one showing more enriched categories related with lipid related pathways. In contrast, cluster C was enriched with pathways related to visual perception and oocyte development signaling pathways. Many TBT specifically over-expressed genes were related to neuroactive ligand receptor interaction signaling pathways, including four receptors involved in neurotransmitter and neuropeptide signaling. Figure 2.1 represents the lipid related GO and KEGG categories extracted from Figure 3 in article I. As pointed before, cluster A (i.e. genes over-represented after 24 h in the three treatments) was the one showing higher number of genes involved in the obtained enriched categories, as lipid catabolic process, triglyceride homeostasis, fatty acid beta-oxidation and glycolipid biosynthetic process, as well as low-density lipoprotein receptor class A. Lipoproteins are the primary mediators of cholesterol and lipid transport, composed of a hydrophobic core where TG and CE are stored and a hydrophilic shell composed of phospholipids, cholesterol, and amphipathic apolipoproteins (Delk et al., 2020). Thus, the obtained enriched lipid categories pointed that lipid homeostasis was disrupted after exposure to the three reported EDCs. Other affected pathways were related to energy metabolism, molting, development, reproduction and life-span functions.

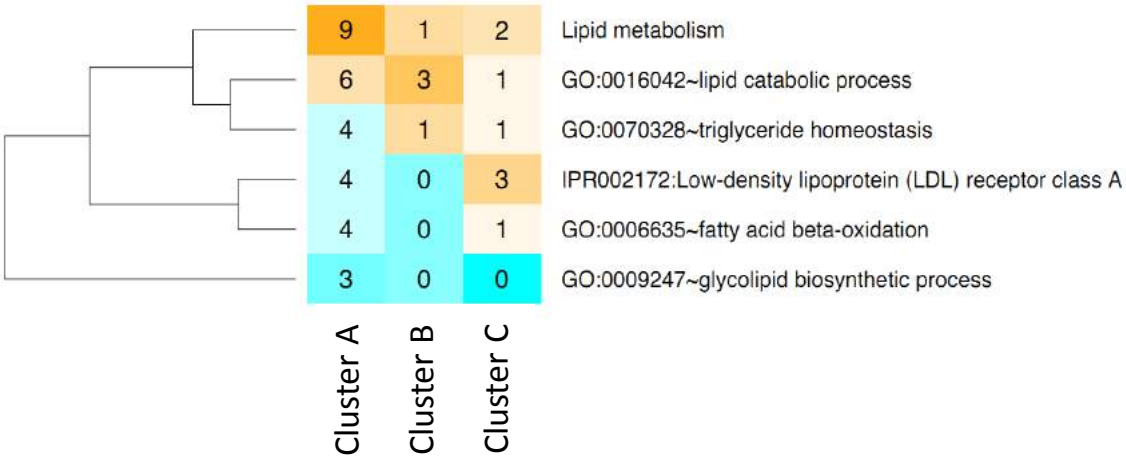


Figure 2.1. Detailed view of GO and KEGG pathways directly related with lipid metabolism, obtained from Figure 3, article I. Only categories with significant enrichment in at least one of the treatments are shown. Colors correspond to the relative importance of each cluster in the total distribution of the genes belonging to each category. Orange and cyan correspond to genes over- or under-represented in each particular cluster, where the actual number of hits for each cluster and categories is indicated with black numbers.

Ultraspiracle (UPS), also known as RXR in *Daphnia*, and the nuclear HR96 receptor were under-expressed upon exposure to the studied compounds after 24 h. *Daphnia* RXR heterodimerizes with EcR (André et al., 2014; Lenaerts et al., 2019) and probably with MfR (Kakaley et al., 2017; LeBlanc et al., 2013; Miyakawa et al., 2013), being the regulatory complex that modulates storage lipid metabolism (Jordão et al., 2016a). In addition, the HR96 nuclear hormone receptor can be involved in lipid homeostasis in *Daphnia* and may be modulated by FA and certain contaminants (Karimullina et al., 2012; Sengupta et al., 2016). There is reported evidence that TBT, and probably juvenoids, can act as ligands of RXR in *Daphnia* (Jordão et al., 2016a; Wang et al., 2007; Wang and LeBlanc, 2009) and some FA and PP of HR96 (Karimullina et al., 2012). Both transcription factors involved in lipid homeostasis were under-expressed after 24 h of exposure of the three compounds, what suggest a probable lipid disruption.

In order to test the observed transcriptomic effects of the tested compounds related to lipid homeostasis, a lipidomic strategy was performed in the scientific article II. A suspected screening targeted approach was applied in order to assess the effects of BPA and the two juvenoids (MF and PP) at different dosses on *D. magna* females' lipidome during their first reproductive cycle and of their eggs, and also to determine the TG profile of the algae used to feed the experimental *D. magna* individuals. In this case, the effect produced by TBT in the lipidome was not tested as it was previously reported in a previous publication (Jordão et al., 2015). To the previously tested exposure times in scientific article I (0 time or 8 h and 24 h of exposure), 48 h of exposure was also studied in order to assess the EDCs effects in the entire *Daphnia's* reproductive cycle.

Lipidomic analyses were performed by means of RPLC coupled to a TOF MS previously optimized in other study (Gorrochategui et al., 2014). Lipidomic results were validated by means of TLC. Results from both techniques were significantly correlated, although in general terms TLC underestimated the TGs and overestimated the PCs. However, there is also the possibility that TOF MS approach overestimated TGs and underestimated PCs. Nevertheless, equivalent abundance values were obtained for PE, PG, PS, and SM comparing results from both techniques, but with TLC it was no possible to determine DGs or MGs that coelute in a single spot together with pigments. On the other hand, with TLC it was possible to determine the amount of cholesterol that cannot be measured with the UHPLC-TOF method. Despite being a quick technique to qualitatively characterize changes in lipid families, this method shows higher matrix effects than MS-based lipidomic approaches due to its low selectivity, as well as it is

less sensible and accurate, producing the under- or overestimation of some lipid families. However, TLC represents an effective method to validate high throughput lipidomic data and also to determine the integrity of lipid samples, since if lipids have been degraded a big FAs' spot can be observed. Lipidomic MS-based applied methodology allow the identification of 234 individual lipids. One of its limitations is that it does not yield MS/MS spectra information, and thus it is not possible to know the exact identity of the lipid. The lipid category and its functional group, number of carbons and number of unsaturations are determined, but not the position of the FA acyl chains within the functional group or the position of the determined unsaturations. Sengupta et al., (2016) performed a QqQ MS-based lipidomic study and reported that PCs represent about 75% of glycerophospholipids in *D. magna* juveniles. In contrast, in article II PCs represented just a 45% of the glycerophospholipids with similar levels of both PC and PE. This suggests that improvements can be made in this lipidomic method to be able to determine a greater number of individual lipids within each family. Despite this, UHPLC-TOF MS method is an effective technique to obtain lipidomic profiles composed of a wide range of lipids, and thus offers a powerful tool in supplying a global map of the lipidome response after exposure to contaminants.

Given the large number of samples reported in the article II, in the clustering representation reported in Figure 3 within the referred publication, the group average of three replicates per condition was shown and not the individual samples. As a proof of the good reproducibility of the results and the little variability within each treatment, Figure 2.2 represents a dendogram of the females and egg triplicate samples where hardly any dispersion and a good sample grouping can be observed.

Article II reported the effects of BPA and MF, PP at two different concentrations each (i.e. low (L) and high (H)). Both juvenoids, MF and PP, reduced dramatically fecundity, which is in line with other studies (Olmstead and Leblanc, 2002; Olmstead and LeBlanc, 2003; Wang et al., 2005). The studied compounds perturbed lipid profiles that are crucial in reproduction and development, enhancing the accumulation of glycerolipids into lipid droplets in reproductive active *D. magna* females and confirming previous results (Jordão et al., 2016b). Lipidomic analyses showed lipid dynamics during *Daphnia'*s reproductive cycle, reflecting changes in storage lipids (TG, DG and cholesterol (CHL)), glycerophospholipids and SMs (Goulden and Place, 1990; Sengupta et al., 2016; Tessier et al., 1983). Reproductively active *Daphnia* females accumulate large quantities of storage lipids and glycerophospholipids ingested from food into lipid droplets and cell membranes. High amounts of storage lipids and

glycerophospholipids are allocated to the eggs and glycerophospholipids are additionally used in the formation of the new adult carapace.

Lipidomic analyses of female *D. magna* showed major effects of both MF and PP, promoting the accumulation of TGs and differential effects on DGs and glycerophospholipids. Larger levels of TGs in females exposed to the higher concentration in MF, coupled with the low levels of DG, PC, PE, LPC, and LPE, may indicate that an important amount of glycerophospholipids and DGs were used to synthesize TGs, whereas enhanced levels of TGs in PP female exposures, but not of DGs or PCs and PEs, probably means that TGs were synthesized directly from the acylation of fatty acids (Arrese and Soulages, 2010; Pol et al., 2014). Glycerolipids invested in reproduction were the lowest in females exposed to juvenoids, which was linked with the reported decreased fecundity produced by these compounds. This is in line with literature, that described juvenoids to reduce fecundity in *Daphnia* (LeBlanc and Medlock, 2015; Olmstead and LeBlanc, 2003). The studied juvenoid compounds also promoted the accumulation of TGs not invested in reproduction in post-reproductive females, which can be advantageous for survival under adverse conditions. The lipidomic profile of females exposed to BPA showed lesser effects than the other compounds, differing from MF and PP in the levels of LPC and LPE, which decreased with the exposure time. This could be explained by the inhibition of lysophospholipases that hydrolyzed glycerophospholipids to their corresponding lysophospholipids (e.g. PC to LPC or PE to LPE) (Kabarowski, 2009; Kohlmeier, 2015). This type of effects has been previously reported for aromatic phosphate compounds although not in BPA (Jiang et al., 2011). This inhibitory effect should have increased PC and PE levels, but this effect was not observed in our study. This could be probably due to the fact that PC and PE are cross-linked with many other metabolic pathways and reactions that may be compensating that effect. BPA did not disrupt fecundity, although it caused smaller investment of TGs into eggs, which was also reported in previous studies with TBT (Jordão et al., 2015). Lower lipid investment in eggs can have detrimental health effects in the progeny (Gliwicz and Guisande, 1992; Wacker and Martin-Creuzburg, 2007). This similar effect between TBT and BPA could point that both EDCs act throughout similar mechanisms of action, disrupting the RXR signaling. This mechanism has been previously reported after TBT exposure in *Daphnia* (Jordão et al., 2015) and is pruposed as one of the mechanism by which BPA produces obesity in mammals (Boucher et al., 2014).

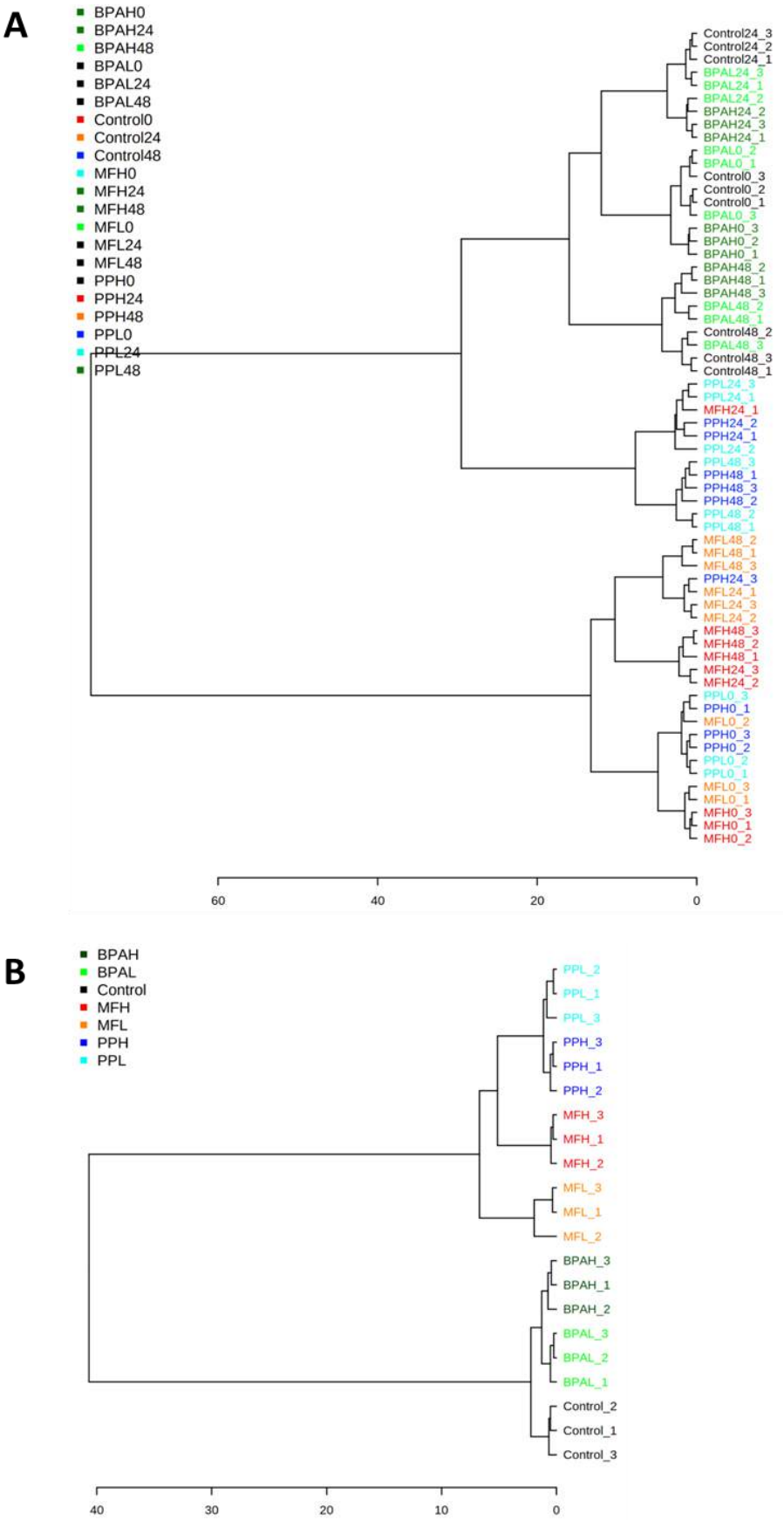


Figure 2.2. Dendogram of female (A) and egg (B) triplicate samples performed using Pearson Pearson's distance measure and Ward's clustering algorithm.

Many studies so far have been carried out to reveal the role of dietary lipid intake as a source of essential FAs governing energy balance, food intake, growth, reproduction and health (Maradonna and Carnevali, 2018). Relationship between TG profiles in algae used as *Daphnia*'s food and the females were observed, pointing that females incorporte FAs from the diet into its TGs and invest more polyunsaturated FAs to the eggs. In fact, individual lipids represented in Figure 3 (from scientific article II) and belonging to TG family, have all between 5 and 9 unsaturations. Previous studies also reported that there is a close link between the FA composition of algae and that of adults and *Daphnia*'s eggs (Brett et al., 2006; Goulden and Place, 1990; Wacker and Martin-Creuzburg, 2007), which agrees with reported results in article II. It has been reported that unsaturated FA present in TGs are preferentially used as a source of energy in crustaceans (Brett et al., 2006), whereas the highest unsaturated ones are used for growth and reproduction and are enriched in eggs (Ginjupalli et al., 2015). This is also in line with the reported evidence by Wacker and Martin-Creuzburg pointing that *Daphnia* allocates high amounts of long-chain polyunsaturated FA to eggs (Wacker and Martin-Creuzburg, 2007). In summary, lipidome analyses showed low levels of TGs and higher of glycerophospholipids in females compared to control treatments, whereas those in the juvenoid treatments had higher levels of TGs and low levels of glycerophospholipids. In contrast, the opposite trend was observed in the produced eggs.

Regarding to the global effect in *Daphnia*'s lipidome, the effect produced by juvenoids compounds seems to be higher, clustering BPA samples with control ones at different exposure times with only differences in some specific lipid families. In multivariate analysis the large differences observed between some treatments can mask more subtle differences. As a proof of principle, Figure 2.3 show the PLS-DA analysis of control and BPA samples at both tested concentrations after 48h of exposure, explaining the variation of individual lipid across BPA treatments and showing that despite producing lesser effects than juvenoids, BPA exposures also disrupt *Daphnia*'s lipid homeostasis.

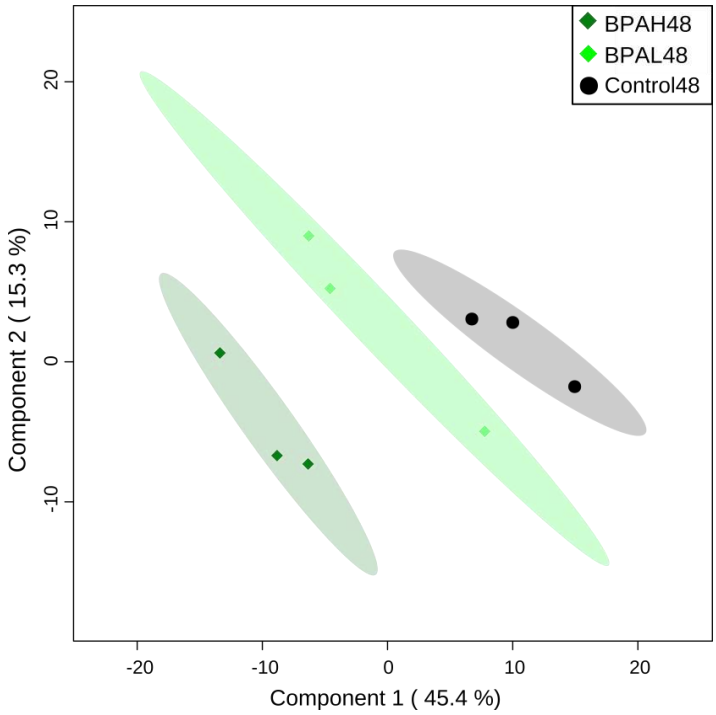


Figure 2.3. Partial least square discriminant analysis (PLS-DA) of control samples and BPAL and BPAH samples after 48 h of exposure. The two obtained components explained 60.7% of the total variance.

When article II was published, the bioinformatic tools necessary to perform an enrichment analysis with the information provided by the applied lipidomic technique were not available. One year later, lipid ontology (LION) enrichment analysis bioinformatic tool was developed (Molenaar et al., 2019). This tool enables to search for enriched LION terms in a lipidomic subset, containing detailed lipid classification based on LipidMaps, biophysical data, lipid functions and/or organelle associations. In order to clarify the reported lipidomic results within scientific article II, enrichment analysis of the obtained data has been performed. Enrichment analyses have been carried out for significant lipids obtained from PLS-DA analysis and represented in Figure 3 from the article II. Each significant lipid was assigned to a cluster (corresponding to those obtained from the hierarchical clustering analysis) and an enrichment analysis was performed per lipid cluster using the whole data set as background. Enrichment significance was set at P-value<0.05. All data manipulation and statistical analysis were performed using homemade scripts in R. Network and heatmap graphs regarding to enrichment results were obtained using packages gplots, reshape2 and igraph R packages.

Obtained results for both females and eggs samples are shown in Figure 2.4 and 2.5, respectively, and complete enrichment results are displayed in Table 2.2. The obtained results were in line with the conclusions obtained from the clustering analysis within the published paper. In female samples, lipid cluster 1 corresponds to enriched categories mainly of glycerophospholipids (PCs, PEs, LPCs, and LPEs), meanwhile lipid cluster 2 was enriched with glycerolipids, mainly TGs (concomitant with the enrichment of lipid storage and lipid droplet LION categories) and of membrane components (mainly DGs and glycerophospholipids). Endoplasmic reticulum (ER) LION category was enriched in both clusters, represented by mainly glycerophospholipids. ER is involved in the synthesis of proteins and *de novo* synthesis of lipids (Balla et al., 2020), which may be associated with vitellogenin, a key component of *D. magna* eggs (Kato et al., 2004). More LION categories appeared enriched in egg samples. Lipid cluster 1 within the published heatmap appeared particularly enriched with different glycerolipids, performing functions as lipid droplet and storage (TGs), lipid mediated signaling (DGs) and membrane component (DGs and SM). In contrast, lipid cluster 2 appeared enriched in membrane component lipids (PEs, LPCs and LPEs), glycerolipids (PEs, LPCs and LPEs), that belong also to the endoplasmic reticulum category, and lysophospholipids (LPCs and LPEs).

Most of the glycerophospholipid categories (in this case PC, PE and PG) included in the lipid cluster 2 (Figure 2.4) contained polyunsaturated FAs, with acyl chains with between 3 and 10 unsaturations. This cluster has lipids whose concentrations increased in juvenoid samples and decreased in control and BPA samples. An alteration of the composition of the lipids conforming the cellular membranes can have harmful consequences, since polyunsaturated phospholipids confer higher melting point, intrinsic curvature and fluidity to the membranes than saturated phospholipids, altering their structure and functionality (Hashimoto and Hossain, 2018). An increase in the degree of acyl chain saturation can be explained by substrate preferences of the glycerophospholipid biosynthetic enzymes and accumulation of acyl-CoA intermediates affecting upstream reactions (de Kroon et al., 2013).

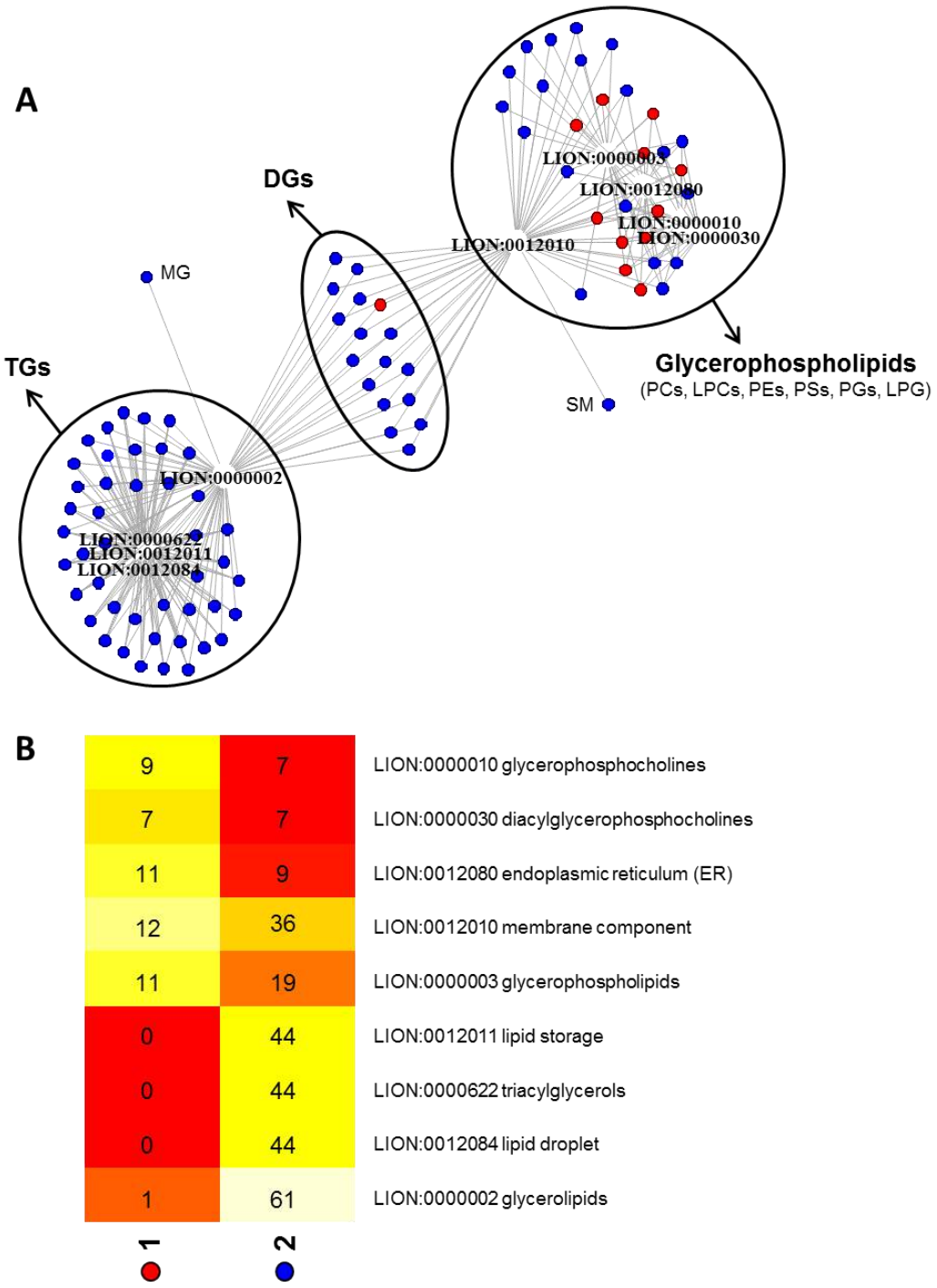


Figure 2.4. Enriched Lipid Ontology (LION) terms for *D. magna* female samples, distributed in clusters. (A) Network representation of significant lipids obtained from PLS-DA (VIP score higher than 1) according to their adscription to functional modules (LION ontology description for each term are given as nodes). Only categories with at least five total hits and a p-value<0.05 are represented, and general and redundant modules were omitted. Lipids are represented by dots, colored by clusters obtained from heatmap in Figure 3A within the published article: red (cluster 1) and blue (cluster 2). (B) Distribution of significant lipids among the different LION categories (row s) and clusters (columns). Numbers indicate the absolute number of lipids for each module and clusters, and colors represent the relative importance of lipids associated with each term for each cluster (heat code, from red (few) to white (most)). Specific represented enrichment results obtained per cluster with their P-values and FDR values are provided in Table 2.2.

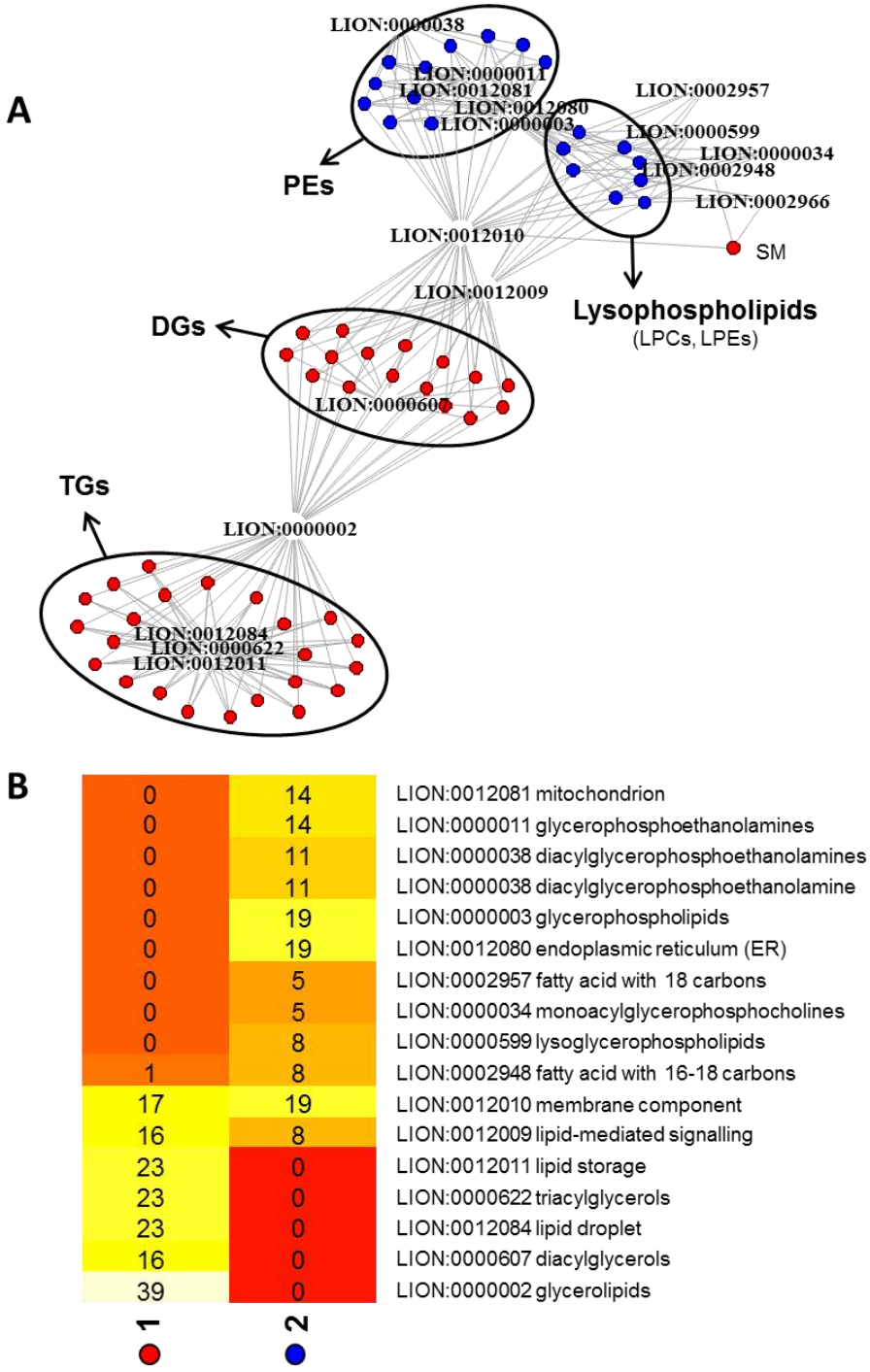


Figure 2.5. Enriched Lipid Ontology (LION) terms for egg samples, distributed in clusters. (A) Network representation of significant lipids obtained from PLS-DA (VIP score higher than 1) according to their adscription to functional modules (LION ontology description for each term are given as nodes). Only categories with at least five total hits and a p-value<0.05 are represented, and general and redundant modules were omitted. Lipids are represented by dots, colored by clusters obtained from heatmap in Figure 3B within the published article: red (cluster 1) and blue (cluster 2). (B) Distribution of significant lipids among the different LION categories (row s) and clusters (columns). Numbers indicate the absolute number of lipids for each module and clusters, and colors represent the relative importance of lipids associated with each term for each cluster (heat code, from red (few) to white (most)). Specific represented enrichment results obtained per cluster with their P-values and FDR values are provided in Table 2.2.

Table 2.2. Specific represented enrichment results obtained per cluster with the number of lipids annotated, significant and expected per category, and their P-values and FDR values. Only enrichment results with FDR<0.05 are shown. FDR: false discovery rate; LION: lipid ontology

LION: Term	Description	Annotated	Significant	Expected	P-value	FDR
			Females			
			Cluster 1			
0012080	endoplasmic reticulum (ER)	20	11	2.58	3.00E-08	1.41E-06
0000010	glycerophospho-cholines	16	9	2.06	2.10E-06	3.29E-05
0000003	glycerophospholipids	30	11	3.87	8.50E-06	9.99E-05
0012010	membrane component	48	12	6.19	0.00017	1.33E-03
0000030	diacylglycerophospho-cholines	14	7	1.81	0.0002	1.34E-03
			Cluster 2			
0000002	glycerolipids	62	61	54	1.30E-05	0.000447
0000622	triacylglycerols	44	44	38.32	0.00022	0.002068
0012011	lipid storage	44	44	38.32	0.00022	0.002068
0012084	lipid droplet	44	44	38.32	0.00022	0.002068
			Eggs			
			Cluster 1			
0000622	triacylglycerols	23	23	15.59	6.20E-06	5.11E-05
0012011	lipid storage	23	23	15.59	6.20E-06	5.11E-05
0012084	lipid droplet	23	23	15.59	6.20E-06	5.11E-05
0000607	diacylglycerols	16	16	10.85	0.00057	3.76E-03
			Cluster 2			
0000003	glycerophospholipids	19	19	6.12	7.20E-16	1.19E-14
0012080	endoplasmic reticulum (ER)	19	19	6.12	7.20E-16	1.19E-14
0000011	glycerophospho-ethanolamines	14	14	4.51	8.70E-10	5.74E-09
0012081	mitochondrion	14	14	4.51	8.70E-10	5.74E-09
0000038	diacylglycerophospho-ethanolamines	11	11	3.54	2.70E-07	1.48E-06
0012010	membrane component	36	19	11.59	6.20E-06	2.92E-05
0000599	Lysoglycero-phospholipids	8	8	2.58	3.40E-05	1.12E-04
0002948	fatty acid with 16-18 carbons	9	8	2.9	0.00025	6.87E-04
0000034	monoacylglycero-phosphocholines	5	5	1.61	0.00232	4.25E-03
0002957	fatty acid with 18 carbons	5	5	1.61	0.00232	4.25E-03
0002967	polyunsaturated fatty acid	4	4	1.29	0.00852	1.34E-02
0000042	monoacylglycerophos-phoethanolamines	3	3	0.97	0.02981	4.47E-02
0002966	fatty acid with less than 2 double bonds	5	4	1.61	0.03329	4.78E-02

Results obtained from both articles point some facts related to the way in which BPA, MF, PP and TBT affect molecular processes associated to lipid metabolism and homeostasis, and how their exposure disrupt the dynamics of some lipid classes in *Daphnia* adult females and their allocation to eggs. However, in some cases relating the activity of an enzyme to the subsequent effect on a particular category of lipids is complicated due to the high number of interconnections and cross-molecular processes of lipids. The specific expression of some DEGs related to lipid metabolic pathways or lipid related proteins (Figure 2.6) can be linked to some of the observe effects in the lipidome. Some DEGs were involved in the *de novo* synthesis of neutral lipids: LPGAT, CG6567, CG4729, CG6847, CG5966, Thiolase, CG8552, LP4 or Mag. The activity of some of them can be related to the reported lipid disruptions. CG6567 is a lysophospholipase that hydrolizes LPC and its expression can explain the reported BPA effect in lysophospholipids profile. This gene was under-expressed after 8h of exposures, producing an increase in LPC, and returned to basal levels at 24 h, meaning that LPC decreased till normal levels similar to those of control *Daphnias*. CG5966 and CG6847 are two triacylglycerol lipases. Both gene expression decreased in PP exposures after 8 h, which produced an increase in TG levels. In contrast, CG5966 was over-expressed in TBT samples after 8 h, and both CG5966 and CG6847 after 24 h, explaining the lower TG levels than controls during the first hours of the intermolt period at that concentration reported by Jordão (Jordão et al., 2015) prior to the produced TG increase after 48 h. Lip4 (or Mag) is also a triacylglycerol lipase and thus is involved in TG degradation. Lip4 expression increased a lot after 24 h in the three tested EDCs. This gene is involved in the maintenance of cholesterol and TG homeostasis in *Drosophila* (Sieber and Thummel, 2012). The increased expression of Lip4 at 24 h can explain reported decreased TG levels in TBT *D. magna* (Jordão et al., 2015), and the increased level of cholesterol by PP. Lip4 has been reported as a direct target for the *Drosophila* and *Daphnia* HR96 nuclear receptors (Sengupta et al., 2017; Sieber and Thummel, 2012), whose transcription was also disrupted in exposures after 24 h. Thiolase, an enzyme with phospholipid transporter and oxidoreductase activity, was overexpressed at 24 h for all the studied compounds. This gene has been reported to belong to the PPAR signaling pathway in vertebrates (Ahmad et al., 2013; Chamouton et al., 2010; Stern et al., 1953). Effects of EDCs in vertebrates have been mostly related to the activation of the RXR and the PPAR, key regulators in several processes related with lipid metabolism and homeostasis (Lempradl et al., 2015; Yang et al., 2015). CG8552 is a phospholipase that hydrolyze the reaction from PC to LPC. The transcription of this gene diminished after 24 h of PP and TBT exposures. Lower

levels of LPC than control samples and to a lesser extent higher levels of PC were observed in PP samples after 24 h, in line with the observed transcriptomic effect.

Other genes that belong to glycerophospholipid pathways were differentially expressed, as CdsA that belongs to the Lands lipid pathway, or Pect, from the Kennedy pathway. CdsA in involved in DG biosynthesis (Blunsom et al., 2018), whose transcription decreased for all treatments at 24 h. DG appeared also disrupted in every treatment at that exposure time, particularly increased by TBT exposures (Jordão et al., 2015). In reported lipid cluster 2 of females' samples (Figure 2.4), results point enrichment of glycerolipids and membrane component LION categories, in which DG are involved, indicating a disruption of the homeostasis of this lipid family. Pect is a phosphoethanolamine cytidylyltransferase and is the main regulatory enzyme in *de novo* biosynthesis of PE from ethanolamine and diacylglycerol by the Kennedy pathway (Pavlovic and Bakovic, 2013). Pect transcriptional levels increased in BPA and TBT samples after 24 h, although the expected lipidomic effects were not observed. In contrast, the mRNA abundance of Pect decreased in PP and TBT samples at 8 h, in line with the decreased PE in both treatments, and the increased DG in TBT samples reported by Jordão. Other genes related to lipid pathways were also deregulated, as norpA, that catalyzes the production of DG and inositol 1,4,5-trisohosphate (Bloomquist et al., 1988), and some other genes that are related with PI, lipid family that was not determine within this lipidomic study, as Pi3K21B, sl, Inos, or with steroids (Sc2), terpenoids (CG33671) or sphingolipids and glycosphingolipids (CDase, alpha4GT1, 4GalNAcTB, Brn, C1GalTA), as well as CG31522, DESAT1, CG11162 and CG4500, FA elongase, desaturase, hydroxylase and synthetase, respectively (Adams, 2000; de Paula et al., 2018).

CDase, CG6472 and CG13282, represented in Figure 2.6A, were some of those genes specifically upregulated for TBT exposures. CDase hydrolyzes the sphingolipid ceramide into sphingosine and free FAs (Rohrbough, 2004), although the effects of its overexpression cannot be evaluated regarding lipidome disruption because these lipid families were not determine within this study. CG6472 and CG13282 are two triacylglycerol lipases, whose transcription was increased in TBT samples explaining the lower levels of TG in these samples.

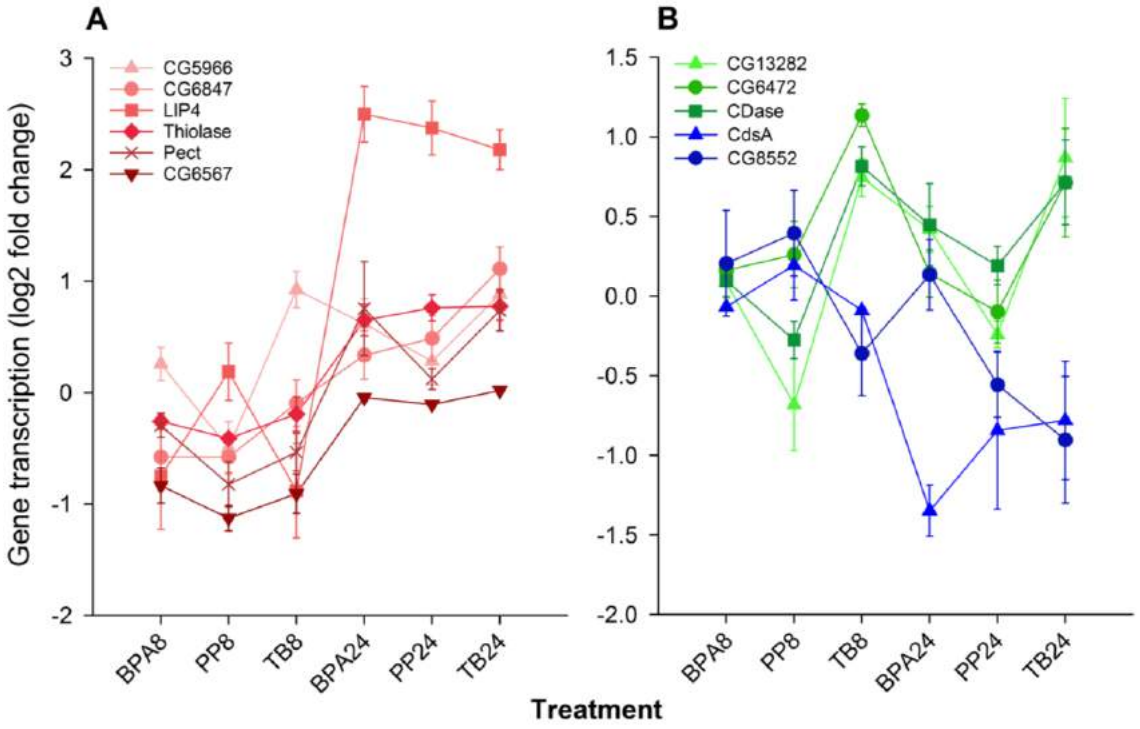


Figure 2.6. Transcription patterns across treatments and time points (Mean SE, N=3) of selected DEGs related to lipid metabolic pathways and discussed within this section. Data is scaled to its time control. DEGs belonging to clusters A (A), and B and C (B) are depicted, respectively, in red, green and blue.

The present study has allowed relating effects produced by the reported EDCs at transcriptomic level with effects produced at the lipidome. Nevertheless, some improvements can be implemented to better understand the mechanism of action of these contaminants, such as lipidomic analysis by MS/MS, which allows to identify precisely the identity of the lipids knowing their specific structure, as well as the analysis of other lipid categories such as ceramides, PI or PA, that according to transcriptomic effects, seems to be affected after the exposure to these compounds.

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Chapter 3

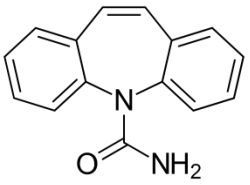
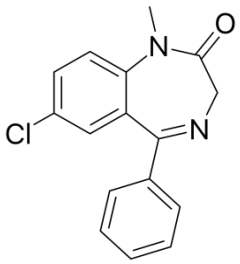
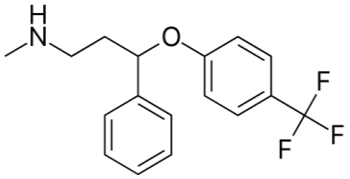
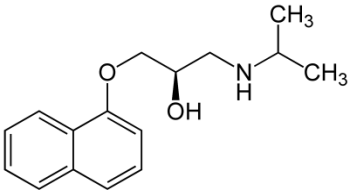
Effects of environmental relevant concentrations of neuroactive pharmaceuticals on transcriptomic and lipidomic *Daphnia magna* responses

3.1. Introduction

The growing environmental concern about pharmaceuticals and neuroactive compounds that contaminate ecosystems was highlighted in Chapter 1. Pharmaceutical products represent an impotant group of ECs, because of their widespread presence in environmental and even drinking water and their potential to produce adverse impacts on ecosystems and humans at low doses (Richardson and Kimura, 2017). In aquatic ecosystems, organisms are normally exposed to complex mixtures of chemicals and it is relatively uncommon to find sites polluted with only one substance (Lydy and Austin, 2004; Villanueva et al., 2014; Walker, 2001). The accurate prediction of chemical interactions in mixtures remains a priority topic in aquatic environmental toxicology, especially for when it results into synergistic toxicity (Cedergreen, 2014). During the last decades, there has been an increase in the occurrence of psychiatric disorders, thus triggering a particular increase in the use of neuroactive pharmaceuticals (Calisto and Esteves, 2009). As a consequence of this extensive application, together with their tendency of persistence and accumulation, this compounds can reach water concentrations of ng/L to µg/L. Thus, aquatic organisms are continuosly exposed to complex multicomponent pharmaceutical mixtures. Nevertheless, relatively little is still known about their consequences on freshwaters ecosystems compared to other pollutants (Bottoni et al., 2010; Fabbri, 2015; Fent et al., 2006; Hughes et al., 2013; Richardson and Kimura, 2017). Despite all the evidence of environmental risk, pharmaceutical compounds currently receive minimal consideration by regulators and policy makers (Farré et al., 2008). This means that assessing the risks of exposure to low doses of human prescribed pharmaceuticals and their mixtures is a prioritized research need, and hence environmental toxicology studies should focus on studying their subtle effects (i.e. neuroendocrine disruptive responses), specially on water ecosystems as one of the major environmental compartments affected by their continued discharge.

Within the present chapter, the effects of four neuroactive pharmaceuticals at different environmentally relevant concentrations (from 0.001 to 1 µg/L) were studied, which structures and described modes of action in humans are summarized in Table 3.1.

Table 3.1. Neuroactive pharmaceuticals tested within this chapter. Their structures, applications and described mode of action are detailed.

Neuroactive pharmaceutical	Structure	Applications	Mode of action
Carbamazepine (CBZ)		Treatment of epilepsy and bipolar disorder	Modulation of voltage-gated sodium channels (Gambeta et al., 2020)
Diazepam (DZP)		Treatment of anxiety and muscle spasms	Allosteric binding at the GABA-$_A$ receptor (Nutt and Malizia, 2001)
Fluoxetine (FX)		Antidepressant	Selective serotonin reuptake inhibitor (SSRI) (Xue et al., 2016)
Propranolol (PR)		Hypertension treatment	β-adrenergic receptor antagonist (Kalam et al., 2020)

Carbamazepine (CBZ) is an anticonvulsant and mood stabilizing drug mainly prescribed to control epilepsy and bipolar disorder. CBZ acts modulating voltage-gated sodium channels, causing inhibition of action potentials and decreased synaptic transmission, although it has been also described its binding to other ion channels, such as voltage-gated calcium channels (Gambeta et al., 2020). Diazepam (DZP) is an anxiolytic benzodiazepine used for anxiety and muscle spasms treatments. DZP, as other benzodiazepines, exerts its effects by facilitating the activity of GABA at various sites. Specifically, benzodiazepines bind at an allosteric site at the interface between the α and γ subunits on GABA-$_A$ receptor chloride ion channels, that leads to an increase in the frequency at which the chloride channel opens, producing an enhanced conductance of chloride ions. This shift in charge leads to a hyperpolarization of the neuronal membrane and reduced excitability of the neuron (Nutt and Malizia, 2001). Fluoxetine (FX), commonly known as being the active component of Prozac, is a selective serotonin reuptake inhibitor (SSRI) antidepressant. SSRIs act increasing

deficient serotonin by inhibiting its reuptake, which is postulated as the cause of depression (Xue et al., 2016). Propranolol (PR) is a competitive β-adrenergic receptor antagonist prescribed to treat hypertension. It exerts its response by competitively blocking β-1 and β-2 adrenergic stimulation in the heart, which is typically induced by epinephrine and norepinephrine (Kalam et al., 2020).

Due to their extensive and increasing use, these compounds are widely found in the aquatic environment. CBZ, fairly persistent in water, is the single most widely studied and detected compound in Europe and North America (Hughes et al., 2013). It has been reported in freshwater ecosystems worldwide at average concentrations of 174.2 ng/L and up to 11,561 ng/L (Hughes et al., 2013), similar to the 183 ng/L average concentrations in European rivers, reaching 11.5 µg/L in rivers areas close to wastewater effuents (Zhou et al., 2019). DZP has been reported at concentrations up to 33.6 ng/L in freshwater ecosystem (Hughes et al., 2013), and in concentrations ranging between 4 and 40 ng/L in Spanish urban rivers (Mendoza et al., 2014; Valcárcel et al., 2012). FX has been measure at average concentrations of 17.8 ng/L in freshwater ecosystems with concentrations up to 596 ng/L (Hughes et al., 2013) and total concentrations of SSRIs in aquatic systems were measured in the range of 840 ng/L to 3.2 µg/L (Metcalfe et al., 2010; Vasskog et al., 2008). Propranolol, which is also quite persistent in water, has been detected on average at 68 ng/L in European surface water, with maximum values of 0.59 µg/L (Zhou et al., 2019).

The effects of these highly prescribed human pharmaceuticals showed in this chapter were studied on the crustacean *D. magna* at environmental relevant concentrations in order to better understand single effects and mixture interactions in nontarget aquatic species. Many of the described human targets of these compounds can be also found in this crustacean, as serotonin, voltage-gated sodium channels or GABA receptors (Gunnarsson et al., 2008), meaning that this species is suitable for assessing the efects of these drugs. Previous studies showed that these compounds enhanced reproduction and alter phototactic behavior at environmental relevant concentrations in *D. magna* (Campos et al., 2019, 2016; Rivetti et al., 2016). FX has been described to promote the accumulation of serotonin in the central nervous system of *D. magna*, to increase aerobic and sugar catabolim and to deregulate serotonergic and some lipid signalling pathways (Campos et al., 2013, 2016; Jordão et al., 2016). Linked to recent advances in gene manipulation techniques (i.e. CRISPR/Cas9), it is possible to genetically manipulate organisms as *Daphnia*, generating transgenic organisms (Nakanishi et al., 2014). Within this chapter, in order to help clarifying the mode of action of FX, the lipidome of modified CRISPR/Cas9 *D. magna* with mutations

in the tryptophan hydrolase (TRH) gene enzyme (rate limit enzyme in serotonin synthesis) was also determined. Contrary to FX exposed samples, these genetically modified TRH knockout *Daphnia* have been previously reported to reproduce less (Rivetti et al., 2018) and to have down-regulated molecular processes related to serotonergic, eicosanoid and lipid metabolism (Campos et al., 2019). The reported human mode of action of PR as β-blocker was confirmed in *Daphnia*, producing the transcriptional deregulation of the β-adrenergic pathway and also of the JH, which is known to be involved in the regulation of the reproduction (Jeong et al., 2018b). All the tested drugs have been recently found to produce the accumulation of eicosanoids (Garreta-Lara et al., 2018). Eicosanoids are FA that may be also implicated in *Daphnia* reproduction and some of them (e.g.prostaglandines) are derived from arachidonic acid and other PUFAs, components of other lipids as glycerophospholipids (Seifi et al., 2017). Although being reported to have different modes of action, all the selected neuroactive pharmaceuticals have been previously reported to affect reproduction, about which there is reported evidence to be related with lipid metabolism. Furthermore, due to the close link between the hormonal system and the nervous system, endocrine efects (i.e. lipid disruption) should be tested for potential neurotoxic efects (Legradi et al., 2018). Nevertheless, little is known about how these compounds affect the lipid dynamics and about the mechanisms by which this happen.

3.2. Experimental section and results

Throughout this chapter an integrative approach is presented linking neuroactive pharmaceutical effects at different levels of biological organization, as organism response (i.e. reproduction), gene expression (transcriptomics) and the subsequent lipid metabolism disruption (lipidomics). Findings are presented divided into two publications:

- Scientific article III:

  Fuertes, I., Campos, B., Rivetti, C., Pinã, B., Barata, C., 2019. Effects of Single and Combined Low Concentrations of Neuroactive Drugs on *Daphnia magna* Reproduction and Transcriptomic Responses. Environ. Sci. Technol. 53. https://doi.org/10.1021/acs.est.9b03228

The main objective of this work was to study the molecular mechanisms by which carbamazepine, diazepam and propranolol affect reproduction in *D. magna* at environmentally relevant concentrations by analyzing the reproductive and microarray transcriptomic responses of individuals exposed to each single compound and to their mixture. The study of transcriptional responses in single and combined exposures

allowed to assess whether or not the studied compounds acted similarly or dissimilarly. High throughput microarray results were further validated by qRT-PCR.

- Scientific article IV:

  Fuertes, I., Piña, B., Barata, C., 2020. Changes in lipid profiles in *Daphnia magna* individuals exposed to low environmental levels of neuroactive pharmaceuticals. Sci. Total Environ. 139029. https://doi.org/10.1016/j.scitotenv.2020.139029

The aim of this study was to assess the effects of low environmental concentrations of propranolol, carbamazepine, diazepam, and in this case also fluoxetine, on the lipidome of *D. magna* females during their first reproductive cycle. Also, the hypothesis that serotonin may be involved in lipid dynamics was tested by the analysis of the lipidome of genetically tryptophan hydrolase gene knockout clones. Lipidomic analyses were assessed by a suspected screening strategy UHPLC-TOF MS. Reproduction effects were also determined.

3.2.1. Scientific article III

Effects of Single and Combined Low Concentrations of Neuroactive Drugs on *Daphnia magna* Reproduction and Transcriptomic Responses.

Fuertes, I., Campos, B., Rivetti, C., Pinã, B., Barata, C., 2019
Environ. Sci. Technol. 53.

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Effects of Single and Combined Low Concentrations of Neuroactive Drugs on *Daphnia magna* Reproduction and Transcriptomic Responses

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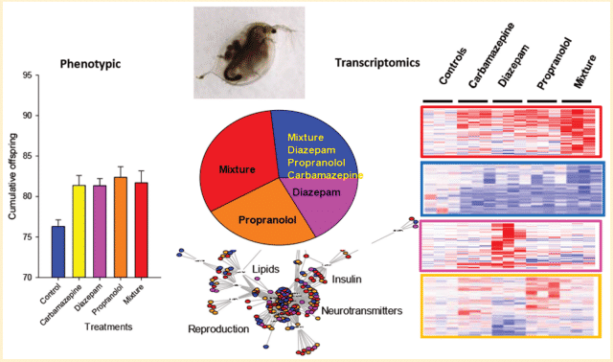
Supporting Information

ABSTRACT: Assessing the risk of neuroactive pharmaceuticals in the environment requires an understanding of their joint effects at low concentrations across species. Here, we assessed reproductive and transcriptional effects of single and ternary equi-effective mixture exposure to propranolol, diazepam, and carbamazepine on the crustacean *Daphnia magna* at environmentally relevant concentrations. The three compounds enhanced reproduction in adults and induced specific transcriptome changes in preadolescent individuals. Comparison of the results from single exposures to a ternary equi-effective mixture of the three compounds showed additive action. Transcriptomic analyses identified 3248 genes affected by at least one of the treatments, which were grouped into four clusters. Two clusters (1897 gene transcripts in total) behaved similarly, appearing either over- or under-represented relative to control, in all single and mixture treatments. The third and fourth clusters grouped genes differently transcribed upon exposure to diazepam and propranolol, respectively. Functional transcriptomics analysis indicated that the four clusters shared major deregulated signaling pathways implicated on energy, growth, reproduction, and neurologically related processes, which may be responsible for the observed reproductive effects. Thus, our study showed additive effects at the transcriptional and physiological level and provides a novel approach to the analysis of environmentally relevant mixtures of neuroactive compounds.



INTRODUCTION

Environmental monitoring surveys routinely confirm that organisms in the environment are continuously exposed to complex multicomponent pharmaceutical mixtures in the ng/L range.[1] This means that assessing the risks of exposure to low doses of human prescribed pharmaceuticals and their mixtures is a prioritized research need.[2] Pharmaceuticals are molecules designed to have low toxicities but high biological activities and specificities; thus, ecotoxicological studies should focus on studying their subtle specific effects (i.e., endocrine disruptive responses) of single exposure and mixtures. It has been reported that often endocrine disruptors act nonmonotonically,[3] which prevents the implementation of the classical mixture toxicity framework that relies on both the mechanism of toxic action and good estimates of concentration−response curves.[4] However, an appropriate combination of experimental designs and molecular mechanistic studies may allow the evaluation of additive and nonadditive effects of mixtures, even when their mixture constituents behave in a nonmonotonically manner.

According to mixture toxicity evaluation, the joint additive effects of similar and dissimilar acting chemicals can be predicted using the concepts of concentration and response addition, respectively.[4] The former considers that mixture constituents can be replaced by their equi-effective fractions. Consequently, the contribution of each mixture constituent to the combined effect of the mixture is proportional to its concentration and activity potency.[4] The response addition concept considers that each mixture constituent acts independently in different target sites; therefore, joint toxic effects can be estimated by multiplying the probabilities of responses.[5] Joint effects of multicomponent contaminant mixtures at environmentally relevant concentrations are often assumed to be additive, although nonadditive synergic or antagonistic effects can also occur,[6−8] which may be explained by interactive or independent action joint activated pathways among mixture constituents.

Here, we study the effects of single and ternary mixtures of three neuroactive pharmaceuticals (diazepam, carbamazepine, and propranolol) on the crustacean *Daphnia magna* at environmentally relevant concentrations in order to better

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understand mixture interactions of these highly prescribed neuroactive drugs in nontarget aquatic species. It is highly probable that these three drugs may coexist in the environment and interactions may occur, as diazepam, carbamazepine, and propranolol are widely used to treat anxiety, epilepsy, neuropathic pain, and high blood pressure. This is also corroborated by their continuous detection during biomonitoring campaigns in European surface waters.[9,10] Concentrations of diazepam ranging between 4 and 40 ng/L have been reported in Spanish urban rivers.[11,12] Carbamazepine, fairly persistent in water, can be found in European river waters at 183 ng/L average concentrations, reaching 11.5 μg/L in rivers areas close to wastewater effluents.[10] Propranolol, also quite persistent in water, has been detected on average at 68 ng/L in European surface water, with maximum values of 0.59 μg/L.[10]

According to the read-across hypothesis, a drug will have an effect in nontarget organisms only if the molecular targets (such as receptors and enzymes) have been conserved.[13] Previous reports show that environmentally relevant concentrations of antidepressants, anxiolytic drugs, and β-blockers may alter the behavior of fish, mollusks, and crustaceans.[14−16] Our studies in the crustacean *D. magna* showed that the selective serotonin reuptake inhibitor (SSRI) fluoxetine, the β-blocker propranolol, the anticonvulsive compound carbamazepine, and the anxiolytic drug diazepam acted similarly by enhancing reproduction at environmentally relevant concentrations, despite having distinct pharmacological target sites.[15,17,18] Human targets of antidepressants, anxiolytic, and neuropathic drugs, including SSRIs, drugs blocking voltage-gated sodium channels and/or GABA agonists, and certain antihypertensive compounds, are highly conserved across vertebrates, and 61% of them are also found in *Daphnia*.[19] Little is known, however, about the mechanisms by which these chemicals promote reproduction in *D. magna*.[15]

Reported information on the mechanisms of action of neuroactive chemicals in *Daphnia* species is limited to SSRI and β-blockers. It has been described that the primary mechanism of action of SSRIs in Daphnia is promoting the accumulation of serotonin in the central nervous system, as in human.[18] However, SSRIs have also been shown to exert other effects in *D. magna* individuals such as increasing aerobic and sugar catabolism and ultimately enhancing reproductive investment.[18,20] The β-blocker propranolol decreased heart rate in *Daphnia* and deregulated the transcription of genes belonging to the β-adrenergic pathway: cAMP-dependent protein kinase (PKA), myosin related genes, gene related to apoptosis, and calcium/calmodulin-dependent kinases.[21] Interestingly, propranolol also deregulated genes involved in the juvenile hormone signaling pathway,[21] which is known to regulate reproduction in crustaceans.[22] Despite the lack of understanding of the gene signaling pathways regulated by diazepam and carbamazepine in *Daphnia*, it has been recently found that the abovementioned drugs, together with the SSRI fluoxetine, upregulate eicosanoids that may be also implicated in *Daphnia* reproduction.[23]

The main objective of this work is to study the molecular mechanisms by which propranolol, carbamazepine, and diazepam increase reproduction in *D. magna* at environmentally relevant concentrations by analyzing the reproductive and transcriptomic responses of individuals exposed to each single compound and to their mixtures. We selected previously reported low effect concentrations (LOEC) on reproduction for single exposures (0.001, 0.1, and 1 μg/L for propranolol, diazepam, and carbamazepine, respectively) since these drugs act nonmonotonically,[15] and hence, it is not possible to estimate an accurate concentration−response effect. Furthermore, in order to assess the joint combined effects, we used a ray design using a constant mixture ratio proportional to LOEC, which is suited for comparing responses with concentration addition and also allows interpretation according to the response addition concept.[4] Our initial hypothesis is that the three compounds enhance reproduction in *D. magna* acting in a similar manner on key molecular signaling pathways. Thus, we expect their joint effects on reproduction and gene transcription to be a priory additive and predicted by the concentration addition concept. Nevertheless, the alternative hypotheses that the three compounds may affect different molecular targets and act either additively following response addition and nonadditively will be also considered. The study of transcription responses in single and combined exposures will allow assessment whether or not the studied compounds acted similarly or dissimilarly. In addition, the recent completion of the *D. magna* reference transcriptome allows studying the whole gene transcriptome profiling.[24] Following that, we use a new custom microarray that includes probes from the full transcriptome of *D. magna*.[25,26] Here, we apply a supervised ANOVA-PLS procedure[27] to elucidate and compare coregulatory gene networks among individual and mixture treatments to assess shared or distinct functional biological processes. The use of curated evolutionary-conserved adverse signaling KEGG pathways for environmental neurotoxicants[28] allowed us to assess specific biological processes linked with reported phenotypic effects. Finally, we also discuss the utility of combining phenotypic and transcriptomic responses in mixture toxicity evaluation to assess both joint effects and their underlying mechanism of action.

MATERIAL AND METHODS

Studied Compounds and Chemicals. Diazepam (CAS-No 439-14-5; analytical standard, purity 99%), carbamazepine (CAS-No 298-46-4; analytical standard, purity 99%), and propranolol hydrochloride (CAS-No 318-98-9; analytical standard, purity 99%) were purchased from Sigma-Aldrich (USA/Netherlands). All other chemicals were analytical grade and were obtained from Merck (Germany).

Experimental Animals. Parthenogenetic cultures of a single clone of *D. magna* (clone F) were used in all experiments. The photoperiod was set to a 14 h light:10 h dark cycle, and the temperature was set at 20 ± 1 °C. Individual cultures were maintained in 100 mL of ASTM hard synthetic water at high food ration levels (5×10^5 cells/mL of *Chlorella vulgaris*, respectively) following Barata and Baird.[29]

Reproduction Experiments. Reproduction experiments follow previous procedures[30,31] and aimed to determine single and joint effects of ternary mixtures at different concentrations of the studied chemicals. For single exposures, we selected concentrations that previously were demonstrated to maximize the observed increase in reproduction,[15] hereafter referred as LOEC: 1, 0.1, and 0.001 μg/L for carbamazepine, diazepam, and propranolol, respectively. Ternary mixture combinations followed a ray fixed ratio design in which exposure levels were selected to include constant equitoxic (LOEC) mixture ratios and four different mixture effect levels. Further details are provided in the Supporting Information.

Transcriptomic Experiments. Treatments included the same single exposure concentrations as for reproduction tests

and the mixture combination dosed at 1/3 of the single LOEC, hereafter referred to as DZP, CBZ, PR, and M for diazepam, carbamazepine, propranolol, and the mixture, respectively. Further details are provided in the Supporting Information.

Transcriptomic Analyses. RNA extraction, labeling, and microarray analyses were performed as previously described.[25,26] Complete protocols are described in the Supporting Information. For microarray hybridization and qPCR analyses, we selected three and four replicates per treatment, respectively, having high RNA quality (RIN >9, Agilent 2100 Bioanalyzer).

Raw microarray data from this study have been deposited at the Gene Expression Omnibus Web site (www.ncbi.nlm.nih.gov/geo/), with accession number GSE131587.

Gene Model Annotation. The optimal interpretation of transcriptional changes requires an actualized functional annotation of the genes. The 41317 probes included in the 8x60 K Agilent array[26] were functionally reannotated into 16293 unique *D. magna* genes merging the previous existing information[25] with the updated annotation from wfleabase (http://wfleabase.org).

Chemical Analyses. Physicochemical water quality and test concentrations were monitored in freshly and old test solutions. Results indicate good water quality conditions. Measured concentrations of test solutions were quite stable and close to nominal values. Further information is in the Supporting Information.

Data Analysis. *Fecundity and Related Life History Traits.* Statistical significance across treatments was analyzed using one-way ANOVA. Treatment differences were further compared by post-hoc Tukey's and Dunnett's tests. Prior to analysis, we confirmed that our data meet the ANOVA assumption of data normality and variance homoscedasticity.[32] Predicted joint effects of fecundity of mixture treatments are in the Supporting Information.

Gene Expression Analysis. Raw microarray data were analyzed using Gene Spring GX v13.0 software (Agilent, USA). Fluorescence data were normalized using quantile normalization and baseline transformation to the median of all samples across the probes belonging to the same gene. The quantile 95 of the added 3500 negative probes was calculated, and this value was assumed as being the fluorescence background noise value of each sample.

We used ANOVA-PLS (analysis of variance-partial least square; lmdme package in R v. 1.0.136, R Core Team) to perform the differential expression analysis between all experimental conditions. The use of multivariate analyses (like the PLS-ANOVA) instead of multiple univariate tests (one ANOVA test for each gene) is becoming increasingly popular for transcriptomic studies because they facilitate the analysis of large data sets in which the number of variables (genes or transcripts in this case) is much larger than the number of samples.[27,33] ANOVA-PLS was performed on the normalized data scaled to the control set and $\log_2$ transformed, considering each one of the treatments (including controls) as an independent class. Genes showing significant variations among the classes were selected as differentially expressed genes (DEGs) for further analysis. The observed variations in selected DEGs were further confirmed by quantitative real-time PCR (qRT-PCR).

Hierarchical clustering and PAM (partition around medoids) clustering analysis were performed using the packages gplots, fpc, and cluster in R. Significant differences between scaled values of all genes included in each cluster were assessed by one-way ANOVA followed by post-hoc Tukey's B tests ($P < 0.05$) to identify statistically different subsets of samples using foreign and agricolae R packages. Further graphs, including heatmaps, were obtained using the R gplots package.

The robustness of the above clustering approach was checked using a Venn diagram analysis using genes whose transcriptional levels deregulated at least 1.5-fold relative to the control treatment.

Functional Gene Analysis. Functional analysis of selected genes was performed using a custom analysis based on gene annotations from wFleaBase (http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/Genes/function/cddrps-dapmaevg14.gotab4), with some manually curated additional annotations. This information was used to construct a binary table containing all possible gene/GO (gene ontology) category combinations. The relative enrichment of a specific GO category in a given subgroup of genes (let us say, a given cluster) and the significance of such enrichment were explored by the hypergeometric distribution, using the whole data set as the universe. The final P values were corrected for multiple tests using the Benjamini–Hochberg (FDR) post-hoc correction. Enrichment significance was set at $P < 0.05$. All data manipulation and statistical analysis were performed using homemade scripts in R. R scripts and the associated data files used for functional gene analysis are available on request.

To further characterize the function of the deregulated genes, we searched for enriched curated evolutionary-conserved adverse signaling KEGG pathways for environmental neurotoxicants[28] as well as for those that might be associated with metabolic routes related to observed changes in reproduction (lipid, arachidonic acid, steroid, terpenoid, and sugar metabolism). We combined KEGG annotations from putative orthologs in different well-annotated species, including *Daphnia pulex* (dpx) and several insect species such as *Drosophila melanogaster* (dme), *Aedes aegypti* (aag), and *Apis mellifera* (ame), as well as from the KEGG reference data set (ko), to include more deregulated genes in the functional analysis.

Validation of Microarray Results by qPCR. Microarray results were validated by qRT-PCR using nine selected DEGs. Further details of selected genes, primers, and qPCR methods are shown in the Supporting Information.

RESULTS

Effects on Fecundity and Related Life History Traits. Females treated with single exposures of all three compounds (carbamazepine, diazepam, and propranolol) as well as those exposed to their 1/3 ternary mixtures significantly ($P < 0.05$) produced more offspring than the control groups (Figure 1, treatment effect, $F_{3,73} = 3.12$). Females exposed to the 1/3 mixture combination showed an enhancement in reproduction as predicted by the concentration addition concept (open square above M/3 treatment). Mixtures having greater and lower concentration levels than that of M/3 did not significantly produce more offspring than those of controls (Figure 1). Furthermore, for the mixture M, it was possible to predict joint effects according to the response addition concept, which were greater than observed values. There were no significant ($P < 0.05$, one-way ANOVA) treatment effects for body length or age at first reproduction. The sizes of the neonates produced by females exposed to M/3 treatments

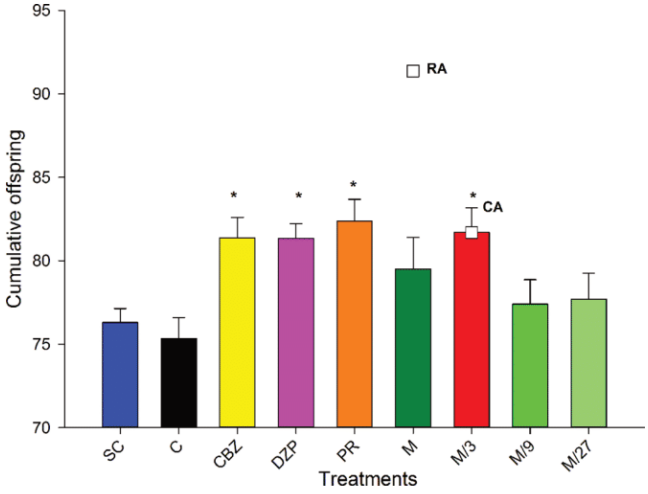

Figure 1. Cumulative reproductive responses of *D. magna* exposed to 1, 0.1, and 0.001 μg/L carbamazepine (CBZ), diazepam (DZP), and propranolol (PR), respectively, and four mixture combinations (M) of the studied compounds. Mixture constituents were dosed at 1, 1/3, 1/9, and 1/27th of their single concentration. SC and C correspond to solvent control and control treatments, respectively. Values are mean ± SE, N = 10. Asterisks indicate significant differences to solvent control treatments following ANOVA and Dunnett's test. White squares on top of M and M/3 treatments are joint predicted levels following the response (RA) and concentration addition (CA) concepts, respectively.

were only smaller than those of controls or exposed to the M treatment. Further details are depicted in Table S3.

Determination and Classification of Deregulated Probes and Genes. ANOVA-PLS identified 3248 unique genes differentially transcribed in at least one of the four experimental subsets relative to the rest (normalized transcription values of DEGs are in the Supporting Information). PAM clustering defined four clusters: A, B, C, and D, including 866, 1031, 792, and 559 genes, respectively (Figure 2A). Cluster A included genes whose mRNA levels decreased in *D. magna* individuals exposed to the selected contaminants and to their mixture, whereas clusters B, C, and D included genes upregulated upon exposure to the mixture, PR, and DZ, respectively (Figure 2B). Notice, however, that cluster B also grouped upregulated genes from females exposed to single treatments (Figure 2B). PAM analysis showing the four defined clusters is presented in Figure S1. The observed patterns of expression for several selected DEGs were confirmed by qRT-PCR (Figure S2). The use of Venn diagrams with genes whose transcriptional levels were deregulated at least 1.5-fold relative to control treatments showed equivalent clusters (Figure S3). The mixture treatment clustered the majority of upregulated genes (342 out 678) with 175 of genes shared with single exposure treatments. DZP, PR, and CBZ included 150, 124, and 38 unique genes, respectively. For a total of 473 downregulated genes, 330 were grouped by the mixture treatment and 248 were shared between the mixture and single treatments. Only 83, 82, 22, and 7 genes were unique for DZP, M, PR, and CBZ treatments, respectively.

Functional Characterization. The deregulated genes within each of the four clusters were used for the functional enriched gene ontology (GO) analysis. Up to 35, 27, 30, and 17 significant (P < 0.05) enriched gene signaling pathways

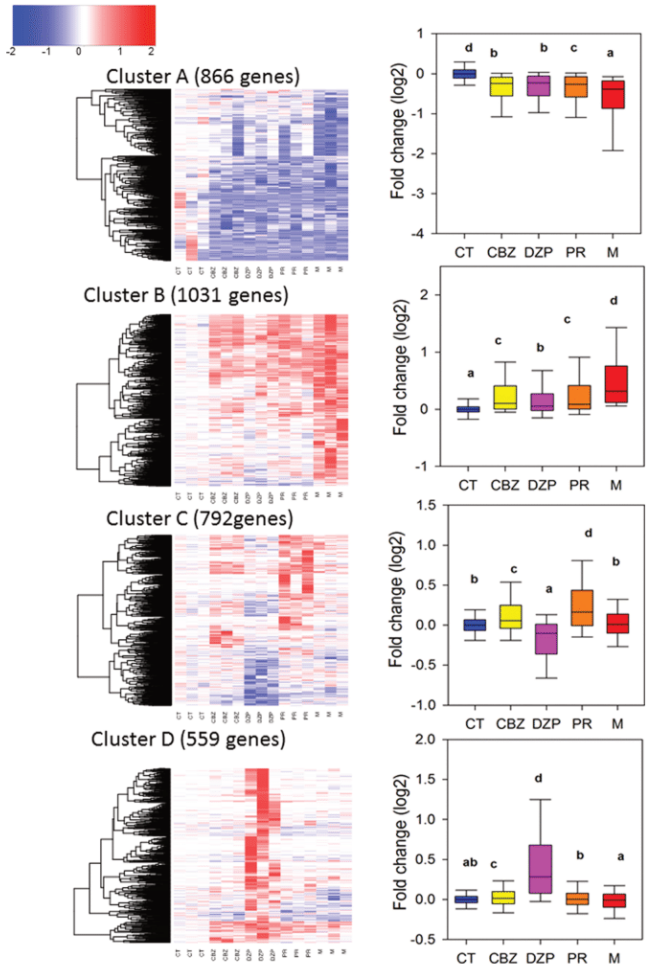

Figure 2. Transcription patterns of differentially expressed genes (DEGs) belonging to the four clusters identified by the partition around medoid cluster analysis. Left panels show hierarchical clustered heatmaps; colors indicate overexpressed (red) or underexpressed (blue) DEGs relative to the controls. The right panel shows box plots of the distribution of the corresponding transcription values. Boxes indicate ranges from the first to the third quartiles, the thick lane indicates the median, and the whiskers indicate the 10 and 90th percentiles. Letters in the box plots represent significantly different sets of values (ANOVA + Tukey's). Gene expression for the exposure treatments is presented relative to the average of the controls.

were obtained for clusters A, B, C, and D, respectively (complete results are in the Supporting Information). Full sets of genes associated with each GO category in each cluster are also depicted in the Supporting Information. Figure 3 shows the distribution of DEGs among the different enriched GO pathways and clusters. The network representation of the most important GO categories is shown in Figure S4. Most deregulated genes from the four clusters could be associated with four functional categories (membrane, protein phosphorylation, proteolysis, and Zn binding), with some minor categories selectively enriched in genes belonging to different clusters. For example, cluster B was especially enriched in genes belonging to nine categories, five of them shared with cluster C. Categories enriched in cluster B included lipid metabolism, sodium ion transport and channel activity, and transferase activity. Common enriched categories for clusters B and C were the extracellular region, chitin metabolism, hydrolase and sulfotransferase activities, and steroid hormone

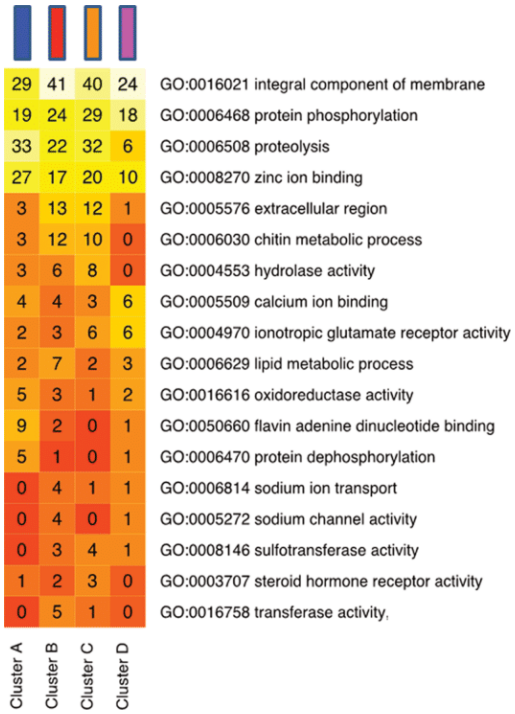

Figure 3. Distribution of DEGs among the different GO functional categories and clusters (columns). Only categories with at least five total hits are represented; redundant modules were simplified to the one with the highest number of hits. Numbers indicate the absolute number of DEGs for each module and cluster, and colors (heat code, from red (few) to white (most)) represent the relative importance of genes associated with each pathway for each cluster.

receptor. Few categories were enriched in clusters C and D, being glutamate receptor activity enriched in both clusters and calcium ion binding only enriched in cluster D. Cluster A was enriched in genes belonging to flavin adenine dinucleotide binding and protein dephosphorylating. Up to 35 significant ($P < 0.05$) enriched KEGG signaling pathways were obtained for the deregulated genes (Supporting Information). These included curate neurotoxic evolutionary-conserved adverse signaling pathways[28] as well as those associated with reproduction[26,34] (lipid, steroid, terpenoid, insulin, and sugar metabolism). We added an additional one, "arachidonic acid", which we consider important to explain reproductive effects,[23] despite being only marginally significant ($0.05 < P < 0.1$). The full set of genes associated with each KEGG category and enzymatic/reaction in each cluster is also depicted in the Supporting Information. Figure 4A shows a network representation of the most important KEGG categories in which transcripts (dots) are labeled by the cluster they belong; a quantitative representation of the same network is shown in Figure 4B. The results showed that all categories were related to some extent. Most categories involving neurotransmitter, depression, circadian clock, and reproductive signaling pathways clustered together, which means that they shared many genes. Lipid metabolism related pathways, such as fatty acid, glycerophospholipid, glycerolipid, arachidonic acid metabolism, and longevity regulatory pathways, were located in the periphery of the previous cluster and shared genes with neurological and reproductive signaling pathways. The outermost layer included genes involved in starch, sucrose, steroids, and *Daphnia* terpenoid metabolic pathways. Cluster A was enriched in genes belonging to steroid biosynthesis, whereas both clusters A and B were enriched in glutamanergic, serotonergic and adrenergic signaling pathways, long-term depression, oocyte meiosis, and glycerolipid metabolic functional categories. In addition, cluster B was enriched in pathways related to insulin, progesterone mediated oocyte maturation, GABAergic synapse, longevity, fatty acid, and ovarian steroidogenesis. Cluster C was only enriched in starch and sucrose metabolism, and cluster D was especially unenriched in genes belonging to cholinergic and GABAergic synapse and lipid metabolic pathways.

DISCUSSION

The results presented in this study indicated that exposure to the three tested pharmaceuticals (carbamazepine, propranolol, and diazepam) at environmentally relevant concentrations disrupts to a greater extent common to chemical-specific gene signaling pathways related to important neurological and reproductive functions, which translates phenotypically into increased reproduction rates in *Daphnia*. Exposure of *D. magna* individuals to equi-effective concentrations of single and the combined ternary mixture treatment (M/3, Figure 1) of the tested neuroactive pharmaceuticals produced similar enhancing effects on reproduction, which were close to joint effect estimates following the concentration addition concept. Alternatively, joint effects on reproduction of mixture treatments, whose constituents were dosed at higher equi-effective concentrations (M, Figure 1) did not affect reproduction, were match lower than response addition estimates. Mixture treatments with lower equi-effective concentrations (M/9, M27 in Figure 1) also did not affect reproduction. These results are in line with previous findings that reported nonmonotonic reproductive responses of these compounds at the studied environmentally relevant concentrations.[15] There were no treatment effects on body length or age at first reproduction but females exposed to the equitoxic mixture treatment (M/3) produced the smallest offspring. Smaller offspring will be less tolerant to food shortage conditions;[29] thus, there was a fitness cost of producing more offspring in the mixture (M/3). In a previous study,[15] it was reported that the three studied chemicals delay reproduction and hence fitness, but these effects occurred at higher concentrations than those used here.

Transcriptomic results revealed similar patterns of gene transcription and similar deregulated signaling pathways across single and mixture treatments in two out of the four gene clusters. One of the clusters (A) included 866 genes downregulated in all single and mixture exposures. Cluster B grouped 1031 genes that were upregulated in all treatments but have the greatest fold changes in mixture exposures. Deviation from additivity of the transcriptional profiles of mixtures may indicate that joint transcription profiles in mixtures represent not only the additive sum of individual compounds fingerprints but also new activated signaling pathways that lead to a stronger response.[35−39] Clusters C and D included 792 and 559 genes, respectively, upregulated upon propranolol and diazepam treatments. This means that the transcription of 1897 genes from clusters A and B behaving similarly across single and mixture exposures exceeded the number of genes deregulated in single treatments (1351; clusters C and D). Previous microarray and RNAseq studies describing transcriptional activity of mixtures acting nonadditively have reported marked differences in differentially transcribed genes between

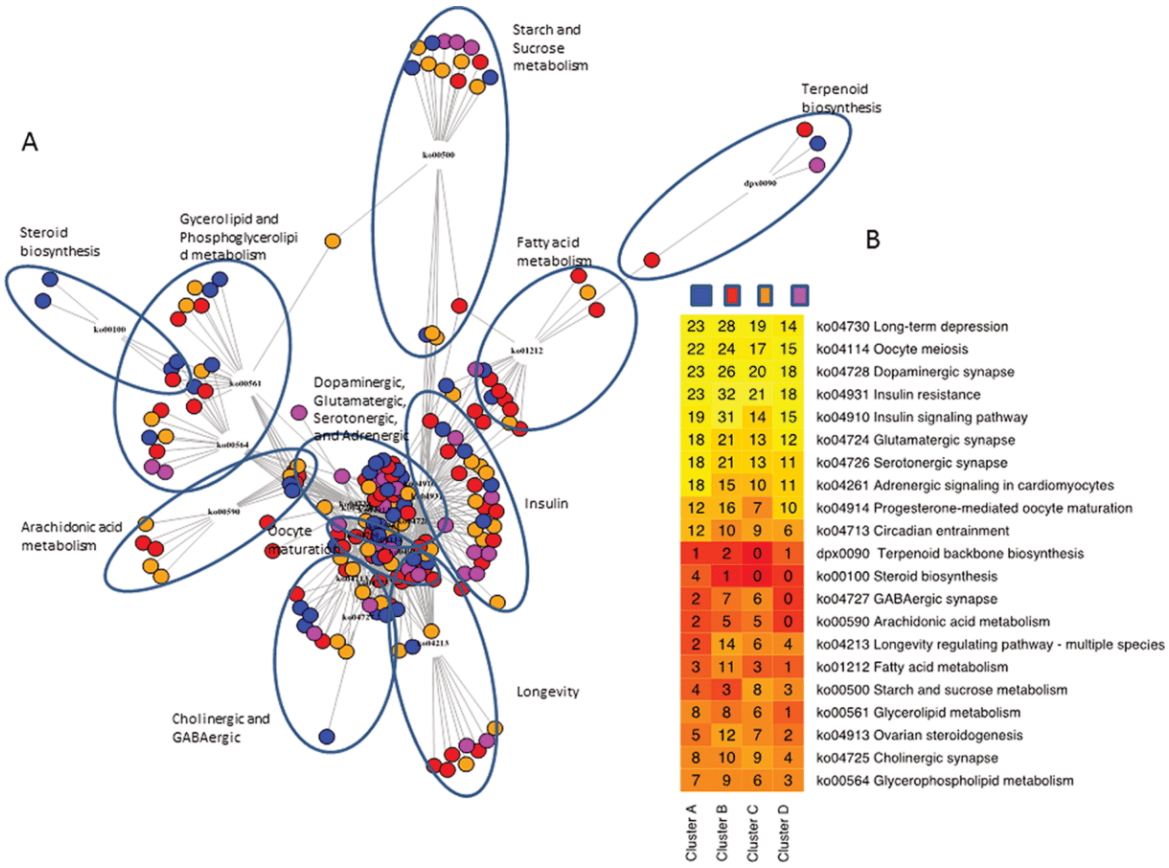

Figure 4. Functional analyses of KEGGs, distributed in clusters (A). Network representation of DEGs according to their adscription to functional modules (KEGG codes for each module are given as nodes). DEGs are represented by dots, colored by clusters as in Figure 3. (B) Distribution of DEGs among the different functional modules (rows) and clusters (columns). General and redundant modules were omitted. Numbers indicate the absolute number of DEGs for each module and clusters; colors (heat code, from red (few) to white (most)) represent the relative importance of genes associated with each pathway for each cluster.

mixture and single exposures.[35,38] Therefore, our results suggest that single and mixture treatments deregulated more genes in a similar than in a dissimilar manner.

Transcriptomic patterns of genes can be somewhat misleading since different genes can coparticipate in the same pathway or function.[40] The analysis of the functional categories enriched in DEGs belonging to the different clusters also reinforces the argument that the three tested chemicals and their mixture shared common mechanisms of action related to neurological processes, growth, and reproduction. These shared DEGs represent a set of genes potentially including biomarker candidates of accelerated reproduction (or delay of first brood). Gene ontology analysis indicated that four categories related to membrane, protein phosphorylation, proteolysis, and Zn-binding agglutinated most deregulated genes. KEGG analysis of the metabolic pathways potentially affected by our treatments in *Daphnia* identified 24 out of the 30 KEGG pathways reported in zebrafish embryos exposed to 12 known chemicals with neurodevelopment and neuroendocrine effects.[28] This finding reinforces the argument that many human drug targets are conserved between vertebrates and arthropods,[19] supporting the interspecies and inter-phyla read-across hypothesis.[13] The KEGG analysis showed that all clusters shared many genes involved in neurotransmitter, depression, circadian clock, and reproductive signaling pathways, but clusters A and B were more enriched in general stress gene responses. For example, cluster B alone or in combination with cluster A was enriched in genes belonging to energy, growth, and reproductive functional categories (chitin metabolism, steroid biosynthesis, oocyte meiosis, progesterone-mediated oocyte maturation, ovarian steroidogenesis, fatty acid and glycerolipid metabolism, longevity and insulin signaling pathways) but also in more specific neurological related processes (glutamanergic, serotonergic, adrenergic signaling pathways, and Na-ion transport). Comparatively, fewer functional processes were enriched exclusively across the single-exposure treatment clusters. Cluster C, which appeared linked to propanolol treatment, was enriched in genes belonging to starch and sucrose metabolism and steroid hormone and glutamate receptor activities, whereas cluster D, associated with diazepam-treated samples, was enriched in genes from the calcium-ion binding and glutamate receptor activity pathways.

Available knowledge of the neurological control of reproduction in crustaceans is limited mostly to decapods where biogenic amines act in regulating reproduction as neuroregulators and neurohormones.[41] Indeed, serotonin and dopamine act by influence on the ovarian maturation: serotonin induces the release of GSH from thoracic ganglion, whereas dopamine reduces thoracic ganglion GSH. In insects, serotonin neurons are known to stimulate insect ecdysteroid and juvenile hormone production, both responsible for controlling oogenesis and vitellogenesis.[42] In the non-decapod crustacean *D. magna*, effects of serotonin on growth and

reproduction have been studied by experimental exposures to SSRIs as well as by analyzing serotonin knock out mutants. *D. magna* individuals chronically exposed to low levels of fluoxetine had higher amounts of serotonin in the brain and reproduced to a greater extent.[18] Conversely, individuals having the tryptophan hydrolase gene mutated (which is the rate-limiting enzyme for serotonin biosynthesis) lacked serotonin and showed decreased growth and reproductive rates.[17] In the present study, genes belonging to the dopaminergic and serotonin signaling pathways were particularly enriched in cluster B followed by clusters A and C in decreasing order. Furthermore, the network representation of enriched KEGG pathways showed a tight clustering of serotonin and dopaminergic signaling pathways with reproductive hormone related genes (oocyte, progesterone, ovarian steroidogenesis), which indicates that these pathways shared many genes.

Insulin signaling pathways were also enriched in decreasing order from clusters B to A, C, and D. In *Drosophila* insulin signaling regulates growth[43] and in knock out *D. magna* lacking serotonin several genes of this pathways were downregulated.[26] This means that we cannot discard the involvement of this signaling pathway in reproduction. Observed upregulation of chitin metabolic genes in clusters that grouped single and mixture treatments also indicated a potential link with reproduction since chitin metabolism is involved in morphogenesis, molting, and growth in insects.[44] In *Daphnia*, several transcriptomic studies have also related chitin metabolism to effects on growth and reproduction.[26,45−47]

Glycerolipid and glycerophospholipid pathways were enriched in clusters A, B, and C, the arachidonic acid one in clusters B and C, and fatty acid metabolism in cluster B, having more upregulation genes than downregulated ones, which agrees with the involvement of lipid and arachidonic acid metabolism in *Daphnia* reproduction.[23,46] Glycerophospholipids are important components of cell membranes but also are the reservoir of highly polyunsaturated fatty acids like arachidonic acid[48] whose derivative products (i.e., eicosanoids) may act as endogenous signaling molecules that regulate reproduction in *Daphnia*.[49] Glycerolipids are key energy storage reserves in *Daphnia* for molting and egg provisioning.[48] In a previous study conducted with two (DZP, CBZ) of the three studied compounds, it was reported that these compounds increased the concentration of 12 eicosanoids in exposed females.[23] This means that the common lipid deregulated pathways in clusters A and B can be related to the observed reproductive effects. Future research should specifically address effects of the three studied drugs on lipids and lipid related enzymatic activities.

The *Daphnia* terpenoid KEGG pathway, which is involved in the biosynthesis of the crustacean juvenile hormone,[22] was also significantly enriched having three genes upregulated in clusters B and D. This means that upregulation of the juvenile hormone pathway by the studied neuroactive drugs may also be involved in the observed effects on reproduction.

Recent mixture toxicity reviews have demonstrated that, in most cases, joint effects of environmentally relevant complex multicomponent mixtures are additive and can be safely estimated according to the concentration addition concept.[6,7] Therefore, this straightforward analysis can be a powerful tool in the mixture assessment, assuming that the effects of common gene sets do not deviate from additivity. Furthermore, our approach provides a further support for interspecies and inter-phyla read-across in transcriptional fingerprints, and thereby, it may facilitate chemical safety assessment.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b03228.

Details of experimental procedures and further explanation of results (PDF)

Normalized transcription values of DEGs (XLSX)

Significant enriched gene signaling pathway (XLSX)

Full sets of genes associated with each GO category in each cluster (XLSX)

KEGG signaling pathways for deregulated genes (XLSX)

Full sets of genes associated with each KEGG category and enzymatic/reaction in each cluster (XLSX)

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Notes

The authors declare no competing financial interest.

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Supplementary Material: scientific article III

Effects of Single and Combined Low Concentrations of Neuroactive Drugs on *Daphnia magna* Reproduction and Transcriptomic Responses.

Fuertes, I., Campos, B., Rivetti, C., Pinã, B., Barata, C., 2019
Environ. Sci. Technol. 53.

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METHODS

Reproduction tests. Mixtures were dosed at similar and at 1/3, 1/9 and 1/27 fraction of single exposure LOECs (Altenburger et al., 2003). Experiments started with neonates (< 24 h old) exposed until their fourth brood (approx. 21-23 days at 20˚C) to the selected treatments. Animals were exposed individually to the tested chemicals in 100 mL of ASTM hard water at the food ration of 5 x 10^5 cells/mL of *C. vulgaris*. The same concentration of ethanol (50 µL/L) was used in all treatments as a carrier solvent and a solvent treatment was also included as a control. Each treatment was replicated 10 times. The test medium was changed every other day. For each individual, female age at first reproduction, adult body length at first reproduction, total offspring production and the body length of at least 10 neonates from the third brood were analyzed. Body length measurements were performed from the head to the base of the spine using the ImageJ software (http://rsb.info.nih.gov/ij/) with a Nikon stereoscope microscope (SMZ 150, Nikon, Barcelona, Spain).

Transcriptomic experiments. Experiments started with newborns (<12h old) reared in groups of five in 200 mL of ASTM hard water at high food ration conditions (5 x 10^5 cells/mL of *C. vulgaris)* and chronically exposed to the selected treatments per quintuplicate. Likewise the reproduction experiment, desired concentrations for each chemical treatment were obtained using ethanol (adjusted to 50 µL/L) as a carrier. An solvent treatment containing only ethanol (50 µL/L) was also included as a control. The test media was changed every other day. Animals were exposed until their third molt (four days), which is the period were *Daphnia* allocation of energy resources into reproduction begins (Campos et al., 2018). At this point, the five replicates containing five individuals each, were preserved in RNA later according to manufacturer's instructions, snap frozen in liquid N_2 and preserved at -80^{o}C for transcriptomic studies.

RNA Extraction. Total RNA was isolated from the samples using Trizol reagent (Invitrogen, USA) and following manufacturer's protocols with slight modifications. After RNA isolation, DNAse treatment was performed according to manufacturer's protocols, followed by a double phenol-chloroform extraction and a further chloroform-only extraction to achieve further purification. RNA was precipitated using sodium acetate and 100% ethanol, being re-suspended in RNAsefree water. Obtained RNA was quantified and its quality was checked in a NanoDrop D-1000 Spectrophotometer (NanoDrop Technologies, USA). Samples presenting a ratio 230/260-260/280 between 1.9-2.1 were selected. RNA integrity was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Only the samples showing RIN values above 9 were used for microarray analysis.

Microarrays analysis. A 8 x 60 K Agilent array containing the full set of the 41,317 gene models (Orsini et al., 2016) representing the full transcriptome of *Daphnia magna* was used. This platform was designed following a previous 4 x 180 K one that contained four probes per gene model and which was tested across seven life-stages (Campos et al., 2018). The 8 x 60 K new platform included 39,000 probes belonging to unique genes scoring the maximum fluorescence signal in at least five stages and the two best probes having the highest signal for the remaining 2,317 genes, which showed a less consistent signalling pattern across life-stages. Further e-array-based quality controls were added, resulting in a microarray with 50,000 probes, as well as an extra 3,500 negative probes computer generated. This was printed on an 8 x 60 K format (Agilent 079797design; GPL23826).

Three replicates per treatment were used. One µg of total RNA was used for each hybridization. cDNA synthesis, labelling, amplification and hybridizations were performed following the manufacturer's kits and protocols (Quick Amp labelling kit; Agilent, Palo Alto, CA). The Agilent one-color Microarray Based Gene Expression Analysis v6.5 was used for microarray hybridizations according to the manufacturer's recommendations. Microarray images were generated by an Agilent high-resolution C microarray scanner. Data was resolved from microarray images using Agilent Feature Extraction software v10.7. Raw microarray data from this study have been deposited at the Gene Expression Omnibus Web site (www.ncbi.nlm.nih.gov/geo/) with accession number GSE131587.

Validation of microarray results by qRT-PCR. Nine differentially expressed genes were selected to validate high throughput transcriptomic data. Three genes implicated in lipid metabolic pathways (Dapma7bEVm003969, an apolipoprotein

homologous to the *Drosophila melanogaster* Apoltp; Dapma7bEVm003203, a fatty acyl-CoA reductase homologous to the *D. melanogaster* CG8306); Dapma7bEVm003259, a lipase 3 homologous to the *D. melanogaster* CG31871); three receptor related genes (Dapma7bEVm000011, a LDL receptor related protein 1 homologous to the *D. melanogaster* LRP1; Dapma7bEVm004624, an ecdysone-induced protein homologous to *D. melanoaster* NR1I2; Dapma7bEVm006088, a regulator of G-protein signalling homologous to the *D. melanogaster* RGS20); a cuticular gene (Dapma7bEVm000605; homologous to the *D. melanogaster* obstructor-B(obst-B); a sodium channel protein (Dapma7bEVm006748, homologous to the *D. melanogaster* pickpocket 28 -ppk28); and a sodium- and chloride-dependent GABA transporter (Dapma7bEVm003613, homologous to the *D. melanogaster* SLC6A5). The gene G3PDH (glyceraldehyde 3-phosphate dehydrogenase) was used as an internal control (house-keeping) (Campos et al., 2013) as its mRNA levels did not change across samples. Primers for each of these genes were designed with Primer Express® Software v3.0.1 (Thermofisher, USA) and are provided in Table S1. Amplification efficiencies were ≥ 90% for all tested genes as described by Pfaffl (2002). qRT-PCR was performed according to manufacturer's protocols using four experimental replicates per treatment.

Relative mRNA abundances were calculated from the second derivative maximum of their respective amplification curves (Cp, calculated from technical triplicates). To minimize errors on RNA quantification among different samples, Cp values for target genes (Cptg) were normalized by Cp values for G3PDH in each sample. Changes in mRNA abundance in samples from different treatments were calculated by the ΔΔCp method (Pfaffl, 2001), using corrected Cp values from treated and non-treated samples.

Prediction of joint effects. For the mixture treatment "M" joint effects could be predicted following the response addition concept following previous procedures (i.e. equation 11 of Faust et al., (2003)) and using the observed effects of single chemical treatments. The selected three chemical concentrations of single treatments enhanced to a similar extent reproduction in *D. magna*, thus theoretically the combination effect of the M/3 would add up to a total effect of about the same (Cleuvers, 2003), which is equivalent to an EC7 = 81.7 offspring.

Chemical analyses. Duplicated water samples of freshly prepared and old (48 h) test solutions were collected at the beginning and end of the tests to determine that pH and oxygen levels were within the limit established by OECD guidelines (OECD,

1981) and to assess the stability of the tested compounds. Dissolved oxygen concentration (DO) was measured using an oxygen electrode model 1302 (Strathkelvin Instruments, Glasgow). pH was measured using an epoxy-body combination electrode, coupled to a Crison micro pH 2001 meter and calibrated with standard pH buffer solutions (Sigma, Madrid, Spain). In all test oxygen levels were within 92 % of saturation and pH values varied between 7.5 and 8.2. Stability of each compound during the tests was confirmed using solid-phase extraction (SPE) and liquid chromatography-tandem mass spectrometry following (Rivetti et al., 2016). Shortly, from single and selected mixture reproduction and transcriptomic tests duplicated water samples of freshly made and old (48 hours) test solutions were collected and pre-concentrated using Oasis HLB SPE cartridges (200 mg), conditioned with 10 mL of methanol followed by 10 mL of water. Five hundred mL of ASTM water were pre-concentrated at a flow rate of 10 mL/min and eluted with 2 x 5 mL of methanol. The eluate was then reduced under nitrogen to almost dryness and reconstituted in 500 µL of methanol. All compounds were measured using LC-ESI-MS/MS (TqDetector, Acquity Waters, USA) following a previous study reporting an analytical method for simultaneous identification of a wide range of pharmaceuticals, with minor changes (López-Serna et al., 2011). Separation was performed by using a Luna C18 (150 mm×2 mm ID, particle size 5 µm, Phenomenex, Torrance, USA) equipped with a SecurityGuard pre-column. The mobile phase composition consisted of binary mixtures with 0.1% formic acid in ACN (A) and 0.1% formic acid in water (B). The gradient of elution started at 5% A , then increased to 40% A in 5 min, 60% A in 10 min, reaching 100% A in 20 min and then return to initial conditions within 5 min. The system was operated at room temperature, the flow rate was set at 200 µL/min and 10 µL were injected. Carbamazepine, diazepam and propranolol were analysed under positive electrospray ionization mode (ESI+). Acquisition was performed in SRM mode using two transitions from $[M+H]^+$ precursor ion to daughter ions to identify each compound. The transitions used as well as the cone voltages and collision energies followed the above mentioned work (López-Serna et al., 2011). Quantification was based on external calibration standard 8 point curves (range between 0.1-1000 µg/L). Limits of detection and quantification (LD, LQ) defined as the minimum detectable amount of analyte with a signal to noise ratio of 3:1 and 10:1, respectively, were 0.14 and 0.51 ng/L for diazepam; 0.08 and 0.023 ng/L for carbamazepine and 0.03 and 0.07 ng/L for propranolol. The data were acquired and processed using the MassLynx v4.1 software package. Measured residue levels of the tested concentrations in freshly prepared and old solutions (Table S2, 0 and 48 h) in single and selected mixture combinations were within 20% of nominal values (14 out of 16 samples) and having the max deviation of

30%. For the sake of clarity, we always refer to nominal values throughout the manuscript.

FIGURES

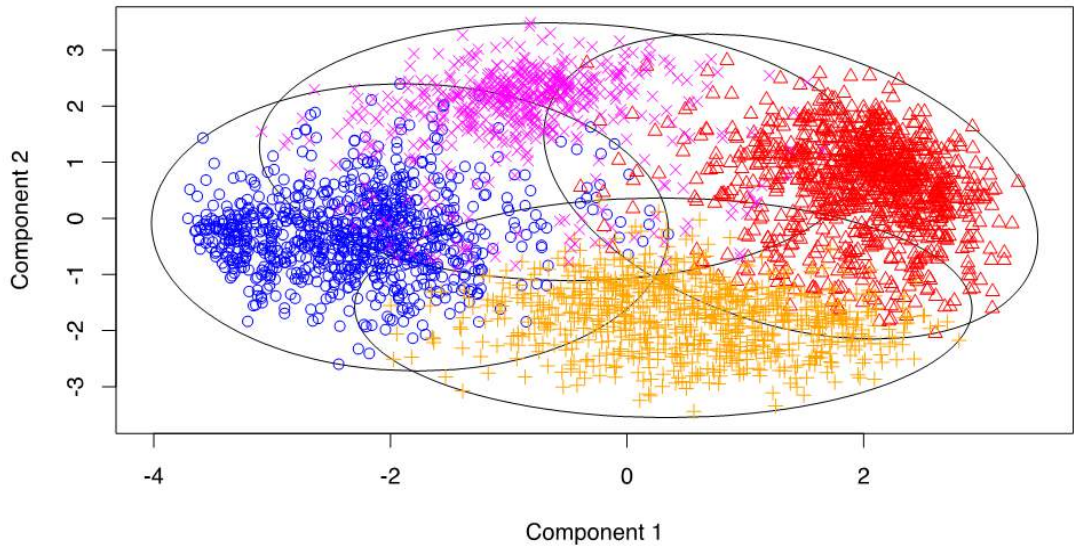

Figure S1. First two components of the partition around medoids cluster analysis. Blue, red, orange and cyan symbols represent genes belonging, respectively, to clusters A, B, C and D. Component 1 and 2 explain 36.48% of the point variability.

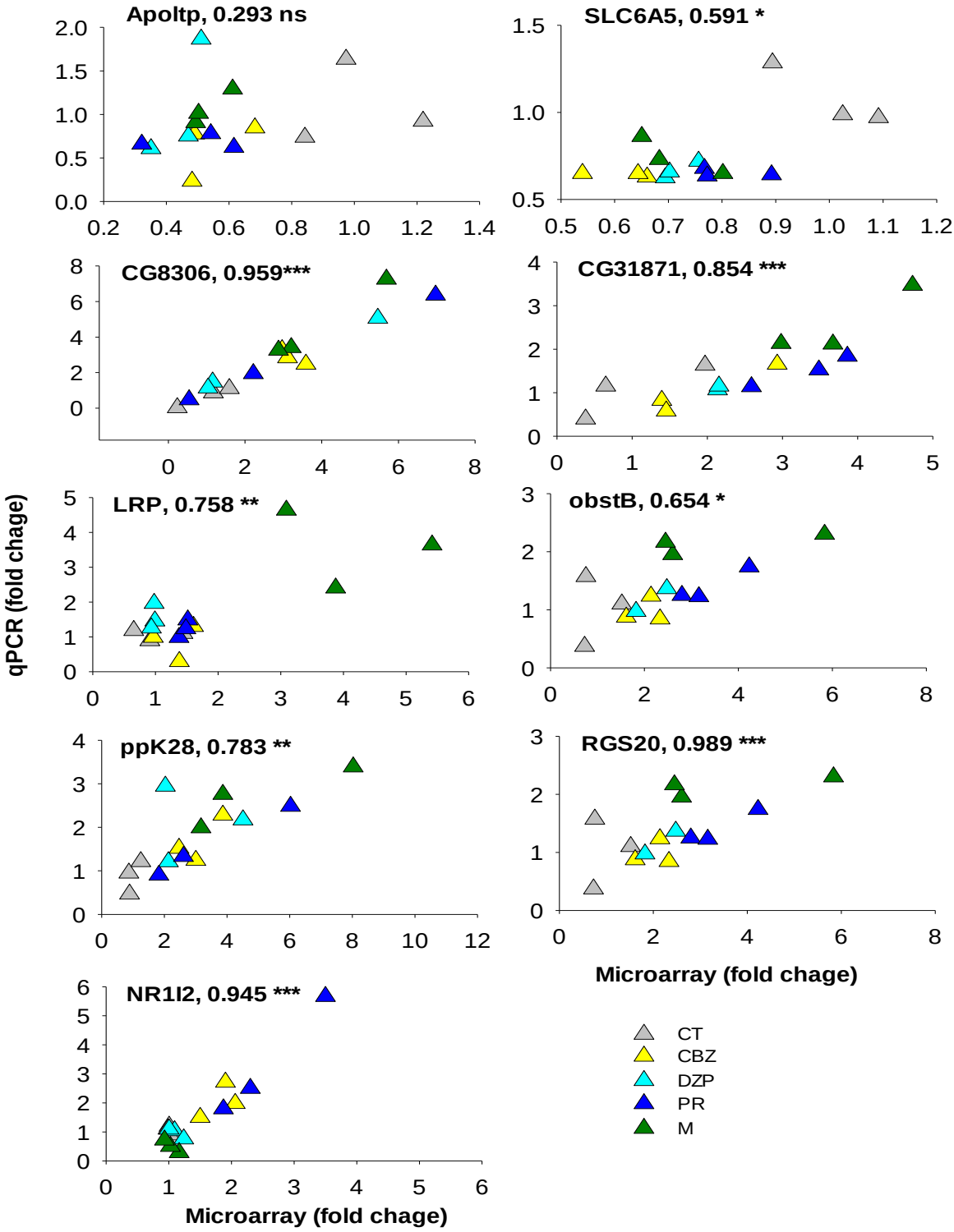

Figure S2. qRT-PCR validation of microarray transcriptomic results for 9 selected de-regulated genes. Results are reported as fold change transcriptomic responses relative to control treatments. Each symbol is a single observation. Numbers following gene names are Pearson correlation coefficients (N=15). ns P>0.05, *0.01<P<0.05, **0.001<P<0.01,***P<0.001.

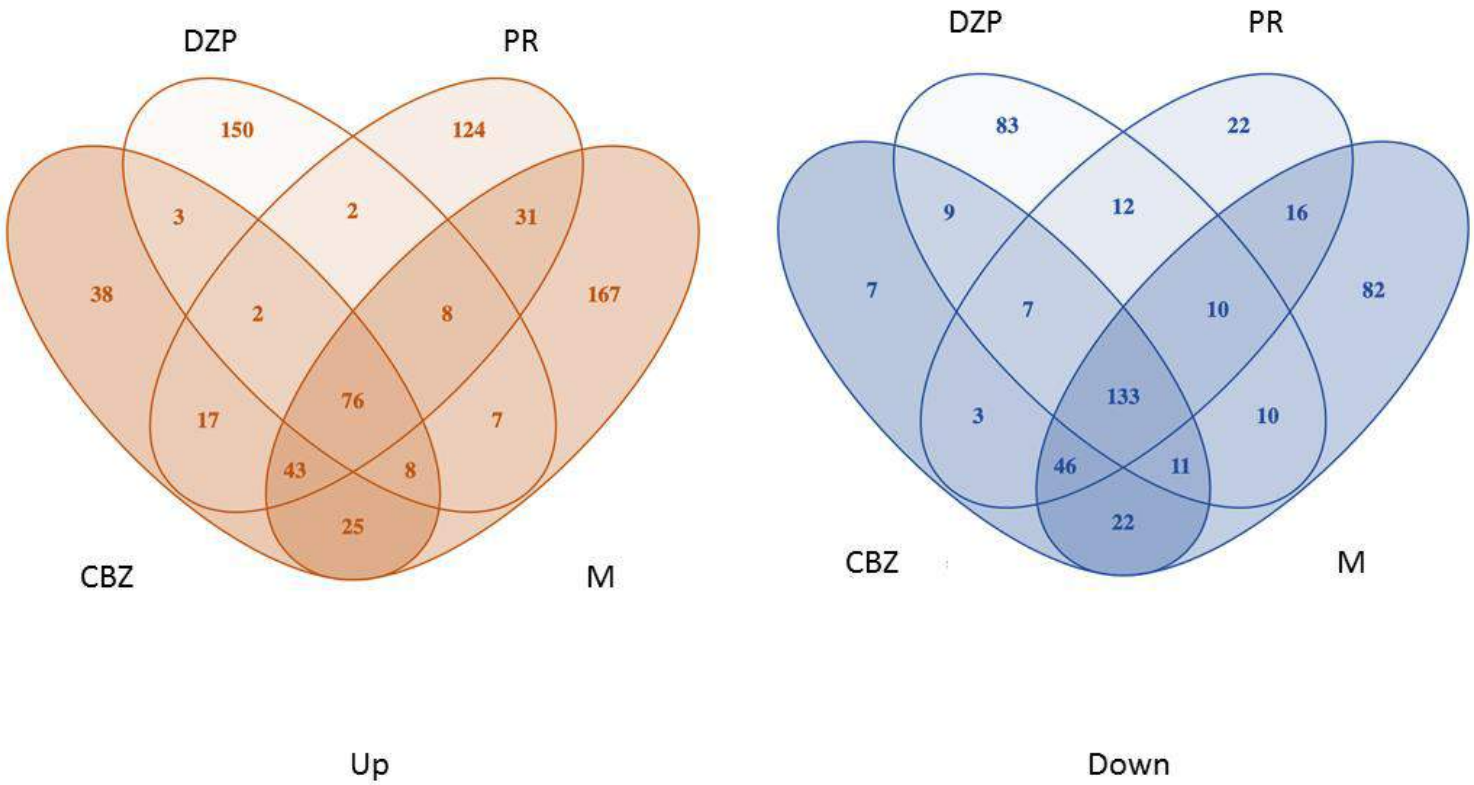

Figure S3. Venn diagrams for up and down regulated genes ≥ 1.5 fold relative to control treatments across the four treatments: diazepam (DZP), carbamazepine (CBZ), propranolol (PR) and the Mixture (M).

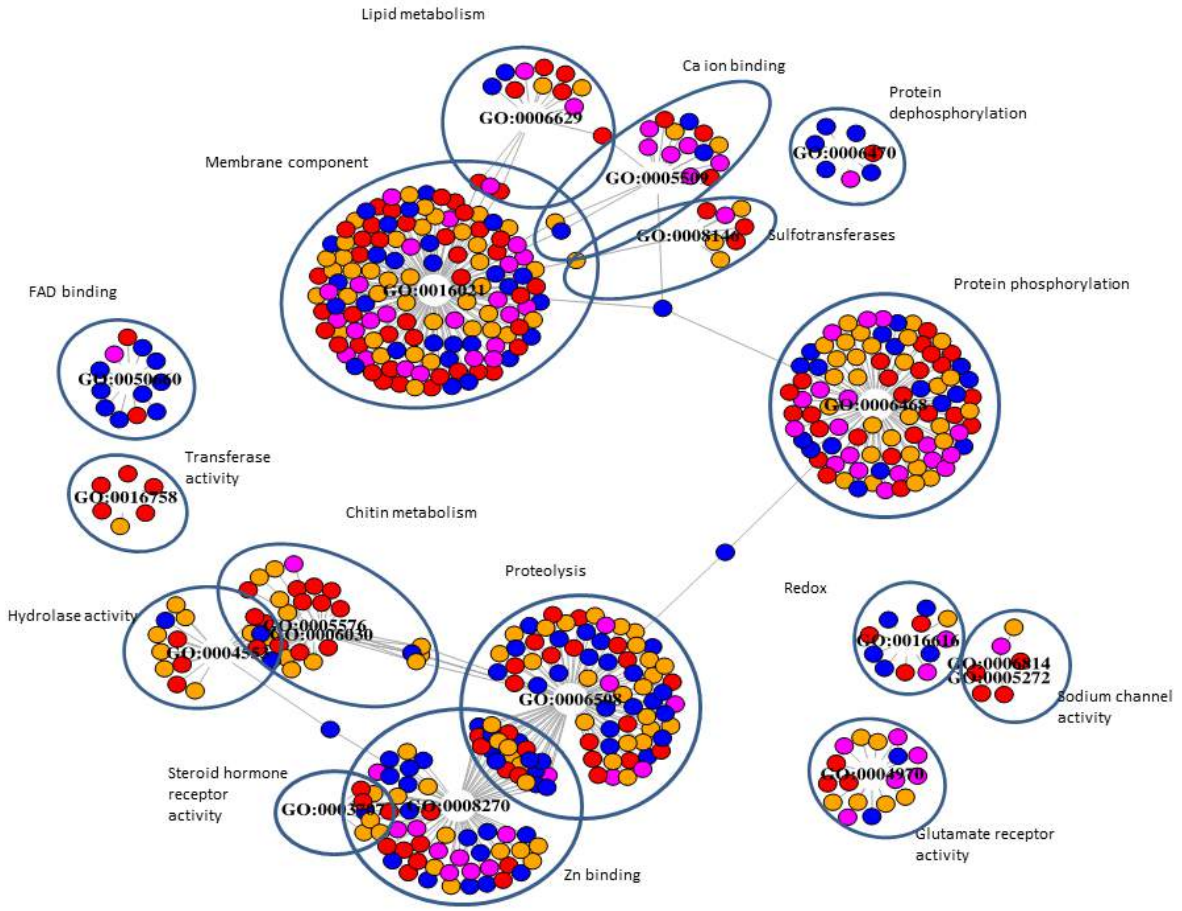

Figure S4. Network representation of DEGs according to their adscription to functional GO categories (codes for each module are given as nodes). DEGs are represented by dots, coloured by clusters. Only modules with at least 5 total hits are represented; redundant modules were simplified to the one with the highest number of hits.

TABLES

Table S1. Primer pairs designed from existing sequences used for amplification of selected *Daphnia magna* partial gene sequences. AZ, amplicon size.

Official gene symbol	Name/function	Acc. Number	*Daphnia* gene	Forward	Reverse	AZ
Apoltp	Apolipoprotein lipid transfer particle (Apoltp)	KZS20461	Dapma7bEVm003969	ACGGAATTGTATGGGTAGAATCGA	TTTGATGAACGGAGAAGAAAACC	71
CG31871	Lipase 3	KZS16460	Dapma7bEVm003259	CAATGTCTGGAAACGAGAGGG	TTCTACACCTGCGGTGTGTGA	71
CG8306	Fatty acyl-CoA reductase	KZS21427	Dapma7bEVm003203	GACGTAACGTCGAGCTCCCT	ATTTTGACGTCCGCGAAATT	71
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase	AJ292555	Dapma7bEVm015323	ACGAGACCCGAAAAACATTCC	CAATGTGAGCATGGGCCTTT	101
LRP1	LDL receptor related protein 1	KZS15516	Dapma7bEVm000011	CAACAGCTGGAATAGGCTCTGACT	TCGAGGGTGAAGAAGTAGAATTACAG	81
NR1I2	Nuclear receptor subfamily 1	KZS08808	Dapma7bEVm004624	GCTGCTGTTCTCCTATCGCC	CGTTCCTGATGCTGACCAATC	71
obstB	Cuticular protein	KZS12557	Dapma7bEVm000605	CCTGAAATAACAGTAGGCAAGTGC	CTTCGTAGCCGGCATTATGAA	71
ppk28	Sodium channel protein Nach	KZS15670	Dapma7bEVm006748	ATCACATAATGGGAAGGACACAAA	CTTTCGTCGTCATTGCGTTTAAT	71
RGS20	Regulator of G protein signalling	KZS19170	Dapma7bEVm006088	AACTCAAAATTCCTGGCAAGACA	AAACTTTGAATTCCACAATGCGT	71
SLC6A5	Sodium and chloride dependent GABA transporter	KZS17977	Dapma7bEVm003613	GCACTTGATGACTGTATAACCCTGA	GAAAATAAAGTCCCTTTAAGCCGG	71

Table S2. Nominal and measured (Mean± SD) concentrations (μg/L) of the tested chemicals in freshly prepared (0 h) and old (48 h) test solutions in single and selected mixture combinations. For mixture treatments we selected two exposure levels for CBZ and DZP (M1, M/3) and one for PR.

Chemical	Nominal concentration	N	Measured (0 h) Mean	Measured (0 h) SD	Measured (48 h) Mean	Measured (48 h) SD
CBZ Single	1	4	0.951	0.042	0.828	0.059
CBZ M1	1	4	0.911	0.032	0.813	0.041
CBZ M/3	0.3	4	0.351	0.013	0.311	0.021
DZP Single	0.1	4	0.107	0.0090	0.091	0.0080
DZP M1	0.1	4	0.112	0.011	0.091	0.0070
DZP M/3	0.03	4	0.0270	0.0050	0.021	0.0090
PR Single	0.001	4	0.00130	0.00050	0.00090	0.00020
PR M1	0.001	4	0.00110	0.00060	0.0010	0.00030

CBZ, DZP and PR are carbamazepine, diazepam and propranolol, respectively.

Table S3. Treatment effects on body length (BL) and age at first reproduction and on the size of offspring (OZ) from the third clutch.

	BL (μm) Mean ± SE		Age (days) Mean ± SE		OZ (μm) Mean ± SE	
SC	3191.3 ± 28.8	a	9.3 ± 0.2	a	901.1 ± 5.8	ab
C	3220.4 ± 22.9	a	9.1 ± 0.1	a	913.6 ± 6.0	b
CBZ	3169.9 ± 13.3	a	9.1 ± 0.1	a	891.3 ± 6.9	ab
DZP	3179.8 ± 18.1	a	9.4 ± 0.2	a	902.4 ± 9.3	ab
PR	3168.2 ± 30.3	a	9.1 ± 0.1	a	892.7 ± 7.4	ab
M	3206.0 ± 25.0	a	9.2 ± 0.1	a	912.3 ± 7.3	b
M/3	3201.6 ± 20.7	a	9.0 ± 0.0	a	883.0 ± 3.5	a
M/9	3178.9 ± 26.2	a	9.2 ± 0.1	a	895.4 ± 5.1	ab
M/27	3185.8 ± 15.2	a	9.0 ± 0.0	a	892.7 ± 5.5	ab

Age at first reproduction was analyzed by non-parametric Kruskal- Wallis tests (K-W =11.1,df= 8, P= 0.1.9) and those on body length using one way ANOVA (BL, $F_{8, 84} = 0.42$, $P = 0.90$; OZ, $F_{8, 76} = 2.51$, $P = 0.018$). Different letters for OZ mean significant ($P<0.05$) differences among groups following Tukey's post hoc test (Zar, 1996).

The reader is referred to the web version of this article (https://pubs.acs.org/doi/10.1021/acs.est.9b03228) in order to access to the following excel data sets:

Table excel 1. Normalized transcription values of DEGs.
Table excel 2. Significant enriched gene signaling pathways.
Table excel 3. Full sets of genes associated with each GO category in each cluster.
Table excel 4. KEGG signaling pathways for DEGs.
Table excel 5. Full sets of genes associated with each KEGG category and enzymatic reaction in each cluster.

3.2.2. Scientific article IV

Changes in lipid profiles in *Daphnia magna* individuals exposed to low environmental levels of neuroactive pharmaceuticals.

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Changes in lipid profiles in *Daphnia magna* individuals exposed to low environmental levels of neuroactive pharmaceuticals

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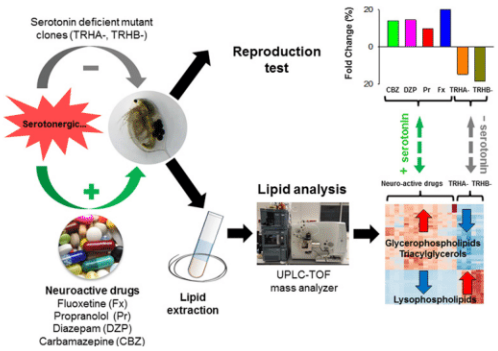
HIGHLIGHTS

- Effects of 4 neuroactive drugs on fecundity and lipids were studied.
- Low concentrations of neuroactive drugs increased fecundity in *Daphnia magna*
- *Daphnia magna* mutated clones lacking serotonin have decreased fecundity
- Females exposed to most drugs had reduced levels of lysophospholipids and increased levels of some glycerophospholipids and triacylglycerol species
- *D. magna* lacking serotonin has the opposite lipid patterns

GRAPHICAL ABSTRACT



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ABSTRACT

Disruptive effects of chemicals on lipids in aquatic species are mostly limited to obesogens and vertebrates. Recent studies reported that antidepressants, anxiolytic, antiepileptic and β-adrenergic pharmaceuticals, with putative distinct mechanisms of action at low environmental relevant concentrations, up-regulated common neurological and lipid metabolic pathways and enhanced similarly reproduction in the crustacean *Daphnia magna*. Conversely CRISPR mutants for the tryptophan hydrolase enzyme gene (TRH) that lack serotonin had the opposed phenotype: the lipid metabolism down-regulated and impaired reproduction. Lipid metabolism is strongly linked to reproduction in *D. magna*. The aim of this study is to test if the above mentioned neuro-active chemicals disrupted common lipid groups and showed also the opposed lipidomic effects as those individuals lacking serotonin. This study used ultra-high performance liquid chromatography/time-of-flight mass spectrometry (UHPLC/TOFMS) to study how neuro-active chemicals (carbamazepine, diazepam, fluoxetine and propranolol) at low (0.1 μg/L) and higher concentrations (1 μg/L) and three CRISPR TRH mutant clones disrupt the dynamics of glycerophospholipids and glycerolipids in *Daphnia* adults. Lipidomic analysis identified 267 individual lipids corresponding to three classes of glycerolipids, eleven of glycerophospholipids, one of sterols and one of sphingolipids, of which 132 and 125 changed according to the chemical treatments or across clones, respectively. Most pharmaceutical treatments enhanced reproduction whereas mutated clones lacking serotonin reproduced to a lesser extent. Except for carbamazepine, most of the tested pharmaceuticals increased some triacylglycerol species and decreased monoacylglycerols, lysophospholipids, sphingomyelins and cholesterol esters in

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exposed females. Opposed lipidomic pattern was observed in mutated clones lacking serotonin. Lipidomic data, thus, indicate a close link between reported transcriptomic and lipidomic changes, which are likely related to serotonin and other neurological signalling pathways.

1. Introduction

Chemical mediated lipid disruptive effects in vertebrates have often been associated to the disruption of peroxisome proliferator-activated receptor (PPARγ) signalling pathway, that together with its heterodimeric partner the retinoid X receptor (RXR), regulate adipocyte differentiation and lipid metabolism in vertebrates (Grün and Blumberg, 2009). The involvement of RXR and two more receptors, the juvenile analogue crustacean receptor methyl farnesoate receptor (MfR) and the ecdysteroid receptor (EcR), have been proposed to affect fat storage accumulation in *D. magna*. There are, however, other mechanisms of endocrine chemical disruption of lipids such as those that alter sex steroids, hypothalamic-pituitary-adrenal axis, thyroid and glucocorticoid hormones (Grün and Blumberg, 2006; Grün and Blumberg, 2009). Of particular interest are those chemicals that interact with central mechanisms that coordinate the whole body response to daily energy intake. The appetite control exerted by the hypothalamic-pituitary-adrenal (HPA) axis plays a critical role regulating energy homeostasis. This means that alteration of neurotransmitter and peptidergic signalling pathways that regulated appetitive or satiety may also disrupt storage lipids. In relation to these, neurological disorders, such as schizophrenia or depression, and some of their treatments (i.e. antidepressant treatments) are known to promote weight body gain in humans (Fava, 2000).

Previous studies reported that the selective serotonin reuptake inhibitor antidepressant fluoxetine increased the accumulation of serotonin in the central nervous system of *D. magna* (Campos et al., 2016), promoted reproduction (Campos et al., 2012) and de-regulated serotonergic and lipid metabolic pathways (Campos et al., 2013). Interestingly, neuro-active compounds such as the β-blocker propranolol, the anticonvulsive compound carbamazepine and the anxiolytic drug diazepam at low environmental concentrations, also promoted reproduction in *D. magna* (Rivetti et al., 2016) and up-regulated the transcription of genes belonging to neurological, glycerolipid and glycerophospolipid signalling pathways (Fuertes et al., 2019a). Conversely, genetically mutated CRISPR tryptophan hydrolase (TRH) *D. magna* individuals lacking serotonin showed the opposite phenotype of fluoxetine exposed individuals: grew and reproduced less (Rivetti et al., 2018) and had down-regulated molecular processes related to serotonergic, eicosanoid and lipid metabolism (Campos et al., 2019). *D. magna* females accumulate from algae diet large quantities of glycerolipids and glycerophospholipids that are invested in the formation of eggs (Fuertes et al., 2018), suggesting that lipid dynamics is strongly related with reproduction. Furthermore, in *D. magna*, eicosanoids, which are lipid mediators formed from polyunsaturated fatty acids (PUFAs), are known to regulate reproduction (Ginjupalli et al., 2015; Heckmann et al., 2008a; Heckmann et al., 2008b; Sengupta et al., 2016). There is evidence that fluoxetine, diazepam and, to a lesser extent, carbamazepine enhance the accumulation of eicosanoids at environmental low concentrations (Garreta-Lara et al., 2018). Pharmacological studies indicated that propranolol not only binds to β-adrenergic receptors but also to 5-HT1 receptors in humans (Tierney, 2001). Accordingly, it is possible to hypothesize that the above mentioned four neuro-active compounds, despite of having disparate modes of action, may act similarly in neurological/cell signalling pathways (i.e. serotonergic, arachidonic) that regulate physiological processes related with reproduction. One of such physiological processes is lipid metabolism. We have previously characterize precisely glycerolipid and glycerophospolipid metabolism and related gene signalling pathways in *D. magna* reproductive individuals exposed to compounds that enhanced the accumulation of storage lipids (Fuertes et al., 2018). The reported results indicated that storage lipid metabolism in *D. magna* was quite similar to that described in *Drosophila*. Nevertheless, little is known about how neuro-active compounds affect the lipid dynamics in *D. magna* reproductive females.

Concentrations of diazepam ranging between 4 and 40 ng/L have been reported in Spanish urban rivers (Mendoza et al., 2014; Valcárcel et al., 2012). Carbamazepine, fairly persistent in water, can be found in European river waters at 183 ng/L average concentrations, reaching 11.5 μg/L in rivers areas close to waste water effluents (Zhou et al., 2019). Propranolol, also quite persistent in water, has been detected on average at 68 ng/L in European surface water, with max values of 0.59 μg/L (Zhou et al., 2019). Surveys in US have reported levels of 12–540 ng/L of fluoxetine, the active ingredient of Prozac, in surface waters and effluents (Kolpin et al., 2002) and total concentrations of SSRIs in aquatic systems were measured in the range of 840 ng/L to 3.2 μg/L (Metcalfe et al., 2010; Vasskog et al., 2008).

The aim of this study is to assess the effects of low environmental concentrations (0.1, 1 μg/L) of the four mentioned contaminants on the lipid profiles of adolescent *D. magna* females during their first reproductive cycle and to test the hypothesis that serotonin may control lipid dynamics.

Genetically tryptophan hydrolase gene knockout clones lacking serotonin were also included to test the role of serotonin controlling lipid dynamics (Rivetti et al., 2018). Lipids were assessed by means of lipidomic analysis by ultra-high performance liquid chromatography (UHPLC) coupled to a time-of-flight mass analyser (TOFMS) (Fuertes et al., 2018; Gorrochategui et al., 2014).

2. Methods

2.1. Chemicals

Fluoxetine hydrochloride (CAS-No 56296-78-7; analytical standard, purity 100%), diazepam (CAS-No 439-14-5; analytical standard, purity 99%), carbamazepine (CAS-No 298-46-4; analytical standard, purity 99%) and propranolol hydrochloride (CAS-No 318-98-9; analytical standard, purity 99%) were purchased from Sigma-Aldrich (USA/Netherlands). All other chemicals were analytical grade and were obtained from Merck (Germany).

2.2. Experimental animals

Parthenogenetic cultures of the *D. magna* clone F were used in experiments with pharmaceuticals. This clone has been maintained in the lab for >20 years and was also used to study transcriptomic changes to the same tested neutron-active compounds (Fuertes et al., 2019b) and lipidomic changes to obesogenic compounds (Fuertes et al., 2018). Three additional CRISPR mutated clones for the tryptophan hydrolase gene originated from clone F, were also used. These included one mono-allelic mutant that have normal levels of serotonin and two bi-allelic mutants without serotonin (Rivetti et al., 2018). Photoperiod was set to 16 h light: 8 h dark cycle, and temperature at 20 ± 1 °C. Bulk cultures of 10 adult females were maintained in 2 L of ASTM hard synthetic water at high food ration levels (5×10^5 cells/mL of *Chlorella vulgaris*), following Barata and Baird (1998). Groups of 100 newborn individuals (<24 h old) obtained from bulk cultures were reared in 2 L ASTM hard water plus algae for 3 days, and then used for exposure

and behavioral assays. Cultures were renewed with new media every other day.

2.3. Experimental procedures

2.3.1. Reproduction experiments

Reproduction experiments follow previous procedures and aimed to determine: (1) the effects of relevant concentrations (0.1 and 1 μg/L), hereafter referred to low and high, of the studied pharmaceuticals; (2) to compare reproductive responses among mutated clones lacking or having serotonin. Previous studies indicated that, within the tested concentration range, the studied chemicals enhanced reproduction and produced important transcriptomic changes in exposed females of clone F (Fuertes et al., 2019a; Rivetti et al., 2016). Experiments with the mutated clones were performed under control (non-exposure) conditions and included the three mutated ones plus the wild type one (clone F). To study effects of pharmaceuticals, animals of clone F were exposed individually to the tested compounds in 100 mL of ASTM hard water at the food ration of 5×10^5 cells/mL of *C. vulgaris*. The same concentration of ethanol (20 μL/L) was used in all treatments as a carrier solvent and a solvent treatment was also included as a control. Culture conditions for the tests with the studied mutated clones were similar to those of pharmaceuticals. Each treatment was replicated 10 times. The test medium was changed every other day. For each individual, total offspring production was analysed.

2.3.2. Lipidomic experiments

All the experiments were carried out according to previously described procedures (Fuertes et al., 2018; Jordão et al., 2015). Experiments with pharmaceuticals were performed at the same concentrations as reproductive assays and aimed to determine effects on the lipid dynamics across an entire adolescent intermoult cycle. Experiment with the mutated clones included the three mutated ones plus the wild type one (clone F). Both types of experiments were started with 4–8-h-old new-born individuals obtained from bulk cultures. The newborns were reared in groups of five in 100 mL of ASTM hard water at high food ration conditions until about 4–8 h before moulting for the third time. At this point, the exposure experiments were initiated, where groups of five juveniles each were exposed/cultured to 500 mL of the test medium. Ethanol at a concentration of 20 μL/L was used as the chemical carrier in the pharmaceutical experiment, and was also used for the controls. The test medium was renewed every 24 h. Animals were sampled just after the fourth moult (i.e. after 72 h of exposure). Before sampling, females were de-brooded according to previous procedures (Fuertes et al., 2018; Jordão et al., 2015).

The physicochemical water quality and test concentrations were monitored in the fresh and old test solutions. Stability of each compound during the tests was confirmed using solid-phase extraction (SPE) and liquid chromatography-tandem mass spectrometry following Rivetti et al. (2016). Further information is provided in the Supplementary materials.

2.4. Lipidomic analysis

Lipidomic analysis of three replicates from each treatment or clone was performed following the procedures described by Fuertes et al. (2018) with minor modifications. Each replicate consisted of a pool of four *D. magna* adults, that were homogenised in 400 μL of chloroform: methanol (2:1, v/v), with 0.01% BHT as an antioxidant. Lipid extraction was performed using similar extraction conditions as described by Folch et al. (1957) with minor modifications, which are explained in further detail in the Supplementary materials. Lipidomic analysis was performed with an Acquity UHPLC system (Waters Corp., Milford, MA, USA) coupled to a Waters/LCT Premier XE TOF analyser operated in positive and negative electrospray ionisation modes. The analytical separation was performed with an Acquity UPLC BEH C8 column (1.7 mm particle size, 10 × 2.1 mm, Waters Ireland, Dublin, Ireland) at 30 °C and a flow rate of 0.3 mL/min. The chromatographic conditions and MS parameters used have been previously reported (Gorrochategui et al., 2014) and are fully explained in the Supplementary materials.

The lipids identified fell into 11 classes of glycerophospholipids (phosphatidylcholine, PC; lysophosphatidylcholine, LPC; ether phosphocholine, PC-O/PC-P; phosphoethanolamine, PE; lysophosphatidylethanolamine, LPE; ether phosphoethanolamine, PE-O/PE-P; phosphatidylglycerol, PG; lysophosphatidylglycerol, LPG; phosphatidylinositol, PI; phosphatidylserine, PS; lysophosphatidylserine, LPS), three of glycerolipids (monoacylglycerols, MG; diacylglycerols, DG; triacylglycerols, TG), one of sterols (cholesterol esters, CE) and one of sphingolipids (sphingomyelins, SM). All were annotated as <lipid subclass> <total fatty acyl chain length>:<total number of unsaturated bonds>, except SM, that were annotated as <total fatty acyl chain length>:<total number of unsaturated bonds in the acyl chain>. In the case of ether phospholipids, no distinction has been determine between plasmanyl- (PC-O or PE-O) and plasmenyl- or plasmalogen (PC-P or PE-P) species, as both types has the same mass and coelute in the chromatogram.

2.5. Data analysis

The effects of pharmaceutical or clone on reproduction and the lipidomic profiles of the major lipid groups were analysed by one-way analysis of variance (ANOVA). Prior to analyses, all data were log transformed to achieve normality and variance homoscedasticity. Significance levels were set at $p < 0.05$ unless indicated otherwise. Tests were performed with IBM-SPSS Statistics version 23 software. Changes in individual lipids across pharmaceuticals and clones in females were further analysed using MetaboAnalyst version 4.0. Data were autoscaled, and partial least squares discriminant analysis (PLS-DA) was carried out to identify clusters of individual lipids similarly affected by the studied chemicals, clones or both. For the latter analysis, lipid concentrations across pharmaceuticals or clones were converted to proportions relative to respective controls or wild type clone. For the three PLS-DA analyses, comparative results were obtained using Principal Component analyses (PCA). Cross validation of the PLS-DA model was carried out (LOOCV), in order to test the accuracy and the predictive ability of the model (Szymańska et al., 2012). Permutation test for testing the hypothesis of no-effect was also done if possible due to the number of variables, with test statistic of prediction accuracy during training and setting the number of permutations in 1000. Taking into account the variable importance in projection (VIP) score values obtained from the PLS-DA, individual lipids with a VIP score higher than 1 were submitted to hierarchical clustering analysis (represented as a heatmap) using autoscaled feature standardisation, Pearson's distance measure, and Ward's clustering algorithm.

2.5.1. Enrichment analysis

Enrichment analysis was carried out for significant lipids obtained from PLS-DA analysis (those with VIP scores > 1) performed with lipids from pharmaceuticals and clones experiments together, proportional to respective controls or wild type clone. Each significant lipid was assigned to a cluster, corresponding to those obtained from the hierarchical clustering analysis, and an enrichment analysis was performed per lipid cluster, using the whole data set as background. The enrichment analysis was performed based on The Lipid Ontology (LION) enrichment analysis web application, a novel bioinformatics tool for lipidomic data that enables to search for enriched LION terms in lipidomic subset, containing detailed lipid classification based on LipidMaps (https://www.lipidmaps.org/) classification, biophysical data, lipid functions and/or organelle associations (Molenaar et al., 2019). General and redundant obtained enrichment categories were omitted for further analysis. The relative enrichment of a specific LION category in each subgroup (cluster) of lipids and the significance of

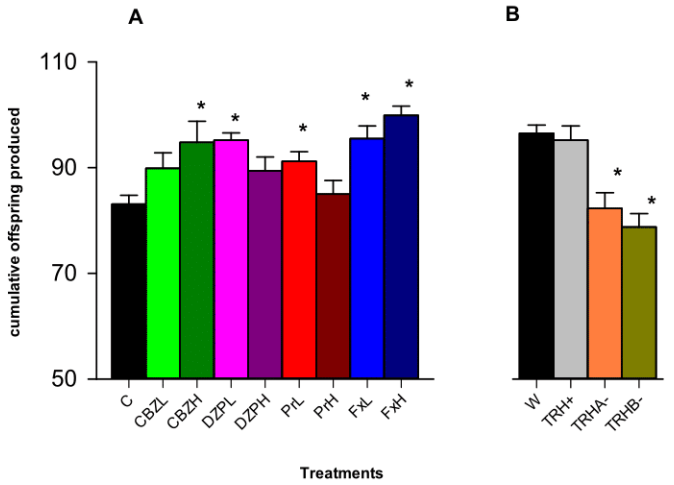


Fig. 1. Cumulative total offspring production (mean ± SE, N = 10) of *D. magna* females exposed to the studied pharmaceuticals (A) and mutated clones (B). Abbreviations are respectively C (Control), CBZ (carbamazepine), DZP (diazepam), Pr (propranolol), Fx (fluoxetine), W (wild type), TRH+ (mono-allelic mutant), TRHA− (bi-allelic mutant A), TRHB− (bi-allelic mutant B). L and H after pharmaceutical name indicate, respectively, low and high concentration treatments. * indicates significant ($p < 0.05$) differences relative to control or wild type clone following ANOVA and Dunnett's test.

such enrichment were explored by the hypergeometric distribution, using the whole data set as the universe. Enrichment significance was set at $p < 0.05$. All data manipulation and statistical analysis were performed using homemade scripts in R. R scripts and the associated data files used for enrichment lipid analysis are available on request. Network and heatmap graphs regarding to enrichment results were obtained using packages *gplots*, *reshape2* and *igraph* R packages.

3. Results

3.1. Effects on fecundity and related life history traits

Females treated with carbamazepine (1 μg/L), diazepam (0.1 μg/L), propranolol (0.1 μg/L) and fluoxetine (0.1, 1 μg/L) produced significantly ($p < 0.05$) more offspring than the control group (Fig. 1, treatment effect, $F_{8,80} = 4.8$). Tryptophan hydrolase bi-allelic mutants (TRHA-, TRHB-) lacking serotonin reproduced less than those with monoallelic mutations (TRH+) or the wild type ones ($p < 0.05$; $F_{3,74} = 12.8$; Fig. 1).

3.2. Effects on the lipid content

Lipidomic analyses allowed us to identify and semi-quantify 267 individual lipids whose calculated and measured mass, elemental composition, mass accuracy error, double-bond equivalent, and retention time were obtained from formula determination tools (elemental composition search) of MassLynx software and are reported in Supplemental materials, Table S2. These comprised 76 TG, 47 PC, 37 DG, 16 PE, 13 CE, 13 PI, 13 PS, 11 LPC, 11 PC-O/PC-P, 9 SM, 8 LPE, 5 PE-O/PE-P, 4 MG, 2 LPS, 1 PG and 1 LPG.

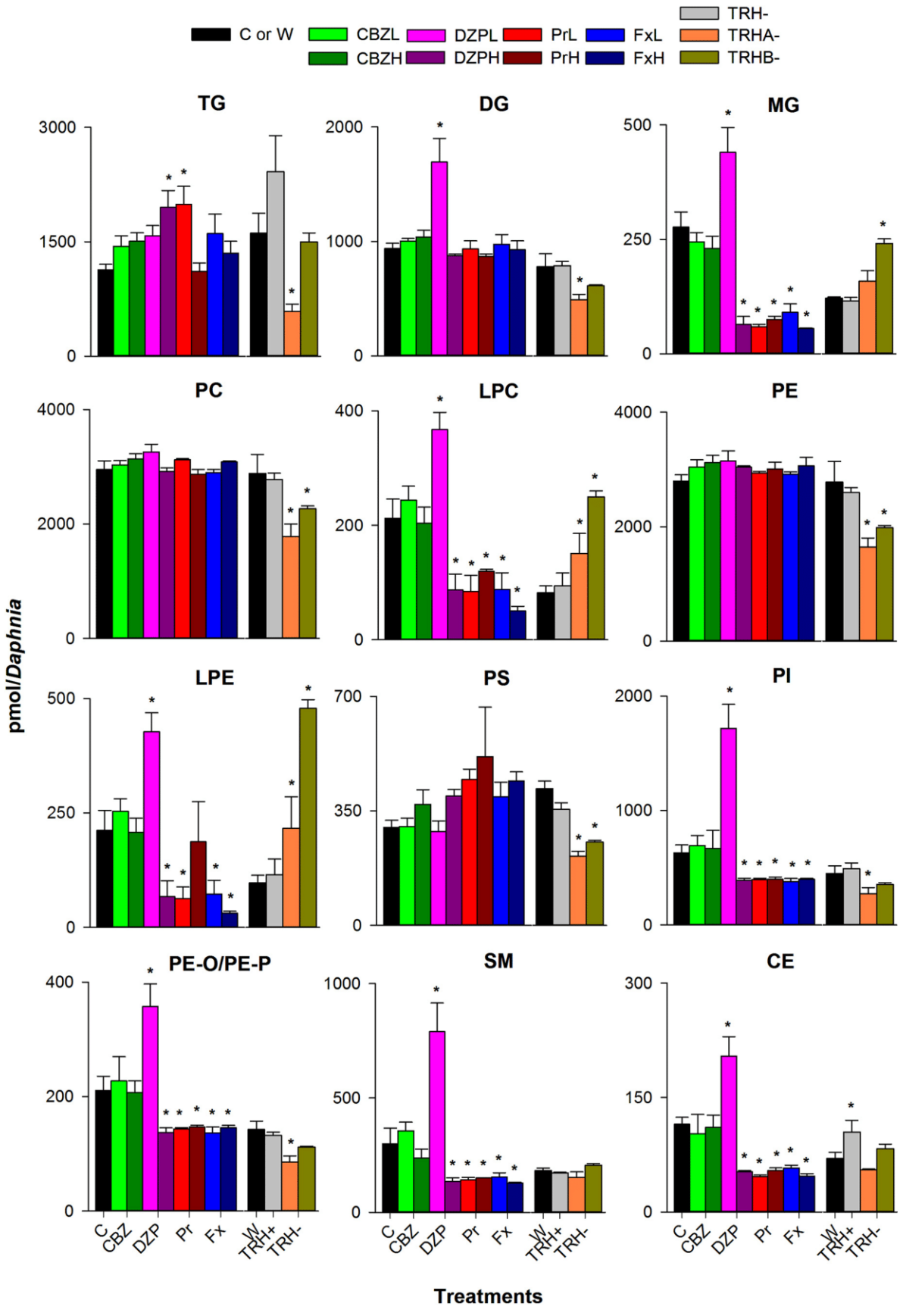
Changes in the lipid contents of *D. magna* females unexposed (control) or exposed to the studied pharmaceuticals in experiment 1, and those from TRH gene mutants in experiment 2 are indicated in Fig. 2 (supporting statistics are provided in the Supplemental materials, Table S4). Pharmaceutical exposures significantly affected ($p < 0.05$) the levels of 9 out of 16 studied lipid classes (Treatment effects, from Supplemental materials, Table S4). The lipid content of females from mutated clones differed from that of the wild type one (clone F) in 11 out of 16 lipid classes. Females exposed to DZPL showed the most conspicuous profiles of lipids having much higher levels of DG, MG, LPC, LPE, PI, PE-O/PE-P, SM and CE than the rest of treatments. Levels of TG were enhanced in DZPH and PrL exposed females. Females exposed to Pr (L and H), Fx (L and H) or DZPH showed lower levels of MG, LPC, LPE, PI, PE-O/PE-P, SM and CE than control ones. Females from both or one of the bi-allelic mutated clones lacking serotonin (TRH− clones) had lower levels of TG, DG, PC, PE, PS, PI, PE-O/PE-P than the wild type clone and greater levels of MG, LPC and LPE.

PLS-DA was performed to explain the variation of individual lipids across pharmaceuticals and mutated clones. In the experiment with pharmaceuticals, the two components obtained from the PLS-DA explained 68.2% of the total variance. Cross validation of the PLS-DA model reported an accuracy of 0.593, Q^2 of 0.718 and R^2 of 0.868. Permutation test reported an empirical p value of 0.003. PLS-DA identified 132 out of 267 lipids having VIP scores > 1. Hierarchical analysis of these 132 lipids is represented as a heatmap in Fig. 3. These lipids clustered into two groups of 8 and 124 lipids, respectively. Individual lipids belonging to each of the two clusters are depicted in Table S5, Supplementary material. The largest group contained mostly glycerophospholipids, DG, MG and few TG. Interestingly 88% of the TG included in the largest cluster group contained saturated fatty acids, which contrast with the 12% found in the total TG analysed. The smallest group only contained 5 PC, 2 PE and 1 TG. The above mentioned lipid clusters separated the studied samples in two groups. One group with the replicates from control, CBZ and DZPL treatments that showed elevated levels of lipids for the larger cluster and low levels of lipids for the smaller one. The second group included Pr, Fx and DZPH treatments that showed the opposite pattern.

In the experiment with the mutated clones the two components obtained from the PLS-DA explained 75.2% of the total variance. Cross validation of the PLS-DA model reported an accuracy of 1, Q^2 of 0.918 and R^2 of 0.999. PLS-DA identified 125 out of 267 lipids having VIP scores > 1. These lipids clustered in two groups of 30 and 95 lipids (individual lipids are depicted in Table S5, Supplementary material). The smaller group was enriched with lysophospholipids (mainly LPC and LPE) and the largest one included PC, PE, PS, PI and DG. Both lipid clusters separated wild type and TRH+ samples from TRH− ones. Samples from TRH− clones had higher levels of lysophospholipids and lower levels of PC, PE, PS, PI and DG that wild type and TRH+ samples.

To further test the assumptions that serotonin may be involved in the observed lipidomic effects, the relative changes of the studied lipids across the studied pharmaceuticals and clones were compared using a PLS-DA combining proportional lipid concentrations from both experiments. The two components obtained from the PLS-DA explained 71.1% of the total variance. Cross validation of the PLS-DA model reported an accuracy of 0.570, Q2 of 0.7200 and R2 of 0.872. Permutation test reported an empirical p value of 0.021. PLS-DA identified 132 out of 267 lipids having VIP scores >1. Hierarchical analysis of these 132 lipids is represented as a heatmap in Fig. 4. These lipids clustered into three groups of 31, 16 and 85 lipids (lipid cluster 1, 2 and 3, respectively). Individual lipids belonging to each of the two clusters are depicted in Table S5, Supplementary material. The first cluster showed up-regulated levels of PC, PE, TG and PS for all replicates from Fx and Pr

Fig. 2. Lipidomic profiles of major lipid classes (mean ± SE, N = 3) of *D. magna* females exposed to the studied pharmaceuticals or the studied mutated clones. For clarity, only those lipids which varied significantly across treatments or clones are depicted. Legend names are explained in Fig. 1. Each represented lipid group corresponds to the sum of the measured individual lipids. Graphs include three glycerolipids (monoacylglycerols, MG; diacylglycerols, DG; and triacylglycerols, TG), seven glycerophospholipids (phosphatidylcholine, PC; lysophosphatidylcholine, LPC; phosphoethanolamine, PE; lysophosphatidylethanolamine, LPE; phosphatidylserine, PS; phosphatidylinositol, PI; ether phosphoethanolamine, PE-O/PE-P), one sphingolipid (sphingomyelin, SM); and one sterol (cholesterol ester, CE). * indicates significant ($p < 0.05$) differences relative to control or wild type clone following ANOVA and Dunnett's test.


C or W
CBZL
CBZH
DZPL
DZPH
PrL
PrH
FxL
FxH
TRH-
TRHA-
TRHB-
TG
DG
MG
PC
LPC
PE
LPE
PS
PI
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Fx
W
TRH+
TRH-
Treatments

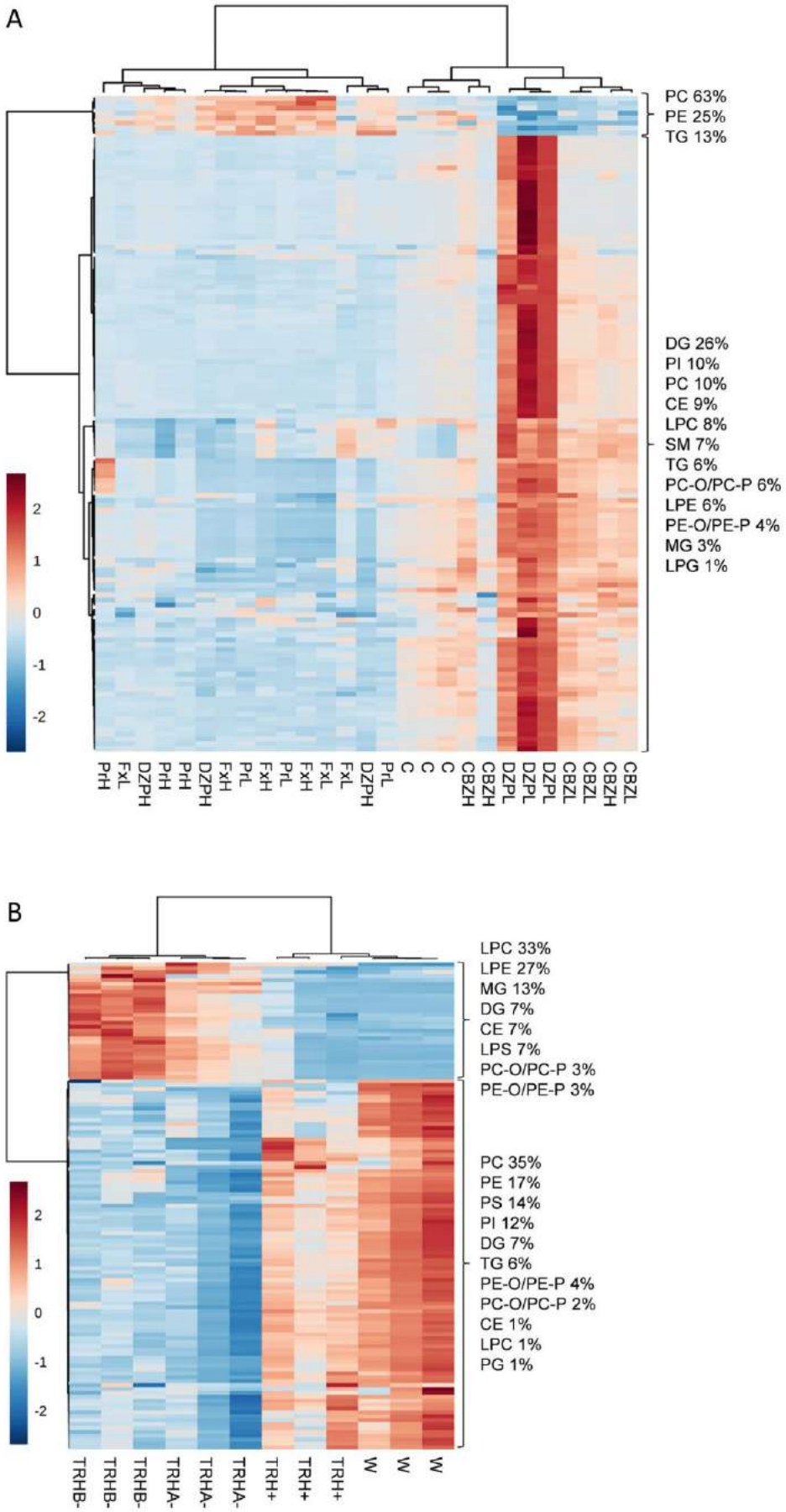


Fig. 3. Heatmap of the clustering results (Pearson's distance measure, and Ward's clustering algorithm) obtained using individual lipids with VIP scores > 1 after PLS-DA analysis, from females exposed to the studied pharmaceuticals (A) and mutated clones (B).

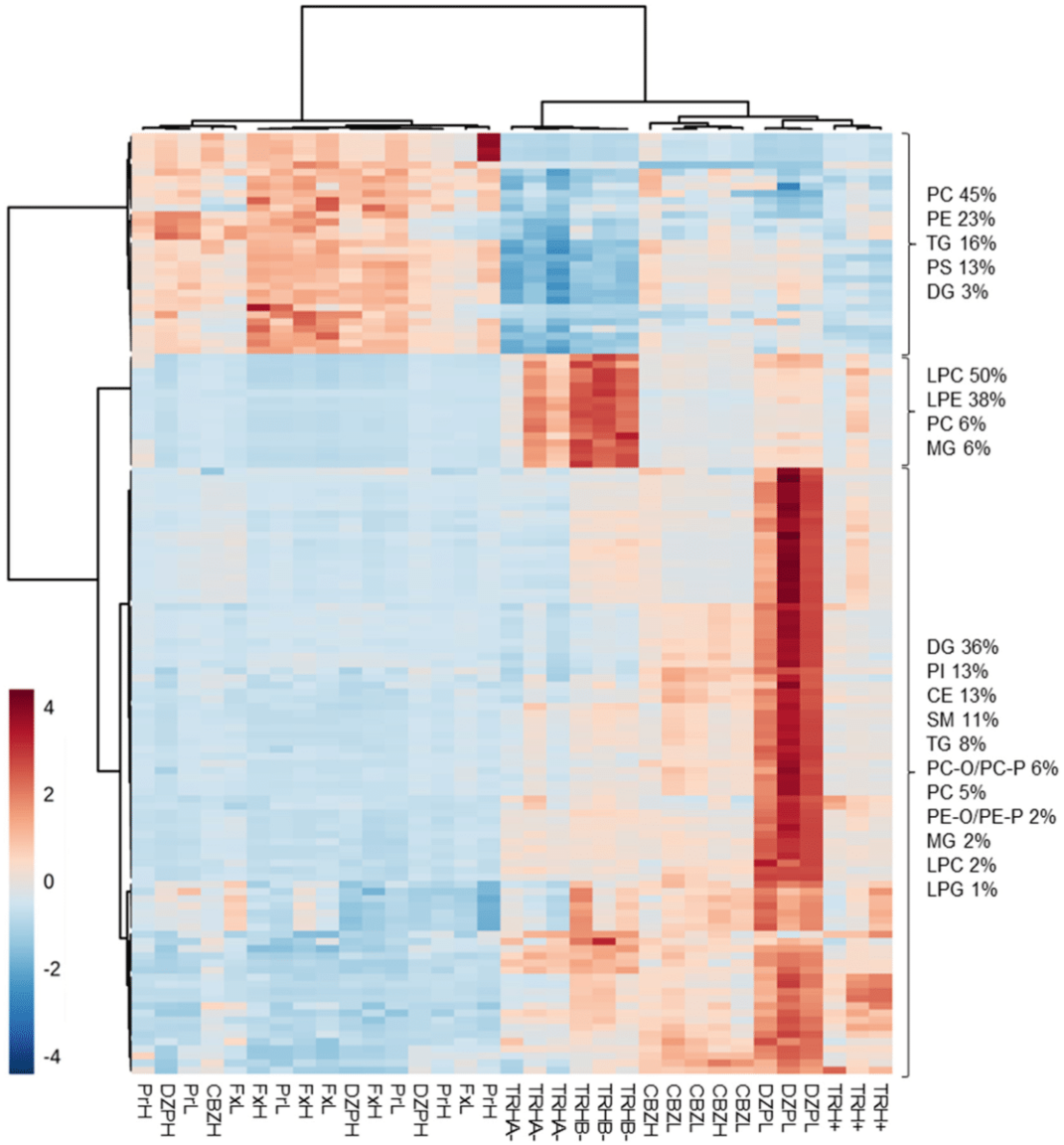


Fig. 4. Heatmap of the clustering results (Pearson's distance measure, and Ward's clustering algorithm) obtained using individual lipids with VIP scores > 1 after PLS-DA analysis, from *Daphnia*females exposed to the studied pharmaceuticals and mutated clones respect to each corresponding control or wild type clone. Lipids clustered intro three groups, of 31, 16 and 85 lipids, named for their interpretation as cluster 1, 2 and 3, respectively.

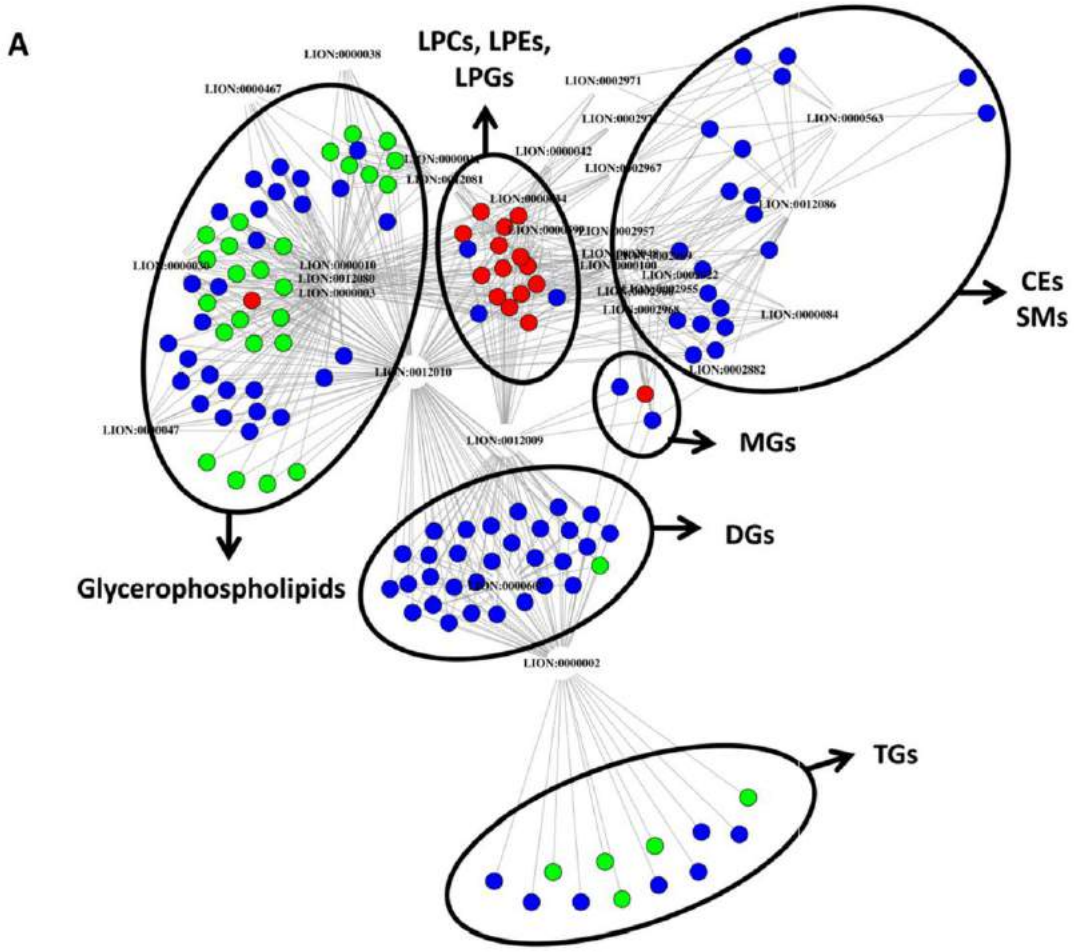
treatments and those of DZPH. The second cluster differentiated mutated clones lacking serotonin from the rest (TRH−) with up-regulated LPE, LPC and MG. The third cluster grouped in order of abundance DG, PI, CE, SM, TG, PC-O/PC-P and some other glycerophospholipids, which were up-regulated in *D. magna* individuals from clone TRH+, those exposed to DZPL and CBZ.

Up to 32 significant ($p < 0.05$) enriched LION signalling pathways were obtained across the three clusters of lipids that were differentially de-regulated among the studied chemicals and clones. Full sets of lipids associated with each LION category in each cluster are also depicted in Supporting information, File S1. Fig. 5A shows a network representation of the most important LION categories, in which individual lipids (dots) are labelled by the heatmap cluster to which they belong. A quantitative representation of the same network is shown in Fig. 5B. The results showed that the obtained represented 27 unique LION categories were almost exclusively lipid classification categories. Glycerophospholipid categories (LION:0000003, LION:0000010, LION:0000030) included lipids mainly from clusters 1 and 3; lysophospholipid categories (LION:0000034, LION:0000042, LION:0000599) almost exclusive of cluster 2; categories regarding to sterols (LION:0000563), sphingomyelins (LION:0000084), diacylglyceriols (LION:000607), phosphatidylinositols (LION:0000047), or ether phospholipids (LION:0000467), mostly with lipids of cluster 3; and glycerolipids category (LION:0000002), including TG, DG and MG, mainly to cluster 3 and to a lower extent, to cluster 1.

4. Discussion

Obtained results evidenced two lipidomic patterns. Some TG species increased in most individuals exposed to the studied pharmaceuticals. Conversely, TRH− knockout clones accumulated less TG and reproduced less. The previous TG changes were positively related with reproduction. Indeed proportional responses of TG and fecundity relative to control treatments were significantly correlated (Pearson correlation = 0.63, $p = 0.04$, $N = 11$; Fig. S1, Supplementary material). *D. magna* females accumulate large quantities of TG to be invested to reproduction, thus it is likely to find a physiological link between TG and reproduction (Goulden and Place, 1990; Jordão et al., 2015; Tessier and Goulden, 1982). Indeed PLS analyses with individual lipids and the LION enrichment analysis showed that some TG species were specifically up-regulated in pharmaceutical treatments. Previously reported

transcriptomic responses evidenced a functional enrichment of glycerolipid metabolism gene signalling pathways in *D. magna* females exposed to propranolol, diazepam and carbamazepine (Fuertes et al., 2019a), up-regulation of lipid metabolic genes in females exposed to fluoxetine (Campos et al., 2013) and down regulation of lipid metabolism in knockout TRH− clones (Campos et al., 2019). Thus, the reported



B

0	6	7	LION:0002967	polyunsaturated fatty acid
0	5	6	LION:0002969	monounsaturated fatty acid
0	4	5	LION:0002977	fatty acid with 3-5 double bonds
0	4	10	LION:0002968	saturated fatty acid
0	9	7	LION:0002957	fatty acid with 18 carbons
0	13	13	LION:0000100	fatty acid with 18 carbons or less
0	13	11	LION:0002948	fatty acid with 16-18 carbons
0	9	16	LION:0002966	fatty acid with less than 2 double bonds
0	14	3	LION:0000599	lysoglycerophospholipids
0	8	2	LION:0000034	monoacylglycerophosphocholines
0	6	0	LION:0000042	monoacylglycerophosphoethanolamines
7	6	4	LION:0012081	mitochondrion
7	6	3	LION:0000011	glycerophosphoethanolamines
14	1	4	LION:0000030	diacylglycerophosphocholines
7	0	0	LION:0000038	diacylglycerophosphoethanolamines
0	0	11	LION:0000563	steryl esters
0	0	11	LION:0000047	diacylglycerophosphoinositols
0	0	9	LION:0000084	ceramide phosphocholines (sphingomyelins)
0	0	7	LION:0000467	contains ether-bond
1	0	30	LION:0000607	diacylglycerols
0	0	20	LION:0012086	endosome/lysosome
25	15	30	LION:0000003	glycerophospholipids
21	15	29	LION:0012080	endoplasmic reticulum (ER)
14	9	15	LION:0000010	glycerophosphocholines
1	15	35	LION:0012009	lipid-mediated signalling
6	1	39	LION:0000002	glycerolipids
26	15	69	LION:0012010	membrane component
1	2	3		

transcriptomic effects of the tested drugs and clones agree with the observed responses of TG profile.

The second lipidomic pattern was that females exposed to propranolol, fluoxetine or high levels of diazepam (those replicate-treatments of cluster 1 in Fig. 4) showed high levels of the more significant glycerophospholipids (PC, PE, PS) and depletion of MG, DG, LPC, LPE, PI, PE-O/PE-P, SM and CE, whereas mutated clones lacking serotonin had mostly the opposite patters, with enhanced levels of MG, LPC and LPE. Glycerophospholipids are an important source of arachidonic and other long chain polyunsaturated fatty acids, which in *Daphnia* have a key role in regulation growth and reproduction (Ginjupalli et al., 2015; Heckmann et al., 2008a; Heckmann et al., 2008b; Sengupta et al., 2016). Jordão et al. (2015) also reported that glycerophospholipids together with TG increased dramatically during the reproductive cycle to be allocated to eggs. Thus the observed high levels of glycerophospholipids found in females having also enhanced levels of reproduction are in line with previous reported studies. Interestingly an enriched LION category related with propranolol, fluoxetine or high levels of diazepam (cluster 1 in Fig. 5B) included endoplasmatic reticulum-associated lipids, which is involved in the synthesis of proteins and de novo synthesis of lipids (Balla et al., 2020), which may be associated with vitellogenin, a key component of *D. magna* eggs (Kato et al., 2004). Other enriched LION categories were those related with PI and PE, which are involved in the synthesis of other glycerophospholipids (Eibl, 1980).

On the other hand, decreased lysophospholipids in *D. magna* females with enhanced reproduction may indicate an activated synthesis of glycerophospholipids (Koizumi et al., 2010; Niu et al., 2008), which can either be allocated as lipid structural components of eggs or further metabolized to DG, which are precursors of TG, the major lipid component of eggs (Pol et al., 2014). Moreover, the observed decreased levels of MG in females exposed to most of the studied neuro-active drugs may also indicate that this lipid group is also being used to synthetize TG, being DG intermediaries in the process (Pol et al., 2014). Most of the genes encoding for the previous lipid metabolic routs were found to be up-regulated by compounds that enhanced storage lipid accumulation in *D. magna* (Fuertes et al., 2019b). Conversely, the observed reduction of glycerophospholipids and enhanced levels of lysophospholipids in TRH knockout clones lacking serotonin (TRH−) can be associated with reduced allocation of lipids to reproduction. Increased levels of lysophospholipids are indicative of increased hydrolysis of related glycerophospholipids by phospholipases, which have been reported to occur in patients with psychiatric disorders (Noponen et al., 1993). Indeed many enriched LION categories that included lysophospholipids and MG were related with the fatty acids, glycerolipid and glycerophospholipid metabolism for mutant samples (Fig. 5B), which means that these pathways were altered in *D. magna* mutants lacking serotonin.

Lipid profiles of PI were reduced in most treatments, high concentration of diazepam, and both concentrations of propranolol and fluoxetine, which may indicated an enhanced metabolism of this lipid group. Polyphosphoinositides, which are produced by the phosphorylation of PI, have important cell signalling functions regulating lipid distribution and metabolism, ion channels, pumps and transporters (Abel et al., 2001; Balla et al., 2009). One of the mechanisms of action of antidepressants and propranolol is the PI turnover. In this signalling system, agonist binding to serotonergic, α-adrenergic, and muscarinic receptors activated via receptor-coupled G_q pathways PI-specific phospholipases C (PI-PLC) that catalyze the hydrolysis of phosphatidyl-inositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG) (Dwivedi et al., 2002; Khorchid et al., 1999). Both IP_3 and DG act as second messengers regulating intracellular Ca^{2+} mobilization and activating protein kinases. It has been previously reported de-regulation of 4 gene enzymes belonging to this pathway in *D. magna* females exposed to lipidogenic compounds (Fuertes et al., 2019b). Thus the observed reductions of PI in *D. magna* females exposed to the studied neuro-active drugs are in line with previous reported studies.

Another study, focused on *Daphnia* glycerophospholipids, found that compounds that reduced the metabolism of sphingomyelin (SM) into ceramides or sphingosine (important signalling compounds), thereby promoting SM accumulation, impaired reproduction (Sengupta et al., 2017). In line with the previous study, the opposite pattern is presented upon exposure to high levels of diazepam, propranolol and fluoxetine (decreased levels of SM), indicating that these drugs were also enhancing reproduction by promoting ceramides or sphingosine.

Observed reduction of cholesterol esters in *D. magna* females exposed to most of the tested drug treatments can be related to an enzymatic inhibition of acyl-coenzyme A: cholesterol-*O*-acyltransferase (ACAT) activity, which catalyzes the esterification of cholesterol with fatty acids (Pol et al., 2014). The above mentioned effects have been reported in human cultured fibroblasts exposed to propranolol (Mazière et al., 1990). In a previous transcriptomic study *D. magna* ACAT like gene (Dapma7bEVm010251), homologous to the ACAT *Drosophila* one (Dana\GF18181), was down-regulated by similar exposures to propranolol, diazepam and carbamazepine (results shown in Fig. S2, Supplementary information). Therefore cholesterol ester results are in line with previous reported findings.

In summary, the results reported in this study indicated that both or at least one of the studied treatments of diazepam, propranolol and fluoxetine showed increased levels of some lipids from the major glycerophospholipid groups, of some specific TG species and reduced levels of MG, SM and CE. Conversely, knockout clones lacking serotonin had opposite responses. Whether or not the observed similar or contrasting lipidomic patterns are the results of the observed reproductive effects or are related to common neurological mechanisms that control both lipid metabolism and reproduction have to be evaluated with caution. Recently reported transcriptomic results for some of the tested drugs at similar low concentrations (diazepam, carbamazepine, propranolol) found up and down-regulation of similar neurological signalling pathways such as the GABAergic, dopaminergic, serotonergic, glutamatergic, cholinergic and adrenergic ones. Diazepam, carbamazepine and propranolol also de-regulated insulin, arachidonic acid and steroid biosynthesis signalling pathways (Fuertes et al., 2019a). The above mentioned shared neurological pathways contrast with the putative primary/secondary mode of action of these drugs (GABAergic for diazepam and carbamazepine, adrenergic or serotonergic for propranolol). The same trend has been reported for serotonin manipulation experiments in *D. magna*. Fluoxetine treated *D. magna* females showed enhanced brain serotonin activity and the up-regulation of serotonergic but also of the arachidonic and insulin signalling pathways (Campos et al., 2013; Campos et al., 2016; Garreta-Lara et al., 2018), whereas the same signalling pathways were down-regulated in knockout TRH − *D. magna* clones lacking serotonin (Campos et al., 2019). Furthermore, recent evidence indicates that fluoxetine and similar acting antidepressants at low doses do not act as selective serotonin reuptake inhibitors but they increased brain neuro-steroid content activating GABA receptors in mice (Pinna et al., 2006). This means that at the low and environmentally relevant concentrations the studied drugs

Fig. 5. Enriched Lipid Ontology (LION) terms, distributed in clusters. (A) Network representation of significant lipids obtained from PLS-DA (VIP score higher than 1) according to their adscription to functional modules (LION ontology description for each term are given as nodes). Only categories with at least five total hits and a p-value < 0.05 are represented, and general and redundant modules were omitted. Lipids are represented by dots, coloured by clusters obtained from heatmap in Fig. 4: green (cluster 1), red (cluster 2) and blue (cluster 3). (B) Distribution of significant lipids among the different LION categories (rows) and clusters (columns). Numbers indicate the absolute number of lipids for each module and clusters, and colours represent the relative importance of lipids associated with each term for each cluster (heat code, from red (few) to white (most)). Specific enrichment results obteined per cluster with their P-values and FDR values, as well as LION term lipid associations, are provided in Supplementary Material.

may target alternative neurological signalling pathways. Despite that the serotonergic signalling pathway seems a priori a good candidate provided the apparent contrasting lipidomic patterns between the two fluoxetine treatments and the two TRH knockout clones, the combined contribution of complementary neurological signalling pathways is likely to regulate lipidomic and fecundity responses in *D. magna*.

CRediT authorship contribution statement

Inmaculada Fuertes: Investigation, Methodology, Formal analysis, Writing - original draft. **Benjamín Piña:** Formal analysis, Software, Writing - review & editing. **Carlos Barata:** Project administration, Conceptualization, Supervision, Resources, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.139029.

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Supplementary Material: scientific article IV

Changes in lipid profiles in *Daphnia magna* individuals exposed to low environmental levels of neuroactive pharmaceuticals.

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METHODS

Lipid extraction. Lipid extractions were performed as described by Folch et al., (1957) with minor modifications following previous work (Fuertes et al., 2018). Three replicates were extracted for each treatment or mutated clone, consisting each sample of a pool of four *D. magna* individuals. Briefly, samples were homogenate in 400 µL of chloroform:methanol (2:1, v/v) with 0.01% BHT as an antioxidant. Homogenates were centrifuged 15 min at 14,000 rpm to remove the insoluble parts of the samples. One hundred µL of homogenized sample were mixed with 1 mL of chloroform:methanol (2:1, v/v) and 250 µL of potassium chloride (0.88%). Internal standards (200 pmol) for different lipid species were also added (Supplemental Material, Table S1). Samples were centrifuged 10 min at 2,500 rpm before phase separation, where the aqueous phase was discarded. This liquid-liquid extraction was carried out twice. The organic fraction was dried under N_2. Lipid extracts were reconstituted in 500 µL methanol and dried under N_2 again. Samples were reconstituted with 150 µL UPLC grade-methanol, centrifuged, and transfer to a chromatographic vial for its analysis.

Lipidomic analyses. Lipidomic analyses were performed following previous work (Fuertes et al., 2018; Jordão et al., 2015) with an Acquity UHPLC system (Waters, USA) coupled to a Waters/LCT Premier XE time-of-flight (TOF) mass analyzer operated in positive and negative electro spray ionization (ESI) mode. The analytical separation was performed with an Acquity UPLC BEH C8 column (1.7 mm particle size, 10×2.1 mm, Waters, Ireland) at 30°C and a flow rate of 0.3 mL/min. Chromatographic conditions and MS parameters have been previously reported (Gorrochategui et al., 2014). Full-scan spectra from 50 to 1,800 Da were acquired, and individual spectra were summed to produce data points each of 0.2 sec. Mass accuracy at a resolving power of 10,000 and reproducibility were maintained by using an independent reference spray (Lock Spray Waters). Mobile phases used were A,

methanol:2 mM ammonium formiate:0.2% formic acid and B, water:2 mM ammonium formiate:0.2% formic acid. Throughout the analysis sequence of the samples, a mix of standards was analyzed to assure the lack of retention time drifts.

Glycerolipids (MG, DG, TG), cholesterol esters (CE) and some glycerophospholipids and sphingolipids (PC, LPC, PC-O/PC-P and SM) were detected under positive ESI as their ammonium and hydrogen adducts, respectively, while PE, LPE, PE-O/PE-P, PG, LPG, PI, PS and LPS were detected under negative ESI as their hydrogen adducts. Identification and relative semi-quantification of lipids was carried out using the ion chromatogram obtained for each compound using 0.05 Da windows. Positive identification of lipids was based on three criteria: the accurate mass measurement, with a minimum mass error (<5 mg/L) respect to the measured m/z ratio of the monoisotopic peak considering possible adducts; its relative retention time, combing the retention time of the internal standard used for each lipid family with the retention time of the individual lipid of interest, and taking into account the changes in the retention time due to the number of carbons and of unsaturations; and the correct isotopic distribution of the main adduct of each lipid. The LipidMaps database and MassLynx software (Waters, USA) were used to identify individual lipids from specific exact masses. Relative semi-quantification was done by comparison of peak areas in extracted ion chromatograms between expected lipids and its corresponding internal standards. Repeatability, expressed as intra-day relative standard deviation calculated for all lipid standards, was satisfactory.

A total of 267 individual lipids were identified and semi-quantified by UHPLC-TOF ESI positive and negative mode. These included 11 classes of glycerophospholipids (phosphatidylcholine, PC; lysophosphatidylcholine, LPC; ether phosphocholine, PC-O/PC-P; phosphoethanolamine ,PE; lysophosphatidylethanolamine, LPE; ether phosphoethanolamine, PE-O/PE-P; phosphatidylglycerol, PG; lysophosphatidylglycerol, LPG; phosphatidylinositol, PI; phosphatidylserine, PS; lysophosphatidylserine, LPS), three of glycerolipids (monoacylglycerols, MG; diacylglycerols, DG; triacylglycerols, TG), one of sterols (cholesterol esters, CE) and one of sphingolipids (sphingomyelins, SM). All were annotated as <lipid subclass> <total fatty acyl chain length>:<total number of unsaturated bonds>, except SM, that were annotated as <total fatty acyl chain length>:<total number of unsaturated bonds in the acyl chain>. In the case of ether phospholipids, no distinction has been determine between plasmanyl- (PC-O or PE-O) and plasmenyl- or plasmalogen (PC-P or PE-P) species, as both types has the same

mass and coelute in the chromatogram. Elemental composition of each of the individual lipids measured in *Daphnia* is reported in Table S2.

Chemical analyses. Duplicated water samples of freshly prepared and old (48 h) test solutions were collected at the beginning and end of the tests to determine that pH and oxygen levels were within the limit established by OECD guidelines (OECD, 1981) and to assess the stability of the tested compounds. Dissolved oxygen concentration (DO) was measured using an oxygen electrode model 1302 (Strathkelvin Instruments, Glasgow). pH was measured using an epoxy-body combination electrode, coupled to a Crison micro pH 2001 meter and calibrated with standard pH buffer solutions (Sigma, Madrid, Spain). In all tests, oxygen levels were within 91 % of saturation and pH values varied between 7.6 and 8.1. Stability of each compound during the tests was confirmed using solid-phase extraction (SPE) and liquid chromatography-tandem mass spectrometry following Rivetti et al., (2016). Shortly, selected reproduction and lipidomic tests duplicated water samples of freshly made and old (48 hours) test solutions were collected and pre-concentrated using Oasis HLB SPE cartridges (200 mg), conditioned with 10 mL of methanol followed by 10 mL of water. Five hundred mL of ASTM water were pre-concentrated at a flow rate of 10 mL/min and eluted with 2 x 5 mL of methanol. The eluate was then reduced under nitrogen to almost dryness and reconstituted in 500 µL of methanol. All compounds were measured using LC-ESI-MS/MS (TqDetector, Acquity Waters, USA) following a previous study reporting an analytical method for simultaneous identification of a wide range of pharmaceuticals, with minor changes (López-Serna et al., 2011). Separation was performed by using a Luna C18 (150 mm×2 mm ID, particle size 5 µm, Phenomenex, Torrance, USA) equipped with a SecurityGuard pre-column. The mobile phase composition consisted of binary mixtures with 0.1% formic acid in ACN (A) and 0.1% formic acid in water (B). The gradient of elution started at 5% A , then increased to 40% A in 5 min, 60% A in 10 min, reaching 100% A in 20 min and then return to initial conditions within 5 min. The system was operated at room temperature, the flow rate was set at 200 µL min−1 and 10 µL were injected. Carbamazepine, diazepam, propranolol and fluoxetine were analyzed under positive electrospray ionization mode (ESI+). Acquisition was performed in SRM mode using two transitions from $[M+H]^+$ precursor ion to daughter ions to identify each compound. The transitions used as well as the cone voltages and collision energies followed the above mentioned work (López-Serna et al., 2011). Quantification was based on external calibration standard 8 point curves (range between 0.1-1000 µg/L). Limits of detection and quantification (LD, LQ) defined as the minimum detectable amount of analyte with a signal to noise ratio of

3:1 and 10:1, respectively, were 0.14 and 0.51 ng/L for diazepam; 0.08 and 0.023 ng/L for carbamazepine, 0.03 and 0.07 ng/L for propranolol and 1.27 and 3.97 ng/L for fluoxetine . The data were acquired and processed using the MassLynx v4.1 software package.

Measured residue levels of the tested concentrations in freshly prepared and old solutions (Table S3, 0 and 48 h) were within 20% of nominal values (14 out of 16 samples) and having the max deviation of 30%. For the sake of clarity, we always refer to nominal values throughout the manuscript.

TABLES

Table S1. List of internal standard added before lipid extraction in order to perform relative semi-quantification of each family of lipids. All were purchased from Avanti Polar Lipids.

Lipid class	Lipid name	Formula	Ionization
Glycerolipids	MG 17:0	$C_{20}H_{40}O_4$	$[M+NH_4]^+$
	DG 1,3-17:0 D5	$C_{37}H_{67}D_5O_5$	$[M+NH_4]^+$
	TG 1,2,3-17:0	$C_{54}H_{104}O_6$	$[M+NH_4]^+$
Glycerophospholipids	PC 16:0 D31-18:1	$C_{42}H_{51}D_{31}NO_8P$	$[M+H]^+$
	LPC 17:0	$C_{25}H_{52}NO_7P$	$[M+H]^+$
	PE 16:0 D31-18:1	$C_{39}H_{45}D_{31}NO_8P$	$[M-H]^-$
	LPE 17:1	$C_{22}H_{44}NO_7P$	$[M-H]^-$
	PS 16:0 D31-18:1	$C_{40}H_{44}D_{31}NO_{10}PNa$	$[M-H]^-$
	LPS 17:1	$C_{23}H_{43}NO_9PNa$	$[M-H]^-$
	PG 16:0 D31-18:1	$C_{40}H_{45}D_{31}O_{10}PNa$	$[M-H]^-$
	LPG 17:1	$C_{23}H_{44}O_9PNa$	$[M-H]^-$
Sphingolipids	SM 12:0	$C_{35}H_{71}N_2O_6P$	$[M+H]^+$
Sterols	CE 17:0	$C_{44}H_{78}O_2$	$[M+NH_4]^+$

Table S2. Elemental composition of glycerophospholipids, glycerolipids, sphingolipids and sterols species found in *D. magna* lipid samples, calculated by mass accuracy within error of 5 mg/L, with atom constraints and with -0.5 ≤ DBE ≤ 50.0. DBE: double-bond equivalent. Elemental composition of neutral glycerolipids and sterols refer to their ammonium adducts detected under ESI (+), and elemental composition of glycerophospholipids and sphingolipids refer to their hydrogen adducts detected under ESI (+) for PC, LPC, PC-O/PC-P and SM, and under ESI (-) for PE, LPE, PE-O/PE-P, PG, LPG, PI, PS and LPS.

This table is reported within the annexes section, annex II.

Table S3. Nominal and measured (Mean± SD) concentrations (μg/L) of the tested chemicals in freshly prepared (0 h) and old (48 h) test solutions in selected treatments.

Chemical	**Nominal**		**Measured (0 h)**		**Measured (48 h)**	
		N	Mean	SD	Mean	SD
CBZ	0.1	4	0.121	0.032	0.828	0.059
CBZ	1	4	0.891	0.052	0.813	0.041
DZP	0.1	4	0.351	0.013	0.311	0.021
DZP	1	4	0.107	0.0090	0.091	0.0080
Pr	0.1	4	0.112	0.011	0.091	0.0070
Pr	1	4	0.0270	0.0050	0.021	0.0090
Fx	0.1	4	0.00130	0.00050	0.00090	0.00020
Fx	1	4	0.00110	0.00060	0.0010	0.00030

CBZ, DZP, Pr and Fx are carbamazepine, diazepam, propranolol and fluoxetine, respectively.

Table S4. One way ANOVA results for the major lipid groups of Figure 2 comparing pharmaceutical treatments and clones. For clarity only degrees of freedom and Fisher's coefficient are depicted.

	Pharmaceutical exposure experiment				**Clone experiment**			
	Treatment	Error			Clone	Error		
Lipid	df	df	F	P	df	df	F	P
TG	8	18	2.7	0.036	3	8	7.2	0.012
DG	8	18	13.6	<0.001	3	8	4.8	0.033
MG	8	18	23	<0.001	3	8	18.9	0.001
PC	8	18	2.2	0.081	3	8	6	0.019
LPC	8	18	16.3	<0.001	3	8	11.8	0.003
PE	8	18	0.9	0.519	3	8	6.9	0.013
LPE	8	18	9.1	<0.001	3	8	18.8	0.001
PS	8	18	1.8	0.154	3	8	28.4	<0.001
LPS	8	18	0.7	0.672	3	8	9.6	0.005
PI	8	18	12.2	<0.001	3	8	3.7	0.06
PC-O/PC-P	8	18	1.7	0.17	3	8	2.7	0.119
PE-O/PE-P	8	18	11.1	<0.001	3	8	7	0.013
SM	8	18	16.8	<0.001	3	8	2.5	0.136
CE	8	18	14.5	<0.001	3	8	5.3	0.027

Table S5. Individual lipids shown in Figure 3 and 4 (pharmaceuticals, mutated clones and both) obtained after PLS-DA analysis of all individual lipids in MetaboAnalyst. Only lipids with a VIP score higher than 1 are depicted. Lipids are given in the table in the same order that is represented in Figures 3 and 4.

Pharmaceuticals		Mutated clones		All samples	
Lipid	**VIP score**	**Lipid**	**VIP score**	**Lipid**	**VIP score**
Cluster 1		**Cluster 1**		**Cluster 1**	
PC 30:4	1.1585	PC-O 40:6/PC-P 40:5	1.0442	PS 38:0	1.1656
PE 34:4	1.0316	CE 20:4	1.3472	PS 38:3	1.1804
PE 34:5	1.0279	DG 36:2	1.0918	PS 38:1	1.2327
PC 40:4	1.2099	CE :205	1.2122	PS 38:2	1.1866
PC 38:2	1.0355	DG 34:0	1.4825	PC 40:8	1.2325
PC 40:8	1.0857	LPS 18:1	1.4376	PC 40:2	1.4153
PC 40:2	1.2050	LPE 20:1	1.5555	PC 38:2	1.2720
TG 60:4	1.1508	LPG 18:1	1.5524	PC 38:3	1.2190
Cluster 2		LPE 16:0	1.5053	PC 40:4	1.5287
DG 34:2	1.3178	LPE 20:3	1.5315	PC 30:4	1.4035
DG 36:3	1.3342	LPE 18:3	1.5305	PC 32:5	1.1369
DG 36:4	1.3236	LPE 18:1	1.5614	TG 60:4	1.3995
DG 30:1	1.3352	LPE 18:2	1.5473	TG 60:5	1.0961
DG 28:0	1.3774	MG 16:0	1.4659	TG 54:4	1.1120
DG 32:1	1.3529	MG 18:0	1.4369	TG 54:5	1.0463
DG 34:1	1.3376	MG 18:1	1.5006	PE 36:3	1.0570
PI 38:4	1.3285	MG 18:2	1.4602	PE 36:5	1.0311
PI 38:5	1.3108	LPC 20:3	1.5146	PE 32:3	1.0722
DG 32:2	1.2742	LPE 18:0	1.4784	PC 34:5	1.1669
DG 34:3	1.2626	LPS 20:0	1.4830	PC 34:4	1.0839
DG 34:4	1.2668	LPE 16:1	1.4362	PC 32:3	1.0207
DG 36:5	1.2758	LPC 18:1	1.5508	PC 36:3	1.0052
DG 36:6	1.2744	LPC 20:4	1.5459	PE 36:2	1.1417
DG 30:3	1.2483	LPC 18:2	1.5360	PC 36:2	1.0857
DG 32:4	1.1995	LPC 20:5	1.5233	TG 58:14	1.1826
DG 32:5	1.2387	LPC 16:1	1.5187	DG 38:4	1.0113
DG 34:5	1.2454	LPC 18:3	1.5471	PC 40:5	1.0635
DG 34:6	1.2469	LPC 16:0	1.5425	PC 34:6	1.0354
DG 32:3	1.2330	LPC 14:0	1.5339	PE 34:6	1.1836
DG 30:2	1.3168	LPC 18:0	1.5177	PE 34:4	1.2562
DG 44:9	1.2447	**Cluster 2**		PE 34:5	1.2127
PE-O 34:4 /PE-P 34:3	1.2176	CE 20:2	1.0306	**Cluster 2**	
PC 28:1	1.1225	PE-O 38:4/PE-P 38:3	1.2643	LPE 16:1	1.3713
DG 42:10	1.3081	PE-O 34:3/PE-P 34:2	1.1074	LPE 16:0	1.3260
DG 36:2	1.4071	PS 34:1	1.2545	LPC 18:3	1.2207

DG 32:0	1.4175	PC 38:3	1.2021	LPC 16:1	1.2171
DG 30:0	1.4112	PC 38:4	1.0133	LPC 16:0	1.1605
DG 36:0	1.4223	PC 38:2	1.3602	PC 44:10	1.0606
DG 36:1	1.4157	PC 40:5	1.5125	LPC 20:3	1.1334
DG 40:1	1.3864	PC 40:1	1.3599	LPC 18:0	1.0410
PC 44:10	1.3881	PC 38:1	1.4531	LPC 18:2	1.0705
DG 40:0	1.4251	PC 40:2	1.4263	LPC 18:1	1.0888
DG 38:0	1.4179	PC 36:7	1.2994	LPC 20:4	1.0631
PI 36:6	1.3736	PC-O 38:4/PC-P 38:3	1.1517	MG 18:1	1.1331
PI 32:2	1.3537	PE 32:1	1.3452	LPE 18:3	1.0231
PI 34:4	1.3462	PE 32:2	1.2886	LPE 18:0	1.2054
PI 32:3	1.3313	TG 44:2	1.0761	LPE 18:1	1.1663
PI 34:1	1.3498	TG 42:2	1.0650	LPE 18:2	1.1049
PI 34:3	1.3366	TG 44:4	1.0101	**Cluster 3**	
PI 36:4	1.3555	DG 38:4	1.3077	PC 28:1	1.0100
PI 36:2	1.3534	PS 36:5	1.2037	DG 44:9	1.1020
PI 36:3	1.3524	DG 44:10	1.0549	DG 36:4	1.2334
PI 36:5	1.3853	LPC 14:1	1.2434	DG 36:3	1.2308
PI 34:2	1.3760	TG 58:11	1.0306	DG 34:2	1.2173
PC-O 34:1/PC-P 34:0	1.3753	TG 58:6	1.0832	DG 34:3	1.1288
SM 22:0	1.4037	PC 30:3	1.0032	DG 30:2	1.3461
SM18:0	1.3982	PC 32:5	1.0419	DG 32:1	1.3143
SM 20:0	1.3976	PC 30:4	1.1693	DG 30:1	1.2912
SM 16:0	1.3783	PC 30:2	1.2224	DG 32:4	1.0644
SM 22:1	1.3764	PC 32:3	1.1801	DG 30:3	1.1815
SM 24:0	1.3905	PS 36:1	1.3766	DG 36:6	1.2842
SM 20:1	1.3953	PE 36:1	1.4378	DG 34:6	1.2209
SM 14:0	1.3901	PE 32:4	1.3558	DG 32:5	1.1682
PC-P 40:6	1.3783	PC 40:6	1.4640	DG 34:5	1.2027
DG 38:1	1.4085	PE 36:3	1.4323	DG 32:3	1.1796
PC 42:10	1.3769	PE 36:4	1.3548	DG 32:2	1.1738
TG 54:1	1.0680	PC 36:2	1.3987	DG 36:5	1.2405
TG 48:0	1.0427	DG 44:11	1.4163	DG 34:4	1.1847
TG 38:0	1.2270	DG 44:12	1.4070	PI 36:6	1.1527
TG 44:0	1.1665	DG 42:11	1.3585	PI 36:4	1.1016
TG 46:0	1.1506	PC 32:4	1.2807	PI 34:1	1.0899
TG 36:0	1.1326	PE 36:2	1.4268	PI 36:2	1.1020
TG 40:0	1.1751	PE 34:4	1.3862	PI 36:3	1.0970
TG 42:0	1.1685	PC 34:4	1.3573	PI 36:5	1.1748
LPE 20:3	1.0896	PC 36:1	1.3574	PI 34:2	1.1707
LPE 18:3	1.2987	PE 34:2	1.3839	PI 32:2	1.0175
LPE 18:2	1.3048	PC 32:1	1.3226	PI 32:3	1.0018
LPE 18:1	1.2939	PC 40:9	1.4362	PC 38:4	1.0366
LPE 16:1	1.3551	PC 34:6	1.3110	PC –O 38:2/PC-P	1.0927

				38:1	
LPE 16:0	1.3858	PC 40:4	1.3375	PC-O 36:1/PC-P 36:0	1.2409
LPE 18:0	1.3785	PS 36:4	1.3829	PE-O 30:1/PE-P 30:0	1.2854
PC 44:2	1.1463	PC 38:6	1.3777	PE-O 30:0	1.2629
PS 34:2	1.1918	PC 42:10	1.2237	PC-O 34:1/PC-P 34:0	1.4178
DG 38:2	1.3333	PS 36:2	1.2365	SM 20:0	1.4469
LPG 18:1	1.3183	PC 30:1	1.2017	SM 18:0	1.4213
LPC 18:0	1.3968	PE 34:1	1.3176	SM 20:1	1.3668
LPC 18:2	1.3804	PC 32:2	1.3162	SM 22:0	1.3947
LPC 18:3	1.3803	PC 34:5	1.3004	SM 14:0	1.3932
LPC 16:0	1.3849	PS 34:3	1.2463	DG 38:1	1.3628
LPC 14:0	1.3825	PE 34:6	1.2084	PC 42:10	1.3001
LPC 20:5	1.3171	PC 36:5	1.2773	MG 18:2	1.2517
LPC 20:4	1.3280	PC 36:3	1.3826	SM 24:1	1.3018
LPC 18:1	1.3736	PE 36:5	1.3601	SM 24:0	1.2499
LPC16:1	1.3639	PC 34:1	1.2739	SM 16:0	1.3485
PC-O 38:3/PC-P 38:2	1.2545	PC 34:2	1.3171	SM 22:1	1.3217
CE 20:5	1.2362	PC 36:4	1.3156	PI 38:5	1.3772
PC 36:6	1.1015	PC 38:5	1.3802	PI 38:4	1.3752
PC 34:3	1.3078	PE 32:3	1.3599	DG 34:1	1.4883
PC 38:7	1.2497	PS 36:3	1.3522	DG 30:0	1.4715
PC-O 34:3/PC-P 34:2	1.2166	PC-O 44:4	1.3740	DG 28:0	1.4372
MG 18:0	1.1958	PE 34:	1.3291	DG 36:2	1.4477
PS 34:1	1.0319	PE 36:6	1.2792	DG 36:0	1.4745
PS 36:3	1.1577	PS 38:3	1.4104	DG 36:1	1.5081
PS 36:2	1.0106	PS 38:2	1.3875	DG 32:0	1.4916
PC-O 38:4/PC-P 38:3	1.1444	PS 38:1	1.3971	DG 40:1	1.4086
PE-O 38:4/PE-P 38:3	1.0493	PS 38:0	1.2784	DG 40:0	1.4989
PS 34:3	1.1583	PG 34:3	1.2601	DG 38:0	1.4044
PC 32:0	1.2115	PS 38:4	1.3870	TG 38:0	1.1712
PC-O 36:1/PC-P 36:0	1.3137	PE 34:5	1.3491	TG 54:1	1.1451
PC 28:0	1.2040	PS 34:2	1.0088	TG 36:0	1.1442
CE 16:1	1.3104	TG 58:14	1.0596	TG 46:0	1.2037
CE 20:4	1.3708	DG 38:0	1.0286	TG 40:0	1.2318
CE 18:0	1.3885	PI 36:6	1.0712	TG 42:0	1.2791
CE 16:0	1.3976	DG 42:10	1.0397	TG 44:0	1.2501
CE 18:1	1.3970	PE-O 30:0	1.1282	PC-O 34:0	1.1766
CE 18:2	1.3918	PI 34:3	1.0837	CE 20:5	1.2709

DG 34:0	1.3920	PI 36:5	1.1747	LPC 20:5	1.5279
CE 20:3	1.3669	PI 34:4	1.2347	CE 20:4	1.5846
MG 18:1	1.3988	PE-O 30:1/PE-P 30:0	1.2307	DG 34:0	1.6516
MG 16:0	1.3826	PI 32:2	1.134	MG 16:0	1.6242
PC 38:4	1.3796	PI 32:3	1.0426	CE 16:2	1.3629
PE-O 34:3/PE-P 34:2	1.3081	PI 34:1	1.2487	CE 16:3	1.3229
CE 18:3	1.3916	PI 36:4	1.2143	CE 18:2	1.3600
MG 18:2	1.3925	PI 36:2	1.2341	CE 18:3	1.3256
CE 16:2	1.3825	PI 34:2	1.2414	CE 16:1	1.3829
CE 16:3	1.3565	PI 36:3	1.2372	CE 20:3	1.5586
PE-O 30:1/PE-P 30:0	1.4116			CE 18:0	1.5572
PE-O 30:0	1.3975			CE 18:1	1.4612
LPC 20:3	1.3909			CE 16:0	1.4266
PC-O 38:2/PC-P 38:1	1.3632			DG 38:2	1.3481
PC 30:0	1.3661			LPC 14:0	1.4933
PC 34:0	1.3633			LPG 18:1	1.2807
SM 24:1	1.3412			PC-O 34:3/PC-P 34:2	1.1536
				PC 38:7	1.1314

The reader is referred to the web version of this article (https://doi.org/10.1016/j.scitotenv.2020.139029) in order to access to the following excel data set:

Table excel 1. LION term associations and enrichment results per cluster.

FIGURES

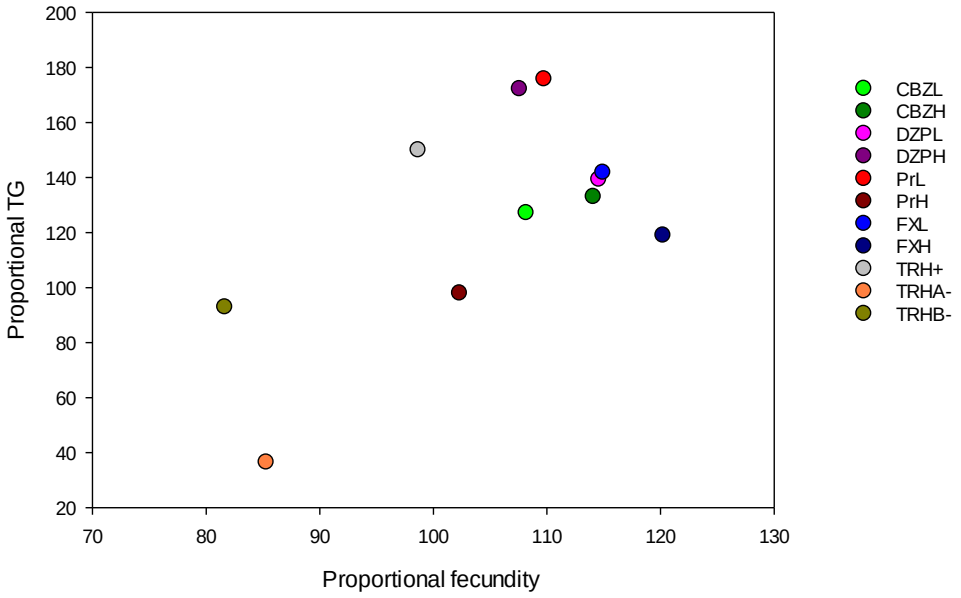

Figure S1. Bi-plot of fecundity versus TG levels of *D. magna* females exposed to the studied compounds and clones. Results are % relative to respective controls or wild type clone.

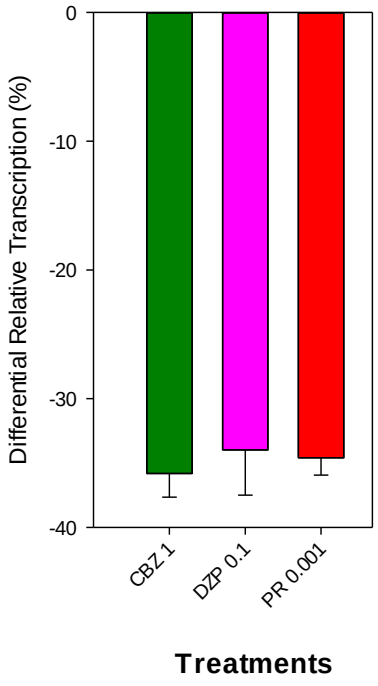

Figure S2. Differential gene transcription responses relative to control treatments (Mean SE, N=4) across the studied drugs and clones for ACAT gene. Data has been obtained from previous studies (Fuertes et al., 2019). Control treatments values are 0. Negative values indicated down-regulation. In the horizontal axis concentrations (μg/L) are depicted after the chemical name.

Chapter 3

3.3. Further discussion and final remarks

Assessing the risks of exposure to environmentally relevant concentrations of human prescribed pharmaceuticals and their mixtures is a prioritized research need. For this reason, in this chapter an integrative approach has been presented studying neuroendocrine disrupted effects produced by neuroactive pharmaceuticals at different levels of biological organization, as organism response (i.e. reproduction), gene expression (transcriptomics) and the subsequent lipid metabolism disruption (lipidomics) in *D. magna*.

In the scientific article III, the reproductive and transcriptional effects of environmental relevant concentrations of the pharmaceuticals CBZ, DZP and PR, and to a equimolar mixture of them, were determined. Effects on the gene expression of these ECs were assessed by means of the same custom-made microarray platform used in the previous chapter, designed from the complete set of gene models representing the whole transcribed genome of *D. magna* (Campos et al., 2018). Selected concentrations for single exposures where those that maximize reproduction: 1, 0.1 and 0.001 µg/L for CBZ, DZP and PR, respectively.

Mixture effects on reproduction were assessed at different equi-effective constituent concentrations, showing non-monotonic reproductive responses, in line with previous reported results (Rivetti et al., 2016). For transcriptomic study, the mixture having greater effects on reproduction was selected, which was that whose constituents were dosed at 1/3 of their individual exposures: 0.03, 0.3 and 0.0003 µg/L for CBZ, DZP and PR, respectively. The three tested ECs enhanced reproduction at the tested environmental relevant concentrations and induced specific transcriptome changes in *D. magna* individuals, showing additive action by the comparison of the results from single and mixture exposures. Transcriptomic analyses identified 3,248 DEGs by at least one of the treatments, which were grouped into four clusters. Two clusters gathered DEGs of either over- or under-represented mRNA levels relative to control in every treatment, and the third and fourth clusters grouped DEGs upon exposure to DZP and PR, respectively. Interestingly in the two first clusters that grouped the majority of the DEGs (1,897) transcriptomic effects for the three individual treatments and the mixture followed the same trend, being the mixture the treatment with the greatest effects. This may indicate that joint transcription profiles in mixtures represent not only the additive sum of individual compounds fingerprints, but also new activated signaling pathways that lead to a stronger response (Brun et al., 2019; Garcia-Reyero et al., 2012). It has been demonstrated that in most cases, joint effects

of environmental relevant complex mixtures are additive and can be safety estimated according to the concentration addition concept (Altenburger et al., 2018; Backhaus, 2014).

Functional analysis was further performed not only with *Drosophila* homologous, but also combining KEGG annotations from putative orthologs in other well-annotated species, including *Daphnia pulex* and other insect species such as *Aedes aegypti* and *Apis mellifera*. The four DEGs clusters shared major deregulated signaling pathways implicated on energy, growth, reproduction and neurologically related processes, which may be responsible for the observed reproductive effects. Obtained results indicated that the three tested pharmaceuticals disrupted, at environmental relevant concentrations, gene signaling pathways related to neurological (e.g. serotonergic and adrenergic signaling pathways, and cholinergic, dopaminergic, GABAergic and glutamatergic synapse) and reproductive functions (e.g. chitin metabolism, oocyte meiosis, progesterone mediated oocyte maturation, ovarian steroidogenesis and insulin signaling pathways), in line with the obtained increased fecundity after exposure to these compounds. PR is a competitive β-adrenergic receptor antagonist (Kalam et al., 2020) that has been described to decreased heart rate in *Daphnia* and deregulated the transcription of genes belonging to the β-adrenergic pathway (Jeong et al., 2018a). Nevertheless, obtained results did not point only to a specific disruption of adrenergic signaling in cardiomyocytes but to a boarder range of effects. In addition, CBZ is described to modulate voltage-gated sodium channels (Gambeta et al., 2020), however sodium channel GO category was not specifically enriched for CBZ exposure. In contrast, DZP is described as binding to the GABA-$_A$ receptor in an allosteric manner (Nutt and Malizia, 2001). GABAergic synapse appeared enriched and specifically under-expressed in samples after DZP exposure. It has been reported that phospholipase-C/calcium/calcineurin-signaling cascade converts the initial enhancement of GABA-$_A$ receptors by benzodiazepines to a long-term downregulation of GABAergic synapses, which is in line with obtained results (Nicholson et al., 2018). Furthermore, previous studies have observed metabolomic disruption of different neurotransmitters after exposure to these pharmaceuticals, reporting reduced GABA in DZP samples, together with lower levels of dopamine and histamine. This study also reported higher amounts of norepinephrine, an adrenergic neurotransmitter, produced by PR (Rivetti et al., 2019).

Different lipid-related pathways were enriched in the majority of clusters including the GO categories lipid metabolic process, glycerolipid and glycerophospholipid pathways, terpenoid backbone biosynthesis, the arachidonic acid

or the fatty acid metabolism KEGG pathways. The arachidonic acid metabolism categories contained mainly over-expressed transcripts, in line with the involvement of lipid and arachidonic acid metabolism in *Daphnia* reproduction (Ginjupalli et al., 2015) and with the reported alteration of different oxylipins belonging to arachidonic, linoleic and eicosapentaenoic acid metabolic pathways in *Daphnia* individuals exposed to the same pharmaceuticals (Garreta-Lara et al., 2018). *Daphnia*'s terpenoid backbone biosynthesis KEGG pathway is involved in the biosynthesis of the crustacean JH (LeBlanc, 2007). The transcription of the genes that constitutes this pathway was over-represented, pointing that this upregulation of JH pathway may also be involved in the observed effects on reproduction by these pharmaceuticals.

There is reported evidence of the interconnected nature of the endocrine and nervous systems. The central nervous system innervates the endocrine glands through direct axonal contact. Hormonal signals from the endocrine system influence the nervous system with effects based on environmental exposures (Manley et al., 2018). CBZ, DZP, FX and PR have been reported to disrupt oxylipins (especially eicosanoids) in *D. magna*, which are lipid mediators formed from polyunsaturated fatty acids (PUFAs) and also known to regulate reproduction (Garreta-Lara et al., 2018). Therefore, and due to the transcriptomic effects observed in different lipid-related pathways after exposure to the tested drugs, a disruption in lipid homeostasis is likely. In order to assess this, in the scientific article IV the effects of low concentrations of CBZ, DZP, PR, and also of FX, on the lipidome of *D. magna* females during their first reproductive cycle were assessed. FX was reported to produced up-regulation of lipid metabolic genes (Campos et al., 2019, 2013) and also to increase serotonin in *D. magna* (Campos et al., 2016; Rivetti et al., 2019). The hypothesis that serotonin may be involved in lipid dynamics was also tested by the analysis of the lipidome of genetically tryptophan hydrolase (TRH, rate limiting enzyme for serotonin biosynthesis) gene knockout clones, that showed down-regulated FA and lipid metabolsim gene pathways (Campos et al., 2019).

Regarding to the lipid extraction procedure, in chapter 2 a extraction based on Christie et al. procedure was applied, by a liquid-liquid separation using chloroform:methanol (2:1, v/v) but without phase separation (Christie, 1985). In this chapter a extraction procedure based on Folch's method was applied, performing also a chloroform:methanol 2:1 (v/v) lipid extraction but with a phase separation step afterwards (Folch et al., 1957). Although a quantitative and qualitative study was not carried out and no analytical parameters were determined to compare both extractions, in Figure 3.1 a qualitative comparison of the number and type of lipids determined by

both methods is displayed. Some lipids (55) were not measured after the application of Folch's method, probably lost during the phase separation step. However, it was possible to determine new lipid classes (i.e. CE, PC-P, PC-O, PE-P, PE-O and PI) present in lower concentrations in biological matrices, most likely due to the reduction of the number of other analytes and interferences in the biological matrix after phase separation. In this way, it was possible to have a wider vision of *Daphnia*'s lipidome increasing the number of lipid families determined, although at the expense of the number of individual lipids in some other lipid family, such as DG, PC or PE.

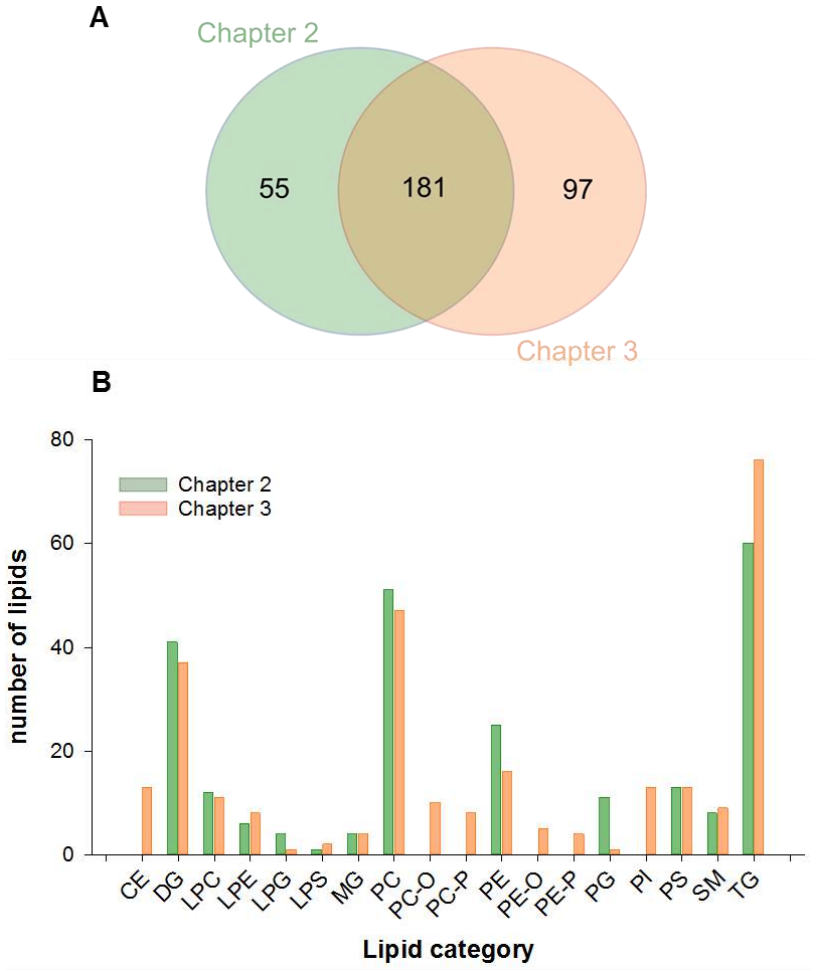

Figure 3.1. Comparison of lipids obtained by the different extraction methods applied through this thesis. A: Venn diagram of the total number of individual lipids determined in chapter 2 (Christie, 1985) and chapter 3 (Folch et al., 1957), and B: number of lipids extracted per lipid family.

Lipidomic analyses revealed disruption in different lipid families. Some TG species increased in most individuals exposed to the studied pharmaceuticals, in contrast to the lower accumulation of TG in TRH knock out clones, which also had lower reproduction. LION enrichment analysis showed that some TG species were

specifically enhanced in pharmaceutical treatments, which agree with the detected transcriptomic effects. *D. magna* exposed to PR, FX and higher dose of DZP showed higher levels of some glycerophospholipids (i.e. PC, PE, PS) and depletion of MG, DG, LPC and LPE, whereas mutated clones lacking serotonin showed the opposite effect. Glycerophospholipids are an important source of arachidonic and other long chain polyunsaturated FA, which in *Daphnia* have a key role in regulation growth and reproduction (Ginjupalli et al., 2015; Heckmann et al., 2008a, 2008b; Sengupta et al., 2016). The observed high levels of glycerophospholipids found in females having also enhanced levels of reproduction were in line with results reported in chapter 2. Furthermore, clusters corresponding to samples from exposures to PR, FX, and DZP at the higher dose present the peculiarity of, although being the amount of measured saturated and mono-unsaturated TGs relatively small, having a great amount of them as significant lipids down-regulated, whereas the amount of polyunsaturated PC and PE was enhanced. This could indicate a preferential hydrolysis of saturated or monounsaturated TG to FA that are oxidized in β-oxidation. Furthermore, the reported alteration of PC and PE membrane lipids can be a signal of harmful consequences, since polyunsaturated phospholipids confer higher melting point, intrinsic curvature and fluidity to the membranes than saturated phospholipids, altering their structure and functionality (Hashimoto and Hossain, 2018). Decreased levels of SM were also observed after exposure to DZP, PR and FX. There is reported evidence that compounds reducing metabolism of SM into ceramides or sphingosine (thus promoting SM accumulation) impaired reproduction (Sengupta et al., 2017). In contrast to this, in scientific article IV decreased SM was observed, enhancing reproduction by promoting ceramides or sphingosine.

The apparent different lipidomic disruption behavior between these pharmaceuticals at low and high tested concentration, especially for DZP, FX and PR is not surprising, as well as the reproductive effect of DZP and PR at the lowest tested dose but not at the highest one. Non-monotonic responses have been previously reported for these compounds (Rivetti et al., 2016). Many studies have point the non-monotonic response of different compounds, typical for endocrine disruptors, due to the complicated dynamics of receptor occupancy and saturation (Uchtmann et al., 2020). Furthermore, there is also an increased number of studies showing that effects of antidepressants at low concentrations do not follow a monotonic response (Fong and Ford, 2014; Ford and Fong, 2016), acting specifically on their target sites, whereas at high concentrations became toxic and hence impair survival, growth and reproduction.

Lipid profiles of PI were reduced in most treatments: at high concentration of diazepam and both concentrations of PR and FX. Polyphosphoinositides, which are produced by the phosphorylation of PI, have important cell signaling functions regulating lipid distribution and metabolism as well as ion channels, pumps and transporters (Balla et al., 2009), which is in line with transcriptomic enrichment of sodium ion transport and sodium ion activity. Furthermore, the mechanism of action of antidepressants and PR has been related with PI turnover. In this signaling system, an agonist binding to serotonergic, α-adrenergic and muscarinic receptors activated via receptor-coupled G_q pathways PI-specific phospholipases C (PI-PLC) is produced, that catalyze the hydrolysis of phosphatidyl-inositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and DG (Dwivedi et al., 2002). Both IP_3 and DG act as second messengers regulating intracellular Ca^{2+} mobilization and activating protein kinases. Transcriptomic deregulation of 4 gene enzymes belonging to this pathway was also observed.

The measured decreased lysophospholipids in *D. magna* females with enhanced reproduction may indicate an activated synthesis of glycerophospholipids, which is in line with transcriptomic results, where enrichment of glycerophospholipid pathways was determined, with a particular group of DEGs over-represented. This fact also supported the decreased levels of MG in females exposed to most of the tested neuroactive pharmaceuticals, which may also indicate that this lipid group is also being used to synthetize TG (Pol et al., 2014). This is in line with the over-expression of genes within the glycerolipid pathway. Conversely, the observed reduction of glycerophospholipids and enhanced levels of lysophospholipids in TRH knockout clones lacking serotonin (TRH-) can be associated with reduced allocation of lipids to reproduction and/or to the increased hydrolysis of glycerophospholipids by phospholipases (Kabarowski, 2009; Koizumi et al., 2010). Nevertheless, as the transcriptomic profiles of TRH- have not been determined within this study, increased phospholipase activity cannot be verified.

Links between serotonin and lipids were stablished in article IV. FX, compound described to increase serotonin, increased reproduction, meanwhile TRH knockout clones had the opposed effect. Lipid dynamics are known to be closely linked with reproduction. There is a cross-talk between serotonin receptors and arachidonic acid metabolism (Tournois et al., 1998), which in insects regulates reproduction (Stanley, 2006).In addition, an elevated brain arachidonic acid signaling has been related with deficiencies in serotonin transporters (Basselin et al., 2009). Within this study, opposite effect between reported pharmaceuticals lipidome affection and knockout clones

lacking serotonin was reported. As previously mention, reported transcriptomic results found over- and under-representation of neurological signaling pathways. CBZ, DZP and PR also deregulated insulin, arachidonic acid and steroid biosynthesis signaling pathways. The same trend was reported for serotonin manipulation experiments in *D. magna*. FX treated females showed enhanced brain serotonin activity and the upregulation of serotonergic, arachidonic and insulin signaling pathways (Campos et al., 2016, 2013) but downregulation in TRH- clones (Campos et al., 2019). Nevertheless also GABA receptors have been described to be activated by FX (Pinna et al., 2006). The above mentioned shared neurological pathways reported to be disrupted in this chapter contrast with the putative primary/secondary mode of action described for these drugs. This means that at the low and environmentally relevant concentrations the studied drugs may target alternative neurological signaling pathways. Peripheral serotonin (i.e. serotonin acting as gastrointestinal hormone) has been previously described to be related with lipid metabolism (Watanabe et al., 2011), promoting the efficient storage of energy by enhancing lipid anabolism (Watanabe et al., 2010; Wyler et al., 2017; Yabut et al., 2019). Nevertheless, despite that the serotonergic signaling pathway seems a priori a good candidate to explain the apparent contrasting lipidomic patterns between FX treatments and the two TRH knockout clones, the combined contribution of complementary neurological signaling pathways is also likely to regulate this lipid disruption together with fecundity responses in *D. magna*.

It is difficult to explain disruption in specific lipid categories due to the co-participation of each family in many different pathways. Nevertheless, the specific transcription found for some genes belonging to the glycerolipid and glycerophospholipid metabolism KEGG pathways can explain some of the lipidomic reported changes comparing the exposures at same concentrations between the two articles. These pathways were enriched after exposure to every studied pharmaceutical and the transcription of selected genes is represented in Figure 3.2. Observed reduction of CEs in *D. magna* females exposed to most of the tested drug treatments can be related to the enzymatic inhibition of acyl-coenzyme A: cholesterol-O-acyltransferase (ACAT) activity, which catalyzes the esterification of cholesterol with FA (Pol et al., 2014). This effect has also been reported in human cultured fibroblasts exposed to PR (Mazière et al., 1990). In addition, downregulation of the transcription of lysosomal acid lipase was observed. This enzyme catalyzes the deacylation of TG of endocytosed low density lipoproteins to generate free FAs and cholesterol (Zschenker, 2004), and thus its inhibition could explain detected enhancement of some TG species

after exposure to most of the studied compounds. The transcription of the phospholipase A2 (LPA2), that hydrolyzes glycerophospholipids to lysophospholipids, was downregulated by PR and upregulated by DZP at the lower tested concentration. This can explain the decreased and increased lysophospholipids in PR and DZP, respectively. Triacylglycerol lipase hydrolyzes TG to DG. Its increased transcription in DPZ exposures (at the lower concentration) could explain the reported increased DG. This lipase was downregulated for CBZ and PR, although hardly any effects were detected in their DG profile, which may be due to feedback from other lipid metabolism pathways.

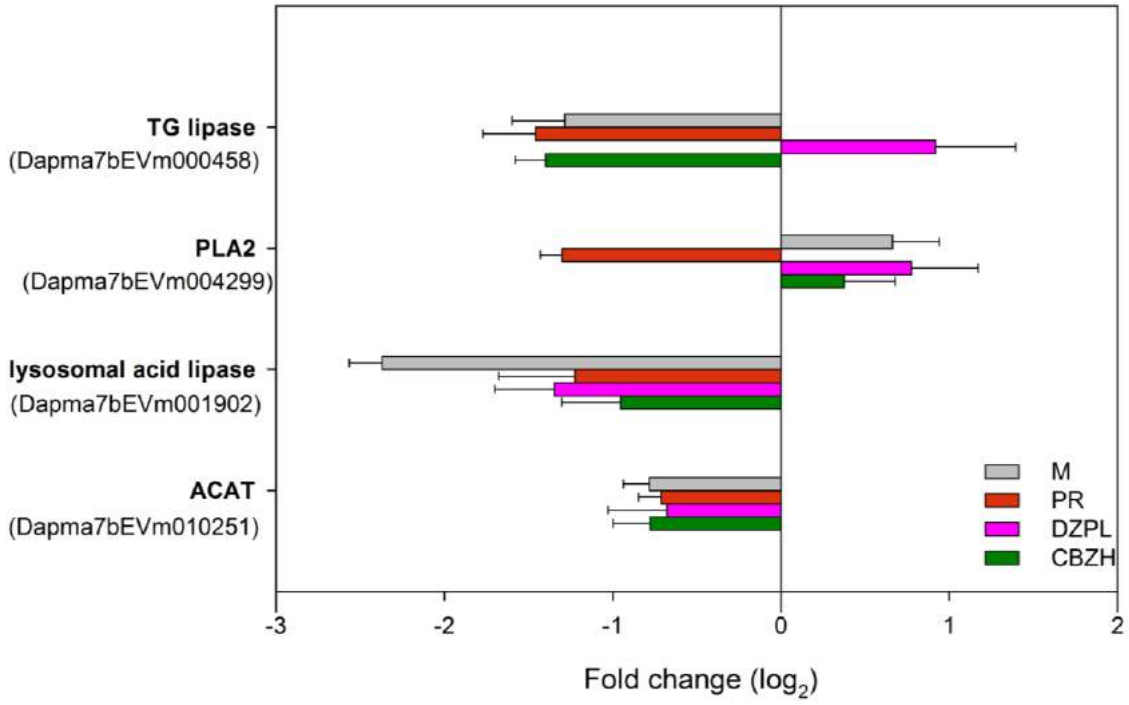

Figure 3.2. Fold change (log2) of four DEGs from glycerolipid and glycerophospholipid metabolism KEGG pathways. Values are mean ±SE (N=3). ACAT: acyl-coenzyme A: cholesterol-*O*-acyltransferase; PLA2: phospholipase A2

The present study has allowed relating effects produced by environmentally relevant concentrations of the reported neuroactive pharmaceuticals at transcriptomic level with effects produced in the lipidome, relating it also with fecundity effects. Transcriptomic and lipidomic results indicated that, although with some peculiarities, these drugs produced similar effects in the neuroendocrine system of *Daphnia* despite having a different described primary mode of action, at least in humans. Furthermore, a relationship between serotonin and lipid metabolism has been stablished, although further research is needed in order to clear up this aspect.

3.4. References

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Chapter 4

Daphnia magna as a model organism for neurotoxicity studies

4.1. Introduction

The number of potential neuroactive pollutants in the environment is increasing significantly, representing a great risk for the environment. Currently neurotoxicity assessment is mostly performed to predict and prevent harm to humans. Animal behavior is fast and sensitive, and its sublethal changes may affect trophic interactions and ecosystem function. Despite the increasing number of reports pointing species showing altered behavior, neurotoxicity assessment for environmental species is not required and therefore mostly not performed (Legradi et al., 2018). Indeed i*n vivo* tests with rodents are still the only accepted test for neurotoxicity risk assessment in Europe (Westerink, 2013). In order to make the neurotoxicity considered in risk assessment, novel test systems are needed. It is necessary to incorporated behavior assays into environmental toxicology testing, and particularly in neurotoxicity studies (Clotfelter et al., 2004; Legradi et al., 2018), and to stablish relationships between behavioral consequences and neuroactive compounds endpoints (Peterson et al., 2017). Recently, some recommendations have been made as a result of identifying necessities to be able to evaluate the effects of neuroactive contaminants. These include the study of the links between biomolecular process like transcriptomic or metabolomic changes and behavioral alterations and to investigate the relationship between known neurotoxic modes of action and the interaction with neurotransmitter receptors (Legradi et al., 2018). The present chapter is framed following these recommendations, presenting effects of different neuroactive chemicals on behavioral response, gene transcription and neurotransmitters.

This study was conducted using *D. magna* as model species, that has the advantage of sharing several vertebrates neurotransmitters and related gene pathways described to be targeted by many neuroactive chemicals (Dircksen et al., 2011; McCoole et al., 2011, 2012a; Weiss et al., 2012). In recent years an increasing number of studies have been conducted relating *Daphnia* behavior and the effect of neuroactive compounds in neurotransmitters (McCoole et al., 2011; Ren et al., 2015; Rivetti et al., 2016; Simão et al., 2019) and the transcriptomic disruption of neurological pathways (An et al., 2018; Christie and McCoole, 2012; Fuertes et al., 2019; McCoole et al., 2011, 2012b, 2012a). Nevertheless, only a very small number of studies have been focused on analyzing the amounts of neurotransmitters in *Daphnia* matrices with analytical techniques (Ehrenström and Berglind, 1988; Gómez-Canela et al., 2019; Rivetti et al., 2019). Furthermore, there are no studies that assessed simultaneously

the effects of neuroactive compounds relating behavioral response to neurotransmitter metabolomic responses or transcriptomic effects.

Neurotransmitters are endogenous metabolites elementary for neurotransmission. Many of them play a role not only as chemical messengers in the brain, but as hormones themselves in the peripheral tissues and being part of the neuroendocrine system regulating energetic metabolism, immunological responses, tissue pigmentation, social behavior, and development, growth and reproduction in various species (Waye and Trudeau, 2011). As stated before, neurotransmitters are described to be affected by many neuroactive chemicals. Furthermore, the wide variety of important functions of neurotransmitters means that they can have a great potential to be altered by environmental pollutants producing the alteration of key organism processes (Legradi et al., 2018; Rock and Patisaul, 2018). In fact, there is extensive research pointing that neurotransmission is able to be altered by different environmental stressors (Andersen et al., 2000; Horzmann and Freeman, 2016). Therefore, there is a growing interest in developing metabolomic strategies that allow the determination of different neurotransmitters for understanding the effect of contaminants on their alteration and how this imbalance alters key organism functions in different biological models (Gómez-Canela et al., 2019; Kim et al., 2020; Rivetti et al., 2019; Tufi et al., 2016; Wirbisky et al., 2015). Although metabolomics studies have evolved and increased in recent years, developing non-targeted strategies where a large number of metabolites are determined, only a few of them have detected and investigated neurotransmitter and related metabolites. Some of these studies have reported gas chromatography analysis techniques, having to deal with derivatization steps of the compounds (Hong et al., 2013; Zhang et al., 2020), which makes the use of liquid chromatography (LC) preferred. In last years, some researches have applied different LC-MS/MS methods for neurotransmitter analysis (Gómez-Canela et al., 2018; Konieczna et al., 2018; Pan et al., 2018; Wang et al., 2019). Due to the high polarity of neurotransmitters, the use of hydrophilic interaction liquid chromatography (HILIC) is suitable, without having to apply any derivatization step to increase neurotransmitter retention, as required in RPLC applications (Park et al., 2013; Tufi et al., 2015). In chapter 1 some of the advantages of HILIC chromatographic separation for metabolomic studies have already been presented. HILIC has been used in the study included in this chapter in order to optimize a metabolomic targeted approach by UHPLC coupled to a triple quadrupole (QqQ) mass analyzer.

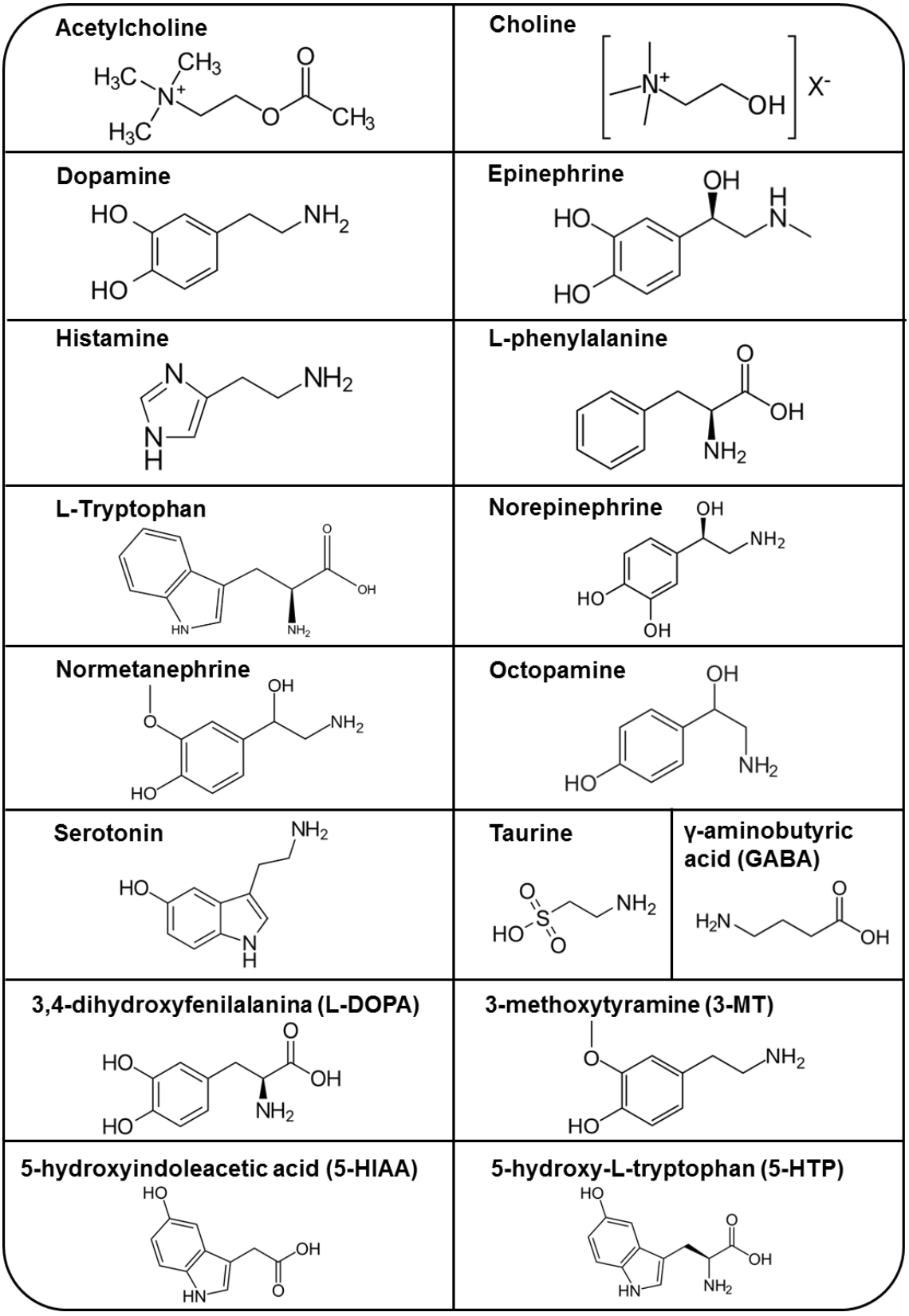


Figure 4.1. Neurotransmitters analyzed within article V in the present chapter.

The neurotransmitters analyzed in this chapter are detailed in figure 4.1. They belong to the most important neurotransmitter systems, as well as some of their precursors and metabolites. Their biosynthetic and metabolomic pathways are illustrated in Figure 4.2. The key neurotransmitter in the cholinergic system is acetylcholine, the major neurotransmitter in the parasympathetic nervous system and at neuromuscular junctions. It is formed from acetyl-CoA and choline via choline acetyltransferase (ChAT), and has been implicated in arousal, reward, and learning and memory. Histamine is released from histaminergic neurons, and within the central nervous system (CNS), is associated with wakefulness, feeding, learning and memory (Horzmann and Freeman, 2016). In the serotonergic neurons, amino acid L-tryptophan is converted to 5-hydroxytryptophan (5-HTP) by the action of the enzyme tryptophan hydroxylase, and subsequently converted to the neurotransmitter serotonin by the enzyme aromatic amino acid decarboxylase (AAAD). Serotonin can be then metabolized to 5-hydroxyindoleacetic acid (5-HIAA). It has been associated with motor function, circadian rhythms, arousal and depression (Horzmann and Freeman, 2016). Phenylalanine is the precursor of tyrosine, and thus of dopamine, norepinephrine, epinephrine and octopamine. In the dopaminergic neurons, tyrosine hydroxylase produces 3,4-dihydroxyfenilalanina (L-DOPA), that is converted by AAAD to the catecholamine dopamine, that can be metabolized to 3-methoxytyramine (3-MT). In adrenergic neurons, dopamine is converted to norepinephrine by dopamine beta-hydroxylase and can be further converted by phenylethanolamine-N-methyltransferase to epinephrine, or by catechol O-methyltransferase to normetanephrine (Horzmann and Freeman, 2016). Octopamine is closely related to norepinephrine, and synthesized by an homologous pathway in the biosynthetic pathways for catecholamines and trace amines, and it has been implicated in regulating aggression in invertebrates as *Drosophila* (Zhou et al., 2008). In some invertebrates, adrenergic signaling is considered absent and analogous functions being performed by octopamine and its precursor tyramine (Bauknecht and Jékely, 2017). Norepinephrine and epinephrine have not been unequivocally identified in *Drosophila*, although low concentrations have been detected in some other insect species, meanwhile crustaceans have been reported to use both signaling pathways (Adamo, 2008; Gallo et al., 2016). GABA is the major inhibitory neurotransmitter in the CNS reducing excitability (Horzmann and Freeman, 2016). Many sedative drugs act by enhancing its effects. Taurine is a β-amino acid present in high concentrations in different areas of the central nervous system, participating in processes as signal transduction, modulation of calcium movement or neurotransmission. It is not considered a classic neurotransmitter because to date a specific receptor has not yet been identified, and its release is

independent of Ca^{2+}, a vital element in the process of neurotransmitter release. It is known that taurine is an agonist of $GABA_A$ receptors (Ochoa-de la Paz et al., 2019).

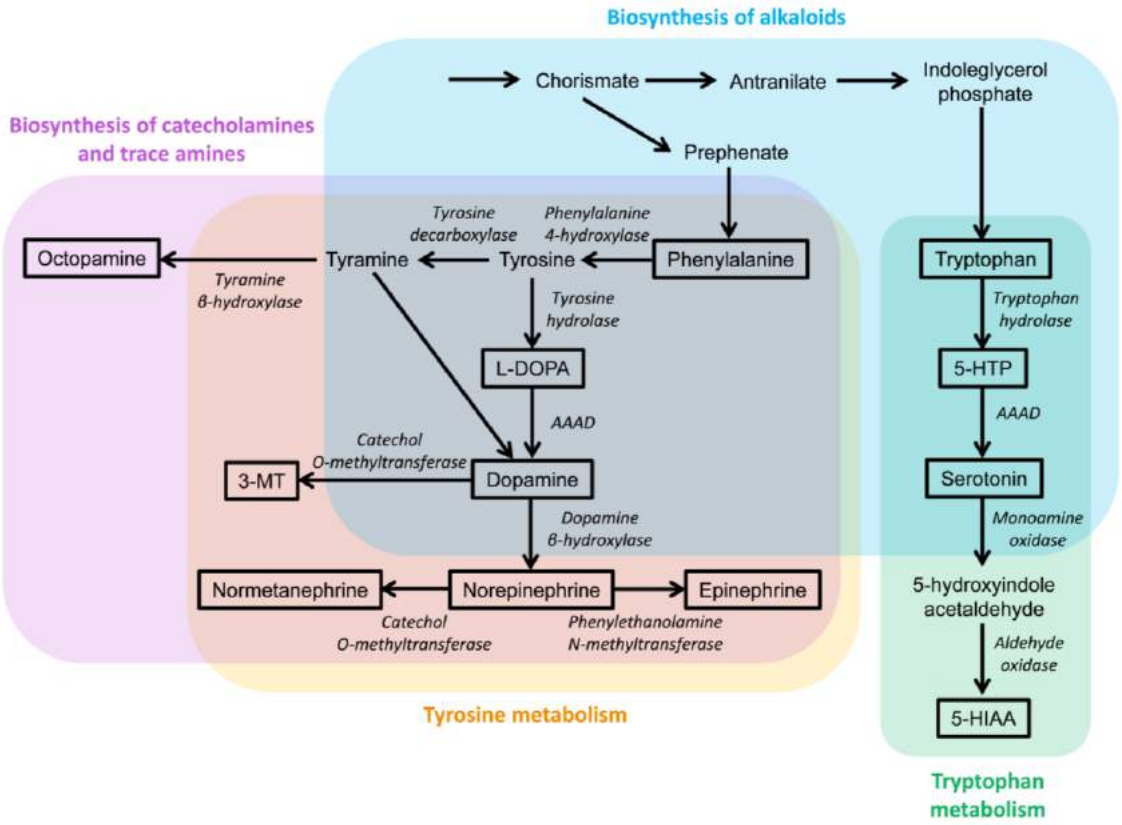


Figure 4.2. Pathway created from detailed KEGG pathways, which related some of the neurotransmitters of interest in this chapter. Black-boxed compounds are those that have been analyzed within scientific article V. In italics, the catalytic enzymes of the reactions of interest are detailed.

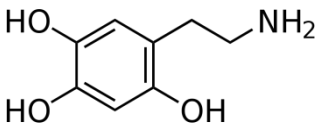
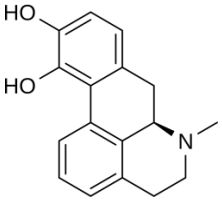
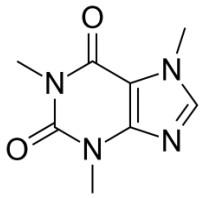
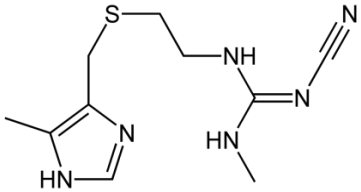
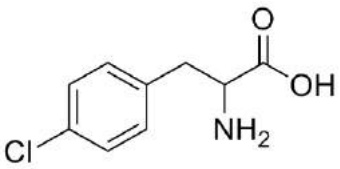
The analysis of a wide range of single chemical compounds is the first critical step in the identification of defense mechanisms pathways that may be shared among stressors, leading to a better understanding of mechanisms (Orsini et al., 2018). Untargeted transcriptomic analysis by whole transcriptome next-generation sequencing (i.e. RNAseq) provides a powerful tool for investigate the organism molecular mechanism after a chemical exposure (Schirmer et al., 2010; Wang et al., 2009), and thus unraveling the effects of neuroactive chemicals. In the present chapter, early transcriptional response after 24 h exposure to selected neuroactive chemicals was assessed. After an initial acclimation phase, genes interacting with the environment generally return to their initial expression point (Eng et al., 2010; Orsini et al., 2018), so it is important to identify the mechanisms of early response. Early response is critical to survival and fitness in later life stages and requires a fine adjustment of the molecular

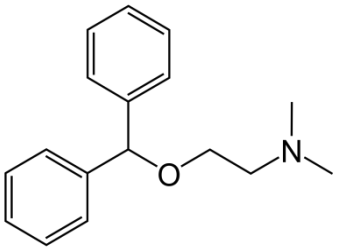
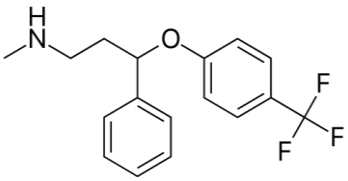
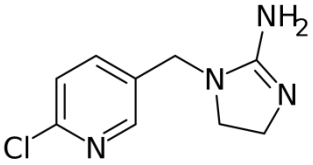
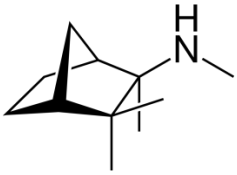
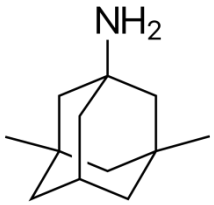
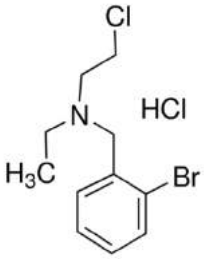
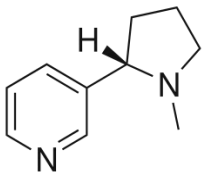
machinery regulating physiological and behavioral responses (Brooks et al., 2011; Orsini et al., 2018). Furthermore, gene transcription responses linked to specific toxicological modes of action are tissue or organ specific, thus toxicological studies should focus in specific organs (i.e. CNS). For this reason, within this chapter the neurotoxic effects were center in the cephalic transcriptome (i.e. *Daphnia*'s head).

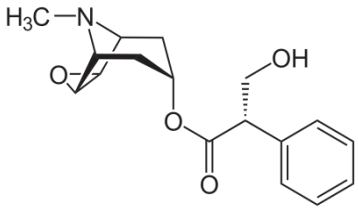
In the present chapter, the effects of several neuroactive chemicals were studied, which structures and described modes of action in humans are summarized in Table 4.1. 6-hydroxydopamine (6OH) and apomorphine (APO) are two compounds described to affect the dopaminergic system. 6OH is a synthetic organic neurotoxic compound used in research for the selective destruction of dopaminergic and noradrenergic neurons (Breese et al., 2005; Feng et al., 2014). APO is a synthetic derivative of morphine used for Parkinson's disease and non-selective agonist of dopamine receptor activating on D_2-like receptors, also described to have affinity for serotonin and adrenergic receptors (Bownik et al., 2018; Jenner and Katzenschlager, 2016). Cimetidine (CIM) and diphenhydramine (DIPH) are two neuroactive compounds described to affect the histaminergic system. CIM is a histamine H_2 receptor antagonist used to treat ulcers (McCoole et al., 2011), and DIPH is a histamine H_1 receptor antagonist used for the treatment of allergies (Berninger et al., 2011). Imidacloprid (IMI), nicotine (NIC), mecamylamine (MEC) and scopolamine (SCO) are compounds that interfere in the cholinergic neurotransmitter system. IMI is the most widely used neonicotinoid insecticide in the world and insect nicotinic acetylcholine receptors (nAChRs) agonist, present in water bodies all over the world at concentrations up to 225 µg/L (Sánchez-Bayo et al., 2016). NIC is a potent neuroactive alkaloid found mainly in the tobacco plant and MEC is an antihypertensive drug, which are described to affect nicotinic acetylcholine receptors nAChRs in an agonist and antagonist manner, respectively. SCO is used to treat motion sickness and postoperative nausea and vomiting, and is reported as being a muscarinic acetylcholine receptors (mAChRs) agonist (M. Faria et al., 2019). Chloro-DL-phenylalanine (PCPA), also known as Fenclonine, and fluoxetine (FX), one of the studied neuroactive pharmaceuticals in Chapter 3, affect the serotonergic neurotransmitter system. PCPA is known to be a tryptophan hydrolase inhibitor (Melissa Faria et al., 2019) and thus an inhibitor in the synthesis of serotonin (Geller and Blum, 1970), and FX, the active component of Prozac, is a selective serotonin reuptake inhibitor (SSRI) antidepressant. SSRIs act increasing deficient serotonin by inhibiting its reuptake, which is postulated as the cause of depression (Rivetti et al., 2016; Xue et al., 2016). N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4) is a compound known to destroy noradrenergic neurons

(Castelino and Ball, 2005). Memantine (MEM) is a neuroactive pharmaceutical used for Alzheimer's disease treatment, that is known to affect glutamatergic system being a N-Methyl-D-aspartate (NMDA) receptor antagonist (Faria et al., 2019; Rogawski and Wenk, 2006) Caffeine (CAFF) is a commonly known psychoactive drug present in coffee, known to disrupt different neurotransmitter systems and to stimulate the central nervous system (CNS) (Steele et al., 2018).

Table 4.1. Neuroactive chemicals tested within this chapter. Their structures, applications and described mode of action are detailed.

Neuroactive chemical	Structure	Applications	Mode of action
6-hydroxydopamine (6OH)		Synthetic organic neurotoxic compound used in research	Selective destruction of dopaminergic and noradrenergic neurons
Apomorphine (APO)		Parkinson's desease treatment	Non-selective dopamine-D_2 agonist (Bownik et al., 2018)
Caffeine (CAFF)		Psychoactive drug present in coffee	CNS stimulant (Steele et al., 2018)
Cimetidine (CIM)		Ulcer treatment	H_2-Receptor antagonist (McCoole et al., 2011)
Chloro-DL-phenylalanine (PCPA)		Human drug also known as Fenclonine	Tryptophan hydrolase inhibitor inhibitor (Melissa Faria et al., 2019)

Diphenhydramine (DIPH)		Allergies treatment	H_1-Receptor antagonist (Berninger et al., 2011).
Fluoxetine (FX)		Antidepressant	Selective serotonin reuptake inhibitor (SSRI) (Xue et al., 2016)
Imidacloprid (IMI)		Neonicotinoid insecticide	Insect nicotinic AChRs agonist (Sánchez-Bayo et al., 2016)
Mecamylamine (MEC)		Antihypertensive drug	Nicotinic AChRs antagonist (M. Faria et al., 2019)
Memantine (MEM)		Treat moderate to severe Alzheimer´s disease	Antagonist of the NMDA receptor (Melissa Faria et al., 2019)
N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4)		Neurotoxin	Destroys noradrenergic neurons (Castelino and Ball, 2005),
Nicotine (NIC)		Alkaloid found in the tobacco	Nicotinic AChRs agonist (M. Faria et al., 2019)

Scopolamine (SCO)		Motion sickness and postoperative nausea and vomiting treatment	Muscarinic AChRs antagonist (M. Faria et al., 2019)

4.2. Experimental section and results

In this chapter an integrative approach is presented linking effects of neuroactive chemicals at different levels of biological organization: organism response (i.e. behavioral response), gene expression (transcriptomics) and neurotransmitter disruption (targeted metabolomics). Results are presented in two scientific articles, the first one already published and the second one about to be submitted:

- Scientific article V:

  Fuertes, I., Barata, C., 2021. Characterization of neurotransmitters and related metabolites in *Daphnia magna* juveniles deficient in serotonin and exposed to neuroactive chemicals that affect its behavior: A targeted LC-MS/MS method. Chemosphere 263, 127814. https://doi.org/10.1016/j.chemosphere.2020.127814

The aim of this study was to develop an appropriate, accurate and sensible method of analysis of neurotransmitters in *D. magna*, which allows the characterization of the vast majority of metabolic pathways related to neurotransmitters, thus enabling to understand the effects of neuroactive compounds. For that purpose, hydrophilic interaction liquid chromatography (HILIC) was applied coupled to a QqQ MS. A targeted metabolomic method LC-MS/MS was developed for the analysis of neurotransmitters in *Daphnia* samples, properly evaluated in terms of comprehensive mass spectral characterization, selectivity, linearity, accuracy, precision and sensitivity. The suitability of this method was validated by their analysis in genetically mutated CRISPR tryptophan hydrolase (TRH) *D. magna* individuals (already studied in chapter 3) that should lack serotonin. In addition, the method was applied to determine the effects of chemical compounds known to affect different neurotransmitter systems. The referred chemicals tested within this study are reported in Table 4.1, with the exception of FX, whose effects on neurotransmitters have already been evaluated in previous studies (Rivetti et al., 2019). These chemicals were also tested for changes in *D. magna* behavior.

- Scientific article VI:

  Fuertes, I., Piña, B., Barata, C., De Schampheleare, K., Asselman, J., 2021. Effects of behavior-disrupting neuroactive chemicals in *Daphnia magna* cephalic transcriptome. To be submitted to Environmental Science and Technology.

The aim of this study was to explore the effects of 6OH, APO, DIPH, FX, MEM and PCPA in *D. magna* linking behavioral alterations and effects on early transcriptomic biomolecular processes in tissues enriched with nervous cells (the head). Untargeted transcriptomic analysis was assessed through RNAseq. High throughput sequencing data was validated by qRT-PCR.

4.2.1. Scientific article V

Characterization of neurotransmitters and related metabolites in *Daphnia magna* juveniles deficient in serotonin and exposed to neuroactive chemicals that affect its behavior: A targeted LC-MS/MS method.

Fuertes, I., Barata, C., 2021.
Environ. Sci. Technol. 53.

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Characterization of neurotransmitters and related metabolites in *Daphnia magna* juveniles deficient in serotonin and exposed to neuroactive chemicals that affect its behavior: A targeted LC-MS/MS method

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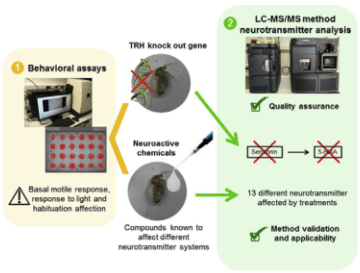
HIGHLIGHTS

- Neurotransmitter metabolites in *Daphnia magna* were determined by a targeted LC-MS/MS, determining up to 17 metabolites belonging to five different neurotransmitter systems.
- The method was validated using animals deficient in serotonin and neuroactive compounds.
- Knockout animals for tryptophan hydrolase gene showed low levels of serotonin related metabolites.
- The 12 tested neuroactive substances affected behavioral escape responses to light.
- Up to 13 metabolites were affected by the studied compounds in an expected way.

GRAPHICAL ABSTRACT



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ABSTRACT

Neurotransmitters are endogenous metabolites that play a crucial role within an organism, at the chemical synapses. There is a growing interest in their analytical determination for understanding the neurotoxic effect of contaminants. *Daphnia magna* represents an excellent aquatic model for these environmental studies, due to its similarities with vertebrates in several neurotransmitters and related gene pathways and because of its wide application in ecotoxicological studies. Within this study, an accurate and sensible method of analysis of 17 neurotransmitters and related precursors and metabolites was developed. The method was validated in terms of sensitivity, reproducibility, precision, and accuracy, and also matrix effect was evaluated. As an independent probe of method validation and applicability, the method was applied to two different scenarios. First, it was used for the study of neurotransmitter levels in genetically mutated tryptophan hydrolase *D. magna* clones, confirming the absence of serotonin and its metabolite 5-HIAA. Additionally, the method was applied for determining the effects of chemical compounds known to affect different neurotransmitter systems and to alter *Daphnia* behavior. Significant changes were observed in 13 of the analyzed neurotransmitters across treatments, which were related to

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https://doi.org/10.1016/j.chemosphere.2020.127814

Neurochemicals
Behavior

the neurotransmitter systems described as being affected by these neurochemicals. These two studies, which provide results on the ways in which the neurotransmitter systems in *D. magna* are affected, have corroborated the applicability of the presented method, of great importance due to the suitability of this organism for environmental neurotoxicity studies.

1. Introduction

Neurotransmitters (NTs) are endogenous metabolites elementary for neurotransmission. NTs play a fundamental task in the organism in response to stress, motor coordination regulation, control of psychomotor, gastrointestinal and homeostatic function and inter-neuronal communication (Matys et al., 2020). In humans, imbalance in neurotransmitters are known to produce a wide range of neurological disorders, such as neuropsychiatric disorders, Alzheimer's disease, attention deficit hyperactivity disorder (ADHD), hyperserotonemia or autism spectrum disorder (ASD) (Engert and Pruessner, 2008; Heyer and Meredith, 2017; Khachaturian, 1985; Kurian et al., 2011). Drugs, pharmaceuticals, chemotherapeutic agents, radiation, food additives, pesticides and heavy metals can affect neurotransmission (Andersen et al., 2000; Horzmann and Freeman, 2016). Thus, there is a growing interest in the analytical determination of neurotransmitters for understanding the effect of contaminants on them and how this imbalance disrupts key organism functions in different biological models (Gómez-Canela et al., 2019; Kim et al., 2020; Rivetti et al., 2019; Tufi et al., 2016; Wirbisky et al., 2015).

Analysis of neurotransmitters in biological matrices, and particularly in small organism such as *Daphnia*, requires efficient extraction methods and precise, reliable, accurate and sensitive analysis techniques, due to their low concentration. The development of different analytical methods for metabolomics studies has been widely expand in recent years (Burgess et al., 2014; Emwas et al., 2019; Fiehn, 2002; Labine and Simpson, 2020; Liu et al., 2019), but only a few of them have detected and investigated neurotransmitter and related metabolites. Some studies have reported gas chromatography analysis techniques, having to deal with derivatization steps of the metabolites (Hong et al., 2013; Zhang et al., 2020), which makes the use of liquid chromatography (LC) preferred. LC coupled to triple quadrupole mass spectrometry (LC-MS/MS) is particularly suitable for the analysis of targeted metabolites because its extraordinary sensitivity and selectivity (Gómez-Canela et al., 2013). In last years, some researchers have applied different LC-MS/MS methods for NTs analysis (Gómez-Canela et al., 2018; Konieczna et al., 2018; Pan et al., 2018; Wang et al., 2019). In this context, and due to the high polarity of neurotransmitters, the use of hydrophilic interaction liquid chromatography (HILIC) has the advantage to retain very polar compounds without applying any derivatization step to increase their retention, as required in reversed-phase LC applications (Park et al., 2013; Tufi et al., 2015). Furthermore, due to its selectivity, HILIC provides greater freedom from matrix effects, compared to reverse phase LC (Van Eeckhaut et al., 2009). For all these reasons, HILIC couple to MS/MS is a promising and reliable technique, which is increasingly being applied in more studies of neurotransmitter analysis in different organism (Danaceau et al., 2012; Olesti et al., 2019; Rivetti et al., 2019; Sardella et al., 2014; Tufi et al., 2015).

The crustacean and aquatic ecotoxicological model organism *Daphnia magna* is a suitable model to study the effects and toxicological consequences of environmental contaminants that produce neurotransmitter disorders. This invertebrate share with vertebrates several neurotransmitters and related gene pathways (Dircksen et al., 2011; McCoole et al., 2011, 2012a; Weiss et al., 2012a). However, there are few studies that focus on analyzing neurotransmitter levels in *D. magna*. Neurotransmitter-related studies in this model specie have been mainly focus in the transcriptomic disruption of neurological pathways (An et al., 2018; Christie and McCoole, 2012; Fuertes et al., 2019; McCoole et al., 2011, 2012b, 2012a) or about the anatomic or functional characterization of its brain (Barry, 2002; Kress et al., 2016; Weiss et al., 2012b). In recent years, studies relating *Daphnia* behavior and the effect of neuroactive chemicals in NTs have been developed (McCoole et al., 2011; Ren et al., 2015; Rivetti et al., 2016; Simão et al., 2019). Only a scarce number of studies have been focused on analyzing the amounts of neurotransmitters in *Daphnia* matrices with analytical techniques, using ion-pair reversed phase liquid chromatography with electrochemical detection (Ehrenström and Berglind, 1988) or by liquid chromatography-mass spectrometry (Gómez-Canela et al., 2019; Rivetti et al., 2019). The previous mentioned studies had certain limitations. The study of Rivetti et al. (2018) was only limited to 8 key neurotransmitters and neglected related metabolites and precursors, and thus provided limited information of metabolomic neurological pathways. Furthermore, in terms of sensitivity, it had relatively high detection limits. The study of Gómez-Canela et al. (2019), despite of characterizing a large number of metabolites, was unable to quantified serotonin, one of the targeted neurotransmitter of many neurochemicals, being only partial validated across pharmaceuticals whose mode of action in non mammalian species is uncertain. Pharmacological treatments are difficult to interpret because they are generally not specific and can have unwanted side effects on other neurotransmitters. Thus, the use of reverse genetic models deficient in specific neurotransmitters offers a more robust way to validate them. Reverse genetics have been widely used to validate gene metabolic pathways (Perkins et al., 2006; Yamada et al., 2003) and to a lesser extent metabolite pathways (Begolo and Clayton, 2016; Bringaud et al., 2015). Recently, using CRISPR genome editing methods, we obtained *D. magna* clones having bi-allelic mutations on the serotonin synthesis rate limiting gene/enzyme tryptophan hydrolase (Rivetti et al., 2018). These mutated clones showed lack of brain serotonin immuno-reactivity and were also deficient in serotonin.

The aim of this study is to develop an appropriate, accurate and sensible method of analysis of a high number of neurotransmitters in *D. magna*, which allows the characterization of the vast majority of metabolite pathways related to neurotransmitters, thus having a fast and reliable tool to be able to understand the effects of neuroactive chemicals. For that purpose, hydrophilic interaction liquid chromatography (HILIC) was applied as reported in previous studies (Rivetti et al., 2019) but developed for a much higher number of targeted metabolites, characterizing the vast majority of neurotransmitter systems, and coupled to a more sensible and reliable MS/MS analyzer.

The neurotransmitters analyzed in this study belong to the most important neurotransmitter systems, as well as some of their precursors and metabolites. These include the major NT of the cholinergic system, acetylcholine, and its precursor choline,

implicated in arousal, reward, and learning and memory (Robinson et al., 2011). Histamine is released from histaminergic neurons and is associated with wakefulness, feeding, learning and memory (Horzmann and Freeman, 2016). In the serotonergic neurons, amino acid L-tryptophan is converted to 5-hydroxytryptophan (5-HTP) by the action of the enzyme tryptophan hydroxylase, and subsequently converted to the neurotransmitter serotonin by the enzyme aromatic amino acid decarboxylase (AAAD). Serotonin can be then metabolized to 5-hydroxyindoleacetic acid (5-HIAA). Serotonin has been associated with motor function, circadian rhythms, arousal and depression (Horzmann and Freeman, 2016). Phenylalanine is the precursor of tyrosine, and thus of dopamine, norepinephrine, epinephrine and octopamine. In the dopaminergic neurons, tyrosine hydroxylase produces 3,4-dihydroxyfenilalanina (L-DOPA), that is converted by AAAD to the catecholamine dopamine, that can be metabolized to 3-methoxytyramine. In adrenergic neurons, dopamine is converted to norepinephrine by dopamine beta-hydroxylase and can be further converted by phenylethanolamine-N-methyltransferase to epinephrine, or by catechol O-methyltransferase to normetanephrine (Horzmann and Freeman, 2016). Octopamine is closely related to norepinephrine, and synthesized by an homologous pathway in the biosynthetic pathways for catecholamines and trace amines, and it has been implicated in regulating aggression in invertebrates as *Drosophila* (Zhou et al., 2008). In some invertebrates, adrenergic signaling is consider absent and analogous functions being performed by octopamine and its precursor tyramine (Bauknecht and Jékely, 2017). Norepinephrine and epinephrine have not been unequivocally identified in *Drosophila*, although low concentrations have been detected in some other insect species, meanwhile crustaceans have been reported to use both signaling pathways (Adamo, 2008; Gallo et al., 2016). GABA is the major inhibitory neurotransmitter in the CNS reducing excitability (Horzmann and Freeman, 2016). Taurine is a β-amino acid present in high concentrations in different areas of the CNS, participating in processes as signal transduction, modulation of calcium movement or neurotransmission. It is also an agonist of $GABA_A$ receptors (Ochoa-de la Paz et al., 2019).

The performance of the LC-MS/MS method was evaluated in terms of comprehensive mass spectral characterization, selectivity, linearity, accuracy, precision and sensitivity. The application of this technique for the analysis of serotonergic metabolites was validated, after performing behavioral assays, by their analysis in genetically mutated CRISPR tryptophan hydrolase (TRH) *D. magna* individuals, which should be deficient in serotonin (Rivetti et al., 2018). Furthermore, the response of the remaining neurotransmitters, and also of serotonergic, ones was studied in *D. magna* individuals exposed to 12 chemicals known to alter the behavior of mammals and fish and modulate the cholinergic, serotonergic, dopaminergic, adrenergic, histaminergic and GABAergic systems (details of selected neurochemicals, their mode of action and reported studies are in Table S1, Supplementary Material).

2. Experimental

2.1. Chemicals and materials

Pure analytical standards of neuroactive chemicals used for the exposition experiments were purchased from Sigma-Aldrich (USA/Netherlands): apomorphine (APO; CAS 41372-20-7; purity ≥98.5%), caffeine (CAFF; CAS 58-08-2; purity ≥99%), chloro-DL-phenylalanine (PCPA; CAS 7424-00-2; purity ≥98.5%), cimetidine (CIM; CAS 51481-61-9; purity ≥98%), 6-hydroxydopamine (6OH; CAS 28094-15-7; purity ≥97%), imidacloprid (IMI; CAS 138261-41-3; purity ≥98%), memantine (MEM; CAS 41100-52-1, purity ≥97%), N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4; CAS 40616-75-9; purity ≥98%), nicotine (NIC; CAS 54-11-5, purity ≥99%) and scopolamine (SCO; CAS 55-16-3; purity ≥90%); except diphenhydramine (DIPH; CAS 147-24-0; purity ≥98%), that was purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and mecamylamine hydrochloride (MEC; CAS 826-39-1, purity ≥98%), that was purchased from Tocris Bioscience (Minneapolis, MN, USA). Detailed information about the studied neurochemicals is depicted in Table S1 (Supplementary Material).

Neurotransmitter and isotope-labelled analytical standards, solvents and acids used for chemical analyses and procedures to prepare stock solutions are described in Supplementary Material and Table S2.

2.2. Experimental animals

Parthenogenetic cultures of a single clone of *D magna* (clone F) were used in the neuroactive chemicals study and also as a wild type control in the mutated clone studies. This clone has been described to have a marked negative phototactic behavior (Simão et al., 2019). Three additional genetically modified CRISPR *D. magna* clones originated from clone F were used to test how mutations in the tryptophan hydrolase (TRH) gene enzyme modulates responses to repetitive light stimuli. These included two clones presenting bi-allelic mutations in the TRH gene enzyme that should lack serotonin (hereafter TRHA-, TRHB-) and a mono-allelic mutant (TRH+) that should have normal levels of serotonin (Rivetti et al., 2018). Further details of cultured conditions are in Supplementary Material.

2.3. Exposures and sample collection

Two sets of experiments were carried out. One with the three mutated clones plus the wild type one (clone F) and the other with *D. magna* clone F exposed to different neuroactive chemicals. *D. magna* juveniles were pre-exposed to the selected chemicals for 24 h, in groups of 12 individuals in 300 mL of test medium plus algae prior to behavioral assays. Following exposures, 10 animals were distributed randomly among 24 well plates (two treatments per plate) without food. Selected concentrations for each tested chemical within this study were far below those impairing survival or swimming. Neurochemicals were initially screened for light stimuli motile responses using the concentrations reported in other studies, detailed in Table S1. Those concentrations having the greatest effect in light stimuli motile response assays were selected for neurotransmitter determination. Detailed information about the studied neurochemicals across experiments and concentrations used is depicted in Table S1 (Supplementary Material). Non-exposed samples (controls) were used in all the experiments. Acetone at 20 μL/L was used as the carrier chemical, and was also added to the controls. Just after behavioral assays, juveniles were collected in groups of five animals, snap-frozen dry in liquid nitrogen and stored at −80 °C until extraction. Additional control samples for blanks, quality controls and matrix standard calibration were also sampled. Similar culturing conditions were used in the experiment with mutated clones but animals were not exposed to any chemical.

2.4. Behavioral assays

The behavioral assay was based in the automatized delivery of visible light stimuli using a DanioVision Observation Chamber (DVOC-0040). Locomotor response of *Daphnia* to these light stimuli

was video tracked and analyzed using the EthoVision XT 9 software (Noldus, Wageningen, The Netherlands). All testing was performed at 20 °C. Light intensity of the stimuli was selected at 50% in DanioVision setting (290 lux), and then, sequences of the stimuli (light) were delivered at 5 s interstimulus interval (ISI). The period of time between stimuli was in darkness (near infrared light). Trials were conducted in 24 well plates, with one 4 day old *D. magna* juvenile in each well containing 1 mL of exposure medium. Before delivering the first stimulus, *Daphnia* individuals were left in the DVOC in darkness (near infrared light) for 20 min to acclimatize. Videos were recorded at 30 frames per second and the locomotion response was analyzed for each individual *Daphnia* by measuring the maximal distance moved per second (mm) within the light period after first stimulus.

2.5. Sample extraction

Samples were extracted following previous studies (Rivetti et al., 2019) with minor modifications, which are described in Supplementary Material.

2.6. UPLC-MS/MS analysis

Targeted neurotransmitters were measured using Ultra Performance Liquid Chromatography couple to tandem mass spectrometry (UPLC-MS/MS), consisting of an Acquity UPLC system (Waters, USA) connected to a Xevo TQ-S triple quadrupole mass spectrometer (Waters, USA). Chromatographic conditions were as reported in previous studies (Rivetti et al., 2019) with minor modifications, which are described in Supplementary Material. Since much higher number of neurotransmitters were measured simultaneously in this study, a first analytical approach with the analysis of a solution containing all the analytical standards at 1 mg/L in ACN:H_2O (50; 50, v/v) was analyzed in order to check peak resolution. Neurotransmitters were measured under positive electrospray ionization (ESI+). Mass spectrometer conditions were similar for the common optimized metabolites to those reported in some previous studies for zebrafish samples (Gómez-Canela et al., 2018) with minor modifications, optimized as described in Supplementary Material. Table S2 (Supplementary Material) displays final optimized conditions. Experimental data were acquired and processed using the MassLynx v4.1 software package (Waters, USA).

2.7. Quality assurance and method validation

Calibration was performed over a concentration range from 0.005 to 1 mg/L, using seven calibration points. Calibration standard curves were prepared into *D. magna* neurotransmitter samples to account for matrix effects (Zhou et al., 2015). Further details are in Supplementary Material. To evaluate the quality and validate the method, linearity, sensitivity, reproducibility and repeatability (inter- and intra-day precision), carry over, recovery and matrix effect parameters were determined as it is indicated in Supplementary Material.

2.8. Data analysis

Likewise it has been described in fish (Faria et al., 2019a, 2019b), *D. magna* responses to repetitive light stimuli are bi-phasic, increasing the response to light stimuli until a maximum response is reached, and then the response decreases due to habituation (Fig. S1A). The relative (versus controls) motile response of *D. magna* juveniles exposed to the tested chemicals varied largely before the first light stimuli (basal activity) and the last 5 light stimuli (late habituation phase), and was quite consistent between the 5 to the 20 stimuli (Fig. S1 B, C, D, E). Accordingly, for each individual replicate per tested neurochemical, the gross mean response before light was determined, from 5 to 20 and from 25 to 30 light stimuli, and compared by one way ANOVA followed by Tukey's post hoc tests and also by Student's t-test. Prior to analyses data was tested to meet the ANOVA assumptions of normality and variance homoscedasticity (Zar, 1996).

Levels of neurotransmitters per *D. magna* samples were normalized per individual (pg/animal) and were compared with those of controls or wild type clone using one-way ANOVA followed by Dunnett's post-hoc tests ($P < 0.05$). Neurotransmitter levels in *D. magna* samples exposed to the studied neuroactive chemicals were normalized with respect to the control of each of the experiments and expressed as fold-change.

Overrepresentation (enrichment) analysis of categorical KEGG annotations (Kanehisa, 2000) for the set of analyzed neurotransmitters was performed using MBrole (López-Ibáñez et al., 2016). KEGG number annotation of each neurotransmitter (Table S2) was submitted as compound set and enrichment analysis was computed using the full MBrole database as background set. Only categories related to KEGG pathways and with false discovery rate (FDR) < 0.05 were considered. Categories with just one hit in set were also excluded.

3. Results and discussion

3.1. Behavioral assays

Fig. 1 shows behavioral responses for TRH mutated clones and *Daphnias* exposed to the tested neuroactive chemicals. Individuals from mutated clones lacking serotonin (TRHA-, TRHB-) moved significantly more ($P < 0.05$) under darkness relative to the wild type clone ($F_{2, 27} = 7.1$) (Fig. 1B) and were habituated to a lower extent ($F_{2, 27} = 7.1$) (Fig. 1F). These results indicate that knocking down TRH gene increases basal activity and decreases habituation to light. Greater locomotor activity in darkness for the same clones lacking serotonin has been previously reported (Rivetti et al., 2018). Reduced habituation in organisms having diminished serotonin levels agrees with reported responses in rodents, fish and invertebrates such as *Aplysia* (Carlton and Advokat, 1973; Conner et al., 1970; Faria et al., 2019a, 2019b; Glanzman et al., 1989).

The tested chemicals affected significant ($P < 0.05$) the motile response of *D. magna* juveniles in dark upon light stimuli ($F_{11, 108} = 66.0$) and later on during habituation ($F_{11, 108} = 48.7$) (Fig. 1). MEM, 6OH, MEC and PCPA treatments increase basal motile responses (Fig. 1A); 6OH, PCPA, NIC and SCO enhanced the response to light (Figure 1C) and 6OH, PCPA, MEC and APO decreased habituation to light (Fig. 1E). The opposite behavior was observed in IMI, MEM and CIM exposed samples. Contrasting effects of the two tested agonists of the nicotinic acetylcholine receptor (nAChR), NIC and IMI, enhancing and decreasing the response to light, respectively, are in line with reported higher affinity of neonicotinoid insecticides (i.e. IMI) for arthropod nAChRs (Tomizawa and Casida, 2003) and results for the zebrafish embryo vibrational startle response assay (Faria et al., 2019a, 2019b). Effects of SCO, antagonist for muscarine AChR, decreasing habituation to light stimuli have also been reported for the rabbit nictitating membrane responses (Harvey et al., 1983). Contrasting effects of the nAChR antagonist MEC and its agonist IMI, decreasing and enhancing habituation, is also in line with effects found in rabbit eye blink (Woodruff-Pak, 2003). Results also indicate that 6OH increased motile activity and decrease habituation, which is in concern with reported findings in rats (Adams and Geyer, 1981; Luthman et al., 1989). CIM

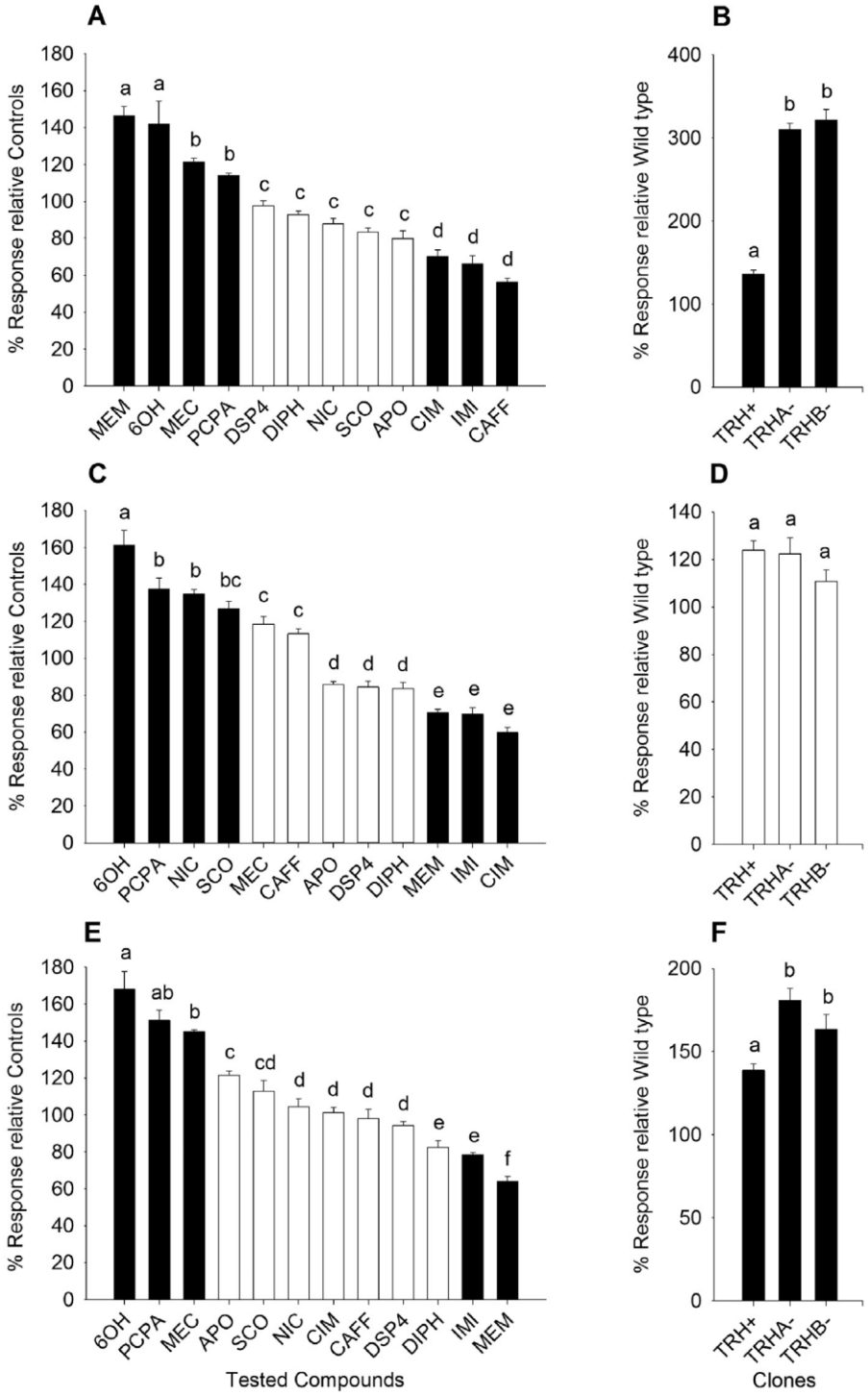

Fig. 1. Locomotion activity (Distance moved relative to controls, %; Mean ± SE, N = 10) of *D. magna* juveniles across repetitive light stimuli following 24 h exposure to the studied neuroactive chemicals (A, C, E) and in mutated clones (B, D, F). Responses are reported during the first dark period (basal motile response: A, B), from the 5th to the 10th light stimuli (response to light: C, D) and from the last 25th-30th stimuli (habituation: E, F). Black bars are treatments or mutated clones significantly ($P < 0.05$) different from the solvent carrier control group or wild type clone, respectively, following one way ANOVA and Dunnett's post-hoc tests. Different letters indicated significant ($P < 0.05$) differences among treatments following ANOVA and Tukey's post-hoc multiple comparison tests.

inhibitory effects on the *D. magna* response to light were also reported elsewhere in *D. pulex* (McCoole et al., 2011).

3.2. LC-MS/MS conditions

Optimization of mass spectrometer conditions were performed by the flow injection analysis (FIA) of standards at 1 mg/L. In order to maximize the method sensitivity, mass spectrometer analysis was carried out in Multiple Reaction Monitoring (MRM) detection mode. Table S2 displays MRM parameters, final transitions used as well as optimized cone voltages and collision energies for each fragment of the 17 targeted neurotransmitters and their 7 isotope

labelled internal standards analyzed, that were similar for those reported in Gómez-Canela et al. (2018) for the common optimized metabolites. In most cases, the protonated molecule was observed as precursor ion, except for dopamine and serotonin that present the loss of an ammonia molecule, and norepinephrine and normetanephrine that formed the protonated ion with the loss of a water molecule as base peak. The entire analyzed compounds showed a good fragmentation pattern. For each one, two fragment ions were optimized and detected following the choice of precursor ion, in accordance with the Commission Decision 2002/657/EC recommendations (European Commission, 2002), except for 5-hydroxy-L-tryptophan (5-HTP). As previously mentioned, chromatographic conditions were those reported in Rivetti et al. (2019), but taking into account the good chromatographic separation of the much larger number of analyzed metabolites. Fig. S2 shows the LC-MS/MS extracted ion chromatograms of the 17 targeted metabolites in a mixed solution at 1 mg/L, showing good resolving peaks for all them.

3.3. Quality parameters and method validation

In order to correct mass analyzer responses and to ensure exact quantification performance, external standard calibration was conducted, correcting the obtained signal per calibration point by an internal standard. Calibration curves were prepared with *D. magna* neurotransmitter extracts with a standard mixture of the targeted metabolites and the isotope labelled internal standards, quantified by subtracting the contribution from the endogenous neurotransmitters within the matrix. Table 1 displays all the quality and validation parameters obtained by LC-MS/MS.

Correlation coefficients (R^2) were equal or higher than 0.99 for most of the studied metabolites in a range from 0.005 to 1 mg/L, except for epinephrine, histamine, serotonin, 5-HIAA and 5-HTP, that was until 0.5 mg/L. Carryover effects were investigated by injecting solvent blanks throughout the sample analytical sequence, and no effect was observed, indicating no carryover during LC-MS/MS runs. Instrumental detection limit (IDL) values ranged between 0.0015 pg and 8.60 pg, being lower than those reported in previous studies for the common targeted metabolites (Gómez-Canela et al., 2018; Rivetti et al., 2019). Method detection limit (MDL) ranged from 0.05 pg/daphnia for 5-HTP and 51.56 pg/daphnia for taurine. These values were within the range reported by Gómez-Canela et al. (2018) but lower than those reported by Rivetti et al. (2019) for the common metabolites. As an example, in the previous study, norepinephrine MDL was reported as 18.56 pg/daphnia whereas here is 0.52 pg/daphnia. Method quantification limit (MQL) range between 0.15 pg/daphnia for 5-HTP and 171.85 pg/daphnia for taurine, and values were also lower to those reported in previous mentioned studies. Intra- and inter-day precision values were lower than 20%, thus indicating a robust method. Method performance was tested using five replicates of *D. magna* samples spiked with 100 μg/L of the neurotransmitter standard mixture and 50 μg/L of the internal standard mixture, recovering the 17 neurotransmitters in the range 51.3% ± 9.1% to 91.7% ± 7.6%. In addition, matrix effect (ME), which may cause suppression or enhancement of the analytes ionization, was also evaluated by comparing for each analytes the peak area from the spiked *D. magna* calibration curve with the signal obtained from the standard solution at the same concentration in solvent. In most cases, no matrix effect was observed, with values between 74.1% and 118.6%, meaning that signal suppression or enhancement was much lower than around 25%, except for 5-HIAA and GABA that showed values a little bit lower (58.6 and 65.0%, respectively). Therefore, quantification with the external procedure, correcting the obtained signal per point by an internal standard, seems adequate to avoid over or underestimation of the calculated analytes concentration (Van Eeckhaut et al., 2009).

3.4. Method suitability and D. magna TRH mutants neurotransmitter profiles

Enriched analyses of the measured neurotransmitters showed a good coverage of some of the most relevant pathways related to neurotransmitters systems, as those related to neuroactive ligand receptor interaction, tyrosine metabolism, biosynthesis of alkaloids, gap junction, tryptophan metabolism or ABC transporters, among others (Fig. 2). Obtained enriched pathways are listed in Table S3 (Supplementary Material).

The presented analytical method was used for determining differences in the neurotransmitter profiles of wild type and genetically mutated CRISPR tryptophan hydrolase (TRH) *D. magna* individuals, including one mono-allelic mutant (TRH+) that should have normal levels of serotonin and two bi-allelic mutants (TRHA- and TRHB-) were no presence of serotonin should be detected.

Table 1
Quality parameters obtained by LC-MS/MS for the targeted metabolites. F: slope; R^2: determination coefficient; IDL: instrumental detection limit; MDL: method detection limit; MQL: method quantification limit.

Targeted metabolites	Linear range (mg/L)	F	R^2	Intra-day precision (% RSD)	Inter-day precision (% RSD)	Recovery (% Mean ± SD)	Matrix effect (% Mean ± SD)	IDL (pg)	MDL (pg/daphnia)	MQL (pg/daphnia)
Acetylcholine	0.005–1	0.34	0.9975	3.32	5.37	63.2 ± 3.3	115.0 ± 15.0	1.58	0.07	0.24
Choline	0.005–1	3.02	0.9952	0.58	2.59	51.3 ± 9.1	78.4 ± 3.3	0.28	0.08	0.28
Dopamine	0.005–1	6.35	0.9989	0.60	1.37	60.0 ± 1.4	115.4 ± 9.4	0.16	0.19	0.64
Epinephrine	0.005–0.5	1.51	0.9948	3.89	4.65	88.1 ± 4.2	118.6 ± 3.9	0.19	0.17	0.57
Histamine	0.005–0.5	6.39	0.9982	2.65	2.65	57.9 ± 2.7	99.2 ± 14.7	0.88	1.25	4.16
L-phenylalanine	0.005–1	0.83	0.9999	4.85	8.56	80.3 ± 8.8	78.9 ± 11.6	0.25	0.46	1.55
L-Tryptophan	0.005–1	6.92	0.9802	9.64	12.39	75.0 ± 3.8	71.9 ± 6.9	0.15	1.76	5.86
Norepinephrine	0.005–1	0.71	0.9949	6.68	12.08	58.5 ± 3.4	74.1 ± 14.2	0.65	0.52	1.72
Normetanephrine	0.005–1	4.81	0.9917	2.19	13.98	56.4 ± 1.2	100.1 ± 6.5	0.17	0.14	0.48
Octopamine	0.005–1	0.45	0.9870	0.92	8.51	63.6 ± 1.7	107.6 ± 5.5	1.11	6.39	21.29
Serotonin	0.005–0.5	8.01	0.9995	2.21	8.50	91.7 ± 7.6	117.8 ± 13.5	0.33	0.96	3.21
Taurine	0.005–1	1.12	0.9848	3.71	4.00	73.3 ± 13.8	102.6 ± 4.8	8.60	51.56	171.85
γ-aminobutyric acid (GABA)	0.005–1	15.28	0.9878	4.33	14.90	60.7 ± 4.9	58.6 ± 9.4	4.12	12.54	41.79
3,4-dihydroxyfenilalanina (L-DOPA)	0.005–1	0.13	0.9914	1.90	4.33	63.0 ± 6.6	83.5 ± 14.3	<0.01	0.73	2.45
3-methoxytyramine (3-MT)	0.005–1	1.98	0.9925	4.25	9.39	70.4 ± 1.9	107.4 ± 14.3	0.04	0.14	0.46
5-hydroxyindoleacetic acid (5-HIAA)	0.005–0.5	1.25	0.9833	11.13	11.10	71.6 ± 9.7	65.0 ± 2.0	0.09	0.52	1.73
5-hydroxy-L-tryptophan (5-HTP)	0.005–0.5	3.18	0.9984	12.90	18.93	69.7 ± 2.3	79.3 ± 12.4	<0.01	0.05	0.15

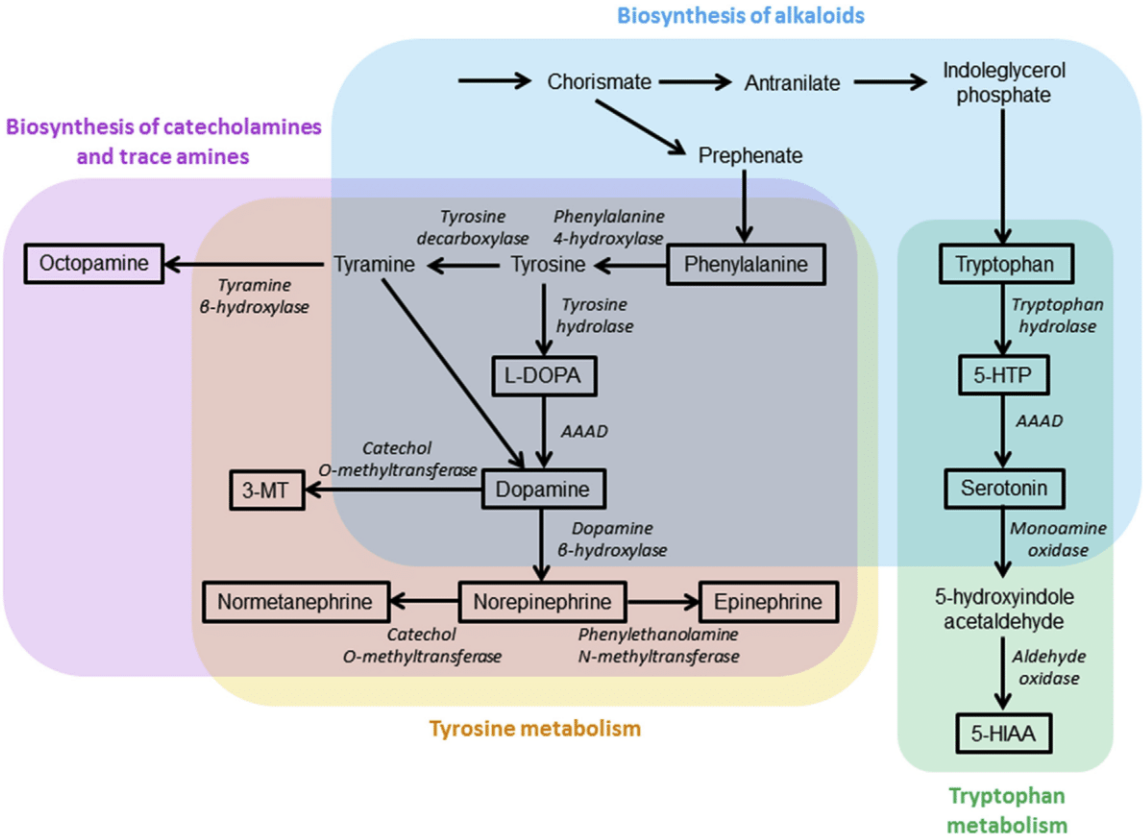

Fig. 2. Pathway created from some of the detailed KEGG pathways obtained after enrichment analysis and listed in Table S3, which related some of the analyzed metabolites. Black-boxed metabolites are those that have been analyzed within this study. In italics, the catalytic enzymes of the reactions of interest are detailed.

Obtained results, represented in Fig. 3, provide an independent probe of method validation. Raw data and ANOVA results for the response of the quantified neurotransmitters and related metabolites or precursors across clones are detailed in Tables S4 and S5 (Supplementary Material). Significant differences ($P < 0.05$) were detected in 9 of the 17 analyzed metabolites. Serotonin was measured in similar amounts in wild type and TRH + samples, and no serotonin was detected in either TRHA- or TRHB- samples. A similar pattern was observed for 5-HIAA and to a lesser extent for tryptophan and 5- HTP, which are respectively, the immediate metabolite and the precursors of serotonin. These results are in line with previous studies. Rivetti et al. (2018), who despite of using a less sensitive method found similar serotonin levels for the wild and TRH + clone and did not detect serotonin in individuals from TRHA- and TRHB- clones. Campos et al. (2019) reported down-regulated molecular processes related to serotonergic metabolism for TRHA- and TRHB- *D. magna* clones.

Contrary to the study of Rivetti et al. (2018) significant differences were detected in 8 more metabolites (acetylcholine, choline, tryptophan, epinephrine, dopamine, phenylalanine, 3-MT and taurine; Fig. 3), suggesting that the disruption in tryptophan metabolism by serotonin may have affected the metabolism of other related neurotransmitters, as the biosynthesis of catecholamines and the tyrosine metabolism, where dopaminergic and adrenergic neurotransmitters are found.

3.5. Neurotransmitter profiles in D magna exposed to neurochemicals

This analytical method was also applied to determine the effects of neuroactive chemicals that were known to affect different neurotransmitter systems and were previously tested to produce changes in *Daphnia* behavior. Of the 17 neurotransmitters analyzed, 13 showed significant differences ($P < 0.05$) across treatments (Fig. 4). Raw data and ANOVA results for the response of the quantified neurotransmitters and related metabolites or precursors across treatments are detailed in Tables S4 and S5 (Supplementary Material). *Daphnia* individuals were exposed to two neurochemicals affecting dopaminergic system: APO and 6OH. APO, known to be a non-selective agonist of dopamine receptor activating D_2-like receptors and to have affinity for serotonin receptors and α-adrenergic receptors (Bownik et al., 2018; Jenner and Katzenschlager, 2016), decreased epinephrine, normetanephrine, tryptophan, taurine and choline. 6OH is used in research for the selective destruction of dopaminergic and noradrenergic neurons (Breese et al., 2005; Feng et al., 2014). In line with that, 6OH exposed *D. magna* individuals showed significant lower levels of dopamine and of norepinephrine, which is synthetized directly from dopamine by dopamine beta-hydroxylase. Also, lower levels of octopamine, neurotransmitter analogous to norepinephrine in invertebrates, and 3-MT, a direct metabolite of dopamine by the action of the enzyme catechol O-methyltransferase, were detected in individuals exposed to 6OH. To a lesser extent and despite not being significantly affected, the concentrations of two possible metabolizing products of norepinephrine, normetanephrine and epinephrine, were reduced. These results are in line with the destruction of the dopaminergic and noradrenergic neurons reported for 6OH (Breese et al., 2005; Feng et al., 2014), showing lower levels in most of the neurotransmitters related with this dopaminergic and noradrenergic system through the tyrosine

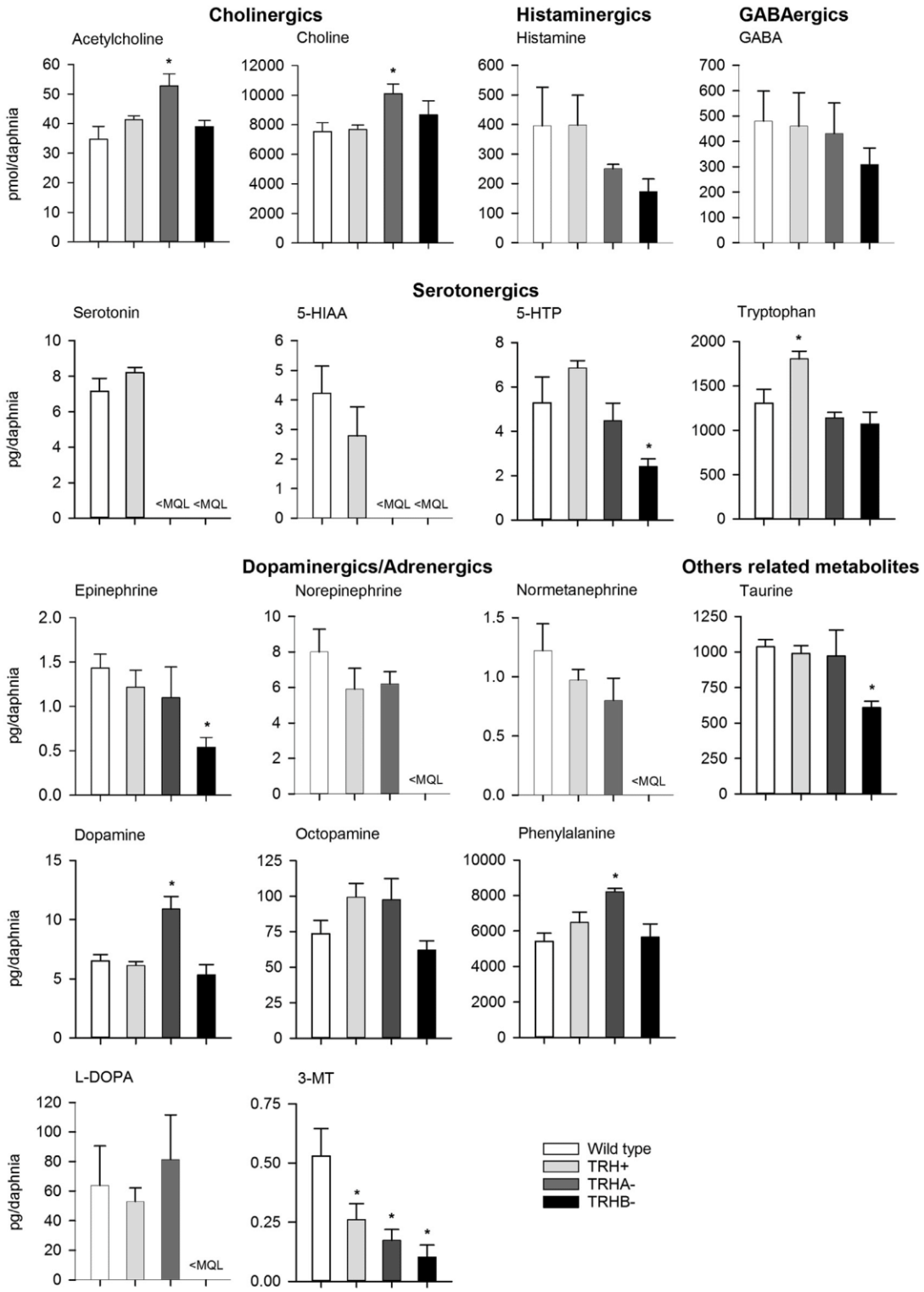

Fig. 3. Responses (Mean ± SE, N = 5) of selected neurotransmitter and related metabolites in females of *D. magna* juvenile clones. TRH+, TRHA-, TRHB- are, respectively, the CRISPR tryptophan hydrolase mono-allelic mutant and the two tryptophan hydrolase bi-allelic mutated clones. * indicates clones significantly ($P < 0.05$) different from the wild type one following one-way ANOVAs and Dunnett's tests. <MQL, below method quantification limit.

metabolism and the biosynthesis of catecholamines. Diphenhydramine (DIPH) and cimetidine (CIM), histamine H_1 and H_2 receptor antagonist, respectively (Berninger et al., 2011; McCoole et al., 2011), decreased epinephrine levels. These results are in line with expectations, since epinephrine is one of the neurotransmitters release upon stress and one of the effects of antihistaminic compounds is to attenuate the neuroendocrine response to stressors (Carrasco and Van De Kar, 2003). Referring to the cholinergic systems, no significant changes were observed in the neurotransmitter profile of *D. magna* samples exposed to SCO,

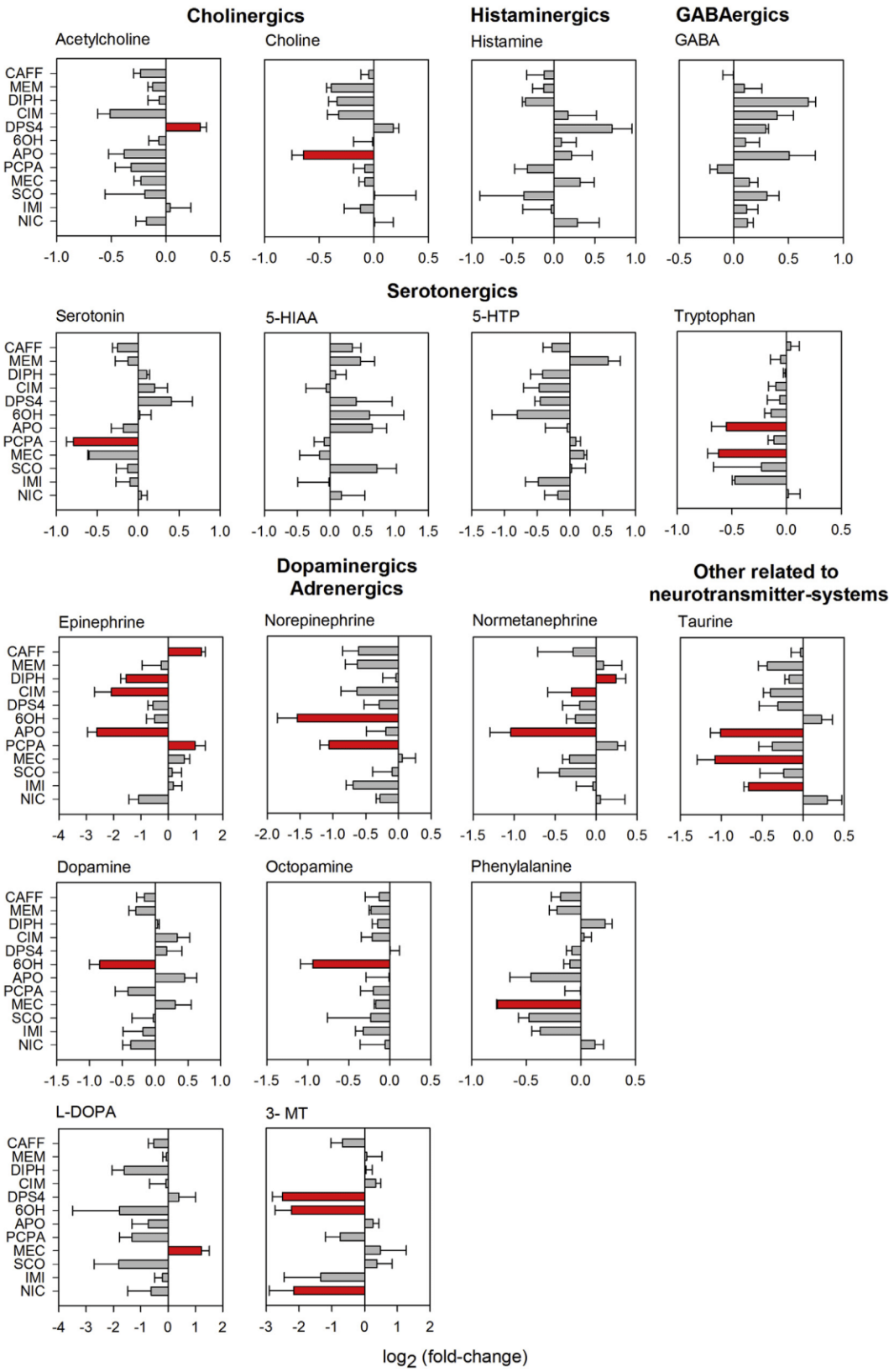

Fig. 4. Responses (log_2 of fold-change relative to control; Mean ± SE, N = 2–4) of the studied neurotransmitter and related metabolites in of *D. magna* juvenile females exposed to selected neuroactive chemicals. Red bars are treatments significantly ($P < 0.05$) different from the solvent carrier control group following one way ANOVA and Dunnett's post-hoc tests. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

although L-DOPA levels decreased more than 50%. *D. magna* exposed to MEC had enhanced levels of L-DOPA and decreased levels of phenylalanine, tryptophan and taurine, whereas those exposed to NIC and IMI showed reduced levels of 3-MT and taurine, respectively. The decrease in 3-MT, the metabolite of dopamine catalyzed by catechol O-methyltransferase (as represented in Fig. 2), suggest down regulation in the activity of this enzyme, whose low activity has been associated with nicotine in humans (Beuten et al., 2006). Observed effects of the studied cholinergic active chemicals on metabolites from the dopaminergic system (phenylalanine and L-DOPA) are in line with the reported cross talk between these two systems in their involvement in cognitive functions (Levin and Simon, 1998). The almost absence of effects in the neurotransmitter profiles of SCO, IMI and NIC, despite the obtained effects on behavior; are in line with reported results for zebrafish embryos (Faria et al., 2019a).

Daphnias were also exposed to DSP4, a compound known to destroy noradrenergic neurons (Castelino and Ball, 2005). No significant changes were observed in neurotransmitters related to adrenergic or noradrenergic neurons, although a slight decrease in epinephrine and, to a lower extent, in norepinephrine and normetanephrine were observed. On the other hand, DSP4 decreased 3-MT, increased acetylcholine and although no significant, enhanced the levels of histamine more than 50%. Enhanced levels of acetylcholine by DSP4 have also been reported in in rats (Magnani et al., 1985) and there is reported information that DSP4 can also affect negatively dopaminergic neurons and hence the associated metabolism (Ross and Stenfors, 2015). The little effects observed of DSP4 on the adrenergic system are in line with the few behavioral effects found for this compound. Despite the observed effects of MEM on *D. magna* behavior (Fig. 1), no effects were observed in the analyzed neurotransmitters. This is probably related to the fact that the primary effect of MEM is to regulate the release of glutamate (Rogawski and Wenk, 2006), which was no analyzed in this study. Caffeine (CAFF), known to stimulate the CNS, just produced a significant increase in epinephrine. This result is in agreement with the reported increase in epinephrine levels produce by caffeine in healthy humans (Flueck et al., 2016). Finally, PCPA decreased serotonin levels, which agrees with its known effect inhibiting TRH activity. TRH inhibition and thus inhibition on serotonin synthesis after exposure to PCPA has also been reported in zebra fish studies (Airhart et al., 2012). Moreover, an increase in epinephrine and a decrease in norepinephrine were also observed. This decrease in norepinephrine is in line with some results obtained in rat studies upon exposure to PCPA (Reader, 1982).

4. Conclusions

A comprehensive optimization of a method for the determination of 17 neurotransmitters belonging to some of the most important pathways related to neurotransmitter systems in *D.magna* samples has been developed and validated in terms of sensitivity, reproducibility, precision, selectivity and accuracy. The improvement of this method in terms of sensitivity with respect to previously developed methods (Rivetti et al., 2019) has been probed. Furthermore, matrix effect was also evaluated for all the analyzed neurotransmitters, which was not observed for practically any compound. This method was further validate by applying it for the study of neurotransmitter levels in genetically mutated TRH *D.magna* samples, confirming the absence of serotonin and its metabolite 5-HIAA in knock out individuals that should not contain serotonin (TRHA- and TRHB- samples), together with lower levels of tryptophan and 5-HTP, which is in concordance with transcriptomic and immunohistochemistry results reported in previous studies (Campos et al., 2019; Rivetti et al., 2018).

Additionally, the method was applied for determining the effects of neuroactive chemicals known to affect different neurotransmitter systems and that altered *Daphnia* behavior. Neurotransmitter profiles in *D.magna* exposed to all these chemicals helped to reaffirm the applicability of the method presented in this work, and shed some light on the mode of action of the tested neurochemicals in *D. magna*. The measurement of not only neurotransmitters but also of their main metabolites and/or precursors provided a robust analytical method that helped to elucidate the mechanisms of action of neuroactive chemicals, one of the major advantages of the proposed method relative to the previous ones (Rivetti et al., 2019).

Credit author statement

Inmaculada Fuertes: Investigation, Methodology, Formal analysis, Writing - original draft; Carlos Barata: Project administration, Conceptualization, Supervision, Resources, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.127814.

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Supplementary Material: scientific article V

Characterization of neurotransmitters and related metabolites in *Daphnia magna* juveniles deficient in serotonin and exposed to neuroactive chemicals that affect its behavior: A targeted LC-MS/MS method.

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METHODS

Chemicals and materials. Neurotransmitter analytical standards and isotope-labelled metabolites were purchased from Sigma-Aldrich (St. Louis, MO, USA), Santa Cruz Biotechnology (Dallas, TX, USA) and Toronto Research Chemicals (Toronto, Ontario, Canada). Further information is in Table S2. Initially, standard stock solutions of all pure standards were prepared at 1000 ng/µL in water or methanol, depending on their solubility. Once prepared, stocks were kept in silanized amber vials at -80 ºC in dark to prevent possible degradation. Working solutions were obtained by dilution of the stock solutions in ACN:H_2O 90:10 v/v. Methanol (MeOH), acetonitrile (ACN) and water (H_2O) HPLC grade were supplied by Merck (Darmstadt, Germany), formic acid (98% purity) was supplied by Fischer Scientific (Loughborough, UK), and ascorbic acid (99% purity) and ammonium formate (purity ≥99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA)

Experimental animals. Photoperiod was set to 16 h light: 8 h dark cycle, and temperature at 20 ± 1 °C. Bulk cultures of 10 adult *Daphnia* females were maintained in 2 L of ASTM hard synthetic water (ASTM, 1994) at high food ration levels ($5x10^5$ cells/mL of *Chlorella vulgaris*), following Barata and Baird (Barata and Baird, 1998). Groups of 100 newborn individuals (< 24 h old) obtained from bulk cultures were reared in 2 L ASTM hard water plus algae for 3 days, and then used for exposure and behavioral assays. Cultures were renewed with new media every other day.

Sample extraction. Briefly, 500 µL of ACN with 0.1% formic acid and 0.02% ascorbic acid were added to samples of five *D. magna* individuals. Every single sample was spiked with 50 µg/L of isotope labelled solution (acetylcholine chloride-d_9, choline chloride-^{13}C, dopamine-d_4 hydrochloride, GABA-d_6, L-phenylalanine-^{13}C, taurine-^{15}N and serotonin-d_4 hydrochloride) as internal standard (internal standard mixture) and samples were shaken using a vortex mixer. Thereupon, samples were homogenized using a bead mill homogenizer

(TissueLyser LT, Qiagen), with three stainless steel beads (3 mm diameter) per sample, at 50 oscillations per min during 60 seconds. Subsequently, samples were centrifuged at 14500 rpm during 10 min at 4ºC. The supernatant was transferred to a new centrifuge tube and left in ice during 30 minutes in order to precipitate possible present proteins. Samples were centrifuged again under same conditions as before, and the supernatant was transferred to a new tube and evaporated to complete dryness under mild nitrogen, when samples were kept at -80ºC until UPLC-MS/MS analysis. On the day of the analysis, samples were re-suspended in 60 µL of ACN:H_2O (50:50, v/v), centrifuged again at 14500 rpm during 10 min at 4ºC, and transferred to an insert amber glass chromatographic vial for its analysis.

UPLC-MS/MS analysis. The chromatographic separation was achieved using a zwitterionic ZIC-cHILIC column (250 x 2.1 mm, 5 µm particle size, Sigma Aldrich) and a ZIC-cHILIC guard column (20 x 2.1 mm, 5 µm particle size, Sigma Aldrich). The mobile-phase composition consisted of a binary mixture with 0.1% formic acid and 10 mM of ammonium fomate in ACN/H2O 90:10 (v/v) (A) and 0.1% formic acid and 10 mM of ammonium fomate in H_2O (B). Gradient elution started at 100% A in the first minute, and decreased to 55% A in the following 15 min. Then, gradient was brought to 35% A during the next 2 min, and held for 2 min. Initial conditions to stabilize the system were attained in 6 minutes, being 26 the total run time. The flow rate was set at 150 µL/min and 10 µL per sample were injected. Neurotransmitters were measured under positive electrospray ionization (ESI+). Mass spectrometer conditions were similar for the common optimized metabolites to those reported in some previous studies for zebrafish samples (Gómez-Canela et al., 2018) with minor modifications. Flow injection analysis (FIA) was performed to obtain the optimum cone voltage (between 1 and 80 V) for the determination of the molecular ion and the optimum collision energies (between 1 and 50 eV) to determine at least two intense fragments if possible. Finally, to identify each metabolite, acquisition was performed in Multiple Reaction Monitoring (MRM) mode using two transitions from precursor ion to product ions, in order to unequivocally identify each metabolite. Table S2 displays final transitions used as well as optimized cone voltages and collision energies for each fragment. Experimental data were acquired and processed using the MassLynx v4.1 software package (Waters, USA).

Quality assurance and method validation. All calibration samples were obtained by pooling *Daphnia* homogenates, extracting them as previously described, and then aliquoted to the same final volume and spiked with standards solution to achieve desired concentration. A mixture containing the seven internal standards was also added to the standard curve at a concentration of 250 µg/L. Because of the presence of endogenous neurotransmitters in the calibration standard curves, *Daphnia* samples without any standard

were also analyzed and subtracted for the calibration points. Sample quantification of all the targeted neurotransmitters was performed by external standard calibration adjusted by internal standard labelled metabolites to correct extraction efficiencies and analytes MS responses, ensuring exact quantification performance. Detailed information on the internal standard used for each metabolite is given in the Table S2, which was assigned based on their molecule structural similarity.

Linearity response was studied over the calibration range, from 0.005 to 1 mg/L, using the seven calibration points, and by calculating the regression coefficient (R^2). Method sensitivity was evaluated by calculating instrumental detection limit (IDL), method detection limit (MDL) and method quantification limit (MQL). IDL was calculated as the concentration that yielded a S/N ratio higher than or equal to three using the lowest concentration standard solution at 0.005 mg/L. MDL and MQL were calculated as the concentration that yielded a S/N ratio higher than or equal to three and ten, respectively, using *D. magna* samples spiked at 100 µg/L. Method precision was assessed by both the reproducibility (inter-day precision) and the repeatability (intra-day precision), expressed as relative standard deviation (%RSD). The intra-day precision was assessed by five consecutive injections of 1 mg/L standard solution, and inter-day precision was determined by measuring the same standard solution during three different days. Solvent blanks were analyzed all throughout the sample analytical sequence to assure no presence of the investigated analytes, indicating no carryover effect during the LC-MS/MS runs.

In addition, recovery studies were performed with five replicates of *D. magna* samples spiked with 100 µg/L of the neurotransmitter standard mixture and 50 µg/L of the internal standard mixture, comparing the results of the theoretical and measured standard concentrations. Furthermore, the matrix effect (ME) was assessed by comparing the peak area of the analyte from the spiked *D. magna* calibration curve with the signal obtained from the standard solution at the same concentration in solvent (ACN/H2O,50:50 v/v) (N=7), following the equation:

$$ME = \frac{(A - B)}{C} \times 100$$

where A is the peak area of each analyte from spiked *D. magna* samples; B is the peak area of each analyte from non-spiked *D. magna* (blanks); and C is the peak area of each analyte in solvent.

TABLES

Table S1. Studied metabolites across experiments, description, putative mode of action and tested concentrations.

Neurotransmitter system	Neurochemical	CAS	Description	Mode of action	Concentration	Reference
Exp 1						
Dopaminergic	Apomorphine (APO)	147-24-0	Synthetic derivative of morphine, used for Parkinson's disease	Non-selective dopamine-D_2 agonist	2 mg/L	(1)
Histaminergic	Cimetidine (CIM)	51481-61-9	Antihistamine drug used to ulcers treatment	H_2-Receptor antagonist	15 mg/L	(3)
	Diphenhydramine (DIPH)	147-24-0	Antihistamine drug used for allergies treatment	H_1 Receptor antagonist	0.6 mg/L	(2)
Exp 2						
Cholinergic	Nicotine (NIC)	54-11-5	Potent neuroactive compound	Nicotinic AChRs agonist	50 μg/L	(4)
Dopaminergic	6-hydroxydopamine (6OH)	28094-15-7	Synthetic organic neurotoxic compound	Destroys dopaminergic and noradrenergic neurons	5 mg/L	(6)
Adrenergic	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4)	40616-75-9	Neurotoxin	Destroys noradrenergic neurons	5 mg/L	(7)
Exp 3						
Cholinergic	Imidacloprid (IMI)	138261-41-3	Neonicotinoid insecticide	Insect nicotinic AChRs agonist	100 μg/L	(4)
	Mecamylamine (MEC)	826-39-1	Neuroactive compound	Nicotinic AChRs antagonist	10 μg/L	(4)
	Scopolamine (SCO)	55-16-3	Neuroactive compound	Muscarinic AChRs antagonist	15 μg/L	(4)
Glutamatergic	Memantine (MEM)	41100-52-1	Treat moderate to severe Alzheimer´s disease	Antagonist of the NMDA receptor	50 μg/L	(5)
Exp 4						
Serotonergic	Chloro-DL-phenylalanine (PCPA)	7424-00-2	Human drug also known as Fenclonine	Tryptophan hydrolase inhibitor	0.5 mg/L	(5)
Multiple	Caffeine (CAFF)	58-08-2	Psychoactive drug	Central nervous system stimulant	1 mg/L	(8)

1 (Bownik et al., 2018), 2 (Berninger et al., 2011), 3 (McCoole et al., 2011), 4 (M. Faria et al., 2019), 5 (Melissa Faria et al., 2019), 6 (Feng et al., 2014), 7 (Castelino and Ball, 2005), 8 (Steele et al., 2018)

Table S2. LC-MS/MS optimized parameters for neurotransmitters (sorted by neurotransmitter system) and internal standards. IS: internal standard; Mw: molecular weight (Da); C.V.: cone voltage (V); C.E.: collision energy (eV).

Neurotransmitter system	Neurotransmitter (acronym)	Labelled IS	Function	Molecular formula	KEGG number	Mw (Da)	Precursor ion (m/z) $[M+H]^+$	C.V. (V)	Fragment ion 1 (m/z)	C.E. 1 (eV)	Fragment ion 2 (m/z)	C.E. 2 (eV)
Dopaminergic/ adrenergic	Dopamine	Dopamine-d^4	NT	$C_8H_{11}NO_2$	C03758	153.2	137 $[M-NH_3]^+$	14	119	14	91	14
	Dopamine-d^4 hydrochloride	-	IS	$C_8D_4H_8ClNO_2$	-	193.6	158	32	141	12	123	18
	Epinephrine	Dopamine-d^4	NT	$C_9H_{13}NO_3$	C00788	183.2	184	22	166	7	107	26
	L-phenylalanine	L-phenylalanine-^{13}C	Precursor dopamine, epinephrine, norepinephrine, octopamine	$C_9H_{11}NO_2$	C00079	165.2	166	24	120	14	103	24
	L-phenylalanine-^{13}C	-	IS	$C_8{}^{13}CH_{11}NO_2$	-	166.1	167	14	120	12	103	24
	Norepinephrine	Dopamine-d^4	NT	$C_8H_{11}NO_3$	C00547	169.2	152 $[M-H_2O+H]^+$	14	135	14	107	14
	Normetanephrine	Dopamine-d^4	Metabolite norepinephrine	$C_9H_{13}NO_3$	C05589	183.2	166 $[M-H_2O+H]^+$	36	149	10	134	14
	Octopamine	Dopamine-d^4	NT	$C_8H_{11}NO_2$	C04227	153.2	154	14	136	9	91	25
	3,4-dihydroxyfenilalanina (L-DOPA)	L-phenylalanine-^{13}C	Precursor dopamine, epinephrine, norepinephrine	$C_9H_{11}NO_4$	C00355	197.2	198	24	181	8	152	14
	3-methoxytyramine (3-MT)	Dopamine-d^4	Metabolite dopamine	$C_9H_{13}NO_2$	C05587	167.2	168	26	151	7	91	24
Cholinergic	Acetylcholine	Acetylcholine-d^9	NT	$C_7H_{16}NO_2^+$	C01996	146.2	147	14	87	12	58	32
	Acetylcholine chloride-d^9	-	IS	$C_7D_9H_7ClNO_2$	-	190.7	155	30	87	10	43	34
	Choline	Choline-^{13}C	Precursor acetylcholine	$C_5H_{14}NO^+$	C00114	104.2	104	50	60	16	45	16
	Choline chloride-^{13}C	-	IS	$C_4{}^{13}CH_{15}ClNO$	-	140.6	141	8	95	14	68	20
GABAergic	γ-aminobutyric acid (GABA)	γ-aminobutyric acid-d^6 (GABA-d^6)	NT	$C_4H_9NO_2$	C00334	103.1	104	26	87	8	69	12
	γ-aminobutyric acid-d^6 (GABA-d^6)	-	IS	$C_4D_6H_3NO_2$	-	109.1	110	30	93	8	49	18

Histaminergic	Histamine	Serotonin-d^4	NT	$C_5H_9N_3$	C00388	111.1	112	42	95	11	68	26
Serotonergic	L-Tryptophan	Serotonin-d^4	Precursor serotonin	$C_{11}H_{12}N_2O_2$	C00078	204.2	205	14	188	7	146	14
	Serotonin	Serotonin-d^4	NT	$C_{10}H_{12}N_2O$	C00780	176.2	160 $[M-NH_3]^+$	56	132	14	115	24
	Serotonin-d^4 hydrochloride	-	IS	$C_{10}D_4H_9ClN_2O$	-	216.6	181	54	164	8	136	22
	5-hydroxyindoleacetic acid (5-HIAA)	Serotonin-d^4	Metabolite serotonin	$C_{10}H_9NO_3$	C05635	191.2	192	34	146	14	91	36
	5-hydroxy-L-tryptophan (5-HTP)	Serotonin-d^4	Precursor serotonin	$C_{11}H_{12}N_2O_3$	C00643	220.2	221	36	204	8	-	-
Other related to neurotransmitter-systems	Taurine	Taurine-^{15}N	NT	$C_2H_7NO_3S$	C00245	125.2	126	36	108	8	44	14
	Taurine-^{15}N	-	IS	$C_2H_7{}^{15}NO_3S$	-	126.1	127	38	109	10	79	18

Table S3. Enrichment result of categorical KEGG annotations for the set of analyzed neurotransmitters using MBrole. Full MBrole database was set as background. Only categories related to KEGG pathways, with more than one hit in set and with false discovery rate (FDR) <0.05 are displayed.

Annotation	set	in set	background	in background	P-value	FDR correction
Neuroactive ligand-receptor interaction	17	9	4557	129	1.77E-10	6.20E-09
Tyrosine metabolism	17	6	4557	76	1.89E-07	3.31E-06
Metabolic pathways	17	15	4557	1455	2.34E-06	2.73E-05
Biosynthesis of alkaloids derived from shikimate pathway	17	6	4557	138	6.51E-06	4.89E-05
Gap junction	17	3	4557	11	6.99E-06	4.89E-05
Betalain biosynthesis	17	3	4557	24	8.32E-05	4.85E-04
Tryptophan metabolism	17	4	4557	81	1.85E-04	9.25E-04
Parkinson's disease	17	2	4557	13	9.97E-04	4.36E-03
ABC transporters	17	3	4557	90	4.15E-03	1.52E-02
Phenylalanine, tyrosine and tryptophan biosynthesis	17	2	4557	27	4.35E-03	1.52E-02
Glycerophospholipid metabolism	17	2	4557	46	1.23E-02	3.91E-02
Glycine, serine and threonine metabolism	17	2	4557	49	1.39E-02	4.05E-02

Table S4. Concentration (in pg/*daphnia*, MEAN±SE) for the neurotransmitters studied in exposed *D. magna*. <MDL: below method detection limit; C: control. Obtained results were corrected with the recovery rates.

CLONES	W	TRH+	TRHA-	TRHB-
Acetylcholine	34.67±4	41.31±1	52.78±4	38.99±2
Choline	7540±599	7694±286	10100±669	8670±946
Dopamine	6.497±0.5	6.121±0.3	10.89±1	5.319±0.9
Epinephrine	1.433±0.2	1.217±0.2	1.097±0.3	0.538±0.1
GABA	480.8±118	460.4±132	431.8±120	309.1±65
Histamine	396.1±130	398.2±101	250.8±14	174.3±42
L-DOPA	63.91±26	52.97±9	81.44±30	<MDL
Norepinephrine	8.003±1	5.900±1	6.202±0.7	<MDL
Normetanephrine	1.223±0.2	0.9716±0.09	0.8005±0.2	<MDL
Octopamine	83.01±3	87.12±3	98.62±8	65.33±7
Phenylalanine	5421±450	6477±586	8209±189	5645±752
Serotonin	7.158±0.7	8.204±0.3	<MDL	<MDL
Taurine	1036±52	990.2±55	971.2±183	606.7±46
Tryptophan	1306±155	1807±85	1140±64	1071±135
3-MT	0.5292±0.1	0.2609±0.07	0.1733±0.05	0.1019±0.05
5-HIAA	4.230±0.9	2.7862±1	<MDL	<MDL
5-HTP	5.293±1	6.860±0.3	4.487±0.8	2.422±0.3

METABOLITES	**Exp 1**				**Exp 2**			
	C 1	APO	CIM	DIPH	C 2	NIC	6OH	DSP4
Acetylcholine	67.19±10	52.27±6	47.48±4	64.98±5	66.92±4	59.61±4	64.38±4	83.37±3
Choline	36705±5223	23684±1767	29593±2012	29254±1573	40605±3667	41906±4591	41139±5129	46008±1611
Dopamine	14.58±3	20.38±4	18.83±2	15.78±0.9	29.63±4	24.71±3	23.44±4	34.08±5
Epinephrine	4.225±0.2	0.7672±0.2	1.393±0.7	1.530±0.2	1.589±0.2	0.8389±0.2	1.211±0.3	1.109±0.1
GABA	376.9±35	606.0±107	545.5±52	658.3±31	931.6±36	1018±40	1014±87	1140±22
Histamine	279.3±2	531.0±100	538.4±138	344.4±10	281.9±4	602.4±96	507.7±69	800.9±147
L-DOPA	115.0±23	86.80±28	136.7±51	84.04±41	219.7±114	263.2±132	204.5±109	354.5±101

Norepinephrine	12.07±0.3	11.26±2	8.134±1	12.15±2	17.76±2	13.68±0.6	8.293±2	14.03±2
Normetanephrine	1.741±56	0.8865±0.2	1.505±0.3	1.690±0.3	2.252±0.4	2.331±0.6	1.659±0.4	2.017±0.3
Octopamine	365.2±56	385.0±81	319.3±31	331.6±16	424.0±85	439.9±72	363.2±78	429.3±34
Phenylalanine	30663±2702	22978±3245	31418±1443	33937±1855	78018±8856	85832±4679	73021±2968	73848±2461
Serotonin	7.984±0.8	7.141±0.7	9.331±1	8.782±0.4	6.867±0.4	7.869±0.9	7.054±0.7	9.479±1
Taurine	1148±192	575.8±47	876.0±53	924.0±95	1246±225	1570±178	1473±152	1049±188
Tryptophan	1842±218	1272±117	1725±82	1906±98	1848±147	1889±130	1681±70	1786±142
3-MT	4.288±1	5.243±0.7	5.505±0.5	4.566±0.6	2.291±0.9	0.7456±0.2	0.5736±0.2	0.4339±0.09
5-HIAA	3.638±0.6	5.860±0.8	3.754±0.8	3.954±0.4	3.041±0.9	3.881±1	5.769±2	4.783±1
5-HTP	3.565±0.3	1.081±0.4	2.004±0.2	1.837±0.4	3.312±0.2	2.824±0.8	2.722±0.6	3.683±0.6
	Exp 3					**Exp 4**		
	C 3	MEM	IMI	SCO	MEC	C 4	PCPA	CAFF
Acetylcholine	83.44±7	76.74±2	87.91±12	75.11±18	71.16±3	67.72±9	55.34±5	63.82±7
Choline	50382±3538	38532±1197	47042±5062	52378±13432	47658±1885	39254±4389	39318±2554	42135±3248
Dopamine	28.81±4	23.69±2	26.97±5	29.09±7	36.10±6	21.37±3	16.60±2	20.91±3
Epinephrine	1.392±0.4	1.621±0.5	4.304±3	1.691±0.4	2.261±0.3	0.5170±0.1	1.132±0.3	1.393±0.3
GABA	993.6±89	1076±113	1082±76	1224±94	1093±60	764.0±56	702.3±55	857.9±117
Histamine	983.0±334	542.9±50	620.5±138	485.1±173	734.3±86	324.1±58	265.4±27	312.2±47
L-DOPA	508.9±78	492.1±45	470.3±93	175.5±97	1209±237	457.4±169	226.1±73	331.0±45
Norepinephrine	15.84±3	10.49±1	9.917±0.8	15.10±3	16.63±2	11.74±3	6.531±1	8.124±1
Normetanephrine	3.592±0.4	3.712±0.5	3.368±0.4	4.512±2	2.694±0.2	1.687±0.2	2.173±0.1	1.720±0.4
Octopamine	487.5±85	416.7±8	391.9±26	442.3±155	433.0±6	363.5±70	324.4±36	343.7±46
Phenylalanine	124547±16950	107493±5056	96825±5233	89873±5991	83376±287	68984±12729	76404±8310	62458±6812
Serotonin	9.166±0.3	8.547±0.9	8.699±0.9	8.404±0.8	6.036±0.05	7.263±1	4.236±0.3	6.510±0.6
Taurine	2783±312	2074±147	1756±65	2408±479	1335±202	1019±160	835.4±91	1048±79
Tryptophan	3314±284	3257±216	2676±236	3302±585	2289±50	1962±239	2003±265	1922±106
3-MT	0.4169±0.02	0.4631±0.1	0.2967±0.2	0.5271±0.2	0.6228±0.3	0.4378±0.1	0.3109±0.09	0.3095±0.08
5-HIAA	3.582±1	5.109±0.7	4.133±1	6.003±1	3.281±0.7	6.865±0.8	5.515±0.5	7.384±0.6
5-HTP	5.360±1	7.899±0.9	6.038±1	8.789±4	8.975±3	3.702±0.6	3.561±0.5	3.121±0.3

Table S5. One way ANOVA results for the response of the quantified neurotransmitters and related metabolites or precursors across clones and the studied neurochemicals experiment.

CLONES		df	F	P				
	Acetylcholine	3,14	5.2	0.013				
	Choline	3,14	3.4	0.049				
	Dopamine	3,14	10.6	0.001				
	Epinephrine	3,14	2.5	0.100				
	GABA	3,14	0.4	0.741				
	Histamine	3,14	1.5	0.251				
	L-DOPA	3,14	0.7	0.590				
	Norepinephrine	3,14	1.8	0.193				
	Normetanephrine	3,14	2.3	0.121				
	Octopamine	3,14	5.4	0.011				
	Phenylalanine	3,14	7.0	0.004				
	Serotonin	3,14	42.3	0.000				
	Taurine	3,14	2.9	0.071				
	Tryptophan	3,14	7.3	0.004				
	3-MT	3,14	5.8	0.009				
	5-HIAA	3,14	1.7	0.212				
	5-HTP	3,14	4.6	0.019				
NEUROCHEMICALS		df	F	P		df	F	P
Exp 1	Acetylcholine	3,12	2.8	0.082	**Exp 2**	3,13	7.4	0.004
	Choline	3,12	3.9	0.037		3,13	0.4	0.787
	Dopamine	3,12	2.9	0.081		3,13	5.4	0.013
	Epinephrine	3,12	13.4	0.001		3,13	2.2	0.139
	GABA	3,12	2.5	0.113		3,13	2.8	0.084
	Histamine	3,12	0.9	0.466		3,13	1.8	0.192
	L-DOPA	3,12	1.9	0.188		3,13	0.3	0.809
	Norepinephrine	3,12	1.1	0.386		3,13	9.6	0.001
	Normetanephrine	3,12	4.8	0.020		3,13	0.7	0.592
	Octopamine	3,12	0.4	0.767		3,13	3.4	0.050
	Phenylalanine	3,12	6.2	0.009		3,13	1.6	0.248
	Serotonin	3,12	1.8	0.198		3,13	2.3	0.121
	Taurine	3,12	8.7	0.002		3,13	1.7	0.214
	Tryptophan	3,12	6.3	0.008		3,13	0.5	0.658
	3-MT	3,12	0.6	0.602		3,13	4.5	0.023
	5-HIAA	3,12	0.4	0.783		3,13	0.9	0.489
	5-HTP	3,12	0.8	0.532		3,13	2.4	0.127
Exp 3	Acetylcholine	4,11	0.5	0.729	**Exp 4**	2,12	1.5	0.257
	Choline	4,11	1.3	0.322		2,12	0.2	0.816
	Dopamine	4,11	0.9	0.498		2,12	1.4	0.290
	Epinephrine	4,11	0.4	0.797		2,12	4.8	0.029
	GABA	4,11	0.6	0.645		2,12	0.8	0.457
	Histamine	4,11	0.5	0.762		2,12	0.6	0.575
	L-DOPA	4,11	10.5	0.001		2,12	1.7	0.233
	Norepinephrine	4,11	3.0	0.064		2,12	3.8	0.051
	Normetanephrine	4,11	0.9	0.515		2,12	0.8	0.477
	Octopamine	4,11	0.4	0.773		2,12	0.2	0.836
	Phenylalanine	4,11	3.5	0.045		2,12	0.8	0.484
	Serotonin	4,11	1.7	0.215		2,12	9.8	0.003
	Taurine	4,11	5.9	0.009		2,12	3.3	0.074
	Tryptophan	4,11	3.1	0.062		2,12	1.7	0.226
	3-MT	4,11	0.6	0.650		2,12	0.7	0.504
	5-HIAA	4,11	0.5	0.712		2,12	3.6	0.058
	5-HTP	4,11	3.8	0.035		2,12	1.6	0.250

FIGURES

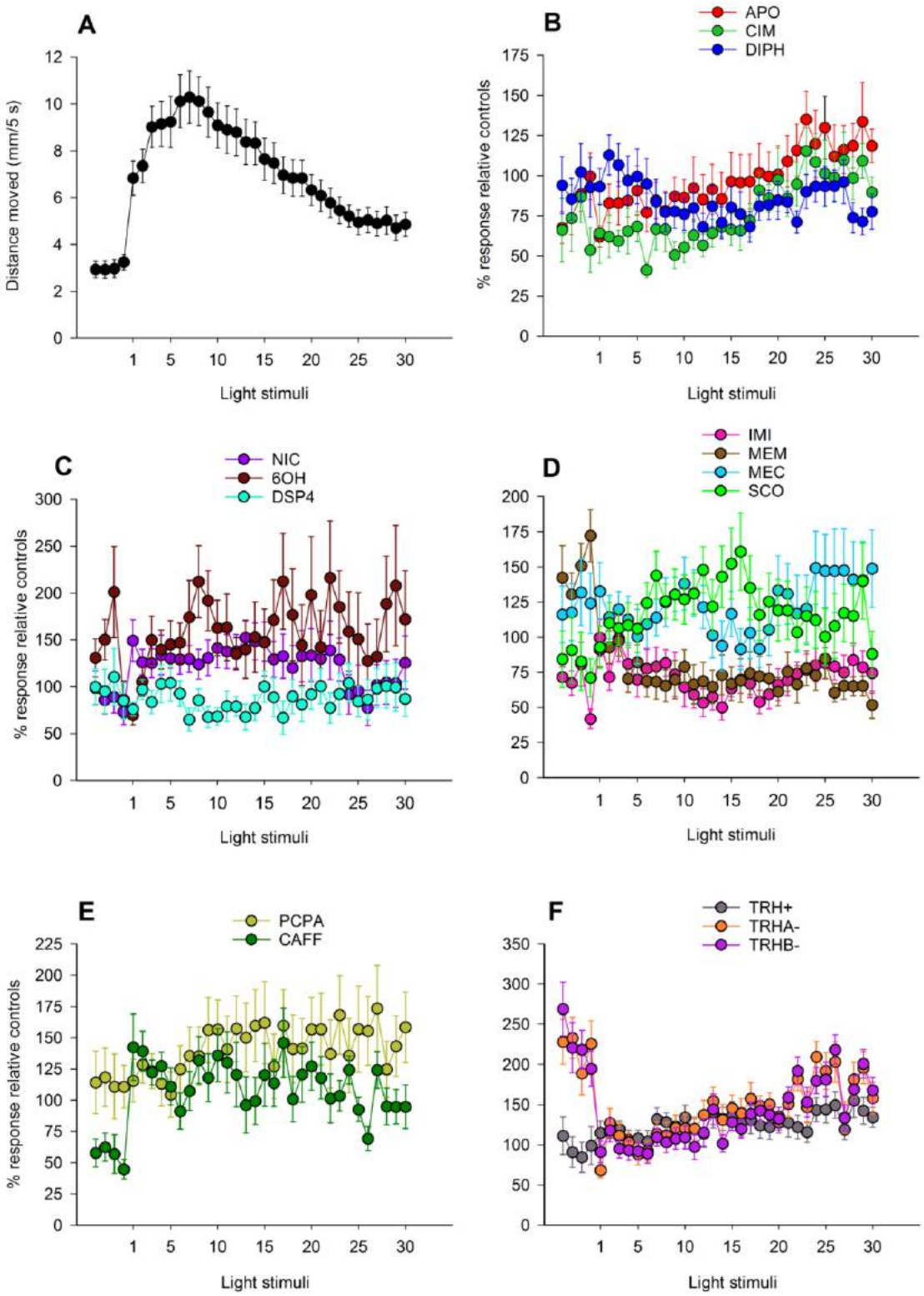

Figure S1. Locomotion activity (Mean ± SE, N= 10) of *D. magna* juveniles across repetitive light stimuli. In graph A is shown the inverted U shape of the typical response to repetitive light stimuli. Graphs B, C, D, E and F show responses relative to control or wild type treatments following 24 h exposure to the studied neuro-active pharmaceuticals (B, C, D, E) or in the studied mutated clones (F). Treatments per graph are grouped according to the performed experiment.

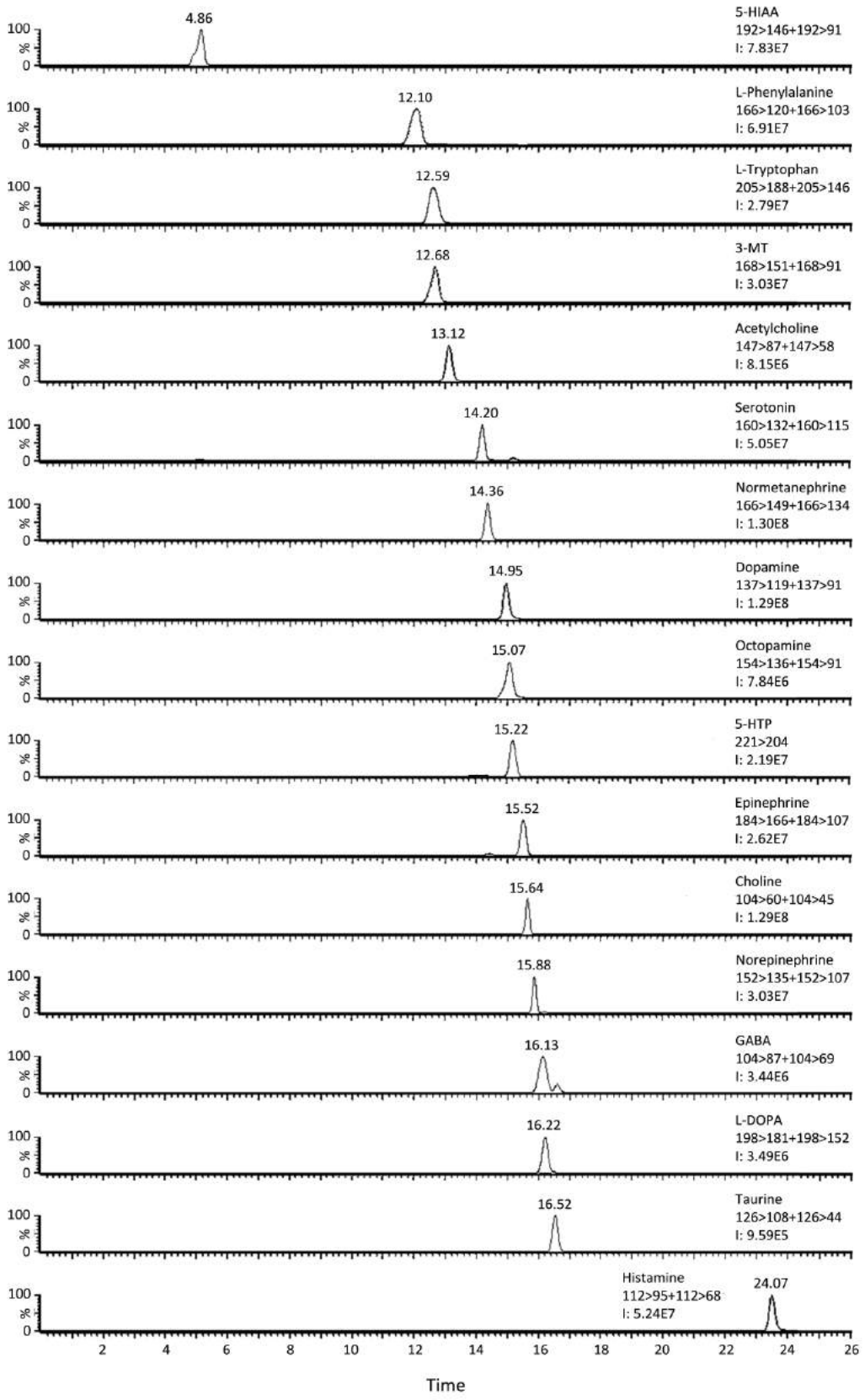

Figure S2. LC-MS/MS extracted ion chromatograms of the 17 targeted metabolites analyzed in *D. magna* matrix at 1 mg/L ordered by retention time. All were analyzed under positive electrospray ionization (ESI+). Metabolite name, transition fragments at MRM and peak intensity are included in the graph.

4.2.2. Scientific article VI

Effects of behavior-disrupting neuroactive chemicals in *Daphnia magna* cephalic transcriptome

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Effects of behavior-disrupting neuroactive chemicals in *Daphnia magna* cephalic transcriptome

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ABSTRACT

Assessing the environmental risk and neurotoxic effect of contaminants, and particularly of neuroactive pharmaceuticals, requires an understanding of the behavioral and transcriptomic effects elicited by these compounds. *Daphnia magna* represents an excellent aquatic model for these environmental studies, due to its wide application in ecotoxicological studies and to the fact that it shares with vertebrates several neurotransmitters and the related gene pathways. Adult *Daphnia magna* were exposed to six chemicals known to affect different neurotransmitter systems for 24 hours, after which behavioral tests were performed and the heads were dissected for gene expression analysis. A total of 1,304 different transcripts showed a change on their relative abundance in at least one of the treatments (DEGs, or differentially expressed genes). Fluoxetine appeared as the treatment affecting more DEGs (585 in total), followed by diphenhydramine (521 DEGs), 6-hydroxydopamine (465 DEGs), apomorphine (451 DEGs), memantine (177 DEGs) and para-chloro-DL-phenylalanine (84 DEGs). Several DEGs were shared between treatments, especially for 6OH and APO. Functional analysis identified gene categories affected by the different treatments. For example, collagen and cuticle metabolism and ECM-receptor interaction pathways were identified as targets for neuroactive chemicals known to affect the dopaminergic system. Furthermore, the expression of two DEGs (beta-hydroxylase and aromatic amino acid decarboxylase) was related to the effect of these neuroactive chemicals on neurotransmitters biosynthetic pathways, supporting the results reported in previous studies. Thus, our study showed effects at the transcriptional and physiological level and provides a novel approach to the analysis of environmental effects of neuroactive compounds.

Keywords: *Daphnia magna* · RNAseq · differential gene expression · behavior · CNS · neurochemical · neurotransmitter · water flea

1. INTRODUCTION

During the last decades, the number of potential neurotoxic or neuroactive chemicals in the environment has increased significantly until representing a substantial risk for the environment. It has been estimated that up to 30% of commercially used chemicals may produce neurotoxic effects, and therefore they represent a topic of environmental relevance (Legradi et al., 2018; Tilson et al., 1995). However, despite the knowledge that many of these types of compounds are found as contaminants in the environment, the study of their effects is restricted to humans and there is a lack of eco-neurotoxic studies in environmentally relevant species. Nowadays, *in vivo* test in rodents are still the only accepted test for neurotoxicity risk assessment in Europe (Westerink, 2013). Therefore, there is an increasing need to develop tools to detect and understand the possible effects of neuroactive compounds both in organisms that inhabit contaminated ecosystems and in environmental model organisms (Legradi et al., 2018).

The crustacean and aquatic ecotoxicological model organism *Daphnia magna (D. magna)* is a suitable model to study the effects and toxicological consequences of neuroactive compounds, sharing with vertebrates several neurotransmitters and related gene pathways (Dircksen et al., 2011; McCoole et al., 2011, 2012a; Weiss et al., 2012). In recent years, this invertebrate has been already used in studies focused in the transcriptomic disruption of neurological pathways (An et al., 2018; Christie and McCoole, 2012; Fuertes et al., 2019a; McCoole et al., 2011, 2012b, 2012a) and in studies relating *Daphnia* behavior to the effect of neuroactive chemicals (Fuertes and Barata, 2021; McCoole et al., 2011; Ren et al., 2015; Rivetti et al., 2016; Simão et al., 2019).

In this study, an integrative approach of behavioral and transcriptomic effects of neuroactive chemicals in *D. magna* is presented. Animal behavioral tests are fast and sensitive, and they describe sublethal changes that may affect trophic interactions and ecosystem functions. Therefore, it is necessary to incorporate them into ecotoxicity testing, and particularly in neurotoxicity studies (Clotfelter et al., 2004; Legradi et al., 2018), and to establish relationships between behavioral consequences and neuroactive compounds' endpoints (Peterson et al., 2017). Whole transcriptome shotgun sequencing by next-generation sequencing provides a powerful tool to investigate the molecular response of organisms to chemical exposures (Schirmer et al., 2010; Wang et al., 2009). After an initial acclimation phase, genes interacting with the environment generally return to their initial expression point (Eng et al., 2010; Orsini

et al., 2018). Early response is critical to survival and fitness in later life stages, and requires a fine adjustment of the molecular machinery regulating physiological and behavioral responses (Brooks et al., 2011; Orsini et al., 2018). Furthermore, gene transcription responses linked to specific toxicological modes of action are tissue or organ specific, thus toxicological studies should focus in specific organs. This is the case for neuroactive compounds, strongly related to the central nervous system. Here, the transcriptomic study was performed in dissected heads of *D. magna* individuals exposed to neuroactive chemicals for 24 hours, at sublethal concentrations determined by behavioral tests.

The neuroactive chemicals used in this study are compounds with known effects on different central nervous system (CNS) neurotransmitters. The effects of these compounds on *D. magna* neurotransmitter profiles as well as on some of their metabolites and precursors were already determined (Fuertes and Barata, 2021; Rivetti et al., 2019). Apomorphine is a synthetic derivative of morphine used to treat Parkinson's disease and non-selective agonist of dopamine receptor activating D_2-like receptors, also described to have affinity for serotonin and adrenergic receptors (Bownik et al., 2018; Jenner and Katzenschlager, 2016). 6-hydroxydopamine is a synthetic organic neurotoxic compound used in research for the selective destruction of dopaminergic and noradrenergic neurons (Breese et al., 2005; Feng et al., 2014). Both compounds produced changes mainly in dopaminergic and adrenergic neurotransmitters in a previous study, as well as in their precursors or metabolites, apomorphine acting as an agonist and 6-hydroxydopamine as an agonist of the dopaminergic system (Fuertes and Barata, 2021). The serotonergic system was targeted by two compounds: para-chloro-DL-phenylalanine, also known as Fenclonine, and described as a tryptophan hydrolase inhibitor and thus an inhibitor in the synthesis of serotonin (Faria et al., 2019b; Geller and Blum, 1970), and fluoxetine, a selective serotonin reuptake inhibitor (SSRI) antidepressant, marketed under the name Prozac. Previous studies reported that para-chloro-DL-phenylalanine decreased serotonin levels and altered levels of some adrenergic neurotransmitters (Fuertes and Barata, 2021), whereas fluoxetine was reported to increase the accumulation of serotonin in the CNS of *D. magna* (Campos et al., 2016; Rivetti et al., 2019), promote reproduction (Campos et al., 2012) and produce lipid disruption (Fuertes et al., 2020). The effects of diphenhydramine were also studied, an histamine H_1 receptor antagonist used for the treatment of allergies (Berninger et al., 2011) and that was reported to decrease epinephrine (Fuertes and Barata, 2021). Finally, memantine is known to affect the

glutamatergic system by being a N-Methyl-D-aspartate (NMDA) receptor antagonist used in Alzheimer's disease treatment (Faria et al., 2019b).

The aim of this study is to shed some light into the effects of these neuroactive compounds in the ecotoxicological model organism *D. magna* linking behavioral alterations and effects on early transcriptomic biomolecular processes in tissues enriched with nervous cells (the head). Thus, the present study provides a novel approach to the analysis of environmental effects of neuroactive compounds.

2. MATERIALS AND METHODS

2.1. Chemical and materials

Pure analytical standards of neuroactive chemicals used for the exposition experiments were of certified high quality grade and were purchased from Sigma-Aldrich (USA/Netherlands): 6-hydroxydopamine (6OH, CAS 28094-15-7, purity ≥97%), apomorphine (APO, CAS 41372-20-7, purity ≥98.5%), fluoxetine hydrochloride (FX, CAS 56296-78-7, purity ≥98%), para-chloro-DL-phenylalanine (PCPA; CAS 7424-00-2; purity ≥98.5%), and memantine (MEM, CAS 41100-52-1, purity ≥97); except diphenhydramine (DIPH, CAS 147-24-0, purity ≥98%), that was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Detailed information about the studied neurochemicals is shown in Table S1 (Supplementary Material). All other chemicals were analytical grade and were obtained from Merck (Germany).

2.2. Experimental animals and culture conditions

Parthenogenetic cultures of a single clone of *D. magna* (clone F) were used in all the experiments. This clone has been described to have a marked negative phototactic behavior (Simão et al., 2019). Photoperiod was set to 16 h light: 8 h dark cycle, and temperature at 20 ± 1 °C. Bulk cultures of 10 adult *D. magna* females were maintained in 2 L of ASTM hard synthetic water (ASTM, 1994) at high food ration levels ($5x10^5$ cells/mL of *Chlorella vulgaris*), following Barata and Baird (Barata and Baird, 1998). Groups of 50 newborn individuals (< 24 h old) obtained from bulk cultures were reared in 2 L ASTM hard water plus algae for 10 days, which coincided with the first release of neonates and the second clutch of eggs allocated into the brood pouch, and then used for exposure and behavioral assays.

2.3. Experimental exposures

The neuroactive chemicals used for this study as well as their exposure concentrations can be found enlisted in Table S1 (Supplementary Material). Groups of

12 *D. magna* adults were exposed to the selected compounds for 24 h in 300 mL of test medium plus algae in 500 mL glass vessels prior to behavioral assays. Non-exposed samples (controls) were used in all the experiments. Acetone at 20 µL/L was used as the carrier chemical, and was also added to the controls.

2.4. Behavioral assays

Following exposures, 6 animals were distributed randomly to 12 well plates (two treatments per plate). Animals did not feed throughout behavioral assays. Selected concentrations for each chemical tested in this study were far below those impairing survival or swimming. Neuroactive chemicals were initially screened for light stimuli motile responses using a broad concentration range ranking from 0.1 to 1000 µg/L. The concentrations having the greatest effect were selected for this study. Elected concentrations correspond to samples from three independent experiments, each one with its corresponding control. Experimental groups and selected concentrations are detailed in Table S1 (Supplementary Material).

Daphnia Photomotor Response Assay (DPMRA) was performed following previous procedures (Bedrossiantz et al., 2020) using an automatized delivery of visible light stimuli from a DanioVision Observation Chamber (DVOC-0040). Photomotor response of *D. magna* to these light stimuli was video tracked and analyzed using the EthoVision XT 9 software (Noldus, Wageningen, The Netherlands). All testing was performed in an isolated behavioral room at 20 °C, where a DanioVision Temperature Control Unit (DVTCU-0011) guaranteed that all trials were performed at the set temperature. Light intensity of the stimuli was selected at 50% in DanioVision setting (290 lux), and then, sequences of the stimuli (light) were delivered at interstimulus selected interval (ISI). The period of time between stimuli was in the dark (near infrared light). Trials were conducted in 12 well plates, with one 10-day-old *D. magna* adult in each well containing 2 mL of exposure medium. Before delivering the first stimulus, *D. magna* individuals were left in the DVOC in the dark (infrared light) for 20 min to acclimate. Videos were recorded at 30 frames per second and the photomotor response was analyzed for each individual animal by measuring the maximal distance moved per second (mm) within the light period after first stimulus.

2.5. *Daphnia* head dissection and RNA extraction

Right after DPMRA behavior assay, *D. magna* were sampled in 500 µL of cool RNA later according to manufacturer's instructions. After 24 hours at 4ºC, the head of the *Daphnia* was dissected, with special attention to eliminate rests of the digestive

tube. Each collected sample corresponded to a pool of 8 heads, which were snap frozen in liquid N_2 and preserved at -80ºC for transcriptomic studies.

Total RNA extraction was performed as previously described (Campos et al., 2018; Fuertes et al., 2019b). Complete procedure is described in Supplementary Material. RNA was extracted from four replicates per condition, from which the three with higher quality were selected for sequencing. Obtained RNA was quantified and its quality was checked in a NanoDrop 8000 Spectrophotometer (NanoDrop Technologies, USA). Samples presenting a ratio 230/260-260/280 between 1.9-2.1 were selected. RNA integrity was checked using an Agilent 2100 Bioanalyzer with RNA Nano Chips (Agilent Technologies, USA). Only the samples showing RIN values above 9 were used for transcriptomic analysis. Prior to sequencing, RNA concentration was measured in a QuBit 2.0 Fluorometer (Invitrogen, USA).

2.6. Transcriptome sequencing

An Illumina paired-end sequencing library was generated from 1 µg of total RNA per sample, using the Truseq stranded messenger RNA Library Prep Kit (Agilent Technologies, USA). Libraries were quantified by quantitative polymerase chain reaction (qPCR), according to Illumina's protocol Sequencing Library qPCR Quantification protocol guide. A DNA 1000 chip (Agilent Technologies, USA) was used to control library's size distribution and quality. In total, 24 RNA sequence (RNAseq) libraries were equimolarly pooled and sequenced on 2 Illumina Hiseq 3000 lanes, generating 2 × 150 base pair reads. All sequencing data was deposited in GEO and is available under accession number GSEXXXXXXXXXX.

2.7. Data analysis

2.7.1. Behavioral data

DPMRA provides two types of results. The first one includes an "enhanced photomotor response" (EPR), until a maximum response is reached. Unlike fish and other vertebrates, in which maximum values are achieved immediately after the first stimuli, *Daphnia* individuals need more than one stimuli to reach the maximum response. Then, during the "habituation" (H) process, the photomotor response to subsequent stimuli decreases (Bedrossiantz et al., 2020). To normalize habituation data, photomotor responses (distance moved) were scaled to maximum values (Faria et al., 2019b). For statistical comparisons between treatments versus their control, the Area Under the Curve (AUC) of both EPR (first 10 stimuli) and H (last 20 stimuli) was

computed following previous studies (Bedrossiantz et al., 2020; Best et al., 2008; Faria et al., 2019a) and analyzed using a one-way ANOVA with treatments as factors. Dunnett's multiple comparison tests were subsequent used for comparing treatments against their control.

2.7.2. Transcriptomic analysis

An average of 25.6 × 10^6 ±1.1 6 × 10^6 reads was generated per sample. Quality of the raw reads was assessed using FastQC version 0.11.8 (Babraham Institute, UK). Reads were dynamically trimmed using Trimmomatic version 0.38 (Bolger et al., 2014) to remove potential Illumina adapter contamination, too short reads and low-quality bases (Phred scale score <20) at the start or end of the read. After that, trimmed reads were mapped against the reference *D. magna* transcriptome (Orsini et al., 2016) using HISAT2 version 2.1.0 (Kim et al., 2015), with an average alignment rate of more than 78%. Then, aligned reads were processed with HTSeq version 0.11.0 (Anders et al., 2015) to generate read counts. The obtained counts were further analyzed within the R statistical environment (R Development Core Team, 2010) using edgeR for statistical analysis (Robinson et al., 2010). Genes with less than 1 count per million in less than two samples were discarded, and the resulting read counts were normalized by applying trimmed means of M-Values (TMM). Gene expression of each treatment was compared with its control using a genewise exact test approach. The final *p* values were corrected using the Benjamini-Hochberg post-hoc correction (false discovery rate, FDR). Significant (FDR<0.05) differences were determined by using Fisher's exact test, identifying the differentially expressed genes (DEGs) per treatment. Functional analysis was performed with this DEGs, annotating them into gene families, gene ontology (GO) categories and Kyoto encyclopedia of genes and genomes (KEGG) pathways, using the available *D. magna* annotation (Orsini et al., 2016) and the entire KEGG database (Kanehisa, 2000). Fisher's exact test was performed to identify enrichment or overrepresentation of gene families, GOs or KEGG pathways within the DEG set (Asselman et al., 2012). Enrichment significance was set at FDR<0.05. Within gene families, uncharacterized proteins were not considered for functional analysis representation. Further graphs were obtained using R environment gplots, pheatmap and intervene (Khan and Mathelier, 2017) packages. In addition, selected human protein sequences were blasted against *D. magna* genome using the online program protein BLAST (blastp algorithm) (McGinnis and Madden, 2004) in order to look for specific genes involved in pathways of interest.

2.8. Data validation by qRT-PCR

Obtained gene expression of DEGs from RNAseq were further confirmed and validated by quantitative real-time PCR (qRT-PCR), using six randomly selected DEGs. Further details of selected genes, primers and qRT-PCR methods are shown in Supplementary Material (Methods and Table S2).

3. RESULTS AND DISCUSSION

3.1. Effects of neuroactive chemicals on *Daphnia* Photomotor Response

A broad range-finder (0.1 to 1000 μg/L for each substance) experimental setup was used to identify concentrations with highly significant effects on *D. magna* photomotor response (experimental groups and selected concentrations are detailed in Supplementary Material, Table S1). Analysis of the obtained responses revealed that the neuroactive chemicals 6OH and MEM decreased EPR, whereas APO and PCPA increased it (statistical details in Table S3). In addition, 6OH and FX increased significantly habituation whereas DIPH and PCPA decreased it (Figure 1, statistical details in supplementary Table S3).

There is reported evidence that dopaminergic agonists, such as APO, increase motile responses, whereas antagonists, like 6OH, decrease them and impair habituation to repeated stimuli in mammals and zebrafish (Carrera et al., 2012; Carvalho et al., 2013; Irons et al., 2013; Nechaev et al., 2006). In *D. magna* it was reported that 6OH impair habituation in 4-day-old juveniles (Bedrossiantz et al., 2020), which is in agreement with our results. FX, that has been reported to promote the accumulation of serotonin (Rivetti et al., 2019), increased habituation having only minor effects on locomotor activities. Conversely, PCPA, that inhibits serotonin synthesis, decreased habituation and increased motile responses to repeated light stimuli. These results agree with previous PCPA responses in *D. magna* juveniles (Bedrossiantz et al., 2020). Reduced habituation in organisms having their levels of serotonin diminished is consistent with reported responses in rodents, fish and the gastropod *Aplysia* (Carlton and Advokat, 1973; Conner et al., 1970; Faria et al., 2019b; Glanzman et al., 1989). Memantine, a NMDA receptor antagonist, decreased dramatically EPR (Figure 1C). This effect is consistent with previous studies in *Daphnia*, zebrafish and mice, showing that memantine reduces motor responses (Best et al., 2008; Faria et al., 2019b; Klamer et al., 2004; Kruchenko et al., 2014). Diphenhydramine, H_1 antihistaminic receptor drug, reduced habituation in *Daphnia* (Figure 1D), which is

consistent with previous reported results in *Daphnia* juveniles (Bedrossiantz et al., 2020) and with the reported somnolence responses of this drug in humans (Kay, 2000).

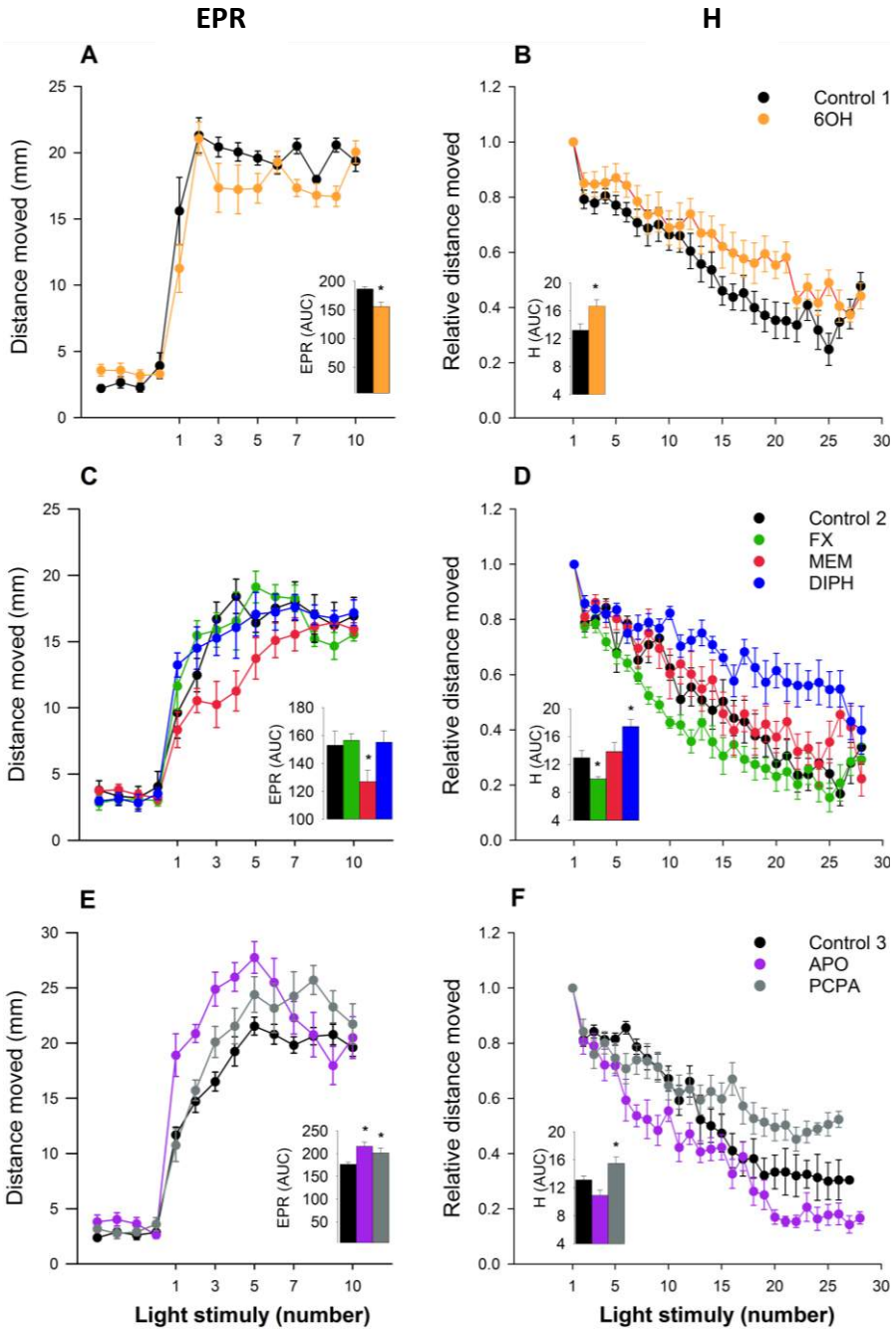

Figure 1. Effect of the tested neurological modulatory compounds on the enhanced photomotor responses (EPR; graphs A, C and E) and habituation (H; graphs B, D and F) processes. Plots of average distance moved ±SE (N=12) against 30 tapping stimuli at 5 s ISI and corresponding bar chards (inlet graphs) of calculated Area Under the Curve (AUC, mean ± SE) for EPR and H phases. EPRs are within the first 10 stimuli, whereas H responses measures the decrease in the distance moved after the maximum response to the light stimulus is reached. H responses are depicted relative to the max (set to 1). Graphs depict data for the three selected experiments: experiment 1 (A and B) control and 6OH (1000 μg/L); 2 (C and D) control, DIPH (1000 μg/L), FX (5 μg/L) and MEM (3 μg/L); 3 (E and F) control, APO (1000 μg/L) and PCPA (40 μg/L). Within AUC bar graphs, * means significant (P<0.05) treatment differences relative to controls following ANOVA and Dunnett's tests.

3.2. Transcriptomic effects produced by neuroactive chemicals

3.2.1. Differential gene expression across different exposures

Up to 1304 unique genes were identified as differentially transcribed (DEGs, FDR<0.05) across the different neuroactive exposures. More specifically, the analysis identified 585, 521, 465, 451, 177 and 84 DEGs for FX, DIPH, 6OH, APO, MEM and PCPA, respectively (Table 1). In general terms, most DEGs were over-represented in the treatments relative to the controls (Figure S2, Supplementary Material). This circumstance was particularly prevalent in the APO-treated samples (95% of DEGs), whereas MEM-treated samples showed almost the same proportion of over- and under-represented DEGs relative to controls (103 vs 74, respectively, Table 1).

Table 1. Number of DEGs (FDR<0.05) per treatment respect to its corresponding control. Number of genes over-represented (up, $log_2FC>0$) and under-represented (down, $log_2FC<0$) with respect to the control is specified.

Nº DEGs	6OH	APO	DIPH	FX	MEM	PCPA
total	465	451	521	585	177	84
up	367	431	425	422	103	68
down	98	20	96	136	74	16

Figure 2A shows the hierarchical clustering of the 1304 unique DEGs, which differentiated in two major clusters. The first one corresponded to APO and 6OH exposures. Both neuroactive chemicals are described as affecting the dopaminergic system, in an agonist or antagonist way, being APO a non-selective agonist of dopamine D_2-like receptors (Bownik et al., 2018; Jenner and Katzenschlager, 2016) and 6OH a selective toxin for dopaminergic neurons (Breese et al., 2005; Feng et al., 2014). Consistently, both compounds elicited opposite transcriptomic responses on *Daphnia* transcriptome in terms of over- or under-representation of the different DEGs (Figure 2A, relative abundances for each DEG and treatment in supplementary Figure S2). Intersection analysis (Figure 2B) identified a large overlap between DEGs affected by APO and 6OH, sharing 202 DEGs unique for these treatments. Common DEGs were mainly annotated as uncharacterized proteins (54% of the total), or as related to cuticle proteins (28%) or to collagen (5%). This contrasting transcriptomic pattern of 6OH and APO was in line with their opposite behavior effects (compare Figures 1A, B with Figures 1E, F).

The second cluster agglutinated the rest of the treatments, and it was subdivided into two sub-clusters, one corresponding to MEM and PCPA, and the other

corresponding to DIPH and FX. Intersection analysis (Figure 2B) identified 200 unique shared DEGs between these two neuroactive chemicals. Functional analysis showed that 50% of these DEGs corresponded to uncharacterized proteins, and the other 50% was split between different categories, such as cuticle proteins, ammonium transporters, arginine/serine-rich proteins or serine protease. FX and PCPA affect serotonin concentration of *D. magna,* as demonstrated in previous studies, by increasing it in the case of FX (Rivetti et al., 2019) or by decreasing it for PCPA (Fuertes and Barata, 2021). This latter effect was related to this inhibitory effect of PCPA on tryptophan hydrolase, the rate-limiting enzyme in serotonin synthesis (Faria et al., 2019b; Geller and Blum, 1970). Both compounds, which obtained a distinctive response in behavioral assays (Figure 1), showed hardly any common DEGs, being restricted to only 6 genes (Figure 2B). This fact suggests that its agonistic and antagonistic effects on serotonin are affected through different genes and metabolic pathways.

Figure 2B represents only the obtained intersections with a minimum of 6 genes due to the large number of intersections with low number of common DEGs. In fact, the intersection corresponding to DEGs detected in all treatments has only two members (although is not shown in Figure 2 because only intersections with more than 5 genes are displayed). Between the larger gene groups represented in Figure 2B were those corresponding to DEGs from a single treatment, being 181 unique DEGs for FX, 141 for DIPH, 133 for APO, 92 for 6OH and 31 for MEM. This suggests a largely specific transcriptomic effect for each treatment.

DEGs obtained from sequencing data were further validated by gene expression analysis of 6 DEGs by qRT-PCR. Results from qRT-PCR correlated significantly ($P<0.05$) with those of RNAseq. For five out of six genes, Pearson correlation values were higher than 0.8 (r=0.59 for obstB, Figure S1, Supplementary Material).

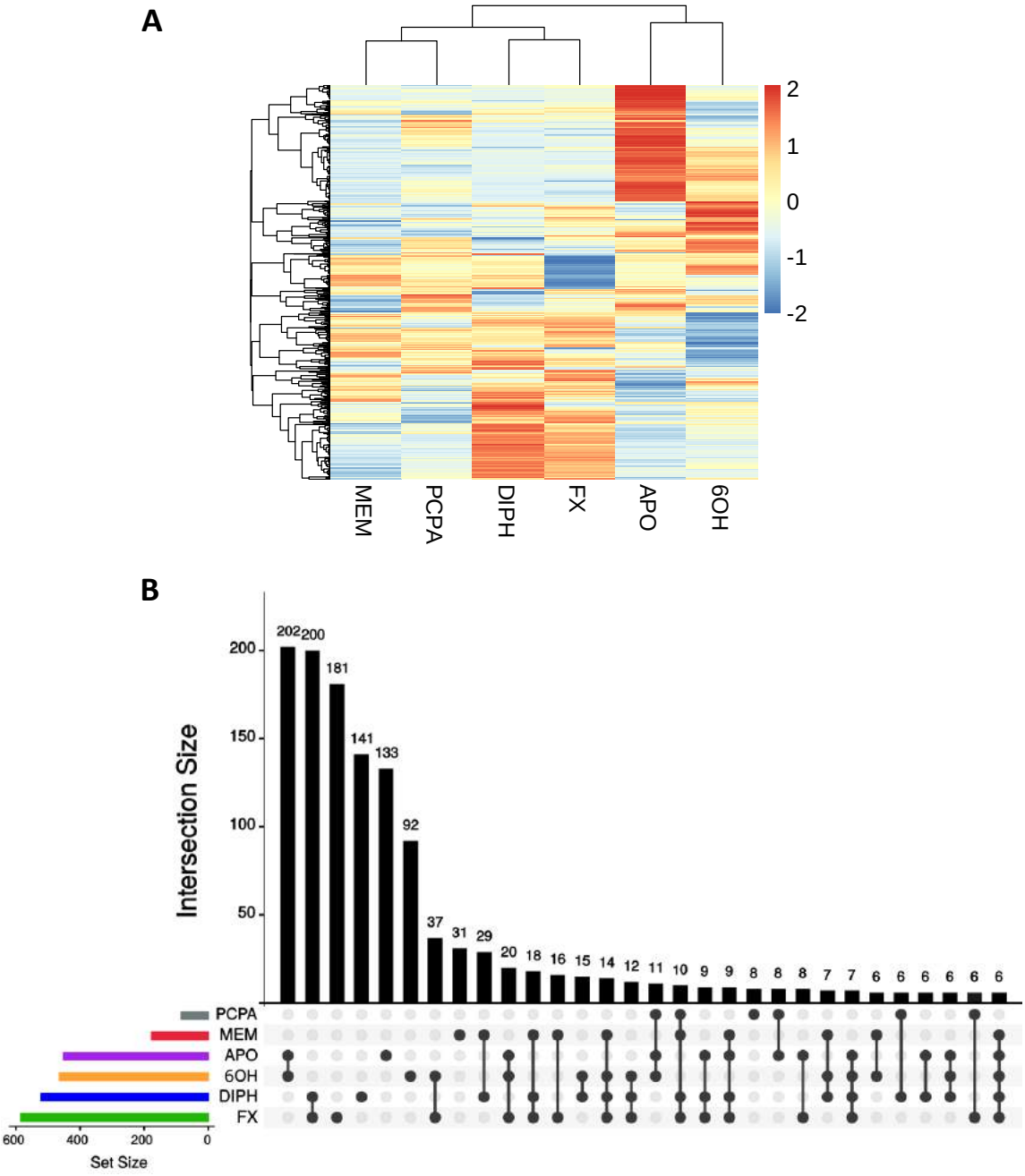

Figure 2. DEGs per treatment. A: Hierarchical clustered heatmap of all DEGs obtained per individual treatment (N=3) respect to its control. In total, 1304 unique genes were differentially expressed between all treatments. Complete clustering method and columns clustered with euclidean distance measure. Colours indicate fold change (log_2), being over- (red) or under-represented (blue) respect to the control. B: UpSet plot of the DEGs intersection of the different neuroactive chemical exposures. Set size corresponds to the DEGs per treatment (N=3) respect to its control. Black bars represent the number of unique common genes related to the compounds whose dots are linked in the diagram below. Only intersections with more than 5 genes are displayed.

3.2.2. Functional relevance of DEGs

Functional analysis showed significant enrichment of several categories levels of functional annotation (gene families, gene ontologies and pathways), suggesting that the corresponding physiological functions were altered by the different treatments (Figure 3, Supplementary Tables S4-S8). Some of these functional categories included gene families related to collagen, cuticle proteins or neurotrophin 1, among others. The distribution of DEGs belonging to these gene families among treatments is represented in Figure 4A.

Collagen gene family was particularly enriched in APO-treated samples, with most members over-represented in samples treated with the dopaminergic effectors 6OH and APO. The gene family showed a pattern of strong upregulation for the majority of the genes upon exposure to 6OH and particularly to APO, which was not present in the other four treatments (Figure 4A). In this regard, is worth noticing that APO is a synthetic derivative of morphine, and that studies in mice reported an increase in the expression of collagen after exposure to morphine, which in turn is associated with a dysfunctional dopaminergic system (Gaweda et al., 2020). Furthermore, a functional relationship between collagen and neuroprotection was reported in different studies (Cheng et al., 2009).

The cuticle proteins gene family (and other cuticle-related gene families), as well as the GO term "structural constituent of cuticle", were also specially enriched upon exposure to 6OH and APO. Again, the majority of the genes were upregulated after exposure to both 6OH and APO. The degree of upregulation showed little variation for 6OH within the gene family while a larger variation in the degree of upregulation could be observed for APO (Figure 4A). Nevertheless, cuticle proteins' gene family was detected and enriched in all treatments. Cuticle proteins has already been described as early response genes to environmental stressors in *D. magna* (Orsini et al., 2018), affecting molting, growth and egg formation. The expression of the genes belonging to these categories was up-regulated respect to its control in all treatments, although it was remarkably high for APO. Cuticle and dopamine signaling and dynamics appears to be mutually related in *Drosophila* and other species, reporting dopamine as cross-linked with cuticle proteins to provide greater structural integrity (Neckameyer et al., 2000; Yamamoto and Seto, 2014; Zhang et al., 2020). This fact could be related to the results obtained in previous studies (Fuertes and Barata, 2021). Despite that only 6OH induced a significant change in the dopamine content of treated animals, all the studied compounds produced disruption of different

catecholamines, such as normetanephrine, norepinephrine or epinephrine, whose direct precursor is dopamine (Fuertes and Barata, 2021).

Neurotrophin 1 gene family was particularly enriched for APO and FX neuroactive exposures, with an overrepresentation of transcripts belonging to this category in both treatments (Figure 4A). Neurotrophins, or neurotrophic factors, are molecules that enhance the growth and survival potential of neurons, and are mediators of synaptic and morphological plasticity (McAllister, 2001; McAllister et al., 1999). Several studies have reported an up-regulation of neurotrophic factors by both APO and FX drugs in mammalian models (APO and FX), suggesting neuroplasticity and neuroprotective effect by the over-expression of these molecules in the brain (Levy et al., 2019; Ohta et al., 2010, 2000; Wang et al., 2008).

There were other gene families or GO terms that showed significant (FDR<0.05) enrichment (Table S4 and S5, Supplementary Material), as serine protease gene family, or proteolysis, protein binding and oxidation-reduction processes GO terms, specially enriched in FX treatment, as well as chitin metabolic process, that was significantly enriched in 6OH, DIPH, FX and MEM exposed samples. Chitin is involved in morphogenesis, molting and growth in insects (Merzendorfer, 2003). Previous studies have linked its metabolism to effects on growth and reproduction in *Daphnia* (Asselman et al., 2018; Campos et al., 2019; Christjani et al., 2016; Fuertes et al., 2019b), so a possible effect on the reproduction after exposure to these compounds cannot be discarded. Apolipoprotein gene family was significantly enriched for FX samples, probably related to the lipid disruption caused by this neuroactive chemical reported in previous studies (Fuertes et al., 2019b).

With respect to the significantly enriched pathways, the different obtained KEGG categories and their distribution among treatments are represented in Figures 3C and 4B. Multiple aspects of brain metabolism, function, and structure are thought to depend on arachidonic acid (Rapoport, 2008). Arachidonic acid metabolism was found to be significant enriched upon APO exposures (Table S6), and genes belonging to this KEGG pathway were over-expressed in APO, DIPH, FX and MEM treatments, but specially in PCPA (Figure 4B). This over-expression in PCPA samples could be related with previously reported significant reduction of serotonin in *D. magna* by this neuroactive chemical (Fuertes and Barata, 2021). There is a cross-talk between serotonin receptors and arachidonic acid metabolism (Tournois et al., 1998), which in insects regulates reproduction (Stanley, 2006), and an elevated brain arachidonic acid signaling has been related with deficiencies in serotonin transporters (Basselin et al.,

2009). Furthermore, the specific enrichment of the arachidonic acid pathway in APO samples has been previously reported in rats, where this neuroactive chemical produce the stimulation of brain arachidonic acid signaling (Bhattacharjee et al., 2008).

ECM (extracellular matrix) - receptor interaction and focal adhesion were two KEGG pathways significantly enriched by all the tested chemicals. DEGs belonging to these pathways were over-represented in all treatments, but especially in samples treated with dopaminergic compounds (6OH and APO) (Figure 4B). Some of the DEGs in these pathways belonged to genes from the collagen family, already described to be affected by dopaminergic compounds. The involvement of collagen in ECM-receptor is known, and have been related with neuromuscular junction and thus with cholinergic synapse (Heikkinen et al., 2020). Interestingly, a disruption in the amount of choline in *D. magna* exposed to APO was observed in previous studies (Fuertes and Barata, 2021). The ECM provides a number of critical functions in the CNS (Franco and Müller, 2011) as well as focal adhesion (Ditlevsen et al., 2007), and have been particularly associated with the dopaminergic system (Ditlevsen et al., 2007; Mitlöhner et al., 2020). ECM has been linked with the control of synaptic activity to regulate neuronal structure and function and thereby impact animal behavior (Kerrisk et al., 2014). In particular, disruption of ECM-receptor interaction pathway has been observed in some studies with rats and 6OH treatments (Li et al., 2019; Sievers et al., 1994).

Given the characteristics of the chemical compounds tested in this study, the results obtained in the enrichment of pathways related to neurotransmitters are of particular importance. Serotonergic synapse pathway appeared significantly enriched for DIPH and FX treatments, and also glutamatergic synapse was significantly enriched for DIPH (Figure 3C and Table S6). Most of DEGs from both categories were the same, due to the fact that both routes have many non-specific genes in common, and thus its distribution in Figure 4B is represented together. DEGs belonging to these neurotransmitters pathways were up-regulated in all treatments. Relationship between FX and serotonin is well reported, being FX a selective serotonin reuptake inhibitor (SSRI) antidepressant able to promote the accumulation of serotonin in the CNS of *D. magna* (Campos et al., 2016; Rivetti et al., 2019). The reported NMDA inhibiting effect of the anti-histaminic drug DIPH (Adolph et al., 2014; Fang et al., 2014) may explain why these DEGs are also related to the glutamatergic synapse. Furthermore, there is also reported information indicating that DIPH may also be a serotonergic chemical (Tanaka et al., 2011).

Other pathways that appeared enriched for some treatments were retinol metabolism and steroid biosynthesis. Retinol metabolites, and particularly retinoic acid, have been reported to have a key function in the development of CNS (Maden, 2001). This pathway appeared enriched in APO and FX treatments, being DEGs from this pathway over-represented for both treatments and also for PCPA. Steroid biosynthesis, which influence in the activity of neurons, synaptic plasticity, behavior or brain homeostasis (Jauregui-Huerta et al., 2010; Ozawa, 2005), was also enriched for both APO and FX exposures. Nevertheless, in this case the two treatments have opposite the effects on the relative abundance of the implicated DEGs, suggesting opposite steroid-related effects. Also starch and sucrose metabolism KEGG pathway was significantly enriched after APO exposure. However, DEGs in this category were under-expressed for 6OH and over-expressed for APO, suggesting some relationship between antagonist and agonist effects on dopamine and sucrose metabolism. Enrichment of other KEGG pathways that has been related with the CNS in *Drosophila*, implicating hippo, p53 or TGF-beta signaling pathways was also observed. Hippo signaling pathway is in charge of maintaining quiescence in *Drosophila* neural stem cells (Ding et al., 2016), and was significantly over-represented in DIPH and FX treatments. P53 and TGF-beta signaling pathways were significantly over-represented in DIPH, FX and MEM. In *Drosophila*, p53 has been related to neuroprotective function, preventing apoptosis, and inducing the expression of synaptic genes in the adult brain (Contreras et al., 2018), and TGF-beta signaling pathways has been linked to neuronal remodeling, neuromuscular junction and axon guidance (Upadhyay et al., 2017). TNF signaling pathway was also enriched in DIPH, FX and MEM treatments, and has been related in humans to have several functions within the CNS, including glutamatergic transmission, and synaptic plasticity and scaling (McCoy and Tansey, 2008).

3.2.3. Effects on specific enzymes related to neurotransmitter pathways

Among all the obtained DEGs, a specific search was made for enzymes involved in neurotransmitter synthesis and metabolism or related genetic pathways, as beta-hydroxylase and aromatic amino acid decarboxylase (AAAD).

In serotonergic neurons, amino acid L-tryptophan is converted to 5-hydroxytryptophan (5-HTP) by the action of the enzyme tryptophan hydroxylase, and subsequently converted to the neurotransmitter serotonin by the enzyme AAAD. In dopaminergic neurons, tyrosine hydroxylase produces 3,4-dihydroxyfenilalanina (L-DOPA), that is converted by AAAD to the catecholamine dopamine. In adrenergic neurons, dopamine is converted to norepinephrine by dopamine beta-hydroxylase and

can be further converted by phenylethanolamine-N-methyltransferase to epinephrine, or by catechol O-methyltransferase to normetanephrine (Horzmann and Freeman, 2016). Also, in the biosynthesis of catecholamines and trace amines pathways, tyramine beta-hydroxylase catalyzes the hydroxylation of tyramine to octopamine (Châtel et al., 2013).

D. magna tyramine and dopamine beta-hydroxylase proteins showed high protein similarity based on BLAST to the same *D. magna* gene (Dapma7bEVm004237), a situation similar to what has been reported for *D. pulex* (McCoole et al., 2012a). This beta-hydroxylase gene appeared as differentially expressed for 6OH, DIPH, FX and MEM exposures, being over-expressed in 6OH samples and under-expressed in DIPH, FX and MEM. In previous studies (Fuertes and Barata, 2021), 6OH exposed samples showed significant lower levels of dopamine and of its metabolites 3-MT, which is explained by the increase of the expression of beta-hydroxylase. Down-regulation of this gene supports the previously reported decrease in epinephrine upon exposure with DIPH and the decrease in norepinephrine in MEM samples (Fuertes and Barata, 2021). Furthermore, down-regulation of beta-hydrolase in FX exposures is in line with the increase in tyramine reported in other previous study (Gómez-Canela et al., 2019).

AAAD protein was identified as the gene product of Dapma7bEVm004237, which in turn is homologous of Ddc gene in *Drosophila melanogaster*. This gene appeared as over-expressed upon exposure to APO, which supports previously reported changes in the concentrations of tryptophan, L-DOPA (both decreased) and dopamine (increased) in *D. magna* exposed to this compound (Fuertes and Barata, 2021). To a lesser extent, AAAD appear also over-expressed in DIPH, FX and MEM samples. Significant changes in neurotransmitters were barely reported in DIPH and MEM samples in a previous reported study, although FX treatment was reported to increase serotonin levels (Rivetti et al., 2019). Over-expression of AAAD has been already reported in *D. magna* studies with fluoxetine (Campos et al., 2013), and it has already been shown to be involved in insect behavior, cuticle maturation, neuronal regulation, pigmentation patterning, and innate immunity (Hodgetts and O'Keefe, 2006). Up-regulation of AAAD in rats exposed to SSRIs has been suggested as a response to the blocking of the re-uptake mechanisms, which leads to low levels of serotonin inside the terminal neuron axon (Choi et al., 2012). AAAD appeared also under-represented in PCPA-treated samples and, to a lesser degree in 6OH-treated ones. This fact is consistent with the significant lower amount of serotonin in PCPA-

treated samples and of dopamine and its metabolite 3-MT in 6OH exposures reported in a previous study (Fuertes and Barata, 2021).

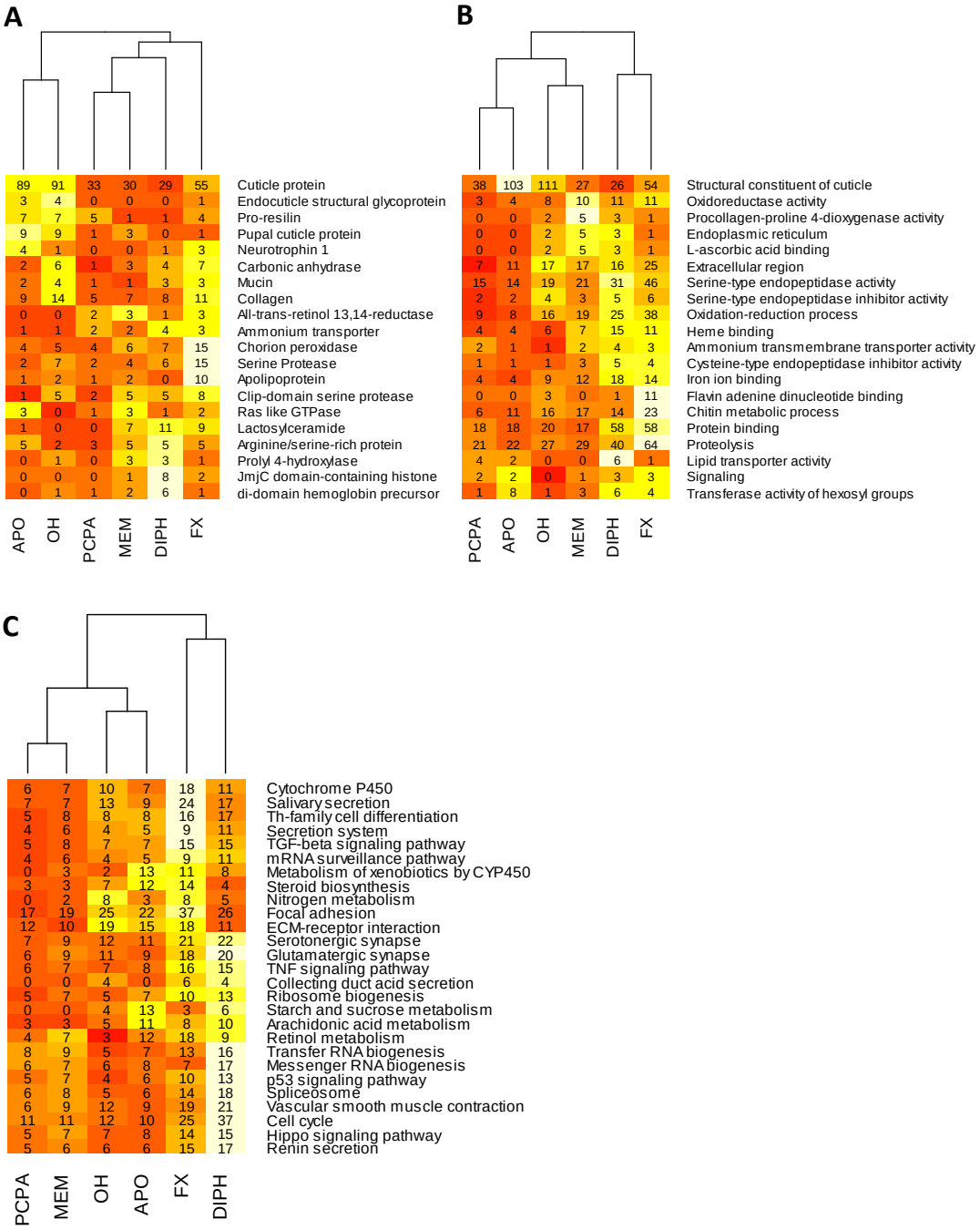

Figure 3. Distribution of DEGs among the different gene families (A), GO categories (B), KEGG pathways (C) and neuroactive treatments (columns). Redundant functional categories were simplified to the one with the highest number of hits. Functional categories were selected as the significant (FDR<0.05) ones for each treatment, and each category was linked to the number of genes belonging to that one in the remaining treatments. Complete functional results are in Tables S4 to S9 (Supplementary Material). Numbers indicate the absolute number of DEGs for each category and treatment, and colours (heat code, from red (few) to white (most)) represent the relative importance of genes associated with each category for each treatment.

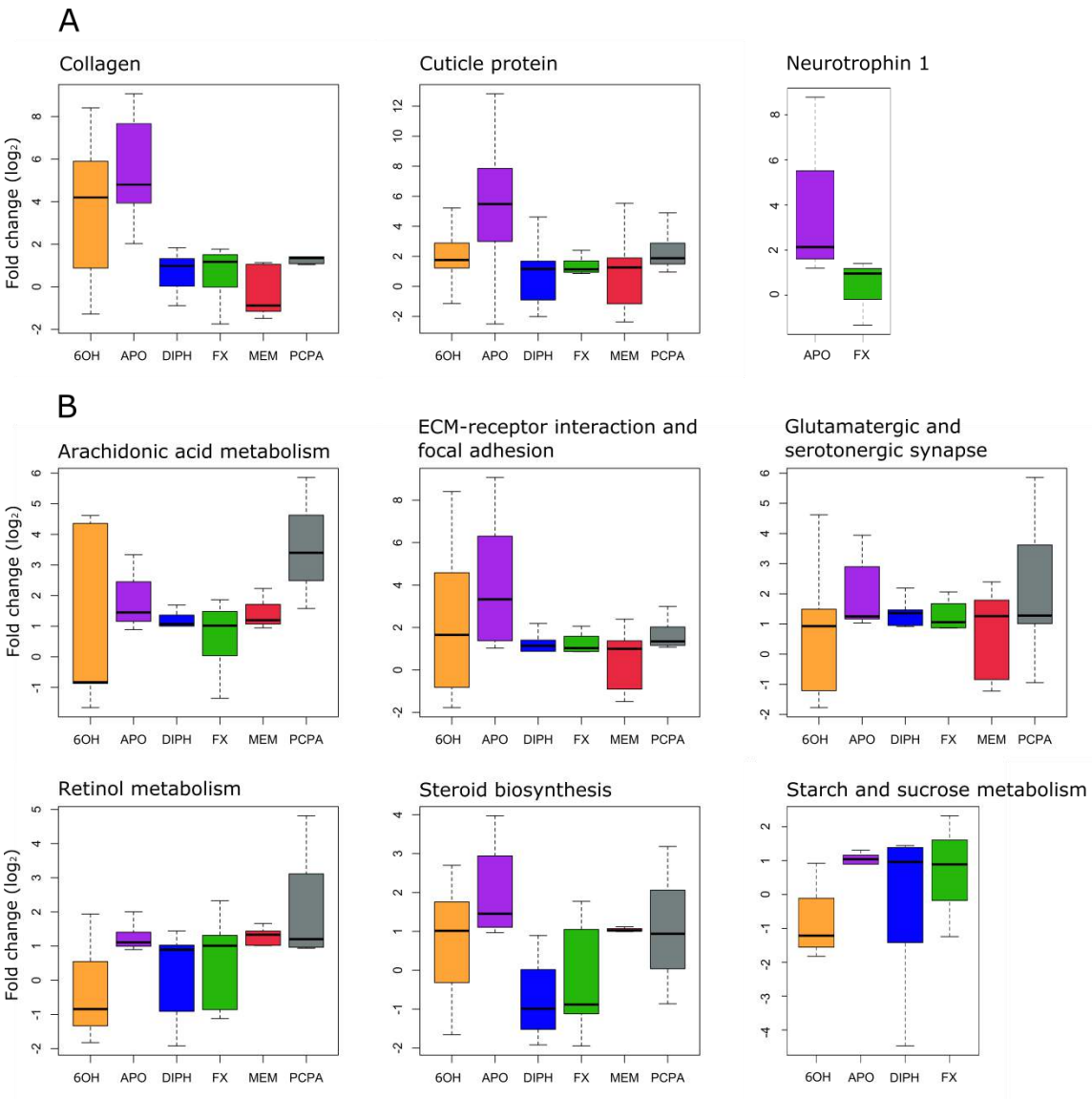

Figure 4. Boxplots of the distribution of DEGs (FDR<0.05) from selected gene families (A) and KEGG pathways (B) per treatment (N=3). Boxes indicate ranges from the first to the third quartiles, the thick lane indicates the median, and the whiskers indicate the 10 and 90th percentiles. Gene expression for the exposure treatments is presented relative to the average of the controls as fold change (log_2).

4. CONCLUSIONS

The integrative approach of behavioral and transcriptomic effects of neuroactive chemicals in *D. magna* presented here is a first step to answer the increasing need to develop tools to detect and understand the possible effects of neuroactive compounds in environmental and model organisms. These analyses have been up to now almost solely performed in mammals, including humans. Our study identified 1304 unique early response genes were identified as DEGs across the different neuroactive

exposures. Commonly affected transcripts in 6OH and APO treatments, an agonist and an antagonist of dopamine, respectively, were identified as the largest DEG overlap between treatments. Different patterns in terms of up- and down- gene expression were identified between both treatments. A common effect on collagen and cuticle genes was identified, being both gene families related to dopamine signaling and dynamics, as well as ECM-receptor interaction and focal adhesion. The second biggest gene intersection corresponded to DIPH and FX treatments that produced similar effects in serotonergic synapse pathway, hippo, p53, TNF or TGF-beta signaling pathways. A specific over-expression of genes belonging to arachidonic acid metabolism was identified for PCPA samples, establishing a possible relationship with its capacity to induce serotonin deficiency in treated animals. However, no common functional categories affected either in a similar or an opposite way enough were identified between FX and PCPA, both compounds affecting serotonin. Although no early transcriptional response of target receptors described for these compounds was identified, two DEGs related with metabolism of neurotransmitters were identified (beta-hydroxylase and AAAD), relating their expression to the effect of these compounds on neurotransmitters, as reported in previous study (Fuertes and Barata, 2021). The study therefore provides the first indications of specific transcriptional mechanisms in a ecotoxicological model organism *D. magna* in response to neuroactive chemicals but further study is needed to elucidate the specific mechanisms of action at the transcriptional level of these compounds in this and other ecotoxicological models.

Acknowledgments

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Supplementary Material: scientific article VI

Effects of behavior-disrupting neuroactive chemicals *in Daphnia magna* cephalic transcriptome.

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METHODS

RNA extraction. Total RNA was isolated from samples using Trizol reagent (Invitrogen, USA) and following manufacturer's protocols with slight modifications. After RNA isolation, DNAse treatment was performed according to manufacturer's protocols, followed by a double phenol-chloroform extraction and a further chloroform-only extraction to achieve further purification. RNA was precipitated using sodium acetate and 100% ethanol, being re-suspended in RNAse-free water. Obtained RNA was quantified and its quality was checked in a NanoDrop 8000 Spectrophotometer (NanoDrop Technologies, USA). Samples presenting a ratio 230/260-260/280 between 1.9-2.1 were selected. RNA integrity was checked using an Agilent 2100 Bioanalyzer with RNA Nano Chips (Agilent Technologies, USA). Only the samples showing RIN values above 9 were used for microarray analysis. Prior to sequencing, RNA concentration was measured in a QuBit 2.0 Fluorometer (Invitrogen, USA).

Validation of RNAseq results by qRT-PCR. Obtained gene expression of DEGs from RNAseq were further confirmed and validated by quantitative real-time PCR (qRT-PCR). Six DEGs from RNAseq results were selected randomly, and are detailed in Table S2. The gene G3PDH (glyceraldehyde 3-phosphate dehydrogenase) was used as an internal control (house-keeping) (Campos et al., 2013) as its mRNA levels did not change across samples. Primers for each of these genes were designed with Primer Express® Software v3.0.1 (Thermofisher, USA) and are provided in Table S2. Amplification efficiencies were ≥ 90% for all tested genes as described by Pfaffl (Pfaffl, 2002). qRT-PCR was performed according to manufacturer's protocols using four experimental replicates per treatment. Relative mRNA abundances were calculated from the second derivative maximum of their respective amplification curves (Cp, calculated from technical triplicates). To minimize errors on RNA quantification among different samples, Cp values for target genes were normalized by Cp values for G3PDH in each sample. Changes in mRNA abundance in samples from different

treatments were calculated by the ΔΔCp method (Pfaffl, 2001), using corrected Cp values from treated and non-treated samples.

FIGURES

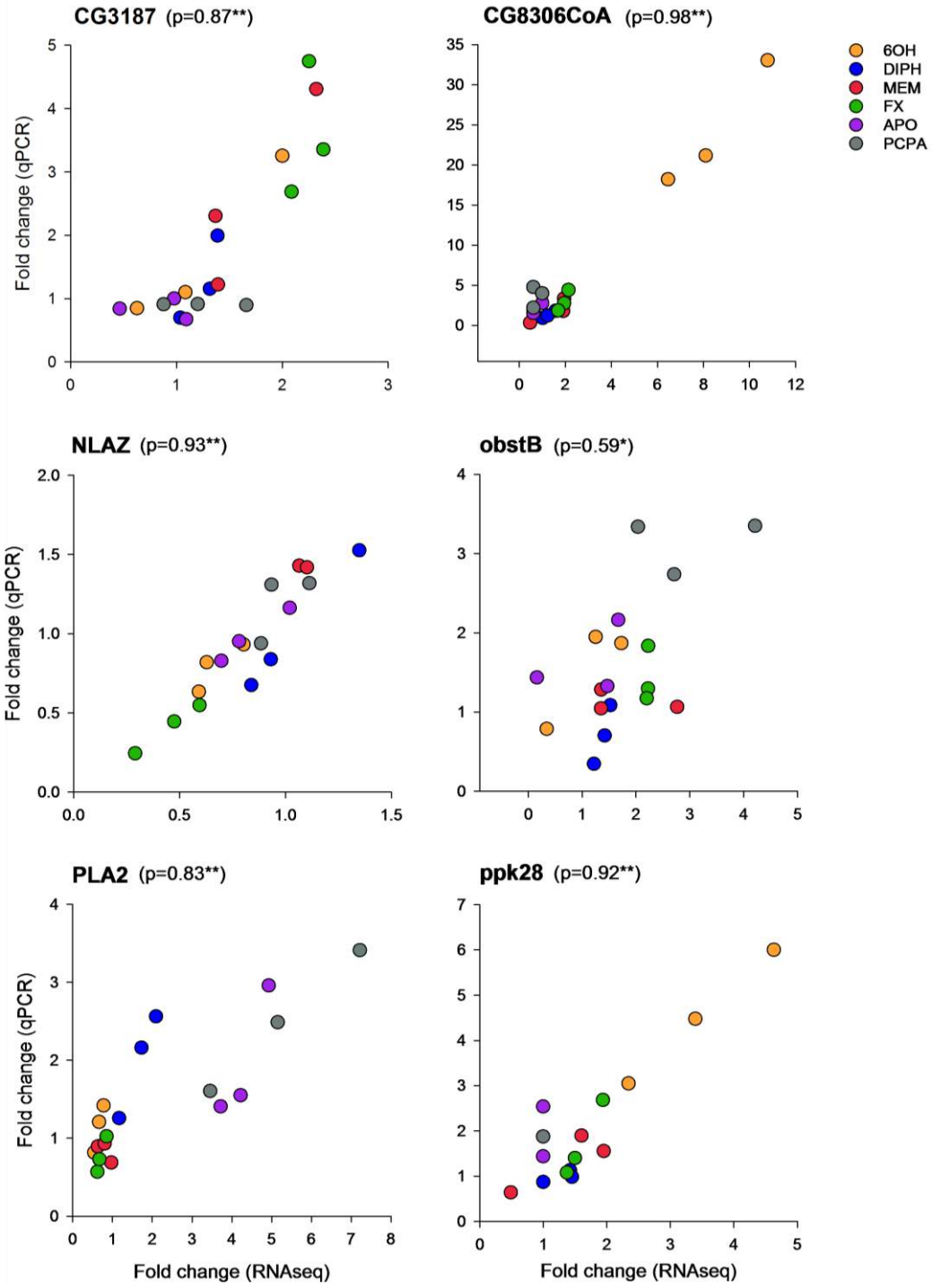

Figure S1. qRT-PCR validation of gene expression RNAseq results for six selected differentially expressed genes. Results are reported as fold change responses relative to control treatments. Each symbol is a single observation. Numbers following gene names are Pearson correlation coefficients (N=18). * 0.01<P<0.05, ** P<0.001.

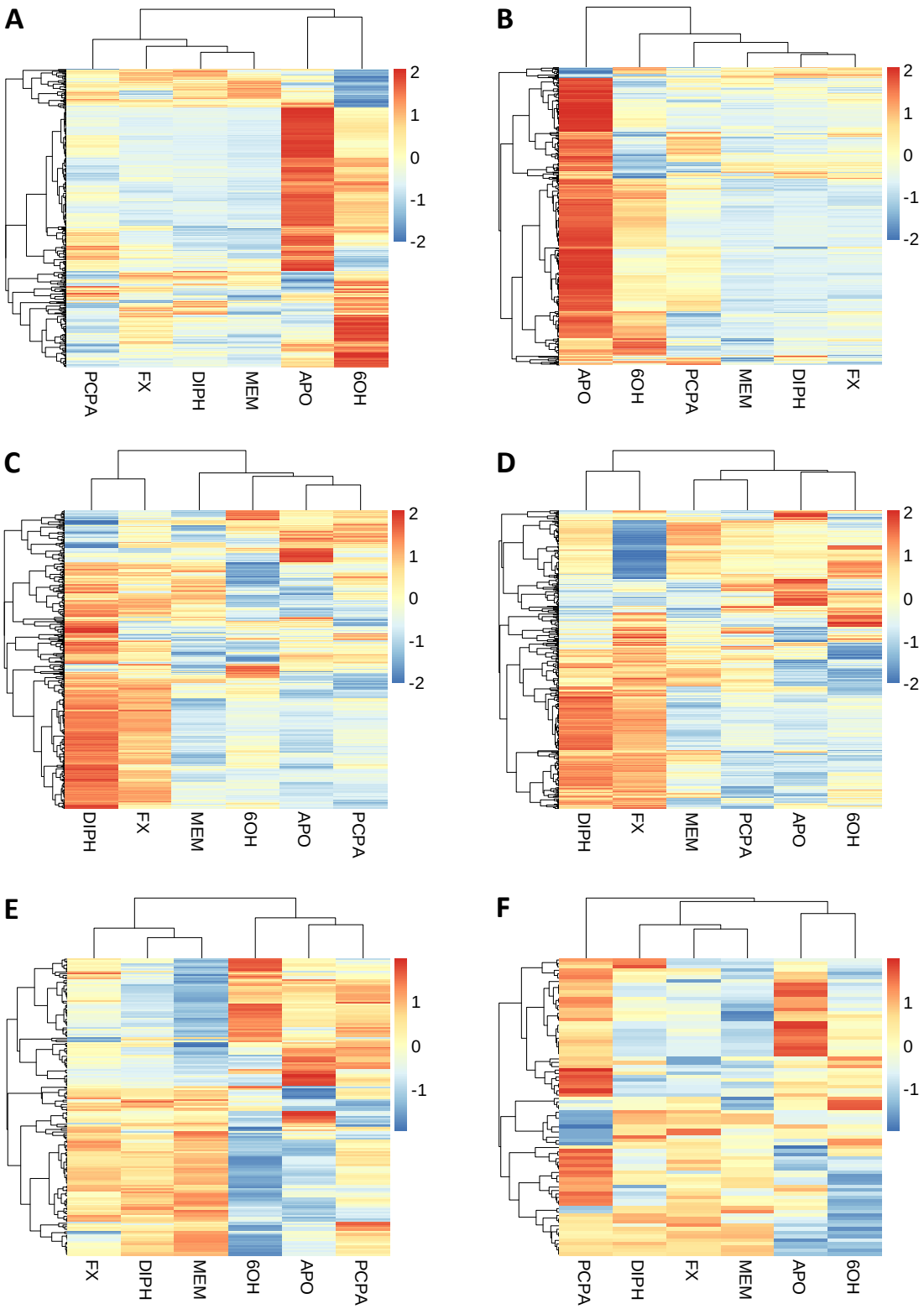

Figure S2. Hierarchical clustered heatmaps of differentially expressed genes (DEGs) obtained per individual treatment respect to its control. Each figure corresponds to A: 465 DEGs from 6OH; B: 451 DEGs from APO; C: 521 DEGs from DIPH; D: 585 DEGs from FX; E: 177 DEGs from MEM; and F: 84 DEGs from PCPA, each one respect to its control and compared to the same genes in the rest of the treatments. Complete clustering method and columns clustered with euclidean distance measure. Colours indicate fold change (log_2), being over-expressed (red) or under-expressed (blue) respect to the control.

TABLES

Table S1. Studied compounds across experiments, description, putative mode of action and tested concentrations.

Neurotransmitter system	Compound	CAS	Description	Mode of action	Concentration
Experiment 1					
Dopaminergic	6-hydroxydopamine (6OH)	28094-15-7	Neurotoxic synthetic organic compound	Destroys dopaminergic and noradrenergic neurons	1000 μg/L (1)
Experiment 2					
Histaminergic	Diphenhydramine (DIPH)	147-24-0	Antihistamine drug	H_1 Receptor antagonist	1000 μg/L (2)
Serotonergic	Fluoxetine (FX)	56296-78-7	Antidepressant	Selective Serotonin Reuptake Inhibitors (SSRIs)	5 μg/L (3)
Glutamatergic	Memantine (MEM)	41100-52-1	Treat moderate to severe Alzheimer´s disease	Antagonist of the NMDA receptor	3 μg/L (4)
Experiment 3					
Dopaminergic	Apomorphine (APO)	147-24-0	Treat Parkinson´s disease	Non-selective dopamine-D_2 agonist	1000 μg/L (5)
Serotonergic	Para-chloro-DL-phenylalanine (PCPA)	7424-00-2	Human drug also known as Fenclonine	Tryptophan hydrolase inhibitor	40 μg/L (4)

1(Feng et al., 2014) , 2 (Berninger et al., 2011), 3 (Rivetti et al., 2016), 4 (Melissa Faria et al., 2019), 5 (Bownik et al., 2018)

Table S2. Primer pairs designed from existing sequences used for amplification of selected *Daphnia magna* partial gene sequences. AZ: amplicon size.

Official gene symbol	Name/function	Acc. Number	*Daphnia* gene	Forward	Reverse	AZ
CG31871	Lipase 3	KZS16460	Dapma7bEVm003259	CAATGTCTGGAAACGAGAGGG	TTCTACACCTGCGGTGTGTGA	71
CG8306	Fatty acyl-CoA reductase	KZS21427	Dapma7bEVm003203	GACGTAACGTCGAGCTCCCT	ATTTTGACGTCCGCGAAATT	71
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase	AJ292555	Dapma7bEVm015323	ACGAGACCCGAAAAACATTCC	CAATGTGAGCATGGGCCTTT	101
obstB	Cuticular protein	KZS12557	Dapma7bEVm000605	CCTGAAATAACAGTAGGCAAGTGC	CTTCGTAGCCGGCATTATGAA	71
ppk28	Sodium channel protein Nach	KZS15670	Dapma7bEVm006748	ATCACATAATGGGAAGGACACAAA	CTTTCGTCGTCATTGCGTTTAAT	71
NLAZ	Neural Lazarillo	KZS14940.1	Dapma7bEVm009537	TCTATAGACACCATAAAAGTTTGGCAA	CACTTTCCCACTTTAAACTAAAACGA	71
PLA2	Phospholipase A2	KZS08312	Dapma7bEVm011274	TGCTCGTCGTCGTTCTTCG	TCCCTGTCGTTGTTGGCTG	81

Table S3. Student's t tests and one-way ANOVA results comparing enhanced photomotor (EPR) and habituation (H) response measured as Areas Under the Curves (AUC) across treatments within experiments. df, t or F and P are degrees of freedom, student t test, Fisher's coefficient and significant levels, respectively.

	df	t or F	P
Experiment 1			
EPR	22	2.5	0.019
H	22	2.7	0.013
Experiment 2			
EPR	3.44	3.200	0.031
H	3.44	10.10	<0.001
Experiment 3			
EPR	2.33	5.6	0.008
H	2.33	9.5	0.001

Table S4. List of genes families enriched for DEGs per treatment, the p-value of the Fisher Exact Test, the number of significant and non-significant genes in the gene family and the Benjamini-Hochberg adjusted p-value (false discovery rate, FDR). Only enrichment results with FDR <0.05 are displayed.

Gene family	p-value	significant	non-significant	FDR
6OH				
Cuticle protein	5.12E-75	91	47	6.69E-72
Collagen	6.04E-10	14	15	2.63E-07
Pupal cuticle protein	1.05E-08	9	4	3.42E-06
Pro-resilin	1.19E-06	7	4	2.59E-04
Endocuticle structural glycoprotein	8.15E-05	4	1	1.52E-02
Mucin	2.32E-04	4	2	3.79E-02
Carbonic anhydrase	3.23E-04	6	10	4.22E-02
APO				
Cuticle protein	7.65E-78	89	49	9.99E-75
Pupal cuticle protein	2.89E-09	9	4	1.26E-06
Pollen-specific leucine-rich repeat extensin protein 1	2.94E-08	6	0	9.60E-06
Pro-resilin	4.40E-07	7	4	1.15E-04
Arginine/serine-rich protein	1.01E-05	5	2	2.21E-03
Collagen	1.80E-05	9	20	3.36E-03
Neurotrophin 1	1.31E-04	4	2	2.14E-02
DIPH				
JmjC domain-containing histone	4.52E-07	8	4	2.35E-04
Cuticle protein	5.39E-07	29	109	2.35E-04
di-domain hemoglobin precursor	5.05E-06	6	2	1.65E-03
Ammonium transporter	3.51E-05	4	0	9.16E-03
Arginine/serine-rich protein	4.95E-05	5	2	1.08E-02

Lactosylceramide	1.55E-04	11	29	2.89E-02
FX				
Cuticle protein	5.73E-20	55	83	3.74E-17
Chorion peroxidase	5.17E-08	15	15	2.25E-05
Apolipoprotein	5.13E-06	10	9	1.67E-03
Clip-domain serine protease	1.01E-05	8	5	2.64E-03
Serine Protease	3.46E-05	15	31	7.53E-03
Collagen	8.12E-05	11	18	1.51E-02
Arginine/serine-rich protein	2.05E-04	5	2	3.34E-02
MEM				
Cuticle protein	1.06E-13	30	108	6.90E-11
Arginine/serine-rich protein	2.76E-06	5	2	1.20E-03
Clip-domain serine protease	1.36E-04	5	8	4.45E-02
Collagen	1.73E-04	7	22	4.53E-02
All-trans-retinol 13,14-reductase	3.02E-04	3	1	4.93E-02
Prolyl 4-hydroxylase	3.02E-04	3	1	4.93E-02
Ras like GTPase	3.02E-04	3	1	4.93E-02
PCPA				
Cuticle protein	5.66E-19	33	105	3.70E-16
Pro-resilin	1.92E-05	5	6	8.35E-03

Table S5. List of GO categories enriched for DEGs per treatment, the p-value of the Fisher Exact Test, the number of significant and non-significant genes in the gene family and the Benjamini-Hochberg adjusted p-value (false discovery rate, FDR). Only enrichment results with FDR <0.05 are displayed.

GO category	p-value	significant	non-significant	FDR
6OH				
structural constituent of cuticle	5.75E-127	111	45	3.79E-124
chitin metabolic process	2.84E-07	16	80	7.22E-05
chitin binding	3.29E-07	16	81	7.22E-05
extracellular region	1.86E-06	17	106	3.07E-04
serine-type endopeptidase activity	2.82E-05	19	162	3.71E-03
proteolysis	1.66E-04	27	317	1.82E-02
APO				
structural constituent of cuticle	5.89E-111	103	53	3.88E-108
transferase activity, transferring hexosyl groups	9.09E-06	8	22	3.00E-03
protein binding	1.02E-04	18	1058	2.24E-02
DIPH				
serine-type endopeptidase activity	6.80E-09	31	150	4.48E-06
structural constituent of cuticle	1.95E-07	26	130	6.42E-05
proteolysis	3.94E-06	40	304	8.65E-04
iron ion binding	5.52E-06	18	83	9.10E-04
heme binding	1.76E-05	15	65	2.26E-03

cysteine-type endopeptidase inhibitor activity	2.05E-05	5	3	2.26E-03
ammonium transmembrane transporter activity	3.80E-05	4	1	3.13E-03
ammonium transport	3.80E-05	4	1	3.13E-03
lipid transporter activity	1.65E-04	6	11	1.21E-02
lipid transport	2.37E-04	6	12	1.56E-02
chitin metabolic process	5.32E-04	14	82	3.00E-02
signaling	5.75E-04	3	1	3.00E-02
chitin binding	5.92E-04	14	83	3.00E-02
extracellular region	8.04E-04	16	107	3.79E-02
FX				
structural constituent of cuticle	2.06E-27	54	102	1.36E-24
serine-type endopeptidase activity	1.98E-17	46	135	6.52E-15
proteolysis	2.55E-16	64	280	5.61E-14
oxidoreductase activity	3.39E-09	11	9	5.58E-07
chitin metabolic process	7.38E-09	23	73	9.73E-07
chitin binding	9.14E-09	23	74	1.00E-06
extracellular region	5.75E-08	25	98	5.41E-06
flavin adenine dinucleotide binding	1.18E-04	11	38	9.74E-03
oxidation-reduction process	1.86E-04	38	302	1.36E-02
cysteine-type endopeptidase inhibitor activity	7.34E-04	4	4	4.65E-02
signaling	8.16E-04	3	1	4.65E-02
serine-type endopeptidase inhibitor activity	8.46E-04	6	14	4.65E-02
MEM				
structural constituent of cuticle	2.75E-14	27	129	1.81E-11
chitin metabolic process	1.22E-09	17	79	3.17E-07
chitin binding	1.44E-09	17	80	3.17E-07
serine-type endopeptidase activity	3.80E-08	21	160	6.26E-06
extracellular region	5.83E-08	17	106	7.69E-06
proteolysis	1.39E-07	29	315	1.52E-05
procollagen-proline 4-dioxygenase activity	8.83E-07	5	3	7.28E-05
iron ion binding	2.53E-05	12	89	1.65E-03
oxidoreductase activity	2.71E-05	10	61	1.65E-03
L-ascorbic acid binding	2.75E-05	5	9	1.65E-03
endoplasmic reticulum	5.72E-05	5	11	3.14E-03
PCPA				
structural constituent of cuticle	6.98E-29	38	118	4.60E-26
serine-type endopeptidase activity	1.36E-05	15	166	4.48E-03
proteolysis	3.19E-05	21	323	7.02E-03

Table S6. List of KEGG pathway enriched for DEGs per treatment, the p-value of the Fisher Exact Test, the number of significant and non-significant genes in the gene family and the Benjamini-Hochberg adjusted p-value (false discovery rate, FDR). Only enrichment results with FDR <0.05 are displayed.

KEGG pathway	p-value	significant	non-significant	FDR
6OH				
ECM-receptor interaction	4.27E-15	19	36	8.28E-13
Focal adhesion	6.66E-06	25	257	6.46E-04
Nitrogen metabolism	3.70E-05	8	32	2.39E-03
Cytochrome P450	2.38E-04	10	69	1.16E-02
Collecting duct acid secretion	1.14E-03	4	11	4.41E-02
APO				
ECM-receptor interaction	8.45E-10	15	40	1.76E-07
Metabolism of xenobiotics by cytochrome P450	8.63E-05	13	88	7.86E-03
Steroid biosynthesis	1.13E-04	12	78	7.86E-03
Starch and sucrose metabolism	1.72E-04	13	95	8.94E-03
Focal adhesion	8.25E-04	22	260	3.03E-02
Retinol metabolism	8.75E-04	12	100	3.03E-02
mRNA surveillance pathway	1.29E-03	5	18	3.84E-02
Arachidonic acid metabolism	3.48E-03	11	104	4.52E-02
DIPH				
RNA surveillance pathway	8.96E-08	11	12	2.19E-05
Hippo signaling pathway	8.53E-06	15	45	1.04E-03
p53 signaling pathway	2.14E-05	13	37	1.42E-03
Secretion system	2.32E-05	11	26	1.42E-03
TNF signaling pathway	1.18E-04	15	59	5.41E-03
Transfer RNA biogenesis	1.33E-04	16	67	5.41E-03
Cell cycle	1.93E-04	37	254	6.72E-03
TGF-beta signaling pathway	2.93E-04	15	65	8.94E-03
Ribosome biogenesis	5.99E-04	13	55	1.62E-02
Serotonergic synapse	7.76E-04	22	132	1.89E-02
ECM-receptor interaction	1.05E-03	11	44	2.13E-02
Spliceosome	1.41E-03	18	103	2.64E-02
Messenger RNA biogenesis	1.66E-03	17	96	2.90E-02
Renin secretion	1.83E-03	17	97	2.91E-02
Glutamatergic synapse	2.02E-03	20	125	2.91E-02
Vascular smooth muscle contraction	2.15E-03	21	135	2.91E-02
Th-family cell differentiation	3.79E-03	17	105	4.62E-02
FX				
ECM-receptor interaction	1.40E-08	18	37	3.42E-06
Cytochrome P450	5.45E-06	18	61	6.65E-04
mRNA surveillance pathway	1.16E-05	9	14	9.41E-04
TNF signaling pathway	3.57E-05	16	58	2.18E-03
Focal adhesion	1.28E-04	37	245	6.25E-03

Collecting duct acid secretion	3.09E-04	6	9	1.06E-02
TGF-beta signaling pathway	3.32E-04	15	65	1.06E-02
Salivary secretion	3.46E-04	24	139	1.06E-02
Steroid biosynthesis	4.71E-04	14	60	1.28E-02
Retinol metabolism	6.40E-04	18	94	1.56E-02
Secretion system	7.31E-04	9	28	1.62E-02
Hippo signaling pathway	1.06E-03	14	66	2.15E-02
p53 signaling pathway	1.90E-03	10	40	3.32E-02
Serotonergic synapse	2.10E-03	21	133	3.42E-02
MEM				
ECM-receptor interaction	3.35E-06	10	45	5.93E-04
mRNA surveillance pathway	3.70E-05	6	17	3.27E-03
p53 signaling pathway	5.41E-04	7	43	1.61E-02
Focal adhesion	5.83E-04	19	263	1.61E-02
Secretion system	6.04E-04	6	31	1.61E-02
Small cell lung cancer	6.04E-04	6	31	1.61E-02
Transfer RNA biogenesis	6.36E-04	9	74	1.61E-02
TGF-beta signaling pathway	2.12E-03	8	72	4.70E-02
PCPA				
ECM-receptor interaction	5.88E-09	12	43	9.29E-07
DNA replication	5.21E-04	7	52	2.82E-02
Focal adhesion	5.35E-04	17	265	2.82E-02
Transfer RNA biogenesis	8.70E-04	8	75	3.44E-02

Table S7. List of selected significant enriched gene families. Enriched significant genes families (FDR<0.05) per treatment were selected, and number of significant DEGs in every treatment is detailed.

Gene Family	6OH	APO	DIPH	FX	MEM	PCPA
All-trans-retinol 13,14-reductase	0	0	1	3	3	2
Ammonium transporter	1	1	4	3	2	2
Apolipoprotein	2	1	0	10	2	1
Arginine/serine-rich protein	2	5	5	5	5	3
Carbonic anhydrase	6	2	4	7	3	1
Chorion peroxidase	5	4	7	15	6	4
Clip-domain serine protease	5	1	5	8	5	2
Collagen	14	9	8	11	7	5
Cuticle protein	91	89	29	55	30	33
di-domain hemoglobin precursor	1	0	6	1	2	1
Endocuticle structural glycoprotein	4	3	0	1	0	0
JmjC domain-containing histone	0	0	8	2	1	0
Lactosylceramide	0	1	11	9	7	0
Mucin	4	2	3	3	1	1
Neurotrophin 1	1	4	1	3	0	0
Prolyl 4-hydroxylase	1	0	3	1	3	0

Pro-resilin	7	7	1	4	1	5
Pupal cuticle protein	9	9	0	1	3	1
Ras like GTPase	0	3	1	2	3	1
Serine Protease	7	2	6	15	4	2

Table S8. List of selected significant enriched GO categories. Enriched significant GO categories (FDR<0.05) per treatment were selected, and number of significant DEGs in every treatment is detailed.

GO category	6OH	APO	DIPH	FX	MEM	PCPA
ammonium transmembrane transporter activity	1	1	4	3	2	2
chitin metabolic process	16	11	14	23	17	6
cysteine-type endopeptidase inhibitor activity	1	1	5	4	3	1
endoplasmic reticulum	2	0	3	1	5	0
extracellular region	17	11	16	25	17	7
flavin adenine dinucleotide binding	3	0	1	11	0	0
heme binding	6	4	15	11	7	4
iron ion binding	9	4	18	14	12	4
L-ascorbic acid binding	2	0	3	1	5	0
lipid transporter activity	0	2	6	1	0	4
oxidation-reduction process	16	8	25	38	19	9
oxidoreductase activity	8	4	11	11	10	3
procollagen-proline 4-dioxygenase activity	2	0	3	1	5	0
protein binding	20	18	58	58	17	18
proteolysis	27	22	40	64	29	21
serine-type endopeptidase activity	19	14	31	46	21	15
serine-type endopeptidase inhibitor activity	4	2	5	6	3	2
signaling	0	2	3	3	1	2
structural constituent of cuticle	111	103	26	54	27	38
transferase activity, transferring hexosyl groups	1	8	6	4	3	1

Table S9. List of selected significant enriched KEGG pathways. Enriched significant KEGG pathways (FDR<0.05) per treatment were selected, and number of significant DEGs in every treatment is detailed.

KEGG pathway	6OH	APO	DIPH	FX	MEM	PCPA
Arachidonic acid metabolism	5	11	10	8	3	3
Cell cycle	12	10	37	25	11	11
Collecting duct acid secretion	4	0	4	6	0	0
Cytochrome P450	10	7	11	18	7	6
ECM-receptor interaction	19	15	11	18	10	12
Focal adhesion	25	22	26	37	19	17

Glutamatergic synapse	11	9	20	18	9	6
Hippo signaling pathway	7	8	15	14	7	5
Messenger RNA biogenesis	6	8	17	7	7	6
Metabolism of xenobiotics by cytochrome P450	2	13	8	11	3	0
mRNA surveillance pathway	4	5	11	9	6	4
Nitrogen metabolism	8	3	5	8	2	0
p53 signaling pathway	4	6	13	10	7	5
Renin secretion	6	6	17	15	6	5
Retinol metabolism	3	12	9	18	7	4
Ribosome biogenesis	5	7	13	10	7	5
Salivary secretion	13	9	17	24	7	7
Secretion system	4	5	11	9	6	4
Serotonergic synapse	12	11	22	21	9	7
Spliceosome	5	6	18	14	8	6
Starch and sucrose metabolism	4	13	6	3	0	0
Steroid biosynthesis	7	12	4	14	3	3
TGF-beta signaling pathway	7	7	15	15	8	5
Th-family cell differentiation	8	8	17	16	8	5
TNF signaling pathway	7	8	15	16	7	6
Transfer RNA biogenesis	5	7	16	13	9	8
Vascular smooth muscle contraction	12	9	21	19	9	6

4.3. Further discussion and final remarks

An integrative approach of the effects of neuroactive chemicals at behavioral, metabolomic neurotransmitter profile and transcriptomic responses has been presented within this chapter, stablishing relationships between different levels of biological response and showing the suitability of *D. magna* as model species for environmental neurotoxicity studies.

In the scientific article V an optimization of a targeted metabolomic LC-MS/MS method for the determination of 17 neurotransmitters, related precursors and metabolization products was developed. With this optimization, it was possible to determine the *D. magna* metabolomic profile for some of the most important pathways related to neurotransmitter systems. The relatively low concentration of these metabolites compared to other biomolecules, such as lipids or proteins, means that an untargeted metabolomic method is not the most appropriate strategy. Typically, untargeted metabolomic studies provide an overall metabolomic profile of different metabolites within a biological sample, but fail to accurately determine metabolites that are presented at low concentrations within organisms, such as neurotransmitters. When applying targeted metabolomics sample preparation can be optimized, reducing the dominance of high-abundance molecules in the analyses. In addition, since all analyzed species are clearly defined, analytical artifacts are not carried through toward downstream analysis (Roberts et al., 2012). In this way, metabolites extraction, the chromatographic separation method and MS parameters were optimized to detect only those of interest and to avoid larger metabolite families. Regarding the extraction procedure and the chromatographic separation method, their optimization was not necessary since it was based on a previous work published in the group (Rivetti et al., 2019). The extraction protocol applied was one to extract polar compounds, where a protein precipitation phase was also performed. In addition, the antioxidant and preservative ascorbic acid was added to reduce the oxidation of labile neurotransmitters such as octopamine (Tufi et al., 2015). Regarding to the chromatographic separation, HILIC separation was implemented, which has the advantage of retaining very polar compounds without the need for derivatization (Park et al., 2013; Tufi et al., 2015). However, triple quadrupole (QqQ) mass spectrometry parameters had to be optimized. The type of ionization used was ESI in positive polarity mode, which due to the chemical and physical nature of the metabolites of interest was more appropriate. ESI has the disadvantage of having strong ion suppression effects when analyzing complex molecular mixtures (Petković et al.,

2001). For this reason, applying a targeted approach extracting and analyzing just the analytes of interest (i.e. neurotransmitters) was also an advantage. Furthermore, the disadvantages of ion suppression and matrix effects can be best controlled when performing a targeted metabolomics experiment through the use of isotope-labeled internal standards for absolute quantitation of metabolite concentrations (Roberts et al., 2012). In this case, both isotope-labeled internal standards and also calibration curves for each analyte of interest were implemented. All the neurotransmitters were quantified using external calibration (calibration curve) but correcting the signal per calibration point by an internal standard to ensure exact quantification. The method was validated in terms of sensitivity, reproducibility, precision, selectivity, accuracy and matrix effects, obtaining good quality results in every case.

The method was applied for two different scenarios. The first one, to the study of neurotransmitter levels in genetically mutated CRISPR TRH *D. magna* samples, confirming the absence of serotonin and its metabolite 5-HIAA in knock out individuals that should not contain serotonin, together with lower levels of serotonin precursors tryptophan and 5-HTP, which were in concordance with transcriptomic and immunohistochemistry results reported in previous studies (Campos et al., 2019; Rivetti et al., 2018). Additionally, the method was applied for determining the effects of neuroactive chemicals known to affect different neurotransmitter systems. Behavioral assays were performed, obtaining different alterations in *Daphnia*'s behavior after exposure. The neurotransmitter metabolomic profile of these animals with altered behavioral responses was determined, helping to reaffirm the applicability of the developed targeted metabolomic method. An important point to be noted is that octopamine and also norepinephrine and epinephrine have been measured in *D. magna* samples, even though in some invertebrates adrenergic signaling is considered absent with analogous functions being performed by octopamine (Bauknecht and Jékely, 2017). Indeed norepinephrine and epinephrine have not been unequivocally identified in *Drosophila* (Adamo, 2008; Gallo et al., 2016). This fact support reported studies that point that crustaceans use both adrenergic and octopamine signaling (Adamo, 2008; Gallo et al., 2016).

Alteration of the gene expression produced by some of the chemicals whose metabolomic neurotransmitter disruption was determined in the previous article was examined in the scientific article VI. Article VI also includes neuroactive chemicals previously studies by members of my group that showed marked changes in cognitive behavior in *D. magna* (Bedrossiantz et al., 2020). Here, an integrative approach of behavioral and transcriptomic effects of these neuroactive chemicals in *D. magna* was

presented. Transcriptomic effects were determined by means of an untargeted transcriptomic approach with RNAseq. This is a rather novel method that requires elevated computational power and a deep knowledge of bioinformatics. For widely applied model species such as zebrafish, mammals or *Drosphila*, there are already bioinformatic pipelines to process and ensemble the thousands of short reads obtained. Nevertheless, for *Daphnia* RNAseq analysis requires in-depth knowledge of bioinformatics and programming, which I acquired in my stage in Guent University, Belgium.

About 1,304 unique early response genes were identified as DEGs across the different neuroactive exposures. This number of DEGs is somewhat limited in order to determine shared pathways among treatments leading to the identification of predictive pathways activated by neuroactive compounds. However, it was possible to identify some relationships between treatments and effects of different functional categories, as those between 6OH and APO, both affecting measured concentrations of different dopaminergic neurotransmitters in article V.

These chemicals increased or decreased differently the transcription of common DEGs. Both compounds affected collagen and cuticle gene families, related to dopamine signaling and dynamics (Weiss et al., 2015). Relationships between cuticle and dopamine have been reported previously. In *Daphnia* it has been described that invertebrate predators induce the development of spines/crests in the carapace by altering the dopaminergic pathways that regulated among other processes collagen and cuticle metabolism (Weiss et al., 2015). This is rater known response of *Daphnia* to invertebrate predators that preferable pray in small organisms (Barata et al., 2002). Development of spines allows *Daphnia* to increase its size and hence to diminish invertebrate predatory pressure (Barata et al., 2002). ECM-receptor interaction and focal adhesion, which are neurological signaling pathways that likely to be related with behavioral responses, were also affected by both 6OH and APO. Indeed these results are in line with the observed opposed behavioral responses of 6OH and APO in *D. magna* in article V. APO and 6OH, which are known to act as agonists and antagonists of dopaminergic receptors, increased and decreased, respectively, the motile response to light in *D. magna* exposed females. 6OH also impaired cognitive behavior decreasing habituation to repetitive light stimuli, which is in concern with results obtained from a previous study (Bedrossiantz et al., 2020). DIPH and FX neuroactive chemicals produced also similar effects in some pathways, as serotonergic, hippo, p53, TNF or TGF-signaling. A specific increased transcription of genes belonging to arachidonic acid metabolism was identified for PCPA samples, establishing a possible

relationship with its effect reducing serotonin due to the cross-talk between serotonin receptors and arachidonic acid metabolism (Tournois et al., 1998) and the reported evidence that an elevated brain arachidonic acid signaling is related with deficiencies in serotonin transporters (Basselin et al., 2009).

It is interesting to note that the observed transcriptomic dissimilar effects of PCPA and FX were supported by neurotransmitter analyses and behavioral responses. The neurotransmitter metabolomic profiles for PCPA samples and those obtained for CRISPR TRH *D. magna* samples, decreased serotonin with hardly any other effect in other neurotransmitters. In a similar way FX and PCPA exposures had the opposite pattern on serotonin, either increasing serotonin in the case of FX (Rivetti et al., 2019) or decreasing it in PCPA exposed samples measured within this chapter, and also had hardly any common DEG between them. This suggests that their different mode of action affect different genes and gene pathways. Indeed, in article V, FX and PCPA produced opposed behavioral patterns: FX increased habituation, whereas PCPA and genetically modifies CRISPR clones in Bedrossiantz et al., (2020) study that lack serotonin, increased habituation.

Other different gene pathways were determined to be affected by different tested compounds, results that corroborated the data obtained regarding the concentration of neurotransmitters in article V. Although FX neurotransmitters were not determined in article V, it was measured in a previous study (Rivetti et al., 2019). Transcriptomic effect obtained for FX, with a significant effect in serotonergic synapse pathway, was in agreement with the increased serotonin reported by Rivetti et al., (2019). Apolipoprotein gene family was significantly enriched for FX samples. Apolipoproteins form the protein component of circulating lipoproteins, and function in lipid transport and modulate its metabolism (Kalani et al., 2020; Su and Peng, 2020). Lipoproteins are the primary mediators of cholesterol and lipid transport, composed of a hydrophobic core where TG and CE are stored and a hydrophilic shell composed of phospholipids, cholesterol, and amphipathic apolipoproteins (Delk et al., 2020). Thus, the obtained differential expression of FX genes related to apolipoproteins can be probably linked to the lipid disruption caused by this neuroactive chemical reported in chapter 3 (scientific article IV), where reduced levels of CEs, lysophospholipids and some phospholipids were reported after exposure to FX at two different concentrations.

The transcription of some of the genes described as human targets for the tested neuroactive chemicals tested was explored in more detail, such as e.g. dopamine receptors, adrenergic receptors, dopamine and norepinephrine transporters

or 5-hydroxytryptamine (5-HT) receptors (described as serotonin receptors (Nichols and Nichols, 2008)). Nevertheless, no early differentially transcriptional response was identified for these target genes or other genes related to the metabolism and interconversion of different neurotransmitters, with the exception of beta-hydroxylase and aromatic amino acid decarboxylase (AAAD). The reactions catalyzed by these enzymes are represented in Figure 4.2 (Chapter 4, Introduction). The transcription of these genes, represented in more detail in Figure 4.3, was found to be related to the results obtained in the metabolomic disruption of neurotransmitters in article V. The increased expression of beta-hydroxylase in 6OH samples can be related to the obtained lower levels of dopamine and of its metabolite 3-MT, and its under-expression in DIPH and MEM samples with the reported decrease in epinephrine and norepinephrine, respectively. In addition, under-expression of beta-hydroxylase in FX exposures is in line with the increase in tyramine reported in other previous study (Gómez-Canela et al., 2019). However, the increased expression of beta-hydroxylase after 6OH exposure should have produced an increased in norepinephrine. However, decreased values of norepinephrine were detected. This suggests that this neurotransmitter must be metabolized by other enzyme through another metabolic route whose differential expression has not been identified.

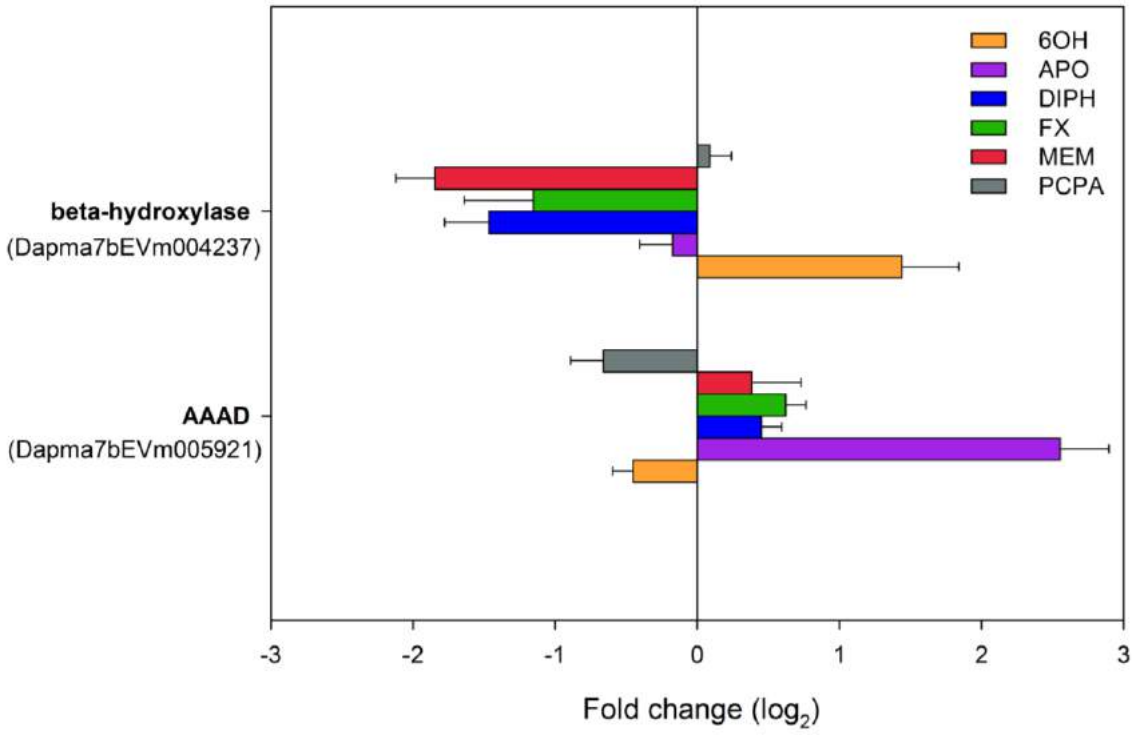

Figure 4.3. Fold change (log_2) of two DEGs, beta-hydroxylase and aromatic amino acid decarboxylase (AAAD), across all neuroactive chemical treatments from article VI. Values are mean ±SE (N=3).

Regarding AAAD, its over-expression in APO samples can be linked with obtained tryptophan and L-DOPA decrease and dopamine increase in *D. magna* exposed to this compound. AAAD was also over-expressed for FX samples, which was in line with obtained neurotransmitter and transcriptomic results in *D. magna* from previous studies (Campos et al., 2013; Rivetti et al., 2019). Obtained under-expression of AAAD in PCPA and 6OH samples was also in agreement with the measured lower amount of serotonin and of dopamine and 3-MT, respectively, in the metabolomic analysis. Therefore the obtained results in both articles within this chapter are in agreement, where different neuroactive chemicals produce changes in neurotransmitters and in the related enzymes that metabolize them.

Despite that it was possible to establish links between effects at metabolomic and transcriptomic level produced by neuroactive chemicals that affect the behavior of *D. magna* in articles V and VI, these studies have some limitations. Among them is the fact that, despite the observed effects of MEM on *D. magna* behavior decreasing motile responses to light in article V, almost no effects were observed in the analyzed neurotransmitters. Nevertheless, this does not mean that MEM cannot affect any neurotransmitter system. MEM is described as an antagonist of the NMDA receptor (Melissa Faria et al., 2019) and thus its primary effect should be to affect glutamate (Rogawski and Wenk, 2006), which was no analyzed in this study.

In addition, concerning the effects on gene transcription, two different aspects could be improved. Between 42.3% and 49.7% of identified DEGs were annotated as uncharacterized proteins. *D. magna* has a poor functional annotation in some lineage-specific genes lacking homology with other assembled genomes, which represents a problem in the interpretation of changes in those DEGs (Campos et al., 2018). So, increasing *D. magna* gene annotation is necessary to improve our understanding of the transcriptomic and subsequent metabolic results. On the other hand, the fact of having observed hardly any effect on target genes, receptors or related gene pathways described as being affected by these neuroactive chemicals does not mean that these effects would not have already occurred or would have occurred later. mRNA molecules have a short lifetime, which indicates that the time-point of measurement is crucial (Asselman et al., 2019). The lack of gene expression at a specific time point can means either than the mRNA is not yet produced or that it has been already translated into functional proteins. For this reason, further studies about the transcriptomic response of this neuroactive chemicals at different time points is recommended in order to be able to determine not only early transcriptional response effect, but also effects at other transcriptional levels.

Finally, the obtained behavioral responses across articles IV and V and that of Bedrossiantz et al., (2020) merit some discussion. One of the problems behind behavioral responses is the lack of consistence. In animals such as mammals, including humans, cognitive behavior varies largely among individuals. Results for 6OH and PCPA decreasing habituation and of MEM decreasing motile responses to light were consistent across the above mentioned experiments. Those of FX varied across article V and the study of Bedrossiantz et al., (2020), and results for APO and DIPH were consistent in article V and Bedrossiantz et al., (2020). This may be related to previous findings that reported that the effects of FX increasing serotonin levels in *Daphnia* depends on the food level (Campos et al., 2016). At high food cultured conditions *Daphnia* ingest large quantities of the serotonin precursor tryptophan and as such it has saturated levels of serotonin (Campos et al., 2016). This means that FX only increase serotonin levels under limiting food conditions when serotonin is not saturated. Thus slight differences in food across experiments may have changed the effects of FX on serotonin. This is not the case for PCPA, which inhibits the synthesis of serotonin from its precursor tryptophan irrespectively of food levels. For DIPH and APO the use of different exposure concentrations in articles V and VI may explain the observed differences as many neuroactive compounds affect non monotonically biological responses, which is in line with their known concentration-specific effects on neuronal receptors (Fong and Ford, 2014).

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Chapter 5

Chapter 5

Conclusions

Chapter 5

Overall, this thesis aimed to prove the importance of integrating omic and conventional toxicological approaches in order to obtain significant information that helps to unravel new toxicity mechanism triggered by ECs on the aquatic environment. Different integrative approaches have been developed to assess EDCs and neuroactive chemicals toxicity by linking effects on reproduction and behavior (individual organism responses), gene expression and its subsequent metabolomic (and thus lipidomic) disruption in the aquatic model organisms *D. magna*.

The following conclusions were achieved:

1. The study of transcription changes associated to enhanced accumulation of storage lipids by BPA, PP and TBT in *D. magna* suggested a common mechanism, identifying affection on energy-related categories, molting and reproduction, as well as different lipid functional categories, linked to an identified under-expression of HR96 and RXR nuclear receptors.

2. The obtained lipidome of *D. magna* confirm previous results indicating that during a reproductive cycle, the levels of glycerolipids and glycerophospholipids increase to be invested into reproduction and the formation of the new carapace. Furthermore, a link between *Daphnia*'s fatty acids and its diet has been established, pointing also a preferential investment of more polyunsaturated fatty acids to its eggs.

3. Juvenoids compounds (MF and PP) promoted the accumulation of triacylglycerols and reduced fecundity and investment of glycerolipids and glycerophospholipids towards reproduction, means while BPA impaired the transfer of triacylglycerols to its eggs in a similar way as reported in previous studies for TBT.

4. The harmful effects of environmental relevant concentrations of the neuroactive pharmaceuticals carbamazepine, diazepam and propranolol at both transcriptional and lipid levels were reported, relating them also with fecundity effects.

5. Deregulation of different signaling pathways was observed in *Daphnia* individuals exposed to environmentally relevant concentrations of the pharmaceuticals carbamazepine, diazepam, propranolol and to their equitoxic mixture. Most of the deregulated genes as well as signaling pathways were shared between treatments and were enhanced in the mixture suggesting similar and additive effects. Deregulated pathways were mainly those implicated on energy, growth,

reproduction and neurologically related processes, which may be responsible for the observed reproductive effects.

6. Carbamazepine, diazepam, propranolol and fluoxetine disrupted different lipid categories, increasing levels of glycerophospholipids and triacylglycerols, and reducing levels of monoacylglycerols, sphingomyelins and cholesteryl esters. The previous results are in concern with transcriptomic and reproductive observed effects. Conversely, genetically *D. magna* knockout clones lacking serotonin showed opposite responses, suggesting a relationship between serotonin and other neurological signaling pathways in regulating lipidomic and fecundity responses in *D. magna*.

7. A comprehensive optimization of a target metabolomic approach applying hydrophilic interaction chromatography coupled to a triple quadrupole mass analyzer was developed in order to determine neurotransmitters belonging to some of the most important pathways related to neurotransmitter systems in *D. magna* samples. This method was validated with exposures to known neuroactive pharmaceuticals and knockout clones lacking serotonin. Results confirmed that the proposed analytical method was able to detect neurotransmitter changes in concern with previous reported results.

8. The suitability of *D. magna* as model species for environmental neurotoxicity studies was further proved combining RNAseq transcriptomic analysis on the dissected heads of *D. magna* exposed to six different neurotransmitter modulators, which produced behavioral effects. Transcriptomic results indicated distinctive altered genes and related signaling pathways across chemicals having differentiated behavioral responses. Furthermore, common disrupted signaling pathways were identified for some compounds described to affect the dopaminergic system, as collagen and cuticle metabolism and ECM-receptor interaction pathways. In addition, the expression of two deregulated genes (beta-hydroxylase and aromatic amino acid decarboxylase) was related to the effect of these neuroactive chemicals on neurotransmitter metabolic pathways. Thus, effects at the transcriptional and physiological level were reported, providing a novel approach to the analysis of environmental effects of neuroactive compounds.

ANNEXES

Annex I, Supplementary Material from scientific article I:

Fuertes, I., Jordão, R., Piña, B., Barata, C., 2019. Time-dependent transcriptomic responses of *Daphnia magna* exposed to metabolic disruptors that enhanced storage lipid accumulation. Environ. Pollut. 249, 99-108. https://doi.org/10.1016/j.envpol.2019.02.102

Table S2. Elemental composition of glycerophospholipids, glycerolipids and sphingolipids species found in *D. magna* lipid samples, calculated by mass accuracy within error of 5 ppm, with atom constraints and with -0.5 ≤ DBE ≤ 50.0 (DBE, double-bond equivalent). Elemental composition of neutral glycerolipids refer to their ammonium adducts detected under ESI (+), and elemental composition of glycerophospholipids and sphingolipids refer to their hydrogen adducts detected under ESI (+) for PC, LPC, LPG and SM, and under ESI (-) for PE, LPE, PS and PG.

Lipid subclass	Lipid specie	Measured mass (m/z)	Elemental composition	Calculated mass (m/z)	Error (ppm)	DBE	RT (min)
GLYCEROPHOSPHOLIPIDS							
PC							
	28:0	678.5066	C36H73NO8P	678.5074	-1.2	1.5	5.77
	28:1	676.4901	C36H71NO8P	676.4917	-2.4	2.5	5.36
	28:2	674.4778	C36H69NO8P	674.4761	2.5	3.5	4.05
	30:0	706.5380	C38H77NO8P	706.5387	-1.0	1.5	6.87
	30:1	704.5234	C38H75NO8P	704.5230	0.6	2.5	6.15
	30:2	702.5069	C38H73NO8P	702.5074	-0.7	3.5	5.52
	30:3	700.4910	C38H71NO8P	700.4917	-1.0	4.5	5.05
	30:4	698.4747	C38H69NO8P	698.4761	-2.0	5.5	4.64
	32:0	734.5692	C40H81NO8P	734.5700	-1.1	1.5	8.25
	32:1	732.5535	C40H79NO8P	732.5543	-1.1	2.5	7.34
	32:2	730.5402	C40H77NO8P	730.5387	2.1	3.5	6.55
	32:3	728.5226	C40H75NO8P	728.5230	-0.5	4.5	5.90
	32:4	726.5066	C40H73NO8P	726.5074	-1.1	5.5	5.30
	32:5	724.4902	C40H71NO8P	724.4917	-2.1	6.5	4.92
	34:0	762.6009	C42H85NO8P	762.6013	-0.5	1.5	9.03
	34:1	760.5844	C42H83NO8P	760.5856	-1.6	2.5	8.68
	34:2	758.5712	C42H81NO8P	758.5700	1.6	3.5	7.74

	34:3	756.5541	C42H79NO8P	756.5543	-0.3	4.5	6.87
	34:4	754.5375	C42H77NO8P	754.5387	-1.6	5.5	6.08
	34:5	752.5245	C42H75NO8P	752.5230	2.0	6.5	5.55
	34:6	750.5060	C42H73NO8P	754.5074	-1.9	7.5	5.05
	36:0	790.6346	C44H89NO8P	790.6326	2.5	1.5	10.28
	36:1	788.6173	C44H87NO8P	788.6169	0.5	2.5	9.97
	36:2	786.6014	C44H85NO8P	786.6013	0.1	3.5	9.06
	36:3	784.5878	C44H83NO8P	784.5856	2.8	4.5	8.18
	36:4	782.5702	C44H81NO8P	782.5700	0.3	5.5	7.27
	36:5	780.5565	C44H79NO8P	780.5543	2.8	6.5	6.46
	36:6	778.5378	C44H77NO8P	778.5387	-.12	7.5	5.77
	36:7	776.5226	C44H75NO8P	7765230	-0.5	8.5	5.39
	38:0	818.6636	C46H93NO8P	8186639	-0.4	1.5	11.53
	38:1	816.6473	C46H91NO8P	816.6482	-1.1	2.5	10.72
	38:2	814.6320	C46H89NO8P	814.6326	-0.7	3.5	9.91
	38:3	812.6163	C46H87NO8P	812.6169	-0.7	4.5	9.06
	38:4	810.6014	C46H85NO8P	810.6013	0.1	5.5	8.50
	38:5	808.5878	C46H83NO8P	808.5856	2.7	6.5	8.37
	38:6	806.5699	C46H81NO8P	806.5700	-0.1	7.5	7.31
	38:7	804.5535	C46H79NO8P	804.5543	-1.0	8.5	6.49
	38:8	802.5401	C46H77NO8P	802.5387	1.7	9.5	5.77
	40:1	844.6772	C48H95NO8P	844.6795	-2.7	2.5	12.32
	40:2	842.6637	C48H93NO8P	842.6639	-0.2	3.5	11.53
	40:3	840.6473	C48H91NO8P	840.6482	-1.1	4.5	10.75
	40:4	838.6324	C48H89NO8P	838.6326	-0.2	5.5	9.84
	40:5	836.6161	C48H87NO8P	836.6169	-1.0	6.5	9.12
	40:6	834.6002	C48H85NO8P	834.6013	-1.3	7.5	8.84
	40:7	832.5851	C48H83NO8P	832.5856	-0.6	8.5	7.93
	40:8	830.5697	C48H81NO8P	830.5700	-0.4	9.5	7.02
	40:10	826.5381	C48H77NO8P	826.5387	-0.7	11.5	6.46
	42:5	864.6477	C50H91NO8P	864.6482	-0.6	6.5	10.44
	42:6	862.6362	C50H89NO8P	862.6326	4.2	7.5	9.69
	44:10	882.6019	C52H85NO8P	882.6013	0.7	11.5	9.06
LPC							
	14:0	468.3082	C22H47NO7P	468.3090	-1.7	0.5	1.95
	14:1	466.2943	C22H45NO7P	466.2934	1.9	1.5	2.42
	16:0	496.3396	C24H51NO7P	496.3403	-1.4	0.5	2.48
	16:1	494.3239	C24H49NO7P	494.3247	-1.6	1.5	2.11

	18:0	524.3710	C26H55NO7P	524.3716	-1.1	0.5	3.17
	18:1	522.3557	C26H53NO7P	522.3560	-.06	1.5	2.73
	18:2	520.3399	C26H51NO7P	520.3403	-0.8	2.5	2.29
	18:3	518.3227	C26H49NO7P	518.3247	-3.9	3.5	1.98
	20:0	552.4021	C28H59NO7P	552.4029	-1.4	0.5	3.48
	20:1	550.3885	C28H57NO7P	550.3873	2.2	1.5	3.23
	20:5	542.3251	C28H49NO7P	542.3247	0.7	5.5	2.29
	22:2	576.4026	C30H59NO7P	576.4029	-0.5	2.5	2.45
PE							
	30:1	660.4609	C35H67NO8P	660.4604	0.8	3.5	6.16
	32:1	688.4918	C37H71NO8P	688.4917	0.1	3.5	7.32
	32:2	686.4764	C37H69NO8P	686.4761	0.4	4.5	6.51
	32:3	684.4612	C37H67NO8P	684.4604	1.2	5.5	5.91
	32:4	682.4441	C37H65NO8P	682.4448	-1.0	6.5	5.25
	34:1	716.5239	C39H75NO8P	716.5230	1.3	3.5	8.70
	34:2	714.5070	C39H73NO8P	714.5074	-0.6	4.5	7.73
	34:3	712.4919	C39H71NO8P	712.4917	0.3	5.5	6.85
	34:4	710.4754	C39H69NO8P	710.4761	-1.0	6.5	6.10
	34:5	708.4587	C39H67NO8P	708.4604	-2.4	7.5	5.57
	36:0	746.5706	C41H81NO8P	746.5700	0.8	2.5	10.27
	36:1	744.5535	C41H79NO8P	744.5543	-1.1	3.5	9.98
	36:2	742.5381	C41H77NO8P	742.5387	-0.8	4.5	9.04
	36:3	740.5227	C41H75NO8P	740.5230	-0.4	5.5	8.13
	36:4	738.5081	C41H73NO8P	738.5074	0.9	6.5	7.26
	36:5	736.4920	C41H71NO8P	736.4917	0.4	7.5	6.41
	36:6	734.4769	C41H69NO8P	734.4761	1.1	8.5	5.75
	38:1	772.5857	C43H83NO8P	772.5856	0.1	3.5	10.64
	38:2	770.5695	C43H81NO8P	770.5700	-0.6	4.5	9.83
	38:3	768.5546	C43H79NO8P	768.5543	0.4	5.5	9.48
	38:4	766.5372	C43H77NO8P	766.5387	-2.0	6.5	9.20
	38:5	764.5232	C43H75NO8P	764.5230	0.3	7.5	8.23
	38:6	762.5079	C43H73NO8P	762.5074	0.7	8.5	7.32
	38:7	760.4901	C43H71NO8P	760.4917	-2.1	9.5	6.48
	38:8	758.4788	C43H69NO8P	758.4761	3.6	10.5	5.78
LPE							
	16:0	452.2773	C21H43NO7P	452.2777	-0.9	1.5	2.49
	16:1	450.2625	C21H41NO7P	450.2621	0.9	2.5	2.12
	18:0	480.3087	C23H47NO7P	480.3090	-0.6	1.5	3.19

	18:1	478.2922	C23H45NO7P	478.2934	-2.5	2.5	2.71
	18:2	476.2780	C23H43NO7P	476.2777	0.6	3.5	2.31
	18:3	474.2625	C23H41NO7P	474.2621	0.8	4.5	1.99
PS							
	34:0	762.5284	C40H77NO10 P	762.5285	-0.1	3.5	6.66
	34:1	760.5134	C40H75NO10 P	760.5129	0.7	4.5	6.04
	36:0	790.5606	C42H81NO10 P	790.5598	1.0	3.5	7.98
	36:1	788.5442	C42H79NO10 P	788.5442	-0.1	4.5	7.04
	36:2	786.5285	C42H77NO10 P	786.5285	0.1	5.5	6.32
	36:3	784.5129	C42H75NO10 P	784.5129	1.7	6.5	5.75
	38:0	818.5911	C44H85NO10 P	818.5911	0.1	3.5	9.36
	38:1	816.5755	C44H83NO10 P	816.5755	1.3	4.5	8.45
	38:2	814.5598	C44H81NO10 P	814.5598	0.4	5.5	7.60
	38:3	812.5442	C44H79NO10 P	812.5442	-1.5	6.5	6.79
	40:0	846.6224	C46H89NO10 P	846.6224	0.5	3.5	10.14
	40:1	844.6068	C46H87NO10 P	844.6068	-3.4	4.5	9.70
	40:2	842.5911	C46H85NO10 P	842.5911	-2.6	5.5	8.89
PG							
	32:1	719.4872	C38H72O10P	719.4863	1.3	3.5	6.63
	32:2	717.4704	C38H70O10P	717.4707	-0.4	4.5	5.88
	34:1	747.5178	C40H76O10P	747.5176	0.3	3.5	7.92
	34:2	745.5025	C40H74O10P	745.5020	0.7	4.5	7.01
	34:3	743.4861	C40H72O10P	743.4863	-0.3	5.5	6.25
	34:4	741.4701	C40H70O10P	741.4707	-0.8	6.5	5.60
	36:4	769.5037	C42H74O10P	769.5020	2.2	6.5	6.54
	36:5	767.4861	C42H72O10P	767.4863	-0.3	7.5	5.91
	36:6	765.4684	C42H70O10P	765.4707	-3.0	8.5	5.28
	42:7	847.5474	C48H80O10P	847.5489	-1.8	9.5	7.48
	44:10	869.5331	C50H78O10P	869.5333	-0.2	12.5	6.79
LPG							
	14:1	455.2403	C20H40O9P	455.2410	-1.5	1.5	2.13
	18:1	511.3028	C24H48O9P	511.3036	-1.6	1.5	3.07
	18:4	505.2560	C24H42O9P	505.2566	-1.2	4.5	1.13
	20:1	539.3328	C26H52O9P	539.3349	-3.9	1.5	3.73
GLYCEROLIPIDS							
TG							
	38:0	684.6133	C41H82NO6	684.6142	-1.3	1.5	12.43

38:1	682.5988	C41H80NO6	682.5986	0.3	2.5	11.96
40:0	712.6452	C43H86NO6	712.6455	-0.4	15	13.40
40:1	710.6303	C43H84NO6	710.6299	0.6	2.5	12.84
40:2	708.6158	C43H82NO6	708.6142	2.3	3.5	12.30
42:0	740.6768	C45H90NO6	740.6768	0.0	1.5	14.25
42:1	738.6604	C45H88NO6	738.6612	-1.1	2.5	13.75
42:2	736.6451	C45H86NO6	736.6455	-0.5	3.5	13.21
42:3	734.6302	C45H84NO6	734.6299	0.4	4.5	12.68
44:0	768.7097	C47H94NO6	768.7081	2.1	1.5	15.03
44:1	766.6915	C47H92NO6	766.6925	-1.3	2.5	14.53
44:2	764.6769	C47H90NO6	764.6768	0.1	3.5	14.03
44:3	762.6617	C47H88NO6	762.6612	0.7	4.5	13.53
44:4	760.6456	C47H86NO6	760.6455	0.1	5.5	13.06
46:0	796.7389	C49H98NO6	796.7394	-0.6	1.5	15.75
46:1	794.7235	C49H96NO6	794.7238	-0.4	2.5	15.28
46:2	792.7086	C49H94NO6	792.7081	0.6	3.5	14.81
46:3	790.6917	C49H92NO6	790.6925	-1.0	4.5	14.31
46:4	788.6757	C49H90NO6	788.6768	-1.4	5.5	13.84
46:5	786.6625	C49H88NO6	786.6612	1.7	6.5	13.28
48:0	824.7708	C51H102NO6	824.7707	0.1	1.5	16.44
48:1	822.7538	C51H100NO6	822.7551	-1.6	2.5	15.97
48:2	820.7386	C51H98NO6	820.7394	-1.0	3.5	15.50
48:3	818.7237	C51H96NO6	818.7238	-0.1	4.5	15.00
48:4	816.7078	C51H94NO6	816.7081	-0.4	5.5	14.56
48:5	814.6915	C51H92NO6	814.6925	-1.2	6.5	14.09
48:6	812.6761	C51H90NO6	812.6768	-0.9	7.5	13.59
50:0	852.8011	C53H106NO6	852.8020	-1.1	1.5	17.17
50:1	850.7859	C53H104NO6	850.7864	-0.6	2.5	16.60
50:2	848.7712	C53H102NO6	848.7707	0.6	3.5	16.19
50:3	846.7531	C53H100NO6	846.7551	-2.4	4.5	15.66
50:4	844.7382	C53H98NO6	844.7394	-1.4	5.5	15.25
50:5	842.7234	C53H96NO6	842.7238	-0.5	6.5	14.81
50:6	840.7074	C53H94NO6	840.7081	-0.8	7.5	14.37
52:0	880.8335	C55H110NO6	880.8333	0.2	1.5	17.95
52:1	878.8180	C55H108NO6	878.8177	0.3	2.5	17.07
52:2	876.8019	C55H106NO6	876.8020	-0.1	3.5	16.82
52:3	874.7866	C55H104NO6	874.7864	0.2	4.5	16.35
52:4	872.7706	C55H102NO6	872.7707	-0.1	5.5	15.91

	52:5	870.7543	C55H100NO6	870.7551	-0.9	6.5	15.50
	52:6	868.7374	C55H98NO6	868.7394	-2.3	7.5	15.13
	54:0	908.8671	C57H114NO6	908.8646	2.8	1.5	18.23
	54:1	906.8482	C57H112NO6	906.8489	-0.9	2.5	17.83
	54:2	904.8353	C57H110NO6	904.8333	2.2	3.5	17.29
	54:3	902.8178	C57H108NO6	902.8177	0.1	4.5	17.01
	54:4	900.8028	C57H106NO6	900.8020	0.9	5.5	16.51
	54:5	898.7859	C57H104NO6	898.7864	-0.6	6.5	16.10
	54:6	896.7732	C57H102NO6	896.7707	2.8	7.5	15.78
	56:0	936.8981	C59H118NO6	936.8959	2.3	1.5	19.11
	56:1	934.8822	C59H116NO6	934.8803	2.0	2.5	18.42
	56:2	932.8652	C59H114NO6	932.8646	0.6	3.5	18.01
	56:3	930.8494	C59H112NO6	930.8490	0.4	4.5	17.45
	56:4	928.8325	C59H110NO6	928.8333	-0.9	5.5	16.91
	56:5	926.8170	C59H108NO6	926.8177	-0.8	6.5	16.70
	58:1	962.9069	C61H120NO6	962.9116	-4.9	2.5	19.36
	58:2	960.8969	C61H118NO6	960.8959	1.0	3.5	18.64
	58:3	958.8812	C61H116NO6	958.8803	0.9	4.5	17.98
	58:4	956.8633	C61H114NO6	956.8646	-1.4	5.5	17.70
	60:4	984.8934	C63H118NO6	984.8959	-2.5	5.5	18.58
	60:5	982.8801	C63H116NO6	982.8803	-0.2	6.5	17.92
DG							
	26:0	502.4459	C29H60NO5	502.4471	-2.4	0.5	5.55
	28:0	530.4792	C31H64NO5	530.4784	1.5	0.5	6.84
	28:1	528.4624	C31H62NO5	528.4628	-0.8	1.5	6.05
	28:2	526.4473	C31H60NO5	526.4471	0.4	2.5	5.46
	30:0	558.5093	C33H68NO5	558.5097	-0.7	0.5	8.24
	30:1	556.4944	C33H66NO5	556.4941	0.5	1.5	7.27
	30:2	554.4779	C33H64NO5	554.4784	-0.9	2.5	6.46
	30:3	552.4623	C33H62NO5	5524628	-0.9	3.5	5.77
	32:0	586.5390	C35H72NO5	586.5411	-3.6	0.5	9.62
	32:1	584.5246	C35H70NO5	584.5254	-1.4	1.5	8.68
	32:2	582.5096	C35H68NO5	582.5097	-0.2	2.5	7.77
	32:3	580.4935	C35H66NO5	580.4941	-1.0	3.5	6.93
	32:4	578.4773	C35H64NO5	578.4784	-1.9	4.5	6.18
	32:5	576.4616	C35H62NO5	576.4628	-2.1	5.5	5.11
	34:0	614.5730	C37H76NO5	614.5724	1.0	0.5	10.41
	34:1	612.5557	C37H74NO5	612.5567	-1.6	1.5	10.00

	34:2	610.5416	C37H72NO5	610.5411	0.8	2.5	9.12
	34:3	608.5244	C37H70NO5	608.5254	-1.6	3.5	8.24
	34:4	606.5092	C37H68NO5	606.5097	-0.8	4.5	7.27
	34:5	604.4936	C37H66NO5	604.4941	-0.8	5.5	6.52
	36:0	642.6031	C39H80NO5	642.6036	-0.9	0.5	11.53
	36:1	640.5877	C39H78NO5	640.5880	-0.5	1.5	10.72
	36:2	638.5687	C39H76NO5	638.5724	-4.8	2.5	10.38
	36:3	636.5557	C39H74NO5	636.5567	-1.6	3.5	9.50
	36:4	634.5411	C39H72NO5	634.5411	0.0	4.5	8.59
	36:5	632.5251	C39H70NO5	632.5254	-0.5	5.5	7.74
	36:6	630.5091	C39H68NO5	630.5098	-1.0	6.5	6.87
	38:0	670.6337	C41H84NO5	670.6349	-1.9	0.5	13.04
	38:1	668.6199	C41H82NO5	668.6193	0.9	1.5	11.85
	38:2	666.6033	C41H80NO5	666.6063	-0.6	2.5	11.10
	38:3	664.5894	C41H78NO5	664.5880	2.1	3.5	10.63
	38:4	662.5714	C41H76NO5	662.5723	-1.5	4.5	10.41
	38:5	660.5559	C41H74NO5	660.5567	-1.2	5.5	9.59
	38:6	658.5410	C41H72NO5	658.5411	-0.2	6.5	8.56
	40:0	698.6646	C43H88NO5	698.6663	-2.4	0.5	13.20
	42:10	706.5408	C45H72NO5	706.5411	-0.4	10.5	6.87
	42:11	704.5245	C45H70NO5	704.5254	-1.3	11.5	6.18
	44:6	742.6365	C47H84NO5	742.6350	2.0	6.5	10.85
	44:10	734.5722	C47H76NO5	734.5724	-0.3	10.5	8.24
	44:11	732.5562	C47H74NO5	732.5567	-0.7	11.5	7.34
	44:12	730.5415	C47H72NO5	730.5411	0.5	12.5	6.55
MG							
	16:0	348.3107	C19H42NO4	348.3114	-2.0	-0.5	2.92
	18:0	376.3414	C21H46NO4	376.3427	-3.5	-0.5	3.64
	18:1	374.3269	C21H44NO4	374.3270	-0.3	0.5	3.51
	20:0	404.3746	C23H50NO4	404.3740	1.5	-0.5	4.11
			SPHINGOLIPIDS				
SM							
	14:0	675.5444	C37H76N2O6P	675.5441	0.4	1.5	5.89
	16:0	703.5744	C39H80N2O6P	703.5754	-1.4	1.5	7.12
	18:0	731.6061	C41H84N2O6P	731.6067	-0.8	1.5	8.59
	20:0	759.6378	C43H88N2O6P	759.6380	-0.3	1.5	10.00
	20:1	757.6243	C43H86N2O6P	757.6224	2.5	2.5	8.84
	22:0	787.6683	C45H92N2O6P	787.6693	-1.3	1.5	11.32

	22:1	785.6545	C45H90N2O6P	785.6537	1.0	2.5	10.41
	24:0	815.7009	C47H96N2O6P	815.7006	0.4	1.5	12.32

Annex II, Supplementary Material from scientific article IV:

Fuertes, I., Piña, B., Barata, C., 2020. Changes in lipid profiles in *Daphnia magna* individuals exposed to low environmental levels of neuroactive pharmaceuticals.Sci. Total Environ. 139029
https://doi.org/10.1016/j.scitotenv.2020.139029

Table S2. Elemental composition of glycerophospholipids, glycerolipids, sphingolipids and sterols species found in *D. magna* lipid samples, calculated by mass accuracy within error of 5 mg/L, with atom constraints and with -0.5 ≤ DBE ≤ 50.0. DBE: double-bond equivalent. Elemental composition of neutral glycerolipids and sterols refer to their ammonium adducts detected under ESI (+), and elemental composition of glycerophospholipids and sphingolipids refer to their hydrogen adducts detected under ESI (+) for PC, LPC, PC-O/PC-P and SM, and under ESI (-) for PE, LPE, PE-O/PE-P, PG, LPG, PI, PS and LPS.

Lipid subclass	Lipid specie	Measured mass (m/z)	Elemental composition	Calculated mass (m/z)	Error (ppm)	DBE	RT (min)
GLYCEROPHOSPHOLIPIDS							
PC							
	28:0	678.5052	C36H73NO8P	678.5074	-3.2	1.5	6.06
	28:1	676.4902	C36H71NO8P	676.4917	-2.2	2.5	5.43
	30:0	706.5381	C38H77NO8P	706.5387	-0.8	1.5	7.29
	30:1	704.5227	C38H75NO8P	704.5230	-0.4	2.5	6.47
	30:2	702.5078	C38H73NO8P	702.5074	0.6	3.5	5.75
	30:3	700.4916	C38H71NO8P	700.4917	-0.1	4.5	5.22
	30:4	698.4752	C38H69NO8P	698.4761	-1.3	5.5	4.77
	32:0	734.5709	C40H81NO8P	734.5700	1.2	1.5	8.70
	32:1	732.5548	C40H79NO8P	732.5543	0.7	2.5	7.79
	32:2	730.5375	C40H77NO8P	730.5387	-1.6	3.5	6.94
	32:3	728.5225	C40H75NO8P	728.5230	-0.7	4.5	6.19
	32:4	726.5065	C40H73NO8P	726.5074	-1.2	5.5	5.56
	32:5	724.4916	C40H71NO8P	724.4917	-0.1	6.5	4.99
	34:0	762.6008	C42H85NO8P	762.6013	-0.7	1.5	10.02
	34:1	760.585	C42H83NO8P	760.5856	-0.8	2.5	9.07
	34:2	758.5706	C42H81NO8P	758.5700	0.8	3.5	8.13
	34:3	756.5550	C42H79NO8P	756.5543	0.9	4.5	7.29
	34:4	754.5375	C42H77NO8P	754.5387	-1.6	5.5	6.47
	34:5	752.5228	C42H75NO8P	752.5230	-0.3	6.5	5.78
	34:6	750.5077	C42H73NO8P	754.5074	0.4	7.5	5.25
	36:0	790.6318	C44H89NO8P	790.6326	-1.0	1.5	11.24

	36:1	788.6163	C44H87NO8P	788.6169	-0.8	2.5	10.42
	36:2	786.6014	C44H85NO8P	786.6013	0.1	3.5	9.51
	36:3	784.5857	C44H83NO8P	784.5856	0.1	4.5	8.6
	36:4	782.5693	C44H81NO8P	782.5700	-0.9	5.5	7.73
	36:5	780.5539	C44H79NO8P	780.5543	-0.5	6.5	6.78
	36:6	778.5389	C44H77NO8P	778.5387	0.3	7.5	6.03
	36:7	776.5228	C44H75NO8P	7765230	-0.3	8.5	5.5
	38:1	816.6488	C46H91NO8P	816.6482	0.7	2.5	11.58
	38:2	814.6331	C46H89NO8P	814.6326	0.6	3.5	10.74
	38:3	812.6179	C46H87NO8P	812.6169	1.2	4.5	9.92
	38:4	810.6006	C46H85NO8P	810.6013	-0.9	5.5	8.98
	38:5	808.5854	C46H83NO8P	808.5856	-0.2	6.5	8.04
	38:6	806.5688	C46H81NO8P	806.5700	-1.5	7.5	7.96
	38:7	804.5549	C46H79NO8P	804.5543	0.7	8.5	7.73
	38:8	802.5386	C46H77NO8P	802.5387	-0.1	9.5	6.78
	38:9	800.5228	C46H75NO8P	800.5225	-0.2	10.5	6.03
	40:1	844.6783	C48H95NO8P	844.6795	-1.4	2.5	12.68
	40:2	842.6666	C48H93NO8P	842.6639	3.2	3.5	11.96
	40:4	838.6340	C48H89NO8P	838.6326	1.7	5.5	10.33
	40:5	836.6170	C48H87NO8P	836.6169	0.1	6.5	9.36
	40:6	834.6011	C48H85NO8P	834.6013	-0.2	7.5	8.51
	40:8	832.5908	C48H81NO8P	830.5700	6.2	8.5	7.44
	40:9	830.5681	C48H79NO8P	828.5543	-2.3	9.5	7.13
	42:10	854.5710	C50H81NO8P	854.5700	1.2	11.5	8.20
	44:2	898.7232	C52H101NO8P	898.7268	-3.7	3.5	13.47
	44:10	882.6011	C52H85NO8P	882.6013	-0.2	11.5	9.54
LPC							
	14:0	468.3082	C22H47NO7P	468.3090	-1.7	0.5	2.01
	14:1	466.2927	C22H45NO7P	466.2934	-1.5	1.5	2.48
	16:0	496.3397	C24H51NO7P	496.3403	-1.2	0.5	2.61
	16:1	494.3235	C24H49NO7P	494.3247	-2.4	1.5	2.23
	18:0	524.3721	C26H55NO7P	524.3716	1.0	0.5	3.27
	18:1	522.3558	C26H53NO7P	522.3560	-0.4	1.5	2.80
	18:2	520.3399	C26H51NO7P	520.3403	-0.8	2.5	2.42
	18:3	518.3244	C26H49NO7P	518.3247	-0.6	3.5	2.11
	20:3	546.3563	C28H53NO7P	546.356	0.5	3.5	3.20
	20:4	544.3398	C28H51NO7P	544.3403	-0.9	4.5	2.80
	20:5	542.3251	C28H49NO7P	542.3247	0.7	5.5	2.42

PC-O/PC-P							
PC-O	32:0	720.5900	C40H83NO7P	720.5907	-1.0	0.5	9.64
PC-O	34:0	748.6201	C42H87NO7P	748.6220	-2.5	0.5	10.89
PC-O/PC-P	34:1/34:0	746.6066	C42H85NO7P	746.6064	0.3	1.5	9.98
PC-O/PC-P	34:3/34:2	742.5749	C42H81NO7P	742.5751	-0.3	3.5	8.26
PC-O/PC-P	36:1/36:0	774.6377	C44H89NO7P	774.6377	0.0	1.5	10.93
PC-O/PC-P	38:2/38:1	800.6530	C46H91NO7P	800.6533	-0.4	2.5	11.30
PC-O/PC-P	38:3/38:2	798.6359	C46H89NO7P	798.6377	-2.3	3.5	10.45
PC-O/PC-P	38:4/38:3	796.6228	C46H87NO7P	796.6220	1.0	4.5	9.65
PC-O/PC-P	40:6/40:5	820.6242	C48H87NO7P	820.6220	2.7	6.5	9.77
PC-P	40:6	818.6064	C48H85NO7P	818.6064	0.0	7.5	9.67
PE							
	32:1	688.4933	C37H71NO8P	688.4917	2.3	3.5	7.74
	32:2	686.4756	C37H69NO8P	686.4761	-0.7	4.5	6.96
	32:3	684.4613	C37H67NO8P	684.4604	1.3	5.5	6.23
	32:4	682.4448	C37H65NO8P	682.4448	0.0	6.5	5.54
	34:1	716.5210	C39H75NO8P	716.5230	-2.8	3.5	9.09
	34:2	714.5059	C39H73NO8P	714.5074	-2.1	4.5	8.15
	34:3	712.4922	C39H71NO8P	712.4917	0.7	5.5	7.30
	34:4	710.4763	C39H69NO8P	710.4761	0.3	6.5	6.36
	34:5	708.4592	C39H67NO8P	708.4604	-1.7	7.5	5.79
	34:6	706.4479	C39H65NO8P	706.4448	4.4	8.5	5.23
	36:1	744.5531	C41H79NO8P	744.5543	-1.6	3.5	10.40
	36:2	742.5398	C41H77NO8P	742.5387	1.5	4.5	9.50
	36:3	740.5219	C41H75NO8P	740.5230	-1.5	5.5	8.59
	36:4	738.5067	C41H73NO8P	738.5074	-0.9	6.5	7.68
	36:5	736.4918	C41H71NO8P	736.4917	0.1	7.5	6.74
	36:6	734.4749	C41H69NO8P	734.4761	-1.6	8.5	6.02
LPE							
	16:0	452.2774	C21H43NO7P	452.2777	-0.7	1.5	2.66
	16:1	450.2615	C21H41NO7P	450.2621	-1.3	2.5	2.25
	18:0	480.3077	C23H47NO7P	480.3090	-2.7	1.5	3.35
	18:1	478.2936	C23H45NO7P	478.2934	0.4	2.5	2.88
	18:2	476.2766	C23H43NO7P	476.2777	-2.3	3.5	2.47
	18:3	474.2614	C23H41NO7P	474.2621	-1.5	4.5	2.15
	20:1	506.3201	C25H49NO7P	506.3247	-4.1	2.5	2.78
	20:3	502.2927	C25H45NO7P	502.2934	-1.4	4.5	2.09
PE-O/PE-P							

PE-O	30:0	648.4964	C35H71NO7P	648.4968	-0.6	1.5	7.27
PE-O/PE-P	30:1/30:0	646.4812	C35H69NO7P	646.4812	0.0	2.5	6.83
PE-O/PE-P	34:3/34:2	698.5139	C39H73NO7P	698.5125	2.0	4.5	8.32
PE-O/PE-P	34:4/34:3	696.4966	C39H71NO7P	696.4968	-0.3	5.5	8.20
PE-O/PE-P	38:4/38:3	752.5610	C43H79NO7P	752.5594	2.1	5.5	9.66
PG							
	34:3	743.4861	C40H72O10P	743.4863	-0.3	5.5	6.64
LPG							
	18:1	509.2881	C24H46O9P	509.2879	0.4	2.5	2.72
PI							
	32:2	805.4865	C41H74O13P	805.4867	-0.2	5.5	6.23
	32:3	803.4716	C41H72O13P	803.4711	0.6	6.5	5.61
	34:1	835.5333	C43H80O13P	835.5337	-0.5	4.5	8.15
	34:2	833.5184	C43H78O13P	833.5180	0.5	5.5	7.27
	34:3	831.5020	C43H76O13P	831.5024	-0.5	6.5	6.49
	34:4	829.4857	C43H74O13P	829.4867	-1.2	7.5	5.86
	36:2	861.5485	C45H82O13P	861.5493	-0.9	5.5	8.62
	36:3	859.5305	C45H80O13P	859.5337	-3.7	6.5	7.83
	36:4	857.5177	C45H78O13P	857.5180	-0.3	7.5	6.80
	36:5	855.5023	C45H76O13P	855.5024	-0.1	8.5	6.05
	36:6	853.4864	C45H74O13P	853.4867	-0.4	9.5	5.45
	38:1	891.5964	C47H88O13P	891.5963	0.1	4.5	10.75
	38:4	885.5505	C47H82O13P	885.5493	1.4	7.5	8.74
	38:5	883.5371	C47H80O13P	883.5337	3.8	8.5	7.90
	38:6	881.5176	C47H78O13P	881.5180	-0.5	9.5	6.86
PS							
	34:1	760.5115	C40H75NO10P	760.5129	-1.8	4.5	8.62
	34:2	758.4966	C40H73NO10P	760.5129	-0.8	5.5	7.68
	34:3	756.4811	C40H71NO10P	758.4972	-0.7	6.5	6.83
	36:1	788.5438	C42H79NO10P	756.4816	-0.5	4.5	9.97
	36:2	786.5294	C42H77NO10P	788.5442	1.1	5.5	9.03
	36:3	784.5134	C42H75NO10P	786.5285	0.6	6.5	8.24
	36:4	782.4986	C42H73NO10P	784.5129	1.8	7.5	7.17
	36:5	780.4825	C42H71NO10P	782.4972	1.2	8.5	6.36
	38:0	818.5908	C44H85NO10P	780.4816	-0.4	3.5	9.78
	38:1	816.5765	C44H83NO10P	818.5911	1.2	4.5	8.87
	38:2	814.5605	C44H81NO10P	816.5755	0.9	5.5	7.99
	38:3	812.5444	C44H79NO10P	814.5598	0.2	6.5	7.02

	38:4	810.5283	C44H77NO10P	812.5442	-0.2	7.5	6.20
LPS							
	18:1	522.2827	C24H45NO9P	522.2832	-1.0	3.5	2.80
	20:0	552.3306	C26H51NO9P	552.3301	0.9	2.5	2.50
			GLYCEROLIPIDS				
TG							
	36:0	656.5833	C39H78NO6	656.5829	0.6	1.5	11.90
	38:0	684.6117	C41H82NO6	684.6142	-3.7	1.5	13.00
	40:0	712.6457	C43H86NO6	712.6455	0.3	1.5	13.94
	42:0	740.6763	C45H90NO6	740.6768	-0.7	1.5	14.82
	42:1	738.6612	C45H88NO6	738.6612	0.0	2.5	14.25
	42:2	736.6455	C45H86NO6	736.6455	5.7	3.5	13.72
	42:3	734.6288	C45H84NO6	734.6299	-1.5	4.5	13.00
	44:0	768.7087	C47H94NO6	768.7081	0.8	1.5	15.57
	44:1	766.6927	C47H92NO6	766.6925	0.3	2.5	15.04
	44:2	764.6761	C47H90NO6	764.6768	-0.9	3.5	14.57
	44:3	762.6622	C47H88NO6	762.6612	1.3	4.5	13.88
	44:4	760.6466	C47H86NO6	760.6455	1.4	5.5	13.31
	46:0	796.7397	C49H98NO6	796.7394	0.4	1.5	16.26
	46:1	794.7241	C49H96NO6	794.7238	0.4	2.5	15.82
	46:2	792.7081	C49H94NO6	792.7081	0.0	3.5	15.28
	46:3	790.6927	C49H92NO6	790.6925	0.3	4.5	14.72
	46:4	788.6771	C49H90NO6	788.6768	0.4	5.5	14.19
	46:5	786.6608	C49H88NO6	786.6612	-0.5	6.5	13.59
	46:6	784.6456	C49H86NO6	784.6455	0.1	7.5	12.97
	48:0	824.7714	C51H102NO6	824.7707	0.8	1.5	16.92
	48:1	822.7556	C51H100NO6	822.7551	0.6	2.5	16.42
	48:2	820.7375	C51H98NO6	820.7394	-2.3	3.5	15.95
	48:3	818.7239	C51H96NO6	818.7238	0.1	4.5	15.42
	48:4	816.7076	C51H94NO6	816.7081	-0.6	5.5	14.94
	48:5	814.6912	C51H92NO6	814.6925	-1.6	6.5	14.47
	48:6	812.6791	C51H90NO6	812.6768	2.8	7.5	13.83
	48:7	810.6606	C51H88NO6	810.6612	-0.7	8.5	13.25
	48:8	808.6448	C51H86NO6	808.6455	-0.9	9.5	12.65
	50:0	852.8022	C53H106NO6	852.8020	0.2	1.5	17.70
	50:1	850.7852	C53H104NO6	850.7864	-1.4	2.5	17.14
	50:2	848.7703	C53H102NO6	848.7707	-0.5	3.5	16.61

50:3	846.7551	C53H100NO6	846.7551	0.0	4.5	16.10
50:4	844.7390	C53H98NO6	844.7394	-0.5	5.5	15.60
50:5	842.7228	C53H96NO6	842.7238	-1.2	6.5	15.16
50:6	840.7075	C53H94NO6	840.7081	-0.7	7.5	14.63
50:7	838.6934	C53H92NO6	838.6925	1.1	8.5	14.10
50:8	836.6779	C53H90NO6	836.6768	1.3	9.5	13.53
50:9	834.6602	C53H88NO6	834.6612	-1.2	10.5	12.91
52:0	880.8333	C55H110NO6	880.8333	0.0	1.5	18.61
52:1	878.8166	C55H108NO6	878.8177	-1.3	2.5	17.93
52:2	876.8022	C55H106NO6	876.8020	0.2	3.5	17.30
52:3	874.7877	C55H104NO6	874.7864	1.5	4.5	16.76
52:4	872.7678	C55H102NO6	872.7707	-3.3	5.5	16.29
52:5	870.7562	C55H100NO6	870.7551	1.3	6.5	15.79
52:6	868.7394	C55H98NO6	868.7394	0.0	7.5	15.29
52:7	866.7234	C55H96NO6	866.7238	-0.5	8.5	14.82
52:8	864.7066	C55H94NO6	864.7081	-1.7	9.5	14.25
52:9	862.6957	C55H92NO6	862.6925	3.7	10.5	13.72
54:1	906.8475	C57H112NO6	906.8489	-1.7	2.5	18.80
54:2	904.8340	C57H110NO6	904.8333	0.8	3.5	18.08
54:3	902.8164	C57H108NO6	902.8177	-1.4	4.5	17.52
54:4	900.8018	C57H106NO6	900.8020	-0.2	5.5	16.92
54:5	898.7843	C57H104NO6	898.7864	-2.3	6.5	16.45
54:6	896.7710	C57H102NO6	896.7707	0.3	7.5	15.98
54:7	894.7526	C57H100NO6	894.7551	-2.8	8.5	15.48
54:8	892.7371	C57H98NO6	892.7394	-2.6	9.5	14.97
54:9	890.7255	C57H96NO6	890.7238	1.9	10.5	14.47
56:2	932.8645	C59H114NO6	932.8646	-0.1	3.5	19.06
56:3	930.8485	C59H112NO6	930.8490	-0.5	4.5	18.30
56:4	928.8323	C59H110NO6	928.8333	-1.1	5.5	17.70
56:5	926.8177	C59H108NO6	926.8177	0.0	6.5	17.08
56:6	924.8024	C59H106NO6	924.8020	0.4	7.5	16.61
56:7	922.7859	C59H104NO6	922.7864	-0.5	8.5	16.14
58:3	958.8795	C61H116NO6	958.8803	-0.8	4.5	19.37
58:4	956.8643	C61H114NO6	956.8646	-0.3	5.5	18.61
58:5	954.8485	C61H112NO6	954.8490	-0.5	6.5	17.05
58:6	952.8353	C61H110NO6	952.8333	2.1	7.5	16.57
58:7	950.8167	C61H108NO6	950.8177	-1.1	8.5	16.07
58:8	948.8041	C61H106NO6	948.802	2.2	9.5	15.66

	58:9	946.7859	C61H104NO6	946.7864	-0.5	10.5	15.13
	58:10	944.7720	C61H102NO6	944.7707	1.4	11.5	14.70
	58:11	942.7545	C61H100NO6	942.7551	-0.6	12.5	14.20
	58:14	936.7078	C61H94NO6	936.7081	-0.3	15.5	11.43
	60:4	984.8961	C63H118NO6	984.8959	0.2	5.5	19.53
	60:5	982.8788	C63H116NO6	982.8803	-1.5	6.5	18.75
	60:6	980.8621	C63H114NO6	980.8646	-2.5	7.5	18.14
DG							
	28:0	530.4799	C31H64NO5	530.4784	2.8	0.5	7.25
	30:0	558.5105	C33H68NO5	558.5097	1.4	0.5	8.70
	30:1	556.4935	C33H66NO5	556.4941	-1.1	1.5	7.73
	30:2	554.4800	C33H64NO5	554.4784	2.9	2.5	6.97
	30:3	552.4651	C33H62NO5	5524628	4.2	3.5	6.06
	32:0	586.5407	C35H72NO5	586.5411	-0.7	0.5	10.11
	32:1	584.5258	C35H70NO5	584.5254	0.7	1.5	9.20
	32:2	582.5095	C35H68NO5	582.5097	-0.3	2.5	8.29
	32:3	580.4941	C35H66NO5	580.4941	0.0	3.5	7.35
	32:4	578.4786	C35H64NO5	578.4784	0.3	4.5	6.50
	32:5	576.4656	C35H62NO5	576.4628	4.9	5.5	5.78
	34:0	614.5714	C37H76NO5	614.5724	-1.6	0.5	11.37
	34:1	612.5558	C37H74NO5	612.5567	-1.5	1.5	10.45
	34:2	610.5405	C37H72NO5	610.5411	-1.0	2.5	9.54
	34:3	608.525	C37H70NO5	608.5254	-0.7	3.5	8.70
	34:4	606.5095	C37H68NO5	606.5097	-0.3	4.5	7.76
	34:5	604.4915	C37H66NO5	604.4941	-4.3	5.5	6.82
	34:6	602.4780	C37H64NO5	602.4784	-0.7	6.5	6.09
	36:0	642.6034	C39H80NO5	642.6036	-0.5	0.5	12.47
	36:1	640.5889	C39H78NO5	640.5880	1.4	1.5	11.68
	36:2	638.5728	C39H76NO5	638.5724	0.6	2.5	10.80
	36:3	636.5563	C39H74NO5	636.5567	-0.6	3.5	9.98
	36:4	634.5401	C39H72NO5	634.5411	-1.6	4.5	9.07
	36:5	632.5246	C39H70NO5	632.5254	-1.3	5.5	8.13
	36:6	630.5093	C39H68NO5	630.5098	-0.6	6.5	7.25
	38:0	670.6345	C41H84NO5	670.6349	-0.7	0.5	13.47
	38:1	668.6218	C41H82NO5	668.6193	3.7	1.5	12.37
	38:2	666.6023	C41H80NO5	666.6063	-2.1	2.5	11.97
	38:4	662.5705	C41H76NO5	662.5723	-2.9	4.5	10.80
	40:0	698.6646	C43H88NO5	698.6663	0.1	0.5	14.50

	40:1	696.6523	C43H86NO5	696.6506	2.4	1.5	13.72
	42:10	706.5396	C45H72NO5	706.5411	-2.1	10.5	7.29
	42:11	704.5247	C45H70NO5	704.5254	-1.0	11.5	6.47
	44:9	736.5899	C47H78NO5	736.5880	2.6	9.5	9.64
	44:10	734.5718	C47H76NO5	734.5724	-0.8	10.5	8.70
	44:11	732.5579	C47H74NO5	732.5567	1.6	11.5	7.79
	44:12	730.5414	C47H72NO5	730.5411	0.4	12.5	6.94
MG							
	16:0	348.3114	C19H42NO4	348.3114	0.0	-1.0	3.05
	18:0	376.3427	C21H46NO4	376.3427	0.0	-1.0	3.74
	18:1	374.3278	C21H44NO4	374.3270	2.1	0.5	3.24
	18:2	372.3115	C21H42NO4	372.3114	0.3	1.5	2.80
			SPHINGOLIPIDS				
SM							
	14:0	675.544	C37H76N2O6P	675.5441	-0.1	1.5	6.19
	16:0	703.5751	C39H80N2O6P	703.5754	-0.4	1.5	7.50
	18:0	731.6079	C41H84N2O6P	731.6067	1.6	1.5	9.04
	20:0	759.6379	C43H88N2O6P	759.6380	-0.1	1.5	10.45
	20:1	757.6224	C43H86N2O6P	757.6224	0.0	2.5	9.30
	22:0	787.6685	C45H92N2O6P	787.6693	-1.0	1.5	11.71
	22:1	785.653	C45H90N2O6P	785.6537	-0.9	2.5	10.86
	24:0	815.7001	C47H96N2O6P	815.7006	-0.6	1.5	12.68
	24:1	813.6849	C47H94N2O6P	813.6850	-0.1	2.5	11.71
			STEROLS				
CE							
	14:1	612.5717	C41H74NO2	612.5720	-0.5	5.5	13.22
	16:0	6426159	C43H80NO2	642.6189	-4.7	4.5	17.17
	16:1	640.6024	C43H78NO2	640.6033	-1.4	5.5	16.42
	16:2	638.5851	C43H76NO2	638.5876	-3.9	6.5	15.79
	16:3	636.5718	C43H74NO2	636.5720	-0.3	7.5	15.16
	18:0	670.6495	C45H84NO2	670.6502	-1.0	4.5	18.18
	18:1	668.6343	C45H82NO2	668.6346	-0.4	5.5	17.30
	18:2	666.6183	C45H80NO2	666.6189	-0.9	6.5	16.61
	18:3	664.6028	C45H78NO2	664.6033	-0.8	7.5	16.04
	20:1	696.6673	C47H86NO2	696.6659	2.0	5.5	18.24
	20:2	694.6504	C47H84NO2	694.6502	0.3	6.5	17.45
	20:3	692.6354	C47H82NO2	692.6346	1.2	7.5	16.83

	20:4	690.6209	C47H80NO2	690.6189	2.9	8.5	16.23
	20:5	688.6036	C47H78NO2	688.6033	0.4	9.5	14.53