**Universidade de Aveiro** Departamento de Biologia

**2016**

**Rita Marisa Gomes Jordão**

# Abordagem integrada para caracterizar novos mecanismos de toxicidade de contaminantes em *Daphnia magna*

# Integrative approach to characterize new mechanisms of toxicity of pollutants in *Daphnia magna*

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Professor Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático no Departamento de Biologia da Universidade de Aveiro e da co-orientação do Doutor Carlos Barata Martí, Investigador Principal no Instituto de Diagnóstico Ambiental y Estudios del Agua do Consejo Superior de Investigaciones Cientificas em Barcelona e do Professor Doutor Marco Filipe Loureiro Lemos, Professor Adjunto no Instituto Politécnico de Leiria.

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Dedico este trabalho ao Luís e á minha família pelo incansável apoio.

**o júri**

presidente

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Rita

**palavras-chave**

*Daphnia magna*, obesogen, ecdisona, juvenóide, metil farnesoato, RXR, EcR, resistência multi-xenobiótica, Hsp70.

**resumo**

Nas últimas décadas, a comunidade científica tem vindo a focar-se no estudo da toxicidade de poluentes químicos persistentes e amplamente distribuídos no ambiente aquático. Normalmente, misturas complexas de poluentes prioritários, contaminantes emergentes, productos da degradaçao dos mesmos e/ou compostos naturais coocorrem em sistemas aquáticos, levando a que a avaliação de risco seja um desafio. Os ensaios de toxicidade padrão realizados atualmente, tais como os baseados em respostas agudas e crónicas podem não ser suficientemente sensíveis para detetar efeitos ao nivel das concentrações consideradas ambientalmente relevantes. Assim, é essencial o desenvolvimento de novas ferramentas para a avaliação de risco ambiental, assim como novas abordagens conceptuais.

O objetivo desta tese é desvendar novos mecanismos de ação de toxicidade em invertebrados aquáticos usando a espécie de crustáceo *Daphnia magna* como organismo modelo. Para tal, foram usados ensaios convencionais combinados com novas abordagens para analisar efeitos específicos sub-letais de poluentes emergentes e clássicos, e possíveis consequências para a saúde dos ecossistemas. O trabalho foi desenvolvido em torno de dois tópicos principais: a caracterização de efeitos obesogénicos causados por desreguladores endócrinos e a caracterização de mecanismos de resistência multi-xenobiótica em clones tolerantes de *D. magna*. Com os resultados obtidos, observámos efeitos de disrupção endócrina no metabolismo lipídico da *D. magna* quando exposta aos compostos emergentes e clássicos, ativando as vias de sinalizaçao dos recetores da ecdisona, da hormona juvenil e RXR. Uma das principais características observadas na desregulação endócrina, incluindo os efeitos obesogénicos, foram os efeitos adversos na qualidade da descendência e o respetivos problemas durante o seu ciclo de vida. Observámos também no segundo tópico sinais de uma estreita associação entre as respostas gerais ao stress e os mecanismos de resistência multi-xenobiótica em *D. magna* e que ambos os mecanismos são geneticamente co-selecionados.

Concluindo, este trabalho apresenta, pela primeira vez, efeitos obesogénicos em *D. magna* e ainda que os mecanismos de resistência multi-xenobiótica podem estar envolvidos no mecanismo geral de tolerância ao stress.

**keywords** *Daphnia magna*, obesogen, ecdysone, juvenoid, methyl farnesoate, RXR, EcR, multixenobiotic resistance, Hsp70.

**abstract**

In the last decades, the scientific community focused on the study of the toxicity of persistent and widely distributed chemical pollutants in the aquatic environment. Typically, complex mixtures of priority pollutants, emerging substances, transformation products and/or natural compounds co-occur in aquatic systems, thus rendering the risk assessment a challenge.

The current standard toxicity assays such as those based on acute and chronic responses may not be sensitive enough to detect the contaminant effects at low environmentally relevant concentrations. Thus, the development of new assessment tools and new conceptual approaches is essential.

Therefore, the aim of this thesis is to elucidate novel mechanisms of action of toxicity in aquatic invertebrate species using the crustacean *Daphnia magna* as a model organism. For that, standard assays combined with new approaches were used for the analysis of specific sub-lethal effects and health consequences of emerging and classical pollutants. The work was developed around two main topics; the characterization of obesogenic effects produced by endocrine disruptors and as well, the characterization of the multixenobiotic resistance mechanisms in tolerant clones of the *D. magna.*

With the obtained results we observed endocrine disrupting effects on the lipid metabolism of *D. magna* when exposed to emerging and classical compounds, activating the ecdysone, the juvenil hormone and RXR receptor signalling pathways. One of the key features of endocrine disruption, including obesogenic effects, was to adversely affect offspring quality and its latter performance during its life cycle. Moreover, in the second topic we observed evidences of a close association between general stress responses and multixenobiotic resistance mechanisms in tolerant clones of *D. magna* and that both mechanisms are genetically co-selectable.

In conclusion, this thesis presents, for the first time, obesogenic efects in *D. magna* and that multixenobiotic resistance mechanisms can be part of a general mechanism of tolerance, key to cope with environmental stress.

***Index***

## *List of figures*

### Chapter I – General introduction and thesis outline



### Chapter II – Obesogenic effects of TBT in *Daphnia magna*

**Chapter III – Compounds altering fat storage in *Daphnia magna***

**Chapter IV – Two-generational effects in *Daphnia magna***



**Chapter V – Multixenobiotic resistance mechanism in *Daphnia magna***

**Chapter VI – Final remarks**

## *List of tables*

### Chapter I – General introduction and thesis outline



### Chapter II – Obesogenic effects of TBT in *Daphnia magna*

**Chapter III – Compounds altering fat storage in *Daphnia magna***

**Chapter V – Multixenobiotic resistance mechanism in *Daphnia magna***



**Annex – Supplemental information and published articles**

### *List of abbreviations*

| | |
|---|---|
| 20E | 20-hydroxyecdysone |
| ABC | ATP binding cassette |
| AMPK | AMP-activated kinase |
| ANOVA | Analysis of variance |
| AR | Androgen receptor |
| ASTM | American Society for Testing and Materials |
| ATP | Adenosine triphosphate |
| bHLH-PAS | basic Helix-Loop-Helix-Per-Arnt-Sim |
| BHT | 2,6-di-tert-butyl-4-methylphenol |
| BPA | Bisphenol A |
| BSA | Bovine serum albumin |
| CA | Concentration addition |
| Ca-AM | Calcein-AM |
| cDNA | Complementary deoxyribonucleic acid |
| CE | Cholesterylesters |
| CHL | Chlorambucil selected clone |
| Cp | Crossing point |
| DEE | Dichlorodiphenyldichloroethylene |
| DEPH | Di(2-ethylhexyl)phthalate |
| Df | Degree of freedom |
| DG | Diacylglycerol |
| DNA | Deoxyribonucleic acid |
| DO | Dissolved oxygen |
| DTT | Dithiothreitol |
| EC | Effect concentration |

| | |
|---|---|
| $EC_{50}$ | Half maximal effective concentration |
| EcR | Ecdysone receptor |
| EDC | Endocrine disrupting compound |
| EDTA | Ethylenediamine tetraacetic acid |
| EM | Emamectin benzoate |
| ER | Estrogen receptor |
| ESI | Electrospray ionization |
| FA | Fatty acyl |
| FEN | Fenarimol |
| FIA | Flow injection analysis |
| FU | Fluorescence units |
| FX | Fluoxetine |
| G3PDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GABA | Gamma-aminobutyric acid |
| GL | Glycerolipid |
| GP | Glycerophospholipid |
| GSH | Reduced glutathione |
| IA | Independent action |
| ICP-MS | Inductively coupled plasma mass spectrometer |
| ISO | International Organization for Standardization |
| IVM | Ivermectin selected clone |
| JH | Juvenile hormone |
| LC50 | Half maximal lethal concentration |
| LC-MS | Liquid chromatography-mass spectrometry |
| LIPID MAPS | Lipid Metabolites and Pathways Strategy |
| LOD | Limit of detection |

| | |
|---|---|
| LOEC | Lowest observed effect concentration |
| LPC | Lysophosphatidylcholine |
| MDR | Multidrug resistance |
| MET | Methoprene-tolerant |
| MF | Methyl farnesoate |
| MfR | Methyl farnesoate receptor |
| MIH | Moult-inhibiting hormone |
| MIT | Mitoxantrone selected clone |
| MRM | Multiple reaction monitoring |
| mRNA | messenger Ribonucleic acid |
| MRP | Multidrug resistance protein (or ABCC) |
| MT | Methoprene |
| MXR | Multixenobiotic resistance |
| NP | Nonylphenol |
| Ns | Not significant |
| OECD | Organisation for Economic Co-operation and Development |
| PBO | Piperonyl butoxide |
| PBS | Phosphate-buffered saline |
| PC | Phosphocholine |
| PCB | Polychlorinated biphenyl |
| PCP | Pentachlorophenol selected clone |
| PE | Phosphatidylethanolamine |
| P-gp | P-glycoprotein (or ABCB) |
| PI | Phosphatidylinositol |
| PK | Polyketide |
| PMSF | Phenylmethylsulfonyl fluoride |

| | |
|---|---|
| PoA | Ponasterone A |
| PP | Pyriproxyfen |
| PPAR | Peroxisome proliferator-activated receptor |
| PPAR*γ* | Peroxisome proliferator-activated receptor gamma |
| PR | Prenol lipid |
| PS | Phosphatidylserine |
| PUFA | Polyunsaturated fatty acid |
| PVDF | Polyvinylidene difluoride |
| qPCR | quantitative real time Polymerase Chain Reaction |
| REACH | Registration, Evaluation, Authorisation and Restriction of Chemicals |
| RNA | Ribonucleic acid |
| RXR | Retinoid X receptor |
| SDS-PAGE | Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis |
| SE | Standard error |
| SL | Saccharolipid |
| SM | Sphingomyelin |
| SP | Sphingolipid |
| SRC | Steroid receptor co-activator |
| SRM | Selected reaction monitoring |
| ST | Sterol lipid |
| TBT | Tributyltin |
| TEB | Tebufenozide |
| TG | Triacylglycerol |
| TOF | Time-of-flight |
| TOR | Target of rapamycin |
| TPT | Triphenyltin |

| | |
|---|---|
| UPLC | Ultra-performance liquid chromatography |
| USEPA | United States Environmental Protection Agency |
| USP | Ultraspiracle |
| VER | Verapamil selected clone |
| WFD | Water Framework Directive |
| WHO | World Health Organization |

# Chapter I

General introduction and thesis outline

## Chapter I – General introduction and thesis outline

### Background

Since the industrial revolution, the efforts of removing anthropogenic pollutants from the natural environment have been unable to keep pace with the increasing amount of waste materials and growing population that further aggravates the situation. This has often resulted in the transformation of lakes, rivers, and coastal waters into sewage depots where the natural biologic balance is severely upset and in some cases totally disrupted (Goldberg *et al.,* 2012). The concepts of "ecosystem health" and "good chemical and ecological status" have been developed, involving a more comprehensive approach to aquatic environments. In this context, assess the potential risks of pollutants released into the environment is an important aspect of regulations dealing with the sustainable management of water resources (Tlili *et al.,* 2015). The European Water Framework Directive (WFD; European Commission, 2000) constitutes one of the most important European Union pieces of environmental legislation dealing with the aquatic compartment. It represents a transformation of the guidelines for water quality assessment and monitoring across all EU Member States in terms of protection and management of inland surface, transitional, coastal and ground waters (Martinez-Haro *et al.,* 2015). The inherent aim of the WFD is to protect and prevent deterioration of European waters on the basis of their ecological community structures and, therefore, 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).

In the last decades, the scientific community was also focused on the evaluation of the toxicity of persistent and widely distributed chemical pollutants in the aquatic environment. Special regulations have been issued for those considered as particularly noxious for the environment, with the ultimate target of their abolishment whenever possible, or, at least, a severe reduction on their production and use. However, the continued discharge of pollutants into aquatic ecosystems all over the world, due to increase of urbanization and continued human population growth are steadily intensifying the aquatic pollution, as wastewater makes up an ever-growing fraction of the flow of lotic ecosystem, especially in urban areas (Rosi-Marshall *et al.,* 2015). The monitoring programs initiated under the WFD have accumulated vast amounts of data on contamination and on the ecological status of surface waters in the European Union (European Environment Agency, 2012). At the same time, a wealth of chemical property and environmental data originated from the REACH (Registration, Evaluation,

Authorisation and Restriction of Chemicals) legislation framework, biocides (European Commission, 1998), plant protection products (European Commission, 2009) and pharmaceuticals (European Commission, 2010) is gradually becoming available. Although toxic effects on aquatic life are regularly observed, it remains a great challenge to link occurrence of chemicals with the ecological status of waters, identify major chemical stressors that would call for risk management measures, and to find efficient solutions for the abatement of pollution-related risks. Typically, complex mixtures of priority pollutants, emerging substances, by- and transformation products, and natural compounds co-occur in aquatic systems, thus rendering a comprehensive assessment challenging (Brack *et al.,* 2015). The emerging pollutants reach the environment from various anthropogenic sources and are distributed throughout environmental matrices. Although great advances have been made in the detection and analysis of trace pollutants during recent decades, due to the continued development and refinement of specific techniques, a wide array of undetected contaminants of emerging environmental concern need to be identified and quantified in various environmental components and biological tissues. These pollutants may be mobile and persistent in air, water, soil, sediments and ecological receptors even at low concentrations. Robust data on their fate and behaviour in the environment, as well as on threats to ecological and human health, are still lacking (Gavrilescu *et al.,* 2015).

The study of these so-called emergent pollutants is included in the priority research guidelines of many environmental and health regulation bodies, such as the World Health Organization (WHO), the United States Environmental Protection Agency (USEPA) or the European Commission (Barceló 2003). A peculiar property of some of these contaminants is that, they are continuously released, thus their negative effects are independent from their persistence in the environment. Although most of these emerging pollutants show low acute toxicity, their potential sub-lethal effects and toxicological mode of action is unknown or only known in humans (Drewes *et al.,* 2005; Kinney *et al.,* 2006; Veldhoen *et al.,* 2006; Woodling *et al.,* 2006; Gibs *et al.,* 2007; Claessens *et al.,* 2013; Cristale *et al.,* 2013; Hutchinson *et al.,* 2013).

Traditionally, ecotoxicological assays have been focused on effects of pollutants at the individual level and assaying endpoints like mortality, reproduction and individual growth (Chevalier et al., 2015). Many of the ecotoxicological assays standardized by organizations like Organisation for Economic Co-operation and Development (OECD) and USEPA focus on individual level effects and do not provide information regarding toxic mechanisms. Often, these standardized toxicity assays such as those based on acute and chronic responses may not be sensitive enough to detect the effects at low

environmentally relevant concentrations. In the future, a better understanding is required to link biological responses to chemical exposure across different levels of biological organization.

## 1 Emerging pollutants

Emerging pollutants refer to a wide range of substances including pharmaceuticals, endocrine disruptors, hormones and toxins, as well as polar pesticides, veterinary products, industrial compounds/by-products, food additives and engineered nanomaterials (Lapworth *et al.,* 2012; Gavrilescu *et al.,* 2015; Ternes *et al.,* 2015).

The mechanisms of action of many classical pollutants (i.e. metals, coplanar aromatic polycyclic hydrocarbons and PCBs, dioxins, estrogenic compounds) in aquatic organisms are known but in case of emerging pollutants little is known about their effects. Emerging pollutants may act in key developmental processes, such as proliferation, apoptosis, or differentiation, and as well impair some specific responses of adult stages such as growth, reproduction and behavioural responses related to the ecological fitness (swimming, foraging and escaping reactions). Other effects may also be measured beyond individuals at the population and community levels using both, laboratory and field assays. Environmental features such as exposure to pollutant mixtures, changes in temperature, food supply and the widespread presence of pathogens are likely to increase due to global change. Besides, new advances in physiology have addressed the importance of natural factors such as temperature, food supply and diseases modulating the lipid composition, the immune response and the neuroendocrine system (Barceló 2003; Lapworth *et al.,* 2012).

The increasing concern on emerging pollutants has urged for the need of novel ecotoxicological procedures in environmental risk assessment. In furtherance of this, the assessment of biological effects of new and classical pollutants for sensitive environmental diagnosis requires the development of new assessment tools and new conceptual approaches.

Hereupon, within this perspective, in this thesis we focused mainly on the analysis of novel specific sub-lethal effects and health consequences of emerging and classical pollutants on crustacean *Daphnia magna*, which is highly sensitive to a wide range of chemicals and is representative of freshwater organisms. Therefore, the work was developed around two main topics, the study of endocrine disrupting compounds (EDCs)

related with disrupting fat storage and the study of multixenobiotic resistance mechanisms in different tolerant clones of *D. magna*.

## 1.1 Endocrine disrupting compounds

Environmental pollution by endocrine disruption in vertebrates and invertebrates is a worldwide environmental problem, but relatively 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 the example of tributyltin-induced reproductive damage and population declines in molluscs (Hutchinson 2002). 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 reproduction. Nevertheless, other toxic effects such as stage or sex related differences in sensitivity (i.e. survival) to EDCs and/or reduced energy resources acquisition may also affect the above mentioned life history traits (Barata *et al.,* 2004). In fact, EDCs can interact with endocrine receptors, affect hormonal levels or/and deregulate their metabolism. An often reported effects of certain EDCs in *Daphnia* include an alteration 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).

In the last years, several laboratories have shown that a variety of environmental EDCs can influence adipogenesis and obesity. Thus, the potential to interact with hormone receptors and/or neuroendocrine signalling pathways leads to a disruption of the lipid homeostasis on organisms (Barata *et al.,* 2004; LeBlanc 2007; Haeba *et al.,* 2008; Wang and LeBlanc 2009; Wang *et al.,* 2011). This class of compounds are called obesogens and can be defined functionally as chemical agents that inappropriately, regulate and promote lipid accumulation and adipogenesis (Grün and Blumberg 2007,2009a). 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 2009a). In vertebrates, the mode of action of some EDCs that show obesogenic effects is known (Grün and Blumberg 2006; Grün *et al.,* 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). Thus, it is necessary to complement ecotoxicology studies with genomic and metabolomic resources in order to unravel the mode of action of EDCs.

### 1.1.1 Nuclear receptors and endocrine signalling

Nuclear receptors are a large family of proteins widely distributed among bilateral animals (*Bilateria*), responsible for the control of a variety of processes, from sexual characteristics and behaviour in vertebrates to different metabolic pathways (oxidative metabolism, lipid imbalance, etc.) or moulting and reproduction in invertebrates. The mechanism of action of nuclear receptors involves their binding to small natural molecules (ligands), and both act as homo- or heterodimers that bind to specific DNA elements in order to elicit transcriptional activation of target genes (Wang *et al.,* 2007; Johnson and O'Malley 2012). More than that, once bound (ligand-receptor), the ligand modify the ability of the nuclear receptor to regulate gene transcription either positively (as an agonist) or negatively (as an antagonist) (Wang *et al.,* 2011). This regulation confers to the receptor the property of modifying the gene expression patterns of the target cells, with dramatic effects at different biological organization levels (i.e. from cell up to individual level) (Wang *et al.,* 2007; Johnson and O'Malley 2012). Given their key role on homeostasis maintenance and behaviour control of organisms, any pollutants able to interfere in this interaction between ligand and receptor, either by affecting the circulating levels of the ligand, or by competing with the natural hormone for the bind site to the receptor, having profound effects on organisms (Wang *et al.,* 2007).

Androgen receptor (AR) in males and the estrogen receptor (ER) and progesterone receptor in females, by binding to sexual hormones, are crucial regulators of steroid endocrine system. Notwithstanding, the nuclear receptors also play an important role in non-steroid endocrine systems. Given the complexity of the non-steroid endocrine systems, the information about their putative disruptors is very incomplete if compared with our knowledge of the “classical” endocrine disruptors, which typically affect sex-related signalling mechanisms (e.g. ER, AR) (Ferré 2004; Grün and Blumberg 2009a). However, there are several pieces of evidence indicating that many compounds are putative thyroid, retinoid X receptor (RXR) and/or peroxisome proliferator-activated receptor (PPAR) disruptors. This occurs because some of these nuclear receptors are recognized as targets of toxicity associated with endocrine-active chemicals (Iguchi *et al.,* 2007).

In vertebrates, improper regulation of lipid homeostasis can result in serious health problems, such as obesity, increased risk of coronary artery diseases, diabetes and related detrimental problems, such as hypertension and lipidemia (Grün and Blumberg 2006; Sharma and Staels 2007). The master nuclear regulators of adipocyte differentiation are the peroxisome proliferator-activated receptor gamma (PPAR*γ*) and the heterodimeric partner RXR that are involved in the regulation of food intake, metabolic efficiency and energy storage (Figure 1.1) (Grün and Blumberg 2007; Santos *et al.,* 2012).

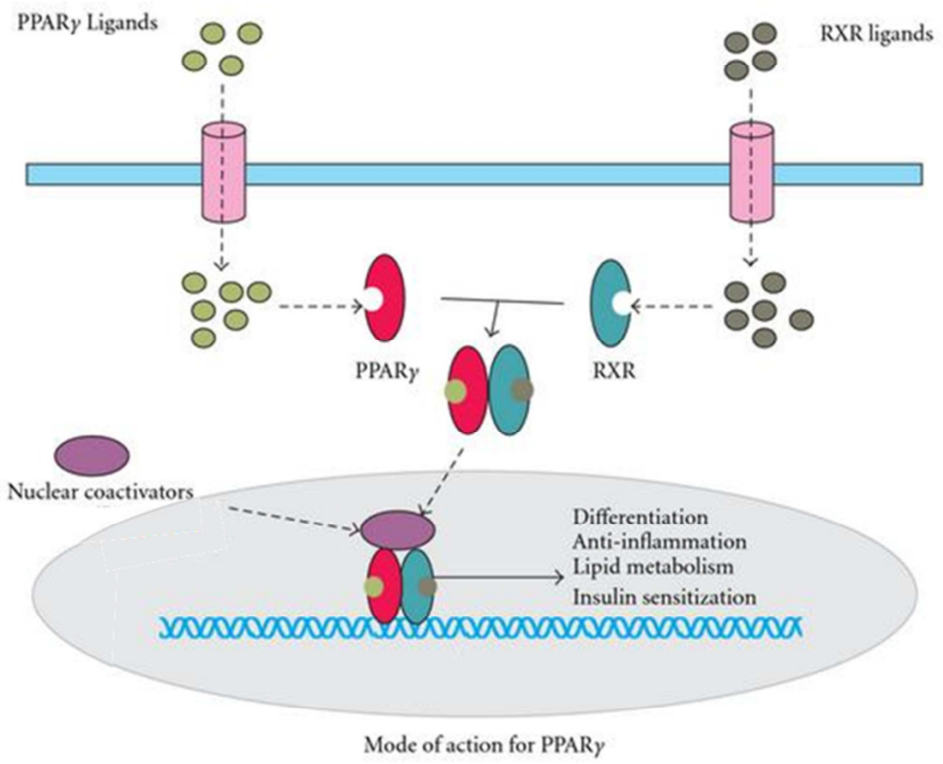


**Figure 1.1** – Schematic diagram of the mode of action for the master adipogenic receptors. PPARγ and RXR form a heterodimer, which is activated by the respective ligands. The activated PPARγ/RXR heterodimer will then be translocated into the nucleus and where it regulates downstream target genes in concert with nuclear receptor co-activators (adapted from Wagner *et al*., (2010)).

### 1.1.2 Endocrine signalling in invertebrates

The PPAR has not been described outside deuterostomes whereas RXR is ubiquitous within metazoans and was originally called ultraspiracle, USP, in insects

(LeBlanc 2007; Santos *et al.,* 2012). In arthropods, ecdysone receptor (EcR) functions as a prototypical heterodimeric transcription factor with RXR (Yao *et al.,* 1993) and the ecdysone hormone binding the complex (as ligand), recruits co-activators that ultimately stimulate gene transcription. In response to ecdysone hormones that bind to EcR, the EcR:RXR complex regulates a variety of activities related to development, growth, and reproduction (LeBlanc 2007; André *et al.,* 2014). The ecdysteroid 20-hydroxyecdysone (20E) is natural ligands of EcR. Another important receptor in arthropods is the Juvenile hormone (JH) receptor, that in crustaceans the JH lost the epoxide group and becoming methyl farnesoate (MF) (Mu and Leblanc 2004). Although it is still unclear how RXR may interact with the JH signalling pathway in arthropods, recent studies identified a putative methyl farnesoate receptor (MfR) complex in *Daphnia pulex* and *D. magna* (LeBlanc *et al.,* 2013; Miyakawa *et al.,* 2013). This complex included the leading candidate for juvenile receptor in insects, the methoprene-tolerant (MET) transcription factor, and the steroid receptor co-activator (SRC), involved in the reception and signal transduction of JH, which are activated by the crustacean 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). Methoprene-tolerant and SRC are proteins that belong to the basic helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) family of transcription factors that are involved in the activation of many receptors (Miyakawa *et al.,* 2013).

*Ecdysteroids function*

Ecdysteroids are signalling molecules that are well characterized with respect to their role in regulating the moulting process (ecdysis). Ecdysteroids are under the negative regulatory control of Moult-inhibiting hormone (MIH). In general, the ecdysteroid levels are low during the intermoult and post-moult periods, while in pre-moult, the concentrations rise and reach a peak shortly before moulting (Mykles 2011). The MIH serves as the linkage between neurological signalling and steroid control of process such as moulting and embryo development. Just prior to moulting, MIH levels decrease resulting in a precipitous increase in circulating ecdysteroid levels. Ecdysis is triggered by the subsequent decline in ecdysteroids back to basal levels. Shedding of the old exoskeleton and its replacement with a larger carapace allows for growth of the organism (LeBlanc 2007).

*Juvenoids function*

Juvenoids, which belong to the class of terpenoids, 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). Methyl farnesoate/JH has many regulatory functions in crustaceans such as reproductive maturation in decapods where it increases production of vitellogenin and eggs; and stimulation of gonadal maturation (increased mass) of both males (testes) and females (ovaries). High levels of MF in reproductive daphnids result in the production of male offspring (LeBlanc 2007).

### 1.1.3 Organotins – obesogenic compounds

Some compounds are known to disrupt specific invertebrate endocrine system, including development and reproductive function. These substances currently serve as reference standards for the scientific community to use in developing screening and test assays, model systems, and investigative approaches to detect chemicals with obesogen effects. One of the most studied obesogenic compounds in vertebrates is tributyltin (TBT) (Janer *et al.,* 2007; Tingaud-Sequeira *et al.,* 2011; Bernat *et al.,* 2014). This compound shows disruptive effects on lipid homeostasis and have been mostly associated with increasing triacylglycerols (TGs) or/and fatty acids in adipocytes or in other tissues (Santos *et al.,* 2012). Since the late 1960s, TBT and other organotins such as triphenyltin (TPT), have been extensively used across the world as biocides in antifouling paints, applied on ship hulls and fishing nets, and as fungicides in agricultural crops (Lyssimachou *et al.,* 2009; Basic *et al.,* 2012). Despite their gradual removal from the market and their prohibition of use, their release into the environment combined with their low solubility in water and high octanol–water partition coefficient has resulted in worldwide contamination of the aquatic environment (Lyssimachou *et al.,* 2009). Both TBT and TPT are potent endocrine disruptors and potent activators of nuclear receptors RXR and PPAR*γ* (Basic *et al.,* 2012). The ability to target both receptors simultaneously should be particularly effective since adipogenic signalling can be mediated through both the RXR and PPAR*γ* components of the heterodimer (Grün and Blumberg 2006). Grün and co-workers reported effects of RXR and PPAR*γ* activation by TBT in the adipogenesis pathway. Tributyltin increased the number of differentiated adipocytes in the 3T3-L1 murine pre-adipocyte cell line and disrupt normal adipogenesis in vertebrate

developmental models (Grün and Blumberg 2006). In molluscs, organotins cause imposex (development of male sex organs in female gastropods), through the activation of the RXR signalling pathways (Nishikawa *et al.,* 2004; Nakanishi 2008). Janner and co-workers reported that long term exposure to 500 ng/L of TBT in the snail *Marisa cornuarietis* increased total lipids and fatty acid methyl esters by two to three fold in females (Janer *et al.,* 2007). Conversely, short term responses of the same snail species to 500 ng/L of TPT decreased lipid levels and fatty acids (Lyssimachou *et al.,* 2009). Despite numerous studies addressing toxicological impacts on various animal species, very few have investigated the lipid perturbation topic outside of gastropods, molluscs and vertebrates. In the urochordate sea squirt *Ciona intestinalis*, *in vitro* exposures of ovaries of this species to TBT reduced total lipid content and changed lipid composition such as reduced TGs, increased phospholipids and long chain polyunsaturated fatty acids (PUFA). Puccia and co-workers proposed that the observed pattern in lipid dynamics could be a general cell adaptive mechanism to pollutants (Puccia *et al.,* 2005).

### 1.1.4 Other compounds as obesogens

Obesogenic effects in vertebrates can also be caused disrupting other endocrine signalling pathways than PPAR (Grün and Blumberg 2009b). Compounds that exhibit endocrine disruption in vertebrates might also affect invertebrate endocrines systems controlling reproduction and/or development. Most of these compounds are thought to interfere with ecdysone at target tissues and others are juvenoid-like compounds that have also been shown to inhibit moulting, likely by interfering with the stimulatory effect of JH (Rodríguez *et al.,* 2007). The vertebrate obesogens bisphenol A (BPA), nonylphenol (NP) and the di(2-ethylhexyl)phthalate (DEPH) have been reported to affect neuropeptide Y expression in the midbrain or the ER and consequently increase fat accumulation (Grün and Blumberg 2009a). For instance, BPA is a component of polycarbonate plastics, widely used in numerous products such as polycarbonate baby bottles, beverage containers, the linings of food cans, dental composites, and sealants. This compound has a potential to bind to the nuclear ER and interact with a variety of other targets in mammalian cells (Wetherill *et al.,* 2007; Basic *et al.,* 2012). In rodents, perinatal exposure to BPA results in a higher body fat percentage by increasing the size of adipocytes at adult age (Riu *et al.,* 2014). Nonylphenol, an industrial detergent degradation product, can promote adipocyte differentiation or proliferation of murine pre-adipocyte cell lines (such as 3T3-L1cells) (Grün and Blumberg 2006). On the other hand some phthalates can negatively affect the

adipose homeostasis (e.g. DEPH) (Basic *et al.,* 2012). Phthalates are a class of compounds that include various perfluorochemicals and plasticizers that are widely used as surface repellents and surfactants (Basic *et al.,* 2012). Other class of compounds such as pharmaceuticals like the selective serotonin reuptake inhibitor fluoxetine (FX) are reported to decrease mammalian fat (Péry *et al.,* 2008; Lemieux *et al.,* 2011). Lemieux and co-workers observed also that fat storage in the invertebrate worm nematode *Caenorhabditis elegans* decreased when worms were exposed to FX (Lemieux *et al.,* 2011). Thus, there are many more compounds than TBT that may disrupt lipid storage. In arthropods, compounds that are interfering with ecdysteroid and juvenoid signalling pathways have also the potential to disrupt lipid homeostasis. The reason is that the ecdysteroids and juvenoids are both the major regulators of moulting, growth, and reproduction in crustaceans. For instance, emamectin benzoate (EM), an insecticide used to control sea lice infestations in fishes, was reported as responsible for an abnormal moulting in arthropods (Waddy *et al.,* 2002). The fact that EM is a GABA-ergic pesticide, and that gamma-aminobutyric acid (GABA) can inhibit the secretion of several eyestalk hormones supports this hypothesis (Rodríguez *et al.,* 2007). Other example is the non-steroidal ecdysteroid agonist tebufenozide (TEB) that has potential for control of pest Lepidoptera larvae, the compound bind to ecdysteroid receptors and provide larvae with premature signal to start synthesizing cuticle before they are competent to moult (Trisyono and Michael Chippendale 1998). Other compound is the agricultural fungicide fenarimol (FEN) that has been shown to have anti-ecdysteroidal activity on *D. magna* (Mu and LeBlanc 2002; Rodríguez *et al.,* 2007). Pesticides that are JH analogues are other class of compounds that disrupt endocrine-regulated processes, such as methoprene (MT) and pyriproxyfen (PP) (Olmstead and LeBlanc 2003). Methoprene is manufactured as an insecticidal that target the juvenile hormone system of insects as a JH agonist. This compound has been used effectively to control mosquitoes and other aquatic insects (Olmstead and LeBlanc 2001). While PP is used in flea and tick control in veterinarian applications, and in fire ant bait, being increasingly recommended for agricultural uses such as the control of white fly on cotton and scale insects on fruit trees (Olmstead and LeBlanc 2003). The crustacean hormone MF is remarkably similar in structure to insect JH, therefore there is reason to suspect that MT and PP and/or its breakdown products can be active in crustaceans (deFur 2004). These compounds have the ability to stimulate male offspring production in daphnids and also inhibit moulting (Wang *et al.,* 2005).

### 1.1.5 Lipids and their relevance

Lipids are essential for energy homeostasis, reproductive and organ physiology, and numerous aspects of cellular biology (Lee *et al.,* 2003). They are central to structural, reserve and signalling processes and directly reflect ecological changes. Lipids play an important role in aquatic environments, the largest part of the biosphere. In aquatic systems, lipids provide the densest form of energy yielding at least two-thirds more energy per gram than proteins or carbohydrates. They are highly reduced compounds, thus, important fuels for oxidation. Lipid energy is transferred from algae to vertebrates via zooplankton and total lipid energy in kilojoules is a predictor of reproductive potential in fish stocks (Marshall *et al.,* 2000; Parrish 2013). The seasonal changes in fatty acids may produce changes in pollutant tissue dynamics, because the fatty acid chain length of dietary lipids greatly influences the solubility and hence systemic bioavailability of ingested lipophilic chemicals. Fatty acids and sterols are also susceptible to oxidative damage leading to cytotoxicity and a decrease in membrane fluidity. The physical characteristics of biological membranes can be defended from the influence of changing temperature, pressure, or lipid peroxidation by altering the fatty acid and sterol composition of the lipid bilayer. Lipid composition plays also a key role in pollutants distribution depending on their polarity. Pollutants alter storage and can disrupt lipid metabolism and homeostasis in somatic organs (Parrish 2013). They are also a solvent and absorption carrier for organic contaminants and thus can be drivers of pollutant bioaccumulation (Laender *et al.,* 2010).

In crustaceans like in other arthropods, lipid storage dynamics varies along the moulting and reproduction cycle that are regulated by the ecdysone and JH signalling pathways (Tessier and Goulden 1982). In *D. magna*, storage lipids have a very large dynamic range of variation and are stored inside special fat cells (similar to adypocites) mainly as TGcerols, being sensitive to food supply conditions (Zaffagnini and Zeni 1986). These special fat cells are distributed along the hemocoel and accumulate storage lipids in large lipid droplets that showed a high dynamic range of variation during an intermoult or reproduction cycle (Tessier and Goulden 1982; Zaffagnini and Zeni 1986). Triacylglycerols contribute to 60-80% of accumulated fatty acids, which are mainly acquired through the diet (Goulden and Place 1990). Adults of *D. magna* reared under high food ration conditions may increase TGs three to six fold during the intermoult cycle (Goulden and Place 1990).

*Lipid classification and biological function*

Lipids are a heterogeneous group of water-insoluble compounds, due to their hydrophobic characteristics. The International Lipid Classification and the Nomenclature Committee, together with the Lipids Metabolites and Pathways Strategy (LIPID MAPS) Consortium, defined eight categories of lipids (Figure 1.2), and divided them into classes and subclasses (Fahy *et al.,* 2011; Rolim *et al.,* 2015). Lipids were classified by their chemically functional backbones and biochemical principles in the categories and classes: Fatty acyls (FA) with the main classes fatty acids and conjugates, octadecanoids, eicosanoids, docosanoides and fatty alcohols; Glycerolipids (GLs) with the main classes monoradylglycerols, diradylglycerols and tiradylglycerols; Glycerophospholipids (GPs) with the main classes glycerophosphocholines, glycerophosphoglycerols, glycerophosphoethanolamines, glycerophosphoglycerophosphates, glycerophosphoserines, and glycerophosphoinositols; Sphingolipids (SP) with the main classes sphingoid bases, ceramides, phosphosphingolipids, neutral glycosphingolipids and acidic glycosphingolipids; Sterol lipids (ST) with the main class the sterols; Prenol lipids (PR) with the main classes isoprenoids; Saccharolipids (SL) with the main classes acrylaminosugars and Polyketides (PK) with the main class the linear polyketides.

The individual lipid classes 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). Lipids are not only energy depots and structure builders in the cell; they also play active roles in membrane functions, and can act as messenger molecules. Phospholipids for example have, among others, the function of forming the double layer that is the basic structure of cell membranes. The PUFA are constituents of a large variety of phospholipids, and provide several important properties such as the fluidity and the flexibility of cellular membranes (Harkewicz and Dennis 2011; Rolim *et al.,* 2015). Lipids define the permeability barrier of cells and organelles, are integral components of multi-subunit protein complexes, provide the solvent within which membrane proteins fold, support and influence membrane associated processes, provide precursors for macromolecular synthesis, and act as regulatory molecular signals (Dowhan and Bogdanov 2008; Dowhan 2009). Uncovering potential roles for lipids using solely biochemical approaches is limited by the lack of inherent catalytic activity so that many putative functions have been based on how a particular lipid affects a reconstituted

biological process (Dowhan 2009). In Table 1.1 there is a short description based on chemical structure and biological function of each class of lipids.

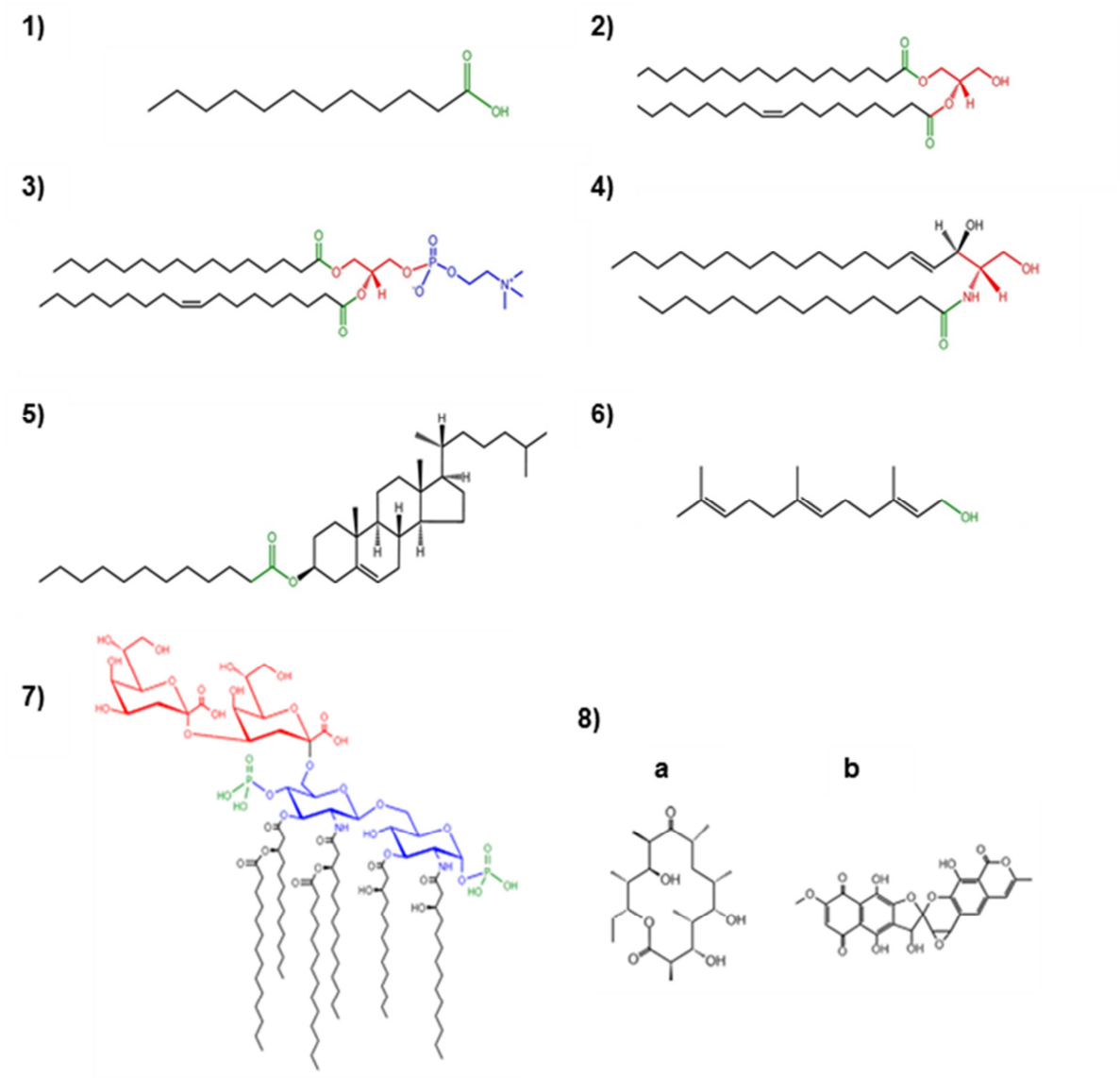


**Figure 1.2** – Representative structure of the eight categories of lipids as defined by the LIPIDMAPS Consortium: (1) Fatty acyls, (2) Glycerolipids, (3) Glycerophospholipids, (4) Sphingolipids, (5) Sterol lipids, (6) Prenol lipids, (7) Saccharolipids, (8) Polyketides. In the classes 1 to 6, the acyl or prenyl chains are oriented horizontally with the terminal functional group on the right (green) and unsubstituted "tail" on the left; 7 is an example of Kdo2-Lipid A structure, that in blue represent the glucosamine residues, in red Kdo residues, acyl chains in black and phosphate groups in green; 8a, macrolide polyketides structure: 6-deoxyerythronolide B and in 8b, the structure of aromatic polyketides: griseorhodin A (Fahy *et al.,* 2011; LIPIDMAPS 2015).

**Table 1.1**– The eight categories of lipids as defined by the LIPID MAPS Consortium. Information obtained from (Fahy *et al.,* 2011; LIPIDMAPS 2015; Rolim *et al.,* 2015).

| Categories | Chemical structure | Biological function |
|---|---|---|
| **Fatty acyls** | Diverse group of molecules synthesized by chain elongation of an acetyl-CoA primer with malonyl-CoA (or methylmalonyl-CoA), groups that may contain a cyclic functionality and/or are substituted with heteroatoms. | The fatty acids can undergo several chemical or metabolic reactions such as oxidation, scission, or polymerization.<br>E.g. Fatty acids and conjugates, Eicosanoids. |
| **Glycerolipids** | They are composed mainly of mono-, di- and tri-substituted glycerols, the most well-known being the fatty acid esters of glycerol (TGs), also known as triglycerides. | Energy storage.<br>E.g. Diacylglycerol (DG) and TG. |
| **Glycero-phospholipids** | Phosphorylated glycerolipids, sometimes called phosphatides but more generally phospholipids, form a diversified group in relation to their molecular structure. | They referred to as phospholipids, are key components of the lipid bilayers, as well as being involved in metabolism and signalling.<br>E.g. Phosphatidylcholines and Phosphatidylethanolamines (PEs). |
| **Sphingolipids** | Complex family of compounds that contain a sphingoid base backbone and a long-chain fatty acyl-CoA, then converted into ceramides, phosphosphingolipids, glycosphingolipids, and other species, including protein adducts. | They have been implicated in numerous intra and extracellular signalling processes as both signalling molecules and secondary messengers.<br>E.g. Sphingomyelins (SMs). |
| **Sterol lipids** | Class with a unique fused ring structure that distinguishes them from classes of cyclic triterpenes, and have a different fused ring stereochemistry and methylation patterns as a consequence of alternative folding of the linear precursor during biosynthesis. | They are important components of membrane lipids along with the GPs and SMs. These lipids mainly act as hormones and signalling molecules.<br>E.g. Cholesterol and derivates. |
| **Prenol lipids** | They are synthesized from the 5-carbon precursor's isopentenyl diphosphate and dimethylallyl diphosphate that are produced mainly via the mevalonic acid (MVA) pathway. | Some biologically important prenol lipids: carotenoids are important simple isoprenoids that function as antioxidants and as precursors of vitamin A, vitamin E and vitamin K. |

| | | |
|---|---|---|
| **Saccharolipids** | Compounds in which fatty acids are linked directly to a sugar backbone, forming structures that are compatible with membrane bilayers. | The most familiar saccharolipids are the acylated glucosamine precursors of the Lipid A component of the lipopolysaccharides in Gram-negative bacteria. |
| **Polyketides** | Many polyketides are cyclic molecules whose backbones are often further modified by glycosylation, methylation, hydroxylation, oxidation, and/or other processes. | They comprise a large number of secondary metabolites and natural products from animal, plant, bacterial, fungal and marine sources, and have great structural diversity. |

### 1.1.6 Lipidomics

Lipidomics is an emerging field of research, which includes complex lipidome analysis. In short, the lipidome is the comprehensive and quantitative description of the lipid species present in an organism. Lipidomics is the systems-level analysis of lipids and factors that interact with lipids. It Involves quantitative experimental and theoretical studies of lipid and membrane self-assembly, lipid-protein interactions, lipid-gene interactions, and the biophysical properties of lipid structure and dynamics, among others. A large number of experiments using sub-cellular, cellular and biochemical approaches in model organisms have advanced our knowledge of lipid biosynthesis, structure and function. Many of these studies included direct measurement of lipid molecules (Wenk 2005,2010; Rolim *et al.,* 2015). Lipidomic procedure consists of a sequence of steps including sample preparation, metabolite extraction, derivatization, metabolite separation, detection, and data treatment. The selection of the steps depends on the type of study (untargeted vs. targeted), kind of sample (e.g. solids vs. liquids), instrumentation to be used for separation (e.g. Gas chromatography vs. Liquid chromatography) and detection method (e.g. Mass spectrometry vs. Nuclear magnetic resonance).

The ultra-performance liquid chromatography (UPLC) is a technology that takes advantage of liquid chromatography principles, separating lipids using columns packed with smaller particles and/or higher flow rates for increased speed, with superior resolution and sensitivity (de Villiers *et al.,* 2006). The UPLC have been used in lipid research for a long time and conditions have been well adapted for the analysis of various classes of lipid compounds, such as sterols, TGs, diacylglycerols and fatty acids (Wenk 2005). This technique is suitable for fingerprinting analysis, considering its speed, robustness, and high sample throughput. The last important topic, is the detection methods, a coupled UPLC with time-of-flight (TOF) mass spectrometry is one option, due to its high-

throughput and comprehensive analysis with minimal sample preparation (Pongsuwan *et al.,* 2008; Wenk 2010).

### 1.1.7 Mixture toxicity

In natural environments, organisms are frequently exposed to mixtures of pollutants (Sharom 1997) and is relatively uncommon to find sites polluted with only one substance (Walker 2001). Some chemicals are highly persistent, some are applied repeatedly or continuously, and others are applied as mixtures to increase efficiency or reduce costs (Marking 1977). Due to the large number of different chemical compounds present in environmental matrices, the individual testing of each component is not fully representative of the total mixture effect. Thus, robust predictive toxicity models are needed, in order to estimate their toxicity with acceptable accuracy (Vighi *et al.,* 2003; Lydy and Austin 2004).

In order to address the effect of a toxic mixture, the observed combined toxic effect can be compared with an expected combined effect calculated from the single component toxicity, using specific reference models. Specifically in environmental toxicology, there are two well established conceptual non-interactive models, termed independent action (IA) and concentration addition (CA), derived from pharmacological sciences. These two models describe general relations between the effects of single substances and their corresponding mixtures, for dissimilar and similar acting chemicals (Barata *et al.,* 2007).

The IA model is a statement about the relationships between the probability of the response, whereas the CA model refers to relative toxicities (De March 1987). In other words, IA addresses the question whether the probability of response of one chemical may be independent from the probability of response of another, while CA addresses the question if the relative toxicity of the mixture is the same as the sum of the toxicities of the individual compounds. In aquatic toxicology the IA and CA have been successfully used to predict non interacting joint effects of mixtures of dissimilar and similar acting compounds (Altenburger *et al.,* 2000; Backhaus *et al.,* 2000; Faust *et al.,* 2000; Scholze *et al.,* 2001; Faust *et al.,* 2003; Barata *et al.,* 2007).

To accurately predict joint effects of mixtures, it is crucial to estimate precisely the predicted values for individual and mixture combinations (Scholze *et al.,* 2001). Predicted values for the studied individual components can be estimated using several approaches and models. One of them is to use the best fit model (Scholze *et al.,* 2001). One of the most used models is the non-linear Hill equation, which according to the rate theory

(Eyring 1935) can be used to describe the inhibition of a biological process as a function of toxicant concentration using a sigmoid type function. When considering mortality values that range between 0 and 100 the Hill model become that of equation 1.1.

$$E(\%inh) = \frac{100}{1+(EC_{50}/x)^{p}} \qquad \text{eq. 1.1}$$

E=effect in %; p=slope; EC=effect concentration; x=concentration (µM)

On the basis of the concentration–response functions of individual compounds, predictions of concentration addition were calculated for mixture containing binary combinations in a definite ratio (based on $EC_{50}$ or $EC_{10}$). A total concentration of the mixture, at which a certain effect is generated, can be calculated using concentration addition according to equation 1.2.

$$ECx_{mix} = \left( \sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \qquad \text{eq. 1.2}$$

In this equation $ECx_{mix}$ is the total concentration of the mixture provoking x% effect; $ECx_i$ is the concentration of component (i) provoking the x% effect, when applied singly; and $p_i$ denotes the fraction of component (i) in the mixture. The calculation of total mixture concentrations for various effect levels lead to a complete iteration of an expected concentration–effect relationship.

The prediction concept IA allows explicit calculation of combined effects according to equation 1.3.

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \qquad \text{eq. 1.3}$$

The effect at the total concentration of the mixture, $E(c_{mix})$, is based on the effects of the components which they generate at concentration (x) at which they are present in the mixture ($E(c_i)$). If the latter is expressed as a fraction ($p_i$) of the total mixture concentration, it holds equation 1.4.

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(p_i c_{mix})) \qquad \text{eq. 1.4}$$

This allows the calculation of an effect expected according to the concept of IA for any concentration of the mixture. These models can be used for understanding the mode of toxic action of obesogens in *D. magna* and identifying the agonists and antagonists of RXR, EcR and MfR receptors.

## 1.2 Multixenobiotic resistance system

Some decades ago oncologists started realizing that many tumours presented an inherent resistance pattern, while other tumours were able to develop resistance throughout the time of treatments, against many of the chemotherapeutic drugs. This phenotype was described as being a multidrug-resistance pattern. After these findings, scientists from over the globe have established that this protection mechanism was due to the presence in the plasmatic membrane of transporter proteins that actively expel various cytotoxic agents from cancer cells, thus allowing the tumours to have an overtime resistance to chemotherapeutic treatments. This mechanism of resistance was named multidrug resistance (MDR) mechanism (Dano 1973; Sarkadi *et al.,* 2006). In the following decades, researchers have been trying to clarify the mechanism behind this resistance and nowadays the role of these transporters is well defined both pharmacologically and in cell biology literature. The MDR is conspicuous to all cells and it was first described in cancer cells that by having overexpressed levels of specific transporters became resistant to treatment with cytostatic drugs.

The MDR system includes many transporters from the ABC (ATP binding cassette) superfamily, being the P-glycoprotein (P-gp or ABCB) and the multidrug resistance associated proteins (MRP or ABCC) the ones having greater environmental relevance (Leslie *et al.,* 2005). In many marine and freshwater organisms represents a general

biological defence mechanism for their protection against both endogenous and environmental toxicants. Similar to the MDR mechanisms in mammalian tumour cell lines, multixenobiotic resistance (MXR) in aquatic organisms is mediated by membrane-bound efflux transporter proteins belonging to the protein superfamily of ABC transporters. These proteins are present in almost all living organisms, from prokaryotes to mammals which suggests they are of ancestral origins (Sarkadi *et al.,* 2006). For example, *in silico* studies of several genomes, have identified many putative transporters, including 48 ABC transporters in the human genome, 41 in *Danio rerio* (Dean and Annilo 2005), 64 in *D. pulex* (Sturm *et al.,* 2009) and 65 in the sea urchin *Strongylocentrotus purpuratus* (Sodergren *et al.,* 2006).

### 1.2.1 ATP binding cassette transporters

In mammals, the ABC superfamily is subdivided into seven sub-families designated ABCA to ABCG. Toxicologically relevant transporters that confer MDR or MXR belong to the ABCB (P-gp), ABCC (MRP 1 - 5) and members of the ABCG family (breast cancer resistance protein; BCRP). The activity of these transporters provides a “first line of defence” against xenobiotics that are immediately effluxed out before entering the cell (phase 0 of cellular detoxification) or compounds that are effluxed from the cell after metabolizing inside the cell (phase 3) (Leslie *et al.,* 2005).

P-glycoprotein and MRP transporters are already described as active in several aquatic organisms including marine mussels (Luedeking and Koehler 2004; Luckenbach and Epel 2005), freshwater mussels (Faria *et al.,* 2011), sea urchins (Kurelec 1992), fish (Bard 2000) and *D. magna* (Campos *et al.,* 2014). Transcriptomic analyses of those transporters show high homology between them and humans (Sturm *et al.,* 2009).

In environmental sciences the majority of the research efforts have been focused in xenobiotic transformation and elimination. These transporters constitute the first and last line of defence against contaminants. In the first line are ABCB (P-gp) transporters and some ABCC (MRP) transporters that efflux out chemicals when enter to the cell. While in the last line of defence, are also known that ABCC transporters efflux out xenobiotic metabolites and conjugates (Epel *et al.,* 2008).

*ABCB family*

In aquatic organisms, the first report of P-gp (MDR1 like) mediated toxicant resistance was in the freshwater mussel *Anodonta cygnea* (Kurelec and Pivčević 1989).

Since then, it has been identified in up to 35 aquatic organisms such as, marine and freshwater mussels, clams, crabs, fishes, worms, sea urchins among others (Bard 2000; Smital *et al.,* 2003; Faria *et al.,* 2011; Campos *et al.,* 2014). P-glycoprotein is a 170 kDa size transporter protein that recognizes an extraordinary wide spectrum of chemicals which is also characteristic of other MXR transporters. The subfamily contains both full and half transporters (Dean and Allikmets 2001; Sturm *et al.,* 2009). P-glycoprotein is remarkably non-specific with respect to its substrates and this lack of specificity is of high adaptive importance generating protection against many novel anthropogenic products.

Contrary to other ABC transporters, P-gp does not efflux metabolites, but the parent compound alone is the transporter substrate. P-glycoprotein localization within the membrane suggests its action on the substrate shortly after it enters the cytoplasm. P-glycoprotein has high affinity to moderated hydrophobic, amphipathic (somewhat soluble in both water and lipids) chemicals of low molecular weight, cationic or neutral, planar with basic nitrogen atoms, and natural products (Gottesman and Pastan 1988; Endicott and Ling 1989; Gottesman *et al.,* 1994). In humans, P-gp proteins and RNA are detected in high levels in liver, kidney, small bowel, colon, pancreas, adrenal cortex, placenta, blood brain barrier tissues (Fojo *et al.,* 1987; Sugawara *et al.,* 1988; Cordon-Cardo *et al.,* 1990; Sugawara 1990). In aquatic invertebrates it has been identified in tissues involved in absorption, secretion and barrier function, such as gills, anterior digestive tract and epidermal tissue of worms, cell membranes of sponges and others (Bard 2000).

*ABCC family*

Multidrug resistance-associated proteins belong to the C family of ABC transporters. ABCC subfamily consists of a diverse group of 190 kDa glycoproteins (Bard 2000; Sturm *et al.,* 2009). The MRP have a very broad substrate specificity, which includes many organic anion conjugates of structurally different compounds such as endogenous substrates that are natural products of the metabolism and xenobiotic metabolites resulting from phase I and II of the biotransformation metabolism. The transport of several of these conjugates has been shown to depend on the presence of reduced glutathione (GSH) (Cole and Deeley 2006). Other functional properties also include iron transport, cell surface receptors and toxin secretion activities. Additionally, some members of the MRP family also contribute to the efflux of unmodified hydrophobic compounds, thus acting in a similar way as P-gp.

### 1.2.2 Chemosensitization

Chemosensitization is the term used to describe the chemical inhibition of multidrug transmembrane transporters that allow cells to efflux out toxic chemicals or/and metabolites. A consequence of chemosensitization is the increased accumulation of xenobiotics and metabolites inside the cell, and therefore enhanced toxic effects. Chemosensitizers can be chemicals that are not necessarily toxic themselves, but are also substrates of the transport system and thus compete with other more toxic xenobiotics saturating the transport system and compromising the MXR defence (Figure 1.3) (Kurelec *et al.,* 2000). Chemosensitizers tend to be more lipophilic than other substrates (Sharom 1997). Xenobiotics that are substrates of the MXR transporters are found experimentally to diffuse very slowly across membranes on the order of minutes to hours when compared to chemosensitizers, which transverse membranes much faster (Eytan and Kuchel 1999). Other chemosensitizers may not be substrates to the transporters pumps, but nevertheless inhibit their activity through some indirect effects (e.g. inhibiting ATP) (Epel *et al.,* 2008).

There is increasing evidence of anthropogenic contaminants acting as chemosensitizers by inhibiting the MXR system that reduce the resistance of aquatic organisms to toxicants. Chemosensitization by environmental contaminants could be a problem also for species that are highly resistant to chemicals due to high efflux transporter activity. A low number of environmentally relevant compounds have been found to act as chemosensitizers, including pesticides, perfluorochemicals, fragrances and polyaromatic hydrocarbons (Minier *et al.,* 1993; Luedeking and Koehler 2004). However, the chemical diversity of these compounds indicates that chemosensitization could in fact be a common effect elicited by environmental chemicals. Research on environmental aspects of chemicals acting as chemosensitizers is still in an early stage, however the available literature indicates that the effect could be important and is underappreciated.

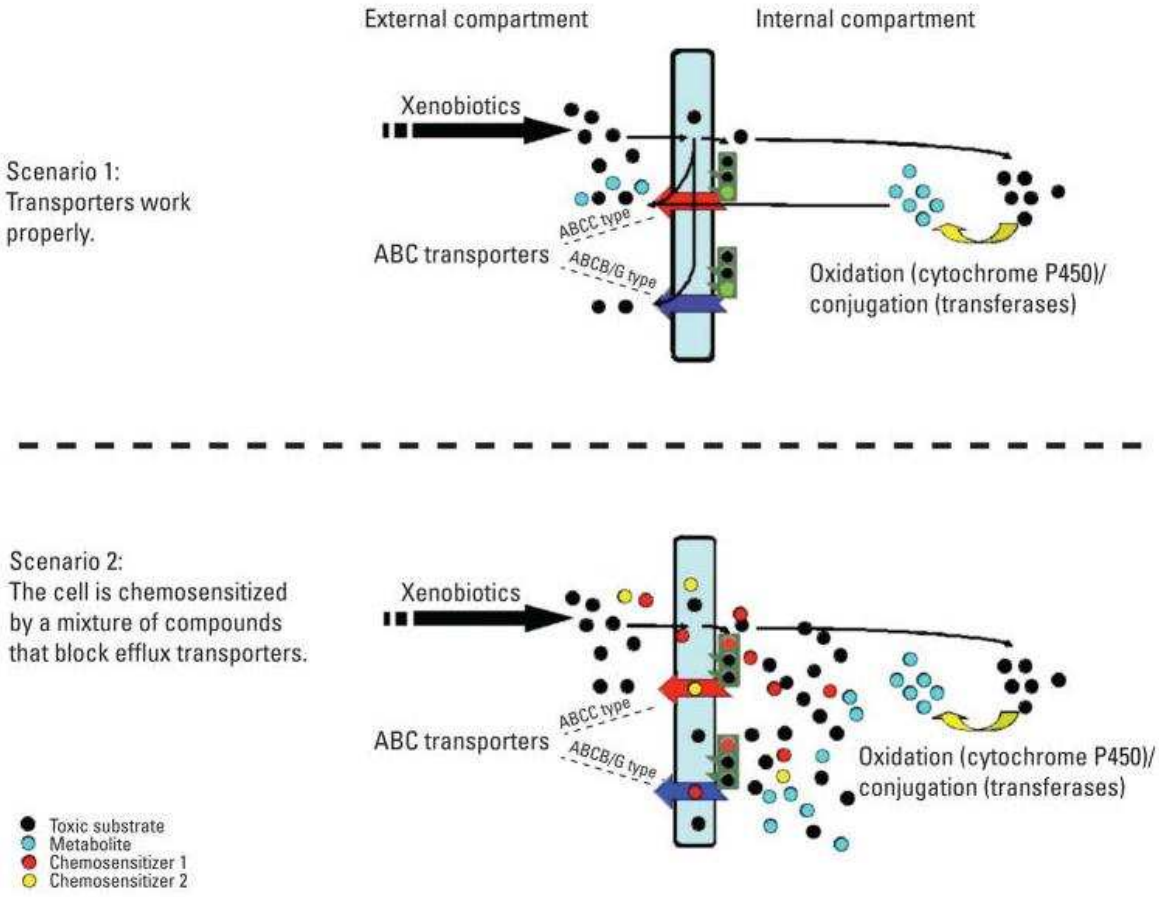


**Figure 1.3** – Scheme of two scenarios of transporter function in a cell. Scenario 1: all transporters work properly, few toxicants remain inside the cell, and those that do are modified by oxidation and conjugation and then extruded; Scenario 2: the cell is exposed to a mixture of chemicals, including some which may act as chemosensitizers. Their inhibition of the transporter activity allows chemical concentration in the cytoplasm to increase, causing toxicity (Epel *et al.,* 2008).

### 1.2.3 MXR in *Daphnia magna*

Studies of the cellular mechanisms of tolerance of organisms to pollution are a key issue in aquatic environmental risk assessment. The MDR mechanism based on the activity of ABC transporters represents an essential cellular defense of marine and freshwater organisms against environmental toxicants. Campos and collegues provided data showing for the first time, evidence on molecular, functional and toxicological levels for an ABC transporter-based toxic defense system in the crustacean *D. magna* (Campos *et al.,* 2014). Using reported genomic sequences of *D. pulex* (Sturm *et al.,* 2009) they

cloned partial complementary DNAs (cDNAs) of an *ABCB*1 ortholog (P-gp) and of MRP gene orthologs *ABCC1-3* like, *ABCC4* and *ABCC5* and found constitutive expression of the respective transcripts in *D. magna* eggs, embryos, neonates and juveniles using quantitative real time polymerase chain reaction (qPCR). Furthermore, they caracterized the efflux activity of MXR transporters in the organisms using fluorescent dyes that are known to be specific substrates of P-gp and MRP pumps (Faria *et al.,* 2011; Campos *et al.,* 2014). However, in this study, the model ABC transporter substrates, inducers and inhibitors did not target *D. magna* transporters with sufficient specificity to distinguish between activities of different ABC efflux transporter types. There was, thus, unclear evidence of the toxicological role of ABCB versus ABCC type transporters. So, more studies comparing MXR responses across tolerant and intolerant individuals to known MXR toxic substrates may offer the possibility to study the functional role of different ABC transporter types.

### 1.3 Integrated test approaches

Test strategies focused on responses across different biological organization levels, from genes to populations and/or multigenerational tests allow us to unravel new potential modes of action of emergent contaminants (Brennan *et al.,* 2006; Ankley *et al.,* 2010). The integrated test approaches, give us the possibility to identifying key-events and effects and then, use this information to design novel tools for environment risk assessment (Ankley *et al.,* 2010).

We will use, simultaneously, multigenerational tests and genomic tools in a context of laboratory induced toxic effects on fitness, physiology and morphology to shed light on the mechanisms operating in laboratory and wild populations potentially affected by similar pollutants. In case of, multigenerational tests is useful for determine the magnification of the toxicity compounds in the subsequent generations, such as increased mortality and decreased fecundity, that otherwise would not be observed (Brennan *et al.,* 2006). Moreover, the use of genomic tools, permit the identification of genes involved in responses to environmental stressors (molecular initiating event) and how they are regulated thus increasing the value of these findings in a *weight-of-evidence* and *read-across* perspective. These approaches, stencilled from Adverse Outcome Pathways framework can have an immediate impact on regulatory decisions (Ankley *et al.,* 2010).

The biological impact of pollutants on ecosystems is the result of a cascade of events that begins with their introduction into the environment. Environmental pollution

affects different biological processes within the exposed organisms causing adverse effects in target tissues, whole organism and eventually in entire ecosystems (Piña *et al.,* 2007). When toxic pollutants enter an organism they trigger physiological responses aimed to maintain homeostasis. The first response is at gene level and then transferred to higher organizational levels until a final, ecosystem level (Van der Oost *et al.,* 2003). A major mechanism underlying almost all the responses to environmental change is mediated by a shift in the transcription of genes.

In 2004 the term "*Ecotoxicogenomics"* was introduced and defined as the integration of genomic based science into ecotoxicology studies (Snape *et al.,* 2004). The first obvious advantage of using genomic information was the increase of the information that we could extract from an experiment, thus allowing for better and more accurate conclusions. This would also allow us to link molecular biomarkers with higher level population and ecosystem responses, and that way anticipate potential risks. This new branch is now developing into a key tool for the assessment of environmental impacts and environmental risk assessment, because of its potential to highlight toxicant specific gene expression patterns (fingerprints) that can be used to identify changes induced by chemicals. Genomic technologies are also having a decisive contribution to the development of new biomarkers, and the determination of new and more accurate mode of action studies (Pina and Barata 2011).

## 1.4 *Daphnia magna* as a model organism

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

The taxonomy classification of *Daphnia magna Strauss*, accordingly to the World register of marine species is as follows:

- Kingdom: Animalia
- Phylum: Arthropoda
- Subphylum: Crustacea
- Class: Branchiopoda
- Subclass: Phyllopoda
- Order: Diplostraca
- Family: Daphniidae
- Genus: *Daphnia* O.F. Muller, 1785
- Species: *Daphnia Magna* Straus, 1820

*Daphnia magna* is a brackish and freshwater organism. Anatomy and morphology are shown in Figure 1.4. Like all crustaceans, it possesses an exoskeleton, consisting of a dimerous chitinous carapace, with multiple tissue layers, surrounding the thorax and abdomen. The carapace is non-elastic, as a consequence of which growth is possible only by regular renewals of it, a phenomenon known as moulting (Ebert and Jacobs 1991). *Daphnia 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 the second antenna, *Daphnia* 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 naupilus eye and the gut opening. Directly in front of the gut opening, there is a pair of maxillae, rigid, which are able to triturate, to a certain degree, the food particles. The gut has an upward orientation in this section, making a U in its higher point, and then directing towards the posterior section of the body. In the head

region, connected to the gut, there is a pair of *caeca*, which are homologous to a hepato-pancreas (Ebert and Jacobs 1991). In the thoracic region are present the filter setae, used as filters, retaining the food particles and directing them to the maxillary glands, epipodites (long regarded as gills, but actually are sites of haemoglobin production), heart and 2 ovaries, flanking the gut; and the brood pouch, an opening on the back of the animals where the embryos will be deposit and develop till birth. The post-abdomen is bent ventrally (as the entire animal) and forward, finishing in a spine. It possesses a claw and spines, which uses to clean the thoracic appendages and carapace, and to keep the eggs in the brood pouch (Ebert and Jacobs 1991; Goldmann *et al.,* 1999).

*Daphnia magna* has a Holarctic distribution, living in small or medium size shallow freshwater ponds with low fish predation pressure (De Gelas and De Meester 2005). It represents a large part of the zoo-planktonic community, being a keystone species in the freshwater ecosystems. It is one of the most important primary consumers, being fundamental to control algae blooms, while is also important as a food source for both invertebrates and vertebrates predators. The presence of *Daphnia* has considerable implications for the maintenance of the ecological quality status of aquatic systems (Hebert 1978). *Daphnia* species are non-selective filter feeders, using their thoracic appendages to create water current through the interior of the carapace using their fine *setae* to retain particles, which are then transported to the *maxillae*. *Daphnia sp.* mainly feed on algae, but will retain all the suspended particles that can be withheld by their filtering apparatus (>1 µm), ingesting any suspended particles (Gillis *et al.,* 2005).

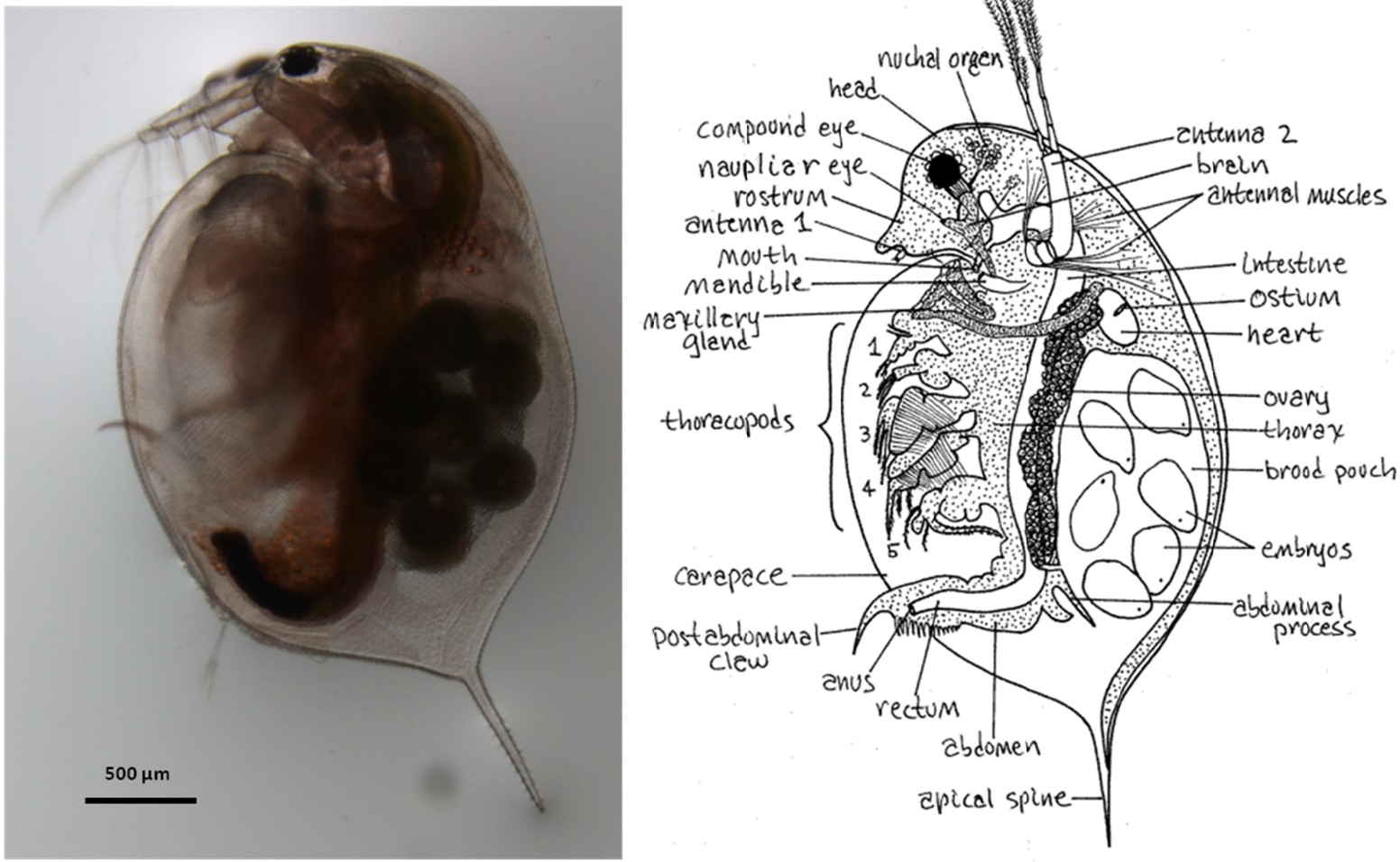


**Figure 1.1** – The functional anatomy of *Daphnia* (Adapted from Richard Fox, Lander University, http://www.ncbi.nlm.nih.gov/books/NBK2042/).

*Daphnia magna* reproduces through cyclical parthenogenesis (Zaffagnini 1987). During the parthenogenic phase, the population is composed exclusively by females, which produce diploid eggs in the ovary (Figure 1.5). Oogenesis is not fully meiotic or strictly mitotic resolving in diploid eggs identical to the mother (Ebert 1994). Even though *Daphnia* mainly reproduces through parthenogenesis, certain environmental triggers like food limitation, high population densities, short photoperiod or desiccation of the habitat can activate sexual reproduction. The males are formed during a special parthenogenic event, and therefore they are genetically identical to the mothers. They are identical to the females in aspect, but are of smaller size, and have the first antenna much more developed. Subsequently, females produce haploid eggs (two per brood) that are deposited in the brood pouch, which are then fertilized by male haploid spermatozoa and then undergo a further development to resting eggs, also called ephippia. These resting eggs are encapsulated by multiple layers, formed by a transformation of the brood pouch in order to protect the eggs inside against adverse environmental conditions. They are able to resist in dormant status for up to hundred years (Jeppesen *et al.,* 2001). When the conditions become favourable, the ephippia develop to parthenogenically reproducing females, and a new population is established.

*Daphnia magna* has been studied for almost three centuries in the fields of taxonomy, physiology and limnology, which has provided useful information for all the modern research. *Daphnia* can be found in freshwater ecosystems all around the world, playing a key ecological role in the food web, resulting in the recognition of the ecological relevance of testing. The lifecycle is ideally suited for experiments, due to its short reproduction time, when compared to most eukaryotic model species: the reproductive maturity is reached within six to ten days and a reproductive batch occur every three days hereafter (Ebert 1992). Through asexual parthenogenic reproduction, it is possible to produce genetically constant clonal lines, hereby reducing genetic variability, which can be a factor of interest in many experiments. On the other hand, different clones can be maintained to create experimental populations with controlled genetic variation. *Daphnia magna* is the most frequently used invertebrate in standard acute and chronic aquatic toxicity testing. Bioassays with *D. magna* are formally endorsed by major international institutions such as OECD and International Organization for Standardization (ISO). Results from these assays have been used in environmental risk assessment screenings of almost all tested substances.

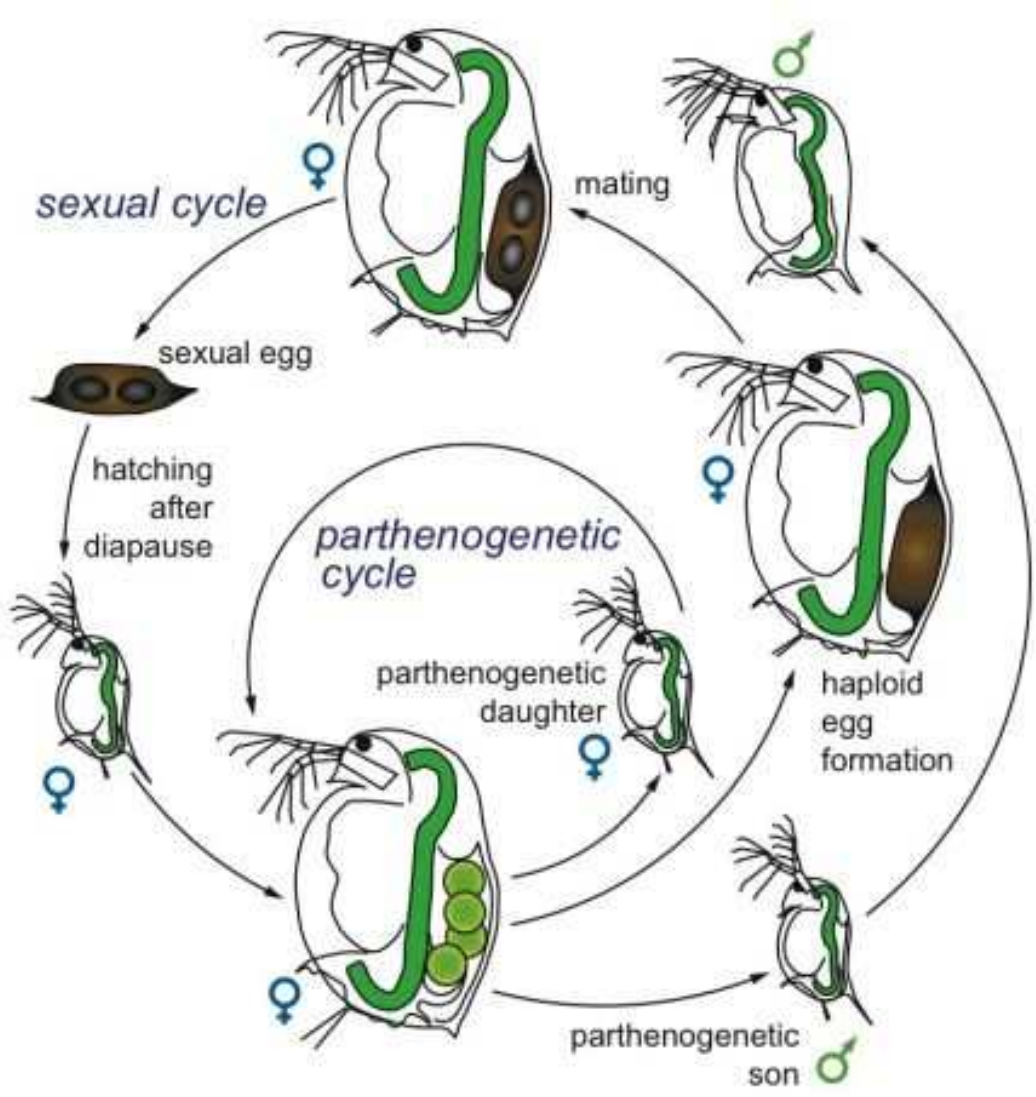


**Figure 1.5** – Diagram of sexual and asexual (parthenogenic) life cycle of a *Daphnia* (Adapted from Ebert 2005, http://www.ncbi.nlm.nih.gov/books/NBK2042/).

### 1.4.1 Life cycle and laboratory conditions

In laboratory, optimal conditions for the *Daphnia* development can be maintained continuously, allowing the elimination of sexual reproduction events and creating the possibility of keeping clones indefinitely. This give to researchers a very important tool that is the possibility of studying an organism that is a perfect genetic clone, with all its implications at the level of reproducibility of experiments and constancy of results between

tests and a good model for studying genomic variations without the problem of normal variation in species with sexual reproduction events.

The life cycle of *D. magna* depends on environmental conditions (Hebert 1978). Under constant, non-stressed conditions, which are pursued in laboratorial tests, it can be summarized as follows. At temperature of 20 °C, a daily photoperiod of approximately 12 h and abundant food, eggs develop in the brood pouch in 3 days. Neonates are released as free-swimming miniature adults. After a juvenile period of 7 to 10 days, in which the animal moults 5 or 6 times, the first brood is born. Succeeding broods are then released every 3 days approximately, which is followed by a moult shortly thereafter. Body size can only increase before the new carapace is hardened, which results in 'saltatory' growth. In a single female, three broods are developing simultaneously. One clutch of eggs develops in the brood pouch, while the second clutch is provisioned in the ovaries. At the same time a third clutch differentiates from nurse cells to oocytes (Zaffagnini 1987). Growth and reproduction can continue until senescence (Enserink 1995).

### 1.4.2 Lipid dynamics in *Daphnia*

Lipids vary during the cladocerans moult and reproductive cycle. 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 reproductive period these lipids are then 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. In cladocerans, storage lipids are primarily TG located in scattered spherical droplets throughout the animal's hemocoel (Sterner *et al.,* 1992). In daphnids, the contains of lipids are between 16 to 41 % of their dry mass (Goulden and Place 1990).

### 1.4.3 Feeding in *Daphnia*

*Daphnia*, like many other cladoceran species, can be characterized as particle filter feeders. The feeding apparatus of *Daphnia* consists of thoracic appendages, the third and fourth pair of appendages carries large filter-like screens which filter the particles from the water. Together with the carapace they pump water, containing food particles, from head to tail through the gape of the carapace. Particles retained by the screens are transported to the ventral food groove and moved towards the mouth. Here, the food is

grinded by the mandibles before egestion takes place (Lampert 1987). *Daphnia* is a relatively passive filter feeder. However, unwanted food, for instance filamentous algae or excess food, can be removed from the food groove by the post-abdominal claws or rejected by the mouthparts (Richman and Dodson 1983). The maximum size of particles entering the carapace is limited by the distance between the edges of the carapace valves. The size of the gape is narrowed in dense algal suspensions, thereby preventing obstruction of the filtering apparatus. This mechanism may increase survival during summer algal blooms (Enserink 1995).

### 1.5 Objectives and thesis outline

The crustacean *D. magna* have been used effectively in ecological and evolution to studying interactions between abiotic and biotic factors and screening the toxicity of chemicals for many years. This characteristic, together with the recent full sequence of its genome, gives the possibility to study and relate molecular mechanisms of toxicity with apical effects occurring at individual and population levels. Herewith, the main goal of this thesis is to use multilevel studies in order to assess endocrine disrupting effects on the lipid metabolism of classical and emerging compounds (chapters 2, and 3), to study reproductive toxic effects that propagate from parents to their progeny causing multigenerational effects (chapter 4) and to further characterize the MXR system in chapter 5. Disruption of lipid metabolism and of the MXR system by chemical contaminants are well known in vertebrates but has seldom be addressed in invertebrates. This means that the new knowledge generated in this thesis may help to “read-across” chemical toxicity using whole animal assays different than vertebrate model species.

This thesis is divided into six chapters, that the first one was an overview of ecotoxicology field, focusing in effects of classical and emerging pollutants in endocrine system and MXR system in invertebrates; and the importance of multilevel studies for evaluation of ecotoxicological effects at different levels, from genes to populations, facilitating the study of mechanisms of action and environmental risk assessment. The other chapters will be described below with the respective objectives:

**Chapter II – Obesogenic effects of TBT in *Daphnia magna***

- Assessment of disruptive effects of the model obesogen TBT on the lipid homeostasis in *Daphnia magna*, along the moulting and reproductive cycle, its genetic control, and health consequences of its disruption.

**Chapter III – Compounds altering fat storage in *Daphnia magna***

- Screening chemicals that act as obesogens, and alter lipid fingerprints in exposed organisms using single and binary exposures of chemicals and known agonists and antagonists of RXR, EcR and MfR receptor signalling pathways.

**Chapter IV – Two-generational effects in *Daphnia magna***

- Assessment of multigenerational effects of putative endocrine disruptors (NP, TBT and piperonyl butoxide) in *Daphnia magna*. Based on evidence that NP and TBT decrease the size of offspring and have detrimental multigenerational effects and piperonyl butoxide is a known cytochrome P450 (CYP) inhibitor and hence can affect the metabolism of ecdysteroids, which are necessary for embryo development in *Daphnia*.

**Chapter V – Multixenobiotic resistance mechanism in *Daphnia magna***

- Characterization of MXR system in tolerant *Daphnia magna* clones selected against an array of chemicals that are known to be substrates of MXR and/or to induce transporter activity;
- Evaluate the response of heat shock proteins in the above selected tolerant clones to distinguish between substrate specific MXR responses from general stress ones.

**Chapter VI – Final remarks**

- General discussion and final conclusions.

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# Chapter II

Obesogenic effects of TBT in *Daphnia magna*

## Chapter II – Obesogenic effects of TBT in *Daphnia magna*

## 2 Obesogens beyond Vertebrates: Lipid Perturbation by Tributyltin in the Crustacean *Daphnia magna* [1]

### 2.1 Abstract

Background: The analysis of obesogenic effects in invertebrates is limited by our poor knowledge of the lipid metabolism's regulatory pathways. Recent data from the crustacean *Daphnia magna* points to three signalling hormonal pathways related to the moulting and reproductive cycles (RXR, JH and ecdysone) as putative targets for exogenous obesogens.

Objectives: The present study addresses the disruptive effects of the model obesogen tributyltin (TBT) on the lipid homeostasis in *Daphnia* along the moulting and reproductive cycle, its genetic control, and health consequences of its disruption.

Methods: *Daphnia magna* individuals were exposed to low and high levels of TBT. Reproductive effects were assessed by life history analysis methods. Quantitative and qualitative changes in lipid droplets during moulting and reproductive cycle were studied using Nile red staining. Lipid composition and dynamics were analysed by UPLC-TOF mass spectrometer. Relative abundances of mRNA from different genes related to RXR, JH and ecdysone signalling pathways were studied by qPCR.

Results and Conclusions: TBT disrupted the dynamics of neutral lipids impairing the transfer of triacylglycerols (TGs) to eggs and hence promoting their accumulation in adult individuals. TBT disruptive effects translated into a lower fitness for offspring and adults. Co-regulation of gene transcripts suggests that TBT activates the RXR, JH and ecdysone receptor signalling pathways, presumably through the already proposed interaction with RXR. This is the first clear evidence of obesogenic effects in a non-vertebrate species.

[1] **Jordão R.**, Casas J., Fabrias G., Campos B., Piña B., Lemos M. F. L., Soares A. M. V. M., Tauler R. and Barata C., (2015). "Obesogens beyond Vertebrates: Lipid Perturbation by Tributyltin in the Crustacean *Daphnia magna*." Environmental Health Perspectives 123(8): 813-819. DOI: 10.1289/ehp.1409163 (article in annex).

## 2.2 Introduction

In mammals, improper control of lipid homeostasis can result in serious health problems, such as obesity, increased risk of coronary artery diseases, diabetes, and related detrimental effects, such as hypertension and lipidemia (Grün and Blumberg 2006; Sharma and Staels 2007). The nuclear receptor PPARγ, together with its heterodimeric partner RXR, are master regulators of adipocyte differentiation, being involved in the regulation of food intake, metabolic efficiency, and energy storage (Santos *et al.,* 2012). Organotins such as TBT are high-affinity ligands of both RXRs and PPARγ (Santos *et al.,* 2012). Organotins stimulate cell differentiation and the expression of adipocyte marker genes, elevate lipid accumulation in several tissues of mice, and increase adipocytes in zebra fish juveniles (Santos *et al.,* 2012). Although PPAR has not been described outside deuterostomes, RXR is ubiquitous within metazoans. Thus, the taxonomic scope for organotin-mediated lipid homeostasis disruption may be wider than initially anticipated.

Recently, Wang and colleagues showed that in the cladoceran crustacean species *Daphnia magna*, RXR is activated by TBT and elicits toxicity by interfering with the ecdysone signalling pathway (Wang and LeBlanc 2009; Wang *et al.,* 2011). Nevertheless, there are no reported results on whether or not lipid profiles are impacted by TBT. In *D. magna* moulting, growth and reproductive functions modulate the quantity and fate of storage lipids, mainly TGs located in spherical lipid droplets inside fat cells scattered throughout the animal hemocoel (Tessier and Goulden 1982; Zaffagnini and Zeni 1986). Lipid droplets and/or TG levels increase during the intermoult cycle and are reduced after being allocated to the moult in juvenile stages or to the moult and egg formation in adult stages (Tessier and Goulden 1982). In adult *D. magna* reared under high-food-ration conditions, TGs may increase from 3- to 6-fold during the intermoult cycle (Goulden and Place 1990). These accumulated lipids are subsequently utilized for reproduction and growth (Tessier and Goulden 1982). Storage lipids are related to starvation tolerance. Neonates with high maternal storage lipids survive longer than those with lower levels (Tessier *et al.,* 1983). Ecdysteroids and juvenoids have a major role in regulating moulting, growth, and reproduction in crustaceans. Ecdysteroids, such as ecdysone, exert effects through the interaction with the EcR, known to heterodimerize with RXR and to bind to the promoters of ecdysone-regulated genes (LeBlanc 2007; Wang and LeBlanc 2009). Ecdysone receptor regulates the expression of a number of genes such as *HR3*, *HR38*, and *HR96* (LeBlanc 2007). This regulatory activity is controlled by RXR (Mu and Leblanc 2004; LeBlanc 2007). Recent findings indicate that the JH receptor in *Daphnia* is

a complex of two nuclear proteins of the bHLH-PAS family of transcription factors: the MET and the SRC protein (Miyakawa *et al.,* 2013). Juvenoids promote expression of hemoglobin genes, such as *Hb2,* and sex-determining genes in the latter stages of ovarian oocyte maturation and supress *HR38* in *Daphnia* (LeBlanc 2007). Tributyltin acts as a disruptor of this regulatory pathway because the EcR:RXR heterodimer can be activated by TBT and juvenoids under the presence of ecdysteroids (Wang and LeBlanc 2009). Thus, it is plausible that TBT may alter lipid homeostasis in the crustacean *D. magna* by interacting with ecdysone and/or the juvenile signalling pathway.

In the present study, we used Nile red to quantify the amount of lipid droplets in whole *D. magna* individuals during the first reproductive instar (the so-called adolescent instar) across food levels and under exposure to TBT. During the adolescent instar, *Daphnia* females provision the first clutch of eggs (Barata and Baird 1998). Life-history effects of the disruption of this provision process by TBT were analysed by testing the tolerance to starvation and life-history performance of adult females exposed during the adolescent instar and of the neonates hatched from eggs provisioned under organotin exposure. Effects of TBT on the lipid profiles in adults and eggs were assessed by lipidomic non-target analysis using UPLC-TOF mass spectrometer (Gorrochategui *et al.,* 2014). To identify hormonal signalling pathways affected by TBT, we studied changes in mRNA abundance on genes related to ecdysone (*EcRB, HR3, HR38,* and *Neverland*), methyl farnesoate (*Hb2, SRC,* and *MET*), and retinoid X receptor (*RXR*) signalling pathways (LeBlanc 2007).

## 2.3 Materials and Methods

### 2.3.1 Chemicals

Tributyltin chloride (TBT; CAS No. 1461-22-9) and Nile red (CAS No. 7385-67-3) were purchased from Sigma-Aldrich, and lipid standards were from Advanti Polar Lipids. All other chemicals were analytical grade and were obtained from Merck.

### 2.3.2 TBT treatments

Tributyltin was dissolved in acetone; the same amount of acetone (< 0.1 mL/L) was used for a solvent control and in all experimental treatments except in the untreated control (control) to account for any carrier effect. Actual TBT concentrations in test

solutions were measured as total tin using a Perkin-Elmer Elan 6000 inductively coupled plasma mass spectrometer (ICP-MS) (Barata *et al.,* 2005), and were confirmed to be within 10 % of nominal concentrations (0.036 and 0.36 µg/L for 0.1 and 1 µg/L doses, respectively).

### 2.3.3 Experimental animals

All experiments were performed using the well-characterized clone F of *D. magna* maintained indefinitely as pure parthenogenetic cultures (Barata and Baird 1998). Individual cultures were maintained in 100 mL of ASTM (American Society for Testing and Materials) hard synthetic water at low and high food-ration levels (*Chlorella vulgaris* Beijerinck, $1 \times 10^5$ and $5 \times 10^5$ cells/mL, respectively), as described by Barata and Baird (1998). Individual animals fed daily with *C. vulgaris* at low and high food rations ($1x10^5$ and $5x10^5$ cells/mL, corresponding to 0.36 and 1.8 µg C/mL, respectively). *Chlorella vulgaris* was grown axenically in Jaworski/*Euglena gracilis* 1:1 medium (CCAP, 1989). Algae were harvested in the exponential phase of growth, centrifuged and then re-suspended in ASTM hard water. The number of algal cells in freshly prepared medium was checked daily from absorbance measurements at $\lambda$ = 650 nm in a dual-beam spectrophotometer (Uvikon 941) using standard calibration curves based on at least 20 data points, with an $r^2 > 0.98$. The culture medium was changed every day, and neonates were removed within 24 hrs. Photoperiod was set to 14 hrs light: 10 hrs dark cycle and temperature at 20 ± 1 °C. Cultures were maintained until females produced the sixth brood and were re-initiated with newborn neonates < 4-12 hrs obtained from third to sixth brood females. Prior to experiments, cultures were acclimated to each food ration conditions during three generations. This procedure allowed obtaining highly synchronized brooding females, which is crucial for minimizing inter-individual variability in storage lipids due to differences in age. From these cultures newborn neonates produced from third to sixth brood females were used for experiments.

### 2.3.4 Experimental design

Experiments were initiated with newborn neonates < 4-8 hrs old obtained from synchronized females cultured individually at high food-ration levels. Groups of five neonates (F0) were reared in 150 mL of ASTM hard water under high food-ration conditions until the end of the third juvenile instar (about 4-8 hrs before moulting for the

third time). At this point, juveniles were used in three sets of experiments using two TBT treatments, 0.1 µg/L (low; TBTL) and 1 µg/L (high; TBTH). Five to ten replicates per treatment were used.

The first experiment, studied effects of exposure to TBT during the adolescent instar (i.e., 3 days) on the life history of these females (F0) through five consecutive clutches. Their first clutch of neonates, exposed during the egg-provisioning stage (F1) was similarly studied during four consecutive clutches. Following exposure to TBT, F1 females were cultured individually under high food conditions without TBT, and their growth and reproduction performance monitored until the fifth clutch. The tolerance of F1 neonates to starving conditions was studied monitoring the time to death of 10 neonates individually cultured in 50 mL of ASTM hard water alone. The medium was renewed every day. Life-history performance of F1 neonates was studied by culturing them individually in 100 mL of ASTM hard water at high food conditions until the release of the fourth clutch. Measured life-history traits were survival, reproduction, body length of each adult instar (including that of the adolescent instar), age at first reproduction, the size of neonates of each clutch, and the population growth rate (*r*) estimated from the age-dependent survival and reproduction rates according to the Lotka equation (Barata *et al.,* 2001).

The second set of experiments (experiments 1 and 2) aimed to study lipid droplet changes across food and TBT treatments using the Nile red assay. In experiment 1, animals were exposed to three food regimes: starving (no food added), low food ($1 \times 10^5$ cells/mL *C. vulgaris*), and high food ($5 \times 10^5$ cells/mL *C. vulgaris*). In experiment 2, animals were exposed to two TBT concentrations (TBTL and TBTH) across low and high food levels. Exposures lasted through all the adolescent instars, and females were sampled just after their fourth moult and having released their eggs into the brood pouch (48 hrs).

The third set of experiments aimed to determine effects of TBTL and TBTH on the dynamics of lipids, lipid droplets, and mRNA levels of selected genes across an entire adolescent intermoult cycle. Experiments were conducted only at high food levels and included five samplings: 0 hrs (just after the third moult), 8 hrs, 16 hrs, 24 hrs, and just after the fourth moult (48 hrs), as shown in Figure 2.1. At each sampling, three and five replicates of 5 individuals were collected and processed for total lipid determination and mRNA gene transcription measurement, respectively, and 10 animals were processed for Nile red determination. At the 48 hrs sampling period, females were de-brooded by gently flushing water into the brood pouch. Obtained eggs and de-brooded females were then collected and used for lipid and gene transcription analyses. Because of the large number

of synchronized animals needed, three different independent but consecutive experiments were performed and used for lipidomic, gene transcription, and Nile red determinations, respectively.

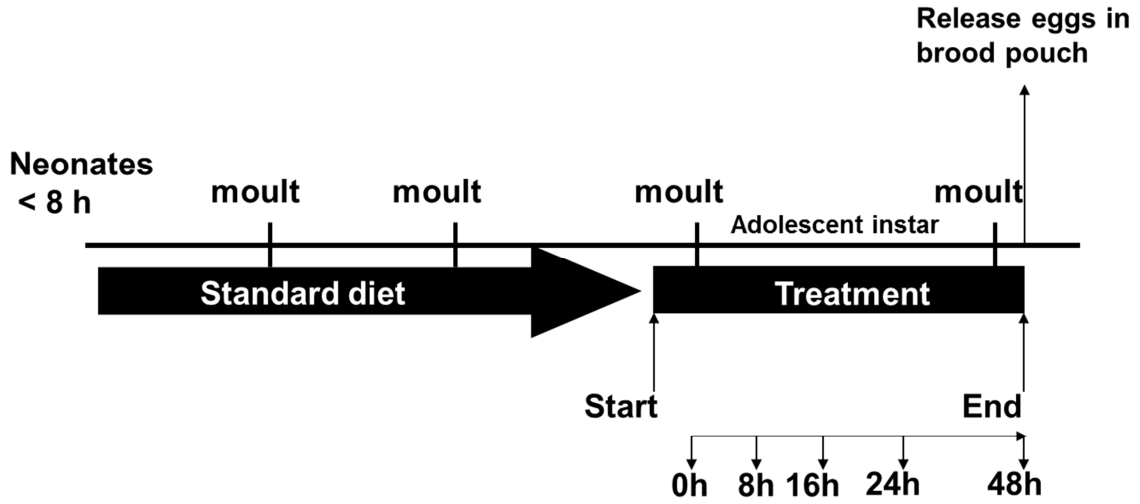


**Figure 2.1 –** Diagram of the test protocol used to expose *D. magna* and measure changes in lipids and lipid droplets. *D. magna* individuals were fed on high food during their growth until 2/3 of their third juvenile instar and then exposed to the studied food treatments, TBT and its solvent carrier during a little bite more than the adolescent instar.

### 2.3.5 Nile red determination

The Nile red stock solution was prepared in acetone and stored protected from light following Tingaud-Sequeira *et al*., (2011). Just before use, the working solution was prepared by diluting the stock solution to 1.5 µM in ASTM hard water. Live individuals were then exposed to Nile red working solution in the dark for 1 hr at 20 °C. After incubation, animals were placed in 100 mL ASTM for 1 min to allow clearance of Nile red residuals. Following clearance, animals were placed individually in 1.5 mL microcentrifuge tubes, the remaining water was removed, and samples were sonicated in 300 µL of isopropanol. The homogenized extract was then centrifuged at 10,000 × *g*. We used 200 µL of supernatant to measure Nile red fluorescence using an excitation/emission wavelength of 530/590 nm and a fluorescence microplate reader (Synergy 2, BioTek). Each treatment had one animal per sample (10 replicates in total). For each quantification and treatment, 10 blanks (animals not exposed to Nile red) were used to account for background levels of fluorescence. After exposure to Nile red, images were taken in the area surrounding the midgut for visualization of lipid droplets. Fluorescence and bright file

images were obtained using a Nikon SMZ1500 microscope and a Nikon Intensilight C-HGFI with a GFP filter (EX 472/30, EM 520/35; Nikon).

### 2.3.6 Lipidomic analyses

Lipidomic analyses were performed as described by Gorrochategui *et al*.,(2014), with minor modifications. Each replicate consisted of a pool of five animals that were homogenized in 500 µL phosphate-buffered saline (PBS), pH 7.4, with 2,6-di-*tert*-butyl-4-methylphenol (BHT; 0.01%) as an antioxidant. Lipid extraction was performed using a modification of Folch's method (Folch *et al.,* 1957). Briefly, 100 µL of the homogenized sample was mixed with 500 µL of chloroform and 250 µL of methanol. Internal standards (200 pmol) were also added (Table 2.1). Samples were heated at 48 °C overnight and dried under $N_2$ the next day. Lipid extracts were solubilized in 150 µL methanol. The liquid chromatography–mass spectrometer (LC-MS) consisted of a Waters Aquity UPLC system connected to a Waters LCT Premier Orthogonal Accelerated TOF Mass Spectrometer (Waters) operated in positive and negative electrospray ionization (ESI) mode. Full-scan spectra from 50 to 1500 Da were obtained. Mass accuracy and reproducibility were maintained by using an independent reference spray (LockSpray; Waters). A 100 mm × 2.1 mm i. d., 1.7 µm C8 Acquity UPLC BEH (Waters) analytical column was used. Further chromatographic details of mobile phases were described by Gorrochategui *et al*., (2014).

Quantification was carried out using the ion chromatogram obtained for each compound using 50 mDa windows. The linear dynamic range was determined by injection of standard mixtures. Positive identification of compounds was based on the accurate mass measurement, with an error < 5 mg/L, and its LC retention time compared with that of a standard (± 2 %).

**Table 2.1** – Internal standards used for the analysis of lipids in *D. magna*, (MW, molecular weight).

| Family | Name | Abbreviation | Molecular Formula | MW | Exact mass |
|---|---|---|---|---|---|
| **Neutral glycerolipids** | Triheptadecanoate 1,2,3-triheptadecanoyl-glycerol | TG C51:0 | $C_{54}H_{104}O_6$ | 849.4 | 848.783 |
| | 1,3 (d5)-diheptadecanoyl-glycerol | DG C34:0 | $C_{37}H_{67}D_5O_5$ | 601.995 | 601.569 |
| | 1-heptadecanoyl-rac-glycerol | MG C17:0 | $C_{20}H_{40}O_4$ | 344.529 | 344.293 |
| **Sterols** | cholest-5-en-3ß-yl heptadecanoate | CE C17:0 | $C_{44}H_{81}O_2N$ | 639.089 | 638.6 |
| **Phospholipids** | 1-hexadecanoyl(d31)-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine | d31 34:1 | $C_{42}H_{51}D_{31}NO_8P$ | 791.267 | 790.772 |
| | 1-hexadecanoyl(d31)-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine | d31PE 34:1 | $C_{39}H_{45}D_{31}NO_8P$ | 749.187 | 748.725 |
| | 1-hexadecanoyl(d31)-2-(9Z-octadecenoyl)-sn-glycero-3-[phospho-L-serine] | d31PS 34:1 | $C_{40}H_{45}D_{31}NO_{10}P$ | 815.179 | 814.697 |
| **Lyso-phospholipids** | 1-(10Z-heptadecenoyl)-sn-glycero-3-phosphoethanolamine | LPE C17:1 | $C_{22}H_{44}NO_7P$ | 465.561 | 465.286 |
| | 1-(10Z-heptadecenoyl)-sn-glycero-3-phospho-L-serine | LPS C17:1 | $C_{23}H_{44}NO_9P$ | 531.552 | 531.257 |
| | 1-heptadecanoyl-sn-glycero-3-phosphate | LPA C17:0 | $C_{20}H_{41}O_7P$ | 446.491 | 446.241 |
| | 1-heptadecanoyl-sn-glycero-3-phosphocholine | LPC C17:0 | $C_{25}H_{53}NO_7P$ | 509.657 | 509.348 |
| **Sphingolipids** | N-lauroyl-D-*erythro*-spingosyl phosphorylcholine | SP C12:0 | $C_{35}H_{71}N_2O_6P$ | 646.922 | 646.505 |

A total of 116 lipids were identified and quantified by UPLC-TOF ESI-positive mode that were distributed as follows: five classes of glycerophospholipids (GPs) [phosphocholine (PC) with 20 lipids, lysophosphatidylcholine (LPC) with 6 lipids, phosphatidylethanolamine (PE) with 9 lipids, phosphatidylserine (PS) with 7 lipids, and phosphatidylinositol (PI) with 3 lipids]; diacylglycerols (DG) with 20 lipids; TGs (TG) with 39 lipids; cholesterylesters (CE) with 4 lipids; and sphingolipids (SP) with 8 lipids. Glycerophospholipids, DG, TG, and CE were annotated as <lipid subclass>, <total fatty acyl chain length> and <total number of unsaturated bonds>. Sphingolipids were annotated as <lipid subclass>, <total fatty acyl chain length> and <total number of unsaturated bonds>.

### 2.3.7 Transcriptomic analyses

Methods of extraction, purification, and quantification of mRNA from the studied genes and their primers follow previous procedures (Campos *et al.,* 2013). Eight genes were selected for representation of different pathways/gene families: *EcRB, HR3, HR38, Neverland, Hb2, RXR, MET,* and *SRC*. The gene glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) was used as an internal control. For each of the genes, primers were designed using Primer Quest (IDT Technologies) and are listed in Table 2.2. Aliquots of 10 ng were used to quantify specific transcripts in a LightCycler® 480 Real-Time PCR system (Roche) using a LightCycler 480 SYBR Green I Master® (Roche). Relative abundance values of all genes were calculated from the second derivative of their respective amplification curve (Cp; crossing point) values calculated by technical triplicates. Crossing point values of target genes were compared with the corresponding reference genes.

**Table 2.2** – Primer pairs designed. Primers have been obtained from existing sequences used for the analyses of gene transcriptomic changes.

| Genes | Accession Number | Forward | Reverse | Amplicon size |
|---|---|---|---|---|
| ***G3PDH*** | AJ292555 | GACCATTACGCTGCTGAATACG | CCTTTGCTGACGCCGATAGG | 100 |
| ***EcRB*** | AB274824 | CACCACAACCAACTGCATTTAC | CCATTSSTGTCAAGATCCCACA | 81 |
| ***HR3*** | FJ755466 | AAGGTCGAGGATGAAGTGCG | AAAGACGCTACTATCGGGCG | 81 |
| ***HR38*** | KM982449 | AGTGAGCGGAGTTCTGGCAG | CGTGACATATAACCCGGAAGC | 81 |
| ***Neverland*** | KM893860 | CAAATGAGGGCAATACGCGT | GATGCTCTCGGCGAGAACAT | 81 |
| ***Hb2*** | AB021136 | CCCAGGTTCTTTTCCGCCTTC | CGGATTGAGGAACATCGGC | 81 |
| ***RXR*** | DQ530508 | GTGTCGAGTGCAAGGACGAG | TTTTCCAGTTGGTTGAATGGG | 100 |
| ***SRC*** | AB698070 | TACTAGGCGTCTTGCTGAATGAA | CCATAATTTGCAAGGCTCCG | 81 |
| ***MET*** | AB698069 | CAAACAGCCAGAGATTACCGG | GCACTGTTGGTTCCAGCATTC | 81 |

### 2.3.8 Data analyses

The effect of food rations, treatment, sampling period, juvenile stage, Nile red fluorescence, lipidomic profiles, mRNA abundance, life history and physiological responses were analysed by two-way and/or one-way analysis of variance (ANOVA). Post-hoc Dunnett's or Tukey's tests were performed to compare exposure treatments with solvent controls. Prior to analyses, all data except survival responses were log transformed to achieve normality and variance homoscedasticity. If not indicated otherwise, significance levels were set at $p < 0.05$. Survival responses were assessed by Wilcoxon-Gehan tests. Tests were performed with IBM-SPSS statistics software, version 19. Lipidomic data were further analysed using cluster and K-means analyses in R (R Core Team 2014) to identify clusters of lipid families similarly affected by TBT.

## 2.4 Results

### 2.4.1 Life-history consequences of exposure to TBT

Table 2.3 shows a summary of life-history effects and tolerance to starvation after short-term TBT exposures in adult females (F0) and their offspring (F1) (for detailed

statistical analysis results, see Table 2.4 and Table 2.5). Females treated with TBT during the adolescent instar (F0) were smaller after moulting (48 hrs sampling point) and showed a significant decrease of the total number of their offspring. These life-history traits translated into a lower reproduction and, consequently, lower population growth rates (*r*). Adolescent females (F0) exposed to high doses of TBT during the period of egg provisioning in their ovaries also produced smaller neonates than their untreated and solvent controls or TBTL–treated counterparts, although this did not affect the tolerance of their offspring (F1) to starvation. Neonates (F1) pre-exposed to either concentration of TBT showed impaired survival and reduced reproduction and population growth rates, even when they grew to adulthood in a TBT-free environment (Table 2.3).

**Table 2.3** – Summary of life-history traits. Values for life history traits (Mean ± SE, $n$ = 10) are for adult females exposed to TBT during their adolescent instar (F0) and for their offspring (F1) that were exposed to TBT during the egg provisioning stage. Abbreviations: F, clutch size; N, neonate size; $r$, population growth rate (days$^{-1}$); S, body length (µm). For each trait, the first number indicates the generation, and the second number refers to the brood or adult instar number. (*) $p < 0.05$ compared with solvent controls, by ANOVA and Dunnett's post-hoc test.

| Traits | Control | Solvent Control | TBTL | TBTH |
|---|---|---|---|---|
| **Parental generation (F0)** | | | | |
| **Adult survival 21 days (%)** | 90 ± 10 | 90 ± 10 | 70 ± 15 | 70 ± 15 |
| **Age at first reproduction (days)** | 9 ± 0 | 9 ± 0 | 9 ± 0 | 9 ± 0 |
| Clutch size (*n*) | | | | |
| F0-1 | 10.3 ± 0.4 | 9.2 ± 0.6 | 9 ± 0.5 | 8.5 ± 0.4 |
| F0-2 | 16.7 ± 0.6 | 17.1 ± 0.7 | 10.8 ± 0.7* | 13.6 ± 1.4* |
| F0-3 | 19.8 ± 0.5 | 19.7 ± 0.3 | 15.3 ± .7* | 15.4 ± 0.7* |
| F0-4 | 27 ± 1.0 | 26.6 ± 1.2 | 27.4 ± 1.1* | 27.1 ± 1.0 |
| F0-5 | 27.7 ± 0.7 | 29.1 ± 0.7 | 23.7 ± 1.5* | 21.1 ± 1.7* |
| Total Offspring | 101.8 ± 1.9 | 101.7 ± 2.2 | 87.6 ± 1.8* | 82.9 ± 2.6* |
| *r*0 | 0.335 ± 0.004 | 0.325 ± 0.009 | 0.313 ± 0.003* | 0.304 ± 0.01* |
| Neonate size (µm) | | | | |
| N0-1 | 732.4 ± 6.2 | 723.6 ± 4.3 | 735 ± 6.5* | 655.3 ± 4.6* |
| N0-2 | 764.5 ± 6.0 | 762.8 ± 10.4 | 766 ± 3.7 | 763 ± 4.8 |
| N0-3 | 793.8 ± 7.7 | 809.6 ± 9.0 | 807.3 ± 5.2 | 801.7 ± 12 |
| N0-4 | 821.4 ± 9.8 | 809 ± 8.7 | 798.7 ± 10.7 | 787.6 ± 8.4 |
| N0-5 | 827.3 ± 10.7 | 834.7 ± 6.3 | 813.6 ± 8.2 | 812.4 ± 4.1 |
| Body length (µm) | | | | |
| S0-1 | 2454.6 ± 13.9 | 2481.1 ± 23.4 | 2407.1 ± 16.2* | 2305.8 ± 23.9* |
| S0-2 | 2618.2 ± 28.4 | 2684.9 ± 42.3 | 2574.3 ± 25.4* | 2445.7 ± 35* |
| S0-3 | 2842.2 ± 22.6 | 2858.2 ± 27.5 | 2861.2 ± 28.9 | 2790.8 ± 17.1 |
| S0-4 | 3145.4 ± 25.6 | 3133.5 ± 44.4 | 3108.3 ± 24.2 | 3134 ± 21.3 |
| S0-5 | 3275.4 ± 20.6 | 3248.4 ± 34.5 | 3274.7 ± 36.4 | 3239.3 ± 19.9 |
| S0-6 | 3377.7 ± 20.2 | 3340.9 ± 29.3 | 3337.6 ± 25.3 | 3344.7 ± 14.1 |
| **First generation (F1)** | | | | |
| N0-1 (µm) | 741.1 ± 13.5 | 755 ± 12.2 | 744.1 ± 9.6* | 641.7 ± 12.0 * |
| **Survival Starvation (days)** | 6.1 ± 1.1 | 5.2 ± 2.4 | 4.5 ± 2.1 | 5.9 ± 1.2 |
| **Juvenile Survival (%)** | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 | 80 ± 13.3 |
| **Adult survival 21 days (%)** | 100 ± 0.0 | 100 ± 0.0 | 60 ± 16.3* | 40 ± 16.3* |
| **Age at first reproduction (days)** | 10.1 ± 0.1 | 10.1 ± 0.1 | 10 ± 0.0 | 10.9 ± 0.4 |
| Clutch size (*n*) | | | | |
| F1-1 | 16.7 ± .7 | 17 ± 1.0 | 17.4 ± 1.1 | 14.8 ± 1.0 |
| F1-2 | 20.1 ± .8 | 22.3 ± .7 | 22 ± 1.2 | 20.2 ± 1.6 |
| F1-3 | 24.9 ± .7 | 25.1 ± .8 | 25.5 ± 1.4 | 22.3 ± 2.1 |
| F1-4 | 27.2 ± .7 | 26.7 ± .6 | 26.8 ± .7* | 20.3 ± 1.8* |
| Total Offspring (*n*) | 88.6 ± 2.1 | 91.1 ± 2.3 | 64.2 ± 11.2* | 48.6 ± 9.9* |
| *r*1 | 0.33 ± 0.0 | 0.34 ± 0.0 | 0.28 ± 0.01* | 0.23 ± 0.02* |
| Neonate size (µm) | | | | |
| N1-1 | 725.3 ± 9.4 | 717.1 ± 6.9 | 712.3 ± 6.2 | 706.6 ± 5.9 |
| N1-2 | 788 ± 9.7 | 778.7 ± 15.1 | 776.3 ± 9.6 | 748.5 ± 17.6 |
| N1-3 | 803.2 ± 8 | 791.7 ± 6.1 | 797.2 ± 4.8 | 810.1 ± 4.0 |
| N1-4 | 866.4 ± 9.6 | 881.1 ± 2.6 | 864.4 ± 6.3 | 8639.2 ± 2.0 |
| Body length (µm) | | | | |
| S1-1 | 2656 ± 63.7 | 2642.3 ± 46.1 | 2608.7 ± 35.6 | 2686.8 ± 50.3 |
| S1-2 | 2686.8 ± 50.3 | 2656 ± 63.7 | 2679.6 ± 44.3 | 2589.6 ± 36.3 |
| S1-3 | 3004.9 ± 43.6 | 3049.1 ± 54.4 | 3021.5 ± 29.1 | 2890.2 ± 52.4 |
| S1-4 | 3202.1 ± 39 | 3204.4 ± 41.9 | 3208.5 ± 38.8 | 3071.9 ± 103 |
| S1-5 | 3297 ± 41.1 | 3337.5 ± 28.8 | 3340.1 ± 25.6 | 3258.3 ± 25.0 |

**Table 2.4** – Univariate parametric and non-parametric ANOVA results (degrees of freedom, df) on Life-History traits. Analyses were performed on survival, age at first reproduction, total offspring produced (T offspring) and population growth rate (*r*) of females treated during the adolescent instar (Parental Generation) and of offspring exposed during their egg provisioning stage (F1 Generation).
ns (not significant, $p > 0.05$), (*) $0.05 < p < 0.01$, (**) $0.01 < p < 0.001$, (***) $p < 0.001$.

| **Parental Generation** | | | | **F1 Generation** | | | |
|---|---|---|---|---|---|---|---|
| | | **df** | **Statistic** | | | **df** | **Statistic** |
| **Survival adult** | Wilcoxon (Gehan) Statistic | 3 | 2.4 ns | Survival starvation | Chi-Square | 3 | 4.3 ns |
| | | | | Survival juvenile | Wilcoxon (Gehan) Statistic | 3 | 6.1 ns |
| | | | | Survival adult | Wilcoxon (Gehan) Statistic | 3 | 13.7** |
| **Age** | Kruskal-Wallis | 3 | 0 ns | Age | Kruskal-Wallis | 3 | 6.4 ns |
| **T offspring** | ANOVA | 3,31 | 19.9** | T offspring | ANOVA | 3,36 | 7.1** |
| ***R*** | ANOVA | 3,36 | 4.6** | r | ANOVA | 3,36 | 7.5** |

**Table 2.5** – Repeated measurement ANOVA results (Fisher's coefficient, F, degrees of freedom, df) on Life-History traits. Brood number was used as a repeated measure and treatment as a fixed factor for clutch and neonate size and body length across broods of females treated during the adolescent instar and of offspring exposed during their egg provisioning stage.
ns (not significant, p > 0.05), (*) 0.05 < *p* < 0.01, (**) 0.01 < *p* < 0.001, (***) *p* < 0.001.

| **Parental Generation** | | | **F1 Generation** | | |
|---|---|---|---|---|---|
| **Clutch size** | **df** | **F** | **Clutch size** | **df** | **F** |
| **Brood** | 1,26 | 851.1** | **Brood** | 1,25 | 213.4** |
| **Treatment** | 3,26 | 15.1** | **Treatment** | 3,25 | 3.2* |
| **Brood x Treatment** | 3,26 | 4.5* | **Brood x Treatment** | 3,25 | 2.1 ns |
| | | | | | |
| **Neonate size** | df | F | **Neonate size** | df | F |
| **Brood** | 1,26 | 368.4** | **Brood** | 1,25 | 357.6** |
| **Treatment** | 3,26 | 12.7** | **Treatment** | 3,25 | 0.6 ns |
| **Brood x Treatment** | 3,26 | 5.8** | **Brood x Treatment** | 3,25 | 1.4 ns |
| | | | | | |
| **Body size** | df | F | **Body size** | df | F |
| **Brood** | 1,28 | 3171.1** | **Brood** | 1,25 | 839.4** |
| **Treatment** | 3,28 | 3.9* | **Treatment** | 3,25 | 0.2 ns |
| **Brood x Treatment** | 3,28 | 8.3** | **Brood x Treatment** | 3,25 | 0.4 ns |

### 2.4.2 Nile red staining of lipid droplets

The complexity of lipid droplet dynamics in *D. magna* is summarized in Figure 2.2 (staining optimization) and Figure 2.3. Nile red staining showed significantly higher levels of fluorescence in females cultured at high food levels than in those reared at low food levels or starved ($F$ 2,27 = 144.1, $p$ < 0.05; Figure 2-3A; the quantification of results are shown in Figure 2.3B). Exposure to TBTH significantly increased Nile red fluorescence in females within ($F$ 2,54 = 55.9, $p$ < 0.05) and across ($F$ 2,54 = 22.7, $p$ < 0.05) food levels at

TBTH, such effects being more pronounced at high food levels (Figure 2.3A,C). The dynamics of lipid droplets during the first reproductive cycle in the presence or absence of TBT is shown in Figure 2.3D. In untreated females (control) or those exposed to TBTL, Nile red fluorescence increased during the intermoult period, peaked at 24 hrs, and decreased just after moulting and releasing of their first brood of eggs (48 hrs). Exposure to TBTH significantly increased Nile red fluorescence, starting at 16 hrs of exposure, reaching a maximal level at 24 hrs (corresponding to twice the levels of control or TBTL samples), and remaining at this high level even after moulting (48 hrs). Statistical analyses showed significant ($p < 0.05$) effects of sampling period ($F_{4,60} = 104.3$), treatment ($F_{2,60} = 31.5$), and their interaction ($F_{8,60} = 10.1$). Whether such changes correspond to enhanced levels of TG was further studied by analysing changes in the whole lipidome.

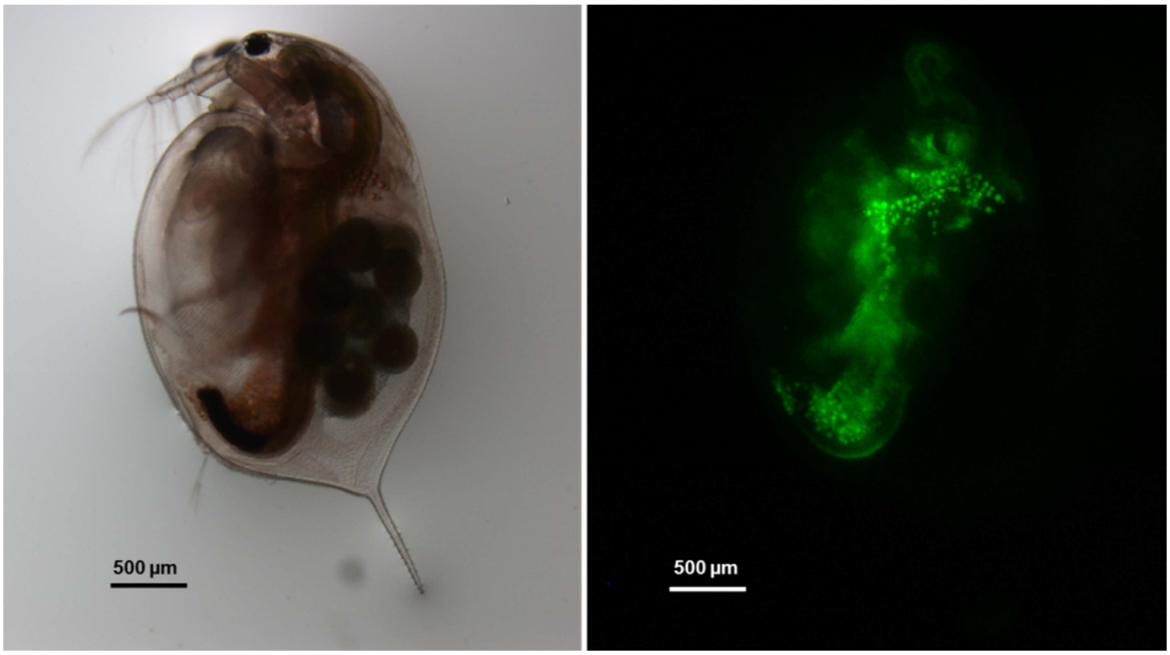


**Figure 2.2** – Optimization of Nile red technique using adolescent females just after moulting in control conditions, total view of individual under fluorescent microscopy.

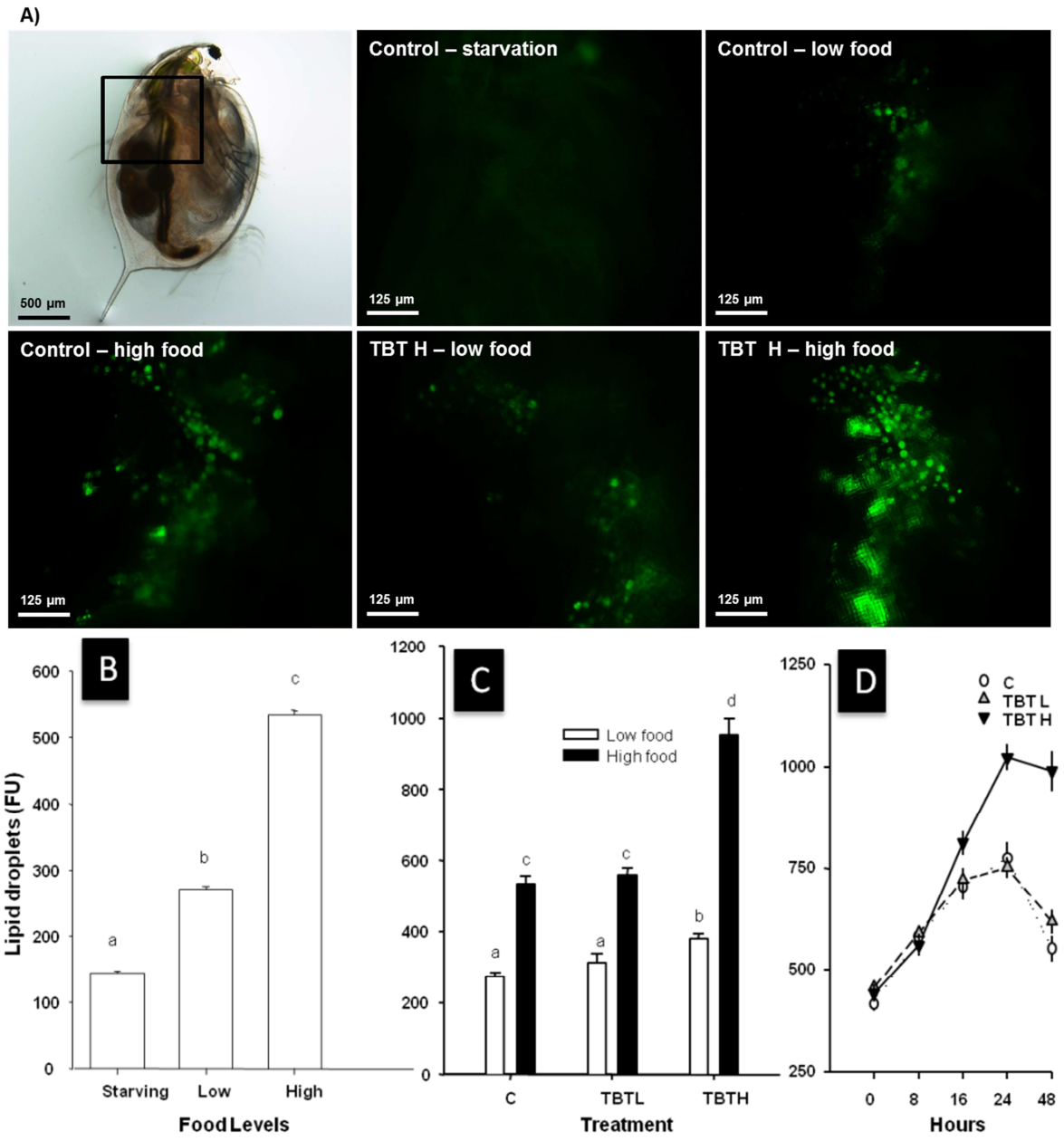


**Figure 2.3** – Quantitative assessment of lipid droplets in *D. magna* individuals. (A) Lateral partial view under fluorescent microscopy of adolescent females just after moulting and releasing the first brood of eggs across different food ration regimes (starving, low food, and high food) and treatments [control and TBTH (1 µg/L)]; top left, bright field microscopy image of a female, with the studied area indicated by a rectangle. Lipid droplets stained with Nile red are in green. (B) Nile red fluorescence [mean ± SE fluorescence units (FU); $n$ = 5–10] in 48 hrs females across starving, low, and high food rations, and (C) across TBTL and TBTH at low and high food rations. (D) Nile red fluorescence (mean ± SE; $n$ = 5–10) measured at different time points within the adolescent instar and just after moulting across TBTL and TBTH, at high food rations. In B and C, different letters indicate significant ($p < 0.05$) differences among food levels or across food levels and TBT treatments, respectively, following ANOVA and Tukey's post-hoc tests.

### 2.4.3 Changes in the lipidome across TBT treatments

Changes in the lipid content of *D. magna* juveniles, unexposed (control) or exposed to TBTL or TBTH during the adolescent instar, are shown in Figure 2.4 (for supporting statistics, see Table 2.6). The sampling time significantly affected ($p < 0.05$) the levels of the nine studied lipid classes within and across TBT concentrations (time and time × treatment effects; see Table 2.6). Levels of TG, DG, CE, and PC in adolescent females increased at the beginning of the instar, peaking at 16–24 hrs, and decreased afterward, reaching the lowest levels just after moulting in de-brooded females (48 hrs). Levels of TG showed the greatest changes, increasing up to 6-fold in control females. Levels of PE, PS, PI, and SP increased during the adolescent instar, usually reaching their highest levels 24 hrs after moulting. In contrast, LPC showed little variation during the adolescent instar. Exposure to TBT affected levels of most lipid classes, with the exception of LPC. Levels of TG, CE, and PC were reduced by TBT exposure during the first 24 hrs of the instar, but they showed increased residual levels just after moulting in de-brooded females. Levels of DG in females exposed to TBT were always higher than those of controls. TBT also reduced the overall levels of lipids belonging to classes of SP, PE, PS, and PI. Unexposed eggs showed levels of TG and CE comparable to the highest levels observed in adult females, whereas PS levels were about 1.5-fold higher than those of de-brooded females just after moulting. In contrast, levels of DG, PC, LPC, SP, PE, and PI were lower in eggs than in adults. Exposure to TBT reduced TG, PC, and PS levels in eggs relative to controls, and dramatically increased CE levels (Figure 2.4). Clustering analysis of individual lipids using K-Means identified four main clusters (Figure 2.5) from which two of them (clusters 2 and 3) were particularly enriched with TG, DG, and CE. Cluster 2 included the most unsaturated TG (Figure 2.6), which were mostly transferred to eggs. Within this cluster, 10 of 26 lipid species had a total fatty acyl chain length ≥ 52 and a total number of unsaturated bonds ≥ 4; thus, they could include the polyunsaturated fatty acids (PUFA) arachidonic acid (20:4) and eicosapentaenoic acid (20:5) combined with two palmitic acids (16:0). Levels of these TG increased in controls through two-thirds of the instar (i.e., 24 hrs), when they were mostly allocated to eggs; their levels were consequently reduced to negligible levels in de-brooded females just after moulting (48 hrs; see Figure 2.6, top). TBTH disrupted this process, making females reach peak levels earlier, maintaining high levels even after moulting, and reducing the amount of these lipids allocated to eggs. Lipid profiles in eggs and females exposed to TBTL showed intermediate levels of disruption. Cluster 3 included the less unsaturated TG (see Figure

2.6, bottom) that were only partially (60%) transferred to eggs in control females. Exposure to TBT (either at the high or low dose) decreased the maximal attained levels of these lipids, and notably reduced their transfer to the eggs (Figure 2.6*,* bottom).

The changes in TG, DG, CE, and PC levels in control and TBTL–exposed females correlated to the observed variations in Nile red fluorescence of lipid droplets in adult females during the instar period (compare Figure 2.3D with Figure 2.4), with Pearson correlation coefficients varying between 0.74 and 0.85 ($p < 0.05$, $n = 10$). However, for individuals exposed to TBTH, this correlation was lost for most lipid classes, except for PC and PS ($r = 0.90$ and 0.95, respectively, $n = 5$).

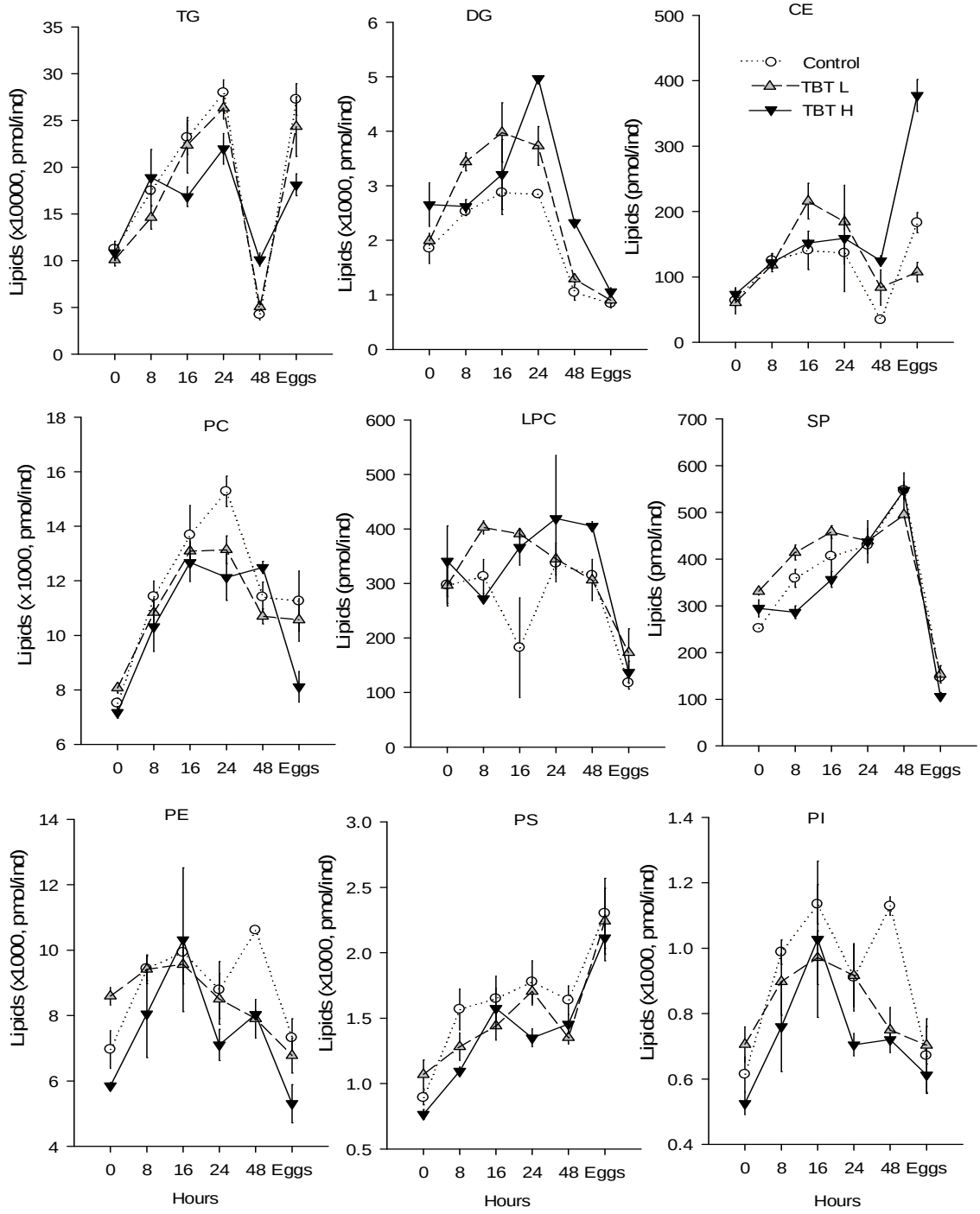


**Figure 2.4** – Lipidomic profiles of major lipid classes (Mean ± SE; *n* = 3) in control, TBTL, and TBTH treatment groups during the adolescent instar in females at 0, 8, 16, and 24 hrs, in de-brooded females just after the fourth moult (48 hrs), and in eggs. Abbreviations: TG, triacylglycerols; DG, diacylglycerols; CE, cholesteryl esters; PC, phosphocholines; LPC, lysophosphatidylcholine; SP, sphingolipids; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.

**Table 2.6** – Two-way ANOVA on lipid classes responses. ANOVA results (Fisher's coefficient, F, degrees of freedom, df) comparing the lipid classes responses across TBT treatments along the adolescent instar, in de-brooded females just after the forth moult and in eggs. For mRNA levels, treatments did not included eggs.
ns (not significant, p > 0.05), (*) 0.05 < *p* < 0.01, (**) 0.01 < *p* < 0.001, (***) *p* < 0.001.

| | **Treatment** | | **Time** | | **Treatment x Time** | |
|---|---|---|---|---|---|---|
| | **df** | **F** | **df** | **F** | **df** | **F** |
| **Lipids** | | | | | | |
| **PC** | 2,36 | 6.5** | 5,36 | 43.6*** | 10,36 | 2.4* |
| **LPC** | 2,33 | 2.1 ns | 5,33 | 22.4*** | 10,33 | 1.0 ns |
| **PE** | 2,36 | 8.4** | 5,36 | 10.2*** | 10,36 | 1.4 ns |
| **PS** | 2,36 | 6.5** | 5,36 | 38.8*** | 10,36 | 1.5 ns |
| **PI** | 2,36 | 7.1** | 5,36 | 10.0*** | 10,36 | 1.0 ns |
| **TG** | 2,36 | 0.6 ns | 5,36 | 112.2*** | 10,36 | 7.9*** |
| **DG** | 2,36 | 14.9*** | 5,36 | 69.8*** | 10,36 | 2.9** |
| **CE** | 2,36 | 12.2*** | 5,36 | 25.3*** | 10,36 | 4.3** |
| **SP** | 2,36 | 10.1*** | 5,36 | 169.3*** | 10,36 | 3.3** |

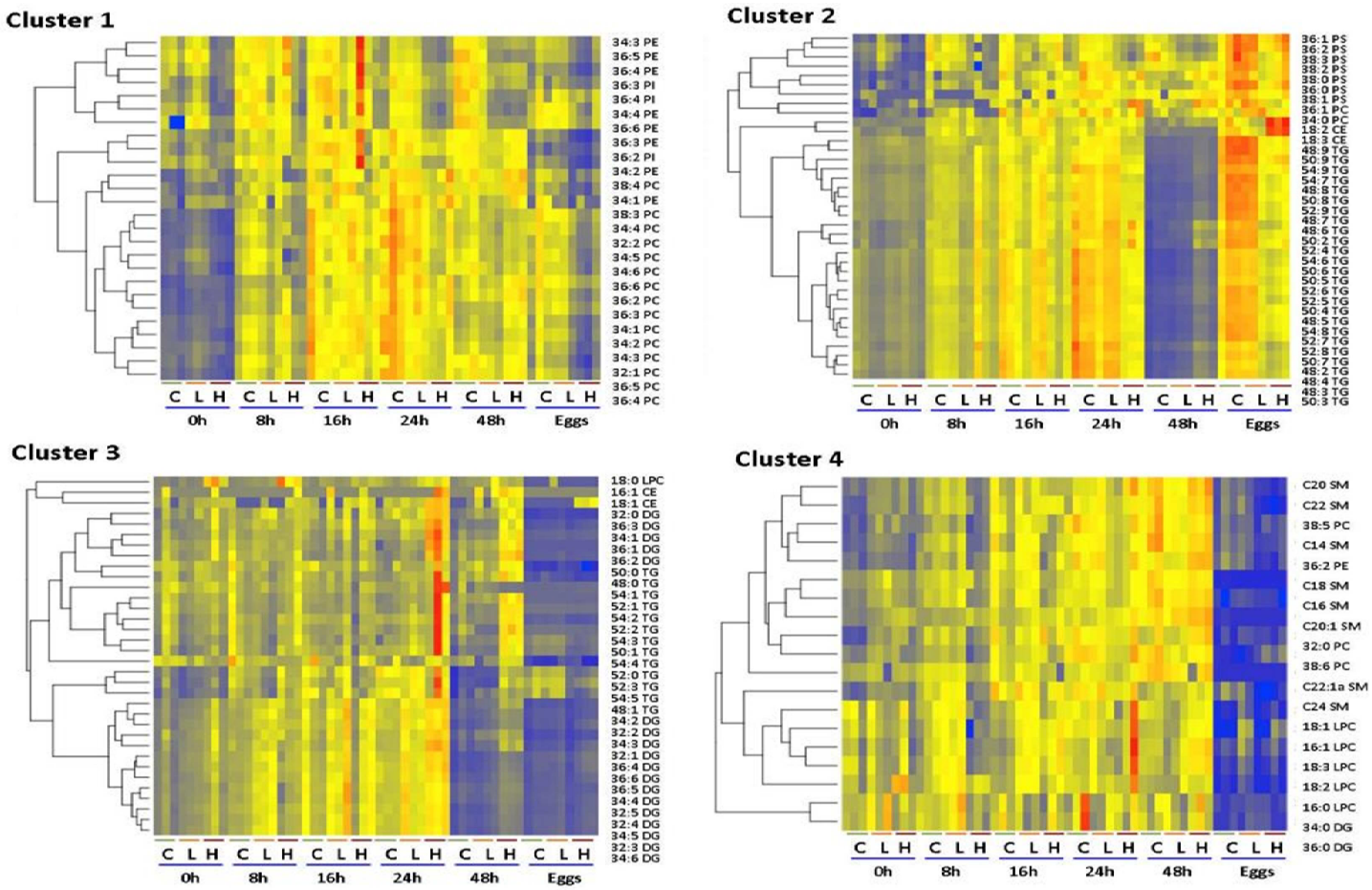


**Figure 2.1** – Heat map and hierarchical clustering (K-Means) of the quantified lipid groups in *D. magna* juveniles along the studied period of the adolescent instar in control (C), TBTL (L) and TBTH (H) treatments. High and low lipid levels are in red and blue and those in yellow unchanged.

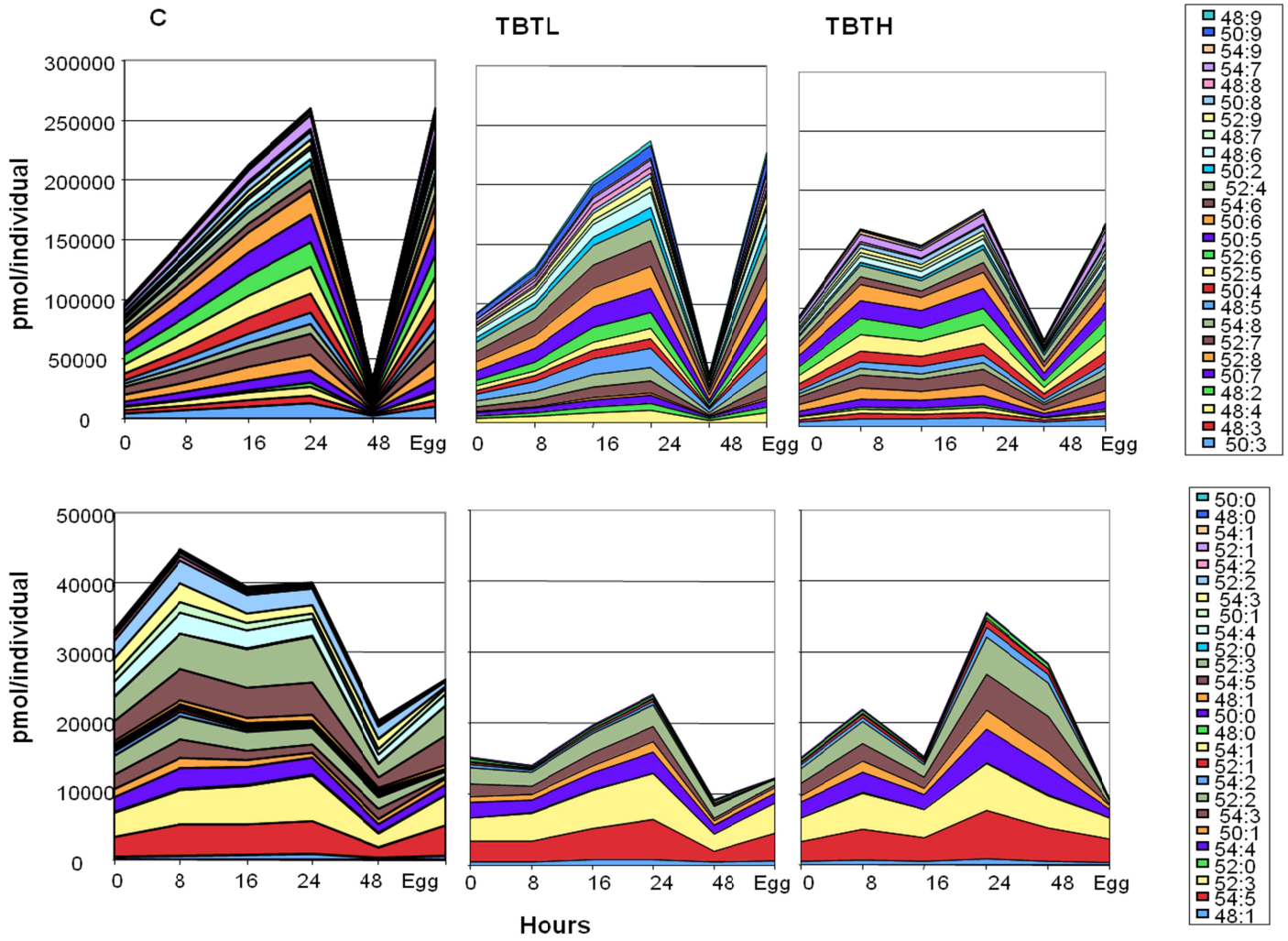


**Figure 2.2**– Mean levels of individual TG differentiated in clusters 2 (upper) and 3 (lower graph panel) depicted in Figure 2.5 for control (C), TBTL and TBTH treatments along the adolescent instar at 0, 8, 16 and 24 hrs and in adults just after the forth moult without eggs (48 hrs) and in eggs. Lipid compounds are depicted in the legends.

### 2.4.4 Gene responses

The profiles of mRNA abundance for the eight genes analysed in this study during the moulting/reproductive cycle in control females are shown in Figure 2.7. Levels of mRNA of *RXR, SRC, EcRB, HR3,* and *Neverland* genes varied during the instar, being highest at 0 and 48 hrs, whereas *HR38, Hb2,* and *MET* levels remained relatively constant throughout the cycle. TBT treatments significantly increased transcript levels of seven of eight of these genes at least in some phases of the moulting/reproductive cycle, including *MET* and, particularly, *Hb2*, which remained relatively constant in control conditions (Figure 2.7; for ANOVA results, see Table 2.7). Affected genes include markers of the ecdysone pathway (*EcRB, HR3, Neverland*), the JH signalling pathway (*MET, SRC*), and the *RXR* gene.

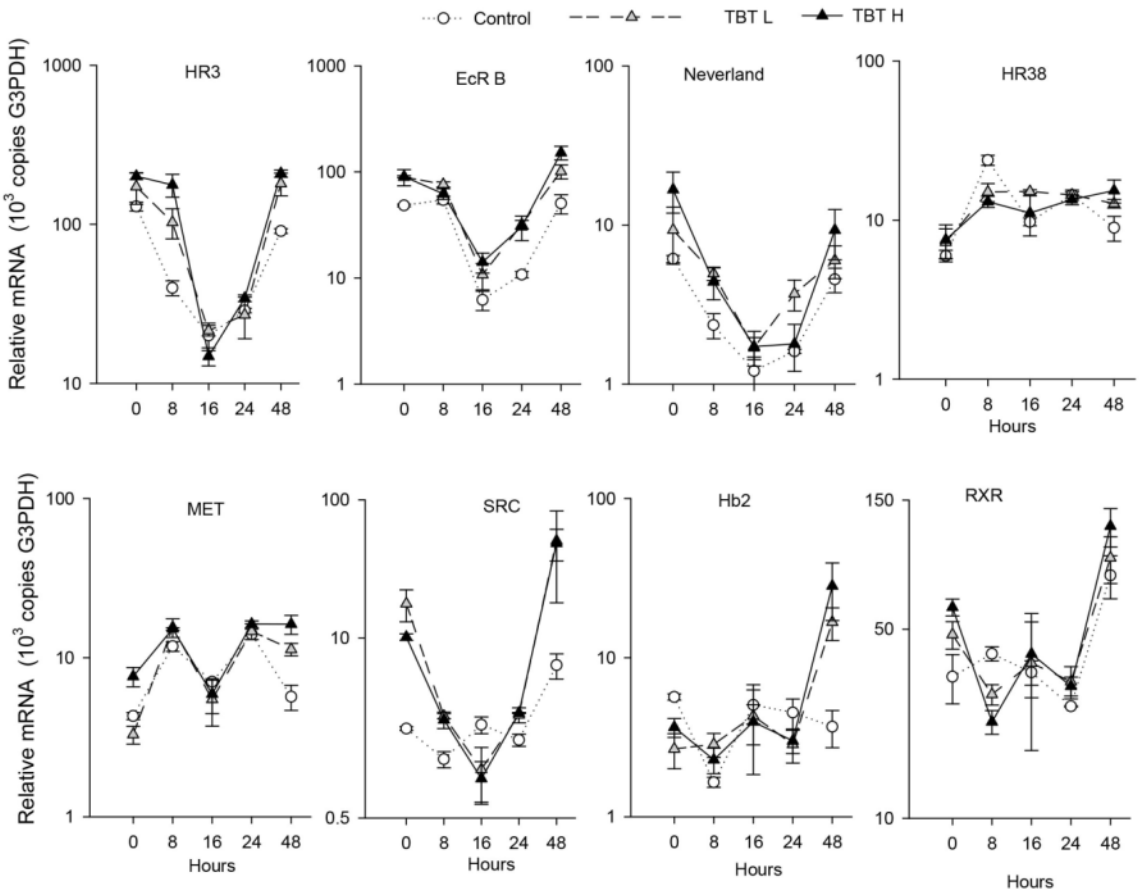


**Figure 2.7** – Transcription patterns (Mean ± SE; $n = 5$) shown by the number of mRNA copies of the eight studied genes (*HR3*, *EcRB*, *Neverland*, *HR38*, *MET*, *SRC*, *Hb2*, and *RXR*), relative to *G3PDH*, across the adolescent instar in females exposed to TBTL (grey triangles), TBTH (black triangles), or the carrier solvent (open circles).

**Table 2.7** – Two-way ANOVA on mRNA gene responses. ANOVA results (Fisher's coefficient, F, degrees of freedom, df) comparing the response of mRNA gene responses across TBT treatments along the adolescent instar, in de-brooded females just alter the forth moult and in eggs. For mRNA levels treatments did not included eggs.
ns (not significant, p > 0.05), (*) 0.05 < *p* < 0.01, (**) 0.01 < *p* < 0.001, (***) *p* < 0.001.

| | **Treatment** | | **Time** | | **Treatment x Time** | |
|---|---|---|---|---|---|---|
| | **df** | **F** | **df** | **F** | **df** | **F** |
| **Genes** | | | | | | |
| ***HR3*** | 2,47 | 4.7* | 4,47 | 30.0*** | 8,47 | 3.1** |
| ***EcR B*** | 2,47 | 17.1*** | 4,47 | 26.8*** | 8,47 | 26.1*** |
| ***Neverland*** | 2,48 | 8.2** | 4,48 | 20.2*** | 8,48 | 1.6 ns |
| ***HR38*** | 2,48 | 0.5 ns | 4,48 | 14.9 *** | 8,48 | 1.9 ns |
| ***MET*** | 2,47 | 6.6** | 4,47 | 29.5*** | 8,47 | 3.1** |
| ***SRC*** | 2,47 | 18.8*** | 4,47 | 78.3*** | 8,47 | 9.9*** |
| ***Hb2*** | 2,48 | 2.7 ns | 4,48 | 23.6*** | 8,48 | 8.1*** |
| ***RXR*** | 2,48 | 1.5 ns | 4,48 | 42.7*** | 8,48 | 3.2** |

## 2.5 Discussion

Life history responses of the progeny of females exposed to TBT showed detrimental effects on fitness. Newborn neonates produced by females exposed to TBTH were smaller than those of unexposed females and suffered a higher mortality during their adulthood, which resulted in lower reproductive output and fitness. Offspring produced by females exposed to TBTL, despite being similar in size to those from unexposed females, also had lower survival during their adulthood, produced fewer eggs, and hence had a lower fitness. Eggs produced by females exposed to TBTL and TBTH also had less TG containing PUFA. These results support previous studies indicating that smaller offspring or those having low levels of PUFA are less fit than larger ones having more PUFA (Tessier and Consolatti 1989,1991; Gliwicz and Guisande 1992; Barata and Baird 1998; Wacker and Martin-Creuzburg 2007). Exposure to TBT during a single reproductive cycle (i.e., 3–4 days) resulted in a long-lasting decrease in the females' fitness and reproductive

capacity for at least five consecutive instars. We concluded that disruptive effects of TBT on lipid metabolism reflected negatively in terms of fitness across the F0 generation and its progeny.

Changes in lipid droplet number and size, and hence in stored TG, were visualized using Nile red in *D. magna* individuals. As expected, lipid droplets were bigger and more abundant in females cultured at high food rations than in those reared at low food or starved. The complex dynamics in *Daphnia* lipid droplets described in this work reflects reported cyclic changes in TG during the reproductive cycle (Tessier and Goulden 1982; Zaffagnini and Zeni 1986; Goulden and Place 1990). Triacylglycerols from ingested food accumulated as droplets in the animal during each intermoult interval until a few hours before moulting. Upon release of the eggs into the brood pouch in adults, lipid droplets decreased as TG become allocated to the formation of the new carapace and eggs.

Lipidomic studies during the adolescent instar showed that quantitative changes in lipid droplets were highly correlated to changes in TG levels, as quantified by LC-MS, supporting the argument that lipid droplet dynamics reflect those of TG in *D. magna* individuals (Tessier and Goulden 1982; Tessier *et al.,* 1983; Goulden and Place 1990). This correlation was lost in females exposed to TBTH. These TBTH–treated animals showed lower TG levels than controls during the first hours of the intermoult period, whereas their after-moulting (48 hrs) TG levels were higher than those from control or TBTL groups. In fact, lipid droplets were higher in females exposed to TBT and did not decrease in de-brooded females just after moult. This discrepancy indicates that there was less transfer of TG to egg provisioning and that TG remained stored as lipid droplets in adults. Consequently, levels of TG in the eggs of exposed females were lower than those from their non-exposed counterparts.

A lipid droplet consists of a core of neutral lipids (TG and CE) surrounded by a monolayer of phospholipid and cholesterol, into which specific proteins are embedded or peripherally associated. Little is known about the formation and metabolism of lipid droplets in *Daphnia*, but there is ample information in *Drosophila,* whose metabolism is in many aspects similar to *Daphnia (Campos et al., 2013)*. *Drosophila* lipogenesis occurs in the fat cells and involves most lipid classes (Arrese and Soulages 2010). Female crustaceans convert a proportion of TG into PC to form lipovitellin, which is the major constituent of egg yolk (Lee *et al.,* 2006). Therefore, DG, TG, CE, GP, and lipid droplets must be physiologically linked during the egg-provisioning period in reproductive females, which may explain their similar pattern of response in Figure 2.4.

Transcription levels of genes from the ecdysone and juvenile hormonal signalling pathways indicate that TBT interacts with different receptors implicated in a variety of regulatory pathways. In the present study, the receptor gene *HR3*, which is an ecdysteroid and TBT-inducible gene in daphnids (Hannas and LeBlanc 2010; Wang *et al.,* 2011), was up-regulated in females exposed to TBT just after moulting at 0, 8, and 48 hrs. This gene response corroborates the findings of Wang *et al*., (2011), indicating that TBT synergizes with endogenous levels of ecdysone to produce endocrine toxicity. The transcription of two additional genes involved in the ecdysone signalling hormonal pathway further showed that TBT disrupted the moult signalling pathway. Transcription patterns of the ecdysone receptor (*EcRB*) and *Neverland* genes also increased in females exposed to TBT just after moulting at 0, 8, and 48 hrs. The *Neverland* gene codifies for an oxygenase-like protein that plays a role in the transport and/or metabolism of cholesterol, and hence it is located upstream in the ecdysone pathway (Rewitz and Gilbert 2008; Gilbert and Rewitz 2009). In the present study, mRNA levels of *EcRB, HR3,* and *Neverland* genes were highest just after moulting, which is consistent with previous reported data and reflects the natural hormonal behaviour during a moult cycle (Kato *et al.,* 2007). The three gene markers selected for the juvenile hormone signalling pathway (*MET, SRC,* and *Hb2*) increased their transcription levels in the presence of TBT just after moulting at 0 and/or at 48 hrs. There is no reported information on gene transcription responses of *MET* and *SRC* in *Daphnia*, but those reported for the hemoglobin gene (*Hb2*) were enhanced by juvenoids (Gorr *et al.,* 2006) (see Figure 2.8 in supporting information). Results of the present study support the argument that TBT also activates the JH receptor pathway, with the effect being greater when ecdysone levels were the highest. Nevertheless, TBT did not induce production of males in *D. magna* (data not shown), a trait characteristic of juvenoids (Wang *et al.,* 2011), which means that TBT was interacting with the JH signalling pathway rather than acting as a juvenoid. Transcription levels of *RXR* mRNA increased in TBT-treated females relative to the controls. We therefore conclude that TBT activates these three signalling pathways, presumably through the already proposed interaction with RXR (Wang *et al.,* 2007; Wang and LeBlanc 2009).

## 2.6 Conclusions

TBT disrupted lipid homeostasis in *D. magna* individuals by impairing the transfer of GP and TG to eggs and consequently increasing the storage of lipids in lipid droplets in

adults. These responses were quite similar to those reported for adipocytes in vertebrates, but their physiological consequences differed. Observed changes in the lipidome in eggs translated into smaller offspring hatched from those eggs that, later in life (during adulthood), showed impaired survival and were consequently less fit. Adult females exposed to TBT during just the adolescent instar had their reproduction and growth impaired in subsequent instars and hence was also less fit. Transcription patterns of the studied genes indicated that TBT activated the transcription of RXR receptor, as it has been reported in gastropods and vertebrates, and altered the ecdysone and JH receptor signalling pathways as reported in other studies. Whether such effects are directly or indirectly related to the observed effects on lipid metabolism and life-history performance requires further study.

## 2.7 Supporting information

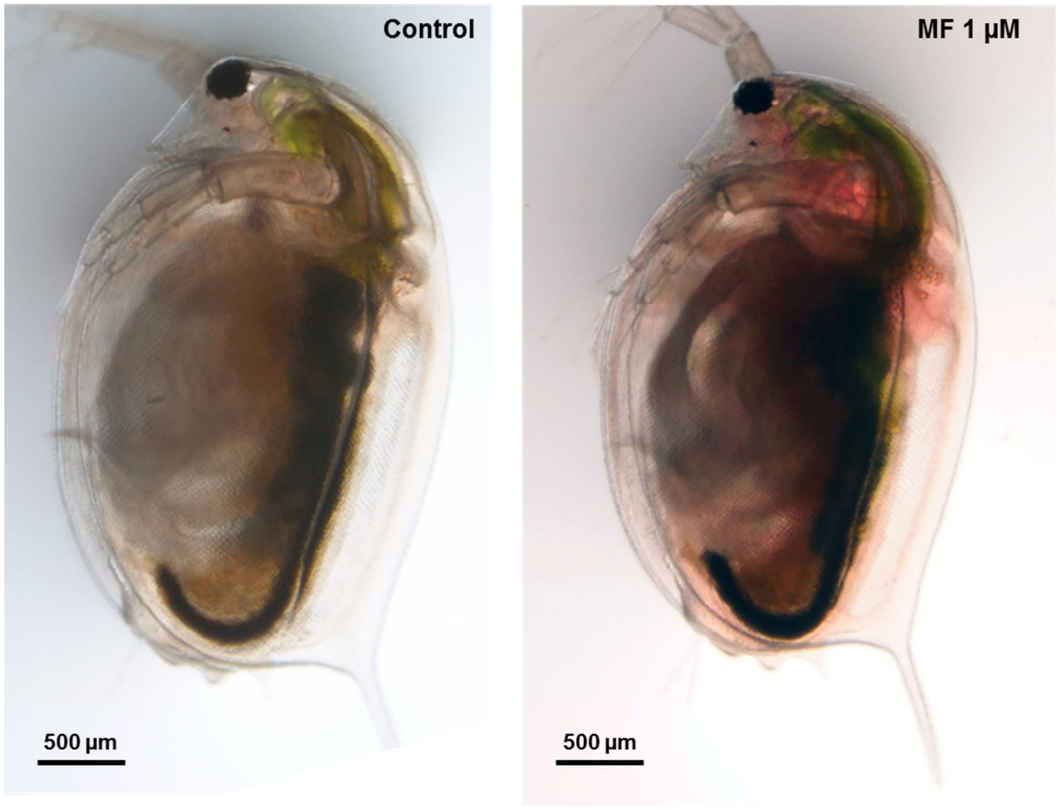


**Figure 2.8** – *Daphnia magna* when exposed to 1 µM of methyl farnesoate (MF) exhibit high levels of hemoglobin, when compared with the control.

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# Chapter III

Compounds altering fat storage in *Daphnia magna*

## Chapter III – Compounds altering fat storage in *Daphnia magna*

# 3 Do obesogens occur in non-vertebrates? - Compounds altering fat storage in *Daphnia magna* [1]

## 3.1 Abstract

The analysis of obesogenic effects in invertebrates is limited by our poor knowledge of the lipid metabolic pathways. A recent study showed that tributyltin (TBT) activated the ecdysteroid, JH and retinoic X receptor signalling pathways, and disrupted the dynamics of neutral lipids in the crustacean *Daphnia magna* impairing the transfer of triacylglycerols (TGs) to eggs and hence promoting their accumulation into lipid droplets in post-spawning females. Tributyltin disruptive effects translated into a lower fitness for offspring and adults. The present study aims to addresses the disruptive effects of existing compounds alone and in mixtures on the lipid droplet dynamics in post-spawning females and their health effects. *Daphnia magna* individuals were exposed 14 chemicals that included vertebrate obesogens (TBT; TPT, triphenyltin; BPA, bisphenol A; NP, nonylphenol; DEPH, di-2-ethylhexyl phthalate), other contaminants known to affect arthropods (PP, pyriproxyfen; FEN, fenarimol; MT, methoprene; EM, emamectin benzoate; TEB, tebufenozide and FX, fluoxetine), as well as the natural hormones methyl farnesoate (MF), 20-Hydroxyecdysone (20E) and ponasterone A (PoA). Reproductive effects were assessed by life history analysis methods. Quantitative changes in lipid droplets were studied using Nile red staining, which showed a close relationship with whole organism levels of TGs. Ten compounds disrupted storage lipids in a concentration related manner enhancing (TBT, MF, PP, BPA, 20E) or decreasing (NP, FEN, EM, MET, FX) their accumulation into lipid droplets in post-spawning females. Joint binary mixture effects indicated that the studied compounds acted on lipid droplets additively and non-additively disruption the signalling pathways of ecdysone, methyl farnesoate and retinoic X receptors. In eight compounds disruptive effects translated into detrimental effects in growth and or reproduction.

**Keywords:** obesogen, lipid disruptor, nuclear receptor, arthropod, reproduction, juvenile receptor

[1]Part of the results of this chapter were published in: **Jordão R.**, Garreta E., Campos B., Lemos M. F. L., Soares A. M. V. M., Tauler R. and Barata C., (2016). "Compounds altering fat storage in *Daphnia magna*." Science of the Total Environment 545–546: 127-136. DOI: 10.1016/j.scitotenv.2015.12.097 (article in annex).

## 3.2 Introduction

In all animals the maintenance of energy reserves is vital, so animals store energy as fat to survive food shortages. When food is not limiting disruption of lipid storage equilibrium may lead to obesity. Recent studies have suggested the involvement of EDCs in the obesity epidemic in mammals (Grün and Blumberg 2006). In many cases, obesogens exposures, in uterus, lead to obesity later in life. Obesity increases the risk of coronary artery diseases, diabetes and related heath detrimental effects, such as hypertension and lipidemia (Grün and Blumberg 2006; Sharma and Staels 2007). There are widely use chemicals that at low doses can promote weight gain. The list is extensive and includes among other, chemicals such as phthalates, BPA, alkylphenols and organotin pesticides (Grün and Blumberg 2009).

Obesogenic effects have often been associated to the disruption of PPARγ signalling pathway that together with its heterodimeric partner RXR, are master regulators of adipocyte differentiation and lipid metabolism in vertebrates (Grün and Blumberg 2009). Although PPAR has not been described outside deuterostomes, a recent study showed that the crustacean *Daphnia magna* responds to the obesogen TBT, through the RXR signalling cascade, thus, increasing the scope of the search for obesogenic effects (Jordão *et al.,* 2015). Tributyltin disrupted the dynamics of neutral lipids impairing the transfer of TGs to eggs and hence promoting their accumulation in lipid droplets inside fat cells in post-spawning adult females. Tributyltin disruptive effects translated into a lower fitness for offspring and adults. Gene transcripts indicated that TBT increases mRNA levels of the RXR gene, and enhanced those of key genes belonging to the signalling pathways of the EcR and MfR (Jordão *et al.,* 2015).

However, there are other mechanisms of endocrine disruption of lipid regulation rather than those based on PPAR and RXR signalling pathways (Grün and Blumberg 2009). Obesogens may also disrupt neuroendocrine signalling pathways that control appetite (i.e. neuropeptide Y that stimulates appetite) and energy homeostasis (TOR, target of rapamycin or AMPK, AMP-activated kinase) among other processes. Psychiatric drugs such as serotonin reuptake inhibitors, BPA, NP and some phthalates also affect the expression of neuropeptide Y an hence change feeding behaviour in rodents (Grün and Blumberg 2009).

*Daphnia magna* is a long established model species in regulatory toxicology with an important role in determining chemical safety criteria around the world, and is the most

commonly used system for ecotoxicological testing worldwide. *Daphnia* shares the most number of genes with human than any other sequenced invertebrate (Colbourne *et al.,* 2011), which also includes several molecular mechanisms that regulate fat storage. *Daphnia* has a functional RXR receptor that is activated with the model obesogen TBT, the cellular fuel gauge mechanisms (TOR), protein kinase complex regulating fatty acid catabolism and glycolysis (AMPK), and neuroendocrine regulators such as insulin, serotonin, dopamine and neuropeptides that regulate food-related behaviour and energy metabolism (Wang *et al.,* 2007; Wang and LeBlanc 2009; Boucher *et al.,* 2010; Dircksen *et al.,* 2011; Campos *et al.,* 2012a; McCoole *et al.,* 2012; Sheng *et al.,* 2012; Campos *et al.,* 2013). *Daphnia magna* also share with insects additional potential mechanisms of fat regulation. In *D. magna* like in other crustacean and arthropods, lipid storage dynamics varied along the moult and reproduction cycle (Tessier and Goulden 1982) that are regulated by the ecdysone and JH receptor signalling pathways (hereafter referred as MF). *Daphnia magna* has a functional HR96 receptor, which in *Drosophila* has a key role in dietary fat utilization and metabolism (Karimullina *et al.,* 2012). Ecdysone, exert its effects through the interaction with EcR, known to heterodimerize with RXR and to bind to the promoters of ecdysone-regulated genes (LeBlanc 2007; Wang and LeBlanc 2009). Recent findings indicate that the MfR in *Daphnia* is a complex of two nuclear proteins of the bHLH-PAS family of transcription factors: the MET and the SRC protein (LeBlanc *et al.,* 2013; Miyakawa *et al.,* 2013). Tributyltin, which is an agonist of RXR together with MF and other juvenoids, enhanced the ecdysteroid-dependent activation of the EcR:RXR heterodimer (Wang and LeBlanc 2009). Transactivation assays showed also that the nuclear HR96 receptor was activated by juvenoids and it was repressed by psychiatric drugs (FX) that targeted neuroendocrine systems (Karimullina *et al.,* 2012). Thus, vertebrate and non-vertebrate EDCs may alter lipid homeostasis in the crustacean *D. magna* by interacting with RXR, EcR, MfR receptors and/or neuroendocrine signalling pathways.

The quantity and quality of ingested food together with body size are key determinats of fat storage in *Daphnia* (Tessier and Goulden 1982; Guisande and Gliwicz 1992; Goulden and Place 1993; Wacker and Martin-Creuzburg 2007; Martin-Creuzburg and von Elert 2009). Food intake also regulates the amount of resources invested to growth and reproduction and is the undelayed mechanisms behind many reported toxic effects on reproduction in *Daphnia* (Barata and Baird 2000; Barata *et al.,* 2004). This means that endocrine disruption of fat storage in *D. magna* and its life-history

consequences have to be assessed at lower concentrations than those impairing growth and food intake (Barata *et al.,* 2004).

The aim of this study is to assess if chemicals alter the storage lipids (i.e. TGs) accumulated in lipid droplets in *D. magna* and its life-history responses. Our departure hypothesis is that many compounds behave phenotypically like TBT (Jordão *et al.,* 2015), altering the transfer of storage lipids to eggs, promoting their accumulation inside lipid droplets in post-spawning females, and hence affecting growth and reproduction. It was hypothesized that vertebrate and non-vertebrate chemicals that are known to interact with RXR, EcR, MfR receptors, and/or neuroendocrine signalling pathways, may alter lipid homeostasis in the crustacean *D. magna*. Nile red fluorescence assay was used to quantify storage lipids inside lipid droplets since this measure was closely related to accumulated TGs in lipid droplets (Jordão *et al.,* 2015). We also aimed to confirm phenotypically, using Nile red fluorescence, previous findings on gene transcription (Jordão *et al.,* 2015) supporting the hypothesis that accumulation of storage lipids inside lipid droplets in *D. magna* is mediated or regulated by RXR, MfR and EcR signalling pathways. Experiments included single and binary exposures of contaminants and known agonists and antagonists of RXR, EcR and MfR. Mixtures combined similar an dissimilar acting chemicals and hence allowed to compare joint responses with those predicted by the concentration and independent action models (Altenburger *et al.,* 2003).

## 3.3 Material and Methods

### 3.3.1 Studied compounds

Studied compounds included three natural hormones (LeBlanc 2007): the juvenile hormone methyl farnesoate (MF, CAS 10485-70-8), the moulting hormones 20-hydroxyecdysone (20E, CAS 5289-74-7) and ponasterone A (PoA, CAS 13408-56-5); pesticides that act as juvenoids like pyriproxyfen (PP, CAS 95737-68-1), methoprene (MT, CAS 40596-69-8); agonists of RXR like tributyltin (TBT, CAS 1461-22-9) and triphenyltin (TPT, CAS 639-58-7) (LeBlanc 2007); the ecdysone agonists emamectin benzoate (EM, CAS 155569-91-8) and tebufenozide (TEB, CAS 112410-23-8) (Retnakaran *et al.,* 2003; Rodríguez *et al.,* 2007); the ecdysone antagonist fenarimol (FEN, CAS 60168-88-9) (Mu and LeBlanc 2004b); contaminants with reported obesogenic effects: nonylphenol (NP, CAS 84852-15-3), bisphenol A (BPA, CAS 80-05-7), di-2-ethylhexyl phthalate (DEHP, CAS 117-81-7) (Grün and Blumberg 2006; Hao *et al.,* 2013), and the fat regulator

fluoxetine (FX, CAS 56296-78-7) (Lemieux *et al.,* 2011). All the compounds were obtained from Sigma Aldrich (U.S.A/Netherlands) except MF, which was supplied by Echelon Bioscience, Utah, U.S.A. For a brief description of compounds see Table SI in Annex.

### 3.3.2 Experimental animals

All experiments were performed using the well-characterized single clone F of *D. magna* maintained indefinitely as pure parthenogenetic cultures (Barata and Baird 1998). Individual cultures were maintained in 100 mL of ASTM hard synthetic water at high food ration levels ($5x10^5$ cells/mL of *Chlorella vulgaris*, respectively), as described in Barata and Baird (1998).

### 3.3.3 Experimental design

Experiments were initiated with newborn neonates < 4-8 hrs old obtained from synchronized females cultured individually at high food ration levels. Groups of five neonates were reared in 100 mL of ASTM hard water under high food ration conditions until the end of the third juvenile instar (about 4-8 hrs before moulting for the third time). At this point, juveniles were exposed individually in 100 mL test medium to selected chemicals and used in lipid droplet and life-history experiments. Exposures were conducted during the adolescent instar, which is the instar where the first brood is formed in the ovaries. Females used for lipid droplet analyses were sampled just after their fourth moult and having released their first clutch of eggs into the brood pouch. For life history trait analyses exposed females were cultured an additional instar in control conditions without pollutants to allow eggs to develop and be released as neonates in the first clutch after the fifth moult (Figure 3.1). The test medium was renewed every other day. The studied chemicals were assayed at concentrations not impairing survival, moulting or food acquisition. Therefore, for each compound preliminary range, finding tests were performed to establish the tested concentration rank, which are indicated in Table 3.1.

The range finding tests were conducted using the same experimental set up as Nile red assays, which means that were initiated with adolescent instar and ended when females just moult and deposited their first brood into their brood pouch. Measured responses included survival, delay on moulting and feeding impairment. Feeding experiments were conducted during the same period but were limited to 24 hrs period within the third and fourth moult. Under our culture conditions females usually enter the

adolescent instar in their third moult. Feeding assays followed previous procedures (Barata *et al.,* 2006). Briefly, groups of five adolescent instar females were transferred into test vessels filled with 100 mL of the appropriate treatment solution plus food. Three replicate test vessels filled with the same culture medium but with no animals were used as blank replicates to measure that algal densities did not increase during feeding assays. The mean initial algal cell concentration of the experimental vessels was measured at the start and end of tests. Cell density was estimated from absorbance measurements at $\lambda$ = 650 nm in a dual-beam spectrophotometer (Uvikon 941) using standard calibration curves based on at least 20 data points, with an $r^2 > 0.98$. Individual feeding rates (cells x animal$^{-1}$ x h$^{-1}$) were determined as the change in cell density during 24 hrs according to the method given by Allen *et al*., (1995) and converted to percentage feeding rates relative to control treatments (%). The median concentration effect estimated was obtained by fitting percentage values to equation 1 setting the Max value to 100 (see Data analyses section).

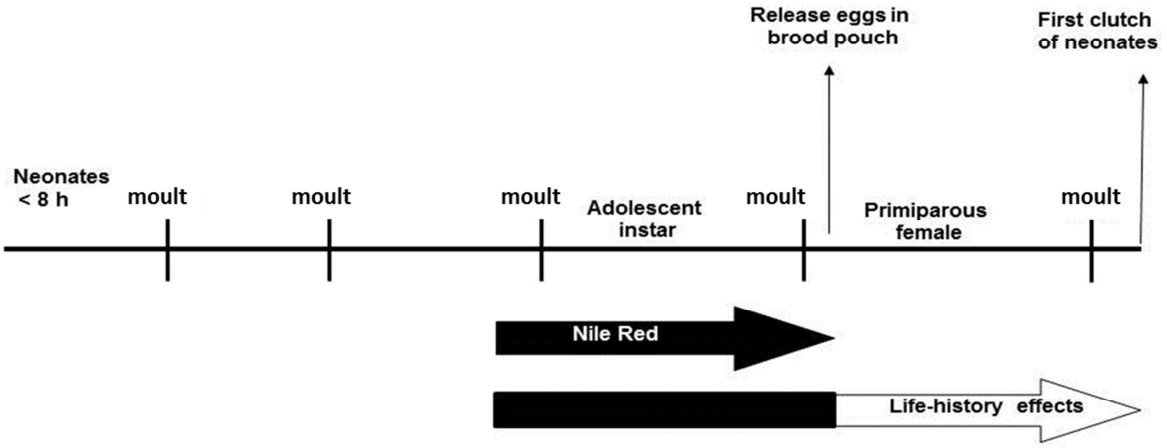


**Figure 3.1** – Test protocol used to expose *D. magna* and to measure changes in Nile red fluorescence and life-history effects. *Daphnia magna* individuals were fed on high food during their growth until 2/3 of their third juvenile instar and then exposed to the studied compounds only during the adolescent instar (black arrows). To get the body length of females at first reproduction and the number and size of neonates released in the first clutch, females were cultured without contaminants an additional instar (white arrow).

**Table 3.1** – Nile red fluorescence assays results of the 14 tested compounds. Includes the tested range, lowest observed effect concentrations (LOEC) based on ANOVA and Dunnet's test, and maximal fluorescence changes relative to controls (Max, %). For 10 compounds $EC_{50}$ and (p) are the regression parameters of fitting Nile red fluorescence to equation 1. All regression models and coefficients were significant ($p < 0.05$) unless stated. Negative Max values mean inhibition of fluorescence relative to unexposed solvent control individuals. *n*, sample size; SE, standard error.

| | | | Nile red fluorescence | | | | |
|---|---|---|---|---|---|---|---|
| | Range | LOEC | Max ± SE | $EC_{50}$ ± SE | p ± SE | $r^2$ | *n* |
| TBT (nM) | 0.003-4.6 | 0.3 | 118 ± 10 β | 2.4 ± 0.2 | 3.7 ± 0.6 | 0.87 | 90 |
| MF (µM) | 0.04-1.0 | 0.2 | 83 ± 14 β | 0.3 ± 0.1 | 1.3 ± 0.3 | 0.72 | 88 |
| BPA (µM) | 1.31-43.8 | 10.9 | 51 ± 15 α | 22.4 ± 2.0 | 2.4 ± 0.5 | 0.67 | 60 |
| PP (nM) | 0.16-9.3 | 0.3 | 49 ± 16 α | 1.2 ± 0.2 | 0.8 ± 0.1 | 0.61 | 70 |
| 20E (µM) | 0.02-1.0 | 0.2 | 49 ± 4 β | 0.3 ± 0.03 | 2.6 ± 0.7 | 0.69 | 78 |
| FEN (µM) | 0.15-0.9 | 0.6 | -29 ± 3 β | 0.5 ± 0.05 | 7.1 ± 6.7 | 0.79 | 40 |
| FX (µM) | 0.05-0.9 | 0.2 | -31 ± 5 β | 0.2 ± 0.05 | 3.0 ± 0.8 | 0.45 | 65 |
| EM (nM) | 0.03-0.2 | 0.3 | -32 ± 4 β | 0.2 ± 0.01 | 9.9 ± 3.0 | 0.64 | 40 |
| NP (µM) | 0.034-0.3 | 0.1 | -32 ± 4 β | 0.1 ± 0.01 | 3.9 ± 1.1 | 0.57 | 40 |
| MT (nM) | 9.7-322.1 | 40.2 | -36 ± 8 β | 41.6 ± 12.2 | 1.1 ± 0.4 | 0.53 | 62 |
| TPT (nM) | 0.6-5.2 | 2.6 | -14 ± 3 α | | | | |
| PoA (nM) | 2.1-21.5 | ns | 3 ± 4 α, ns | | | | |
| TEB (µM) | 0.2-0.9 | ns | 2 ± 4 α, ns | | | | |
| DEPH (µM) | 0.025-0.51 | 0.51 | -34 ± 4 α | | | | |

α, β estimated Max values from observed responses or regression models, respectively. ns, not significant ($p < 0.05$) different than 0.

### 3.3.4 Three sets of experiments were performed

Effects of single exposures on the accumulation of storage lipids inside lipid droplets were performed, using from 4 to 9 concentrations of the 14 studied compounds, which were dosed using acetone as a carrier (0.1 mL/L). A solvent control containing the same amount of acetone (0.1 mL/L) was then included for comparison purposes. None solvent controls were not used since previous studies showed that the tested acetone concentrations had no affect the lipid droplets and life-history responses (Jordão *et al.,* 2015). At the end of exposures, animals were sampled, their body length measured and then processed for lipid droplet quantification using Nile red fluorescence according to previous procedures (Jordão *et al.,* 2015).

The second experiment aimed to determine joint effects of binary mixtures of selected compounds with agonists of the MfR (MF) and RXR (TBT) and with the anti-ecdysteroid (FEN) to test the hypothesis that accumulation of storage lipids inside lipid droplets in *D. magna* is mediated by RXR, EcR and MfR signalling pathways. Mixture combinations included low and high concentration effect responses of selected compounds and followed a two-way ANOVA design that allowed for testing statistically for the null hypothesis that joint responses were additive and predicted by the independent action model, which means that compounds act dissimilarly altering storage lipids accumulation in lipid droplets. Deviations from the null hypothesis were further tested comparing observed joint effects with those predicted by independent action and concentration addition concepts to asses if concentration addition is addictive, antagonistic or synergistic (Altenburger *et al.,* 2003). Pairings included dissimilar acting chemicals enhancing lipid droplet accumulation (TBT/MF, BPA/MF), and similar acting ones (PP/MF). The anti-ecdysteroid FEN was combined with TBT, MF and with the mixture TBT/MF to test the premise that naturally occurring levels of 20E are need it to activate the whole process of storage lipids accumulation into lipid droplets in post-spawning females.

The third experiment studied effects of single exposures to the selected chemicals during the adolescent instar (i.e. 3 days) on the body length of first reproducing females and on the number and size of offspring of the first clutch. Following exposures, females were cultured individually in 100 mL of ASTM hard water at high food conditions without contaminants and the number and size of the offspring released in the first clutch measured (Figure 3.1). Two to three exposure concentrations were selected for each tested chemicals that included measurements of low and high effects on storage lipids

accumulation inside lipid droplets. Treatments were replicated ten times. Body length measurements were performed from the head to the base of the spine using a Nikon stereoscope microscope (SMZ 150, Nikon, Barcelona, Spain) and the ImageJ software (http://rsb.info.nih.gov/ij/).

### 3.3.5 Nile red assay to quantify storage lipids inside lipid droplets

Quantification of storage lipids in the lipid droplets follow previous methods (Jordão *et al.,* 2015). Briefly, Nile red stock solutions were prepared in acetone and stored protected from light. Just before use, the working solution was obtained by dilution of stock solution to 1.5 µM in ASTM hard water. Live individuals were then exposed to Nile red working solution in the dark for 1 hr at 20 ºC. After incubation, animals were place in 100 mL ASTM for 1 min to allow clearance of Nile red residuals. Following clearance, animals were placed individually in 1.5 mL microcentrifuge tubes, the remaining water removed and sonicated in 300 µL of isopropanol. The homogenized extract was then centrifuged at 10,000 x *g.* We used 200 µL of supernatant to measure Nile red fluorescence using an excitation/emission wavelength 530/590 nm and a fluorescence microplate reader (Synergy 2, BioTek, USA). Each treatment had 1 animal per sample (10 replicates in total). For each quantification and treatment, 10 blanks (non-exposed animals to Nile red) were used to account for background levels of fluorescence.

### 3.3.6 Determination of triacylglycerols

Analyses of TGs were restricted to controls and low and high concentrations of TBT, PP, BPA, MF, PP and FEN treatments showing moderate and high effects on Nile red fluorescence. The aim of these assays was to establish a relationship between measured Nile red fluorescence levels and whole body concentrations of TGs. Concentrations of TGs were determined using a commercial Kit (Spinreact S.A., Sant Esteve De Bas, Spain) based on a peroxidase coupled method for the colorimetric detection of TGs followed hydrolysis, phosphorylation of glycerol and oxidation to produce hydrogen peroxide (McGowan *et al.,* 1983). Briefly, pools of five de-brooded adolescent *D. magna* individuals that just released their first clutch of eggs into the brood pouch were homogenized in 250 µL of 0.1 M phosphate buffer saline (pH 7.2) with BHT 0.01 %, as an antioxidant. A volume of the supernatant (20 µL) was then mixed with the assay reagents, incubated in the dark for 10 min and measured spectrophotometrically at 505 nm

according to the manufacturer's protocol. Quantification of TG levels was performed from a triacylglycerol calibration standard curve of 8 points ($r^2$ > 0.99) following the manufacturer's protocol.

### 3.3.7 Chemical analyses

Dissolved oxygen (DO) concentration was measured using an oxygen electrode model 1302 (Strathkelvin Instruments, Glasgow) and 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). Mean oxygen levels were 96 ± 2.4 % of saturation and pH values 7.2 ± 0.2 for all experiments.

The residue analyses of organotin compounds in water in freshly prepared and old (24 hrs) test solutions were restricted to two exposure levels. Actual TPT and TBT concentrations in test solutions of 0.3 and 3 nM were measured as total Sn by means of Perkin Elmer model Elan 6000 ICP-MS (Barata *et al.,* 2005). Stability of the rest compounds during the test was confirmed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Fluoxetine residues were analysed following previous procedures (Campos *et al.,* 2012a) using a Waters 2690 HPLC separations module (Mildford, MA, USA) equipped with a Purospher Star RP-18 end capped column (125 mm × 2.0 mm, particle size 5 µm, Merck, Darmstadt, Germany) connected to a Micromass Quattro triple quadrupole mass spectrometer equipped with a Z-spray electrospray interface (Manchester, UK). The rest of chemical residues were measured using an Acquity UPLC system (Waters, Mildford, MA, USA) connected to a Triple Quadruple Detector Acquity. The analysis was performed on an Acquity UPLC BEH $C_{18}$ (1.7 µm, 2.1 x 100 mm) supplied by Waters (Waters, Mildford, MA, USA). The $C_{18}$ column was equilibrated at 40 °C. Chromatographic separation was carried out with a mobile phase consisting of methanol with 0.1 % formic acid (eluent A) and ultra-pure water with 0.1 % formic acid (eluent B) at a flow rate of 0.3 mL/min. The elution started with 20 % of eluent A, followed by a 4-min gradient to 75 % of eluent A and a 2 mins gradient to 100 % of eluent A, held for 1 min, and then back to the initial conditions within 6 mins. An injection volume of 50 µL was used.

Instrument control, data acquisition and evaluation were performed using MassLynx software (v.4.1) with QuanLynx program (Waters). The ionization source parameters for the analysis were: electrospray source block and desolvation temperature: 150 and 350 °C respectively, capillary and cone voltages: 3 kV and 30 V respectively,

cone nitrogen gas flow and desolvation gas: 43 and 650 L/h. Mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with unit mass resolution on mass analyser. Identification criterion of the target compounds was based on: (a) LC retention of the analyte compared to that of a standard (± 2 %) and (b) the ratio of abundances of two specific precursor ion → product ion transitions (within 10 % of the ratios obtained for the standard). Optimised LC and MS/MS parameters for each analyte were based on reported information (Martínez Vidal *et al.,* 2010; Li and Duan 2011; Liu *et al.,* 2011; Belenguer *et al.,* 2014) and are depicted in Table 3.2. Quantification was based on external calibration standard 8 point curves ($r^2$ > 0.98). Limits of detection and quantification defined as the minimum detectable amount of analyte with a signal to noise ratio of 3:1 and 10:1, respectively, were determined from the spiked water samples. Detection limits are in Table 3.3.

**Table 3.2** – Optimized mean retention time and MS/MS parameters for confirmation/quantification of compounds.

| Chemical | Ionization mode | Mean Retention Time (min) | Cone Voltage (V) | Quantification Transition[a] | Confirmation Transitions[a] |
|---|---|---|---|---|---|
| Ponasterone A | Positive | 2.07 | 28 | 465 → 249 (24) | 465 → 283 (20) |
| Tubefenozide | Positive | 3.29 | 19 | 353 → 297 (7) | 353 → 133 (22) |
| 20-hydroxyecsyterone | Positive | 3.32 | 47 | 481 → 445 (19) | 481 → 371 (14) |
| Nonylphenol | Negative | 4.28 | 46 | 219 → 133 (28) | 219 → 147 (29) |
| Emamectin benzoate | Postitive | 4.31 | 48 | 886 → 158 (33) | 886 → 126 (38) |
| Methoprene | Positive | 4.59 | 18 | 311 → 279 (6) | 311 → 191 (14) |
| Bisphenol A | Negative | 4.63 | 39 | 227 → 133 (40) | 227 → 93 (30) |
| DEPH | Positive | 5.00 | 26 | 391 → 149 (27) | 391 → 113 (8) |
| Fenarimol | Negative | 5.37 | 32 | 329 → 216 (10) | 329 → 110 (19) |
| Piriproxyfen | Positive | 5.71 | 28 | 322 → 96 (20) | 322 → 227 (10) |
| Methyl farnesoate | Positive | 6.97 | 28 | 251 → 95 (27) | 251 → 191 (13) |
| Fluoxetine[b] | Positive | 12.9 | 30 | 310 → 44 (50) | 310 →148(10) |

[a] Collision energies are given in brackets (eV), [b] Data from other analytical method (Campos *et al.,* 2012a)

**Table 3.3** – Nominal and measured concentration levels (nM or µM) of the studied compounds in ASTM hard water in freshly prepared (time 0 hrs) and old test solutions (time 48 hrs). *n* = 4. Limits of detection (LOD) are also reported.

| | | Measured time 0 hrs | | Measured at time 48 hrs | | |
|---|---|---|---|---|---|---|
| Compound | Nominal | Mean | SD | Mean | SD | LOD |
| 20E (µM)[a] | 0.021 | 0.022 | 0.001 | 0.017 | 0.001 | 0.005 |
| | 1.000 | 0.963 | 0.056 | 0.791 | 0.025 | |
| MF (µM)[a] | 0.100 | 0.085 | 0.012 | 0.042 | 0.008 | 0.014 |
| | 0.998 | 0.902 | 0.127 | 0.550 | 0.014 | |
| PoA (nM)[a] | 21.523 | 21.652 | 1.055 | 23.309 | 0.925 | 0.043 |
| MT (µM)[a] | 0.107 | 0.094 | 0.010 | 0.074 | 0.012 | 0.002 |
| | 0.322 | 0.307 | 0.046 | 0.215 | 0.042 | |
| PP (nM)[a] | 5.000 | 4.510 | 0.046 | 3.315 | 0.072 | 0.052 |
| | 10.000 | 9.307 | 0.871 | 8.515 | 0.884 | |
| FEN (µM)[a] | 0.151 | 0.185 | 0.001 | 0.176 | 0.003 | 0.004 |
| | 0.906 | 0.970 | 0.031 | 0.956 | 0.009 | |
| EM (nM)[a] | 0.282 | 0.254 | 0.023 | 0.202 | 0.019 | 0.023 |
| TEB (µM)[a] | 0.213 | 0.120 | 0.003 | 0.149 | 0.005 | <0.001 |
| | 0.851 | 0.686 | 0.032 | 0.750 | 0.006 | |
| NP (µM)[a] | 0.100 | 0.098 | 0.010 | 0.094 | 0.010 | 0.008 |
| | 0.300 | 0.321 | 0.040 | 0.259 | 0.016 | |
| BPA (µM)[a] | 10.951 | 11.025 | 0.153 | 10.520 | 0.982 | 0.009 |
| | 43.804 | 43.959 | 0.652 | 44.270 | 1.522 | |
| DEPH | 100 | 117.215 | 1.062 | 107.215 | 3.810 | <0.001 |
| FX (µM)[b] | 0.129 | 0.125 | 0.008 | 0.121 | 0.010 | <0.001 |
| | 1.000 | 0.946 | 0.054 | 0.913 | 0.005 | |
| TBT (nM)[c] | 0.300 | 0.321 | 0.256 | 0.269 | 0.150 | 0.153 |
| | 3.000 | 2.980 | 0.321 | 2.950 | 0.289 | |
| TPT (nM)[c] | 0.300 | 0.301 | 0.156 | 0.271 | 0.171 | 0.129 |
| | 3.000 | 2.968 | 0.302 | 2.945 | 0.268 | |

Analyses were conducted using an: [a] Acquity UPLC system (Waters, Mildford, MA, USA), [b] Waters 2690 HPLC and [c] ICP-MS. See further explanation in text.

### 3.3.8 Data analyses

Predicted values of studied individual components were estimated considering percentage fluorescence relative to control treatments (R), and by fitting observed responses to the modified non-linear Hill regression model of equation 1.

$$R(c_i) = \frac{Max}{100 + \left(\frac{EC50}{c_i}\right)^p} \qquad (eq.1)$$

Where

$R(c_i)$ – Percentage fluorescent change (%) at concentration $c_i$ relative to controls, which was fixed to 0

Max – maximal fold increase in %.

$c_i$ – concentration of compound (i)

p – is the Hill index

$EC_{50}$ - the concentration of compound that corresponds to 50 % of the maximal response).

In equation 1, regression parameters were estimated by the Least Square Method using the Levenberg-Marquardt algorithm and the software Sigmaplot v13 (Systat Software Inc., San José, California). The standard error of each estimated parameter was then calculated as the standard deviation of the least square estimates.

Adequacy or deviation of joint effects for binary mixture combinations from predicted values considering the concentration addition (CA) and independent action (IA) model were determined following previous procedures (Thienpont *et al.,* 2013). The total effect ($R_{mix}$) caused by a binary mixture with given concentrations of constituents c1 and c2 can be estimate by the CA concept using equation 2.

$$1 = \frac{c1}{F1^{-1}(R_{mix})} + \frac{c2}{F2^{-1}(R_{mix})} \qquad (eq.2)$$

$$\text{with } F_i^{-1} = \frac{EC50_i}{\left[\frac{\max_i}{R_{mix}} - 1\right]^{1/pi}}$$

Where $F^{-1}$ is the inverse function of the individual concentration response regression curves of the mixture constituent (i) (i.e. 1 and 2). The value of $R_{mix}$ satisfying this equation has to be solved iteratively using, for example, the Solver–subroutine in the Microsoft(R) EXCEL Analysis Toolpak. Alternatively, IA predicts for combined effects ($R_{mix}$) of binary stressors were estimated from observed effects in the individual constituents 1 and 2, as follows (Faust *et al.,* 2003):

$$R_{mix} = E(c1) + E(c2) - E(c1)xE(c2) \qquad (eq.3)$$

Where E ($C_i$) is the observed percentage fluorescent change of constituent (i) alone (i.e. 1 and 2) relative to controls.

Testing the null hypothesis of independent action for binary combinations can be implemented by determining the significance of the interaction in the two-way ANOVA's carried out on log transformed observational data. A significant interaction term at the 95 % significance level ($p < 0.05$) found with this ANOVA implies a statistically significant deviation from IA (De Coninck *et al.,* 2013).

## 3.4 Results

### 3.4.1 Contaminant levels in water

Most studied compounds were stable in water having measured residue concentrations at 48 hrs within 20 % of nominal values Table 3.3. Less stable compounds were MF, MT and EM that at 48 hrs had concentrations 40, 70 and 70 % lower than nominal ones, respectively. For the sake of clarity, results are referred to nominal vales.

### 3.4.2 Fat storage alteration

The first criterion for *D. magna* to be considered as an arthropod obesogenic model organism is to provide reasonably accurate evaluations of fat storage alteration caused by chemicals. The second criteria, is to show that storage lipids alteration is cause by an endocrine disruption mechanisms rather than is related to other toxicity mechanisms or altered food intake. We previously showed that fluorescence of Nile red in post-spawning females exposed and non-exposed to TBT was closely related with their TG content (Jordão *et al.,* 2015). In Figure 3.2, we show that there is a good linear relationship ($r^2 = 0.86$, $p < 0.05$, $n = 13$) between mean TG levels and mean Nile red fluorescence in post-spawning females in controls and those exposed to low and high levels of TBT, BPA, PP, MF, 20E and FEN.

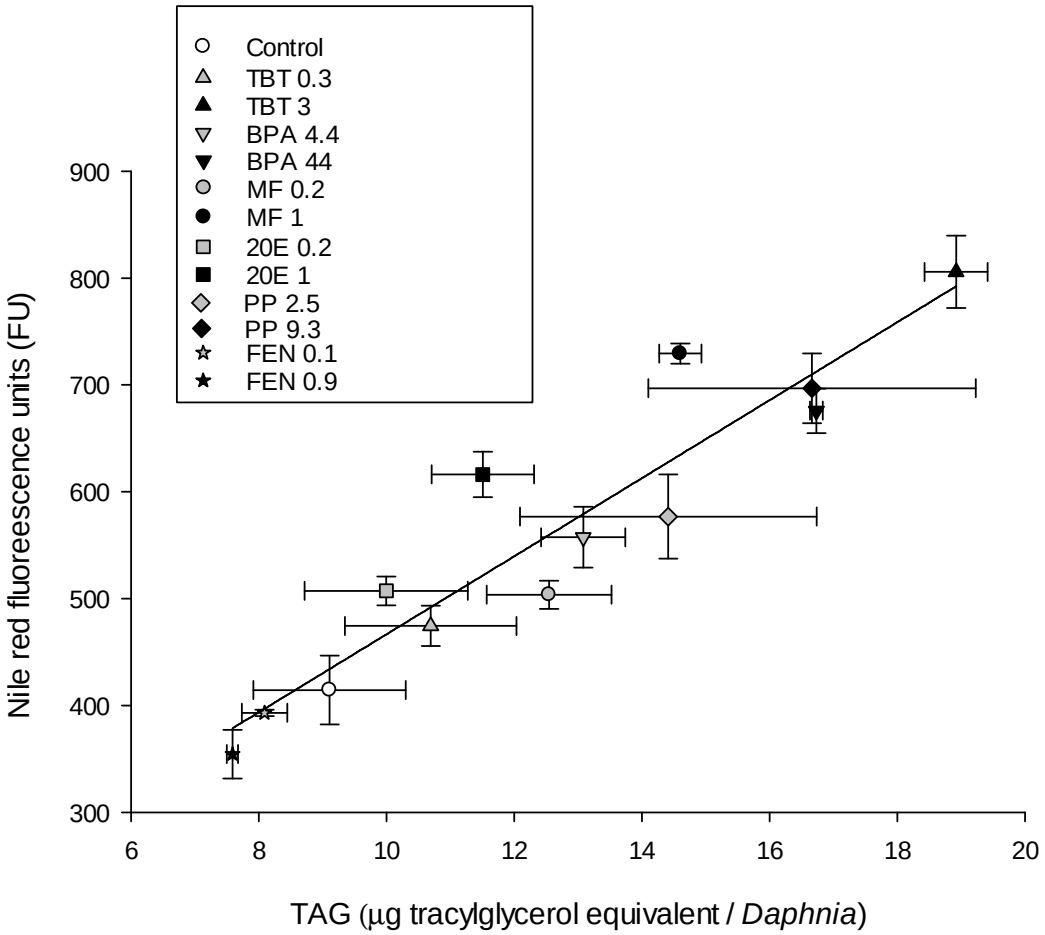


**Figure 3.2** – Relationship between triacylglycerol levels and Nile red fluorescence (FU) of *D. magna* adolescent females. Each symbol is the mean value of five replicates. Errors are SE. In the legend, numbers following symbols indicate concentrations that are in µM except for TBT that are in nM.

None of the tested compounds affected survival at the tested concentrations. Eight out of the 14 tested compounds (MF, PP, 20E, FEN, EM, MT, PoA and TEB) at higher concentrations than those reported in Table 3.1 severely impaired moulting (i.e. Figure 3.7, in supporting information) of exposed females whereas effects on feeding occurred at much higher concentrations (Table 3.4). The remaining four compounds (TBT, TPT, BPA and DEPH) did not affect moulting but impaired food acquisition at higher concentrations than those tested (Table 3.4 and Figure 3.3). Accordingly, for each tested compound we select a concentration range depicted in Table 3.1, that was no lethal, neither impaired, moulting or food intake of exposed *D. magna* females. Within the selected concentration range, the fluorescence of lipid droplets stained with Nile red in *D. magna* individuals exposed to the studied chemicals either increased or decreased relative to the levels of

controls (Figure 3.4; see Figure 3.8 in support information). In 12 out of the 14 compounds tested, fluorescence changes relative to controls were significantly ($p < 0.05$, based on ANOVA tests) different than controls (see LOEC in Table 3.1). Triphenyltin and DEPH despite of reducing the fluorescence of Nile red, also impaired significantly ($p < 0.05$, based on ANOVA) the body length of females at the same concentrations (Table 3.5), therefore, these compounds should be considered false positives since measured fluorescence levels of Nile red are strongly related to body length (Figure 3.5, provides the relationship between fluorescence of Nile red and body length). Body length in the remaining ten compounds was unaffected or affected in the opposite direction (MF, TBT, NP, PP) than changes in fluorescence relative to controls (for further details see those results in Table 3.5), which means that reported disruption of fat storage was independent of measured effects on growth.

Quantitative estimates of storage lipids alteration was performed using Nile red fluorescence changes relative to control treatments and fitting responses to the Hill regression model depicted in equation 1 (Figure 3.4). For BPA and PP it was not possible to reach a max plateau within the studied range and hence max coefficients of equation 1 were parameterized with the observed main values obtained at the highest concentration tested. Fluorescence changes of Nile red relative to controls of selected natural hormones (20E, MF), TBT, BPA and PP increased in a concentration related manner, whereas those of individuals exposed to NP, EM, FX, FEN and MT were decreased. Note that two of the compounds (FEN and EM) had a bi-modal response, enhancing and reducing fluorescence of Nile red at low and high exposure levels, respectively (Figure 3.4). For these two compounds only inhibitory effects were modelled. In all ten compounds fluorescence changes of Nile red across the tested concentrations were accurately fitted by the Hill model ($r^2 \geq 0.5$, $p < 0.001$, Table 3.1). The concentrations at which the studied compounds enhanced or reduced the fluorescence of Nile red (LOEC and $EC_{50}$ in Table 3.1) were over four and two orders of magnitude, respectively Figure 3.4). Tributyltin and BPA were the compounds enhancing the fluorescence of Nile red at the lowest and highest concentrations, respectively. Emamectin benzoate and FEN were the compounds reducing Nile red fluorescence at the lowest and highest concentrations, respectively. Tributyltin increased to a greater extent the fluorescence of Nile red (120 % relative to controls) whereas 20E had the lowest effects (50 %). The inhibitory potential of EM, NP, FX, FEN and MT were quite similar varying from 29 to 36 %.

**Table 3.4** - Concentration effect responses of the studied compounds inhibiting moulting or/and feeding rates (Feeding). Lowest observed effect concentration (LOEC) and $EC_{50}$ are lowest and median concentration effects. SE, are standard errors. Feeding inhibition curves are in Figure 3.3.

| Compound | Moulting (LOEC) | Feeding (LOEC) | $EC_{50}$ | SE |
|---|---|---|---|---|
| TBT (nM) | 9.2 α | 6.1 | 9.3 | 0.4 |
| MF (µM) | 2.1 | 1.3 | 1.6 | 0.03 |
| BPA (µM) | 131.0 α | 65.7 | 99.4 | 3.8 |
| PP (nM) | 18.7 | 50 | 132.2 | 5.5 |
| 20E (µM) | 2.0 | 2 | 8.2 | 3.3 |
| FEN (µM) | 1.8 | 15.1 | 17.6 | 0.2 |
| FX (µM) | 2.9 α | 1.7 | 3 | 0.2 |
| EM (nM) | 0.6 | 1.1 | 2.9 | 0.2 |
| NP (µM) | 0.5 α | 0.5 | 0.5 | 0.03 |
| MT (nM) | 0.6 | 1.3 | 2.1 | 0.07 |
| TPT (nM) | 25.9 | 13.0 | 14.2 | 11.2 |
| PoA (nM) | 43.0 | 86.0 | 376.1 | 108.0 |
| TEB (µM) | 1.7 | nd | nd | nd |
| DEPH (µM) | 6.9 α | 5.1 | 6.9 | 0.3 |

α = No effects of moulting were observed at the highest concentration tested;
nd = no effect on feeding.

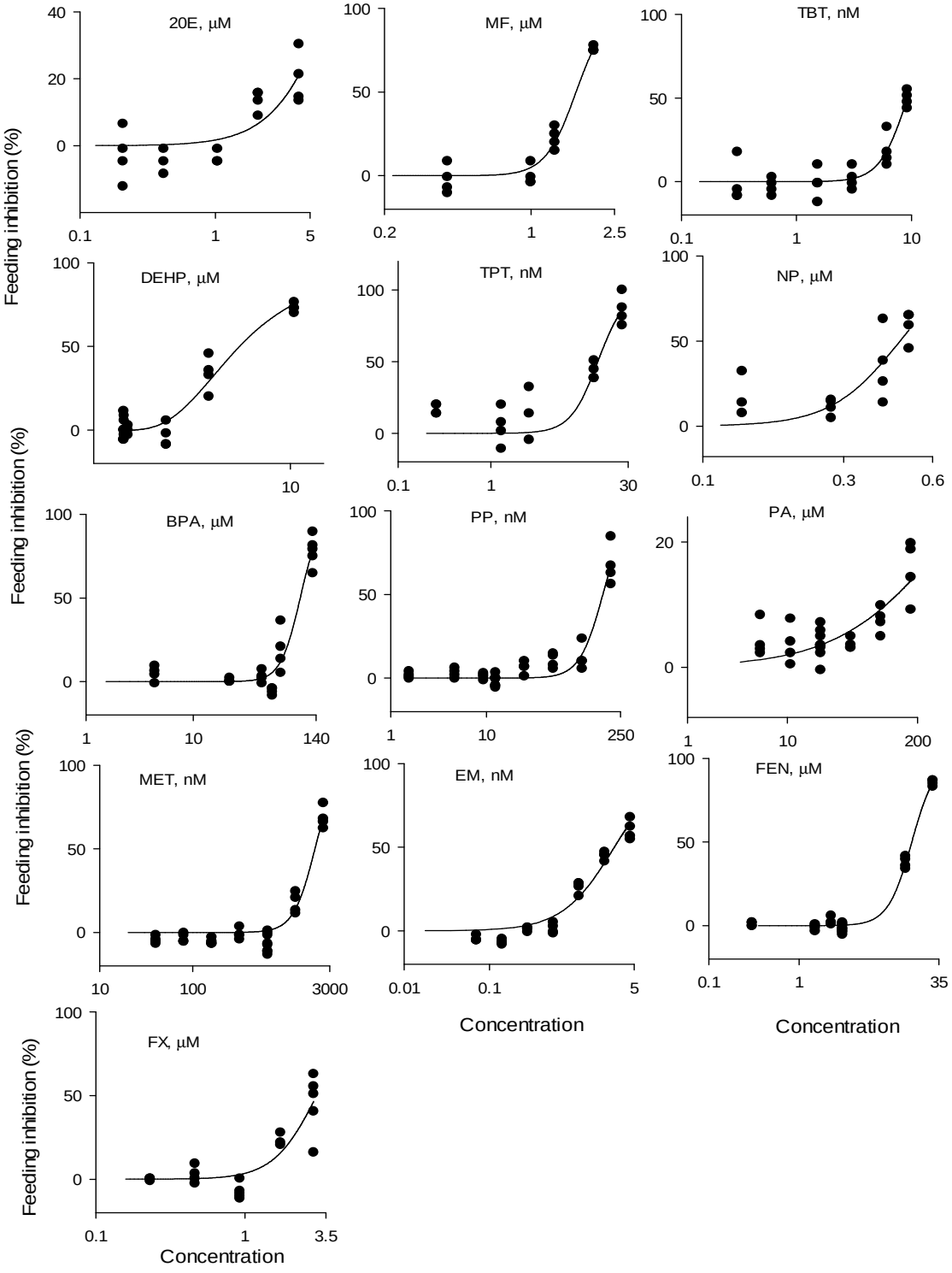


**Figure 3.3** – Feeding inhibition responses of the studied compounds. Each symbol means a single observation. Fitted regression curves are also depicted. TEB did not inhibit feeding at concentrations lower than its water solubility < 3 µM. Axis x is in log scale.

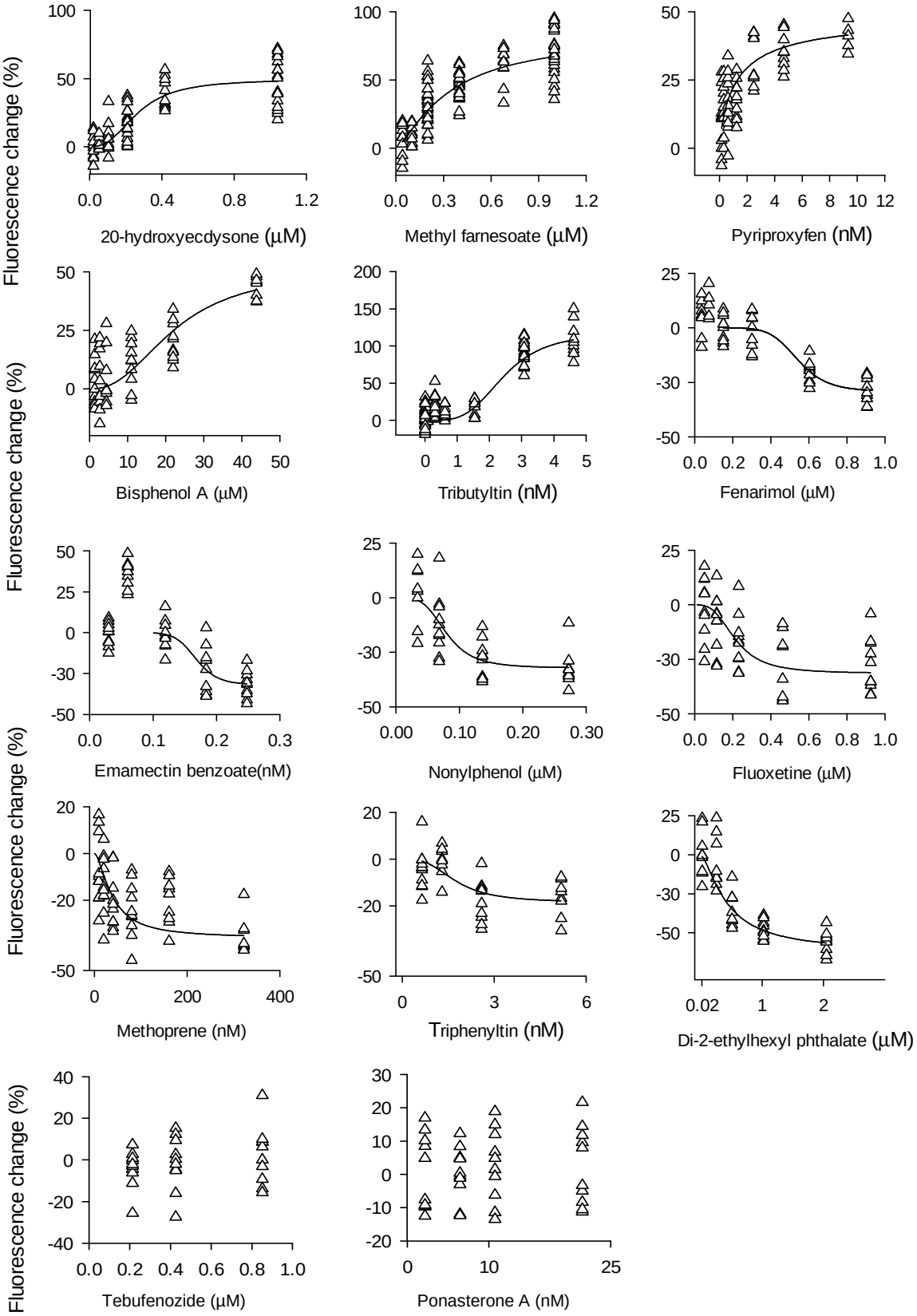


**Figure 3.4** – Nile red fluorescence changes for the 14 studied compounds. Each symbol is a single observation. Fitted regression curves are also depicted.

**Table 3.5** – Body length (Mean ± SE, $n$ = 10) of adolescent females used for Nile red determination. (*) indicates significant ($p < 0.05$) differences from controls (0) following ANOVA and Dunnett's post-hoc test. Concentrations (Conc.) are depicted in µM except for TBT, TPT, PP, MT, and EM that are in nM.

| Compound | Conc. | Length | SE | | Compound | Conc. | Length | SE | |
|---|---|---|---|---|---|---|---|---|---|
| TBT | 0 | 2374.3 | 34 | | TPT | 0 | 2254.7 | 20.3 | |
| | 0.003 | 2373.5 | 15.7 | | | 0.6 | 2261.1 | 19.7 | |
| | 0.009 | 2360.1 | 18.7 | | | 1.2 | 2232.6 | 18.1 | |
| | 0.03 | 2340.3 | 20.9 | | | 2.6 | 2162.6 | 31.3 | * |
| | 0.09 | 2362.4 | 27.1 | | | 5.2 | 2105.2 | 12.7 | * |
| | 0.3 | 2295.2 | 17.9 | * | MT | 0 | 2440.8 | 15.4 | |
| | 0.6 | 2251.5 | 19.9 | * | | 9.7 | 2428.1 | 19.58 | |
| | 1.5 | 2265.9 | 18 | * | | 20.1 | 2448.8 | 21.0 | |
| | 3.1 | 2266 | 33.1 | * | | 40.2 | 2436.0 | 15.6 | |
| | 4.6 | 2264.1 | 15.6 | * | | 80.5 | 2447.2 | 21.5 | |
| MF | 0 | 2272 | 15.1 | | | 161.5 | 2449.5 | 8.6 | |
| | 0.04 | 2313.6 | 10.2 | | | 322.1 | 2398.6 | 15.6 | |
| | 0.1 | 2312.9 | 19.3 | | FEN | 0 | 2400.3 | 33.8 | |
| | 0.2 | 2302.1 | 17.2 | | | 0.06 | 2390.4 | 30.8 | |
| | 0.4 | 2226 | 16.8 | | | 0.1 | 2390.4 | 29.9 | |
| | 0.7 | 2231 | 22.6 | | | 0.3 | 2359.7 | 19.4 | |
| | 1 | 2203.4 | 9.7 | * | | 0.6 | 2392.9 | 43.4 | |
| BPA | 0 | 2438.8 | 26.6 | | | 0.9 | 2413.9 | 33.4 | |
| | 1.3 | 2382.3 | 27.8 | | FX | 0 | 2618.0 | 49.6 | |
| | 2.6 | 2409.7 | 14.7 | | | 0.01 | 2570.5 | 28.2 | |
| | 4.4 | 2392 | 19.8 | | | 0.1 | 2511.4 | 27.9 | |
| | 11.0 | 2362.8 | 26.6 | | | 0.2 | 2614.7 | 20.3 | |
| | 21.9 | 2402.5 | 22.8 | | | 0.5 | 2565.6 | 26.1 | |
| | 43.8 | 2341.3 | 25.5 | | | 0.9 | 2596.8 | 29.5 | |
| PP | 0 | 2389.8 | 26.8 | | EM | 0 | 2493.9 | 6.3 | |
| | 0.2 | 2405.8 | 10.5 | | | 0.03 | 2447.1 | 17.0 | |
| | 0.3 | 2376.7 | 18.4 | | | 0.07 | 2447.4 | 23.5 | |
| | 0.6 | 2400.7 | 16.9 | | | 0.1 | 2488.7 | 7.7 | |
| | 1.2 | 2472.6 | 20 | | | 0.2 | 2470.7 | 11.0 | |
| | 2.5 | 2373.3 | 26.3 | | | 0.3 | 2458.8 | 12.4 | |
| | 4.7 | 2357.3 | 16.7 | | NP | 0 | 2108.1 | 29.5 | |
| | 9.3 | 2307.1 | 14.4 | * | | 0.03 | 2105.9 | 25.1 | |
| 20E | 0 | 2247 | 20.4 | | | 0.07 | 2111.6 | 21.6 | |
| | 0.02 | 2249.3 | 10.8 | | | 0.1 | 2129.2 | 16.4 | |
| | 0.05 | 2227.6 | 19.9 | | | 0.3 | 2254.4 | 25.2 | * |
| | 0.1 | 2222.9 | 11 | | DEPH | 0 | 2328.4 | 24.5 | |
| | 0.2 | 2270.8 | 8.9 | | | 0.02 | 2347.4 | 27.1 | |
| | 0.4 | 2260.3 | 21 | | | 0.25 | 2349.5 | 25 | |
| | 1 | 2274.6 | 15.5 | | | 0.51 | 2229.4 | 21.4 | * |
| | | | | | | 1.02 | 2229.4 | 12.04 | * |
| | | | | | | 2.05 | 2220.7 | 10.0 | * |

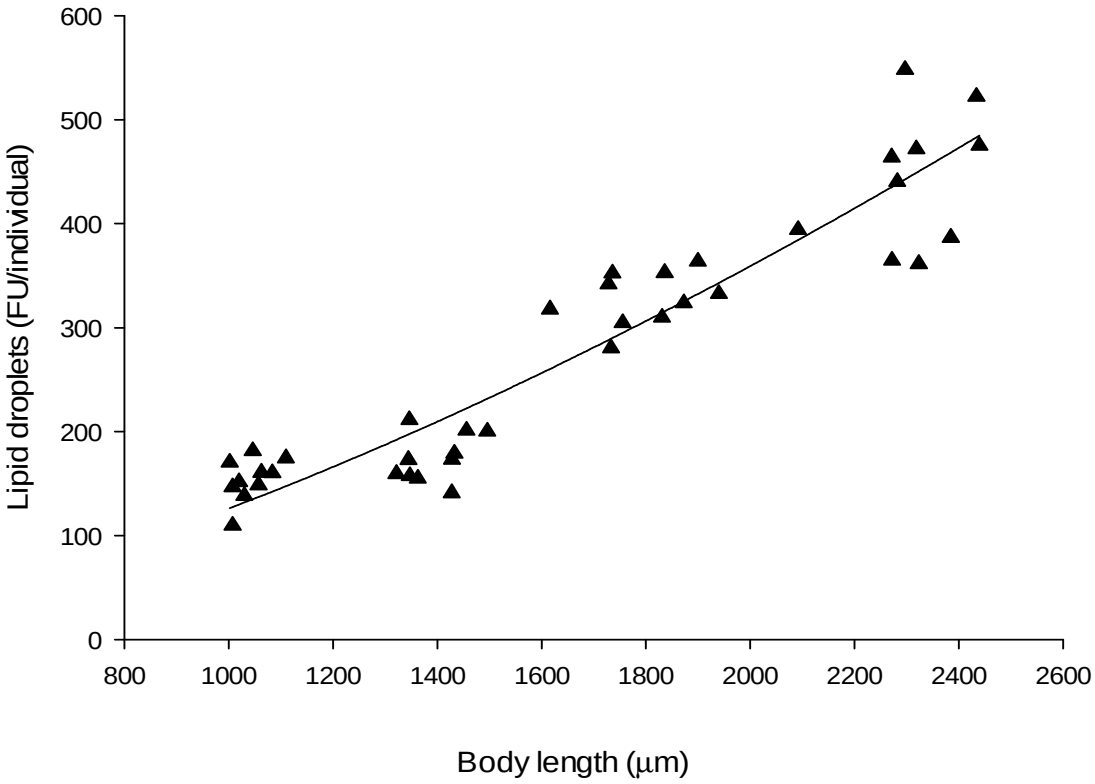


**Figure 3.5** – Lipid droplets expressed as arbitrary fluorescence units (FU) of Nile red versus body length (Mean ± SE, $n$ = 10) of *D. magna* individuals across the first, second, third and fourth instar just after moulting. Each symbol is single data point. The line represents the fitted regression ($y = 3.7\ X^{1.5}$, $r^2 = 0.88$, $n = 40$).

### 3.4.3 Combined effects

Joint effects on the fluorescence of Nile red varied across the studied six binary combinations (Figure 3.6). Two-way ANOVA results performed on individual mixture constituents and their combinations showed non-significant ($p < 0.05$) interaction terms in only two pairings (those of BPA and PP with MF, Table 3.6), which indicates that combined effects were similar to those predicted by IA. Note also that in the pairing of MF and PP, observed combined effects were also no significantly different ($p < 0.05$) than those predicted by CA. Joint effects of the pairing of MF with TBT were additive at low concentrations of TBT and synergic when TBT was dosed at 3 nM. Co-exposure of MF or TBT with FEN and of the combination TBT/MF/FEN always decreased fluorescence levels to a greater extent than expected by IA.

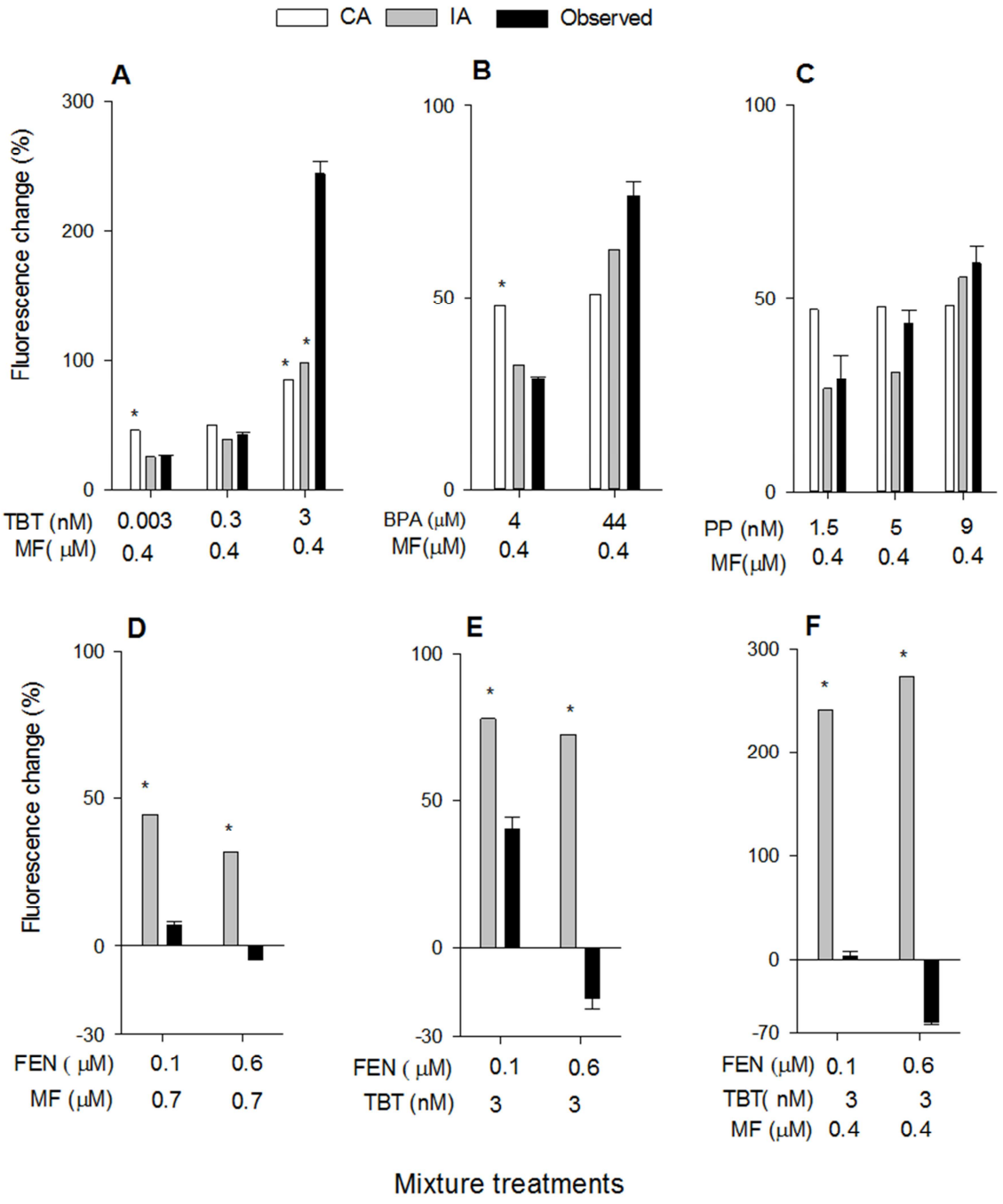


**Figure 3.6** – Joint effects on Nile red fluorescence changes relative to controls (Mean ± SE, *n* = 10) of six binary mixture combinations of TBT, BPA and PP with MF, and of MF, TBT and the mixture TBT/MF with FEN. In each graph their predicted responses following the CA (white bars) and IA (grey bars) models and the observed effects (black bars) are shown. (*) indicated significant ($p < 0.05$) differences between observed and predicted responses following Student's t test after Bonferroni correction for multiple comparisons.

**Table 3.6** – Two-way ANOVA results comparing fluorescence changes of Nile red across single and binary mixture combinations of the tested mixtures. Only degrees of freedom (df), Fisher's coefficient and significant levels are depicted. ns, not significant, $p \geq 0.05$. Single and binary mixture results are depicted in Table 3.7.

| Mixture | | Factor | df | F | *p* |
|---|---|---|---|---|---|
| MF | TBT | MF | 1,72 | 93.2 | <0.001 |
| | | TBT | 3,72 | 264.3 | <0.001 |
| | | MF x TBT | 3.72 | 19.2 | <0.001 |
| | BPA | MF | 1,54 | 53.1 | <0.001 |
| | | BPA | 2,54 | 73 | <0.001 |
| | | MF x BPA | 2,54 | 0.7 | ns |
| | PP | MF | 1,72 | 49.2 | <0.001 |
| | | PP | 3,72 | 42.5 | <0.001 |
| | | MF x PP | 3.72 | 1.4 | ns |
| FEN | MF | MF | 1,54 | 0.4 | ns |
| | | FEN | 2,54 | 86.8 | <0.001 |
| | | MF x FEN | 2,54 | 27.6 | <0.001 |
| | TBT | TBT | 1,54 | 97.1 | <0.001 |
| | | FEN | 2,54 | 92.9 | <0.001 |
| | | TBT x FEN | 2,54 | 23.7 | <0.001 |
| | TBT/ MF | TBT/ MF | 1,54 | 200.0 | <0.001 |
| | | FEN | 2,54 | 395.1 | <0.001 |
| | | TBT/ MF x FEN | 2,54 | 328.4 | <0.001 |

**Table 3.7** – Fluorescence changes of Nile red (%) relative to control treatments (Mean ± SE, *n* = 10) of each mixture experiment used in two-way ANOVA analyses. Concentrations are in µM except for TBT and PP that are in nM. Compound abbreviations are explained in the text. Different letters indicated significant ($p < 0.05$) differences following ANOVA and Tukey's post-hoc test.

| **Mixture constituents** | | **Mean** | **SE** | | **Mixture constituents** | | **Mean** | **SE** | |
|---|---|---|---|---|---|---|---|---|---|
| **MF** | **TBT** | | | | **FEN** | **MF** | | | |
| 0.4 | 0 | 20.7 | 0.8 | ab | 0.1 | 0 | 4.8 | 0.3 | c |
| 0 | 0.003 | 5.7 | 0.3 | a | 0 | 0.7 | 41.8 | 4.5 | d |
| 0.4 | 0.003 | 26.1 | 0.7 | ab | 0.1 | 0.7 | 6.8 | 1.4 | c |
| 0 | 0.3 | 23.8 | 0.8 | ab | 0.6 | 0 | -17.6 | 2.7 | b |
| 0.4 | 0.3 | 42.9 | 1.5 | b | 0.6 | 0.7 | -41.8 | 3.4 | a |
| 0 | 3 | 98.1 | 2.1 | c | | | | | |
| 0.4 | 3 | 244.4 | 9.1 | d | | | | | |
| **MF** | **BPA** | | | | **FEN** | **TBT** | | | |
| 0.4 | 0 | 26.6 | 0.5 | b | 0.1 | 0 | 2.9 | 0.5 | b |
| 0 | 4 | 8.1 | 0.4 | a | 0 | 3 | 77.1 | 5.4 | d |
| 0.4 | 4 | 28.8 | 0.6 | b | 0.1 | 3 | 40.4 | 4.1 | c |
| 0 | 44 | 48.9 | 1.1 | c | 0.6 | 0 | -20.7 | 3.5 | a |
| 0.4 | 44 | 76.6 | 3.7 | d | 0.6 | 3 | -17.4 | 3.2 | a |
| **MF** | **PP** | | | | **FEN** | **TBT/MF** | | | |
| 0.4 | 0 | 15 | 2.9 | b | 0.1 | 0 | 2.165 | 2.7 | b |
| 0 | 1.5 | 8 | 3.4 | ab | 0 | 3/0.4 | 244.4 | 12.9 | c |
| 0.4 | 1.5 | 29.1 | 6.1 | bc | 0.1 | 3/0.4 | 4.1 | 4.5 | b |
| 0 | 5 | 13.3 | 2.9 | b | 0.6 | 0 | -20.4 | 2.477 | b |
| 0.4 | 5 | 43.5 | 3.2 | cd | 0.6 | 3/0.4 | -59.7 | 3 | a |

### 3.4.4 Life-history effects

Females exposed to MF and PP during the adolescent instar were smaller at first reproduction, and produced smaller clutches of male offspring that were larger than female offspring produced by control females (Table 3.8). The rest of treatments only produced female offspring. Bisphenol A and FX did not affect body length, clutch size and offspring size (Table 3.8). Despite that the clutch size of females exposed to 20E were similar than those of control females, they produced larger offspring (Table 3.8). Emamectin benzoate decreased body length of adult females and their offspring, FEN reduced clutch size, and NP reduced the number and size of offspring (Table 3.8). Methoprene dramatically impaired the length and clutch size of first reproducing females and increased or reduced the size of offspring at low and high concentrations, respectively (Table 3.8). Females exposed to TBT were smaller, had similar clutches but produced smaller offspring than those from control treatments (Table 3.8).

**Table 3.8** – Body length, clutch and offspring size (Mean ± SE, *n* = 10) at first reproduction of adult females exposed to selected compounds during their adolescent instar. Results from four different experiments are included. Within each experiment (*) indicates significant ($p < 0.05$) differences from solvent carrier controls (Control) following ANOVA and Dunnett's post-hoc test. Data from TBT (experiment 4) come from a previous study (Jordão *et al.,* 2015). (Conc. = concentration).

| | | Body length (µm) | | | Clutch size | | | Offspring size | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | **Conc.** | **N** | **Mean ± SE** | | **Mean ± SE** | | **N** | **Mean ± SE** | |
| Experiment 1 | | | | | | | | | |
| Control | 0 | 10 | 2517.1 ± 10.9 | | 11.2 ± 0.4 | | 30 | 850.6 ± 5.9 | |
| MF (µM) | 0.2 | 10 | 2561.0 ± 15.9 | | 3.9 ± 0.2 | * | 25 | 888.3 ± 12.5 | * |
| | 0.4 | 10 | 2511.2 ± 15.3 | | 3.9 ± 0.1 | * | 30 | 919.2 ± 6.3 | * |
| | 1.0 | 10 | 2399.9 ± 20.4 | * | 3.3 ± 0.3 | * | 28 | 907.8 ± 6.6 | * |
| PP (nM) | 1.2 | 10 | 2497.4 ± 19.9 | | 4.3 ± 0.2 | * | 26 | 847.5 ± 6.7 | |
| | 4.7 | 10 | 2412.0 ± 25.7 | * | 4.4 ± 0.2 | * | 19 | 869.0 ± 8.4 | |
| | 9.3 | 10 | 2394.7 ± 17.0 | * | 3.9 ± 0.2 | * | 21 | 891.1 ± 7.3 | * |
| BPA (µM) | 4.4 | 10 | 2505.7 ± 14.2 | | 10.2 ± 0.4 | | 25 | 834.8 ± 11.1 | |
| | 43.8 | 10 | 2433.3 ± 32.8 | | 10.4 ± 0.3 | | 24 | 837.3 ± 9.5 | |
| 20E (µM) | 0.1 | 10 | 2535.9 ± 7.6 | | 11.0 ± 0.3 | | 18 | 877.7 ± 7.9 | |
| | 1 | 10 | 2523.4 ± 7.7 | | 10.6 ± 0.3 | | 20 | 906.4 ± 5.3 | * |
| Experiment 2 | | | | | | | | | |
| Control | 0 | 10 | 2553.9 ± 7.0 | | 10.9 ± 0.4 | | 20 | 782.0 ± 9.3 | |
| EM (nM) | 0.1 | 10 | 2525.7 ± 23.6 | | 10.9 ± 0.6 | | 22 | 741.0 ± 7.6 | |
| | 0.2 | 10 | 2473.4 ± 30.8 | * | 9.6 ± 0.8 | | 15 | 715.0 ± 20.4 | * |
| FEN (µM) | 0.1 | 10 | 2494.2 ± 20.6 | | 10.4 ± 0.5 | | 15 | 827.6 ± 16.9 | |
| | 0.3 | 10 | 2513.6 ± 18.8 | | 6.7 ± 0.3 | * | 23 | 755.4 ± 5.1 | |
| | 0.9 | 10 | 2502.6 ± 16.4 | | 6.1 ± 0.4 | * | 15 | 814.4 ± 20.6 | |
| NP (µM) | 0.1 | 10 | 2525.3 ± 18.0 | | 11.1 ± 0.4 | | 18 | 720.1 ± 5.5 | * |
| | 0.3 | 10 | 2499.3 ± 15.0 | | 7.6 ± 0.5 | * | 9 | 692.5 ± 11.1 | * |
| MT(nM) | 9.7 | 10 | 2429.7 ± 24.9 | * | 5.1 ± 0.3 | * | 16 | 878.2 ± 9.8 | * |
| | 322.1 | 10 | 2381.5 ± 20.4 | * | 3.1 ± 0.3 | * | 23 | 690.7 ± 6.6 | * |
| Experiment 3 | | | | | | | | | |
| Control | 0 | 10 | 2537.0 ± 14.6 | | 11.0 ± 0.4 | | 22 | 771.4 ± 8.5 | |
| FX (µM) | 0.2 | 10 | 2531.7 ± 15.6 | | 10.5 ± 0.4 | | 20 | 787.9 ± 8.7 | |
| | 0.9 | 10 | 2510.4 ± 12.6 | | 10.3 ± 0.3 | | 19 | 758.1 ± 10.4 | |
| Experiment 4 | | | | | | | | | |
| Control | 0 | 10 | 2481.1 ± 23.4 | | 9.2 ± 0.6 | | 10 | 723.6 ± 4.3 | |
| TBT (nM) | 0.3 | 10 | 2407.1 ± 16.2 | * | 9.0 ± 0.5 | | 10 | 735.0 ± 6.5 | |
| | 3.0 | 10 | 2305.8 ± 23.9 | * | 8.5 ± 0.4 | | 10 | 655.3 ± 4.6 | * |

## 3.5 Discussion

### 3.5.1 Fat storage disruption

Obtained results indicated that 10 out of the 14 tested compounds enhanced or reduced the accumulation of storage lipids in the lipid droplets in post-spawning females. Altered levels of lipid droplets occurred at lower concentrations than those impairing moulting, growth or food acquisition. This means that the observed altered patterns of storage lipids were unrelated with any measurable effects on energy intake and thus were likely associated with an endocrine disruption mechanisms (Barata *et al.,* 2004). Our tested hypothesis was that storage lipids accumulating in the lipid droplets in post-spawning females were regulated by three hormonal receptor signalling pathways: RXR, EcR and MfR. Agonists of the previous mentioned receptors (MF, PP for MfR; 20E for EcR and TBT for RXR) enhanced the accumulation of storage lipids in lipid droplets in a concentration dependent manner. There is reported evidence showing that BPA in *Daphnia* enhances endogenous juvenoid activity (Mu *et al.,* 2005). Therefore, according to our hypothesis, BPA should also promote lipid droplet accumulation in *Daphnia*. Conversely, MT, which is a weak agonist of MfR in *Daphnia* (Miyakawa *et al.,* 2013) could act antagonistically with endogenous levels of MF on the MfR receptor, decreasing lipid droplets. Previous results, showed that MT co-administered with exogenous MF potentiated the production of male offspring rather than inhibited it (Wang *et al.,* 2005). Thus, it is possible that MT affects differently the MfR signalling pathway than MF and PP do. Fenarimol and NP, which are known to deplete the levels of or alter the metabolism of ecdysone in *D. magna*, respectively decreased lipid droplet formation (LeBlanc *et al.,* 2000; Mu and LeBlanc 2004b). Emamectin benzoate is a GABA-ergic pesticide known to promote ecdysis in decapod crustaceans and insects probably by inhibiting neuropeptides that control the release of ecdysone (Rodríguez *et al.,* 2007). In our study, EM also depleted lipid droplets levels. Binary combination of the two tested juvenoids (MF and PP) and those of BPA with MF enhanced lipid accumulation in lipid droplets additively in a manner also predicted by the CA or IA models. Results obtained with PP and MF support the hypothesis that joint effects of agonists of the MfR (LeBlanc *et al.,* 2013; Miyakawa *et al.,* 2013), should act additively in a CA manner on lipid droplet accumulation. Observed additive effects of BPA with MF also agree with previous reported results that found that the former contaminant enhanced the juvenoid activity of MF (Wang *et al.,* 2005). Joint effects of binary mixtures of MF with TBT indicated that the RXR and the MfR signalling

pathways acted more than additively enhancing storage lipids accumulation in lipid droplets in post-spawning females. This means that these two receptors may act cooperatively in a similar way as PPAR and RXR do in vertebrates (Keller *et al.,* 1993). Conversely, enhancing lipid droplet accumulation by agonists of MfR (MF) and of RXR (TBT) or their combination (TBT/MF) was neutralized or reversed in co-exposures with the ecdysone level inhibitor FEN. These results also support our hypothesis and indicated that observed enhanced levels of lipid droplets by agonists of RXR and MfR are ecdysteroid-dependent.

Serotonin reuptake inhibitors including FX are known to increase serotonin levels and decrease fat storage in *Caenorhabditis elegans* and in humans (Srinivasan *et al.,* 2008; Li and Cheung 2009). In *C. elegans* serotonin regulates fat promoting the β-oxidation of lipids whereas in humans serotonine also regulates fat storage decreasing apetite (Srinivasan *et al.,* 2008). In *D. magna*, FX promoted oxidative catabolism and reproduction without affecting feeding (Campos *et al.,* 2012a; Campos *et al.,* 2013). In this study, FX decreased lipid droplet formation without affecting feeding, which means that this compound is likely to act promoting β-oxidation of lipids in *D. magna*. Therefore, other pathways of lipid disruption than those interacting with the RXR, EcR and juvenile hormone receptors may also affect *D. magna* lipid storage dynamics.

### 3.5.2 Life-history effects

In *Daphnia*, storage resources accumulated during a reproductive instar are allocated to growth and reproduction at the end of that instar (Nogueira *et al.,* 2004). This means that altering the accumulation of storage lipids in female adults and their transfer to eggs may bear important fitness costs since storage lipids are an important source of energy. Previously, we found that exposure to TBT during a single reproductive cycle (i.e. 3-4 days) resulted in smaller females that had a long-lasting detrimental effects on female's reproductive capacity for at least 5 consecutive instars (Jordão *et al.,* 2015). Newborn neonates produced by females exposed to TBT were also smaller than those of unexposed females, and suffered a higher mortality during their adulthood that translated in lower reproductive output and fitness (Jordão *et al.,* 2015). Thus, there was a fitness cost of TBT associated to the allocation of fewer resources to post-spawning females and their offspring. In this study, six (MF, PP, 20E, FEN, EM, MT) out of the ten compounds that alter storage lipids accumulation into lipids droplets, affected the moulting process at higher concentrations, which provides an additional evidence that EcR signalling pathway

is involved in the observed lipid pattern alteration phenomena. Low and high concentrations of juvenoids (MF, PP) that alter the levels of lipid droplets decreased the body length of exposed females, the size of their first brood, changed offspring sex and increased offspring size. Juvenoids, by reducing reproductive investment should favor the accumulation of non-invested resources including storage lipids in post-spawning females. There is reported evidence that at lower concentrations than the tested ones, the studied juvenoids had anti-ecdysteroid activity (Mu and Leblanc 2004a), and reduced clutch size producing all male broods (Olmstead and Leblanc 2002,2003; Wang *et al.,* 2005; Ginjupalli and Baldwin 2013). Thus, juvenoids primarily regulated offspring sex in *Daphnia* and probably also the investment per offspring and the total reproductive investment (LeBlanc *et al.,* 2013). According to LeBlanc *et al*., (2013) activation of the MfR signalling pathway has transgenerational effects allowing further generations of *D. magna* populations to recover from harsh environmental conditions such as low food and or high density. Under food limiting conditions, decreased reproductive output (production of few offspring and males) would reduce population density, increasing the amount of food per capita. Larger offspring also has a greater fitness than smaller ones at limiting food conditions (Tessier and Consolatti 1989,1991; Gliwicz and Guisande 1992), thus our results support previous reported arguments of transgenerational effects of juvenoids in *D. magna*. The juvenoid compound MT despite of reducing lipid droplet formation affected growth, fecundity and at low concentrations offspring size similarly than the other tested juvenoids (MF, PP), but did not change offspring sex. Previous studies have also reported that MT has a poor ability to induce males but that at low concentrations similar to those tested here reduces growth and reproduction (Olmstead and LeBlanc 2001; LeBlanc *et al.,* 2013; Miyakawa *et al.,* 2013). The observed distinctive effects of MT on lipid storage dynamics in lipid droplets and life-history response, support our previous argument that this compound is likely to affect differently the MfR signalling pathway. Concentrations of 20E that enhanced lipid accumulation in lipid droplets also enhanced per offspring reproductive investment as neonates produced in the first clutch were larger than those of controls. There is reported information showing that 20E affects *D. magna* reproduction (Sumiya *et al.,* 2014), but no such studies exist for per offspring reproductive investment. Previous findings indicated that this hormone interacts with the MfR signalling pathway in *Daphnia* (Mu and Leblanc 2004a), thus it is likely that 20E alone or interacting with the MfR, affected also the per offspring reproductive investment as MF and PP did. In our study, FEN decreased the accumulation of storage lipids in post-spawning females and consequently reduced reproductive investment, which agrees with reported reproductive

toxicity results (Mu and Leblanc 2002). Nonylphenol reduced offspring size and at high concentrations also impaired clutch size, thus it is also possible that observed detrimental effects on reproduction were related to the decreasing of storage lipids. At low exposure levels, NP maintains reproduction number while decreasing offspring size and at high concentrations inhibited both the number and size of offspring, which is in line with our previous study (Campos *et al.,* 2012b). Emamectin benzoate reduced the accumulation of storage lipids in lipid droplets and impaired the growth of females and the size of their offspring at concentrations similar to those affecting marine copepods (Willis and Ling 2003), thus it is probable that observed detrimental effects of this compound was related to lower allocation of energy reserves. Bisphenol A and FX did not affect the growth of exposed females, neither the number and size of offspring produced. Reported studies indicated that BPA causes reproductive toxicity in embryos (Mu *et al.,* 2005). In our study, females were only exposed to BPA during the provisioning of the first brood of eggs and hence developing embryos were never exposed to BPA. Previous studies indicated that FX at lower concentrations than those disrupting lipid storage accumulation, increases offspring production de-regulating the neuronal control of reproduction (Campos *et al.,* 2012a; Campos *et al.,* 2012b; Campos *et al.,* 2013). At higher concentrations, however, FX inhibits growth and reproduction probably due to general toxicity mechanisms (Hansen *et al.,* 2008). In our study, FX disrupted storage lipids accumulation at concentrations lying in between those enhancing or inhibiting growth and or reproduction.

In summary, 8 out of 10 of the compounds that altered storage lipids in lipid droplets altered growth and/or reproduction responses. For juvenoids, the observed contrasting effects of MT and of MF and PP, indicated that life-history effects were independent of those observed on storage lipids accumulation. For the remaining compounds, however, increasing or decreasing storage lipids in lipid droplets and effects observed on growth and reproduction are more likely to be physiologically related, but deserved to be further studied. The results reported in this study showed that obesogenic effects similar to those reported in mammalian species do occur and can be quantified using the fluorescent Nile red in *D. magna*. Known mammalian obesogens (TBT and BPA) and fat storage inhibitors (FX) (Grün and Blumberg 2006; Lemieux *et al.,* 2011; Thayer *et al.,* 2012) also enhanced and decreased fat storage in *D. magn*a, respectively. Other mammalian obesogens including DEPH and TPT (Grün and Blumberg 2006) decreased storage lipids into lipid droplets at similar concentrations that also severely impaired growth. Nonylphenol inhibited rather than enhanced storage lipids accumulation in lipid

droplets in *D. magna* individuals. There were also other compounds active in arthropods and with unknown obesogenic effects in mammals (PP, EM, MT and FEN) that disrupted fat storage accumulation in *D. magna.* Single exposures and binary mixtures experiments indicated that the studied compounds disrupted storage lipids interacting with the MfR, RXR and EcR signalling pathways. These results point out that the endocrine disruption of lipid homeostasis can be an important source of toxicity in non-vertebrates.

## 3.6 Supporting information

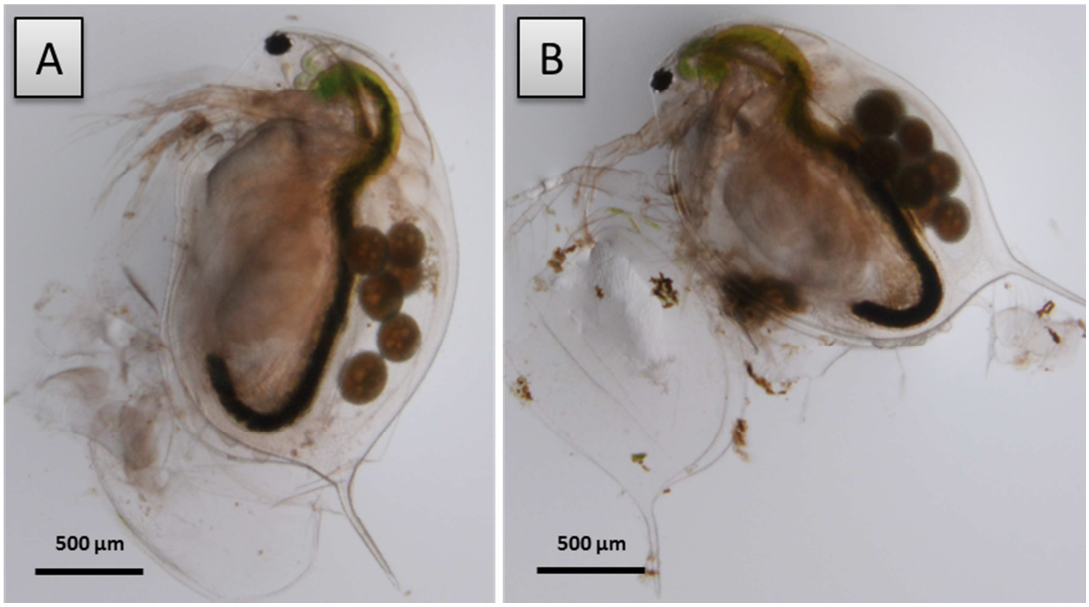


**Figure 3.7** – Moult inhibition observed in individuals of *D. magna* after exposure to 2 µM of 20-hydroxyecdysone (A) or 43 nM of Ponasterone A (B), during 48 hrs.

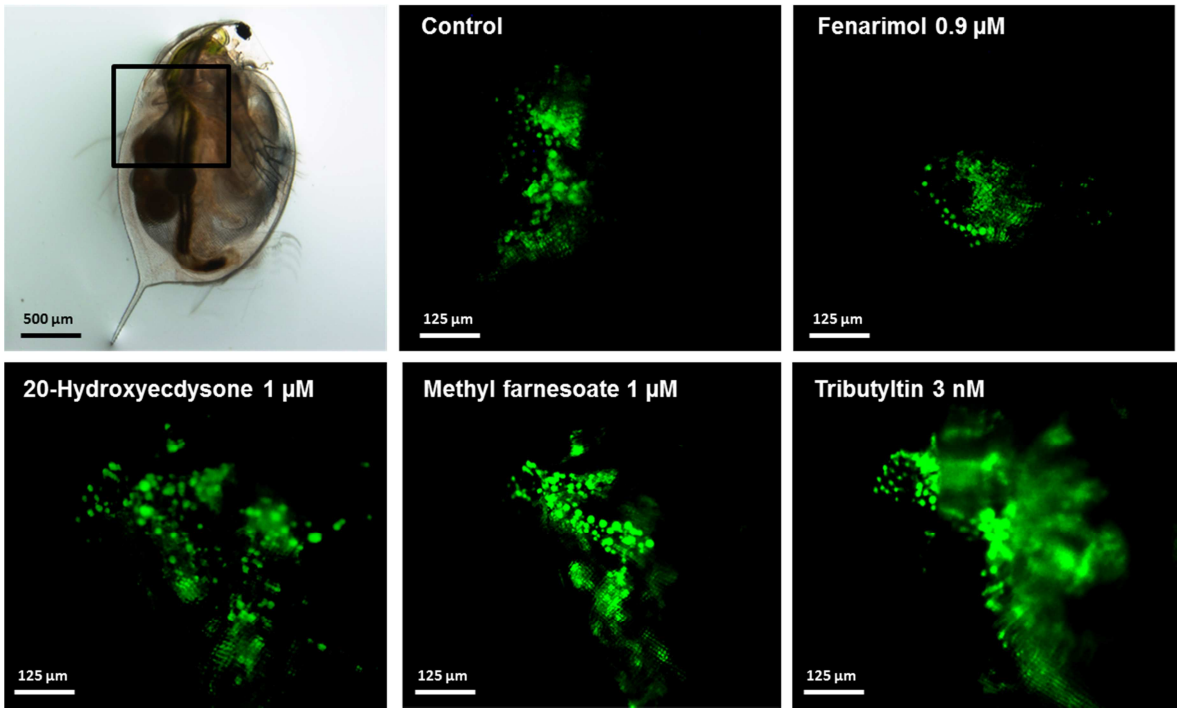


**Figure 3.8** – Nile red lateral partial view under fluorescence microscopy, of adolescent females just after moulting and release of the first brood of eggs into the brood punch of unexposed (control) and exposed individuals to fenarimol, 20-hydroxyecdysone, methyl farnesoate and tributyltin. In the top left, an image of a female and the studied area, taken with the bright field, is also included. Stained lipid droplets with Nile red are in green.

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# Chapter IV

Two-generational effects in *Daphnia magna*

**Chapter IV – Two-generational effects in *Daphnia magna***

# 4 Two-generational effects of contaminants in *Daphnia magna*. Effects of offspring quality [1]

## 4.1 Abstract

The authors set up a protocol to perform a two-generational ring test using the existing guidelines for the *Daphnia magna* reproduction test. It is well known in ecology that the size and quality of offspring varies across the first clutches in *D. magna* and that certain chemicals affect offspring quality. Therefore, the origin of the second generation is an important factor to consider. Two-generational effects across first, second and third clutches were evaluated using 4-nonylphenol; those across first and third clutches were evaluated using tributyltin (TBT) and those across the third clutch were evaluated using piperonyl butoxide (PBO). The compound showing the greatest aggravation of toxic effects between the parental and second generations was PBO followed by 4-nonylphenol, while inter-generational effects of TBT varied across experiments. The studied chemicals affected the quantity and quality of the offspring produced by exposed females of the parental generation, those effects being greater in third-clutch neonates. Therefore, when third-clutch offspring were further exposed, they turned out to be more sensitive than the parental generation. The results are in line with those obtained in multigenerational studies using mammalian tests, which showed that, in many cases, effects on the second generation can be predicted by evaluating the quality of the offspring produced.

**Keywords:** *Daphnia*, reproduction tests, multigeneration, offspring quality, contaminants, life-history analyses.

[1]Campos B., **Jordão R.**, Rivetti C., Lemos M. F. L., Soares A. M. V. M., Tauler R. and Barata C., (2015). "Two-generational effects of contaminants in *Daphnia magna*. Effects of offspring quality." Environmental Toxicology and Chemistry. DOI: 10.1002/etc.3290 (article in annex).

## 4.2 Introduction

Most ecotoxicological test guidelines evaluate the effects observed only within one generation, thus disregarding those potential detrimental effects that may appear across generations. The toxicological potential of contaminants may either decrease or increase across generations, or may even remain unchanged. In many cases, however, pollutants affect both exposed organisms and their progeny; hence adverse effects are likely to become more severe in subsequent generations. Reproductive toxicity in mammals monitors the effects on the parental generation as well as on their offspring (Janer *et al.,* 2007). In fact, there are already standardized test procedures to assess multigenerational effects for mammals and fish (Janer *et al.,* 2007; Nakamura *et al.,* 2015), but there are no equivalent tests for invertebrates, despite the potential their short life cycles offer to cost-effectively monitor multigenerational effects of toxic substances in individuals and populations (Verslycke *et al.,* 2007; Oliveira-Filho *et al.,* 2009a,2009b).

Among existing ecotoxicological assays, the *Daphnia magna* reproduction test is probably the standardized chronic test mostly employed in aquatic toxicology. Several studies suggest that standard *D. magna* reproduction tests may fail to generate sufficient data to predict effects occurring at the population level (Martin *et al.,* 2014). Hammers-Wirtz and Ratte (2000) found that the application of standard *D. magna* reproduction test just to the parental generation was not sufficient to predict population level effects, since impacts on the fitness of the offspring (reflected in their body size at birth) were not assessed. The previous authors found that the chemical-dispersant (dispersogen) decreased the size of the offspring produced, thus impairing their reproductive success. It is well known that the size at birth is a key trait in *D. magna* to predict offspring fitness across food environments (Ebert 1993; Boersma 1997; Barata and Baird 1998) and across contaminants (Enserink *et al.,* 1990; Cox *et al.,* 1992). Therefore, it should be advisable to expand the experimental duration and design to assess impacts to the next generation. Reported information also indicates that certain chemicals decrease offspring quality (i.e. offspring size) and that the size of *D. magna* neonates affects their sensitivity to pollutants (Enserink *et al.,* 1990; Cox *et al.,* 1992; Hammers-Wirtz and Ratte 2000). Therefore, it should be advisable to expand the experimental duration and design of the *D. magna* reproduction test in order to analyse possible impacts across generations.

The *D. magna* reproduction test consists in exposing individuals from birth to adulthood and counting the total number of offspring produced within the first 14 or 21 days (ASTM 1994; OECD 1997). Under non-limiting food conditions and at 20 °C, non-

exposed animals reproduce for the first time at about 8-10 days after birth and produce between three to four clutches. Thus, it should be feasible to extend this protocol to additional generations with just a few amendments.

A crucial issue is the selection of the clutch to start the second generation, since there is ample information indicating that the size and quality of offspring vary across clutches in *D. magna* and that such changes affect life-history performance (Boersma 1997; Barata and Baird 1998). Offspring from the first clutch are smaller and more variable in their size than those produced in latter clutches. Smaller offspring take longer to mature and reproduce and produce fewer and smaller eggs. Thus, it is not surprising to find that test guidelines recommend using offspring from second and later clutches.

In the present study, we first tested the hypothesis that the clutch of neonates selected to start second generation experiments exert an influence over the toxic effects observed across generations. Secondly, we assessed two-generational effects of several chemicals with known effects on offspring quality. Selected compounds included the industrial detergent 4-nonylphenol, the insecticide co-adjuvant PBO and the antifouling insecticide TBT. There is reported evidence that 4-nonylphenol and TBT decrease the size of offspring in *D. magna* (Campos *et al.,* 2012; Jordão *et al.,* 2015) and have detrimental multigenerational effects (Tanaka and Nakanishi 2002; Jordão *et al.,* 2015). Piperonyl butoxide is a well-known and widely used insecticide synergist, known to inhibit the activity of the insect cytochrome P450 detoxification system (Hardstone *et al.,* 2015). Despite the fact that there is no reported information on multigenerational effects of PBO, this compound acts in *Daphnia* in a similar way as 4-nonylphenol, inhibiting steroid hydrolases (Baldwin and LeBlanc 1994), which are necessary for embryo development (LeBlanc *et al.,* 2000). Furthermore, as a broad inhibitor of cytochrome P450 enzymes, PBO can alter additional metabolic pathways in exposed embryos more severely than anti-ecdysteroids like 4-nonylphenol. In zebrafish embryos PBO is embryotoxic (Wang *et al.,* 2012). Therefore, PBO may affect developing embryos and hence reproductive responses of second generation individuals more than anti-ecdysteroids. In this study we tested the premise that offspring size accounted for observed two-generational effects.

## 4.3 Material and Methods

### 4.3.1 Chemicals

The 4-nonylphenol (CAS 104-40-5), tributyltin chloride (TBT) (CAS Number 1461-22-9) and piperonyl butoxide (PBO, CAS 51-03-6) were purchased from Sigma-Aldrich. All other chemicals were analytical grade and were obtained from Merck.

### 4.3.2 Experimental animals and culture conditions

A single clone of *D. magna* (clone F) maintained in our lab for several years, was used. Animals were maintained individually in 100 mL of ASTM hard water at 20 °C and at 16: 8 hrs light: dark photoperiod cycle. Animals were fed with *Chlorella vulgaris* ($5 \times 10^5$ cells/mL, corresponding to 1.8 µC/mL) with the addition of seaweed organic extract as food additive (Barata and Baird 2000). Culture and test media were changed every other day.

### 4.3.3 Experimental design

Experiments were conducted following the *D. magna* reproduction test OECD guidelines (OECD 1997) with minor modifications. Treatments included four (5, 10, 20, 40 µg/L), seven (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 µg/L) and five (50, 100, 200, 400, 800 µg/L) concentrations of 4-nonylphenol, TBT and PBO, respectively. Selected chemical concentrations allowed to fully define concentration-response curves and to estimate low concentration effects. Acetone was used as carrier for the three tested contaminants, and the same amount (< 0.1 mL/L) was added as a solvent control in all experimental treatments to account for any carrier effect.

Once first generation females, hereafter referred as parental generation, released the first, second and third clutch of offspring, we initiated the second generation (F1), hereafter referred to “F11”, “F12” and “F13”, respectively. Individuals from the F1 generation were maintained as those of the parental one for 21 days following also the OECD guidelines (OECD 1997). Two-generational effects of 4-nonylphenol were studied on F11, F12 and F13 while tests on PBO were limited to F13, whereas tests on PBO were limited to F13. Two independent tests were performed with TBT: TBT1, where second generation started with neonates from the first and third clutch (F11 and F13); and TBT2,

where exposure levels were increased and the second generation was initiated only from third brood neonates (F13). Measured life-history traits were: initial and final body length of experimental animals; juvenile and adult survival; age at first reproduction; clutch size and population growth rates (r), estimated from the age specific survival and reproduction rates according to the Lotka equation (Barata *et al.,* 2002b). Body length measurements were performed from the top of the head to the base of the spine using a Nikon stereoscope microscope (SMZ 150, Nikon, Barcelona, Spain) and the ImageJ software (http://rsb.info.nih.gov/ij/).

### 4.3.4 Chemical analyses

Freshly prepared solutions of the three chemicals and aged solutions of 48 hrs were analysed to check for actual concentrations and chemical stability throughout the assays.

Actual TBT concentrations in test solutions were measured as total Sn using the Perkin Elmer model Elan 6000 ICP-MS (Barata *et al.,* 2005). Actual concentrations of 4-nonylphenol and of BPO were measured by UPLC coupled with mass spectrometry following previous methods (Mayer-Helm *et al.,* 2008; Fernández-Sanjuan *et al.,* 2009). In short, 4-nonylphenol was measured using an Acquity UPLC system (Waters, Mildford, MA, USA) connected to a triple quadruple detector Acquity. The analysis was performed on an Acquity UPLC BEH $C_{18}$ (1.7 µm, 2.1 x 100 mm) column supplied by Waters (Waters, Mildford, MA, USA). The column was, equilibrated at 40 °C. Chromatographic separation was carried out using a mobile phase consisting of 0.1 % formic acid (eluent A) and MilliQ water with 0.1 % formic acid (solvent B) at a flow rate of 0.3 mL/min. The elution started with 20 % of solvent A, followed by a 4-mins gradient to 75% of solvent A and a 2 mins gradient to 100 % of solvent A, held for 1 min, and then back to the initial conditions (20 % solvent A) within 6 mins. An injection volume of 50 µL was used. Instrument control, data acquisition and evaluation were performed using MassLynx software (v.4.1) with QuanLynx program (Waters). The ionization source parameters for the analysis were: electrospray source block and desolvation temperature: 150 and 350 °C, respectively, capillary and cone voltages: 3 kV and 30 V respectively, cone nitrogen gas flow and desolvation gas: 43 and 650 L/h. Mass spectrometer was operated in the MRM mode, with unit mass resolution on mass analyser. Identification criterion of the target compounds was based on: (a) LC retention of the analyte compared to that of a standard (± 2 %) and (b) the ratio of abundances of two specific precursor ion to product

ion transitions (within 10 % of the ratios obtained for the standard). Quantification was based on external calibration standard 8 point curves ($r^2$ > 0.98). Limits of detection and quantification defined as the minimum detectable amount of analyte with a signal to noise ratio of 3:1 and 10:1, respectively, were determined from the spiked water samples. Analyses of PBO were performed 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 acetonitrile (A) and 0.1 % formic acid in water (B). The gradient of elution started at 5 % A, then increased to 40 % A in 4 mins, 60 % A in 7 mins, reaching 100 % A in 11 mins and then return to initial conditions within 4 mins. The system was operated at room temperature, the flow rate was set at 200 µL/min and 10 µL were injected. Acquisition was performed in selected reaction monitoring (SRM) mode under positive electrospray ionization (ESI+) using two transitions from [M+H]+ precursor ion to daughter ions. The transitions used as well as, the cone voltages and collision energies were in accordance with (Mayer-Helm *et al.,* 2008). Quantification was based on external calibration standard curve (range between 10-1000 µg/L). Limits of detection and quantification defined as it is described for 4-nonylphenol. The data were acquired and processed using the MassLynx v4.1 software package.

## 4.4 Results

### 4.4.1 Data analyses

One-way ANOVA analyses were performed to compare exposure treatments and controls; and to determine the lowest observed effect concentration (LOEC). Regression analyses were used to determine $EC_{10}$, (10% effect concentration) and median effect concentrations ($EC_{50}$) of the tested chemicals. Regression $EC_{10}$ and $EC_{50}$ estimates were limited to cumulative fecundity and population growth rates, and determined fitting responses to the allosteric decay regression model following previous procedures (Barata *et al.,* 2000b). Analyses of population growth rates were based on jack-knife pseudo-values (Barata *et al.,* 2001). Prior to ANOVA analyses, assumptions of normality and variance homoscedasticity were assessed and data was log transformed when required. Percentage survival and age at first reproduction were analysed by non-parametric Chi-square and Kruskal-Wallis tests, respectively. The LOEC was estimated using one side Dunnett's test or the equivalent test for non-parametric analyses (Zar 1984).

### 4.4.2 Stability of chemicals in water

Measured concentration levels of the three studied compounds in freshly prepared and old test solutions (T0 and T48 hrs) were within 10 % of nominal concentrations, thus from now on our results are referred to nominal values.

### 4.4.3 Two-generational effects

In all assays performed, solvent control responses were not significantly different ($p < 0.05$) from those of controls, thus, the former was to estimate LOECs as indicated in Table 4.1, Table 4.2 and Table 4.3.

Tests performed with 4-nonylphenol aimed to study until which extent the origin of individuals used to initiate second generation exposures had an influence on their toxic response. Effects of 4-nonylphenol on fecundity varied little across generations but those on population growth rates increase in second generation individuals from third brood neonates (F13, Figure 4.1). Lowest observed effect concentration of 4-nonylphenol on population growth rates (LOEC, $EC_{10}$ in Table 4.1) were similar across generations but median effect concentrations ($EC_{50}$) were almost two fold lower in F13 (Table 4.1). Juvenile survival rates were significantly impaired by 4-nonylphenol at 20 µg/L and 40 µg/L ($p < 0.05$, Chi-square tests) in individuals from the parental and F13 generations, while this occurred only at 20 µg/L in those from F11 and at 40 µg/L in those from F12 (Table 4.2). In addition, the final adult body length (Lf) at 40 µg/L in the parental and F11 generations (Table 4.2), was significantly reduced ($p < 0.05$, ANOVA). Only one individual from the F13 generation reached adulthood and reproduced, thus this treatment was not included in the analysis of differences on body length (see missing value for Lf in Table 4.2). The size of the offspring produced by females from the parental generation was reduced significantly ($p < 0.05$, ANOVA) by 4-nonylphenol, as well as in the first and second clutch at 40 µg/L and in the third clutch at 10 µg/L (L0, F11, F12 and F13, in Table 4.2). However, there were no significant detrimental effects of 4-nonylphenol ($p < 0.05$, ANOVA) in the remaining life-history traits (embryo and adult survival, age first reproduction, *data not shown*).

**Table 4.1** – Low and median concentration effects (µg/L) of cumulative fecundity (Fec.) and population growth rates (r) of the studied compounds across generations. Lowest observed effect concentration (LOEC), effect concentration decreasing 10 and 50 % of measured responses relative to controls ($EC_{10}$, $EC_{50}$, respectively). The TBT included two independent experiments (1 and 2). F11, F12, F13 refer to the second generation initiate from neonates from first, second and third clutch of females from the parental generation, respectively. Regression $r^2$ is also depicted. ns, not significant, ($p > 0.05$), (*) $0.05 > p > 0.01$, (**) $p < 0.01$.

| Treatment | Generation | | LOEC | $EC_{10}$ ± SE | $EC_{50}$ ± SE | $r^2$ |
|---|---|---|---|---|---|---|
| 4-Nonylphenol | Parental | r | 40 | 14.0 ± 2.4 | 42.0 ± 3.2 | 0.76** |
| | | Fec. | 40 | 35.7 ± 11.4 | 52.3 ± 17.5 | 0.53** |
| | F11 | r | 20 | 11.0 ± 3.2 | 97.3 ± 27.4 | 0.52** |
| | | Fec. | 40 | 34.5 ± 8.4 | 71.8 ± 22.2 | 0.43* |
| | F12 | r | 40 | 25.5 ± 2.6 | 41.1 ± 1.3 | 0.79** |
| | | Fec. | 40 | 23.1 ± 4.8 | 73.2 ± 14.8 | 0.41* |
| | F13 | r | 20 | 16.2 ± 1.9 | 27.3 ± 1.7 | 0.81** |
| | | Fec. | 40 | 27.3 ± 4.9 | 49.9 ± 7.4 | 0.40* |
| TBT1 | Parental | r | 0.50 | 0.65 ± 0.11 | 5.21 ± 2.32 | 0.59** |
| | | Fec. | 0.05 | 0.43 ± 0.13 | 1.18 ± 0.14 | 0.43** |
| | F11 | r | 0.20 | ns | 7.42 ± 3.20 | 0.40* |
| | | Fec. | 0.05 | ns | 1.35 ± 0.31 | 0.49** |
| | F13 | r | 0.50 | 0.65 ± 0.25 | 9.54 ± 2.30 | 0.40* |
| | | Fec. | 0.05 | ns | 4.89 ± 1.40 | 0.56** |
| TBT2 | Parental | r | 0.10 | 0.15 ± 0.05 | 29.6 ± 12.4 | 0.79** |
| | | Fec. | 0.50 | 0.27 ± 0.07 | 5.61 ± 2.03 | 0.40* |
| | F13 | r | 0.20 | 0.31 ± 0.07 | 2.00 ± 0.41 | 0.54** |
| | | Fec. | 0.20 | 0.24 ± 0.07 | 1.25 ± 0.15 | 0.66** |
| PBO | Parental | r | 200 | 391 ± 57 | 2321 ± 452 | 0.76** |
| | | Fec. | 200 | 215 ± 29 | 514 ± 28 | 0.86** |
| | F13 | r | 50 | 93 ± 19 | 1064 ± 180 | 0.83** |
| | | Fec. | 50 | 16 ± 2 | 339 ± 19 | 0.81** |

**Table 4.2** – Selected life-history trait responses (Mean ± SE, *n* = 10) of *D. magna* individuals exposed to 4-nonylphenol during one (parental) and two consecutive generations using neonates produced in the first (F11), second (F12) and third (F13) clutches. (*) indicate significant differences from solvent control treatments (SC) following ANOVA and Dunnet's post-hoc tests. L0 and Lf are initial and final body length, respectively with standard error.

| Generation | Treatment | L0 (µm) | Juvenile Survival (%) | Lf (µm) |
|---|---|---|---|---|
| Parental | C | 880.3 ± 8.3 | 100 | 3501.5 ± 20.6 |
| | SC | 877.2 ± 9.9 | 100 | 3563.6 ± 35.9 |
| | 5 | 873.3 ± 8.7 | 100 | 3540.4 ± 19.9 |
| | 10 | 871.0 ± 8.1 | 90 | 3570.2 ± 17.4 |
| | 20 | 877.3 ± 7.3 | 60 * | 3507.7 ± 31.7 |
| | 40 | 878.6 ± 11.4 | 50 * | 3297.1 ± 115.2 * |
| F11 | C | 739.3 ± 14.3 | 90 | 3608.4 ± 13.5 |
| | SC | 741.4 ± 15.3 | 100 | 3626.2 ± 31.5 |
| | 5 | 722.4 ± 10.7 | 100 | 3585.4 ± 21.5 |
| | 10 | 716.5 ± 14.6 | 80 | 3556.1 ± 47 |
| | 20 | 727.4 ± 16 | 50 * | 3545.4 ± 26.6 |
| | 40 | 667.5 ± 12.4 * | 70 | 3360.5 ± 61.3 * |
| F12 | C | 810.2 ± 10.3 | 100 | 3564.4 ± 39.6 |
| | SC | 816.0 ± 12.6 | 100 | 3577.0 ± 19.8 |
| | 5 | 809.0 ± 10.1 | 100 | 3589.0 ± 17.7 |
| | 10 | 792.4 ± 9.0 | 100 | 3611.5 ± 21.8 |
| | 20 | 816.6 ± 13.9 | 90 | 3612.8 ± 27.5 |
| | 40 | 772.1 ± 6.5 * | 50 * | 3543.2 ± 37.6 |
| F13 | C | 885.2 ± 12 | 100 | 3554.1 ± 33.7 |
| | SC | 883.3 ± 11.6 | 100 | 3582.9 ± 31.2 |
| | 5 | 825.5 ± 9.3 | 100 | 3554.7 ± 28.1 |
| | 10 | 806.2 ± 12.5 * | 90 | 3537.1 ± 45.3 |
| | 20 | 751.5 ± 12.6 * | 50 * | 3570.3 ± 25.3 |
| | 40 | 765.1 ± 7.5 * | 10 * | |

**Table 4.3** – Selected life-history trait responses (Mean ± SE, *n* = 10) of *D. magna* individuals exposed to TBT and PBO during one (parental) and two consecutive generations (Gen.) using neonates produced in the first (F11) and/or third (F13) clutches. (*) indicate significant differences from solvent control treatments (SC) following ANOVA and Dunnet's post-hoc tests. L0 and Lf are initial and final body length, respectively. TBT1 and TBT2 refer to first and second experiments.

| Gen. | Treat. | L0 (µm) | Lf (µm) | Gen. | Treat. | L0 (µm) | Lf (µm) |
|---|---|---|---|---|---|---|---|
| **TBT1** | **(µg/L)** | | | **TBT2** | **(µg/L)** | | |
| Parental | C | 882.3 ± 10.3 | 3677.9 ± 17.2 | Parental | C | 934.1 ± 10.9 | 3550.8 ± 21.7 |
| | SC | 887.2 ± 14.1 | 3695.0 ± 18.7 | | SC | 933.6 ± 6.8 | 3572.2 ± 23.8 |
| | 0.05 | 870.9 ± 7.6 | 3656.7 ± 15.4 | | 0.01 | 930.1 ± 9.9 | 3539.5 ± 14.6 |
| | 0.1 | 882.2 ± 12.6 | 3636.3 ± 13.5 * | | 0.02 | 913.6 ± 7.5 | 3591.2 ± 22.6 |
| | 0.2 | 887.2 ± 14.1 | 3633.1 ± 8.0 * | | 0.05 | 914.3 ± 7.9 | 3633.3 ± 34.4 |
| | 0.5 | 870.9 ± 7.6 | 3611.1 ± 4.7 * | | 0.1 | 939.1 ± 8.9 | 3542.9 ± 41.1 |
| | 1 | 882.2 ± 12.6 | 3593.1 ± 12.8 * | | 0.2 | 924.3 ± 10.9 | 3568.9 ± 15.4 |
| F11 | C | 738.2 ± 15.3 | 3777.4 ± 26.4 | | 0.5 | 943.6 ± 8.0 | 3502.5 ± 19.7 |
| | SC | 746.9 ± 19.1 | 3762.7 ± 7.9 | | 1 | 924.0 ± 9.0 | 3460.2 ± 12.3 * |
| | 0.05 | 701.7 ± 22.6 | 3691.7 ± 18.9 | F13 | C | 934.1 ± 10.9 | 3449.7 ± 41.8 |
| | 0.1 | 725.9 ± 13.0 | 3733.1 ± 22.4 | | SC | 942.5 ± 3.9 | 3431.6 ± 14.4 |
| | 0.2 | 725.8 ± 13.6 | 3670.0 ± 75.1 | | 0.01 | 926.8 ± 8.1 | 3500.3 ± 19.1 |
| | 0.5 | 709.1 ± 18.6 | 3624.0 ± 17.1 * | | 0.02 | 914.5 ± 8.3 | 3495.1 ± 15.5 |
| | 1 | 643.0 ± 24.6 * | 3514.2 ± 28.8 * | | 0.05 | 903.9 ± 9.2 * | 3470.7 ± 14.0 |
| F13 | C | 878.3 ± 8.3 | 3804.2 ± 42.3 | | 0.1 | 900.7 ± 10.7 * | 3458.4 ± 35.3 |
| | SC | 890.6 ± 10.2 | 3751.9 ± 33.1 | | 0.2 | 896.5 ± 5.5 * | 3432.7 ± 16.0 |
| | 0.05 | 880.9 ± 12.3 | 3773.8 ± 28.4 | | 0.5 | 886.5 ± 11.9 * | 3415.2 ± 15.9 |
| | 0.1 | 873.5 ± 11.4 | 3756.5 ± 44.9 | | 1 | 844.0 ± 11.8 * | 3316.4 ± 13.7 * |
| | 0.2 | 862.6 ± 8.8 | 3742.0 ± 34.2 | | | | |
| | 0.5 | 838.8 ± 9.0 * | 3701.5 ± 75.7 * | | | | |
| | 1 | 832.2 ± 7.9 * | 3668.8 ± 37.5 * | | | | |

| BPO | Treat. (µg/L) | L0 (µm) | Embryo Survival (%) | Age (days) | Lf (µm) |
|---|---|---|---|---|---|
| Parental | C | 901.9 ± 7.0 | 100 | 9.0 ± 0 | 3489.9 ± 16.0 |
| | SC | 905.0 ± 7.8 | 100 | 9.0 ± 0 | 3477.1 ± 18.5 |
| | 50 | 905.7 ± 8.6 | 100 | 9.2 ± 0.1 | 3459.6 ± 21.3 |
| | 100 | 912.0 ± 8.5 | 100 | 9.1 ± 0.1 | 3429.1 ± 18.3 |
| | 200 | 899.5 ± 4.0 | 100 | 9.0 ± 0 | 3416.6 ± 20.4 |
| | 400 | 907.7 ± 8.1 | 96.8 | 9.0 ± 0 | 3464.5 ± 17.1 |
| | 800 | 904.7 ± 7.0 | 54.2 * | 9.0 ± 0 | 3389.8 ± 16.6 * |
| F13 | C | 882.1 ±14.5 | 100 | 9.1 ± 0.1 | 3448.4 ± 31.1 |
| | SC | 884.7 ± 9.9 | 100 | 9.0 ± 0 | 3482.1 ± 21.0 |
| | 50 | 887.4 ± 11.0 | 100 | 9.6 ± 0.2 * | 3444.7 ± 18.3 |
| | 100 | 879.2 ± 9.5 | 100 | 10 ± 0 * | 3437.4 ± 22.2 |
| | 200 | 859.1 ± 13.3 * | 100 | 10 ± 0 * | 3430.6 ± 17.5 |
| | 400 | 843.5 ± 8.7 | 69.8 * | 10.3 ± 0.2 * | 3409.2 ± 24.4 |

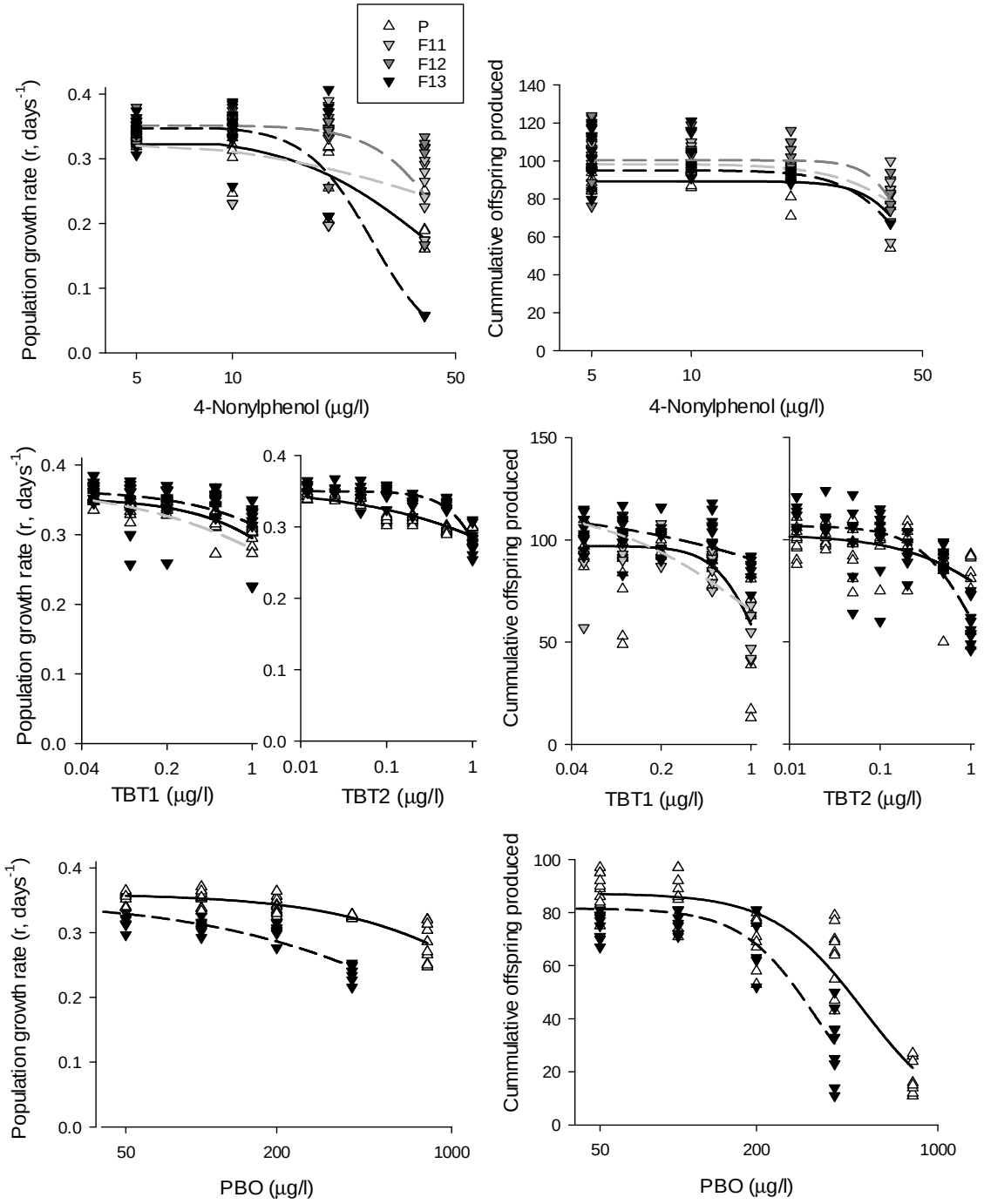


**Figure 4.1** – Cumulative offspring produced and population growth rate responses of 4-nonylphenol (upper panel graphs), tributyltin (TBT, middle panel) and piperonyl butoxide (PBO, lower panel) across generations. TBT1 and TBT2 graphs in the middle panel correspond to first and second experiments, respectively. Each symbol corresponds to a single observation. Lines are fits to the allosteric decay regression model. White symbols correspond to the parental generation. Light and dark grey, and black symbols corresponds to the second generations initiated from first (F11), second (F12) and third (F13) brood neonates, respectively.

Concentration-response curves of TBT on population growth rates and fecundity responses varied across endpoints and experiments rather than across generations (Table 4.1 and Figure 4.1). In the first experiment (TBT1), LOECs for fecundity and population growth rates occurred at 0.05 and at 0.2 to 0.5 µg/L, respectively (Table 4.1).

In the second experiment (TBT2), LOECs for both endpoints were more similar. Levels of LOEC ($EC_{10}$ or LOEC in Table 4.1, Figure 4.1) varied less than two fold across generations but differences in median effect concentrations ($EC_{50}$) were larger (Table 4.1). The $EC_{50}$ in the first experiment increased from parental to F13 whereas the opposite trend was observed in the second experiment. Final adult body length was also affected by TBT exposure in the first experiment. This trait decreased significantly in the parental generation at 0.1 µg/L ($p < 0.05$, ANOVA) as well as in the second generation at 0.5 µg/L (F11 and F13, Table 4.3). In the second experiment, TBT reduced significantly ($p < 0.05$) the final body length (Lf) of both the parental and F13 generations at 1 µg/L. In the first experiment, TBT also reduced significantly ($p < 0.05$, ANOVA) the size of the offspring produced by females from the parental generation in first and third clutches at 1 and 0.5 µg/L, respectively (L0 of F11 and F13, respectively, Table 4.3). In the second experiment, TBT reduced significantly ($p < 0.05$) the size of the offspring produced by females from the parental generation in the third clutch at 0.05 µg/L (L0 of F13, Table 4.3). There were no significant ($p < 0.05$, ANOVA or Chi-square test) effects of TBT in the remaining life-history traits (embryo, juvenile and adult survival, age first reproduction, *data not shown*).

Piperonyl butoxide effects increased dramatically in second generation individuals (Figure 4.1). Low observed effect concentrations and $EC_{50}$ on population growth rates and fecundity were from 1.5 to 13 fold lower in second generation individuals (Table 4.1). Piperonyl butoxide decreased significantly ($p < 0.05$, Chi-square, Kruskal-Wallis and ANOVA) embryo survival at 800 and 400 µg/L in the parental and second generation (F13), respectively; delayed reproduction at 50 µg/L in the second generation, and impaired the final body length at 800 µg/L in the parental generation (Table 4.3). Note that second generation experiments were limited to 0 - 400 µg/L because no surviving neonates were produced by females from the parental generation in the third clutch at higher exposure levels of PBO. Piperonyl butoxide also affected significantly ($p < 0.05$, ANOVA) the size of the offspring produced by females from the parental generation at 200 µg/L (L0 from F13, Table 4.3). The remained traits that included juvenile and adult survival were not significantly affected ($p < 0.05$).

It is important to note that, as expected, L0 of individuals from the parental generation did not vary within experiments in Table 4.2 and Table 4.3 since this

generation was always originated from third to sixth brood neonates of unexposed females.

## 4.5 Discussion

In two of the three studied compounds (4-nonylphenol and PBO), toxic effects were aggravated across generations and were related to observed detrimental traits on offspring quality. For 4-nonylphenol, population growth rate responses provide a better picture of its toxic effects than cumulative fecundity since this compound affected dramatically juvenile survival, which together with fecundity are parameters integrated into population growth rates (Barata *et al.,* 2002a). Indeed, treatment differences in juvenile survival responses measured at the highest concentration tested of 4-nonylphenol (Table 4.1) closely resembled those of population growth rates (Figure 4.1). This means that the observed aggravation of toxic effects of 4-nonylphenol in second generation individuals coming from third brood neonates (F13) was mostly related to high juvenile mortality rates occurring at the highest tested concentration of 40 µg/L (90 %). The previous feature is in line with observed aggravation effects on median effect concentrations ($EC_{50}$) but not at low exposure levels (LOEC, $EC_{10}$). This is, however, an important finding since it indicates that neonates born from third brood mothers exposed to 4-nonylphenol were more susceptible than those born in unexposed females and consequently die before adulthood. There is reported information on increasing sensitivity of 4-nonylpyhenol in second generation individuals (Tanaka and Nakanishi 2002; Brennan *et al.,* 2006). In a previous study we found that 4-nonylphenol at sub-lethal levels increases offspring number at expensed of reducing its size (Campos *et al.,* 2012). Smaller offspring often is less tolerant to pollutants (Enserink *et al.,* 1990; Cox *et al.,* 1992). The present results are, thus, in line with these previous studies. Effects of 4-nonylphenol on offspring size released by exposed females were greatest in the third clutch and, consequently, detrimental effects on fitness related life-history traits such as juvenile survival were also more evident in second generation individuals that were born from this third clutch. Nevertheless, there is reported evidence that at higher concentrations than the ones used here, effects of 4-nonylphenol on offspring quality caused embryo toxicity (LeBlanc *et al.,* 2000). Thus, it is possible that detrimental effects on offspring size coupled with reported embryotoxicity explained the observed two-generational effects.

In TBT experiments, effects on reproduction were apparently greater than those observed on population growth rates since this compound mainly affects reproduction that

contributes logarithmically into the former endpoint (Barata *et al.,* 2002a). Sensitivity to TBT across generations varied across experiments, endpoints and effect concentration levels, irrespectively of the initial size of neonates used to start second generation experiments. Low and high effect concentration levels ($EC_{10}$ and $EC_{50}$) estimated from regression analyses varied largely across endpoints and experiments, due to differences in curve shapes and by the fact that for population growth rate, inhibition effects were moderate (< 30 %). Fecundity responses, however, were inhibited by TBT to values close to 50 % in most treatments. Accordingly, the toxicity of TBT monitored as $EC_{50}$ of cumulative fecundity decreased across generations and clutches used to start second generation (F1) in the first experiment but increased in the second experiment. This apparent contradiction may be related to the fact that the initial size of neonates used to start the second generation TBT experiments was significantly impaired at lower concentrations in the second experiment. Reported multigenerational effects of TBT are contradictory. Oberdörster *et al*., (1998) found that effects of TBT hardly affected reproduction in consecutive generations at sub-lethal concentrations, but enhanced the metabolism of the steroid hormone testosterone. More recently, Jordão *et al.,* (2015) found that adult females exposed to low and high concentrations of TBT for just 3 days produced smaller offspring containing less TGs that survived less and had lower fecundity rates than those from control females. The present results and those reported elsewhere indicate that multigenerational effects of TBT and probably of 4-nonylphenol not only depend on the initial size of individuals used to start second generation experiments, but also on offspring quality.

Exposures to PBO indicate that reproduction and population growth rate responses were equally informative, since this compound affected three traits: fecundity, embryo survival and age at first reproduction, all integrated in population growth rate estimates (Barata *et al.,* 2000a). Two-generational effects of PBO supported the premise that offspring quality is a key factor in determining effects across generations. The decrease in size of the offspring produced by the parental generation was of similar magnitude than those observed for 4-nonylphenol and TBT, but the observed multiple generational effects of PBO were by far the greatest of the three studied chemicals. Piperonyl butoxide is a known cytochrome P450 (CYP) inhibitor and inhibits steroid hydrolases in *Daphnia* (Baldwin and LeBlanc 1994). These enzymes are involved in the biosynthesis and metabolism of ecdysteroids in crustaceans (Mykles 2011), which are crucial for embryo development (Subramoniam 2000). This means that, likewise 4-nonylphenol and to a lesser extent TBT, two-generational effects of PBO are likely to be

related to detrimental effects on both offspring quality and size. LeBlanc *et al.,* (2000) hypothesized that embryotoxicity of 4-nonylphenol was related to deficiencies in some critical developmental components provided to eggs by maternal organisms, such as ecdysteroids, essential fatty acids or TGs. In many crustaceans, ecdysone of maternal origin is packed into eggs largely as polar conjugates (Subramoniam 2000) and 4-nonylphenol inhibits the production of polar metabolites of steroid hormones (Baldwin *et al.,* 1997), thus limiting the amount of ecdysone provided to embryos. However, there is evidence that free ecdysteroids are the predominant ecdysteroids found in eggs of *D. magna* (Martin-Creuzburg *et al.,* 2007). Thus, deficiencies in the maternal transfer of other critical component than ecdysteroid seems to be affected by 4-nonylphenol and probably also by PBO. We found previously (Jordão *et al.,* 2015) that when females were exposed briefly to TBT during only one reproductive event (3 days), the transfer of TGs containing highly PUFA to eggs was impaired. Hence, juveniles developed from these eggs were smaller and had their survival severely impaired later in life, before reproduction.

In the present study, the two compounds having greater two-generational effects (PBO and 4-nonylphenol) affected embryo or juvenile survival. Tributyltin affected juvenile survival only marginally, producing a non-significant decrease from 100 % survival at 1 µg/L in the parental generation to 70 and 80 % survival in the F11 and F13 generations, respectively. Hence, detrimental two-generational effects of TBT were small or negligible. The present results support the argument that detrimental effects on offspring size and quality are needed to obtain multigenerational effects. One possible mechanism of action that may be shared by 4-nonylphenol and PBO is limiting the transfer of some critical development component from the mother to its eggs, making juveniles born from those eggs more vulnerable to further exposures. Further studies are needed to confirm those findings.

## 4.6 Conclusions

The present results showed that aggravation of toxic effects across generations occurred in two out of three chemicals tested: 4-nonylphenol and PBO. Two-generational effects of TBT varied across experiments being ameliorated in one experiment and aggravated in the other. Results obtained for 4-nonylphenol and TBT also indicated that the selection of the clutch to initiate the second generation experiment affected inter-generational effects. For 4-nonylphenol, toxic effects aggravated in second generation individuals coming from third brood neonates but not when first and second brood

neonates were used. For TBT, the opposite trend was observed. Results of PBO were limited to third brood neonates but supported the previous findings obtained with 4-nonylphenol, indicating that the use of third brood neonates improved the detection of inter-generational effects. The studied chemicals affected the quantity and quality of the offspring produced by exposed females, being those effects more obvious in third clutch neonates and, consequently, when these offspring were further exposed, they showed greater sensitivity than the parental generation. These results are in line with those obtained in multigenerational studies in fish and mammalian tests, which evidence that in many cases, detrimental effects on the second generation can be predicted by evaluating the quality of the offspring produced (Janer *et al.,* 2007; Holdway *et al.,* 2008; Schulz *et al.,* 2014).

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# Chapter V

Multixenobiotic resistance mechanism in *Daphnia magna*

# Chapter V – Multixenobiotic resistance mechanism in *Daphnia magna*

## 5 Induction of multixenobiotic defence mechanisms in tolerant *Daphnia magna* clones as a general cellular response to stress [1]

### 5.1 Abstract

Multixenobiotic resistance (MXR) mechanisms were recently identified in *Daphnia magna*. Previous results characterized gene transcripts and efflux activities of four putative ABCB1 and ABCC transporters that were chemically induced but showed low specificity against model transporter substrates and inhibitors, thus preventing to distinguish between activities of different efflux transporter types. In this study, we report the specificity of the induction of ABC transporters and of the stress protein Hsp70 in clones selected to be genetically tolerant to ABCB1 chemical substrates. Clones tolerant to mitoxantrone, ivermectin and pentachlorophenol showed distinctive transcriptional responses of transporter proteins coding genes and of putative efflux dye activity. Expression of Hsp70 proteins also varied across tolerant clones. Clones tolerant to mitoxantrone and pentachlorophenol showed high constitutive levels of Hsp70. Transcriptional levels of the *abcb1* transporter gene and of putative dye efflux activity were also induced to a greater extent in the pentachlorophenol tolerant clone. Observed higher dye efflux activities in exposed individuals from the clone resistant to mitoxantrone were unrelated with transcriptional levels of the studied four *abcc* and *abcb1* transporter genes. Tolerance to ivermectin was not explained by the studied ABC responses and only marginally by expression levels Hsp70 proteins. These findings suggest that Abcb1 induction in *D. magna* may be part of a general cellular stress response.

**Keywords:** Hsp70, induction, P-glycoprotein, MDR, *Daphnia magna.*

[1] **Jordão R.**, Campos B., Lemos M. F. L.,Soares A. M. V. M, Tauler R. and Barata C., (2016). "Induction of multixenobiotic defense mechanisms in resistant *Daphnia magna* clones as a general cellular response to stress." Aquatic Toxicology. 175:132-143 (article in annex).

## 5.2 Introduction

The study of the mechanisms of tolerance to pollution is a key issue in environmental risk assessment. Evidence indicates that the cellular multixenobiotic or multidrug resistance (MDR and MXR, respectively) system in aquatic organisms represents a broad-scale defence mechanism protecting cells and organisms against environmental toxicants dissolved in the water (Kurelec 1992). Multixenobiotic resistance is mediated by membrane-based transport proteins from the ATP binding cassette (ABC) protein family, which acts in a wide variety of chemical-substrates, pumping them out of the cell, thus keeping their levels low in cells. Cellular MXR efflux activities in various aquatic invertebrate species have been so far associated with homologs of mammalian ABCB1 (P-gp) and ABCC (or multidrug resistance protein, MRP) proteins (Bard 2000; Epel *et al.,* 2008). Organisms and cells with potent MXR defences are less sensitive to the toxic impact of compounds that are transporter substrates, because comparatively few of those molecules reach their site of toxic action (Bard 2000; Epel *et al.,* 2008).

Recently, we cloned the putatively MXR related partial *abcb1*, *abcc1/3*, *abcc4* and *abcc5* coding gene sequences[2], which were constitutively expressed in different life stages of the crustacean *Daphnia magna* (Campos *et al.,* 2014). Accumulation of different fluorescent ABC transporter substrates in *D. magna* was increased in the presence of transporter inhibitors, indicating MXR-like efflux activity in tissues from the animals. Enhanced toxicity of toxic transporter substrates co-administered with MXR inhibitors indicated that MXR was involved in tolerance to chemical pollutants in *D. magna*.

Furthermore, expression levels of transcripts of putative MXR related Abc transporters and efflux activity were chemically induced. In the study of Campos *et al*., (2014), however, model ABC transporter substrates, inducers and inhibitors did not target *D. magna* Abc transporters with sufficient specificity to distinguish between activities of different ABC efflux transporter types. There was, thus, unclear evidence of the toxicological role of Abcb versus Abcc type transporters.

Further studies comparing MXR responses across tolerant and intolerant individuals to known MXR toxic substrates offer the possibility to study the functional role of different ABC transporter types. *Daphnia magna* usually reproduces by parthenogenesis in the laboratory, thus it offers the possibility to perform selection

[2] Our nomenclature for gene/protein name is: *abcc*/Abcc for *Daphnia* and *ABCC*/ABCC for human.

experiments to specific toxic MXR substrates and then study how gene transcripts and efflux activities vary across selected tolerant and sensitive clones (Damásio *et al.,* 2007).

Selection of MXR was first observed during cancer treatment and the ABCB1 was found to be the main cause for resistance towards cytotoxic drugs (Juliano and Ling 1976). Later work found that several ABCC (mainly ABCC1-3) were associated with tumour resistance, which is often caused by an increased efflux and decreased intracellular accumulation of natural product anticancer drugs and other agents (Yu *et al.,* 2007). Artificial selection of tolerance to ABCB1 toxic substrates has also been successfully conducted in sub-population of fish cells selected against cytotoxic drugs (Zaja *et al.,* 2008). In whole aquatic animals, however, information is limited to induction experiments. Kurelec and co-workers (1995), exposed marine snails (*Monodonta turbinata*) in the laboratory to Diesel-2 oil and found enhanced Abcb1 activity. Minier and co-workers (1996), also observed increased Abc protein concentration and activity in blood cells of *Mytilus edulis* when the mussels were injected with the ABCB1 substrate vincristine. In Californian and zebra mussels (M*ytilus californianus and Dreissena polymorpha*) induction of gene transcripts and of putative transporter activity of Abcb and Abcc transporters have been reported in larvae and adult gills of individuals pre-exposed to pesticides that are also ABC substrates (pentachlorophenol, mercury and dachtal) as well as to non-substrates (DEE, dichlorodiphenyldichloroethylene; sodium arsenite) (Eufemia and Epel 2000; Tutundjian and Minier 2007; Navarro *et al.,* 2012). Recently, we found that levels of Abc transporter gene transcripts and efflux MXR transporter activity increased in individuals of *D. magna* pre-exposed to mercury, pentachlorophenol or dacthal (Campos *et al.,* 2014). In the previous study, however, it was not clear whether the response was substrate specific or part of a general stress response.

One well-characterized general stress response is the induction of heat shock proteins (Hsps) (Parsell and Lindquist 1994). These proteins, particularly Hsp70, are inducible by a variety of chemical and physical stresses including high temperature, ethanol, metals and oxidizing agents (Parsell and Lindquist 1994). A general response of Hsp70 to various stressors including metals, heat shock and pesticides has been observed in *D. magna* (Bond and Bradley 1995; Haap and Köhler 2009; Mikulski *et al.,* 2011). Several studies showed that long term selection to metallic pollution and naturally occurring stressors such as epibionts are related to changes in heat shock proteins in *D. magna* (Pauwels *et al.,* 2007; Haap and Köhler 2009).

To better understand MXR mechanisms in *D. magna* the aim of this study was to characterize this system in tolerant *D. magna* clones to an array of chemicals that are

known to be substrates of MXR and/or to induce transporter activity. The response of heat shock proteins (Hsp70) was also studied to distinguish between substrate specific MXR responses from general stress ones. Our departure hypothesis was that observed tolerance and co-tolerance across the studied clones to their chemical selectors was related to higher transcriptional and transporter MXR activity or/and due to higher induction levels of Hsp70.

Following previous work conducted in *D. magna* (Barata *et al.,* 2002b; Damásio *et al.,* 2007), the obtained tolerant clones were selected exposing neonates hatched from resting eggs (ephippia) to high concentrations of selected chemicals. Surviving individuals were then maintained as clonal lineages for several generations under non contaminated media and then their acute tolerance assayed following conventional procedures (OECD 1997). Selected chemicals included the cytotoxic drugs that are substrates of mammalian ABCB1 (mitoxantrone) and ABCC transporters (chlorambucil) and toxic to *D. magna* (Campos *et al.,* 2014), verapamil a known ABCB1 inhibitor in mammals, mussels and sea urchins (Yusa and Tsuruo 1989; Eufemia and Epel 2000; Hamdoun *et al.,* 2004; Faria *et al.,* 2011); pentachlorophenol a known inducer of Abcb transporter activity in mussels and *D. magna* (Eufemia and Epel 2000; Campos *et al.,* 2014) and ivermectin, an anthelmintic drug known to be a substrate of MXR pump (Alvarez *et al.,* 2006).

## 5.3 Material and Methods

### 5.3.1 Chemicals

Chemical contaminants (Pestanal analytical standard grade, 99 % purity): mitoxantrone, ivermectin, pentachlorophenol, verapamil, chlorambucil; and dyes: rhodamine B, rhodamine 123 and calcein AM (Ca-AM) and calcein disodium salt, were purchased from Sigma-Aldrich. Acetone, (analytical grade) were supplied by Merck (Darmstadt, Germany).

### 5.3.2 Experimental animals

One laboratory clone of *D. magna* Straus designated as F (Barata *et al.,* 2000), and three field clones tolerant to mitoxantrone, pentachlorophenol and ivermectin designated MIT, PCP and IVM, respectively, were chosen based on their acute sensitivity to those chemicals. Field clones were selected from the few survivors from about 500

neonates (< 12 hrs old) hatched from ephippia and exposed during 48 hrs to high doses of the studied chemical contaminants, which roughly correspond to 2 x 48-$LC_{50}$ of clone F (6 mg/L of mitoxantrone, 6 ng/L of ivermectin, 2 mg/L of pentachlorophenol, 30 mg/L of verapamil, and 80 mg/L of chlorambucil). Despite that some individuals survived to acute exposures of verapamil and chlorambucil, clonal lines obtained from those individuals did not show high tolerance to these chemicals (further details are shown in the "acute response" section in "Results"). Ephippia or resting eggs were collected from rice field sediments of Delta del Ebro River (NE Spain), an area regularly exposed to pesticide runoffs (Hildebrandt *et al.,* 2007). Eggs were preserved in water at 4 °C in darkness and hatched in ASTM hard water at 20 °C under continuous light (Barata *et al.,* 2000). Field clones can be considered genetically different since originated from different resting eggs, which in *D. magna* are produced by sexual reproduction (Barata *et al.,* 2000). Although the exact origin of the selected laboratory clone F is not known, previous studies indicate that this clone have an average tolerance to chemical contaminants (Barata *et al.,* 2000). Thus, differences in ABC gene transcription levels, putative ABC dye efflux rates and protein levels of Hsp70 among these field and laboratory clones can inform us about the role of these two systems (ABC versus heat shock) in the tolerance of *D. magna* to the studied chemicals.

For each clone, two 1 L bulk cultures of five animals were maintained in ASTM hard water as described elsewhere (Damásio *et al.,* 2007). Animals were fed daily with *Chlorella vulgaris* Beijerinck (1 x $10^5$ cells/mL); corresponding to 1.8 µC/mL). The culture medium was changed every other day and neonates were removed within 24 hrs. To ensure that clonal differences in resistance were not due to physiological acclimation, field clones were acclimated to the standard laboratory conditions for more than 10 generations. To minimize maternal effects, all experiments started with 24 hrs old neonates, originated from third to sixth brood females (Barata *et al.,* 2004).

### 5.3.3 Toxicity bioassays

Exposures to the studied chemicals were conducted using three types of bioassays:

(1) Acute toxicity bioassays of each clone against the chemical compound, which was selected (hereafter named "chemical selector") to allow assessing the degree of tolerance.

(2) Acute toxicity bioassays of each clone against all the remained tested chemicals to allow assessing cross-tolerance.

In both assays lethal responses were obtained after 48 hrs of exposure following OECD 202 (OECD 1997) guidelines with minor modifications. Groups of 10 neonates were exposed to 4 or 5 concentrations of the tested chemical in 50 mL ASTM hard water in the absence of food. At the end of the experiments, alive and immobile animals were counted to determine lethal concentration effects.

(3) *In vivo* assays to study MXR and Hsp70 responses of the studied clones to their respective chemical selectors: Groups of five 4 days old juveniles were exposed to their respective chemical selector in 150 mL ASTM hard water for 24 hrs. In each assay the performance of a tolerant clone was compared to that of the laboratory clone F. Assays included an unexposed control and two sub-lethal exposure concentrations equivalent to moderate and high exposures, which were selected from obtained 48 hrs–$LC_{50}$. Acetone (< 0.1 mL/L) was used as a carrier for all contaminants but mitoxantrone, which it was dissolved directly in water. In those chemical treatments in which acetone was used as a carrier, the control treatment also contained the same amount of acetone. Each assay was replicated five times. After pre-exposures, putative transporter efflux activities in the animals were determined with dye efflux assays, transporter transcript levels were quantified with qPCR and levels of Hsp70 using Western blot procedures. Due to sample requirements, Hsp70 experiments followed the same procedure as above with the following modifications: animals were exposed in groups of 20 in 750 mL of ASTM hard water, treatments were replicated twice and limited to a control and the highest concentration tested. For each compound its tolerant clone and clone F were tested together to allow direct comparison of relative protein levels relative to unexposed individuals from clone F. Due to sample size requirements different set of experiments were performed to study gene transcription, transporter activity and protein levels of Hsp70.

### 5.3.4 RNA extraction and qPCR analysis

Transcript abundances of *D. magna, abcb1*, *abcc1/3*, *abcc4* and *abcc5* (GenBank accession nos. KC122929, KC122922-KC122924, respectively), were determined in 4 days old juveniles following a previous study (Campos *et al.,* 2014). For RNA extraction, the 5 juveniles from each replicate were pooled to obtain sufficient RNA. Total RNA was

isolated from *D. magna* tissue using Trizol reagent® (Invitrogen™), according to manufacturer protocols. The RNA concentration was measured by spectrophotometric absorption in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Delaware, DE) and the quality was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). Quantities from 100 ng to 1 µg of DNAse II (Ambion®) treated RNA were retro-transcribed to cDNA using the First Strand cDNA Synthesis Kit (Roche Applied Science®) and stored at -20 ºC. The amounts of cDNA used for qPCR corresponded to 10 ng of the original RNA preparation. qPCR was performed with a LightCycler® 480 Real-Time PCR System using LightCycler® 480 SYBR Green I Master (Roche Applied Science®). The qPCR primers (Table 5.1) were designed with Primer Express software. Sequences of amplicons were confirmed by sequencing using an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems) and by comparing them to the corresponding references in GenBank using NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Campos *et al.,* 2014).

Relative mRNA abundance values were calculated from the second derivative maximum of their respective amplification curve (Cp, values from triplicate assays). RNA abundances of putative reference genes *18S* and *g3pdh* (mRNA) were quantified and tested for stability in the RNA sources used in this work. The minimal variation of Cp values was found for *g3pdh* mRNA, which showed SD values below 1 Cp units in all samples; therefore, this gene was selected as reference gene.

Cp values obtained for the target genes (TG) were compared to the corresponding values of *g3pdh* to obtain ΔCp values (ΔCp = Cp ref- CpTG). qPCR efficiency values for reference and target genes were calculated as described by (Pfaffl 2001) and calculated to be close to 100 %, thus no efficiency corrections were performed. qPCRs were performed with RNA extracts from five replicates, in duplicate per sample. Mean mRNA abundances and standard errors were calculated for each gene from the mean values of each sample.

**Table 5.1** - Primer pairs designed. Primers have been obtained from existing sequences used for the analyses of gene transcriptomic changes (Campos *et al.,* 2014).

| Genes | Forward | Reverse | Amplicon Size |
|---|---|---|---|
| ***ABCB*** | GTATCCAGTGCGGAAGTGGC | ACAGCGTATCGCTATTGCCC | 100 |
| ***ABCC1-3*** | TAGCTCGCGCTCTACTGAGAA | GATCGTCGGTCTCCAGATCG | 100 |
| ***ABCC4*** | CCCGATCCCTTTACGTCGAT | GGTGGCGTCCTACATGAGTGT | 100 |
| ***ABCC5*** | CAGTCCAGTCATCGAGAACGG | TGACGCAACAGAGCTCGG | 100 |
| ***G3PDH*** | GACCATTACGCTGCTGAATACG | CCTTTGCTGACGCCGATAGG | 100 |
| ***18S*** | CGCGAATGGCTCAATAAATCA | CAGTCGAGGCTCGAGTGCA | 100 |

### 5.3.5 Dye efflux assays

ABC transporter activities were determined in 4 days old juveniles using dye accumulation assays following previous procedures (Campos *et al.,* 2014). As proxies for efflux activity in daphnids we used Ca-AM and rhodamine B and 123. Calcein-AM is not fluorescent but forms fluorescent calcein once it reaches the cytosol where it is cleaved by cellular esterases. Calcein-AM is known to be transported by mammalian ABCB1 and ABCC transporters, rhodamine B has greater affinity for ABCB1 transporters, and rhodamine 123 for ABCC transporters (Neyfakh 1988; Homolya *et al.,* 1993; Holló *et al.,* 1994; Essodaïgui *et al.,* 1998; Daoud *et al.,* 2000; Yeheskely-Hayon *et al.,* 2009). Low fluorescence levels of cells/tissues indicate high efflux transporter activity and enhanced fluorescence occurs with low efflux activity. *Daphnia magna* animals were immobilized immediately prior to dye efflux assays by pinching their hearts with a needle and thus stopping filtering activities in all animals used in the experiment. Immobilized *D. magna* juveniles (4 days old) were exposed individually for 1 hr to 2.5, 1.5 and 0.5 µM of rhodamine 123, rhodamine B and Ca-AM (stock dissolved in DMSO), respectively, in 110 mL of ASTM hard water. Assays were replicated 10 times. After the exposure period, animals were transferred to 100 mL clean ASTM hard water for 5 mins to allow depuration of excess dye and the accumulated dye was extracted from tissue by sonication of animals in 1 mL of ASTM hard water. Fluorescence measurements were performed in a microplate fluorescence reader (Synergy 2, BioTek, USA) using excitation/emission

wavelengths of 480/530 nm for Ca-AM and rhodamine 123, and 530/590 nm for rhodamine B. Measurements of each replicate were run in triplicate and were corrected for background fluorescence levels of ASTM hard water. Fluorescence values were then converted into concentration units using a calibration curve based on eight data points from different concentrations of calcein disodium salt (fluorescent dye), rhodamine 123 and rhodamine B. Accumulated dye is reported as pmol/individual.

### 5.3.6 SDS-PAGE and Western blot

For each treatment, two replicates of 4 days old juveniles (*n* = 20) were homogenized on ice with a sonicator in 250 µL of homogenization buffer (10 mM Tris-HCl pH 7.6, 0.5 M sucrose, 0.15 M KCl, 0.1 % Triton X-100, 1 mM dithiothreitol (DTT), 1 mM ethylenediamine tetraacetic acid (EDTA) and 0.1 mM phenantroline), with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 40 µg/mL aprotinin). The homogenate was centrifuged at 6,000 x *g* for 10 mins at 4 °C (Minier *et al.,* 2000). The total protein concentrations were accessed by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford 1976). The supernatants were immediately frozen at −80 °C until further analysis. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot was done essentially as described by Abcam protocols (Abcam, Cambridge, UK). Protein extracts were diluted in equal volume of 2x cracking buffer (250 mM Tris–HCl pH 6.8, 10 % glycerol, 2 % SDS, 100 mM DTT and 0.05 % bromophenol blue) and denatured at 95 °C for 5 mins. Protein extracts (30 µg) were run on 7.5 % gels and transferred to a polyvinylidene difluoride (PVDF) membrane (Roche), for 1 hr at 100 V in transfer buffer (25 mM Tris, 190 mM glycine, 0.1 % SDS and 20 % of methanol), using a Wet/Tank Blotting System (Bio-rad). Membranes were blocked in 3 % BSA/1x TBS-T (0.2 M Tris-HCl pH 7.5, 1.5 M NaCl and 0.05 % Tween-20), for 1 hr and then incubated overnight at 4 °C with a mouse monoclonal primary antibody for the Hsp70 (H5147, from Sigma-Aldrich), diluted 1:1000 in 1 % BSA/1x TBS-T. After, membranes were washed three times for 10 min in 1x TBS-T and incubated for 1 hr at room temperature with an anti-mouse horseradish peroxidase secondary antibody (A4416, from Sigma-Aldrich), diluted 1:2000 in 1 % BSA/1X TBS-T. Membranes were subsequently washed in 1x TBS-T three times for 10 mins and incubated with a SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL, USA). The chemiluminescent signal was captured on an ImageQuant™ LAS 500 (GE Healthcare Life Sciences) and band intensities were

quantified using the Image Studio Lite software version 5.0 (LI-COR Biosciences, Lincoln, NE, USA). The intensity of each band was calculated as its average intensity multiplied by its area after subtracting the background (the pixels average in the neighbourhood of the band).

### 5.3.7 Determination of the test chemicals stability in aqueous test solutions

The stability of mitoxantrone, ivermectin and pentachlorophenol in ASTM hard water over the duration of the toxicity tests was investigated by measuring their concentrations in freshly made aqueous test solutions (time 0) and after 48 hrs. Two concentrations of each compound that corresponded to low and high exposure levels in the MXR and western blot assays were tested. Experiments were conducted under the same conditions as toxicity assays. Dissolved levels of the studied compounds were determined after solid phase extraction (for ivermectin) or directly (for mitoxantrone and pentachlorophenol) by sample injection using an AcQuity UPLC system equipped with a quaternary pump and connected to a triple quadrupole MS/MS system (Waters, USA) following previous procedures (Gómez-Canela *et al.,* 2013). Water samples of ivermectin (500 mL) were extracted using OASIS HLB SPE cartridge (200 mg sorbent/6 mL cartridge, Waters), eluted with methanol containing 5 mM ammonium formate and eluates concentrated to a final volume of 0.5 mL under gentle nitrogen stream. Data was acquired and processed using MassLynx 4.1 software package. A ZORBAX Eclipse XDB-C18 Narrow-Bore column 2.1 x 150 mm, 5 µm from Agilent Technologies (Santa Clara, USA) was used. The mobile phase consisted of solvent A, 5 mM ammonium formate in water, and solvent B, 5 mM ammonium formate in methanol. The initial mobile phase composition was 70 % A and 30 % B (5 min), to 70 % B in 3 min and held for 10 min, and to 100 % B in 3 min. These conditions were held for 4 min and then the initial conditions were regained in 5 min with an equilibration time of 2 min. The flow rate was set at 0.3 mL $min^{-1}$. To optimize ionization and to establish mass spectral features, individual standards were first analysed by flow injection analysis (FIA) in UPLC mass spectrometry in positive electrospray ionization (ESI+) mode for mitoxantrone and in ESI- mode for ivermectin and pentachlorophenol. Full-scan data acquisition was performed scanning from m/z 100 to 600 in profile mode, using a scan time of 2 sec with a step size of 0.1 u and a pause between each scan of 2 msec. Optimized parameters were source temperature (from 125 to 150 ºC), cone voltage (from 5 to 50 V) and collision energy (from 5 to 50 eV). Quantification was based on an external calibration standard curve of eight points ($r^2$ >

0.95). The limits of detection (LOD) were determined as the analyte concentration that gave a signal-to-noise ratio (S/N) of 3, which were 3 µg/L, 0.2 ng/L and 5 µg/L for mitoxantrone, ivermectin and pentachlorophenol, respectively.

### 5.3.8 Statistics and modelling toxic effects

Half maximal lethal concentration effects ($LC_{50}$) were estimated from percentage immobility responses fitted to the Hill regression model of equation 1:

$$E(\%\ immobile) = \frac{100}{1 + (LC_{50} / x)^{p}} \qquad (eq.1)$$

Where *E* is the effect in % of immobile organisms, *p* is the shape parameter; $LC_{50}$ ("lethal concentration 50 %") is the concentration of a test compound causing 50 % mortality of individuals, x is the concentration of the test compound. Regression parameters were estimated by the Least Square Method using the Levenberg-Marquardt algorithm. The standard error (SE) of each estimated parameter was then calculated from the standard deviation of the least square estimates (Zar 1996). Model accuracy was assessed by using the adjusted coefficient of determination ($r^2$) and by analysing the residual distribution.

*qPCR mRNA responses*: One-way ANOVA analyses of relative mRNA expression was performed using ΔCp values, as this parameter followed normal distribution and the variances were homoscedastic, as assessed by the Kolmogorov–Smirnov, Shapiro-Wilk and Bartlett's tests. To facilitate the interpretation of results these values were expressed as mRNA copies of target gene per $10^3$ copies of the reference gene mRNA.

*Dye efflux:* Transporter efflux activity was evaluated comparing the levels of fluorescence across treatments using one-way ANOVA. Lowest observed effect concentration values (LOEC) were estimated with post-hoc Dunnet's test. When required, data was log transformed to improve ANOVA assumptions of normality and variance homoscedasticity.

*Western blot*: Protein levels of Hsp70 of exposed and unexposed individuals from selected clones and those exposed from clone F were compared to unexposed individuals from clone F using one-way ANOVA followed by Dunnett's test (Zar 1996).

Statistics and concentration-response modelling were performed using the SPSS 17 (SPSS Inc., 2002) and Sigmaplot (v 11.0), respectively.

## 5.4 Results and Discussion

### 5.4.1 Stability of test chemicals in aqueous test solutions

Concentrations of ivermectin and pentachlorophenol in unmodified form were stable in ASTM hard water over 48 hrs as measured concentrations at 0 and 48 hrs were close to nominal ones (Table 5.2). Ivermectin was confirmed with fragment ions at m/z 754 and 183 and pentachlorophenol with its precursor ion m/z 265 (Table 5.2). Mitoxantrone was rapidly degraded, the parental compound was absent even in freshly prepared aqueous solutions. The two previously identified transformation toxic products (Gómez-Canela *et al.,* 2013), TP1 and TP2, were stable during the 48 hrs period of the experiment and their sum corresponded to almost 100 % of initial nominal concentrations (Table 5.2).

**Table 5.2** – Nominal and measured concentration levels (mg/L or ng/L) of the studied compounds in ASTM hard water in freshly prepared (time 0) and old test solutions (48 hrs). Protonated molecule of mitoxantrone $[M+H]^+$ was not detected and instead its transformation products TP1 and TP2 (Gómez-Canela *et al.,* 2013) were analysed and results expressed as the sum of both cations. For ivermectin and pentachlorophenol the precursor anion $[M-H]^-$ was the most abundant form. BDL = below detection limit.

| | | | | Time 0 hrs | Time 48 hrs |
|---|---|---|---|---|---|
| Compound | Precursor ion | Fragmented ion | Nominal | Mean ± SD | Mean ± SD |
| Mitoxantrone (mg/L) | 445 | 214 | 1 | BDL | BDL |
| | | | 2 | BDL | BDL |
| Mitoxantrone TP1; TP2 (mg/L) | 268; 282 | 214; 214 | 1 | 0.98 ± 0.03 | 0.95 ± 0.02 |
| | | | 2 | 1.89 ± 0.05 | 1.85 ± 0.07 |
| Ivermectin (ng/L) | 898 | 754; 183 | 3 | 3.10 ± 0.10 | 2.80 ± 0.10 |
| | | | 6 | 5.90 ± 0.20 | 5.40 ± 0.30 |
| Pentachlorophenol (mg/L) | 265 | 265 | 0.5 | 0.48 ± 0.04 | 0.43 ± 0.05 |
| | | | 1 | 1.05 ± 0.08 | 0.96 ± 0.10 |

### 5.4.2 Acute responses

Immobile response-concentration curves and estimated regression parameters of the studied clones are depicted in Figure 5.1 and Table 5.3, respectively. All regression curves depicted on Table 5.3 were significant ($p < 0.05$) and accurately fitted to immobile responses. Only three out of five tested chemicals succeed in selecting tolerant *D. magna* clones relative to the lab one (Figure 5.1 and Table 5.3). The degree of resistance achieved in terms of the quotient between the $LC_{50}$ of selected versus F clone (Damásio *et al.,* 2007) was almost 3 fold for ivermectin, followed by mitoxantrone (1.9 fold) and pentachlorophenol (1.4 fold). Degrees of resistance of 3 to 2 fold can be considered moderate when compared to the six fold reported for resistant clones of *D. magna* selected against insecticides and cadmium in the field (Barata *et al.,* 2002b; Damásio *et al.,* 2007). Lower degrees of tolerance in laboratory selection experiments relative to those observed in the field often occur and are related to the fact that selective pressures in the laboratory are lower (Roush and Mckenzie 1987). In the field, original populations have larger sizes and selective pressures are greater (pesticides often kill over 99 % of the population) (Roush and Mckenzie 1987). Nevertheless, the achieved degrees of resistance in this present study were high enough to allow testing for difference in ABC transporter and Hsp70 responses. Despite that chlorambucil is a known substrate for ABCC transporter and is toxic to *D. magna* (Campos *et al.,* 2014), it failed to be select for tolerance. Observed failure of chlorambucil is probably related to the fact that this compound degrades in water to non-toxic metabolites (Gómez-Canela *et al.,* 2013), thus preventing selection of tolerant individuals against the parental compound. Failure of verapamil, which is also a toxic substrate for ABCB transporter, may be related to the fact that this compound also blocks calcium channels and disrupt the calcium homeostasis in crustaceans (Suarez-Kurtz 1982; Zanotto and Baptista 2011). This means that initial selected organisms were tolerant to chlorambucil metabolites or to calcium deprivation rather than to the parental compounds. Consequently the tolerance of these organisms did not persist across generations. Results from Table 5.3 also indicated that there was some cross-tolerance among the selected clones relative to clone F. Clones resistant to ivermectin and pentachlorophenol, were, respectively, 1.6 times more tolerant to mitoxantrone and ivermectin. Cross-tolerance to metals and to other environmental stressors has been reported in *Daphnia* clones (Agra *et al.,* 2010; Chen and Stillman 2012; Hochmuth *et al.,* 2015). Differential induction of metallothioneins or similar related proteins has been reported to explain metal cross-tolerance in *Daphnia* (Bodar *et al.,*

1990; Barata *et al.,* 2002c; Guan and Wang 2004). Our departure hypothesis was that observed tolerance and co-tolerance to the studied toxic substrates of ABC transporters was related to higher transcriptional and transporter MXR activity or/and due to higher induction levels of Hsp70.

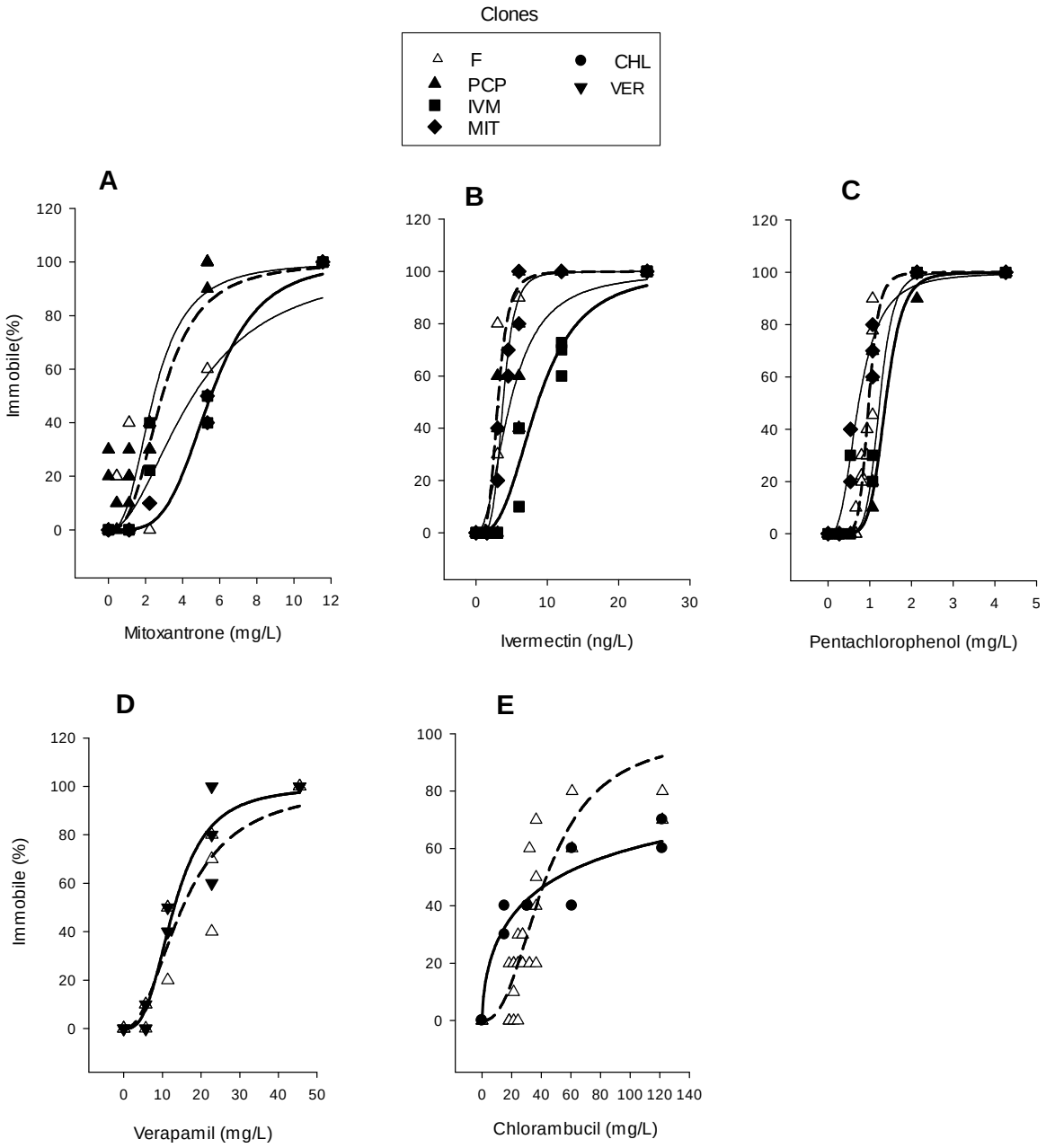


**Figure 5.1** – Concentration-response curves of sensitive (clone F) and tolerant clones (MIT, IVM, PCP, VER, CHL) to mitoxantrone (A), ivermectin (B), pentachlorophenol (C), verapamil (D) and chlorambucil (E), respectively. In each graph, fitted regression lines to responses of resistant and clone F are depicted as thick and dashed lines, respectively, and those of the remaining clones as thin lines. Each symbol corresponds to a single observation.

**Table 5.3** – Acute tolerance (48 hrs - $LC_{50}$) for of the studied clones across the selected chemicals. Results are in mg/L except for ivermectin that are in ng/L. Regression parameters for mortality-regression curves are also depicted. SE, standard error; p, hill index; $r^2$, *r*-square; *n*, sample size. All regressions were significant ($p < 0.05$).

| | | $LC_{50}$ | SE | p | SE | $r^2$ | *n* |
|---|---|---|---|---|---|---|---|
| **Mitoxantrone** | F | 2.89 | 0.310 | 2.78 | 0.67 | 0.95 | 22 |
| | MIT | 5.59 | 0.190 | 4.13 | 0.95 | 0.98 | 12 |
| | IVM | 4.56 | 0.630 | 2.02 | 0.50 | 0.90 | 12 |
| | PCP | 2.44 | 0.230 | 2.67 | 0.62 | 0.91 | 12 |
| **Ivermectin** | F | 3.05 | 0.170 | 4.16 | 1.21 | 0.95 | 18 |
| | IVM | 8.66 | 0.460 | 2.81 | 0.36 | 0.96 | 18 |
| | PCP | 4.82 | 0.650 | 2.14 | 0.56 | 0.85 | 18 |
| | MIT | 3.78 | 0.100 | 4.69 | 0.51 | 0.98 | 20 |
| **Pentachlorophenol** | F | 0.97 | 0.012 | 7.92 | 0.95 | 0.97 | 34 |
| | PCP | 1.38 | 0.042 | 6.75 | 0.72 | 0.99 | 18 |
| | IVM | 1.23 | 0.140 | 7.37 | 2.30 | 0.95 | 14 |
| | MIT | 0.73 | 0.035 | 2.78 | 0.31 | 0.97 | 18 |
| **Verapamil** | F | 15.21 | 1.510 | 2.21 | 0.45 | 0.90 | 18 |
| | VER | 13.04 | 0.820 | 2.92 | 0.49 | 0.95 | 18 |
| **Chlorambucil** | F | 43.51 | 3.330 | 2.39 | 0.41 | 0.75 | 32 |
| | CHL | 51.68 | 8.640 | 0.59 | 0.13 | 0.92 | 12 |

### 5.4.3 Transcriptional and efflux activity results of MXR transporters

The transcriptional results of MXR transporters for the clones MIT, IVM and PCP are depicted in Figure 5.2. Mitoxantrone only affected significantly ($p < 0.05$, $F_{2,12} = 4.4$) the transcription of the *abcb1* gene in individuals from clone F exposed to 1 mg/L (Figure 5.2A). Note also that although not significantly ($p < 0.05$, $F_{2,12} = 2.4$), mitoxantrone also

increased the transcription of gene *abcc1/3* in the clone MIT (Figure 5.2B). Ivermectin decreased the transcription of all studied genes in individuals from clone F ($p < 0.05$; $F_{2,12}$ for *abcb1*, *abcc1/3*, *abcc4* and *abcc5* were 14.3, 128.6, 48.1 and 96.7, respectively, Figure 5.2C) but did not affect those of clone IVM ($p < 0.05$; $F_{2,18}$ for *abcb1*, *abcc1/3*, *abcc4* and *abcc5* were 1.9, 0.5, 1.0 and 0.5, respectively; Figure 5.2D). Pentachlorophenol increased the transcription levels of the *abcb1* gene in individuals from clones F ($p < 0.05$, $F_{2,12} = 6.8$; Figure 5.2E) and PCP ($p < 0.05$, $F_{2,12}=26.9$, Figure 5.2F) and decreased those of *abcc5* in the latter clone ($p < 0.05$, $F_{2,12} = 5.8$; Figure 5.2F). Basal transcription levels of the studied clones (those of unexposed individuals from Figure 5.2) varied significantly ($p < 0.05$, $F_{3,38} = 6.17, 7.38, 14.31, 45.96$ for *abcb1*, *abcc1/3*, *abcc4* and *abcc5*, respectively).

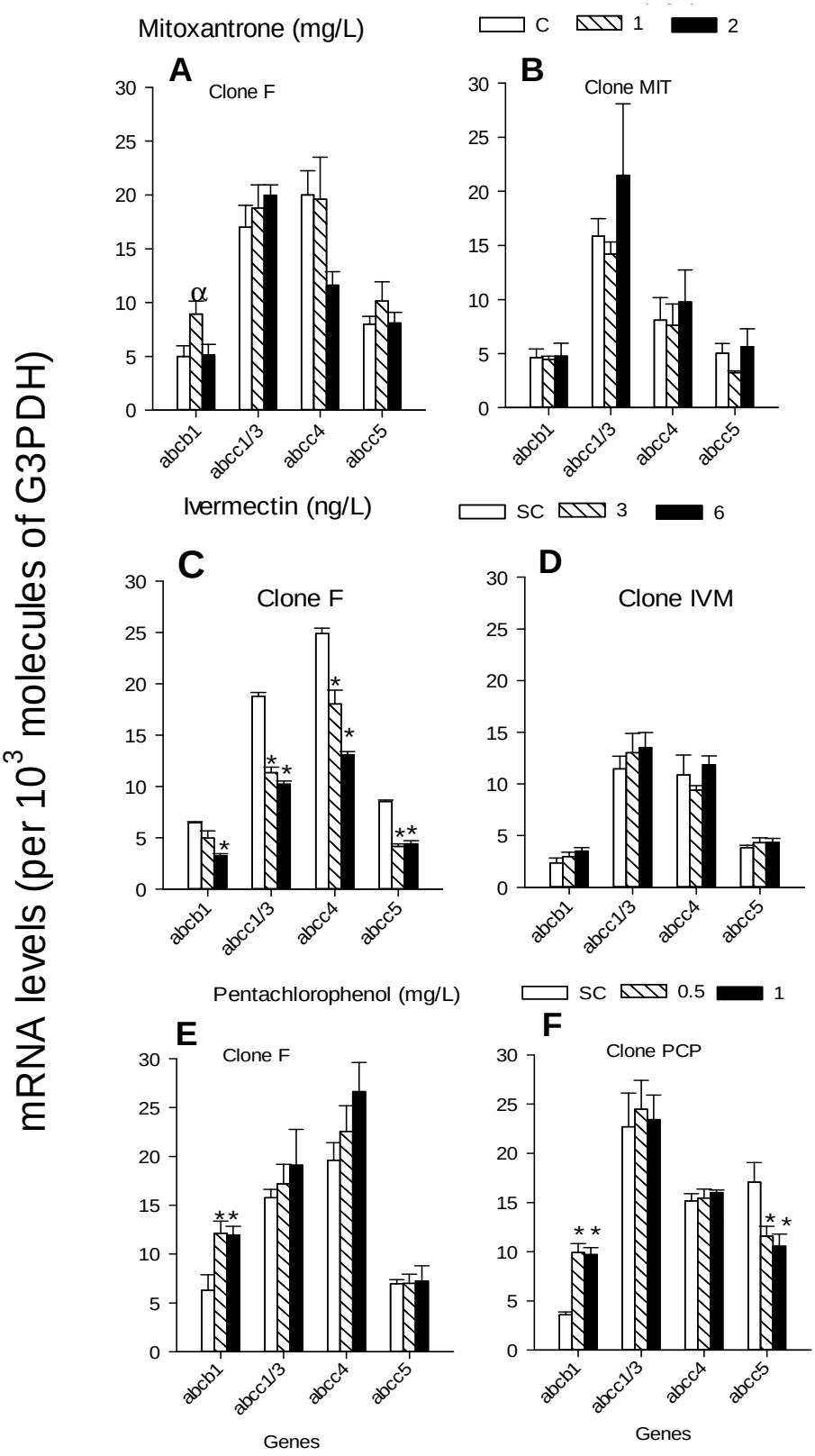


**Figure 5.2** – Induction of ABC transporter gene transcripts (Mean ± SE, *n* = 5) in *D. magna* juveniles from sensitive (clone F in graphs A, C and E) and tolerant clones MIT (B), IVM (D) and PCP (F) pre-exposed for 24 hrs to mitoxantrone (A and B), ivermectin (C and D) and pentachlorophenol (E and F). Within each graph (α) or (*) indicate significant

($p < 0.05$) differences from control (C) or solvent control (SC) treatments, respectively, following ANOVA and Dunnett's tests. Each graph is an independent experiment.

Dye efflux transported activity across the studied contaminants were limited to the Ca-AM dye since previous work did not show clear difference in specificity among this dye and rhodamine ones (Campos *et al.,* 2014). Accumulated levels of calcein decreased in individuals of clone F exposed to mitoxantrone and pentachlorophenol and increased in those exposed to ivermectin ($F_{7,59} = 4.8$; Figure 5.3A). Mitoxantrone also decreased accumulated calcein in individuals of clone MIT ($F_{2,16} = 8.6$; Figure 5.3B) and pentachlorophenol did so in those from clone PCP ($F_{2,26} = 8.6$; Figure 5.3D). Accumulated levels of calcein in unexposed individuals were significantly lower ($F_{3,32} = 5.76$) in clone IVM than in the rest of clones (Figure 5.3). Clonal differences in basal dye efflux activity, however, has to be interpreted with caution since the body length of juveniles used for dye efflux assays with Ca-AM were smaller in tolerant clones relative to F ($p < 0.05$; $F_{3,96} = 27.8$). Mean size (± SE, $n = 25$) of clones F, MIT, IVM, PCP were 2309.1 ± 12.9, 2269.2 ±19.8, 2266.3 ±15.1 and 2112.6± 9.1 µm, respectively. To corroborate the previous finding we repeated dye efflux experiments in unexposed juveniles using rhodamine B and 123. In these experiments we used juveniles of similar size ($p < 0.05$, $F_{3,76} = 1.36$), allowing those from tolerant clones to grow an additional day (6 days). Accumulated levels of rhodamine B and 123 showed also significant ($p < 0.05$) difference among clones ($F_{3,33} = 5.16$, $F_{3,34} = 6.25$, respectively). For rhodamine B individuals from clone PCP had higher levels of accumulated dye (Mean ± SE, $n = 10$, 2.12 ± 0.13 pmol/individual) than the rest of clones (1.48 ± 0.11, 1.72 ± 0.13 1.76 ± 0.08 pmol/individual for clones F, MIT and IVM, respectively). For rhodamine 123 clone IVM had higher accumulated levels of dye (Mean ± SE, $n = 10$, 7.04 ± 0.63 pmol/individual) than the rest of clones (4.12 ± 0.0.30, 4.25 ± 0.48, 5.11 ± 0.73 pmol/individual for clones F, MIT and PCP, respectively).

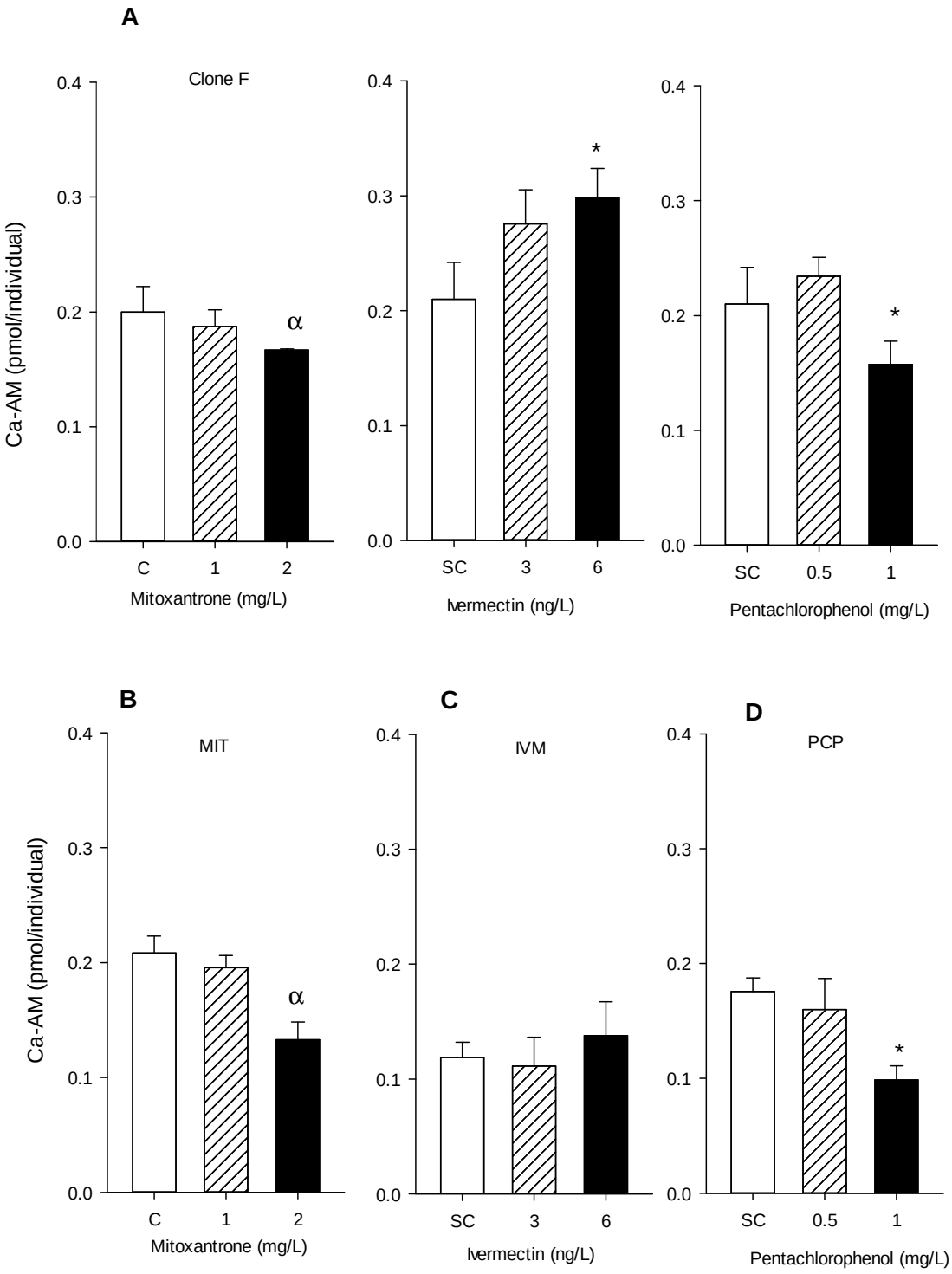


**Figure 5.3** – Induction of ABC transporter activity (Mean ± SE, *n* = 10) in *D. magna* juveniles from sensitive (clone F in graph A) and tolerant clones MIT (B), IVM (C) and PCP (D) pre-exposed for 24 hrs to mitoxantrone (A and B), ivermectin (A and C) and pentachlorophenol (A and D). Within each graph (α) and (*) indicated significant ($p < 0.05$) differences from control (C) or solvent control (SC) treatments, respectively, following ANOVA and Dunnett's test. Each graph is an independent experiment.

Lower accumulated levels of calcein indicate higher dye efflux transporter activity. This means that mitoxantrone and pentachlorophenol enhanced the transporter activity in their respective tolerant clones and in clone F, whereas ivermectin inhibited dye efflux transporter activity of individuals from clone F. Calcein-AM is considered a substrate of ABCB1 and ABCC transporters (Holló *et al.,* 1994; Essodaïgui *et al.,* 1998), thus it is possible to relate efflux activity of this dye with transcription levels of both *abcb1* and *abcc D. magna* genes. Mitoxantrone increased the transcription of gene *abcb1* in clone F, marginally enhanced mRNA levels of gene *abcc1/3* in the MIT clone, and increased transporter activity of Ca-AM in both clones. Therefore, efflux activity of Ca-AM but not those of gene transcription support tolerance results to mitoxantrone between clones MIT and F. Mitoxantrone is detoxified by ABCB1 transporters (Taylor *et al.,* 1991), and accordingly induced to a greater extent transporter activity of dye in the resistant clone MIT relative to the sensitive clone F (36 vs. 16 %, respectively, Figure 5.3A and B). Ivermectin reduced transcriptional levels of gene transporters and also inhibited transporter activity in clone F. Effects of ivermectin on clone F were probably related to toxicity since the tested concentrations were close to its $LC_{50}$. Ivermectin did not affect the transcription of gene transporters nor dye efflux rates in the tolerant clone IVM. This means that tolerance to ivermectin in the IVM clone was unrelated to any of the MXR responses analysed.

Results for pentachlorophenol, which is considered to be detoxified by ABCB1 transporters (Eufemia and Epel 2000; Navarro *et al.,* 2012; Campos *et al.,* 2014), agree with expectations. Pentachlorophenol enhanced mRNA levels of abcb1 89 % and 172 % in *D. magna* individuals from clones F and PCP, respectively (Figure 5.2E, F), and induced Ca-AM transporter activity in these clones 21 and 44 %, respectively (Figure 5.3A and D).

Basal transcription levels and efflux activity against Ca-AM, rhodamine B and 123, were poorly related with most gene transcripts across clones. Only mean clonal transcript levels of gene *abcb1* measured in unexposed individuals were inversely correlated with accumulated levels of rhodamine B (Pearson coefficient of - 0.6, $n = 4$, $P = 0.4$) and rhodamine 123 (Pearson coefficient of - 0.93, $n = 4$, $P = 0.07$). Note that the low sample size ($n = 4$) of these comparisons prevented these correlations to be significant ($p < 0.05$). The specificity of both rhodamine dyes (B and 123) for ABCB1 and ABCC type transporters, respectively, is controversial, since it depends on the substrate concentration and species (Lee *et al.,* 1994; Eytan *et al.,* 1997; Daoud *et al.,* 2000; Smital *et al.,* 2000; Ferreira *et al.,* 2005). Several studies have used either rhodamine B or rhodamine 123 to

measure transporter activity of ABCB1 transporter proteins in different organisms and cells (Yu 1999; Eufemia and Epel 2000; Smital *et al.,* 2000; Zaja *et al.,* 2008). Accordingly, our results agree with previous studies since high transcriptional levels of *abcb1* gene transporters were positively related with high efflux dye activity of rhodamine dyes. Nevertheless, levels of gene transcripts or of dye efflux activity in unexposed individuals were not higher in tolerant clones relative to clone F, which means that observed tolerance was not related to high constitutive levels of ABC transporters.

### 5.4.4 Hsp70

The levels of Hsp70 protein expression across treatments and clones are shown in Figure 5.4 and quantification of Western blot bands in Table 5.4. Hsp70 levels did not changed in unexposed and exposed individuals of clone F (Figure 5.4A, B and C, Table 5.4) and were higher ($p < 0.05$) in clones MIT ($p < 0.05$, $F_{3,4} = 17.6$, Figure 5.4A, Table 5.4) and PCP ($F_{3,4} = 12.0$, Figure 5.4C, Table 5.4) irrespectively of the treatment. In the clone IVM the levels of Hsp70 marginally increased in individuals exposed to ivermectin but the high variability observed between replicates prevented this increase to be significantly different than that of unexposed individuals from clone F ($p < 0.05$, $F_{3,4} = 3.1$; Figure 5.4B, Table 5.4). Results of Hsp70 protein expression indicated that the tolerant clones MIT and PCP had higher constitutive levels of stress proteins whereas individuals of clone IVM induced Hsp70 when exposed to ivermectin. These findings support those obtained by previous authors in mussels (Eufemia and Epel 2000; Tutundjian and Minier 2007) and indicated that in *D. magna* selection of clones having enhanced levels of MXR may be part of a general stress response. In the previous studies (Eufemia and Epel 2000; Tutundjian and Minier 2007), however, observed effects were phenotypic, whereas in our study had a genetic basis. This means that tolerant genotypes having enhanced levels of general stress and MXR exist in natural populations of *D. magna* and can be selected by toxic compounds that are substrates of ABC transporters. In our study, we were able to isolate one tolerant genotype within 500 potential ones, which is in the same order of occurrence than those reported in *D. magna* for metals, organophosphorus and pyrethroid insecticides (Barata *et al.,* 2002a; Barata *et al.,* 2002b; Damásio *et al.,* 2007; Hochmuth *et al.,* 2015). Considering that there are many contaminants that interact with ABC transporters (Smital *et al.,* 2004), there is a strong potential for those compounds to select out sensitive genotypes, which in a long term may reduce the genetic diversity of natural populations increasing their extinction probability (Medina *et al.,* 2007).

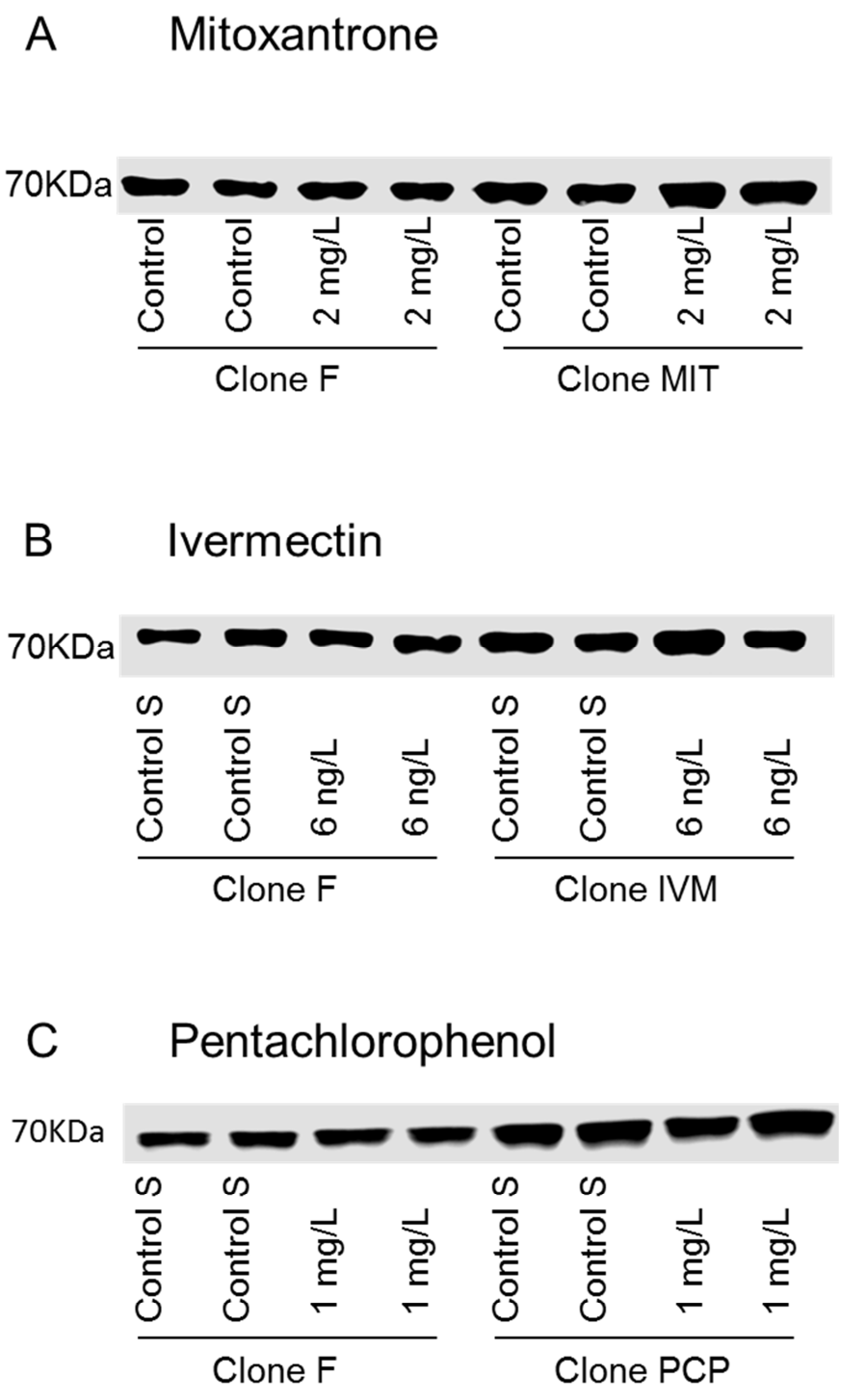


**Figure 5.4** – Western blots for Hsp70 of *D. magna* juveniles from clone F (A, B and C) and tolerant clones MIT (A) , IVM (B) and PCP (C) exposed and unexposed to the studied contaminants. Each graph corresponds to a single experiment. Within each Western blot, band intensities in control (C) or solvent control (CS) treatments from individuals of clone F was compared to those of other treatments as indicated in Table 5.4.

**Table 5.4** – Relative levels of Hsp70 (Mean ± SE, *n* = 2) expressed in percentage intensity of Western blot bands relative to control (C) or solvent control (SC) treatments. Within each column (α) or (*) indicate significant ($p < 0.05$) differences from Clone F control or solvent control treatments, respectively following ANOVA and Dunnetts's test.

| Treatment | Mitoxantrone | Treatment | Ivermectin | Treatment | Pentachlorophenol |
|---|---|---|---|---|---|
| | Mean ± SE | | Mean ± SE | | Mean ± SE |
| Clone F, C | 100.0 ± 10.4 | Clone F, SC | 100.0 ± 9.5 | Clone F, SC | 100.0 ± 6.6 |
| Clone F 2 mg/L | 95.5 ± 6.4 | Clone F 6 ng/L | 109.3 ± 1.3 | Clone F 1 mg/L | 97.3 ± 10.2 |
| Clone MIT, C | 149.4 ± 7.5 α | Clone IVM, SC | 166.8 ± 20.5 | Clone PCP, SC | 204.7 ± 11.5 * |
| Clone MIT 2 mg/L | 150.5 ± 1.6 α | Clone IVM 6 ng/L | 242.1 ± 70.3 | Clone PCP 1 mg/L | 223.6 ± 35.0 * |

In summary, the results reported in the present study showed that mitoxantrone, ivermectin and pentachlorophenol were able to select genetically tolerant clones. Tolerance to mitoxantrone and pentachlorophenol was related to high constitutive levels of Hsp70. Tolerance to pentachlorophenol was also due to high transcriptional levels of *abcb1* gene transporter and of putative dye efflux activity in exposed organisms. Observed higher Abc transporter activity in exposed individuals of the clone tolerant to mitoxantrone was unrelated with transcriptional levels of the studied *abc* gene transporters. Tolerance to ivermectin was not explained by the studied MXR responses and only marginally by induction levels Hsp70 proteins.

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# Chapter VI

Final remarks

# Chapter VI – Final remarks

## 6 General discussion and final conclusions

### 6.1 General discussion

All papers included in this thesis address emerging responses with the aim of elucidating novel mechanisms of action of toxicity in aquatic invertebrate species using the crustacean *Daphnia magna* as a model organism. Obesogenic effects including their health consequences in a non-deuterostome species was characterized for the first time in *D. magna* using first TBT and then a large set of chemical contaminants in chapters 2 and 3. These studies fulfil the first objective of the thesis that was to assess endocrine disrupting effects on the lipid metabolism of classical and emerging compounds. One of the key features of endocrine disruptors including obesogenic compounds is to adversely affect offspring quality, which was assessed in chapter 4. Finally in chapter 5, we provide further evidence that there is a close association between general stress responses and multixenobiotic resistance mechanisms in *D. magna* and that both mechanisms are genetically selectable.

#### 6.1.1 Obesogenic effects in *Daphnia magna*

*Daphnia* species are well known to have a large dynamic range of storage lipids, mainly TGs that are acquired from the diet and are accumulated inside lipid droplets during the inter-moult cycle. The TGs are invested to moult and reproduction at the end of each instar (Tessier and Goulden 1982b; Tessier *et al.,* 1983; Goulden and Place 1990,1993). The lipid dynamic cycle has been extensively described and used by ecologists to infer the feeding status of populations in the field (Tessier and Goulden 1982a). Storage lipids in *Daphnia* species are closely related to growth, reproduction and tolerance to starvation (Tessier *et al.,* 1983). These features make *Daphnia* an ideal model system to study toxic effects on lipid homeostasis. In chapter 2, we developed and validated an “obesogenic marker” based on the accumulation of lipid droplets measured with the *in vivo* dye Nile red using a well-known model obesogen, TBT. Lipid droplet dynamics are complex and vary largely across the moulting cycle so we first set up a period where intra and inter-individual variation in lipid droplets were the lowest. The best period was just after moult and releasing the eggs into the brood pouch. We select for that assay primiparous females (females that produce the first clutch of eggs), to minimize

inter-individual variation since environmental noise on phenotypic responses increases with time (Barata and Baird 1998). Changes in the amount of lipid droplets measured as the fluorescence of Nile red decreased in females cultured at low food levels or starved, increased exponentially during the first hours of the intermoult period and decreased again at the end of the reproductive cycle. Observed Nile red fluorescence changes, thus, matched perfectly the previous described conspicuous cycle of storage lipids in *Daphnia* and its variation across food levels (Tessier and Goulden 1982a; Tessier *et al.,* 1983). We then related Nile red fluorescence with changes in the whole lipidome during the inter-moult cycle and across TBT treatments using UPLC-TOF. Our results showed that lipid droplet dynamics were closely related to that of TGs in unexposed females and that individuals exposed to TBT accumulated more lipid droplets and also had more TGs. A further analyses of up to 116 different lipids indicated that TBT enhanced the accumulation of polyunsaturated TGs in *Daphnia* females. Lipidomic analyses also showed that females exposed to TBT allocated less lipids to eggs and specifically less polyunsaturated TGs. Eggs containing less storage lipids developed into smaller neonates that had lower survival rates during their adulthood and hence had a lower reproductive success. Thus in chapter 2, we were able to relate disruptive effects on lipid metabolism with detrimental transgenerational effects, which is a typical detrimental obesogenic health effect in humans and in other mammalian models. Another objective of chapter 2 was to study the molecular mechanism of action of TBT disrupting lipid homeostasis in *Daphnia*. We hypothesized the involvement of three nuclear master receptors: RXR, the EcR and the JH receptor, which in crustaceans is the MfR. Transcriptomic results of an array of gene markers from the previous nuclear receptors indicated that TBT up-regulated the three receptor signalling pathways during the moult cycle.

Following the results obtained in chapter 2 we further investigated the mechanisms of action of chemicals disrupting lipid homeostasis in chapter 3. We tested two hypotheses. The first one aimed to screen other chemical pollutants disrupting lipid storage dynamics in *Daphnia* mimicking those observed with TBT. The second one aimed to study how EcR, RXR and MfR modulated lipid homeostasis using mixture combinations with known agonists and antagonists of EcR, RXR and MfR. Results obtained in chapter 2 indicated that agonists of EcR (20E), RXR (TBT) and MfR (MF and PP); and BPA increased the accumulation of storage lipids in a concentration-related manner. Conversely, MT, which is a weak agonist of MfR in *Daphnia* (Miyakawa *et al.,* 2013), FEN and NP that are known to deplete the levels of or alter the metabolism of ecdysone in *D. magna*, respectively (LeBlanc *et al.,* 2000; Mu and LeBlanc 2004b). Likewise, EM that is

known to alter ecdysis in decapod crustaceans (Rodríguez *et al.,* 2007) and FX, decreased lipid droplets. The previous results indicated that lipid storage dynamics in *D. magna* is promoted by agonists of three nuclear receptors and that anti-ecdysteroids inhibited the whole process. Observed inhibitory effects of FX are likely to be explained by a different mechanism, the $\beta$-oxidation of lipids, which is in line with the effects observed in vertebrates and *Caenorhabditis elegans* (Srinivasan *et al.,* 2008; Li and Cheung 2009).

To better understand the regulatory pathway of storage lipids we tested several binary combinations involving agonists of the three nuclear receptors and the ecdysteroid antagonist FEN. Joint effects of binary combinations of juvenile and RXR agonists were additive or more than additive. All pairings involving the anti-ecdysteroid compound FEN depleted dramatically lipid droplet formation. These results support the findings of LeBlanc and co-workers that RXR and ecdysone receptors dimerized in *D. magna* and that the resulting heterodimer can be activated by TBT and juvenoids when ecdysone is present (Figure 6.1A) (Wang and LeBlanc 2009). Furthermore, the suppressive effects observed by FEN indicated that empty EcR acts as dominant co-suppressor of the EcR:RXR heterodimer impairing the transcription of genes involved in lipid metabolism. In *Drosophila* unbound EcR also acts as a dominant co-suppressor of transcription whereas unbound RXR do not. According to Wang *et al*., (2009) ligands of RXR and the JH receptor could activate the EcR:RXR dimer and consequently in our lipid assay their combination will produce an additive joint effect. Joint effects of mixtures of MF and TBT on the accumulation of storage lipids, however, were more than additive. The former results can only be explained considering the involvement of two different receptors acting cooperatively as it is depicted in Figure 6.1B: the above mentioned EcR:RXR heterodimer receptor and the JH receptor complex (Miyakawa *et al.,* 2013). The last complex included the leading candidate for juvenile receptor in insects, the MET transcription factor, and the SRC, which is activated by the crustacean MF and other juvenoids. Therefore, our results provide experimental evidence that the nuclear receptors RXR, EcR and probably that of the JH regulate lipid droplet formation. Observed joint additive effects of the two tested juvenoids (MF and PP) support our argument that the JH receptor is also involved in the regulation of storage lipids in *D. magna*. We are now conducting further experiments combining mixtures among different agonists with gene expression assays to confirm the model that the two receptor complexes regulate lipid storage dynamics in *Daphnia*.

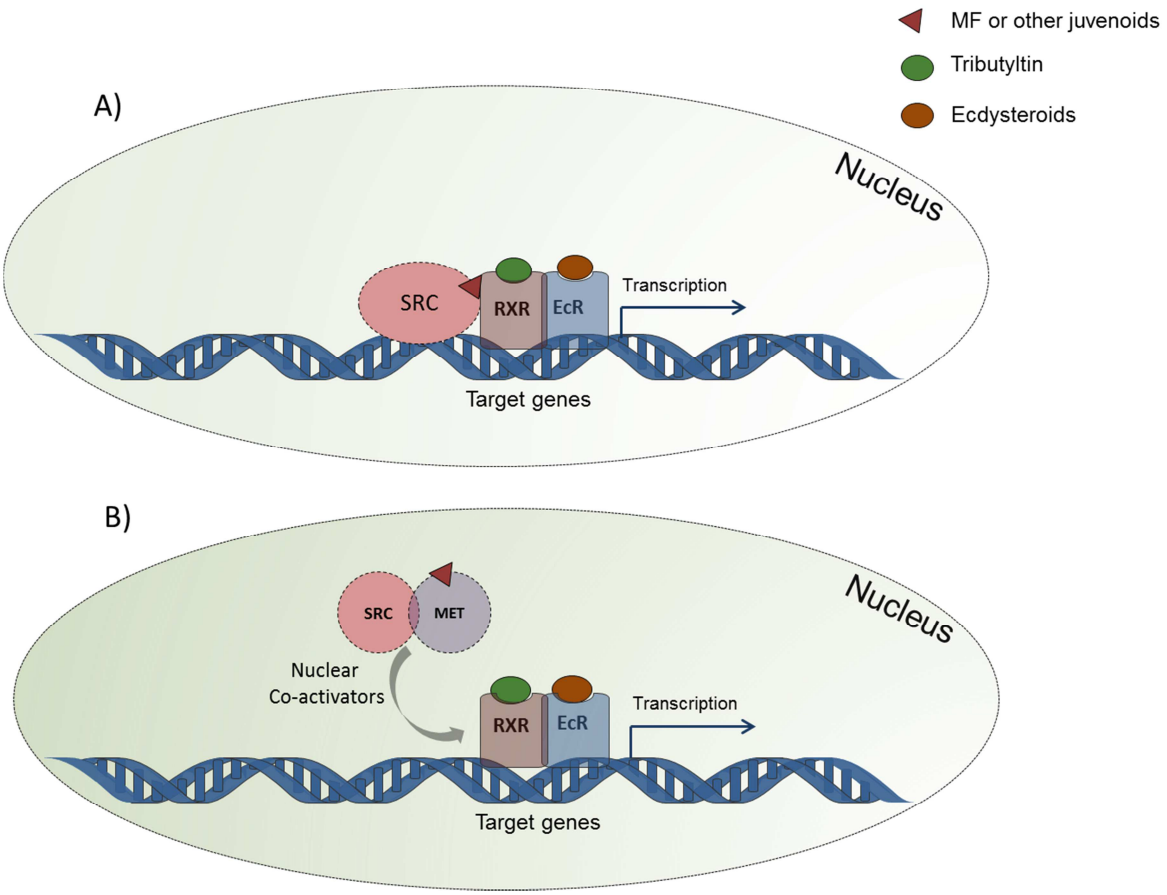


**Figure 6.1** - Putative EcR:RXR and SRC:MET receptor complexes signalling pathways that regulate lipid homeostasis in *D. magna*: A) EcR and RXR form a heterodimer, which is activated by the respective ligands in the presence of ecdysteroids and the co-activator SRC, according with Wang and LeBlanc (2009). B) EcR and RXR form a heterodimer, which is activated by the respective ligands in the presence of ecdysteroids and the nuclear co-activator SRC:MET complex is activated by MF or other juvenoids, according with Miyakawa *et al.,* (2013). EcR – ecdysone receptor; RXR – retinoid X receptor; SRC – steroid receptor co-activator; MET – Methoprene-tolerant; MF – methyl farnesoate.

### 6.1.2 Toxic effects on offspring quality

Toxicity effects on offspring quality in *Daphnia* has been largely neglected provided that ASTM (2012) and OECD (2012) reproduction test guidelines only consider effects on the female reproductive output. This is surprising since mammalian reproduction toxicity tests specifically address effects on both female reproductive output and on their progeny. In this thesis, we specifically studied the toxic effects of offspring quality in three chapters: Chapters 2, 3 and 4. In chapter 2, we showed that the obesogenic compound TBT impaired the transfer of lipids to eggs and hence decreased the reproductive performance

of females hatched from those eggs. In Chapter 3, we found that 8 out of the 10 compounds that altered storage lipids in lipid droplets, also altered clutch size and the size or/and sex of the offspring produced by exposed females. Concentrations of the juvenoids MF and PP that affected storage lipids also decreased the body length of exposed females, the size of their first brood, changed offspring sex and increased offspring size. Juvenoids by reducing reproductive investment should favor the accumulation of non-invested resources including storage lipids in reproductive females. There is reported evidence that at lower concentrations than the tested ones, the studied juvenoids reduced clutch size producing all male broods (Olmstead and Leblanc 2002,2003; Wang *et al.,* 2005; Ginjupalli and Baldwin 2013). Concentrations of 20E that enhanced lipid accumulation in lipid droplets also enhanced per offspring reproductive investment as neonates produced in the first clutch were larger than those of controls. There is reported information showing that 20E affects *D. magna* reproduction (Sumiya *et al.,* 2014), but no such studies exist for per offspring reproductive investment. Previous findings indicated that this hormone interacts with the MfR signalling pathway in *Daphnia* (Mu and LeBlanc 2004a), thus it is likely that 20E alone or interacting with the MfR affected also per offspring reproductive investment. Compounds that decrease the accumulation of storage lipids such as FEN, NP and EM reduced growth and/or total offspring reproductive investment; which agree with energy allocation expectations, since females had less resources to be invested to growth and reproduction. Surprisingly, BPA and FX did not affect the growth of exposed females, neither the number nor the size of offspring produced. Future research needs to study life-history consequences of lipid disruption with more detail.

In chapter 4, we studied the toxic effects in two consecutive generations, which allowed assessing toxic effects on both the parental and offspring generation. We limited the study to three chemicals with known endocrine disruptive effects: 4-nonylphenol and PBO, two compounds that impart ecdysteroid metabolism and TBT that affected lipid metabolism as showed in chapter 2. Ecdysteroids are crucial for embryo development, thus the first two compounds should affect offspring quality. One of the traits that determine offspring quality in *Daphnia* is offspring size, which is strongly affected by the maternal phenotype (i.e. mother size). Young mothers that reproduce for the first time are smaller and hence produce smaller offspring than older mothers. Smaller offspring take longer to reproduce and are less tolerant to toxic pollutants. Offspring size usually stabilizes after the third clutch. Thus, to account for the maternal phenotype, we studied the effects on the offspring generation coming from first, second and third clutches. The

results obtained indicated that toxic effects on the offspring generation varied across clutches being more evident when the second generation was initiated using third clutch juveniles. This was related with the toxicant effects on offspring size and probably on the quality of this offspring, and was more evident in third clutch juveniles. There is ample evidence showing that maternal effects diminished in latter clutches since older mothers are similar in size than young ones (Boersma 1995,1997). Thus, there is more phenotype variability among offspring from first broods (Barata and Baird 1998). Our data agree with previous arguments. The three tested contaminants decreased both the number and size of the offspring produced and as a result increased their toxic effects in the offspring generation. The greatest effects were observed for PBO, which is known to inhibit P450 enzymes, many of them involved in key metabolic pathways such as the ecdysone and JH metabolism hydrolases (Baldwin and LeBlanc 1994). Therefore, results obtained from the first three chapters of this thesis allowed relating endocrine disruptive effects of chemicals with effects on offspring quality and its latter performance in life.

### 6.1.3 General stress response and multixenobiotic resistance mechanisms in *Daphnia magna*

Recently, several studies found that aquatic organisms have an additional mechanism of detoxification. This is the so called MXR (or MDR) mechanism. This mechanism was first described in cancer cells that became tolerant to therapeutic drugs, mostly cytotoxics (Juliano and Ling 1976). Later work found that several ABCC (mainly ABCC1-3) were associated with tumour resistance, which is often caused by an increased efflux and decreased intracellular accumulation of natural product anticancer drugs and other agents (Yu *et al.,* 2007). Since the discovery that these transporters also occur in many aquatic species, they have been exhaustively studied (Kurelec 1992). Organisms and cells with potent MXR defences are less sensitive to the toxic impact of compounds that are transporter substrates, because comparatively few of those molecules reach their site of toxic action (Bard 2000; Epel *et al.,* 2008). One of the questions still not solved on these transporters is to explain why several species have high activities of those transporters whereas others do not. In cells, selection experiments demonstrated that it is possible to select cell phenotypes expressing high levels of these transporters, mainly P-glycoprotein ones, by exposing them to toxic drugs that are also substrate of those transporters (Zaja *et al.,* 2008). There is, however, no clue on which are the selection mechanisms in whole organisms. Previous studies performed on mussels have shown

that pre-exposure to ABC transporter substrates induced P-glycoprotein levels and transporter activity (Eufemia and Epel 2000; Tutundjian and Minier 2007; Navarro *et al.,* 2012). Some of these studies also found that observed induction was related to a general response to stress since Hsp70 was also induced and factors like temperature also induced ABCB1 transporter proteins and activity (Eufemia and Epel 2000; Tutundjian and Minier 2007). The previous studies, however, did not demonstrate that it is possible to genetically select organisms having high activities.

Previous results obtained by members of my research team characterized gene transcripts and efflux activities of four putative Abcb1 and Abcc transporters in *D. magna* that were chemically induced but showed low specificity against model transporter substrates and inhibitors, thus preventing to distinguish between activities of different efflux transporter types (Campos *et al.,* 2014). In chapter 5, we planned to study more deeply the specificity of previous characterized ABC transporters comparing the response of genes and of transporter activity of different clones selected to be tolerant against different toxic substrate of ABC transporters. As a second objective we aimed to study if selection of tolerant genotypes was also associated with selection of general stress tolerant ones. Clones tolerant to mitoxantrone and pentachlorophenol showed high constitutive levels of Hsp70. Transcriptional levels of the *abcb1* gene transporter and of putative dye efflux activity were also induced to a greater extent in the pentachlorophenol tolerant clone. Observed higher dye efflux activities in exposed individuals from the clone resistant to mitoxantrone were unrelated with transcriptional levels of the studied four *abcc* and *abcb1* gene transporters. Tolerance to ivermectin was not explained by the studied ABC responses and only marginally by the expression levels of Hsp70 protein. These findings suggest that Abcb1 induction in *D. magna* may be part of a general cellular stress response.

## 6.2 Final conclusions

**1.** The Nile red assay is a cost effective method to monitor and quantify storage lipids into lipid droplets in *D. magna* across developmental instars, food levels and toxic chemicals;

**2.** Tributyltin disrupted the dynamics of neutral lipids in lipid droplets impairing the transfer of TGs to eggs and hence promoting their accumulation in adult individuals. This is the first clear evidence of obesogenic effects in a non-vertebrate species;

**3.** Tributyltin disruptive effects translated into a lower fitness for offspring and adults;

**4.** Co-regulation of gene transcripts suggests that TBT activates the ecdysone, juvenil hormone and RXR receptor signalling pathways, presumably through the already proposed interaction with RXR;

**5.** Disruptive effects of TBT on lipid metabolism were not unique. Eight compounds also disrupted storage lipids in a concentration related manner enhancing (MF, PP, BPA and 20E) or decreasing (NP, FEN, EM and FX) their accumulation into lipid droplets in reproductive females;

**6.** Joint binary mixture effects on storage lipids indicated that juvenoids and RXR acted through two different receptors in a cooperative manner, and that the inactivation of EcR acted as a dominant co-repressor. The proposed mechanism of regulation of lipid droplet accumulation is in line with recent studies which indicate that in *D. magna* the EcR dimerized with RXR and that this dimer is activated by agonists of RXR and juvenoids when ecdysone is present. *Daphnia magna* also have an active JH receptor that is activated by junenoids;

**7.** In eight compounds disruptive effects on lipid metabolism translated into detrimental effects in growth and or total and per offspring reproductive investment;

**8.** It is possible to develop a two generation reproduction test from existing OECD protocols with a just one amendment: to start the second generation using third broods neonates coming from exposed females from the first generation since they provided the more consistent and greater differences across generations;

**9.** The three studied chemicals, PBO, 4-nonylphenol and TBT, affected the quantity and quality of the offspring produced by exposed females of the first generation, being those effects greater in the third clutch neonates. Thus, observed negative effects on the second generation were related to detrimental effects on per offspring reproductive investment (offspring size);

**10.** Within outbreed field populations of *D. magna* it is possible to select genotypes tolerant to mutixenobiotic toxic substrates. Two of the three selected tolerant genotypes

showed also high constitutive levels of stress proteins (Hsp70) whereas only one genotype had elevated transcriptional levels of P-glycoprotein (abcb1) and associated transporter activity. These results indicated that the multixenobiotic resistance mechanisms can be part of a general stress mechanism of tolerance.

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# Annex

Supplemental information and published articles

**Table SI** – Brief description of studied compounds in chapter III.

| Compounds | Name | Description | Mode of action / Function |
|---|---|---|---|
| **Organotins** | Tributyltin (TBT) and Triphenyltin (TPT) | Biocides, agricultural fungicides, wood preservatives, disinfecting agents, marine antifouling agents, textile industry, slimicides and plasticizers. | Endocrine-disrupting properties in vertebrates and invertebrates organisms; Agonists RXR. |
| **Ecdysteroid hormones** | 20-hydroxyecdysone (20E) | Major biologically active ecdysteroid in insects and crustaceans that binds to the EcR; possible modulator of the action of juvenoids. | Regulation of development, growth and reproduction in crustaceans. |
| | PonasteroneA (PoA) | Insect hormone found in some crustaceans and many plants. | Involved in regulating metamorphosis. |
| **Juvenoid hormone (JH)** | Methyl farnesoate (MF) | Synthesized by crustaceans mandibular organs and is present in the hemolymph; the chemical structures nearly identical to that of insect JH III. | Promotion of moult cycle, stimulate male sex determination in daphnids. |
| **Pesticides** | Pyriproxyfen (PP) | Used in flea and tick control in veterinarian applications, and in fire ant bait; increasingly recommended for agricultural uses such as the control of white fly on cotton and scale insects on fruit trees. | Pesticidal insect growth regulators, juvenoid mimic. JH agonist. |
| | Methoprene (MET) | Disrupt specific physiological processes of target insects; typically perturbed enzymatic and hormonally regulated processes that are relatively specific to insect physiology. | Pesticidal insect growth regulators, juvenoid mimic. JH agonist. |
| | Emamectin benzoate (EM) | Avermectin used in agriculture and aquaculture industries in worldwide. | GABAergic pesticide can cause premature moulting in an arthropod. Ecdysone agonist. |
| **Fungicide** | Fenarimol (FEN) | Agricultural fungicide that is known to inhibit cytochrome P450 enzymes and may accordingly interfere with ecdysone synthesis. | Ecdysone antagonist. |
| **Insecticides** | Tebufenozide (TEB) | Produced to control pest Lepidoptera. | Ecdysone agonist. |
| **Pharmaceutical** | Fluoxetine (FX) | Antidepressant, which acts by inhibiting the re-uptake of serotonin. | Involved in many hormonal and neuronal mechanisms and it is also important in functions such as food intake. Fat regulator. |
| **Ubiquitous environmental contaminants/ reported obesogenic effects in vertebrates** | Nonylphenol (NP) | Industrial detergent degradation product. | Altered metabolism of endogenous steroids (such as ecdysone), resulting in perturbations in their provision to the newly produced eggs. |
| | Bisphenol A (BPA) | Component of polycarbonate plastics is widely used in numerous products such as polycarbonate baby bottles, beverage containers, the linings of food cans, dental composites, and sealants. | Interact with the estrogen receptor as an agonist and elicit estrogen-like activity *in vivo*. |
| | Di(2-ethylhexyl) phthalate (DEPH) | Classes of chemicals that include various perfluoroalkyl compounds and phthalate plasticizers that are widely used as surface repellents and surfactants. | Class of chemicals known as peroxisome proliferators. |