LETHAL AND SUBLETHAL EFFECTS OF PHTHALATES IN WESTERN CLAWED FROG

LÉTALITÉ ET EFFETS SOUS-LÉTAUX DES PHTHALATES CHEZ LE XÉNOPE TROPICAL

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by

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ii. Abstract and résumé

Phthalates are compounds used in a variety of polymers to increase their flexibility and are now ubiquitous in the environment. As few studies have focussed on the adverse effects of these chemicals in aquatic species, this project aimed at elucidating the effects of phthalates in amphibians. Western clawed frog (*Silurana tropicalis*) tadpoles were acutely exposed to water spiked with monomethyl (MMP), dimethyl (DMP) and dicyclohexyl (DCHP) phthalates. DMP and DCHP were found to be embryotoxic; mortality, malformations and developmental delay were induced in treated larvae. In juvenile frogs, chronic exposures to MMP led to increased mortality, accelerated metamorphosis and more recurrent male phenotypes. However, MMP did not have considerable adverse health effects in tadpoles nor adult frogs. Moreover, analysing the expression of selected genes increased our understanding of the mechanism of phthalates. This is the first study to thoroughly investigate the effects of phthalates in the entire amphibian life cycle (egg to adult stage). These assays provided valuable data that will help better assess and manage the risks brought on by the production and the use of phthalates in the Canadian environment.

Les phthalates sont des composés utilisés dans une variété de polymères afin d'accroître leur flexibilité et sont maintenant omniprésents dans l'environnement. Comme peu d'études ont caractérisé les effets de ces produits chimiques chez les espèces aquatiques, ce projet avait pour but d'élucider les effets des phthalates chez les amphibiens. Des têtards du Xénope tropical (*Silurana tropicalis*) ont été exposés à de l'eau contaminée avec du phthalate monométhylique, du phthalate diméthylique et du phthalate dicyclohexylique. Les phthalates diméthylique et dicyclohexylique se sont révélés embryotoxiques; les composés ont induit de la mortalité, des malformations et des délais de développement chez les larves. Chez les grenouilles juvéniles, les expositions chroniques au phthalate monométhylique ont mené à de la mortalité, à l'accélération de la métamorphose et à des phénotypes masculins plus fréquents. Le phthalate monométhylique, par contre, n'avait pas d'effets considérables chez les têtards ni chez les grenouilles adultes. De plus, l'analyse de l'expression d'une série de gènes a accru notre compréhension du méchanisme d'action des phthalates. Cette étude est la première à enquêter de manière approfondie les effets des phthalates sur l'ensemble du cycle de vie des amphibiens (œuf à adulte). Ces expériences ont fourni de précieuses données qui aideront à mieux évaluer et gérer les risques associés à la production et l'utilisation des phthalates dans l'environnement canadien.

Table of contents

i. Acknowledgments 2
ii. Abstract and résumé 3
iii. List of tables 6
iv. List of figures 7
v. List of acronyms 9
Chapter 1 12
1.1 Introduction 13
1.2 Literature review 14
Chapter 2 42
2.1 Introduction 43
2.2 Materials and methods 45
2.3 Results 52
2.4 Discussion 65
2.5 Conclusion 70
Chapter 3 71
3.1 Introduction 72
3.2 Materials and methods 73
3.3 Results 78
3.4 Discussion 89
3.5 Conclusion 92
Chapter 4 93
4.1 Ecological significance of the results 94
4.2 Recommendations for Environment Canada and Health Canada 95
4.3 Recommendations pertaining to current protocols 95
4.4 Future research directions 97
4.5 Conclusion 97
5. Summary 98
References 99
Appendix 1 – Genes that were not disrupted by MMP acute exposures in a biologically significant manner 138

Appendix 2 – Genes that were not disrupted by DMP acute exposures in a biologically significant manner..... 141

Appendix 3 – Genes that were not disrupted by DCHP acute exposures in a biologically significant manner..... 144

Appendix 4 – Genes that were not disrupted by the juvenile exposure to MMP.. 146

Appendix 5 – Genes that were not disrupted by the adult exposure to MMP 148

iii. List of tables

Table 2.1 Genes of interest for the acute exposures……..…51
Table 2.2 Experimental concentrations before and after 24 h of exposure with Western clawed frog larvae……..56
Table 2.3 Effect of monomethyl (MMP), dimethyl (DMP) and dicyclohexyl (DCHP) phthalate spiked water on malformation of Western clawed frog larvae after 72 h exposures……....58
Table 2.4 Effect of monomethyl (MMP), dimethyl (DMP) and dicyclohexyl (DCHP) phthalate spiked water on development of Western clawed frog larvae after 72 h exposures…….…...59
Table 2.5 Summary of the adverse health effects of monomethyl (MMP), dimethyl (DMP) and dicyclohexyl (DCHP) phthalates in Western clawed frog larvae after 72 h exposures…….…..61
Table 2.6 Summary of the effect of monomethyl (MMP), dimethyl (DMP) and dicyclohexyl (DCHP) phthalate spiked water on the expression of thyroid hormone, sex steroid, cellular stress and transcription regulation-related genes in Western clawed frog larvae after 72 h exposures……..62
Table 3.1 Experimental concentrations before and after 3 d of exposure with Western clawed frogs…….....81
Table 3.2 Effect of monomethyl phthalate (MMP) spiked water on Western clawed frog morphological measurements after an 11 w exposure……....85
Table 3.3 Summary of the effect of monomethyl phthalate (MMP) spiked water on the expression of thyroid hormone, sex steroid, cellular stress and transcription regulation-related genes in Western clawed frog livers after juvenile and adult exposures……...….87

iv. List of figures

Figure 1.1 Biological degradation of phthalates……..……..17

Figure 1.2 Examples of the known effects and mechanisms of action of phthalates (PAE) for each organisational level……...…..…...39

Figure 1.3 Phthalate mono and diesters affect the thyroid hormone, growth hormone, sex steroid and stress axes…….….40

Figure 1.4 Median lethal concentrations (LC_{50}s) of phthalate diesters across aquatic trophic levels……..….....41

Figure 2.1 Schematic representation of the experimental design of acute exposures to monomethyl (MMP), dimethyl (DMP) and dicyclohexyl (DCHP) phthalates in the Western clawed frog…….…….50

Figure 2.2 Effect of monomethyl (MMP; A), dimethyl (DMP; B) and dicyclohexyl (DCHP; B) phthalate spiked water on mortality of Western clawed frog larvae after 72 h exposures…….…..57

Figure 2.3 Effect of dicyclohexyl phthalate (DCHP) spiked water on cement gland development in Western clawed frog larvae after 72 h exposures……..…...60

Figure 2.4 Effect of monomethyl phthalate (MMP) and dimethyl phthalate (DMP) spiked water on the expression of peroxisome proliferator-activated receptor alpha (*pparα*) and deiodinase type 1 (*dio1*) in Western clawed frog larvae after 72 h exposures……..……..…....63

Figure 2.5 Effect of dicyclohexyl phthalate (DCHP) spiked water on the expression of deiodinase type 1 (*dio1*), aromatase (*cyp19*), steroidogenic acute regulatory protein (*star*), heat shock protein 70 (*hsp70*) and peroxisome proliferator-activated receptor gama (*pparγ*) in Western clawed frog larvae after 72 h exposures……..….64

Figure 3.1 Schematic representation of the experimental design of chronic juvenile and adult exposures to monomethyl phthalate (MMP) in the Western clawed frog……...77

Figure 3.2 Effect of monomethyl phthalate (MMP) spiked water on survival of Western clawed frogs after an 11 w exposure……....…...82

Figure 3.3 Effect of monomethyl phthalate contaminated water on Western clawed frog metamorphosis during a juvenile exposure…….83

Figure 3.4 Effect of monomethyl phthalate (MMP) spiked water on Western clawed frog body length after (A) an 11 w and (B) a 51 w exposure.84

iv. List of figures (continued)

Figure 3.5 Effect of monomethyl phthalate (MMP) spiked water on Western clawed frog sex ratios after an 11 w exposure……..……..86

Figure 3.6 Effect of monomethyl phthalate (MMP) spiked water on the expression of heat shock protein 70 (*hsp70*) in Western clawed frogs after a 51 w exposure……...…….....……..88

Figure A1.1 Effect of monomethyl phthalate (MMP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures...138

Figure A1.2 Effect of monomethyl phthalate (MMP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures...139

Figure A1.3 Effect of monomethyl phthalate (MMP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures...140

Figure A2.1 Effect of dimethyl phthalate (DMP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures...141

Figure A2.2 Effect of dimethyl phthalate (DMP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures...142

Figure A2.3 Effect of dimethyl phthalate (DMP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures...143

Figure A3.1 Effect of dicyclohexyl phthalate (DCHP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures...144

Figure A3.2 Effect of dicyclohexyl phthalate (DCHP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures...145

Figure A4.1 Effect of monomethyl phthalate (MMP) spiked water on the gene expression in Western clawed frogs after 11 w exposures……...146

Figure A4.2 Effect of monomethyl phthalate (MMP) spiked water on the gene expression in Western clawed frogs after 11 w exposures……147

Figure A5.1 Effect of monomethyl phthalate (MMP) spiked water on the gene expression in Western clawed frogs after 51 w exposures……148

Figure A5.2 Effect of monomethyl phthalate (MMP) spiked water on the gene expression in Western clawed frogs after 51 w exposures……149

v. List of acronyms

17β-HSD	17β Hydroxysteroid dehydrogenase
3β-HSD	3β Hydroxysteroid dehydrogenase
AMA	Amphibian metamorphosis assay
AR	Androgen receptor
βACTIN	Beta actin
BCL2	B cell leukemia/lymphoma 2
BID	BH3-interacting domain death agonist
BMP15	Bone morphogenetic protein 15
BMP4	Bone morphogenetic protein 4
BPA	Bisphenol A
BzBP	Benzyl butyl phthalate
C	Water-only control
CAT	Catalase
CMP	Chemical Management Plan
COX	Cyclooxygenase
CRHR1	Corticotropin releasing hormone 1
CYP11A1	Cytochrome P450 side-chain cleavage
CYP17A1	Cytochrome P450c17
CYP19	Aromatase
CYP3A	Cytochrome P450 3A
CYP3A2	Cytochrome P450 3A2
CYP3A4	Cytochrome P450 3A4
DBP	Dibutyl phthalate
DCHP	Dicyclohexyl phthalate
DEHP	Di-(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DHCR7	7-Dehydrocholesterol reductase
DHT	Dihydrotestosterone
DIDP	Diisodecyl phthalate
DINP	Diisononyl phthalate
DIO1	Deiodinase type 1
DIO2	Deiodinase type 2
DIO3	Deiodinase type 3
DMP	Dimethyl phthalate
DMSO	Dimethyl sulfoxide
DNOP	Dioctyl phthalate
DPeP	Dipentyl phthalate
E1	Estrone
E2	Estradiol
EC_{50}	Median effective concentration

EF1α	Elongation factor 1 alpha
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
FETAX	Frog Embryo Teratogenesis Assay: *Xenopus*
FGF10	Fibroblast growth factor 10
FGFR2	Fibroblast growth factor receptor 2
FSHβ	Follicle-stimulating hormone β
GC-MS	Gas chromatography-mass spectrometry
GH	Growth hormone
GHR1	Growth hormone receptor 1
GHR2	Growth hormone receptor 2
GPR30	G protein-coupled estrogen receptor 1
GPX	Glutathione peroxidase
HSF2	Heat shock factor 2
HSP	Heat shock protein
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
IGF-1	Insulin-like growth factor 1
IGF-1Rα	IGF-1 receptor alpha
IGF-1Rβ	IGF-1 receptor beta
IGF-2	Insulin-like growth factor 2
IGFBP-5	Insulin-like growth factor binding protein 5
INSL3	Insulin-like hormone 3
LC_{50}	Median lethal concentration
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LOEC	Lowest observable effect concentration
MBP	Monobutyl phthalate
MBzP	Monobenzyl phthalate
MCIG	Minimal concentration to inhibit growth
MEHP	Mono-(2-ethylhexyl) phthalate
MMP	Monomethyl phthalate
MOA	Mechanism of action
MPR	Membrane progesterone receptor
NCoR	Nuclear corepressor
NUR77	Nerve growth factor IB
OTX2	Orthodenticle homeobox 2
P53	Tumor protein 53
PAX-6	Paired box protein
PBR	Peripheral benzodiazepine receptor
PPARs	Peroxisome proliferator-activated receptors
PPARα	Peroxisome proliferator-activated receptor alpha

PPARγ	Peroxisome proliferator-activated receptor gama
PTGS2	Prostaglandin-endoperoxide synthase 2
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
RPL8	Ribosomal protein L8
RXR	Retinoid X receptor
SC	Solvent control
SF-1	Steroidogenic factor 1
SMRT	Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor
SOD	Superoxide dismutase
SRB1	Scavenger receptor class B-1
SRC-1	Steroid receptor coactivator-1
SRD5A2	Steroid 5 alpha reductase type 2
StAR	Steroidogenic acute regulatory protein
SULT2A1	Sulfotransferase dehydrogenase 2A1
T	Testosterone
T2	3,3'-Diiodothyronine
T2AH	Testosterone 2α-hydroxylase
T3	Triiodothyronine
T4	Thyroxine
T6BH	Testosterone 6β-hydroxylase
TH	Thyroid hormone
TI	Teratogenic index
TR	Thyroid hormone receptor
TRα	Thyroid hormone receptors alpha
TRβ	Thyroid hormone receptors beta
TSH	Thyroid-stimulating hormone
UGT	UDP-glucuronosyltransferase
VTG	Vitellogenin
XEMA	*Xenopus* metamorphosis assay

Chapter 1

Introduction and literature review

1.1 Introduction

Versatility, robustness and low cost render plastics the material of choice for many applications. Polymers that are not processable or useful in their natural form are mixed with plastic additives. This broad category of chemicals includes flame retardants, surfactants, blowing agents and plasticisers, among others. Plasticisers are the most common plastic additives and provide polymers with flexibility by lowering the glass transition temperature (Daniels, 2009). However, plasticisers are often not covalently bound to the polymer and thus slowly diffuse and evaporate out of the material (Munksgaard, 2004; Nara et al., 2009; Demir and Ulutan, 2013). Consumers typically use plastic items only once before disposing of them. The alarming amount of waste and the plasticisers' ability to leach out of polymers lead to environment contamination (Fromme et al., 2002). Plasticisers are ubiquitous and numerous studies have confirmed the presence of plasticisers and their metabolites in air, soil, water and animal and human body fluids (Horn et al., 2004). Furthermore, several studies have shown that these compounds have toxic effects at all levels of organisation in mammals. This chapter reviews the literature on the adverse effects of plasticisers in mammals and aquatic species.

1.2 Literature review

Today's large number of plastic applications has called for a greater variety of plasticisers. Bisphenol A (BPA) and phthalates are the most important plasticisers. A multitude of studies provided evidence for the detrimental effects of plasticisers on the regulation and function of many axes. The following sections present the pathways of entry of the main plasticisers in the environment and discuss their sublethal effects on the thyroid hormone (TH), sex steroid and stress axes in vertebrates and invertebrates. Lethal effects are also reviewed. A particular focus is given to amphibian literature. In addition, new research directions were identified.

1.2.1 Plasticiser uses and pathways of entry in the environment

BPA is a highly produced plastic monomer and plasticiser. About 1,150,000 metric tons were produced in the Europe Union in 2005-2006 (reviewed in Oehlmann et al., 2008). BPA has good cross-linking properties hence it is a plasticiser of choice (Alonso-Magdalena et al., 2006). Unbound monomers remain after polymerisation and can be released (Brotons et al., 1995). The temperature (Tan and Mustafa, 2003), pH (Xu et al., 2011) and fat content (Lopéz-Cervantes and Paseiro-Losada, 2003) of compounds in contact with plasticised polyvinyl chloride modulate the leaching. BPA has been shown to leach from dental sealants (Olea et al., 1996), tin cans (Brotons et al., 1995) and food contact items (Biles et al., 1997; reviewed in Vandenberg et al., 2007; Fasano et al., 2012). These exposure pathways lead to the detection of BPA in adult and fetal serum (reviewed in Vandenberg et al., 2007), urine (Calafat et al., 2005; Hauser et al., 2007), breast milk (Sun et al., 2004) and adipose tissue (Fernandez et al., 2007). All uptaken BPA is immediately metabolised into inactive metabolites and excreted in urine. The environment is exposed via disposal or recycling of products containing BPA. The amount of BPA leaching from plastic wastes can be as high as 139 mg/kg (Yamamoto and Yasuhara, 1999). Sewage and plastic leachate then lead to surface water and sediment contamination (Fromme et al., 2002). A few days are necessary for BPA to degrade in aerobic environments, whereas BPA does not degrade in anaerobic environments (reviewed in Rykowska and Wasiak, 2006; and in Staples et al., 1998).

Phthalates are certainly the most important type of plasticisers. Phthalates have been used in polyvinyl chloride since 1926 (Oehlmann et al., 2009) to render it flexible, pliable and elastic, but are now used in many other plastic types and in higher concentrations. Phthalate diesters all have a central ring and two esters in common (Fig. 1.1). *Ortho*-phthalates have the esters on consecutive carbons and are the most abundant isomers; therefore the prefix *ortho* is generally not used. *Iso*-phthalates have their R groups in position 1 and 3, while *tere*-phthalates exhibit chains on opposite carbons. As with BPA, phthalates are not irreversibly bound to the matrix and therefore diffuse and evaporate out of the polymer. Food (Bradley et al., 2013), food packaging (Cao, 2010; Fasano et al., 2012), alcoholic beverages (Leitz et al., 2009), PVC flooring (Carlstedt et al., 2012), cosmetics/personal care products (Shen et al., 2007; Sathyanarayana et al., 2008), blood/intravenous solution storage bags (Kim et al., 1976; Monfort et al., 2012; Štrac et al, 2013) and medicinal products/dietary supplements (Hernández-Díaz et al., 2009; Kelley et al., 2012) expose humans to phthalates. Phthalates are also indoor pollutants and are found in the air and dust (Otake et al., 2004; reviewed in Weschler and Nazaroff, 2008). Alkyl chains in the esters can vary greatly, affecting the properties of the phthalate. Longer chains equate higher molecular weights, which correlates to better plasticiser retention and slower migration rates. This explains the current trend towards replacing di-(2-ethylhexyl) phthalate (DEHP) by diisononyl phthalate (DINP; Koch et al., 2007). After being uptaken, *ortho*-phthalate diesters are rapidly degraded to phthalate monoesters (Fig. 1.1) and are then excreted in urine and body fluids. Monoesters harbor one ester and one carboxylic acid on the aromatic ring. Metabolites are used for biomonitoring and epidemiological studies (Blount et al., 2000; Huang et al., 2007; Koch et al., 2007; Mazzeo et al., 2007; Aylward et al., 2009; reviewed in Wittassek et al., 2011; Mieritz et al., 2012). Wastewater treatment plants do not effectively remove plasticisers from the influent, meaning that contaminated urine and other influents represent a significant source of phthalates and their metabolites in the environment (Soliman et al., 2007; Barnabé et al., 2008; Clara et al., 2010; Kusk et al., 2011). Considering phthalates' ability to migrate from plastics, their widespread manufacture and their high concentration in plastics, disposal of plastics is a major source of contamination via landfill leachate (Zheng et al., 2007). This results in phthalates being ubiquitous in the environment (Michael et al., 1984; Suzuki et al., 2001; Fromme et al., 2002; Teil et al., 2007; Blair et al., 2009). For example, DEHP can be found in river sediment at levels as high as 110 mg/L (Horn et al., 2004). In presence of microbes, phthalates have half-lives ranging from a few hours to a few months in surface water, marine water and soil (reviewed in Staples et al., 1997b).

Molecular structure affects the toxicity of phthalates. On top of playing a role in plasticiser retention, alkyl chain length appears to influence the severity of the adverse health effects. Aromatic ring substitution also plays a role in adverse health effect. *Iso*-phthalates usually have biological effects similar to their *ortho* isomer. On the other hand, many studies concluded that *tere*-phthalates are less toxic than their *ortho* counterparts (Barber and Topping, 1995; Faber et al., 2007a; Deyo, 2008; Wirnitzer et al., 2011). In addition, toxicity seems to be impacted by plasticiser metabolisation. Monoesters are thought to be the active metabolites inducing the toxicity observed in diester studies (Gray et al., 2000; Saillenfait et al., 2001; Ema, 2002; Shen et al., 2011). In rodents, secondary-step metabolites are suspected to be even more toxic than the parent compounds or the primary metabolites (Latini, 2005).

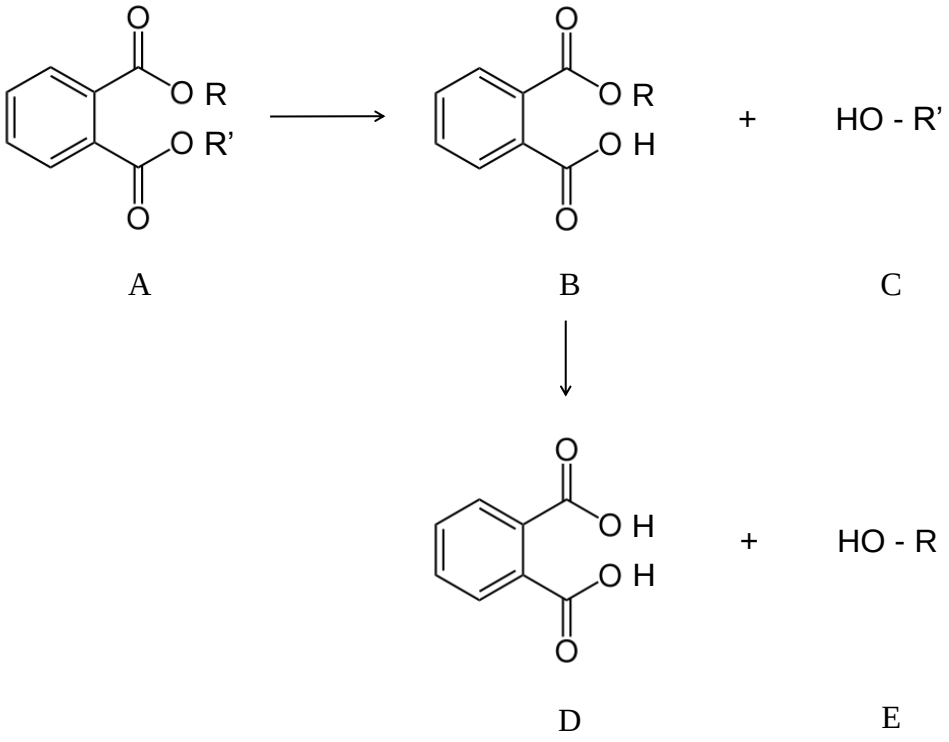

FIGURE 1.1 Biological degradation of phthalates. *Ortho*-phthalate diesters (A) have an aromatic ring and two esters on adjacent carbons. Diesters are metabolised into phthalate monoesters (B) and alcohol (C). Further degradation yields phthalic acid (D) and alcohol (E). The resulting alcohols are of added concern due to their high volatility. For most *ortho*-phthalates, the degradation is incomplete and results in a mixture of monoesters, alcohols and phthalic acid. Adapted from Horn et al., (2004) and Liang et al. (2008).

1.2.2 PPARs as central mechanisms of action

Peroxisome proliferator-activated receptors (PPARs) play a role in fatty acid oxidation and adipose tissue atrophy (Hurst and Waxman, 2003). Studies have shown that BPA and phthalates can affect the expression of PPARs (Fig. 1.2). An in vitro study conducted by Phrakonkham et al. (2008) demonstrated that mouse embryo fibroblasts exposed to 18 mg/L BPA for two days resulted in increased *pparγ2* expression. Likewise, *pparα* and *pparγ* transcripts were increased in DEHP treated rats (Xu et al., 2010). Mono-(2-ethylhexyl) phthalate (MEHP) increased the expression of *pparα* and *pparβ*, but reduced the expression of *pparγ* in human liposarcoma cells (Campioli et al., 2011). Additionally, MEHP upregulated PPARγ target genes in 3T3-L1 cells (Feige et al., 2007). On top of disrupting *ppar* mRNA levels, phthalates can affect their protein expression (Lee et al., 2007; Xu et al., 2010) and can activate them (reviewed in Lapinskas et al., 2005; and in Rusyn et al., 2006). The α isoform was found to be activated by metabolites such as MMP, MEHP, monobenzyl phthalate (MBzP), mono-*sec*-butyl phthalate and 2-ethylhexanoic acid, albeit PPARα was not activated by DEHP (Maloney and Waxman, 1999; Hurst and Waxman, 2003). Similarly, DEHP did not activate PPARγ, whereas monoesters including MEHP, MBzP and mono-*sec*-butyl phthalate activated mouse, human and 3T3-L1 preadipocytes PPARγ (Maloney and Waxman, 1999; Hurst and Waxman, 2003; Feige et al., 2007). Since monoesters and alcohols can activate PPARs, it appears as if metabolites are the active ingredients in diesters. In low molecular weight phthalates, diester hydrolysis occurs rapidly. Phthalates harboring long alkyl chains such as DEHP are hydrolysed slowly and are therefore less potent than the corresponding monoesters (Gray et al., 1983; Maloney and Waxman, 1999).

PPARs can form heterodimers with the retinoid X receptor (RXR) and function as metabolic ligand sensors for lipophilic hormones, fatty acids and their metabolites. The heterodimers also play a transcription regulation role by binding and transactivating peroxisome proliferator response elements located in the 5' regulatory region of peroxisome proliferator-activated genes (Hurst and Waxman, 2003). Therefore, altering PPARs transcript levels, protein expression or activation can lead to disrupted transcription of PPAR-mediated genes. When the expression of fatty acid metabolism-related genes is altered, for example, peroxisome proliferation takes place (Schoonjans et al., 1996). Although phthalates were shown to affect PPARs, as mentioned in the previous paragraph, DEHP and DINP were found not to induce peroxisome proliferation in mammals (Lington et al., 1997; Kurata et al., 1998). Molecular structure plays a role in peroxisome proliferation. Alcohols, a product of diester hydrolysis, induced very little peroxisomal response (Gray et al., 1983; Maloney and Waxman, 1999). Contrarily, a study by Keith et al. (1992) showed that 2-ethylhexanol was a peroxisome

proliferator. Di(2-ethyhexyl) terephthalate was shown not to induce proliferation in rats (Topping et al., 1987; Barber and Topping, 1995), possibly because metabolisation does not lead to the formation of monoesters (Topping et al., 1987). Dimethyl terephthalate, dibutyl terephthalate and di-(2-ethylhexyl) terephtalate can be completely hydrolysed, yielding two moles of alcohol per mole of phthalate diester. *Ortho*-phthalates are not completely hydrolysed when metabolised, which could explain the difference in toxicity (Faber et al., 2007b; Wirnitzer et al., 2011). In addition, branched phthalates were shown to be more potent peroxisome proliferators than their straight chain analogs (Gray et al., 1983; Mann et al., 1985). In sum, metabolisation, alkyl chain length, ring substitution and branching of the alkyl chain affect the ability of a phthalate to induce peroxisome proliferation.

PPARs' transcription factor function is believed to be the main mechanism of action (MOA) behind plasticiser-induced transcriptional changes (Gazouli et al., 2002), reproductive organ toxicity (Hurst and Waxman, 2003, reviewed in Latini et al., 2008), oxidative stress (Lee et al., 2007) and mortality (Abbott et al., 2007). Although a lot of research effort has been focused on PPARs, it is important to continue to characterise the molecular, cellular and organismal effects of phthalates, along with other possible MOAs. Sublethal and lethal effects of plasticisers are presented in the following sections.

1.2.3 Development impairment via the thyroid and growth hormone axes

Since cellular responses to stress can first be detected with transcriptional changes, it is important to review the effects of BPA and phthalates on gene expression. One of the axes disrupted by plasticisers is the TH system. The latter plays a crucial role in the regulation of development, metabolism, heart function and psychoneurological wellbeing (Hofmann et al., 2009). Thyrotropin-releasing hormone is first released by the hypothalamus. Thyrotropin-releasing hormone, along with circulating concentrations of triiodothyronine (T3) and thyroxine (T4), control the rate of thyroid-stimulating hormone (TSH) release in the pituitary gland. In turn, TSH induces the synthesis of T4 in the thyroid gland. Deiodinases (*dio1*, *2* and *3*) can convert T4 to T3 or reverse T3. Reverse T3 and T3 are then transformed into 3,3'-diiodothyronine (T2; Stoker at al., 2004). The physiological effects of T3 and T4 are mediated through the binding to nuclear thyroid hormone receptors alpha and beta (TRs; TRα and TRβ; Flood et al., 2013). Theoretically, TH homeostasis can be disrupted at several points of the pathway, but contaminants have been shown to act primarily by producing hypothryroidism. This could involve inhibition of iodide uptake, inhibition of T4 synthesis, upregulation or downregulation of deiodinases or increased T4 catabolism (Degitz et al., 2005).

BPA has been shown to disrupt the expression of TH-related genes in mammals and aquatic species. BPA downregulated the expression of most T3-response genes in the African clawed frog (Heimeier et al., 2009). Likewise, Iwamuro et al. (2006) showed that BPA decreased the gene expression of *tr*s and *rxrγ* in African clawed frog tail culture. RXR is necessary for the formation of heterodimers with TRs and for transcription regulation. The authors suggested that BPA can bind to TR (Iwamuro et al., 2003). Interestingly, BPA was shown to be a weak ligand for Sprague Dawley rat liver TR, but could not activate it (Moriyama et al., 2002). Similarly, Kitamura and collaborators (2002 and 2005a) showed that BPA did not inhibit the binding of T3 to TR in rat pituitary cell lines. The binding of BPA to TRs is reviewed by Zoeller (2005). Despite BPA's weak binding to TR, the plasticiser is a potent inhibitor of T3 binding to human TH-binding proteins (Ishihara et al., 2003). In addition, clear TH-related effects have been observed at the individual level. The aforementioned effects of BPA at the transcriptional level affected metamorphosis in treated tadpoles. BPA hindered the T3-induced intestinal remodeling (Heimeier et al., 2009), blocked the T3-dependent resorption of the tail and shortened the interocular distance (Iwamuro et al., 2003; Imaoka et al., 2007). Similarly, treated African clawed frogs were one or two developmental stages behind control animals (Iwamuro et al., 2003) and exhibited shorter body length at 4.6 mg/L (Sone et al., 2004). Likewise, tail length, total length and body weight were all reduced in Japanese medaka and swordtail fish chronically treated

with BPA concentrations as low as 2 µg/L (Yokota et al., 2000; Kwak et al., 2001). Sheep exposed to BPA prenatally also had a lower birth weight (Savabieasfahani et al., 2006). A contradictory result was obtained in a chronic study exposing the New Zealand mudsnail to 5 µg/L BPA; the plasticiser increased the mean shell height (Jobling et al., 2003). In addition, BPA delayed hatching, yolk absorption and first feeding by about seven days in juvenile rainbow trout. The authors suggested that this result was attributable to an increase in vitellogenin (*vtg*) mRNA and a decrease in growth hormone (GH)-related gene expression. A shift in energy allocation from somatic growth to vitellogenesis was also suggested (Aluru et al., 2010). Delayed hatching was observed in zebrafish experiments as well (Duan et al., 2008). Altogether, BPA was shown to delay development and to reduce offspring weight and size in a range of species by affecting the transcription of TH and GH-related genes.

Similarly to BPA, three phthalates were shown to disrupt the expression TH-related genes in amphibians (Fig. 1.3). Benzyl butyl phthalate (BzBP) impeded the T3-induced increase in *trβ* transcript and therefore exhibited T3-antagonist activity in African clawed frog tadpoles (Sugiyama et al., 2005). Dibutyl phthalate (DBP) and its metabolite monobutyl phthalate (MBP) disrupted the expression of four genes at concentrations as low as 2 mg/L in African clawed frogs; *trβ* and *rxrγ* transcripts were both decreased, while *tshα* and *tshβ* were upregulated (Shen et al., 2011). Likewise, the testicular expression of *trα1* was upregulated in rats treated with DBP (Lee et al., 2007). Invertebrate endocrine systems are drastically different from those of vertebrates. Ecdysone is a steroid hormone controling the development, moutling and metamorphosis of insects. The ecdysone receptor belongs to the superfamily of nuclear hormone receptor which includes TRs, among others. In chironomid larvae, 0.1 mg/L BzBP increased the expression of the ecdysone receptor, while 100 mg/L DEHP decreased it (Planelló et al., 2011). A mammalian two-hybrid assay by Shen et al. (2011) shed light on a possible MOA. DBP and particularly its metabolite MBP enhanced the interactions between TR and the co-repressor Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor (SMRT) in a dose-dependent manner. SMRT is a transcriptional coregulatory protein that represses transcription pathways. Therefore, DBP and MBP could impair the TH system by suppressing TH-related transcripts (Shen et al., 2011).

On top of their effects at the transcriptional level, phthalates were shown to affect the TH system at the translational level. In female rats for example, exposure to DEHP for 14 days yielded an increase in serum T3 and T4 (Gayathri et al., 2004). Moreover, BzBP was shown to potentiate the T3-induced proliferation of rat pituitary GH3 cells (Ghisari and Bonefeld-Jorgensen, 2009). Hyperactivity of the thyroid gland has been suggested as a MOA (Gayathri et al., 2004). Indeed, Wenzel and collaborators (2005) used a rat cell culture to demonstrate that

phthalates increased iodide intake by increasing the activity of the sodium/iodide symporter. Although iodide is necessary for THs synthesis, phthalate exposure does not necessarily lead to increases in TH production. In men for example, urinary phthalate diester and monoester levels were inversely associated with serum T4 and T3 (Huang et al., 2007; Meeker et al., 2007). These results are in agreement with studies conducted with the African clawed frog; BzBP exhibited T3-antagonist activity in vivo and DCHP, BzBP and DBP displayed T3-antagonist activity in a T3-dependent luciferase assay (Sugiyama et al., 2005). The latter could be due to a displacement of T3 from the TR; DEHP is a potent inhibitor for bullfrog TR ligand-binding domain and was deemed to be four orders of magnitude less potent than T3 (Ishihara et al., 2003). DEHP also decreased the proteic expression of TRα1 in rats (Lee et al., 2009). These effects on the TH axis have repercussions at the individual level. Phthalates affected rodent development and their potency depended on the length of the alkyl chains. Short (methyl and ethyl) and long (octyl, nonyl, decyl) phthalates had low or no developmental toxicity in rodents. Contrarily, medium weight phthalates such as DIBP decreased body weight in both pregnant rats and pups (Field et al., 1993; Saillenfait et al., 2006). On the other hand, numerous studies have provided evidence for short, medium and long chained phthalates deleterious effects on aquatic animals' development. DEHP delayed the hatching time of Japanese medaka embryos (Chikae et al., 2004a). DEHP also had detrimental effects on zebrafish oocyte maturation. The authors advanced that this was due to an increase in the protein expression of bone morphogenetic protein 15 (*bmp15*), leading to a decrease in luteinizing hormone receptor (*lhr*) and membrane progesterone receptors (*mpr*) gene expression (Carnevali et al., 2010). Additionally, Zhou et al. (2011a) exposed abalone embryos to DMP, DBP, DEHP, diethyl phthalate (DEP) and dioctyl phthalate (DNOP) and noted that metamorphosis was suppressed in the 0.2 to 2 mg/L range. Two experiments conducted in African clawed frog are in accordance with these results; phthalates and their metabolites decelerated metamorphosis in tadpoles (Dumpert and Zietz, 1984; Shen et al., 2011). DBP and MBP also significantly reduced the interocular distance and whole body length in African clawed frog tadpoles in subchronic exposures (Lee et al., 2005; Shen et al., 2011). An interlaboratory study exposed African clawed frog tadpoles to phthalic acid for 96 hour and determined that the minimal concentration to inhibit growth (MCIG) was 8,310 mg/L (Bantle et al., 1999), suggesting that this secondary metabolite is not as potent as mono and diesters. In sum, intermediate weight phthalates suppressed development in rodents, while all alkyl chain lengths affected mollusc, fish and amphibian development.

Development is also regulated by the GH axis. Studies documenting the effects of plasticiser on this system are scarce. Aluru et al. (2010) showed that BPA disrupted the expression of six genes involved in the endocrine regulation of growth and development in juvenile rainbow trout; both insulin-like growth factor

1 and 2 (*igf-1* and *2*), their receptor IGF-1 receptor alpha and beta (*igf-1rα* and *β*) and both growth hormone receptors 1 and 2 (*ghr1* and *2*) were downregulated by BPA. Contrarily, genes of the insulin-like growth factor family were upregulated in Wolffian ducts of rats exposed to DBP. Both *igf-1* and *igf-2*, *igf-1r* and insulin-like growth factor binding protein 5 (*igfbp-5*) mRNA abundance was increased. The expression of other growth regulator genes such as bone morphogenetic protein 4 (*bmp4*), fibroblast growth factor 10 (*fgf10*) and fibroblast growth factor receptor 2 (*fgfr2*) was increased as well (Bowman et al., 2005). Clear MOAs for plasticisers' effects on the GH axis have yet to be elucidated. In sum, plasticisers impede with development by disrupting the expression of TH and GH-related genes, by binding to TRs, by influencing THs levels and by enhancing the interactions between TR and a co-repressor. Since plasticisers affect the TH axis, and providing that crosstalk between the TH and the androgen axes has been demonstrated (Flood et al., 2013), it is not surprising that plasticisers also affect sex steroids.

1.2.4 Reproduction alterations mediated by estrogen receptors, aromatase and testosterone

BPA's deleterious effect on the sex steroid axis are thoroughly characterised, including at the transcriptional level. BPA has been shown to affect mRNA levels of genes coding for receptors and enzymes, among others. A 2 µg/kg body weight dose increased the expression of both estrogen receptors in newborn mice (*er*s; *erα* and *erβ*; Kawai et al., 2007), whereas only *erβ* was upregulated in other rodent studies (Akingbemi et al., 2004b; Phrakonkham et al., 2008). A concentration of 0.23 mg/L upregulated *er* mRNA in African clawed frog tadpoles exposed for two weeks (Levy et al., 2004). Olea et al. (1996) showed that BPA increased the expression of another receptor, *mpr*, in vitro in MCF7 human breast cancer cells. BPA also disrupted the expression of *vtg*, the gene coding for a precursor protein of egg yolk essential in ovary growth and oocyte production. The expression of *vtg* was increased in treated primary cultured hepatocytes of male African clawed frog and swordtail fish (Kloas et al.,1999; Kwak et al., 2001). Likewise, rainbow trout exposure to 30 mg/L BPA as oocytes increased *vtg* transcript level in their adult life (Aluru et al., 2010). Finally, aromatase (*cyp19*) was downregulated in rat Leydig cells treated with 2.28 ng/L BPA (Akingbemi et al., 2004b). This enzyme is crucial in the biosynthesis of estrogens from androgens. On top of its effects at the transcriptional level, BPA has estrogenic effects in yeast, rodents and human cell lines. BPA increased β-galactosidase activity in a yeast two-hybrid assay, showing the estrogenicity of the plasticiser (Ike et al., 2002). Mice exposed to BPA during gestation displayed more ERα and ERβ in the brain (Kawai et al., 2007). In addition, BPA can bind to both human ERα and ERβ (Brotons et al., 1995; Olea et al.,1996; Kuiper et al., 1998; Stroheker et al., 2004;

Kitamura et al., 2005b). The chemical can also bind to the three zebrafish ERs (Gibert et al., 2011), the African clawed frog ERs (Lutz and Kloas, 1999; Suzuki et al., 2004) and the giant ramshorn snail's estradiol (E2) and testosterone (T) binding sites, which resemble receptors (Oehlmann et al., 2006). In H295R human adrenocortical carcinoma cells, BPA increased progesterone, estrone (E1) and E2 concentrations. Similarly, E2 levels were increased in treated mouse urogenital sinuses (Arase et al., 2011). Contrarily, rats exposed to BPA experienced E2 levels reduction due to an inhibition of CYP19 activity (Akingbemi et al., 2004b). Finally, this plasticiser induced VTG production in zebrafish (reviewed in Segner et al., 2003), fathead minnows (Sohoni et al., 2001), rainbow trout (Christiansen et al., 1998) and African clawed frog (Nomura et al., 2006). In addition to its effects on the female reproductive axis, BPA exhibits antiandrogenic properties. BPA decreased luciferase induction by dihydrotestosterone (DHT) in MCF-7 cells, meaning it displays anti-androgenic activity (Stroheker et al., 2004) and inhibited the production of androstenedione and T in H295R cells (Zhang et al., 2011). A reduction in T synthesis was also observed in rats fed BPA, which was possibly due to the decreased protein expression of cytochrome P450c17 (CYP17A1), an important steroidogenic enzyme (Akingbemi et al., 2004b). BPA also successfully inhibited the activity of DHT in mouse fibroblast cell line NIH3T3 Kitamura et al. (2005b), repressed the action of DHT in a yeast-based assay (Sohoni and Sumpter, 1998) and inhibited the binding of DHT to the androgen receptor (AR) in yeast (Lee et al., 2003). Salian et al. (2009) has shown that perinatal exposure of male rats to BPA reduced the protein expression of steroid receptor coactivator-1 (SRC-1) and nuclear corepressor (NCoR). Since these testicular steroid receptor coregulators can either activate or repress gene expression, they have a major impact on the regulation of the reproductive axis. In sum, BPA is mostly estrogenic and antiandrogenic in yeast, aquatic species, rodents and human and act by binding to ER, repressing coregulators and by impeding with androgen production.

The fragile balance between androgens and estrogens was reported to be disrupted by BPA exposure. BPA has been identified as a potential human sex hormone-binding globulin ligand, meaning BPA could displace endogenous sex steroids from binding sites and disrupt the balance between androgens and estrogens (Déchaud et al., 1999). BPA can disrupt the endocrine balance in a second way; sex hormone metabolism was affected by exposure to the plasticiser. Firstly, BPA is thought to decrease E2 metabolism (Jurgella et al., 2006; Zhang et al., 2011). BPA also suppressed UDP-glucuronosyltransferase (UGT) activities, UGT2B1 protein and *ugt2b1* mRNA in adult male rats and therefore affected the glucuronidation of sex hormones (Shibata et al., 2002). Likewise, BPA significantly decreased the activity of a few male-specific cytochrome P450 isoforms such as testosterone 2α-hydroxylase (T2AH) and testosterone 6β-hydroxylase (T6BH) in treated rats (Hanioka et al., 1998), meaning that T metabolism was decreased. Furthermore, BPA could affect nongenomic steroid

actions by binding to steroid membrane receptors such as the G protein-coupled estrogen receptor 1 (GPR30; Thomas and Dong, 2006). Binding to nongenomic steroid receptors can provoke Ca^{2+} influx and lead to prolactin secretion (Watson et al., 2007). Studies have characterised a few ways in which BPA can disrupt the balance between androgens and estrogens, which could explain some of the undermentioned effects on reproduction.

A meta-analysis conducted by Carlsen and collaborators (1992) described a decrease in seminal volume and sperm concentration worldwide since the 1940s. Today, fertility is also affected by an increasing rate of testicular cancer, a high incidence of reproductive organ malformations and a decreasing boy to girl ratio (reviewed in Eertmans et al., 2003). In the upcoming section, the influence of BPA on reproduction is reviewed. First, several studies reported that sperm was greatly affected by BPA. The percentage of dogwhelk snails with sperm stored in their vesicular seminalis was decreased by BPA exposure (Oehlmann et al., 2000). Similarly, at 1.75 µg/L, male brown trout exhibited poor semen quality; the density, motility rate and swimming velocity were all reduced (Lahnsteiner et al., 2005). Sakaue et al. (2001) raised the possibility of BPA being an estrogen antagonist during rat spermatogenesis. The authors observed a decrease in daily sperm production in treated rats, suggesting that BPA might prevent E2 from inhibiting germ cell apoptosis. Furthermore, BPA reduced testis weight and penis length in rats and in giant ramshorn snails, respectively (Sakaue et al., 2001; Oehlmann et al., 2006). Similarly, BPA inhibited gonadal growth in both male and female fathead minnows at concentrations as low as 0.64 mg/L (Sohoni et al., 2001). Effects on estrous cyclicity, female sexual maturity and egg production were also observed, although BPA was found to be about 10 to 1000-fold less potent than endogenous estrogens (reviewed in Richter et al., 2007). Suffolk ewe lambs exposed to BPA prenatally showed progressive loss of estrous cyclicity. The authors suggested that a decrease in luteinizing hormone (LH) production or release might be the cause of these observations (Savabieasfahani et al., 2006). Interestingly, another study conducted on ewe lambs concluded that chronic BPA exposure reduced LH pulse frequency and amplitude (Evans et al., 2004). Likewise, female rats exposed to BPA exhibited decreased plasma LH levels and permanently altered estrous cyclicity patterns. These effects have been documented in females treated with exogenous T during development. In these cases, T affected nervous system development via its local aromatisation to E2. Since LH surges control the release of oocytes, alterations of estrous cyclicity might limit reproductive fertility and may decrease overall reproductive success. (Rubin et al., 2001). Indeed, 1.75 µg/L BPA was sufficient to delay ovulation by two weeks, while 5 µg/L completely impeded ovulation in brown trout (Lahnsteiner et al., 2005). In addition, premature vaginal opening was noticed in treated rats (Ashby and Tinwell, 1998). On top of its effects on the estrous cycle and sexual maturity, BPA was shown to affect the egg production in aquatic species. Egg production

was enhanced in treated giant ramshorn snails, dogwhelk snails, New Zealand mudsnails and in copepod (Andersen et al., 1999; Oehlmann et al., 2000; Jobling et al., 2003; Oehlmann et al., 2006). The increase in egg production in the giant ramshorn snail was blocked by antiestrogens, suggesting that BPA is estrogenic (Oehlmann et al., 2006). Contrarily, chronic treatment with BPA inhibited fathead minnow egg production (Sohoni et al., 2001). Taken all together, studies show that BPA inhibits sperm production, reduces gonad size, deregulates estrous cyclicity and affects egg production in invertebrates and vertebrates.

Unlike BPA, studies have shown that phthalates can disrupt the expression of genes involved in cholesterol transport and steroidogenesis. Before cholesterol can be uptaken in the cell for steroidogenesis, double bonds are to be removed by the enzyme 7-dehydrocholesterol reductase (DHCR7). The expression of *dhcr7* is found to be reduced in rats exposed to a single high dose of MEHP (10 mg/kg; Lahousse et al., 2006). The scavenger receptor class B-1 (SRB1) is responsible for transporting high-density lipoprotein cholesteryl esters into the cell and its expression was downregulated by DBP and DEHP in rats (Barlow et al., 2003; Lehmann et al., 2004; Thompson et al., 2004; Borch et al., 2006). Once the cholesterol has crossed the cellular membrane, the steroidogenic acute regulatory protein (StAR) and the peripheral benzodiazepine receptor (PBR) transport it to the inner mitochondrial membrane. The expression of the former is reduced by DEHP, MEHP, DBP and dipentyl phthalate (DPeP) in rats (Barlow et al., 2003; Lehmann et al., 2004; Thompson et al., 2004; Borch et al., 2006; Lahousse et al., 2006; Hannas et al., 2011). The expression of *pbr* can be up or downregulated by phthalates in rodents (Gazouli et al., 2002; Lehmann et al., 2004; Borch et al., 2006) and is believed to be mediated by PPARα (Gazouli et al., 2002). The first step of steroidogenesis involves the transformation of cholesterol into pregnenolone by the enzyme cytochrome P450 side-chain cleavage (CYP11A1). DBP, DEHP and DPeP were all shown to reduce the expression of *cyp11a1* in rats (Shultz et al., 2001; Barlow et al., 2003; Lehmann et al., 2004; Thompson et al., 2004; Borch et al., 2006; Hannas et al., 2011). Progestagens are then transformed into androgens by CYP17A1, which mediates both 17α hydroxylase and 17,20 lyase activities. The gene expression of *cyp17a1* was lowered in rats exposed to DBP and MEHP (Shultz et al., 2001; Barlow et al., 2003; Lehmann et al., 2004; Thompson et al., 2004; Lahousse et al., 2006). The product of the 17,20 lyase transformation is dehydroepiandrosterone. The latter is transformed into androstenedione by the enzyme 3*β* hydroxysteroid dehydrogenase (3β-HSD). DBP decreased the expression of *3β-hsd* in rats (Barlow et al., 2003; Lehmann et al., 2004). The enzyme 17β hydroxysteroid dehydrogenase (17β-HSD) has the ability to transform androstenedione to T, and E1 to E2. The congeners 11 and 12 of this enzyme were found to be downregulated in abalone embryos exposed to DMP or DBP (Zhou et al., 2011a; Zhou et al., 2011b). Finally, T can be transformed into E2 by the enzyme CYP19. Dramatic decreases in *cyp19* transcript levels were

observed in a human adrenocortical carcinoma cell line, in rats and in mice treated with DEHP and MEHP (Lovekamp and Davis, 2001; Noda et al., 2007; Lee et al., 2009; Gupta et al., 2010a; Xu et al., 2010). This is thought to be mediated through the induction of the nuclear receptor 4A subfamily (Noda et al., 2007). On top of disrupting cholesterol transport and steroidogenesis-related genes, phthalates lowered the expression of insulin-like hormone 3 (*insl3*). The protein is produced by Leydig cells and is essential for development of the gubernaculum, which aids in the descent of both testes and ovaries from the pararenal through the abdomen (reviewed in Klonisch et al., 2004; Lehmann et al., 2004). DBP, DEHP, BzBP and DPeP reduced the expression of *insl3* in rodents (Lehmann et al., 2004; Wilson et al., 2004; Borch et al., 2006; Hannas et al., 2011). In addition, the expression of receptors such as the steroidogenic factor 1 (*sf-1*), *lhr* and *mpr* were decreased, while *ar* mRNA content was increased in rats and zebrafish exposed to phthalates (Bowman et al., 2005; Borch et al., 2006; Lee et al., 2007; Carnevali et al., 2010). Finally, a 500 mg/kg dose of DEHP increased the transcription of *vtg* in male zebrafish (Uren-Webster et al., 2010). The transcriptional effects of phthalates on the sex steroid axis was reviewed by Euling et al. (2013). Briefly, phthalates mostly have a deleterious effect on the expression of cholesterol transport proteins, steroidogenic enzymes, receptors and other proteins.

The aforementioned transcriptional changes then led to changes in proteic expression. Studies completed with female rats and cultured rat granulosa cells concluded that DEHP and its metabolite MEHP decreased the proteic expression of CYP19 (Lovekamp and Davis, 2001; Xu et al., 2010). In addition, Wistar rat male pups exposed to DEHP during gestation and lactation showed decreased CYP19 activity at low doses and increased activity at high doses (Andrade et al., 2006). CYP19 activity was also reduced in a human cell line treated with MEHP, which is believed to be due to a rapid increase in nerve growth factor IB (*nur77*) mRNA and protein levels, a member of the nuclear receptor 4A subfamily (Noda et al., 2007). Since CYP19 is an important enzyme in the biosynthesis of estrogens, phthalate exposure might result in disturbances of the normal balance between androgens and estrogens. Indeed, increases in E2, follicle stimulating hormone and LH were observed in rats exposed to DBP and DEHP (O'Connor et al., 2002; Akingbemi et al., 2004a). Contrarily, DEHP and MEHP decreased E2 levels in rats and mice, which is likely due to the decrease in CYP19 protein expression (Lovekamp and Davis, 2001; Gupta et al., 2010a; Xu et al., 2010). Numerous studies have shown the estrogenic activity of phthalates in cell lines, fish, amphibians and mammals. Phthalates can bind human ERα (Ohashi et al., 2005), rat ERs (Zacharewski et al., 1998), rainbow trout ERs (Knudsen and Pottinger, 1999) and African clawed frog ERs (Lutz and Kloas, 1999: Suzuki et al., 2004). Using a yeast-based ER transcription assay, Kang and Lee (2005) showed that BzBP is weakly estrogenic. Finally, the production of VTG was affected by phthalate exposure in rainbow trout, medaka, common carp and zebrafish (Christiansen et al., 1998; Patyna and

Cooper, 2000; Barse et al., 2007; Carnevali et al., 2010). VTG production is a sensitive biomarker of estrogenicity in fish and suggest an activation of the ER signaling pathway (Sumpter and Jobling, 1995; Aluru et al., 2010). In most cases, phthalates are feminising and act by reducing the expression and activity of CYP19. In addition to their estrogenic activity, phthalates are antiandrogens. As seen with BPA, reductions in T production were observed in treated dogs (Pathirana et al., 2011), rabbits (Higuchi et al., 2003) and rodents (Oishi and Hiraga, 1980; Jones et al., 1993; Shultz et al., 2001; Lehmann et al., 2004; Thompson et al., 2004; Wilson et al., 2004; Borch et al., 2006; reviewed in Foster, 2006; Pereira et al., 2006; Lee et al., 2007; Mahood et al., 2007; Howdeshell et al., 2008; Lee et al., 2009; Noriega et al., 2009; Hannas et al., 2011). For example, rat pups exposed to MBP during gestation exhibited a mean testicular T content of about 6% of that of the control group (Shono et al., 2000). Other androgens such as androstenedione and DHT were decreased in rats and rabbits exposed to DBP (Shultz et al., 2001; O'Connor et al., 2002). Unlike other antiandrogens, phthalates do not act by binding to the AR; in vitro studies showed that DEHP and MEHP do not display affinity for the human AR at concentrations up to 10 μM (Parks et al., 2000). As mentioned previously, phthalates modify the gene expression of enzymes and transport proteins involved in T synthesis. A second possible MOA for the adverse effects on reproduction is the increase in T metabolism. Crago and Klaper (2012) showed that the reduction in T observed in DEHP treated fathead minnow was not due to an inhibition of steroidogenic gene expression nor an increase in peroxisome proliferator-related transcripts. An increase in follicle-stimulating hormone β (*fshβ*) mRNA also showed that feedback from the pituitary was not repressed. The gene expression of phase I-metabolising enzyme cytochrome P450 3A4 (*cyp3a4*) and phase II-metabolising enzyme sulfotransferase dehydrogenase 2A1 (*sult2a1*), on the other hand, was increased, providing evidence for increased steroid catabolism. Similarly, Patyna and collaborators (2006) provided evidence for increased T hydroxylase activity in Japanese medaka treated with DINP or diisodecyl phthalate (DIDP). DEP increased cytochrome P450 3A2 (CYP3A2) content in male rats, which hydroxylates T at the 6β-position (Fujii et al., 2005). Another antiandrogenic effect is the decrease in INSL3 secretion in dog testes treated with MBP or MEHP (Pathirana et al., 2011). In addition, the activity of 5α-reductase, the enzyme transforming T into the more potent DHT, was inhibited by DBP and DEHP in the common carp (Thibaut and Porte, 2004). Overall, phthalate esters have been shown to be estrogenic and antiandrogenic. The former is mediated mostly by CYP19, while the latter is accomplished through a reduction in cholesterol transport in mammals and an increase in T metabolism in aquatic species.

Phthalates disrupt reproduction in males of many species. Several papers review the effects of phthalates in mammalian reproduction (Ema, 2002; Hotchkiss et al., 2008; Talsness et al., 2009; Makris et al., 2013). Main and collaborators

(2006) analysed breast milk from a Danish-Finnish cohort. The milk contained six phthalate monoesters. These metabolites had antiandrogenic effects on male infants reproductive hormones. Serum sex hormone-binding globulin, LH to free T ratio and LH level were all positively correlated with the metabolites, while free T was negatively associated with MBP. The authors suggested that phthalates adversely affected Leydig cell function, which resulted in a reduced biologic androgen effect (Main et al., 2006). In rats and rabbits, BzBP, DBP and DEHP reduced sperm synthesis, sperm concentration, sperm motility, ejaculate volume and number of motile sperms, among others (Higuchi et al., 2003; Tyl et al., 2004; Aso et al., 2005; Gray et al., 2009; Lee et al., 2009; Giribabu et al., 2012). When abalone sperms were exposed to 100 µg/L DMP, a fertilisation rate of 38.5% was achieved, compared to approximately 80% in control animals. The authors provided evidence for a decrease in ATPase activity, meaning that sperms had less energy to propel themselves and penetrate into the eggs (Zhou et al., 2011b). Exposed animals also suffered from altered sex accessory organs; a multitude of studies provided evidence for phthalates' effect on the anogenital distance, the distance from the anus to the anterior base of the penis. This distance was decreased in rodents exposed to BzBP, DCHP, DBP, DEHP, DPeP and dipropyl phthalate (Mylchreest et al., 1998; Mylchreest et al., 1999; Mylchreest et al., 2000; Parks et al., 2000; Tyl et al., 2004; Aso et al., 2005; Hoshino et al., 2005; Jarfelt et al., 2005; Gray et al., 2009; Yamasaki et al., 2009; Hannas et al., 2011; Saillenfait et al., 2011). Furthermore, MBzP decreased the anogenital distance in treated rats and may be responsible for the effects seen with BzBP (Ema et al., 2003). The reduction in anogenital distance was believed to be due to a decreased T production during the critical stage of reproductive tract differentiation (Parks et al., 2000). It was shown that anogenital distance is positively correlated with fertility, sperm density and mobile sperm count in men. Therefore, the anogenital distance is associated with fatherhood and may predict male reproductive potential (Eisenberg et al., 2011). In addition, phthalates inhibited transabdominal testicular descent in rats, a T-dependent event (Mylchreest et al., 1998; Shono et al., 2000; Ema et al., 2003; Saillenfait et al., 2006; Saillenfait et al., 2011). Other frequent results of phthalate exposure are permanent retention of nipples (Mylchreest et al., 1999; Mylchreest et al., 2000; Tyl et al., 2004; Hoshino et al., 2005; Jarfelt et al., 2005; Gray et al., 2009; Yamasaki et al., 2009; Hannas et al., 2011), delayed preputial separation (Mylchreest et al., 1999; Aso et al., 2005; Yamasaki et al., 2009) and delayed pubertal onset in male rodents (Tyl et al., 2004; Noriega et al., 2009). Testicular lesions have also been observed in treated rats (Foster et al., 1981; Tyl et al., 2004; Gray et al., 2009) and in African clawed frog (Lee and Veermachaneni, 2005). In addition to these adverse effects, phthalates reduced the weight of androgen-dependent organs such as the prostate and the testes in rats (Srivastava et al., 1990; Parks et al., 2000; Jarfelt et al., 2005; Gray et al., 2009; Noriega et al., 2009). This effect was also believed to be due to the decreased T production (Parks et al., 2000). Phthalates and their monoester metabolites induced atrophy of the testes and

of the seminiferous tubules (Foster et al., 1981; Lake et al., 1982; Mylchreest et al., 1998; Kasahara et al., 2002; Aso et al., 2005; Hoshino et al., 2005; Yamasaki et al., 2009). Three possible MOAs were put forward. The first one involves the induction of oxidative stress which injures mitochondrial function. The latter would then be followed by the release of cytochrome *c*, thereby inducing apoptosis of spermatocytes (Kasahara et al., 2002). The second explanation involves a decrease in sorbitol dehydrogenase activity, fructose level and phospholipids levels (Fukuoka et al., 1989). The last MOA suggests that phthalates act on T and DHT levels, as mentioned in the previous paragraph (Mylchreest et al., 1998). Testicular atrophy and lesions were not observed in rats treated with -tert monoesters, providing evidence for the effect of branching in testicular toxicity (Foster et al., 1981). Similarly, ring substitution plays a role in reproductive damage. For example, di(2-ethylhexyl) terephthalate did not adversely affect rat reproduction (Faber et al., 2007a). This compound was also found not to induce peroxisome proliferation (Topping et al., 1987; Barber and Topping, 1995). The antiandrogenic properties observed in in vivo studies are thought to be related with PPARα, rather than the AR (Gazouli et al., 2002). Ward et al. (1998) refuted this hypothesis; the group used PPARα-null mice to conclude that the testicular toxicity of DEHP was independent of PPARα (Ward et al., 1998). Other authors suggested that the testicular toxicity may be mediated by other PPAR form (Hurst and Waxman, 2003). Wilson and collaborators (2004), on the other hand, showed that lesions were associated with decreased *insl3* transcript. Altogether, studies suggest that phthalates' antiandrogenic proprieties are mainly triggered by the decreased T production, which could be due to PPAR-dependent transcriptional changes.

Unsurprisingly, female reproduction was also affected by phthalate exposure. Puberty and vaginal opening were both delayed in treated rats (Tyl et al., 2004; Fujii et al., 2005). Similarly, Frederiksen et al. (2012) showed an association between phthalate in urine and delayed pubarche in girls. Estrous cycle duration was prolonged by DEHP in treated rats (Xu et al., 2010). Likewise, a reproductive study by Hoshino et al. (2005) showed that DCHP prolonged the estrous cycle in F0 female rats by reducing the body weight gain. In addition, ovary weight was decreased in rats treated with DEHP (Xu et al., 2010). Phthalates also affected fecundity at concentrations as low as 3 µg/L; exposure decreased the number of eggs or youngs produced by medaka and daphnids (Mayer and Sanders, 1973; McCarthy and Whitmore, 1985; DeFoe et al., 1990; Patyna and Cooper, 2000). Moreover, female zebrafish exposed to 40 µg/L DEHP for three weeks produced 1% of the embryos laid by control animals. This reduction could be due to a decrease in prostaglandin-endoperoxide synthase 2 (*ptgs2*) expression. The latter codes for the enzyme essential for the ovulation process, cyclooxygenase (COX; Carnevali et al., 2010). In sum, phthalates affected male and female reproduction in various ways and the MOAs for the effects observed in females remain unclear.

Both BPA and phthalates have been shown to skew sex ratios and induce hermaphrodites. In a medaka study for example, 1.8 mg/L BPA treatment yielded only females (Yokota et al., 2000). Approximately 62-70% females were found in African clawed frog tadpoles exposed to 0.23 mg/L BPA (Kloas et al., 1999; Levy et al., 2004). Pickford et al. (2003) reported different results. In their study, exposing African clawed frogs to 49.7 µg/L BPA did not affect sex ratios. Furthermore, 0.01 µg/L DEHP skewed the sex ratio towards females in medaka (Chikae et al., 2004a). On top of skewing ratios, some animals exposed to plasticisers harbor both testicular tubular and ovarian follicle elements. For example, exposing African clawed frog tadpoles to 0.23 mg/L BPA resulted in two intersexed testes (Levy et al., 2004). BPA concentrations as low as 0.01-1.82 mg/L induced testis-ova in treated medaka (Yokota et al., 2000; Metcalfe et al., 2001). This effect could be encountered in the environment since surface waters were reported to contain as much as 21 µg/L BPA (reviewed in Crain et al., 2007). Similarly, DBP induced intersex in males Japanese medaka (Patyna and Cooper, 2000). Ohtani et al. (2000) showed that treating genetical male Japanese wrinkle frogs with 2.78 mg/L DBP during gonadal differentiation led to ovarian formations in 17% of the gonads. Altogether, these studies show that plasticisers behave like estrogens; E2 and ethinyl estradiol are well known for affecting sex ratios in amphibians (Miyata et al. 1999; Levy et al., 2004; Pettersson et al., 2006; Pettersson et al., 2007; Hu et al., 2008; Lutz et al., 2008). The MOA for BPA involves its binding to ER and the upregulation of ER mRNA levels (Levy et al., 2004). However, the MOA for phthalates is still unclear. Phthalates could act by affecting CYP19 activity (Andrade et al., 2006), then disrupting the balance between androgens and estrogens.

1.2.5 Plasticiser-induced endocrine stress and cellular stress lead to malformations

After being uptaken by the cell, cholesterol can be utilised to produce sex steroid, as mentioned in the previous section, or it can be transformed into corticosteroids. The latter are hormones secreted by the adrenal cortex when organisms experience stress. Several aforementioned studies demonstrated that phthalates downregulate the expression of cholesterol transport and steroidogenesis genes such as *dhcr7*, *srb1*, *star*, *pbr*, *cyp11a1*, *cyp17a1* and *3β-hsd* (Shultz et al., 2001; Gazouli et al., 2002; Barlow et al., 2003; Lehmann et al., 2004; Thompson et al., 2004; Borch et al., 2006; Lahousse et al., 2006; Hannas et al., 2011), but little research efforts have been focused on corticosteroid-specific genes. In rat mammary gland, BzBP upregulated the expression of corticotropin releasing hormone 1 (*crhr1*; Moral et al., 2007). Plasticisers have contradicting effects on stress hormones in fish and mammals. Six phthalates failed to displace the native ligand from rainbow trout liver and brain cortisol receptor in vitro (Knudsen and Pottinger, 1999). Moreover, female rats exposed to DEHP for 14 days experienced a decrease in cortisol (Gayathri et al., 2004). Similarly, production of cortisol and corticosterone was inhibited in a concentration-dependent manner in H295R human adrenocortical carcinoma cells exposed to BPA (Zhang et al., 2011), whereas rainbow trout had significantly higher basal plasma cortisol level when exposed to BPA as oocytes (Aluru et al., 2010). Endocrine stress has not been characterised as extensively as plasticiser-induced cellular stress, which is presented in the upcoming section.

The major function of peroxisomes is the breakdown of long fatty chains through β oxidation. This in turn produces peroxide by direct transfer of electrons to oxygen. Cellular iron could then catalyse the Haber-Weiss reaction, which consists in the production of the highly reactive hydroxide anion from peroxide. Such reactive oxygen species (ROS) are highly reactive toward biological macromolecules, especially DNA. Peroxide and hydroxide can damage DNA by degrading it, breaking strands, crosslinking DNA, inducing scissions, leading to chromatid breaks, exchanging chromatids and inducing unscheduled DNA synthesis. The excessive production of hydrogen peroxide or the diminished rate of hydrogen peroxide degradation by catalase (CAT) are mutagenic and may lead to cancers (Feinstein et al., 1978; Warren et al., 1982; Abdellatif et al., 1991). By increasing peroxisome proliferation, phthalates increase peroxisomal β oxidation (Isenberg et al., 2000; Isenberg et al., 2001), increase ROS production (Kasahara et al., 2002; Pereira et al., 2006; Erkekoglu et al., 2012), induce DNA damage in sperm and testes (Duty et al., 2003; Hauser et al., 2007; Lee et al., 2007) and are associated with breast cancer in Mexican women (Lopéz-Carillo et al., 2010). In sum, phthalates disrupt the oxidative stress balance by activating peroxisomes,

which produces ROS. Fatty chain oxidation is not the only source of ROS. Superoxide radicals are sometimes a by-product of ATP production during oxidative phosphorylation. Xanthine oxidase, NADPH oxidases and cytochromes P450 can also produce superoxide. The cell has a defence system against oxidative stress. This involves antioxidants such as CAT, superoxide dismutase (SOD), glutathione peroxidase (GPX) and thiols, among others. On top of disrupting the ROS balance, phthalates alter the cells' ability to cope with ROS by inducing transcriptional changes. For example, the expression of *gpx* and cytochrome P450 3A (*cyp3a*) was upregulated by five phthalates in treated abalones (Zhou et al., 2011a). Moreover, DEHP and BzBP induced a two and four-fold increase in the expression of heat shock protein 70 (*hsp70*), respectively (Planelló et al., 2011). Heat shock proteins (HSPs) are chaperone proteins which assist the correct folding of nascent and prevent protein aggregation in the event of stress (reviewed in Gupta et al., 2010b). BzBP also upregulated the expression of heat shock factor 2 (*hsf2*) in rat mammary gland (Moral et al., 2007). These transcriptional changes correlate with disrupted proteic expression and activity. SOD activity was found to be increased in rats exposed to DBP and decreased in kidneys of rats exposed to DEHP (Lee et al., 2007; Erkekoglu et al., 2012). Similarly, CAT activity was increased by DBP, DHP and DNOP and decreased by DEHP in rats (Mann et al., 1985; Lee et al., 2007). DEP and DEHP both depleted glutathione content in rat tissues (Kasahara et al., 2002; Pereira et al., 2006; Erkekoglu et al., 2012). GPX activity was either increased by DBP or decreased by DEHP in rats (Lee et al., 2007; Erkekoglu et al., 2012). Thiol and ascorbic acid content was reduced in kidney and testes of rats exposed to DEHP (Kasahara et al., 2002; Erkekoglu et al., 2012). Ultimately, the cells' battle against ROS was lost in rats treated with MEHP; the monoester induced the release of cytochrome *c* and induced apoptosis in the testes (Kasahara et al., 2002). Although BPA-induced cellular stress was not well characterised, phthalates' effects on the mechanisms of defence against ROS have been investigated.

Cellular stress induced by contaminants is hypothesised to induce malformations in aquatic species (Ankley et al., 2004; Mussi and Calcaterra, 2010). BPA exposure therefore resulted in malformations in various animals (reviewed in Staples et al., 1998). Treatment of the giant ramshorn snail with BPA induced malformations in the female genital system. Animals displayed additional female sex organs, malformations of the oviducts and enlargement of pallial accessory sex glands (Oehlmann et al., 2000). Tail deformities and cardiac edema were encountered in zebrafish exposed for 24 hours (Duan et al., 2008). A similar experiment showed that zebrafish and African clawed frog embryos exposed to 5.7 and 1.1 mg/L BPA, respectively, displayed abnormalities of the otoliths, structures required for normal balance and gravity sensing. Since neither E2, ER antagonists, nor THs modified the BPA response, the authors concluded that the malformation was independent of ERs or TRs (Gibert et al., 2011). Other authors reported

malformations in amphibians. Sone and collaborators (2004), for example, noticed microcephaly, flexure, edema, or abnormal gut coiling in 44% of African clawed frog embryos treated with 4.6 mg/L of BPA. Similar studies were conducted by Oka et al. (2003) and Iwamuro et al. (2003). The authors observed developmental abnormalities such as crooked vertebrae and defects of the head and abdomen in treated African clawed frog. Since these results were not inhibited by the addition of E2, the authors also concluded that the malformations were due to non-estrogenic activities on developmental processes (Oka et al., 2003). At a slightly higher concentration, 11 mg/L, African clawed frog tadpoles exhibited eye malformations and scoliosis. The eye malformations were believed to be due to a decrease in paired box protein (*pax-6*) transcript, a key regulator of eye morphogenesis (Imaoka et al., 2007). In sum, studies demonstrated the teratogenecity of BPA and concluded that the plasticiser did not act via ER or TH. Perhaps further research on cellular stress could explain BPA-induced malformations.

As seen with BPA, phthalate exposures resulted in a variety of terata in aquatic species and mammals. Many studies reported reproductive abnormalities, mainly in male rodents, resulting from phthalate exposure (Mylchreest et al., 1998; Mylchreest et al., 1999; Gray et al., 2000; Mylchreest et al., 2000; reviewed in Gray et al., 2001; Tyl et al., 2004). Phthalates decrease the expression of proteins that are necessary for steroidogenesis, leading to a decreased T synthesis. This decrease in T is the origin of the reported reproductive malformations (Parks et al., 2000). In addition, other body parts such as sternebrae, ribs and eyes have been found to be malformed by phthalates (Saillenfait et al., 2006). Two studies reported phthalate-induced abnormalities in African clawed frog in the low mg/L range. A chronic study showed that 0.1 mg/L of DBP was sufficient to induce sexual malformations including seminiferous tubular dysgenesis in males (Lee and Veermachaneni, 2005). Acutely exposing African clawed frog embryos to 15 mg/L DBP resulted in a 100% malformation rate. Animals harbored abnormally coiled guts and cardiac abnormalities. The median effective concentration (EC_{50}) was found to be 0.98 mg/L (Lee et al., 2005). In abalone gametes, DMP exposures led to yolk sac edema, irregular cleavage and asymmetrical splits. Concentrations as low as 0.1 mg/L induced an approximate 25% malformation rate (Zhou et al., 2011b). In another study by the same group, phthalate concentrations varying from 0.2 to 10 mg/L induced 30 to 45% abnormality rates in treated abalones. The malformations included yolk leakage, yolk sac edema and reduction of body pigmentation. The authors suggested four possible MOAs: phthalates could affect osmoregulation, induce oxidative stress, damage the extraembryonic membrane structure or disrupt endocrine-related gene expression (Zhou et al., 2011a). The phthalates' molecular structure may affect the teratogenesis of the compounds. Phthalate monoesters induced the same types of malformations as the corresponding diester and seemed to be more or equally potent in rodents (Ema et

al., 1994; Ema et al., 1996; Ema et al., 1999; Saillenfait et al., 2001; reviewed in Ema, 2002). Phthalic acid, on the other hand, was shown to be non-teratogenic in African clawed frog tadpoles; an EC_{50} of 6,620 mg/L was found by Bantle and collaborators (1999). A similar result was found in rats; phthalic acid was not teratogenic in rat pups (Ema et al., 1997). Cyclic phthalates were also shown to be less potent than their straight chain analogs in rats (Saillenfait et al., 2009). Altogether, several studies showed that phthalates and their metabolites can induce a series of malformations in molluscs, amphibians and rodents. Reproductive teratogenicity is believed to be induced via the PPAR-mediated decrease in T, whereas other malformations are likely due to cellular stress.

1.2.6 Molecular structure of phthalates affects plasticiser lethality

Sublethal effects such as severe malformations, excessive cellular stress and apoptosis could lead to mortality. Unsurprisingly, BPA exposure resulted in mortality in certain aquatic species. A median lethal concentration (LC_{50}) of 16.8 mg/L BPA was reported for zebrafish embryos. A 25 mg/L concentration induced 100% mortality in the embryos (Duan et al., 2008). Rainbow trout oocytes seemed less sensitive to BPA. When oocytes were exposed to 100 mg/L for three hours and allowed to develop in clean water for 400 days, the exposure induced 30% mortality (Aluru et al., 2010). In swordtail fish, the 72 hour LC_{50} was found to be 17.9 mg/L BPA (Kwak et al., 2001). In addition to fish, BPA was shown to induce mortality in aquatic insects. Two studies showed that the 48 hour LC_{50} for daphnids was approximately 10 to 12.8 mg/L (Ike et al., 2002; Hirano et al., 2004). The sensitivity of the mysid shrimp and the copepod, on the other hand, were about 10-fold higher than that of daphnids; the 48 and 72 hour LC_{50}s were 1.34 mg/L and 0.96 mg/L, respectively (Andersen et al., 1999; Hirano et al., 2004). Mihaich et al. (2009) showed that the 96 hour LC_{50} in the midge and the giant ramshorn snail were 2.7 and 2.24 mg/L, respectively. The same group also exposed amphipods to BPA for 42 days and found an LC_{50} of 0.78 mg/L. BPA also induced mortality in amphibians. In African clawed frog embryos, the 48 hour LC_{50} was 4.79 (Iwamuro et al., 2003). A 96 hour exposure to 4.6 mg/L induced 42% mortality, while 6.8 mg/L resulted in 100% mortality (Sone et al., 2004). Oka et al., (2003) obtained similar results. The authors observed that African clawed frog embryos died rapidly during the gastrula stage when exposed to 9.1 mg/L of BPA. A chronic experiment exposing few days old African clawed frog tadpoles until metamorphosis showed that the plasticiser had no observable effect on mortality (Pickford et al., 2003). Some of these drastic deleterious effects could be encountered in the environment considering that BPA concentrations as high as 17.2 mg/L were recorded in Japanese landfill leachates (Yamamoto et al., 2001).

BPA metabolites showed very low acute toxicity in aquatic insects compared with the parent molecule, providing evidence that biodegradation can remarkably reduce BPA's toxic effects (Ike et al., 2002). In summary, BPA is acutely toxic at concentrations of 1.34-17.9 mg/L and chronically lethal in the 0.50-0.78 mg/L range in aquatic species.

Phthalate-induced mortality has been reported in a multitude of species. In addition, ring dove eggshell thickness was decreased by DBP (Peakall, 1974), suggesting a putative decrease in offspring survivability. In rodents, mortality was induced by DBP (Mylchreest et al., 1998), DIDP (Hushka et al., 2001; Cho et al., 2008), di-2-ethylhexyl terephthalate (Faber et al., 2007a), DIBP (Saillenfait et al., 2006), BzBP and its metabolite MBzP (Ema et al., 1996; Ema et al., 1999). Post-implantation loss occured when pregnant rats were exposed to the low dose of 0.75 g/kg/d DBP (Ema et al., 1994). The MOA of phthalate-induced mortality in rodents might involve PPARs. An experiment with PPARα knockout mice showed that perfluorooctanoic acid-induced postnatal mortality required PPARα expression, while early prenatal lethality was independent of PPARα expression (Abbott et al., 2007). A similar MOA is conceivable with phthalates in rodents. Additionally, phthalates were shown to induce mortality in aquatic invertebrates, fish and amphibians. In fish, the 96 hour LC_{50} values for DMP varied from 29 to 121 mg/L (Adams et al., 1995). The heavier DEP was slightly more toxic than DMP and induced 50% mortality at concentrations of 12 to 50 mg/L in fish (Adams et al., 1995; Ghorpade et al., 2002; Barse et al., 2007). DBP was even more lethal, with LC_{50}s varying from 0.35 to 6.5 mg/L (Mayer and Sanders, 1973; McCarthy an Whitmore, 1985; DeFoe et al., 1990; Adams et al., 1995; reviewed in Burridge and Haya, 1995). *Ortho, iso* and *tere*-isomers seemed to be equally toxic in fathead minnow, with 96 hour LC_{50}s of 0.85, 0.9 and 0.61 mg/L, respectively (DeFoe et al., 1990). The 96 hour LC_{50}s for BzBP were 0.82 and 1.5 mg/L in rainbow trout and fathead minnow (Adams et al., 1995). In rainbow trout, the DEHP metabolites 2-ethylhexanoic acid and 2-ethylhexanol had a 96 hour LC_{50} of 150 and 27 mg/L, respectively (Horn et al., 2004). Studies conducted on aquatic invertebrates yielded lethal concentrations that varied over wider ranges. DMP was less toxic in aquatic invertebrates than in fish; the LC_{50} varied from 28.1 to 377 mg/L, with amphipods being the most sensitive species (Adams et al., 1995; Call et al., 2001; Jonsson and Baun, 2003). The metabolite MMP was less toxic to daphnids than the corresponding diester; the 48 hour LC_{50}s were 473 and 284 mg/L, respectively (Jonsson and Baun, 2003). Similarly to fish exposures, DEP was more toxic to aquatic invertebrates than DMP. Concentrations of 4.21 to 131 mg/L induced 50% mortality in the various species (Adams et al., 1995; Call et al., 2001; Jonsson and Baun, 2003). Once again, amphipods were the most sensitive organisms (Call et al., 2001). Monoethyl phthalate was less lethal than DEP in daphnids (Jonsson and Baun, 2003). Likewise, DBP was beyond an order of magnitude more potent than MBP in daphnids (Jonsson and Baun, 2003). Exposures with aquatic invertebrates

yielded LC_{50}s varying from 0.5 to 16.5 mg/L for DBP with the mysid shrimp being the most sensitive species (Mayer and Sanders, 1973; McCarthy an Whitmore, 1985; DeFoe et al., 1990; Adams et al., 1995; reviewed in Burridge and Haya, 1995; Call et al., 2001; Jonsson and Baun, 2003). Defoe et al. (1990) studied the chronic effect of dibutyl *ortho*, *iso* and *tere*-phthalates on daphnids. The *ortho* isomer seemed to be less toxic than the other two isomers; the LC_{50}s were 1.92, 0.2 and 0.46 mg/L respectively (Defoe et al., 1990). No LC_{50} was found for DEHP in aquatic invertebrates, but the 48 hour LC_{50} for its metabolites MEHP, phthalic acid, 2-ethylhexanol and 2-ethylhexanoic acid in daphnids were 3.47, 103, 100 and 120 mg/L, respectively (Jonsson and Baun, 2003; Horn et al., 2004). Bulkier phthalates such as monodecyl phthalate and BzBP induced 50% mortality in aquatic invertebrates at 0.46-11.1 mg/L (Call et al., 2001; Jonsson and Baun, 2003) and in shiner perch at 0.51 mg/L (Ozretich et al., 1983). Data for amphibians is scarcer. A DBP concentration of 27.83 mg/L was lethal in Japanese wrinkled frog tadpoles; all specimens died in less than five minutes (Ohtani et al., 2000). A DBP concentration of 14.5 mg/L induced 50% mortality in a 96 hour African clawed frog exposure (Lee et al., 2005). DBP concentrations as low as 10 and 14.5 mg/L induced 16% and 50% mortality in African clawed frog in five week experiments (Lee and Veermachaneni, 2005). In addition, a study showed that DEHP bound to sediment inhibited moorfrog eggs hatching (Larsson and Thurén, 1987). The metabolite phthalic acid was found not to be harmful in African clawed frog tadpoles; the 96 hour LC_{50} was 9,280 mg/L (Bantle et al., 1999). In sum, exposure to phthalate diesters or their metabolites is lethal for aquatic invertebrates, fish and amphibians.

The length of the alkyl chains has a colossal effect on the toxicity of phthalates (Fig. 1.4). Several studies showed that aquatic toxic tends to increase with increasing chain length (Adams et al., 1995; Rhodes et al., 1995; Call et al., 2001; Jonsson and Baun, 2003). Indeed, toxicological studies conducted on the bluegill sunfish yielded 96 hour LC_{50}s of 50 mg/L, 16.7 mg/L and 0.48-1.2 mg/L for DMP, DEP and DBP, respectively (Mayer and Sanders, 1973; Adams et al., 1995; reviewed in Burridge and Haya, 1995). Phthalates with longer alkyl chains have higher LC_{50}s (Planelló et al., 2011). One of the possible explanations for the difference in toxicity between short and long alkyl chained phthalates is the difference in degradation rate. DBP degrades 16 times faster than DEHP in catfish liver (Mayer and Sanders, 1973). Therefore, DBP can be metabolised to less toxic metabolites such as phthalic acid and MBP. Indeed, high phthalic acid concentrations were necessary to induce lethality in African clawed frog tadpoles (Bantle et al., 1999) and in daphnids (Jonsson and Baun, 2003). In addition, Jonsson and Baun (2003) found that MMP, MEP, MBP and phthalic acid were less potent than their parent compounds in daphnids. Lipophilicity is a second possible explanation for the difference in toxicity between light and heavy compounds; the octanol-water partition coefficient (K_{ow}) increases as the number of carbon atoms

increases. Lipophilicity could influence the ease with which a compound penetrates tissues and cells (Ito et al., 2012). The greater the bioavailability, the greater potential for adverse health effects. Heavy phthalates harboring six or more carbons per alkyl chain, on the other hand, are acutely nontoxic when tested within their water solubility range (McCarty and Whitmore, 1985; Adams et al., 1995; reviewed in Staples et al., 1997a; Jonsson and Baun, 2003; Staples et al., 2011). Several toxicological studies using these bulky phthalates reported lethality at concentrations well beyond their maximal water solubility. McCarthy and Whitmore (1985) suggested that phthalates interact with dissolved organic matter in the water when dissolved beyond their solubility point. The interactions make the contaminants unavailable for uptake and thus the animals are exposed to concentrations much lower than the total water concentration, leading to an overestimation of LC_{50}s. In summary, lighter phthalates are acutely toxic to aquatic species, while heavier ones are more difficult to solubilise and are therefore acutely nontoxic.

To summarise, three main chains of events are happening in phthalate treated animals (Fig. 1.2 and 1.3). Firstly, plasticisers disrupt the TH and GH axes and therefore affect development, but the MOAs are not completely characterised yet. Secondly, a decrease in cholesterol transport to the mitochondrion is experienced and could be mediated by PPARs. The reduction in cholesterol intake leads to reduced T production, thereby inducing reproductive organ malformations, reproductive defects and decreased fertility. In the third chain of event, the activation of PPARs, the increased fatty acid oxidation and the reduced ability to cope with the augmented ROS production lead to apoptosis, malformation and mortality in treated animals.

Several of the sublethal and lethal effects reported here are bound to happen in the environment, considering that environmental concentrations are occasionally higher than the concentrations tested in reported studies. It is therefore important to continue to identify the effects of various plasticisers on more species. Such novel data is presented in chapters 2 and 3; the acute and chronic effects of phthalates on African clawed frog mortality and sublethal endpoints are described. The results are then discussed in chapter 4.

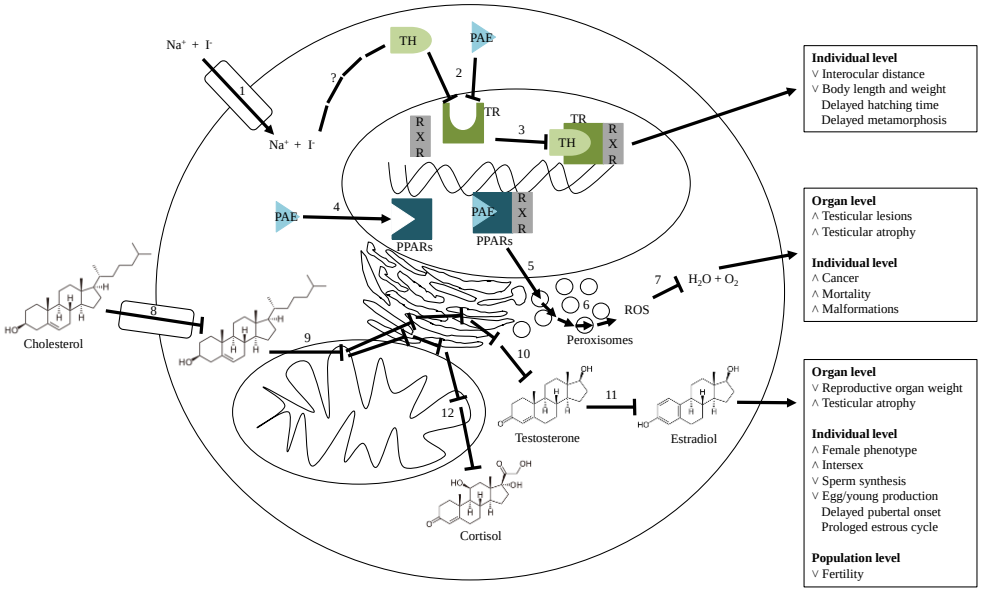

FIGURE 1.2 Examples of the known effects and mechanisms of action of phthalic acid ester (PAE) for each organisational level. (1) PAE increase the activity of the sodium/iodide symporter, leading to increased iodide uptake. Although iodide is necessary for thyroid hormone (TH) production, the effects of PAE on TH synthesis are unclear. (2) PAE impede with the binding of TH to thyroid hormone receptors (TR) by binding to TR themselves. (3) The gene expression of retinoid X receptor (RXR) is reduced by PAE, thus reducing the capacity to form heterodimers with TR. By doing so, the regulation of TH-related genes is disrupted, leading to effects at the individual level. (4) Peroxisome proliferator-activated receptors (PPARs) gene and protein expressions are increased. PAE can also activate PPARs. (5) After forming heterodimers and binding to response elements, peroxisome proliferation is increased. (6) Fatty acid oxidation then lead to the formation of reactive oxygen species (ROS). (7) Transcription, translation and activity of scavenger is decreased, leading to detrimental effects at the organ and individual levels. (8) PAE reduce cholesterol transport by reducing scavenger receptor class B-1, (9) steroidogenic acute regulatory protein and peripheral benzodiazepine receptor mRNA levels. (10) The gene expression of a series of enzymes necessary for steroidogenesis is repressed, leading to lower testosterone levels. (11) The gene expression, protein expression and activity of aromatase are also decreased, resulting in lessened estradiol synthesis. These effects then induce changes at the organ, individual and population levels. (12) Finally, cortisol levels are reduced in treated animals.

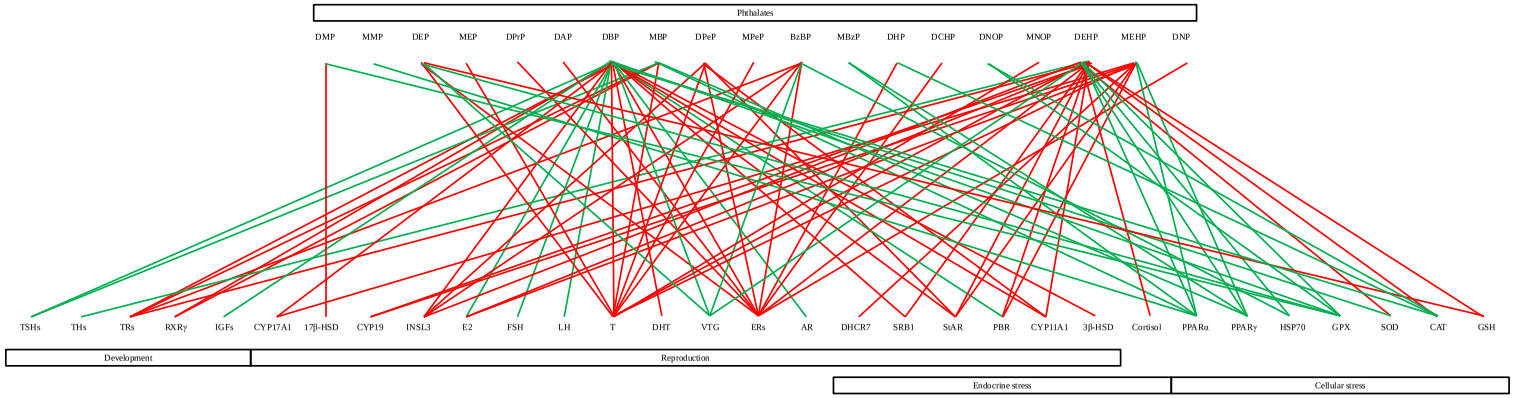

FIGURE 1.3 Phthalate mono and diesters affect the thyroid hormone, growth hormone, sex steroid and stress axes. Decreases in transcription, translation and activity are represented in red. Inhibition of endogenous hormone binding to receptors is also represented in red, whereas opposite effects are represented in green. Note the scarce data on DMP, MMP and DCHP. Acronyms can be found in section v, except the followings: DAP: diallyl phthalate, DHP: dihexyl phthalate, DNP: dinonyl phthalate, DPrP: dipropyl phthalate, GSH: glutathione, MEP: monoethyl phthalate, MNOP: mono-*n*-octyl phthalate, MPeP: monopentyl phthalate.

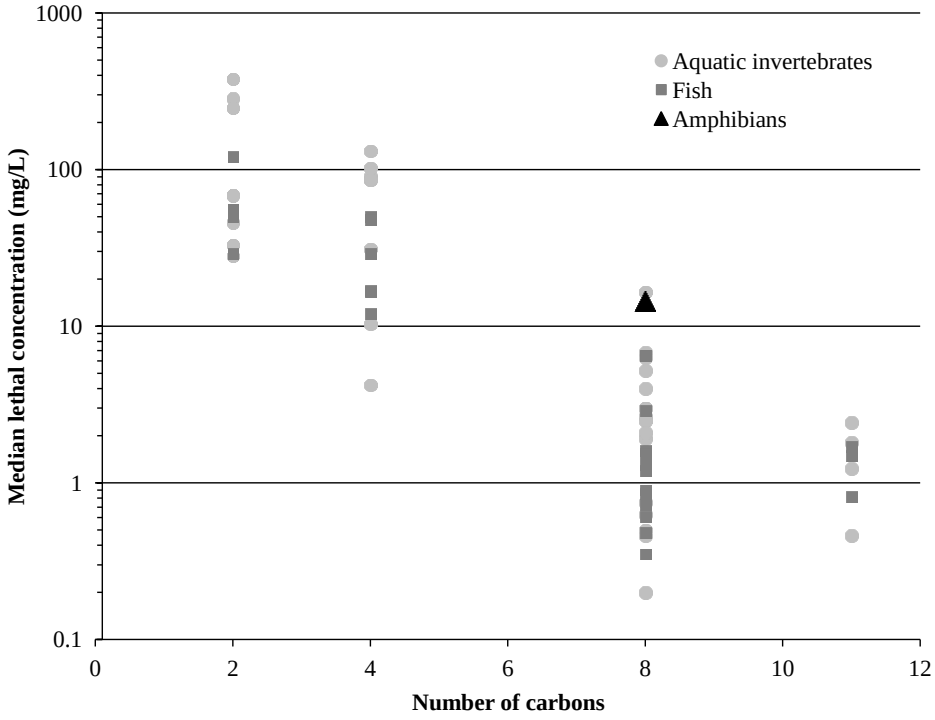

FIGURE 1.4 Median lethal concentrations (LC_{50}s) of phthalate diesters across aquatic trophic levels. In this figure, the number of carbons refers to the total amount of carbon atoms in the two alkyl chains of a given phthalate diester. This figure demonstrates that the hypothesis formulated by Adams et al. (1995), Rhodes et al. (1995), Call et al. (2001) and Jonsson and Baun (2003) may be correct; toxicity of phthalate diesters increases with lipophilicity. In general, phthalates seem to be less toxic to aquatic invertebrates than to fish. Note the sparse amphibian data. Adapted from Mayer and Sanders (1973), McCarthy and Whitmore (1985), DeFoe et al. (1990), Adams et al. (1995), Burridge and Haya (1995), Staples et al., (1997a), Andersen et al. (1999), Call et al. (2001), Kwak et al. (2001), Ghorpade et al. (2002), Ike et al. (2002), Iwamuro et al. (2003), Jonsson and Baun (2003), Hirano et al. (2004), Lee et al. (2005), Barse et al. (2007), Duan et al. (2008) and Mihaich et al. (2009).

Chapter 2

Lethal and sublethal effects of acute exposures to phthalates in the Western clawed frog larvae

2.1 Introduction

Plasticisers such as phthalates are added to polymers in order to increase their flexibility. The latter is achieved by lowering the glass transition temperature (Daniels, 2009). Phthalates have two esters bound to a central ring. These compounds are not covalently bound to the polymer matrix and can therefore leach out of consumer products such as food packaging (Fasano et al., 2012), PVC flooring (Carlstedt et al., 2012) and dietary supplements (Hernández-Díaz et al., 2009). As a result, phthalates are found in many environmental strata including air (up to 1.2 $\mu g/m^3$; reviewed in Weschler and Nazaroff, 2008), surface water (up to 180 µg/L; Horn et al., 2004) and sediment (up to 487.3 µg/kg; Vitali et al., 1997). Several studies have demonstrated that phthalates have adverse effects in aquatic species and mammals. Mortality (Adams et al., 1995; Mylchreest et al., 1998; Jonsson and Baun, 2003) and malformations (Gray et al., 2001; Lee et al., 2005; Zhou et al., 2011b) were observed in treated animals. One of the proposed MOAs is through the induction of cellular stress (Lee et al., 2007; Planelló et al., 2011; Zhou et al., 2011a). In addition, plasticisers impair reproduction in treated organisms (Tyl et al, 2004; Aso et al., 2005; Makris et al., 2013). Phthalates have been shown to have endocrine disrupting properties. Treated rats exhibited several conditions, such as altered sex steroid-related gene expression (Barlow et al., 2003; Borch et al., 2006; Noda et al., 2007; Carnevali et al., 2010; Hannas et al., 2011), lowered E2 production (Xu et al., 2010), reduced T synthesis (Borch et al., 2006) and increased T metabolism (Fujii et al., 2005). Phthalates also exhibit TH-antagonist activity in vertebrates. Among others, urinary diester and monoester concentrations are negatively correlated with THs levels in humans (Meeker et al., 2007). Additionally, phthalates decelerate TH-dependent metamorphosis in amphibians (Shen et al., 2011). The proposed MOA for these endocrine effects include the disruption of TH-related genes (Sugiyama et al., 2005; Lee et al., 2007; Shen et al., 2011).

In light of the previous literature review (Mathieu-Denoncourt et al., in preparation a), there is a critical lack of information on the effects of plasticisers in non-mammalian species. Given that plasticisers are constantly released into the environment and that these chemicals are mainly found in water bound to particles, it is imperative to pay closer attention to the outcomes of plasticiser exposure in aquatic species. Considering that percutaneous passage is much greater in amphibians than in mammals (Quaranta et al., 2009) and that amphibian populations are in decline (Stuart et al., 2004), the effects of plasticisers on amphibians must be further investigated. A great deal of research effort has been focused on DEHP, DBP and BzBP, but it is important to characterise the effects of understudied compounds such as those that are part of Canada's Chemical Management Plan (CMP) priority assessment list (Canada Gazette, 2013). The goal

of the CMP is to collect information on chemicals whose impacts on the environment are unknown. The phthalates that are part of the CMP may be less studied, but have been shown to be in the environment at concentrations comparable to those of DEHP, DBP and BzBP (Hendriks et al., 1994; Suzuki et al., 2001; Clara et al., 2010; Zhang and Shen, 2010). Therefore, the current chapter aimed at elucidating the effects and the MOAs of phthalates that are part of the CMP in amphibians. The effects of acute exposures to MMP, DMP and DCHP on Western clawed frog embryo mortality, malformations and development were investigated. In addition, the expression of a suite of genes involved in reproduction, TH axis, cellular stress and transcriptional regulation was assessed.

Medium to high molecular weight phthalates such as DCHP are used in the manufacturing of PVC, while DMP is utilised mostly in cellulose ester-based plastics (Staples et al., 1997b). DMP has an estimated half-life of 360 hours in water (Mackay, 2001) and is metabolised to the monoester MMP (Albro and Moore, 1974). Monoesters are thought to be the active metabolites responsible for the toxicological effects observed during diester exposures (Gray et al., 2000; Saillenfait et al., 2001; Ema, 2002; Shen et al., 2011). Based on the toxicity of similar compounds, it was hypothesised that MMP, DMP or DCHP concentrations above 1.0 mg/L would induce malformations and concentrations above 15 mg/L would result in increased mortality (Lee et al., 2005). Since MMP and DMP are less lipophilic than DCHP, higher mortality rates should be encountered in the DCHP acute exposure (Fig. 1.4). Development was hypothesised to be hindered at concentrations as low as 0.1 mg/L (Lee et al., 2005). Finally, according to the literature, phthalates were hypothesised to decrease the expression of TH-related genes (Sugiyama et al., 2005; Shen et al., 2011) and sex steroid-related genes (Lovekamp and Davis, 2001; Lehmann et al., 2004; Bowman et al., 2005), while cellular stress response genes were expected to be upregulated by phthalates (Planelló et al., 2011).

2.2 Materials and methods

2.2.1 Animal care and breeding

Animal care was performed according to the guidelines of the Animal Care Committee of Queen's University (Kingston, ON, Canada) and the Canadian Council of Animal Care. Adult husbandry was performed as described in Mathieu-Denoncourt et al. (2014). Adult Western clawed frogs (*Silurana tropicalis*) were reared in dechlorinated and aerated water from the Queen's University Animal Care Facility. Lighting was maintained at a 12 h light and 12 h dark cycle starting at 0700 AM. Breeding was conducted as per Soriano et al. (2014). Briefly, one day prior mating, 11 pairs of adults were injected with 12.5 units of human chorionic gonadotropin (standard grade, 3150.0 IU/mg potency, CAS 9002-61-3, Calbiochem, San Diego, California, USA) into the dorsal lymph sac to initiate amplexus and ovulation. The animals were kept in different aquariums and pH of the aquariums was adjusted to 5.8-6.0 with HCl. Twenty-four h after the first injection, the pH of the aquariums was re-adjusted and animals were injected with 200 units of chorionic gonadotropin. Pairs were then introduced in the breeding chambers and were kept in the dark. Eggs were collected and pooled. Embryos were dejellied to facilitate handling. This was accomplished by gentle swirling for 2 min in a 2% w/v L-cysteine (> 99%, CAS 52-90-4, Acros Organics, Fair Lawn, New Jersey, USA) solution adjusted to pH 8.1 with NaOH. The rearing media was prepared according to the American Society for Testing and Materials publication E1438-98 Standard Guide for Conducting the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX; American Society for Testing and Materials, 1998). The FETAX media was made of 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2H_2O$ and 75 mg $MgSO_4$ per L of dechlorinated water (American Society for Testing and Materials, 1998). Gentamycin sulphate (CAS 1405-41-0, Fisher Scientific, Fair Lawn, New Jersey, USA) was added to every jar at a concentration of 0.04 mg/L for the whole duration of the exposure. This antibiotic was used for its broad antimicrobial spectrum activity (Changez et al., 2004).

2.2.2 Experimental design

Three acute toxicity tests for rapid screening of phthalates were performed as described in FETAX (American Society for Testing and Materials, 1998). Briefly, FETAX is a whole embryo assay used for the evaluation of a chemical's developmental toxicity using African clawed frog tadpoles. Western clawed frog larvae have been shown to be a suitable species in FETAX (Fort et al., 2004; Mathieu-Denoncourt et al., 2014). According to a type III fugacity model, the three phthalates of interest are mainly found in the water, bound to particles (Mackay, 2001); therefore water exposures were conducted. Western clawed frog larvae were reared in FETAX solution spiked with MMP (98%, CAS 4376-18-5, Acros Organics, Fair Lawn, New Jersey, USA), DMP (99%, CAS 131-11-3, Acros Organics, Fair Lawn, New Jersey, USA) and DCHP (99%, CAS 84-61-7, Acros Organics, Fair Lawn, New Jersey, USA). Concentrations varying from the µg/L range to the highest experimental solubility point were chosen in order to determine lethal and sublethal effects (Fig. 2.1). MMP and DMP were dissolved in water to obtain nominal concentrations of 0.24, 2.4, 24, 240, 2400 mg/L and 0.115, 1.15, 11.5, 115, 1150 mg/L, respectively. DCHP was solubilised in 0.82% dimethyl sulfoxide (DMSO; 99.9%, CAS 67-68-5, Fisher, Fair Lawn, New Jersey, USA) to obtain 0.6, 6, 60 and 600 mg/L. A BPA (≥ 99%, CAS 80-05-7, Sigma-Aldrich, St. Louis, Missouri, USA) concentration of 3.4 mg/L dissolved in 0.01% v/v DMSO was used as positive control of gene expression disruption (Sone et al. (2004). DMSO was used in previous African clawed frog acute studies at concentrations up to 1% v/v and did not induce toxic effects at the organismal level (Osano et al., 2002; Lee et al., 2005; Burýšková et al., 2006; Gillardin et al., 2009). Furthermore, DMSO can be employed at a final concentration of up to 1.1% according to the American Society for Testing and Materials (1998). Two water-only controls were conducted; one for BPA and MMP and one for DMP and DCHP. Each treatment was tested in 6-10 replicates of 30 mL of FETAX solution containing 18-50 Western clawed frog gastrulae (Nieuwkoop and Faber 1992; NF11-12) each. Dead embryos were removed and recorded daily. Rearing media was renewed every 24 h to ensure that the oxygen levels remained high. Temperature was kept between 23.8 and 27.0 °C. The pH was kept between 6.4 and 8.4 and was adjusted with NaOH if needed. The exposures lasted 72 h, until the developmental stage NF46 was reached.

2.2.3 Water and tissue sample collection

For each treatment, rearing media samples (n = 2) were collected before the introduction of embryos (time 0) and 24 h later. Media samples were preserved in dark vials at -20 ºC until gas chromatography-mass spectrometry (GC-MS) analysis was performed. At exposure completion, a subset of surviving tadpoles was preserved in formalin (10%, CAS 50-00-0, Fisher Scientific, Fair Lawn, New Jersey, USA). Fixed tadpoles were kept in dark vials at room temperature until malformation and developmental delay analysis. Number of animals collected varied among treatments due to animal availability at the end of the exposure. Pools of ten animals (n = 8-9 pools) were preserved at -80 ºC for further gene expression analysis.

2.2.4 Media analysis

The experimental concentrations were determined by GC-MS in Dr. Yargeau's Laboratory (McGill University, QC, Canada). Briefly, deuterated isotopes were used as surrogates for the native compounds to aid in the quantification. GC-MS analyses were performed using a Trace GC Ultra gas chromatograph equipped with a Triplus As injector and coupled to a Polaris Q (Thermo Fisher Scientific, Waltham, MA, USA). The accuracy of the method was determined with internal standards (BPA: 99.9%, CAS 80-05-7, Supelco, Sigma-Aldrich, St. Louis, Missouri, USA; DCHP: 99.9% CAS 84-61-7, Supelco, Sigma-Aldrich, St. Louis, Missouri, USA; DMP: 99.9%, CAS 131-11-3, Supelco, Sigma-Aldrich, St. Louis, Missouri, USA; MMP: 99.5%, CAS 4376-18-5, Supelco, Sigma-Aldrich, St. Louis, Missouri, USA). Detection limits were 0.07, 0.24, 0.003 and 0.03 mg/L for BPA, MMP, DMP and DCHP, respectively. Results are reported based on experimental concentrations.

2.2.5 Morphological analysis

Tadpoles were staged using the system suggested by Nieuwkoop and Faber (1992). Malformation and development analyses were carried out on the fixed tadpoles under a dissecting microscope (Stereomaster, Fisher Scientific, Fair Lawn, New Jersey, USA) and were performed blinded to avoid observer bias. Types of malformations and signs of developmental delay were recorded based on the Atlas of Abnormalities (Bantle et al., 1998). Eyes, tails, hearts, guts, gills, head and face were verified for malformations. Eye malformations included reduction in size, asymmetric formation, incomplete separation from the brain and cyclops. Tail shortening and flexure were also recorded as malformations. Moreover, failure of the heart to coil properly, displacement of the heart along with edema and blistering were counted. Signs of developmental delay included failure of the choroid fissure to close, missing eye cup, presence of cement glands and incomplete gut coiling. Body length was used as an indicator of growth inhibition and was measured with the microscope Nikon SMZ18 (Nikon, Chiyoda-Ku, Tokyo) along with the software NIS Elements (Nikon, Chiyoda-Ku, Tokyo). Samples were coded to avoid bias during tadpole length measurement.

2.2.6 Gene expression

Whole larvae were used in order to allow for the understanding of overall gene expression in early development. Each treatment was tested in 4 to 9 replicates of pools of 10 whole embryos. Tissues were sonicated with the Ultrasonic Dismembrator-150T (Thermofisher, Ottawa, ON, CA). RNA was extracted and purified using the e.Z.N.A. Total RNA Kit I (Omega Bio-Tek, Norcross, GA, USA). Nucleic acid quality and concentration were measured with a NanoDrop-2000 spectrophotometer (Thermofisher, Ottawa, ON, Canada). Genomic DNA contamination was removed with the RQ1 RNase-free DNase kit (Promega, Madison, WI, USA). Complementary DNA was prepared from 2 µg of total RNA with the GoScript Reverse Transcription System (Promega, Madison, WI, USA) and the PCR Vapo.protect Mastercycler pro S (Eppendorf, Hamburg, Germany). Finally, transcriptional changes were investigated using real-time quantitative polymerase chain reaction (qPCR). The GoTaq qPCR Master Mix (Promega, Madison, WI, USA) was used, which includes the BRYT Green and carboxy-X-rhodamine dyes. The specific forward and reverse primers for the genes of interest are found in Table 2.1. The Agilent Mx3005P (Agilent Technologies, Inc, Santa Clara, CA, USA) thermocycle program included a hot-start activation at 95 °C for 2 min, then 40 cycles of denaturation at 95 °C for 15 s and annealing at 58-62 °C and for 1 min. Dissociation occurred at 95 °C for 1 min, then 58-62 °C

for 30 s and finally 95 °C for 30 s. Exact annealing temperatures are found in Table 2.1. For each qPCR assay, a negative template control and a negative reverse transcriptase control were included to ensure the absence of contamination. Standard curves were prepared by serial dilutions (1:4) starting at 100 ng. Samples, controls and standard curves were run in duplicate. Efficiency and R^2 were kept between 84.8-138.5% and 0.900-1.000, respectively. The threshold for each gene was automatically calculated by the MxPro Software and was corrected manually. Since certain housekeeping genes changed among treatments, each acute assay was normalised to housekeeping genes that were not altered by that particular phthalate. Transcripts for DMP treatments were normalised to the quantified relative expression of elongation factor 1 alpha (*ef1α*) and ribosomal protein L8 (*rpl8*), while MMP and DCHP results were normalised to *ef1α* and beta actin (*βactin*), respectively.

2.2.7 Statistical analysis

In order to determine if phthalate concentrations at time 0 were statistically different from time 24 h, two-tailed case one (paired) t-tests were performed for each treatment. One-tailed case three (unequal variance) t-tests were performed to compare each treatment to the water control. The *p*-values were corrected with the Bonferroni adjustment. Mortality, malformations and signs of developmental delay were analysed using Fisher's exact tests. Lilliefors tests determined that tadpole length data and the expression of some genes were not normally distributed. Tadpole length was analysed using Kruskal-Wallis tests followed by two-tailed Dunn's procedures with the Bonferonni correction. Gene expression was analysed using either one-way ANOVAs and two-sided Dunnet's tests or Kruskal-Wallis tests and two-tailed Dunn's procedures with the Bonferonni correction. Probit analysis was used to determine the LC_{50}s and EC_{50}s. The teratogenic index (TI) was calculated as described in Bantle et al. (1999). In all scenarios, *p*-values of less than 0.05 were considered statistically significant. A threshold of ± 500 µm was considered biologically significant for tadpole length and fold changes higher than 0.8 were deemed biologically significant for gene expression. Statistical analyses were completed using GraphPad (GraphPad Software Inc, San Diego, CA, USA) and R (R Core Team, 2013). The Global Validation of Linear Models Assumptions package was used in R (gvlma; Pena and Slate, 2014).

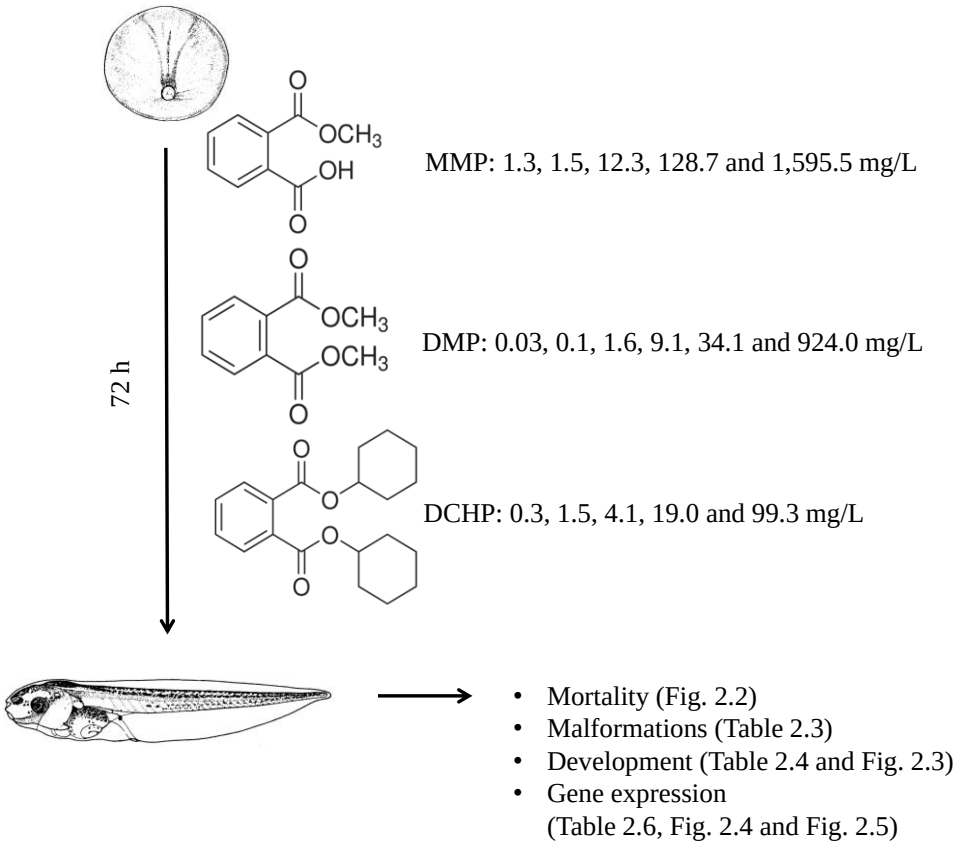

FIGURE 2.1 Schematic representation of the experimental design of acute exposures to monomethyl (MMP), dimethyl (DMP) and dicyclohexyl (DCHP) phthalates in the Western clawed frog. Animals were treated from Nieuwkoop and Faber (NF) 12 to NF46 (72 h). The experimental concentrations are presented above and are based on results reported in table 2.2.

TABLE 2.1 Genes of interest for the acute exposures. For each gene, primer sequences, annealing temperature (°C), amplicon size (bp) and primer concentrations (nM) are provided. Elongation factor 1 alpha (*ef1α*), ribosomal protein L8 (*rpl8*) and beta actin (*βact*) were used as normalising genes. Primer sequences were first reported by Langlois et al (2010) except steroidogenic acute regulatory protein (*star*), heat shock protein 70 (*hsp70*), heat shock protein 90 (*hsp90*) and tumor protein 53 (*p53*), which are described in Soriano et al. (2014) and peroxisome proliferator-activated receptor alpha (*pparα*) and peroxisome proliferator-activated receptor gama (*pparγ*), which were developed in this study. *ar*: androgen receptor, *cyp19*: aromatase, *dio1*: deiodinase type 1, *dio2*: deiodinase type 2, *dio3*: deiodinase type 3, *srd5a2*: steroid 5 alpha reductase type 2, *trβ*: thyroid hormone receptor beta.

Gene function	Target gene	Primer direction	Primer sequence (5'-3')	Annealing tempera-ture (°C)	Amplicon size (bp)	Primer concen-tration (nM)
Miscella-neous	*ef1α*	F	GGCAATGTGGCTGGTGATAG	62	98	150
		R	GCACTAATCTGTCCTGGGTGA			150
	rpl8	F	CCCTCAACCATCAGGAGAGA	62	117	450
		R	TCTTTGTACCACGCAGACGA			450
	βact	F	CTGCTTCTTCTTCATCATTGGA	62	88	200
		R	TCTGGACATCTAAACCGCTCA			200
Thyroid hormone	*trβ*	F	ATCCAACACAGCAAAGGTATTTTC	62	106	300
		R	GTAATGACTGCCCCACATTGC			300
	dio1	F	GTAAGGACACCAACTGAGCAA	58	171	315
		R	GCTGCAACCGTCACTAACAA			315
	dio2	F	GTGTTGCCGACTTTGTGTTG	60	112	275
		R	CGTTCTTCTTGGTTTCTGTGCT			275
	dio3	F	TCGGAACTGAGGATGTGGT	60	199	175
		R	ATGCCCAAGGAGATGAGTG			175
Sex steroid	*ar*	F	TGACAACAACCAACCAGACA	62	96	600
		R	GCCTTTGCCCACTTTACAAC			600
	cyp19	F	GAATCCCGTGCAGTATAACAGC	62	118	112.5
		R	ACAGGTCTCCTCTTGATTCCATAG			112.5
	star	F	GAGCAGAAAGGCACAAACCC	58	105	350
		R	TTCCAGCCACTAAGCCTCTC			350
	srd5a2	F	ACCAGAAGGGAAGCACACAA	62	120	650
		R	CCATAAGCAGCAGGATAAGTGA			650
Cellular stress	*hsp70*	F	GGCCACGCTGTTACCAATGCTGTT	60	179	300
		R	ACATTGCGCTCTCCTCTGGTTCCT			300
	hsp90	F	AGGACCAACAGATGGAGGAGGATGT	58	246	450
		R	TGGTGAGGGTTCGGTCTTGCTT			450
	p53	F	GCTGCTTTGAGGTTCGTGTG	58	99	350
		R	TCTTCCCGTTGGGTTTCAGG			350
Trans-cription regulation	*pparα*	F	CAGGACACTAAGGACGCACC	58	109	300
		R	GGATGGCTCTGGCTTTCACTT			300
	pparγ	F	CGCAAACCCTTCTCCGACTT	58	90	600
		R	TATCGCCAAGTCGCTGTCGT			600

2.3 Results

2.3.1 Experimental phthalate concentrations

GC-MS analyses showed that controls were either not contaminated or contained traces of phthalates (Table 2.2). Neither BPA, MMP nor DMP degraded significantly within 24 h. The latter suggests that animals were exposed to a constant concentration throughout the assay. The experimental BPA concentration (3.0 ± 0.3 mg/L) was very close from the expected value (3.4 mg/L). The lowest MMP concentration (1.3 ± 1.1 mg/L) was not statistically significant from the water-only control, which could result from the large detection limit. The four highest experimental MMP concentrations were lower than the nominal values, but were statistically different from the water control (1.5, 12.3, 128.7 and 1,595.5 mg/L). Measured DMP concentrations were between 1.2 and 14.4 times lower than nominal values. Two concentrations were significantly different from the water control; 1.6 mg/L and 924.0 mg/L. The 9.1 mg/L and 34.1 mg/L are associated with high SD and are therefore not significantly different from the water control. DCHP concentrations were found to be lower than the nominal concentrations (0.3, 1.5, 4.1, 19.0 and 99.3 mg/L). At 1.5 mg/L, a significant 92.6% decrease in DCHP concentration was observed, presumably due to degradation. The other treatments did not significantly degrade within 24 h. Statistical analyses showed that 99.3 mg/L was the only treatment that statistically differed from the solvent control. Biological endpoints mentioned below, on the contrary, clearly showed that 0.3, 1.5, 4.1 and 19.0 mg/L induced adverse health effects that were significantly different from controls. Internal standards allowed to determine that recovery was 7.9 to 10.9% different from the expected value for BPA, 4.5 to 27.4% for MMP, 7.1 to 15.2% for DMP and 1.3 to 3.1% for DCHP.

2.3.2 Mortality

Mortality rates of all controls were below 10%, as recommended by the American Society for Testing and Materials (1998; Fig. 2.2). Exposure to MMP did not induce tadpole mortality, but both DMP and DCHP were significantly toxic to frog larvae. The highest DMP treatments (9.1 mg/L, 34.1 mg/L and 924.0 mg/L) induced 3.8%, 88.1% and 100% mortality, respectively. The three highest DCHP concentrations also significantly induced mortality when compared to the solvent control. All the animals from the 99.3 mg/L group died, whereas the 19.0 mg/L and 4.1 mg/L treatments lead to 94.7% and 5.5% lethality, respectively (Fisher's exact test; $p < 0.0001$ and $p = 0.0022$). Most of the mortality occurred between the 24^{th} and 48^{th} hour of the DCHP exposure. LC_{50} could not be calculated for MMP, as lethality was low within the concentration range tested (Table 2.5). DMP and DCHP had LC_{50}s of 11.9 mg/L and 5.5 mg/L, respectively.

2.3.3 Malformation

In our study, abnormalities were separated into malformations and developmental delay signs. Malformation rates for the water controls were slightly above the recommended 10% (American Society for Testing and Materials, 1998), with a mean of 12.0%. MMP, the positive control and 0.01% v/v DMSO did not have significant effects on malformation (Table 2.3). The malformation rate in DCHP's solvent control was statistically different from that of the water-only control (36.1%). DMP concentrations ranging from 0.1 mg/L to 34.1 mg/L induced malformation rates varying from 22.8 to 97.4% (Fisher's exact test; $p < 0.03$ in all four treatments). Edema and blistering were the most frequent type of abnormality and were augmented in a concentration-dependent manner from 6.0 to 100%. At 34.1 mg/L, at least half of the specimens exhibited deformities such as flexure of the tail, improperly coiled heart, improperly coiled gut and flattened/prominent forehead. A lowest observable effect concentration (LOEC) of 0.03 mg/L was found for DMP due to tail malformations (Table 2.5). Similarly to DMP, DCHP was teratogenic in the 4.1-19.0 mg/L range. All of the organisms from these groups were malformed (Fisher's exact test; $p < 0.0001$ for both treatments). Edemas (up to 100%), non-apparent heart (94.1%), absent gills (94.1%), improperly coiled guts (79.6%) and tail flexure (82.4%) were significantly more frequent in DCHP treated tadpoles. Edemas yielded a LOEC of 1.5 mg/L DCHP. None of the animals from the 19.0 mg/L DCHP group exhibited malformed gut, since 100% of the guts were severely underdeveloped (Table 2.4). DMP and DCHP had EC_{50} values of 3.3 mg/L and 0.5 mg/L, while TI values were 3.6 and 11.0, respectively.

2.3.4 Development

The positive control and solvent controls did not have a significant impact on the frequency of developmental delay signs except 0.82% v/v DMSO (Table 2.4). MMP disrupted tadpole development at 1,595.5 mg/L. At that concentration, incomplete coiling of the gut was increased to 25.5%. In embryos, guts are first straight and then start folding at NF42. At NF46, between 2 and 2.5 revolutions can be observed, with a tight "S" shaped loop on the ventral side of the animal (Nieuwkoop and Faber, 1992). Tadpoles from the highest MMP concentration appeared to be less than one developmental stage (NF46) behind water control animals. MMP did not affect body length in a biologically significant manner. Contrarily to its metabolite, DMP had an important effect on tadpole development in the 0.1 to 34.1 mg/L range. Nearly all the animals from the 9.1 and 34.1 mg/L groups were showing at least one sign of underdevelopment (Fisher's exact test; $p < 0.0001$ in both cases). The most recurrently affected body parts were the eyes

and cement glands. In healthy tadpoles, eyes anlagen start showing at NF20. Grey eye cups are then observed at NF29-30. At NF32, eye cups are horse shoe shaped and continue developing in a circular motion until both ends meet at NF35-36. At this point, the choroid fissure is nearly closed (Nieuwkoop and Faber, 1992). Cement glands are mucus secreting organs found under the mouth. Being attached to a solid support enables the embryos to conserve energy and appear less obvious to predators (reviewed in Sive and Bradley, 1996). The gland starts losing pigments and regressing at NF43 (Nieuwkoop and Faber, 1992). Therefore, the developmental delay signs noted in the 34.1 mg/L DMP group indicate that the animals were approximately one developmental stage (NF45) behind water control animals. Similarly to MMP, DMP did not disturb the mean tadpole length in a biologically significant fashion. DCHP augmented the rate of underdeveloped tadpoles at 4.1 and 19.0 mg/L (Fisher's exact test; $p = 0.0002$ and $p < 0.0001$, respectively). Since DCHP affected mainly the coiling of the gut and the regression of the cement gland, organisms from the 4.1 mg/L treatment seemed to be three developmental stages (NF43) behind water-only control animals (Fig. 2.3). All of the animals from the 19.0 mg/L DCHP treatment were found to display at least one sign of underdevelopment and bared incompletely coiled guts. In addition, no cement glands were scored in the highest DCHP treatment due to the severity of the development retardation, resulting in a five developmental stages delay (NF41) when compared to water controls. The reductions in mean tadpole lengths in the 4.1 and 19.0 mg/L treatments were biologically significant (Kruskal-Wallis test, $p < 0.0001$ for both treatments) and yielded a MCIG of 4.1 mg/L for DCHP (Table 2.5).

2.3.5 Gene expression

In order to investigate possible MOAs for the aforementioned adverse effects, gene expression analyses were conducted. MMP disrupted the expression of *pparα* at 12.3 mg/L (4.8-fold increase; Kruskal-Wallis; $p = 0.0090$; Table 2.6 and Fig. 2.4) in a biologically significant manner. Therefore, the LOEC for MMP is 12.3 mg/L. DMP disrupted the expression on *dio1*; a 2.1-fold increase in mRNA level was observed at 9.1 mg/L DMP (one-way ANOVA; $p = 0.0002$). The expression of *dio1* was also augmented by DCHP in a dose-dependent manner (Fig. 2.5). The latter was significant at 1.5 mg/L and 4.1 mg/L (3.4 and 4.1-fold; one-way ANOVA; $p < 0.0001$). In addition, DCHP disrupted the expression of genes involved in the transcriptional regulation, sex steroid and cellular stress axes. The expression of *pparγ* was upregulated at 1.5 mg/L DCHP (1.1-fold; one-way ANOVA; $p = 0.0001$). The same concentration increased transcripts levels for *cyp19* by 0.9-fold (one-way ANOVA; $p < 0.0001$). The two highest DCHP concentrations upregulated *star* (1.0 and 0.9-fold, respectively; Kruskal-Wallis; $p = 0.0040$). Finally, *hsp70* mRNA levels were higher at 4.1 mg/L (0.9-fold; Kruskal-Wallis; $p = 0.0009$). At a concentration of 0.01% v/v, DMSO increased the expression of *star* and *hsp70*, whereas 0.82% v/v DMSO did not induce any change. Genes which were not disrupted in a biologically significant manner are presented in Appendices 1, 2 and 3.

TABLE 2.2 Experimental concentrations before and after 24 h of exposure with Western clawed frog larvae. Data are expressed as mean ± SD. Two-tailed case one (paired) t-tests confirmed that the chemicals do not significantly degrade between water changes. Multiple case three (unequal variance) one-tailed t-tests with the Bonferroni corrections were performed to compare the mean of each treatment to the water controls. Asterisks indicate statistically significant differences between treatments and water-only controls, while the symbol ‡ indicates statistically significant differences between treatments and solvent controls. BPA: bisphenol A, DCHP: dicyclohexyl phthalate, DMP: dimethyl phthalate, MMP: monomethyl phthalate, SC: solvent control (0.82% v/v DMSO).

Chemical	Nominal concentration (mg/L)	Mean experimental concentration at time 0 h (mg/L ± SD)	Mean experimental concentration after 24 h exposure (mg/L ± SD)	Mean experimental concentration (mg/L ± SD)
BPA	3.4	3.2 ± 0.4	2.9 ± 0.3	3.0 ± 0.3
MMP	0	0.2 ± 0.1	0.7 ± 0.9	0.4 ± 0.6
	0.24	1.9 ± 1.4	0.7 ± 0.4	1.3 ± 1.1
	2.4	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0 *
	24	10.8 ± 0.8	13.8 ± 1.2	12.3 ± 1.9 *
	240	87.9 ± 21.4	169.5 ± 0.7	128.7 ± 48.7 *
	2,400	1,741.5 ± 436.3	1,449.5 ± 214.3	1,595.5 ± 327.4 *
DMP	0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	0.115	0.0 ± 0.0	0.0 ± 0.0	0.03 ± 0.0
	1.15	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
	11.5	1.8 ± 0.0	1.5 ± 0.3	1.6 ± 0.3 *
	38	8.2 ± 5.8	10.0 ± 10.1	9.1 ± 6.8
	115	23.7 ± 24.9	44.4 ± 1.9	34.1 ± 18.7
	1,150	916.5 ± 23.3	939.0[a]	924.0 ± 21.0 *
DCHP	SC	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
	0.6	0.5 ± 0.1	0.1 ± 0.0	0.3 ± 0.2
	6	2.7 ± 0.0	0.2 ± 0.0[b]	1.5 ± 1.4
	23	7.0 ± 1.3	1.2 ± 0.2	4.1 ± 3.5
	60	31.8 ± 4.0	6.3 ± 1.1	19.0 ± 14.9
	600	132.2 ± 34.6	66.3 ± 11.3	99.3 ± 43.5 ‡

[a]No standard deviation could be calculated, as there was only one sample. [b]In this treatment, significant DCHP degradation was observed.

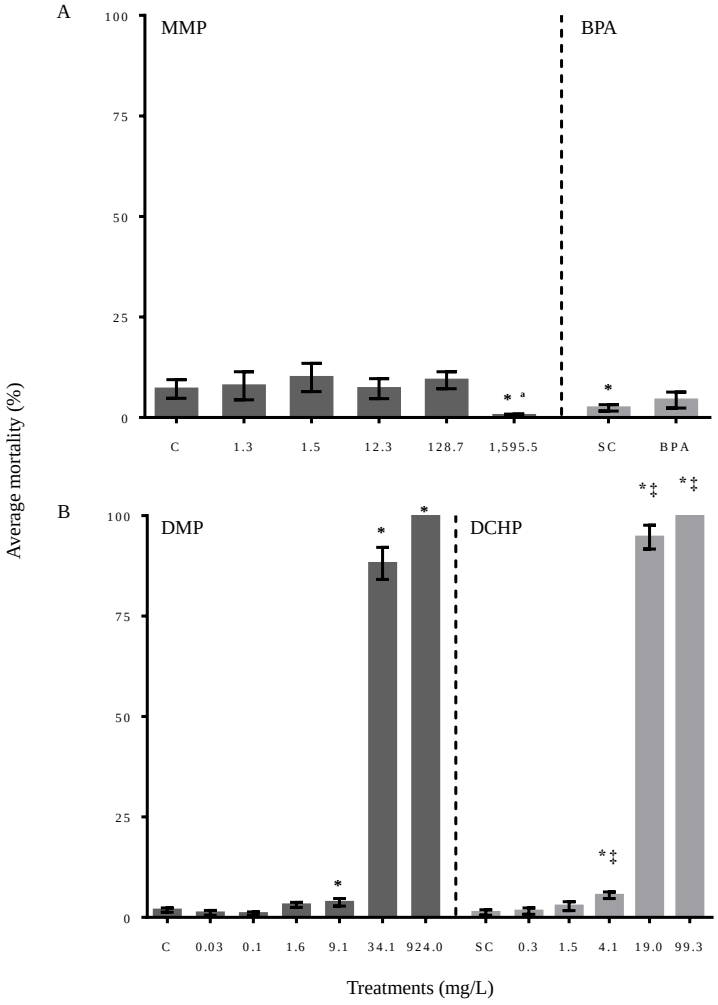

FIGURE 2.2 Effect of monomethyl (MMP; A), dimethyl (DMP; B) and dicyclohexyl (DCHP; B) phthalate spiked water on mortality of Western clawed frog larvae after 72 h exposures. Data are expressed as mean ± SD. Each chemical was tested in 6-10 replicates of 18-50 embryos. Data were analysed using Fisher's exact tests ($p < 0.05$). Asterisks indicate statistically significant differences between treatments and water-only controls, while the symbol ‡ indicates statistically significant differences between treatments and solvent controls. (a) The experimental pH was adjusted to 7.2. BPA: 3.0 mg/L bisphenol A as positive control, C: water-only control, SC: solvent control for BPA (0.01% v/v DMSO), or for DCHP (0.82% v/v DMSO).

TABLE 2.3 Effect of monomethyl (MMP), dimethyl (DMP) and dicyclohexyl (DCHP) phthalate spiked water on malformation of Western clawed frog larvae after 72 h exposures. Data are expressed as percentage of malformed animals per treatment. Percentage was calculated from a subset of animals randomly collected in each treatment (n = 17-226). Data were analysed using Fisher's exact tests ($p < 0.05$). Asterisks indicate statistically significant differences between treatments and water-only controls, while the symbol ‡ indicates statistically significant differences between treatments and solvent controls. BPA: 3.0 mg/L bisphenol A as positive control, C: water-only control, SC: solvent control for BPA (0.01% v/v DMSO), or for DCHP (0.82% v/v DMSO).

Treatments (mg/L)		**Malformed individuals % (*n*)**	**Malformations observed (%)**						
			Eye	Tail	Heart	Gut	Gills	Head & face	Edema & blistering
BPA	SC	15.2 (5)	6.1	12.1	6.1	0.0	0.0	0.0	6.1
	3.0	7.2 (5)	4.3	4.3	0.0	0.0	0.0	1.4	0.0
MMP	C	12.8 (6)	8.5	10.6	2.1	0.0	0.0	0.0	2.1
	1.3	14.3 (7)	8.2	6.1	0.0	2.0	0.0	2.0	4.1
	1.5	12.0 (6)	6.0	8.0	2.0	0.0	0.0	0.0	4.0
	12.3	20.8 (10)	6.3	14.6	4.2	0.0	0.0	0.0	4.2
	128.7	15.6 (7)	8.9	6.7	2.2	2.2	0.0	2.2	11.1
	1,595.5[a]	11.8 (6)	5.9	3.9	2.0	0.0	0.0	2.0	0.0
DMP[b]	C	11.2 (10)	10.1	2.2	0.0	3.4	0.0	0.0	0.0
	0.03	15.5 (35)	6.6	9.3 *	3.1	4.9	1.3	1.3	4.4
	0.1	22.8 (38) *	6.6	14.4 *	4.2	9.0	4.8	3.0	6.0 *
	1.6	27.2 (41) *	7.3	13.2 *	1.3	6.6	0.0	0.0	9.3 *
	9.1	62.9 (141) *	5.4	56.3 *	6.7 *	22.3 *	3.6	4.0	17.0 *
	34.1	97.4 (37) *	10.5	65.8 *	89.5 *	57.9 *	10.5 *	52.6 *	100.0 *
DCHP[b]	SC	36.1 (56) *	24.5 *	20.6 *	10.3 *	8.4	2.6	2.6	2.6
	0.3	21.8 (32) ‡	6.8 ‡	15.0	4.8	4.1	3.4	0.7	3.4
	1.5	40.0 (60)	12.0 ‡	4.7 ‡	7.3	5.3	4.7	0.7	33.3 ‡
	4.1	100.0 (216) ‡	6.5 ‡	11.6 ‡	7.9	79.6 ‡	5.1	6.0	100.0 ‡
	19.0	100.0 (17) ‡	23.5	82.4 ‡	94.1 ‡	0.0	94.1 ‡	5.9	29.4 ‡

[a]The experimental pH was adjusted to 7.2. [b]Animals from the 924.0 mg/L DMP and 99.3 mg/L DCHP were not screened for malformations since all the animals died.

TABLE 2.4 Effect of monomethyl (MMP), dimethyl (DMP) and dicyclohexyl (DCHP) phthalate spiked water on development of Western clawed frog larvae after 72 h exposures. Signs of developmental delay are expressed as percentage of underdeveloped animals per treatment. For each treatment, a subset of 17-226 animals was randomly collected. Data were analysed using Fisher's exact tests ($p < 0.05$). Length is expressed as mean ± SD. For each treatment, 17-50 embryos were measured. Data were analysed using the Kruskal-Wallis test followed by two-tailed Dunn's procedures with the Bonferonni correction ($p < 0.05$). Asterisks indicate statistically significant differences between treatments and water-only controls, while the symbol ‡ indicates statistically significant differences between treatments and solvent controls. BPA: 3.0 mg/L bisphenol A as positive control, C: water-only control, SC: solvent control for BPA (0.01% v/v DMSO), or for DCHP (0.82% v/v DMSO).

Treatments (mg/L)		**Underdeveloped individuals % (*n*)**	**Signs of developmental delay observed (%)**			**Mean tadpole length (µm ± SD)**
			Eye	Cement gland	Gut	
BPA	SC	12.1 (4)	0.0	0.0	12.1	5,462.0 ± 349.0
	3.0	4.3 (3)	0.0	0.0	4.3	5,499.9 ± 302.4
MMP	C	6.4 (3)	2.1	0.0	6.4	5,427.6 ± 346.6
	1.3	8.2 (4)	2.0	0.0	6.1	5,665.2 ± 467.4 *
	1.5	4.0 (2)	0.0	0.0	4.0	5,736.2 ± 285.0 *
	12.3	8.3 (4)	0.0	0.0	8.3	5,603.2 ± 325.9
	128.7	0.0 (0)	0.0	0.0	0.0	5,639.9 ± 435.2 *
	1,595.5[a]	25.5 (13) *	0.0	0.0	25.5 *	5,071.2 ± 262.0 *
DMP[b]	C	4.5 (4)	1.1	2.2	2.2	5,668.5 ± 398.0
	0.03	9.3 (21)	2.2	5.8	3.1	5,807.0 ± 260.1
	0.1	22.2 (37) *	3.6	14.4 *	10.2 *	5,974.8 ± 233.2 *
	1.6	17.2 (26) *	2.6	11.3 *	4.0	5,840.2 ± 366.6 *
	9.1	100.0 (224) *	27.7 *	99.6 *	42.4 *	5,256.4 ± 890.9 *
	34.1	97.4 (37) *	100.0 *	92.1 *	44.7 *	- [c]
DCHP[b]	SC	27.7 (43) *	12.3 *	14.8 *	11.6 *	5,617.5 ± 389.2
	0.3	25.9 (38)	5.4	15.6	16.3	5,499.3 ± 473.2
	1.5	34.7 (52)	4.7 ‡	23.3	13.3	5,400.6 ± 222.2
	4.1	47.2 (102) ‡	6.9	33.3 ‡	18.1	4,674.5 ± 377.4 ‡
	19.0	100.0 (17) ‡	17.6	0.0	100.0 ‡	3,723.1 ± 442.3 ‡

[a]The experimental pH was adjusted to 7.2. [b]Animals from the 924.0 mg/L DMP and 99.3 mg/L DCHP were not measured nor screened for developmental delay signs since all the animals died. [c]No animal could be measured since surviving tadpoles were not fixed in formalin.

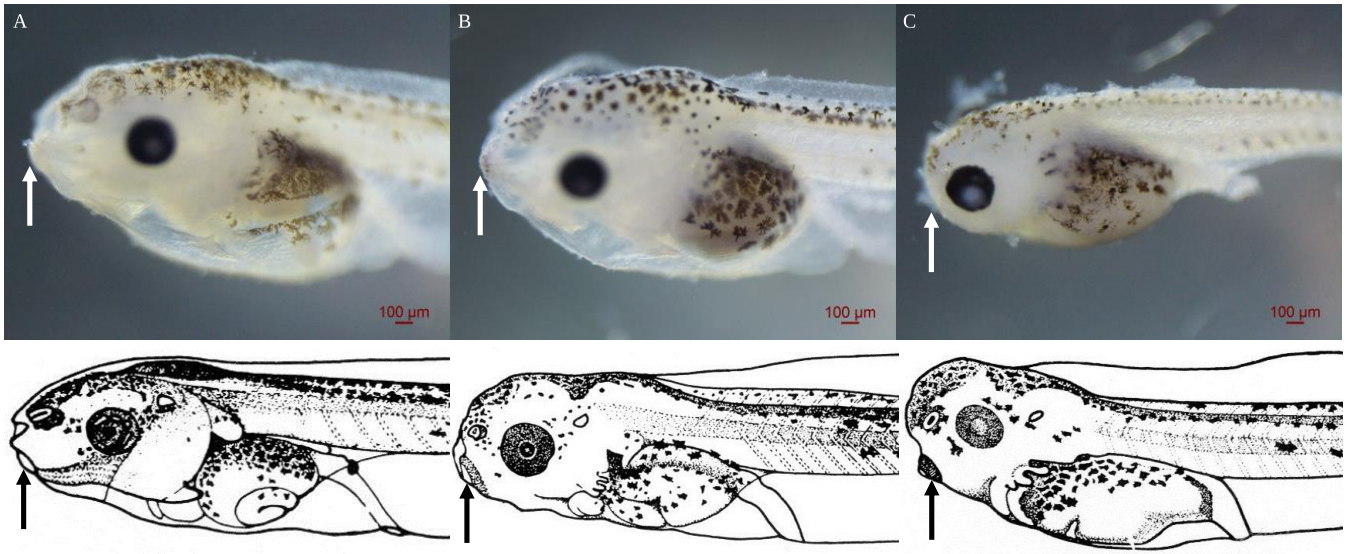

FIGURE 2.3 Effect of dicyclohexyl phthalate (DCHP) spiked water on cement gland development in Western clawed frog larvae after 72 h exposures. (A) At 72 hours post fertilization (NF46), cement glands are fully regressed in control animals, as depicted in the drawing. (B) Delayed development was detected at 4.1 mg/L DCHP; cement glands were grey and protruding. Animals from this treatment were at NF43. (C) At 19.0 mg/L DCHP, tadpole development was so severely affected that cement glands were not developed at all. Guts resembled those of NF41 animals. Red bars represent 100 µm. Photographs were taken with the microscope Nikon SMZ18 (Nikon, Chiyoda-Ku, Tokyo) and the software NIS Elements (Nikon, Chiyoda-Ku, Tokyo). Drawings were adapted from Xenbase.org.

TABLE 2.5 Summary of the adverse health effects of monomethyl (MMP), dimethyl (DMP) and dicyclohexyl (DCHP) phthalates in Western clawed frog larvae after 72 h exposures. Median effective concentrations (EC_{50}s) were derived from malformation results. Mean tadpole lengths were used to calculate minimum concentrations to inhibit growth (MCIGs). LC_{50}: median lethal dose, LOEC: lowest observable effect concentration, TI: teratogenic index.

Chemical	LOEC (mg/L)	LC_{50} (mg/L)	EC_{50} (mg/L)	TI	MCIG (mg/L)
MMP	12.3	-	-	-	> 1,595.5
DMP	0.03	11.9	3.3	3.6	> 9.1
DCHP	1.5	5.5	0.5	11.0	4.1

TABLE 2.6 Summary of the effect of monomethyl (MMP), dimethyl (DMP) and dicyclohexyl (DCHP) phthalate spiked water on the expression of thyroid hormone, sex steroid, cellular stress and transcription regulation-related genes in Western clawed frog larvae after 72 h exposures. Biologically significant results (fold change ≥ 0.8) are presented below with the fold change and an arrow representing the direction of the change. Each treatment was tested in 4 to 9 replicates of 10 whole embryos. Data were analysed using one-way ANOVAs and two-sided Dunnet's tests or Kruskal-Wallis tests and two-tailed Dunn's procedures with the Bonferonni correction ($p < 0.05$). Elongation factor 1 alpha (*ef1α*), ribosomal protein L8 (*rpl8*) and beta actin (*βact*) were used as housekeeping genes to normalise the expression of target genes. *ar*: androgen receptor, BPA: 3.0 mg/L bisphenol A as positive control, C: water-only control, *cyp19*: aromatase, *dio1*: deiodinase type 1, *dio2*: deiodinase type 2, *dio3*: deiodinase type 3, *hsp70*: heat shock protein 70, *hsp90*: heat shock protein 90, *p53*: tumor protein 53, *pparα*: peroxisome proliferator-activated receptor alpha, *pparγ*: peroxisome proliferator-activated receptor gama, SC: solvent control for BPA (0.01% v/v DMSO), or for DCHP (0.82% v/v DMSO), *srd5a2*: steroid 5 alpha reductase type 2, *star*: steroidogenic acute regulatory protein, *trβ*: thyroid hormone receptor beta.

Treatments (mg/L)		Thyroid hormone				Sex steroid				Cellular stress			Transcription regulation	
		trβ	*dio1*	*dio2*	*dio3*	*ar*	*cyp19*	*star*	*srd5a2*	*hsp70*	*hsp90*	*p53*	*pparα*	*pparγ*
BPA	SC	-	-	-	-	-	-	∧ 4.3	-	∧ 3.2	-	-	-	-
	3.0	-	-	-	-	-	-	-	-	-	-	-	-	-
MMP	1.3	-	-	-	-	-	-	-	-	-	-	-	-	-
	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-
	12.3	-	-	-	-	-	-	-	-	-	-	-	∧ 4.8	-
	128.7	-	-	-	-	-	-	-	-	-	-	-	-	-
	1,595.5[a]	-	-	-	-	-	-	-	-	-	-	-	-	-
DMP[b]	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-
	1.6	-	-	-	-	-	-	-	-	-	-	-	-	-
	9.1	-	∧ 2.1	-	-	-	-	-	-	-	-	-	-	-
DCHP[b]	SC	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-
	1.5	-	∧ 3.4	-	-	-	∧ 0.9	∧ 1.0	-	-	-	-	-	∧ 1.1
	4.1	-	∧ 4.1	-	-	-	-	∧ 0.9	-	∧ 0.9	-	-	-	-

[a]The experimental pH was adjusted to 7.2. [b]Gene expression analyses were not performed for 34.1 mg/L DMP, 924.0 mg/L DMP, 19.0 mg/L DCHP and 99.3 mg/L DCHP since all or most of the animals died.

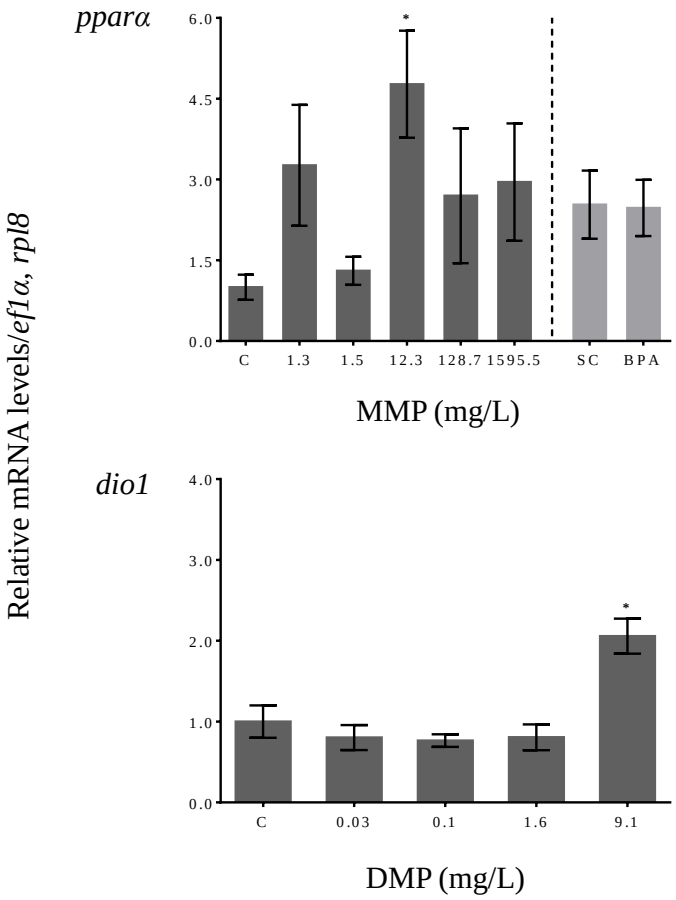

FIGURE 2.4 Effect of monomethyl phthalate (MMP) and dimethyl phthalate (DMP) spiked water on the expression of peroxisome proliferator-activated receptor alpha (*pparα*) and deiodinase type 1 (*dio1*) in Western clawed frog larvae after 72 h exposures. Data are presented as mean ± SEM. Each treatment was tested in 4 to 9 replicates of 10 whole embryos. Data were analysed using one-way ANOVAs and two-sided Dunnet's tests or Kruskal-Wallis tests and two-tailed Dunn's procedures with the Bonferonni correction ($p < 0.05$). Elongation factor 1 alpha (*ef1α*) and ribosomal protein L8 (*rpl8*) were used as control genes to normalise the expression of target genes. The scale of the Y axis varies among genes. Asterisks indicate statistically significant differences between treatments and the water-only controls. C: water-only control, SC: solvent control for BPA (0.01% v/v DMSO).

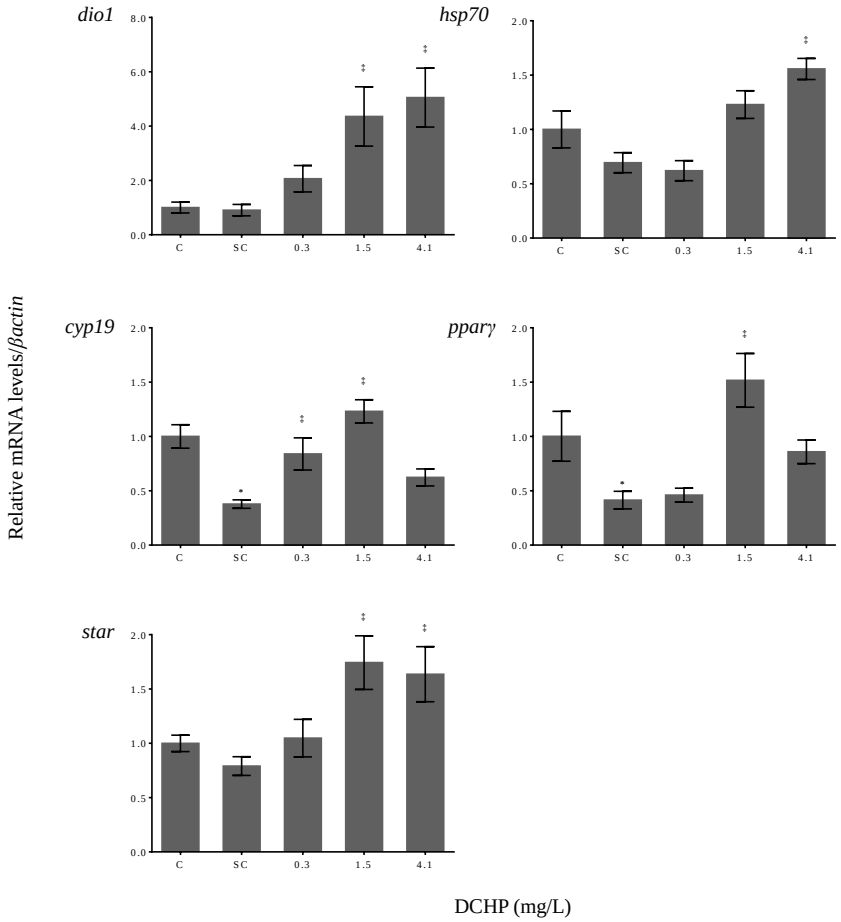

FIGURE 2.5 Effect of dicyclohexyl phthalate (DCHP) spiked water on the expression of deiodinase type 1 (*dio1*), aromatase (*cyp19*), steroidogenic acute regulatory protein (*star*), heat shock protein 70 (*hsp70*) and peroxisome proliferator-activated receptor gama (*pparγ*) in Western clawed frog larvae after 72 h exposures. Data are presented as mean ± SEM. Each treatment was tested in 4 to 9 replicates of 10 whole embryos. Data were analysed using one-way ANOVAs and two-sided Dunnet's tests or Kruskal-Wallis tests and two-tailed Dunn's procedures with the Bonferonni correction ($p < 0.05$). Beta actin (*βact*) was used as control gene to normalise the expression of target genes. The scale of the Y axis varies among genes. Asterisks indicate significant differences between the water-only control and the solvent control, while the symbol ‡ indicates statistically significant differences between treatments and the solvent control. C: water-only control, SC: solvent control (0.82% v/v DMSO).

2.4 Discussion

Some phthalates from the CMP are commonly found in water bodies (Hendriks et al., 1994; Suzuki et al., 2001; Clara et al., 2010; Zhang and Shen, 2010; Teil et al., 2007), however their effects on amphibians have not been characterised yet. This chapter aimed at investigating the lethal and sublethal effects of MMP, DMP and DCHP on Western clawed frog larvae. In this study, it was demonstrated that the plasticisers DMP and DCHP are toxic to tadpoles at relatively high concentrations. DCHP significantly induced mortality at concentrations beyond 4.1 mg/L. This effect was comparable to the effects of other high molecular weight phthalates such as BzBP and monodecyl phthalate in daphnids (Jonsson and Baun, 2003). In fish and other aquatic insects, however, BzBP was found to be more toxic than DCHP. Amphipods survivability was affected at 0.5 mg/L BzBP (Call et al., 2001), while LC_{50}s of 0.8 and 1.5 mg/L BzBP were reported for rainbow trout and fathead minnow (Adams et al., 1995). At the same lethal concentrations, DCHP increased the presence of malformations in tadpoles. Edemas were the most common abnormalities, followed by heart and gill deformities. Similarly, studies reported that the plastic additives BPA, tetrabromobisphenol A and phthalic acid led to edemas in African clawed frog and zebrafish embryos (Bantle et al., 1999; Sone et al., 2004; McCormick et al., 2010). Cardiac abnormalities were also more frequent in tadpoles treated with 15 mg/L DBP (Lee et al., 2005). There is a greater potential for the larvae to be malformed at sublethal concentrations when TI values are above 1.5 (Dawson et al., 1989; Rayburn et al., 1991; Bantle et al., 1999). Since DCHP had a TI of 11.0, it is considered to pose a teratogenic hazard. The increases in lethality and malformations observed in the DCHP treatments were concurrent with augmentations in *hsp70* mRNA levels. HSPs are chaperone proteins which play essential roles in correct folding of nascent and prevention of protein aggregation. HSPs are highly sensitive to slight stresses and are believed to be suitable early warning indicators of cellular stress (reviewed in Gupta et al., 2010b). Transcript levels of *hsp70* were also increased with DEHP and BzBP in midges (Planelló et al., 2011). In addition, BzBP was found to increase *hsf2* mRNA levels in rats (Moral et al., 2007). Such proteins play major roles in regulating transcription of *hsp*s (reviewed in Prahlad and Morimoto, 2008). Altogether, our transcriptomics findings suggest that DCHP induced cellular stress, which is presumably contributing to the organismal effects reported above.

While DCHP is more lipophilic and is believed to enter the organisms more easily, DMP also induced mortality and abnormalities in tadpoles, but to a lesser extent. Mortality was increased by DMP in a concentration range similar to that reported by Adams and collaborators (1995) in fish, i.e. from 29 to 121 mg/L. Likewise, DMP concentrations varying from 28.1 mg/L to 284 mg/L were found to induce 50% mortality in aquatic insects (Call et al., 2001; Jonsson and Baun, 2003). In another phthalate diester study, a DBP concentration of 27.8 mg/L induced 100% mortality in Japanese wrinkled frog tadpoles (Ohtani et al., 2000), which is well beyond 924.0 mg/L for DMP (this present study). This data suggests that DMP could be less toxic than DBP. At sublethal DMP concentrations, increases in tadpole malformation were also noted. As seen with DCHP, edema and blistering were the most frequent types of abnormality, followed by improper coiling of the heart. The third most frequently affected body part was the tail, unlike in DCHP treated animals. Tail deformities have been reported in zebrafish treated with BPA (15 mg/L; Duan et al., 2008) and in Argentine common toad embryos exposed to the herbicide paraquat (2.5 mg/L; Mussi and Calcaterra, 2010). Since DMP induced different types of abnormalities and did not significantly affect the expression of *hsp70*, DMP is hypothesised to act via a different pathway than DCHP. Other cellular stress-related genes may mediate the embryotoxicity. For example, *p53* codes for a protein that functions as a transcription factor which triggers the inhibition of abnormal cell growth and contributes to DNA repair (reviewed in Albrechtsen et al., 1999; and in Zilfou and Lowe, 2009). However, the expression of *p53* was not disrupted by DMP exposure. Other genes involved in the cellular stress signaling cascade will remain to be investigated.

Phthalates have also been shown to interfere with the male reproductive status in vertebrates. In rats, DCHP induced endocrine-mediated effects including reduced anogenital distance, nipple retention and prolonged preputial separation (Yamasaki et al., 2009). Such adverse effects are thought to be mediated by lowered T levels during the critical stage of reproductive tract differentiation (Parks et al., 2000), which is in turn believed to be a result of decreased *star* expression (Barlow et al., 2003; Lehmann et al., 2004; Thompson et al., 2004; Borch et al., 2006; Lahousse et al., 2006; Hannas et al., 2011). In this study, the expression of *star* was also altered in tadpoles. DCHP, however, led to an increase in mRNA levels. Similarly, the transcription of this gene was stimulated in mouse Leydig tumor cells treated with MBP (Hu et al., 2013) and in DBP treated rats (Ryu et al., 2008). Hu et al. (2013) suggested that changes in *star* transcription may result from a phthalate-induced increase in DNA binding of SF-1. SF-1 regulates the transcription of *star* by binding to response elements within the *star* promoter (Sandhoff et al., 1998). Other transition factors are known to regulate *star* transcription. Among others, PPARγ is capable of modifying the proteic expression of StAR in mouse cell lines (Kowalewski et al., 2009). In our study, DCHP

increased *pparγ* mRNA abundance. Similarly, transcription and translation of this gene were augmented in rats treated with DEHP or DBP (Lee et al., 2007; Xu et al., 2010). Nonetheless, DCHP increased the gene expression of *star*, suggesting that cholesterol transport may be altered. Notwithstanding the fact that gonads are absent in NF46 tadpoles, disruption of *star* transcription may affect latter life events such as sex differentiation and reproduction (Mathieu-Denoncourt et al., in preparation c).

Since PPARs are transcription factors for several other genes, their disruption by phthalates could lead to changes in the transcription of other important genes. In rats and humans, PPARs downregulate the expression of *cyp19* (Lovekamp-Swan et al., 2003; Kwintkiewicz et al., 2010; Xu et al., 2010). In several rodent studies, phthalates affect the reproductive axis by decreasing the gene and protein expression and activity of CYP19 (reviewed in Mathieu-Denoncourt et al., in preparation a). Interestingly, 1.5 mg/L DCHP increased the expression of both *pparγ* and *cyp19*, suggesting that DCHP affects the amphibian reproductive system differently from rodents. Similarly, raises in *pparγ* transcription were concurrent with increased *cyp19a* mRNA levels in tributyltin treated salmon (Pavlikova et al., 2010). Such disruption of *cyp19* could result in increased aromatisation. Feminisation in frogs could then result from the increased estrogens levels (Pettersson et al., 2007; Lutz et al., 2008). DMP and its monoester, however, did not affect the expression of *cyp19*.

In the present study, MMP, DMP and DCHP delayed tadpole development, as measured by shorter body lengths and higher occurrence of developmental delay signs. Similarly, DEHP reportedly suppressed zebrafish oocyte and medaka embryos development (Chikae et al., 2004a; Carnevali et al., 2010). African clawed frog tadpoles acutely exposed to 0.1 mg/L or 15 mg/L DBP also experienced significant reductions in body lengths (Higuchi et al., 2001; Lee et al., 2005). It is well known that amphibian development is highly dependent on THs (reviewed in Brown and Cai, 2007). Although the thyroid gland is not functioning in NF46 tadpoles, studies have provided evidence for TH-related gene expression, deiodinase activity and quantification of THs during African clawed frog embryogenesis (Morvan Dubois et al., 2006; Duarte-Guterman et al., 2010). In order to better understand the mechanistic behind phthalate-induced developmental delay, an investigation of TH-related gene expression was performed. Although DMP and DCHP did not alter the expression of *trβ*, the diesters significantly upregulated *dio1*. Interestingly, DCHP upregulated the expression of *dio1* at concentrations lower or equal to those that delay tadpole development. DIO1 can activate THs by converting T4 to T3, but can also inactivate THs by catalysing the two following reactions: T3 can be metabolised to the less potent T2 or T4 can be transformed into reverse T3 and finally into T2. This suggests that phthalate exposure can reduce the levels of active THs, thereby contributing to tadpole

developmental delay. In addition, as both TR and PPARs heterodimerise with RXR to regulate transcription, it is possible that a change in RXR level alter their activity. Indeed, MBP and DBP have been shown to reduce *rxrγ* transcription in African clawed frog tadpoles and to enhance the interactions between TR and SMRT in a mammalian two-hybrid assay (Shen et al., 2011). SMRT is a corepressor that facilitates the recruitment of histone deacetylases and thereby assists nuclear receptors such as TR in the downregulation of target gene expression (Nagy et al., 1997). This data suggests that DBP and its metabolite may have repressed TR's transcriptional activity by lowering RXR level. In addition, glucocorticoids have been shown to suppress development in amphibians and Atlantic salmons (Wright and collaborators, 1994; reviewed in Hayes, 1997; Eriksen et al., 2006). Stress hormones promote survival, but incur costs such as reduced growth (reviewed in Denver, 2009). Glucocorticoids are cholesterol metabolites and the rate-limiting step in their synthesis is cholesterol transport to the mitochondrial inner membrane by proteins such as StAR (Cherradi et al., 1997; Tsujishita and Hurley, 2000). Although glucocorticoids were not measured in Western clawed frog tadpoles, the increase in *star* transcription suggests that DCHP may have led to augmented cholesterol transport and thus a putative raise in glucocorticoid production. Altogether, this work suggests that phthalates inhibited tadpole development by potentially altering the thyroid signaling pathway or by inducing endocrine stress.

In addition, signs of developmental delay induced by DCHP were different from those observed in DMP treatments. There was a high incidence of underdeveloped eyes in DMP treated organisms. Imaoka and collaborators (2007) suggested that eye abnormalities resulted from decreases in the expression of *pax-6* and of the Notch-related gene *esr-1*. Furthermore, retinoic acid signaling could be altered by phthalates (Dufour et al., 2003; Shen et al., 2011). RXR-TR heterodimers are essential for eye development in amphibians. RXR ligands have been shown to induce eye abnormalities in zebrafish and African clawed frog embryos (Minucci et al., 1996; Minucci et al., 1997). DCHP, on the other hand, impaired the development of the cement gland. Sive and Bradley (1996) have suggested that orthodenticle homeobox 2 (*otx2*) may be responsible for cement gland formation in African clawed frogs. Microinjections of *otx2* mRNA led to the formation of ectopic cement glands in African clawed frog embryos (Blitz and Cho, 1995; Pannese et al., 1995). This opens additional research directions in phthalate toxicogenomics.

Prior to conducting the acute phthalate exposures, MMP was predicted to be more embryotoxic than DMP and DCHP, since monoesters have been shown to be equally or more potent than the corresponding diesters in rodents (reviewed in Mathieu-Denoncourt et al., in preparation a). However, in the current study, development was altered at a very high MMP concentration and the expression of one gene only was disrupted. The increase in *pparα* mRNA levels did not mediate the expression of other target genes, but might have affected endpoints that were not studied here. Our results demonstrated that acute Western clawed frog exposure to MMP did not induce mortality, malformation nor disruption of most of the studied genes. Although this finding is in contradiction with our hypothesis, it is in agreement with the findings of Jonsson and Baun (2003). The authors showed that MMP was less acutely toxic than DMP to daphnids. The same trend was also observed for MEP, MBzP and MBP (Jonsson and Baun, 2003). Our findings provide further evidence that, in aquatic species, monoesters are less toxic than their parent compounds.

In this work, BPA was used as a positive control of endocrine disruption since it has been shown to induce changes in the expression of sex steroid and TH-related genes in rodent cell cultures (2.3 ng/L; Akingbemi et al., 2004b), African clawed frog cell culture (0.2 mg/L; Iwamuro et al., 2006) and tadpoles (2.3 mg/L; Heimeier et al., 2009). Western clawed frog tadpoles may be less sensitive to BPA than African clawed frog tadpoles. BPA's solvent was also shown to be inappropriate to conduct acute exposures with Western clawed frog embryos. Although the FETAX protocol allows the use of DMSO at concentrations up to 1.1% v/v (American Society for Testing and Materials, 1998), this solvent carrier induced malformations, which may be a result from cellular stress. A microarray analysis performed by Sumida et al. (2011) showed that DMSO disrupted the expression of genes related to glutathione metabolism and oxidative phosphorylation in human and rat cells cultured in 0.1-0.8% v/v DMSO. In our study, DMSO was used at much lower concentrations and yet induced adverse health effects at the organismal level. In addition, DMSO increased the expression of *star* and *hsp70* when compared to water-only controls. However, this did not prevent the quantification of phthalates' effects in treated tadpoles. Reviewing the FETAX procedure by including the Western clawed frog and by taking into consideration its greater sensitivity to toxicants would be desirable, since there is an increase in the use of this species in ecotoxicogenomics.

2.5 Conclusion

This work is important since MMP, DMP and DCHP have been found in rivers at concentrations as high as other well-characterised toxic phthalate esters such as BzBP, DBP and DEHP (Hendriks et al., 1994; Suzuki et al., 2001; Clara et al., 2010; Zhang and Shen, 2010). Our findings demonstrate that acute exposures to high diester concentrations lead to mortality, malformations and developmental delays. DCHP was found to be more potent and it affected the transcription of several genes involved in cellular stress, reproduction and development. Considering that MMP did not induce adverse health effects at lower concentrations, this monoester could be considered as a replacement for toxic plasticisers although chronic exposures should be attempted first (chapter 3).

Chapter 3

Lethal and sublethal effects of chronic exposures to monomethyl phthalate in Western clawed frogs

3.1 Introduction

Phthalates are additives that render polymers flexible by reducing the glass transition temperature (Daniels, 2009). Phthalates leach out of the polymer matrix and are ubiquitous in the environment (Blair et al., 2009). Degradation leads to one of the two esters being broken down to a carboxylic acid and an alcohol. Resulting monoesters are thought to be responsible for the diester's adverse effects (Gray et al., 2000; Saillenfait et al., 2001; Ema, 2002; Shen et al., 2011). Both phthalate diesters and the corresponding monoesters affect reproduction (Foster et al., 1981; Mylchreest et al., 1998), the sex steroid axis (Lovekamp and Davis, 2001; Xu et al., 2010) and the TH system (Shen et al., 2011). Moreover, a previous amphibian exposure to MMP showed that this monoester could delay tadpole development (Mathieu-Denoncourt et al., in preparation b). Taking into consideration the scarcity of literature tackling chronic effects of phthalates on amphibians and bearing in mind that the acute assay did not allow for a complete understanding of the effects of MMP on later life events such as metamorphosis and reproduction, this chapter aimed at assessing the chronic effects of MMP on Western clawed frog.

In this study, Western clawed frog were exposed to water spiked with MMP until completion of metamorphosis or until sexual maturity (Fig. 3.1). Survival, malformations and sex ratios were evaluated. A series of morphological measurements enabled to determine the chronic effect of MMP on Western clawed frog development. Finally, the expression of sex steroid, TH, cellular stress and transcription regulation-related genes in frog liver allowed to shed light on the MOAs. Survivability and malformation rates were hypothesised to remain unaffected by MMP (Mathieu-Denoncourt et al., in preparation b). Metamorphosis was predicted to be decelerated by the positive control (BPA; Heimeier et al., 2009) and hastened by chronic MMP exposures since phthalates were shown to upregulate TH-related genes in tadpoles (Mathieu-Denoncourt et al., in preparation b). Finally, BPA and MMP were expected to skew sex ratios towards females (Kloas et al., 1999; Yokota et al., 2000; Chikae et al., 2004a; Levy et al., 2004). Considering that PPARs are one of the proposed MOAs for phthalates' toxicity and considering that monoesters tend to activate PPARs (Maloney and Waxman, 1999; Hurst and Waxman, 2003; Feige et al., 2007), MMP was hypothesised to disrupt the expression of *pparα* and *pparγ* (Xu et al., 2010).

3.2 Materials and methods

3.2.1 Animal care and breeding

Animal care was performed as described in Mathieu-Denoncourt and collaborators (in preparation b). Breeding was performed using three pairs of Western clawed frog (*Silurana tropicalis*). Gentamycin sulphate (CAS 1405-41-0, Fisher Scientific, Fair Lawn, New Jersey, USA) was used as antibiotic at a concentration of 0.04 mg/L for the first three days of the experiment in order to prevent microbial propagation.

3.2.2 Experimental design

Western clawed frog larvae were exposed to water spiked with MMP until juvenile stage (11 w) and until adulthood (51 w) in a procedure similar to the amphibian metamorphosis assay (AMA) and the *Xenopus* metamorphosis assay (XEMA). Briefly, these assays involve the exposure of premetamorphic tadpoles to thyroid system disrupting chemicals for 21 or 28 d. The primary endpoint is the developmental stage to which the organisms developed at the completion of the exposure (Opitz et al., 2005; Jagnytsch et al., 2006; United States Environmental Protection Agency, 2009a; reviewed in Miyata and Ose, 2012). The Western clawed frog has been shown to be a suitable species for XEMA (Mitsui et al., 2006) and is a species suggested for the AMA (United States Environmental Protection Agency, 2009a). In the present study, the exposures started when healthy Western clawed frog tadpoles reached the developmental stage Nieuwkoop and Faber 46 (NF; Nieuwkoop and Faber, 1992). MMP (98%, CAS 4376-18-5, Acros Organics, Fair Lawn, New Jersey, USA) was dissolved in water to obtain 0.24, 24 and 240 mg/L. A positive control of endocrine disruption, 3.4 mg/L BPA (≥ 99%, CAS 80-05-7, Sigma-Aldrich, St. Louis, Missouri, USA) in 0.01% v/v DMSO (99.9%, CAS 67-68-5, Fisher, Fair Lawn, New Jersey, USA) was also tested. A solvent control consisting of 0.01% v/v DMSO and a water-only control were conducted. The volume of exposure varied in an attempt to maintain an animal density of 1 g/L. Therefore, each treatment was tested in one replicate of 9 to 35 L containing 39 to 100 animals each according to the weight of the animals. Rearing media and chemicals were completely changed three times per week. Dead animals were removed and recorded daily. Animals were fed commercial tadpole food (Sera Micron, Heinsberg, Germany) from NF46 to NF60, then commercial frog food (Nasco, California, USA). Temperature and pH varied from 21.9 to 26.4

ºC and 5.3 to 8.6, respectively. The exposure was concluded at 11 w, when all of the water-only control animals reached NF66. This stage marks the end of metamorphosis, right after the TH climax (NF62; Yaoita and Brown, 1990; Glennemeier and Denver, 2002; Krain and Denver, 2004; Brown and Cai, 2007). At the completion of the juvenile exposure, 29 animals from the water-only control and 0.24 mg/L MMP, respectively, were randomly selected for the adult exposure. Each treatment was tested in one replicate of 9 to 60 L. Rearing media was entirely changed three times per week. Temperature and pH varied from 22.1 to 26.8 ºC and 7.4 to 9.0, respectively. The exposures were brought to an end when animals reached sexual maturity, at 51 w of age.

3.2.3 Water and tissue sample collection

Since test solutions were replaced three times per week, water changes were separated by a maximum of 3 d. Media samples from the positive control and 240 mg/L MMP were aliquoted at time 0 and 3 d later in order to measure experimental concentrations. Samples were conserved in dark vials at -20 ºC for later GC-MS analysis. After the completion of the exposures, animals were sacrificed by immersion in 2% w/v ethyl 3-aminobenzoate methanesulfonate (MS-222; 98%, CAS 886-86-2, Sigma-Aldrich, St. Louis, Missouri, USA) followed by decapitation. Livers were sampled in both 11 w and 51 w old animals, since this tissue plays an important role in xenobiotic detoxification. Tissues were preserved at -80 ºC until further gene expression analysis.

3.2.4 Media analysis

As experimental concentrations for 0, 0.24, 24 and 240 mg/L MMP have been determined previously and were shown not to degrade within 24 h (Mathieu-Denoncourt et al., in preparation b), experimental concentrations were determined for 240 mg/L MMP and 3.4 mg/L BPA only. GC-MS was performed in Dr. Yargeau's Laboratory (McGill University, QC, Canada) using a Trace GC Ultra gas chromatograph equipped with a Triplus As injector and coupled to a Polaris Q (Thermo Fisher Scientific, Waltham, MA, USA). Internal standards (BPA: 99.9%, CAS 80-05-7, Supelco, Sigma-Aldrich, St. Louis, Missouri, USA; MMP: 99.5%, CAS 4376-18-5, Supelco, Sigma-Aldrich, St. Louis, Missouri, USA) allowed to determine the precision of the method. Detection limit was 0.07 mg/L and 0.24 mg/L for BPA and MMP, respectively. Results are reported based on the experimental concentrations determined in table 2.2.

3.2.5 Morphological analysis

Morphological measurements were taken throughout the experiments and at exposure completion. Each week, the number of surviving animals having reached specific developmental stages was recorded. These stages were the formation of hind limbs (NF52), the occurrence of front limbs (NF60), the reduction in head width (NF62) and the resorption of the tail (NF66). A subset of animals (n = 6-10) were measured every week; for whole body length from NF46 to NF66 and then for snout-to-vent length following NF66. Body length was used to determine the MCIG. Before sacrificing the animals, snout-to-vent length, hind limb length and interocular distance were measured using a digital caliper (Fisher Scientific, Fair Lawn, New Jersey, USA) to the nearest 0.1 mm. Body weight and liver weight were obtained during dissections with a Pioneer scale (± 0.1 mg; Ohaus, Pine Brook, New Jersey, USA). Animals were observed for malformations. Phenotypical sex determination was assessed with gross morphology under a dissecting microscope (Stereomaster, Fisher Scientific, Fair Lawn, New Jersey, USA). Sex was assessed based on the size of the gonads, presence of oviducts or sperm ducts, texture of the gonads and presence of eggs. Sex was confirmed following triple-blinded analysis using pictures of the gonads (Sony DSC-W35, Kōnan Minato, Tokyo, Japan).

3.2.6 Gene expression

Gene expression analysis was performed on livers of 11 w and 51 w old animals, as described in Mathieu-Denoncourt et al. (in preparation b). Since gender was hypothesised to affect the expression of sex steroid-related genes in sexually mature frogs, gene expression analysis was completed on male and female livers of the 51 w assay separately. Each treatment was tested in 4-10 replicates of one liver. RNA was extracted with TRIzol (Ambion RNA, Life Technologies, Invitrogen, Carlsbad, CA, USA). Transcriptional changes were investigated using a qPCR approach. Efficiency and R^2 were kept between 80.0-132.6% and 0.868-1.000, respectively. The specific forward and reverse primers of the genes of interests are presented in Table 2.1. The chronic effects of MMP on the gene expression of *dio1*, *cyp19* and *srd5a2* were not investigated. Since certain housekeeping genes changed among treatments, the two chronic experiments were normalised to a different housekeeping gene. Transcript levels from the 11 w assay were normalised to the quantified relative expression of ribosomal protein L8 (*rpl8*), while results from the adult exposure were normalised to ornithine decarboxylase (*odc*), as their expression did not significantly change amongst treatments. The forward primer sequence for *odc* was 5'-TGAATGATGGCGTGTATGGA-3' and

the reverse sequence was 5'-GTCCCCAAATGCTGCTTG-3'. The annealing temperature for *odc* was 62 °C, while its amplicon size and primer concentration were 120 bp and 150 nM, respectively.

3.2.7 Statistical analysis

In order to determine if test chemicals significantly degraded between water changes, two-tailed case one (paired) t-tests were performed for 240 mg/L MMP and 3.4 mg/L BPA. Experimental concentrations determined for the chronic exposures were also compared to the concentrations reported in the previous chapter using two-tailed case three (unequal variance) t-tests. The *p*-values were adjusted with the Bonferroni correction. Survival, malformation and developmental stages were analysed using Fisher's exact test. Normality and homoscedasticity were assessed using Lilliefors tests and Levene tests. Developmental data from the 11 w exposure was analysed with parametric and non-parametric tests. Body weight and liver weight were Log_{10} transformed, but non-transformed data are presented in tables. Body weight, liver weight and snout-to-vent length were analysed using one-way ANOVAs. Comparison between treatments was completed with two-sided Dunnet's tests. Since body length data was not normally distributed, a two-way ANOVA with time as a second variable could not be performed as suggested by Aluru et al. (2010). Therefore, body length data was analysed separately for each week using Kruskal-Wallis tests. Hind limb length and interocular distance were also analysed using Kruskal-Wallis tests. The latter was followed by two-tailed Dunn's procedures with the Bonferroni correction. Development results from the adult exposure were analysed using F-tests and case two (equal variance) two-tailed t-tests. Sex ratios from both chronic exposures were analysed using Pearson's chi squared test with Yates' continuity correction. Square root, Log_{10} and natural Log transformations were performed on gene expression results that were not normally distributed. Significant changes in gene expression data were investigated with one-way ANOVAs with two-sided Dunnet's tests (11 w) or Tukey's tests (51 w), or with Kruskal-Wallis tests followed by two-tailed Dunn's procedures with the Bonferroni correction. For all comparisons, *p*-values of less than 0.05 were considered statistically significant. Calculations were completed both with GraphPad (GraphPad Software Inc, San Diego, CA, USA) and R (R Core Team, 2013). The Global Validation of Linear Models Assumptions package was used in R (gvlma; Pena and Slate, 2014).

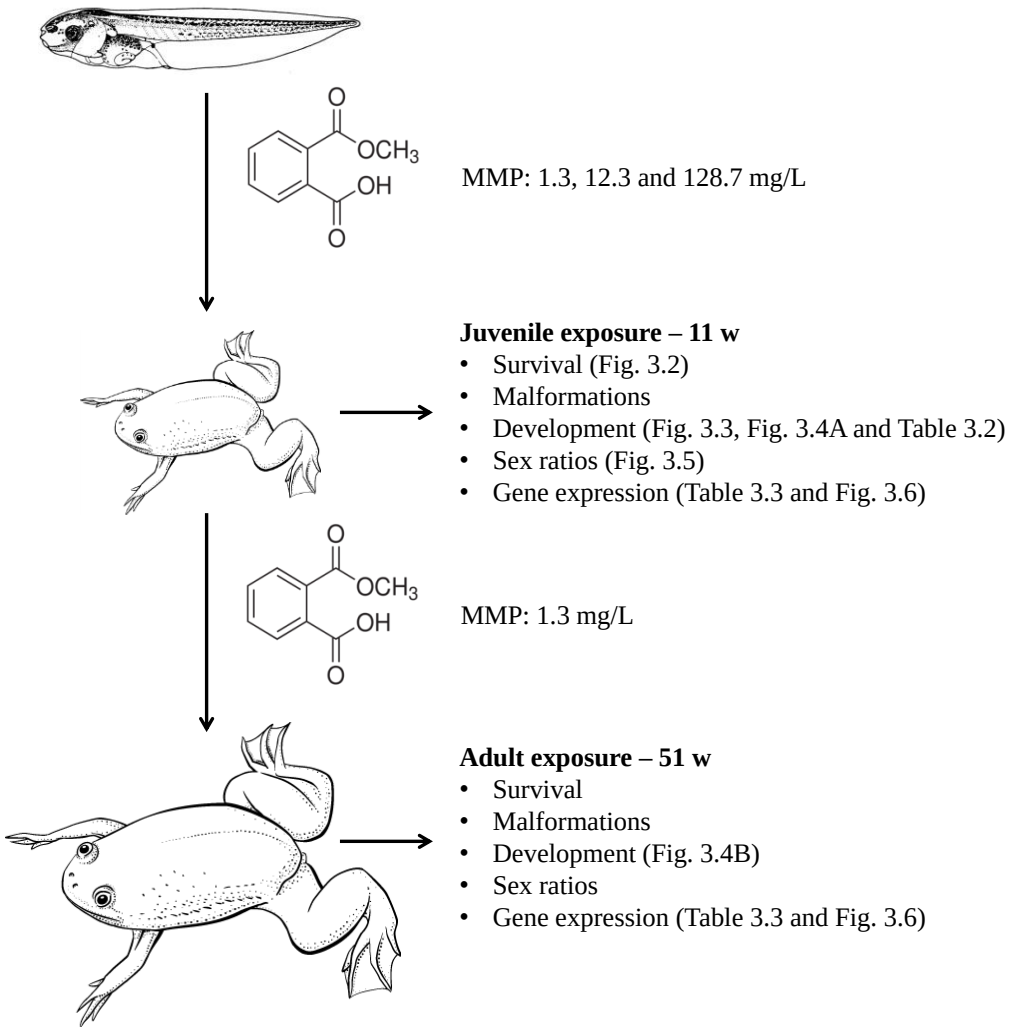

FIGURE 3.1 Schematic representation of the experimental design of chronic juvenile and adult exposures to monomethyl phthalate (MMP) in the Western clawed frog. Animals were treated from NF46 to NF66 (11 w), or from NF46 to sexual maturity (51 w). For consistency, concentrations shown above are experimental and were determined in Mathieu-Denoncourt and collaborators (in preparation b).

3.3 Results

3.3.1 Experimental phthalate concentrations

Statistical analyses showed that MMP and BPA did not significantly degrade within 3 d (Table 3.1), suggesting that animals were exposed to constant chemical concentrations throughout the assays. The mean experimental value for the 240 mg/L group was similar to the expected value i.e. 234.3 mg/L. The experimental BPA concentration (1.4 mg/L) was about 2.4 times lower than the nominal value. Internal standards indicated that recovery was 6 to 15.6% different from the expected value for MMP and 7.9 to 10.9% different for BPA. Experimental concentrations measured in samples from the chronic exposures did not significantly differ from concentrations determined in the acute assays. Therefore, the experimental concentrations presented in Table 2.2 were used throughout the manuscript to ensure consistency.

3.3.2 Survival and malformation

In the juvenile exposure, all three tested MMP concentrations significantly decreased survivability (Fig. 3.2). MMP reduced survival by 22.2 to 31.0%. In addition, the positive control also induced lethality in juveniles. The lowest survival rate was 66.7% and was observed at 3.0 mg/L BPA. No additional mortality occurred between week 11 and the end of the adult exposure (data not shown). Neither 11 w nor 51 w experiments significantly induced malformations (data not shown).

3.3.3 Development

Juvenile exposures to MMP hastened Western clawed frog development. Animals from 128.7 mg/L group reached NF52 before water control animals, which was significant at week 5 (Fig. 3.3A). All three MMP treatments increased the occurrence of NF60 on weeks 4 to 6 when compared to the water control (Fig. 3.3B). All of the animals from the 128.7 mg/L group displayed front limbs at week 7, while only 64.8% of water-only control juveniles were at NF60 at that time (Fisher's exact test; $p < 0.0001$). Similarly, all four MMP groups reached NF62 significantly faster than controls on weeks 5 to 7 (Fig. 3.3C). On the 8^{th} week, heads of all of the animals from the highest MMP concentrations were narrower

than the trunks, whereas only 68.1% of water control juveniles had reached this stage (NF62; Fisher's exact test; $p < 0.0001$). The same trend was observed with NF66 on weeks 6 to 8 (Fig. 3.3D). The entire 128.7 mg/L MMP treatment completed metamorphosis within 9 w, while animals from the water-only control completely metamorphosed within 11 w. The second group to be entirely metamorphosed was the positive control, on week 10. Generally, BPA had a favorable effect on development; animals from the positive control reached NF60, NF62 and NF66 before the solvent control. In addition, DMSO decelerated the onset of those same three developmental stages when compared to the water-only control.

Morphological measurements were significantly affected by juvenile exposure to MMP. A significant reduction in body length was found at the highest MMP concentration on week 7 (55.7% reduction; Kruskal-Wallis; $p = 0.003$; Fig. 3.4A). At exposure completion, mean snout-to-vent length and mean hind limb length were found to be increased at 128.7 mg/L when compared to the water-only control (5.7% and 9.8% increase; Table 3.2). BPA along with its solvent control increased mean liver weight in juveniles by 38% and 32.1%, respectively, when compared to the water control. Western clawed frog body length (Fig. 3.4B) and other morphological measurements were not affected by the adult exposure (data not shown).

3.3.4 Sex ratios

Juvenile exposure to MMP significantly altered phenotypical sex ratios (Fig. 3.5). MMP skewed sex ratios towards males in a concentration-dependent manner. The respective male to female ratios were 13:28 for the water-only control, 17:23 for the lowest MMP concentration, 21:19 for 12.3 mg/L MMP, 27:13 for the highest phthalate concentration, 10:10 for the solvent control and 5:15 for the positive control. The trend was significant at 128.7 mg/L with 67.5% males (Pearson's chi squared test; $p = 0.0027$). Adult exposure to 1.3 mg/L MMP did not affect sex ratios (data not shown). A male to female ratio of 13:16 was observed in the water control, whereas a ratio of 11:18 was obtained from the 1.3 mg/L MMP group. None of the treatments induced intersex.

3.3.5 Gene expression

While juvenile exposure to MMP did not significantly affect gene expression in livers, adult exposures disrupted the expression of one gene (Table 3.3). A statistically significant difference in the expression *hsp70* was observed between control males and treated males (0.4-fold; one-way ANOVA; $p = 0.0041$; Fig. 3.6). Genes that were not significantly affected are presented in Appendices 4 and 5.

TABLE 3.1 Experimental concentrations before and after 3 d of exposure with Western clawed frogs. Media analysis was performed on the positive control and the 240 mg/L monomethyl phthalate (MMP) treatments only. Two-tailed case one (paired) t-tests confirmed that the chemicals do not significantly degrade between water changes. Experimental concentrations were compared with those from the acute exposures (case three two-tailed t-tests). Since there is no significant difference between the two data sets, concentrations presented in Table 2.2 were used throughout the manuscript to ensure consistency. Data are expressed as mean ± SD. BPA: bisphenol A.

Chemical	Nominal concentration (mg/L)	Data set	Mean experimental concentration at time 0 (mg/L ± SD)	Mean experimental concentration after exposure (mg/L ± SD)	Mean experimental concentration (mg/L ± SD)
BPA	3.4	24 h	3.2 ± 0.4	2.9 ± 0.3	3.0 ± 0.3
		3 d	2.2 ± 1.2	0.7 ± 0.0	1.4 ± 1.1
MMP	240	24 h	87.9 ± 21.4	169.5 ± 0.7	128.7 ± 48.7
		3 d	214.5 ± 41.7	254.0 ± 171.1	234.3 ± 104.2

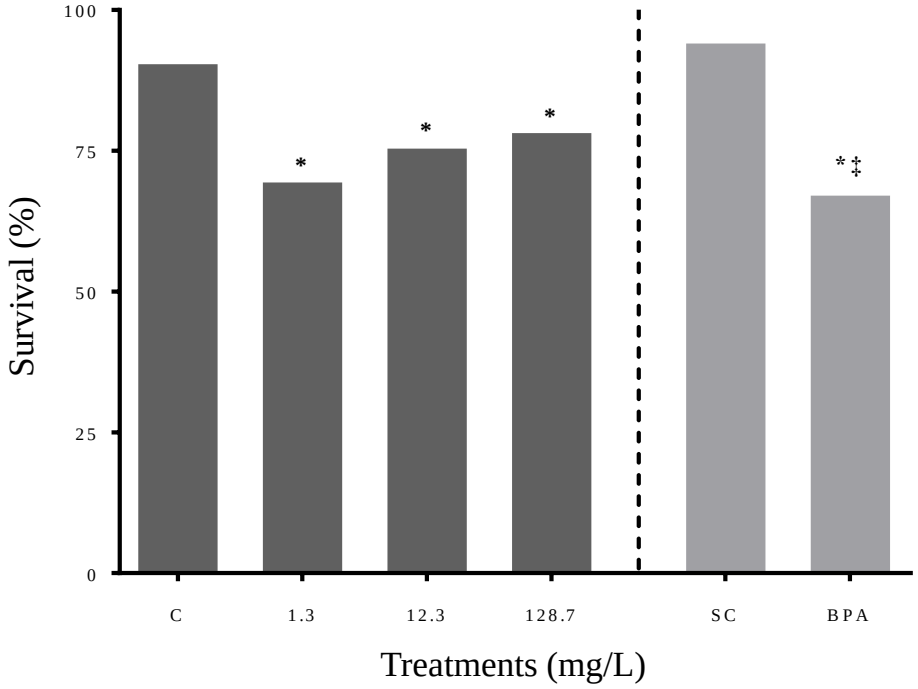

FIGURE 3.2 Effect of monomethyl phthalate (MMP) spiked water on survival of Western clawed frogs after an 11 w exposure. Data are expressed as percentage. Each treatment contained 39 to 100 animals. Data were analysed using Fisher's exact tests ($p < 0.05$). Asterisks indicate statistically significant differences between treatments and the water-only control while the symbol ‡ indicates a statistically significant difference between the positive control and the solvent control. BPA: 3.0 mg/L bisphenol A as positive control, C: water-only control, SC: solvent control for BPA (0.01% v/v DMSO).

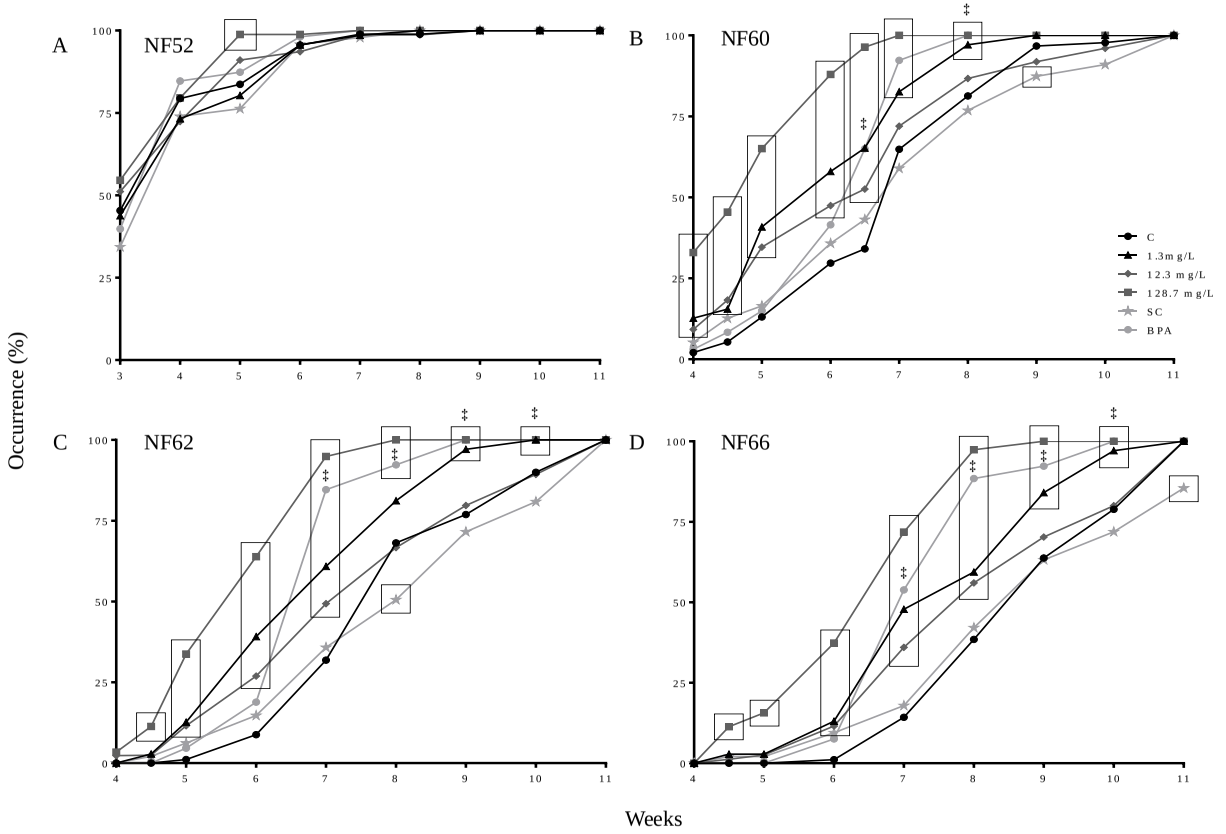

FIGURE 3.3 Effect of monomethyl phthalate contaminated water on Western clawed frog metamorphosis during a juvenile exposure. Metamorphosis starts by the occurrence of hind limbs (NF52; A), then the development of front limbs (NF60; B), a change in head width (NF62; C) and finally the regression of the tail (NF66; D). Data are expressed as percentage of animals that have reached each developmental stage. Each replicate contained 39 to 100 animals. Data were analysed using Fisher's exact tests ($p < 0.05$). Boxes indicate statistically significant differences between treatments and the control while the symbol ‡ indicates statistically significant differences between the positive control and the solvent control. BPA: 3.0 mg/L bisphenol A as positive control, C: water-only control, SC: solvent control for BPA (0.01% v/v DMSO).

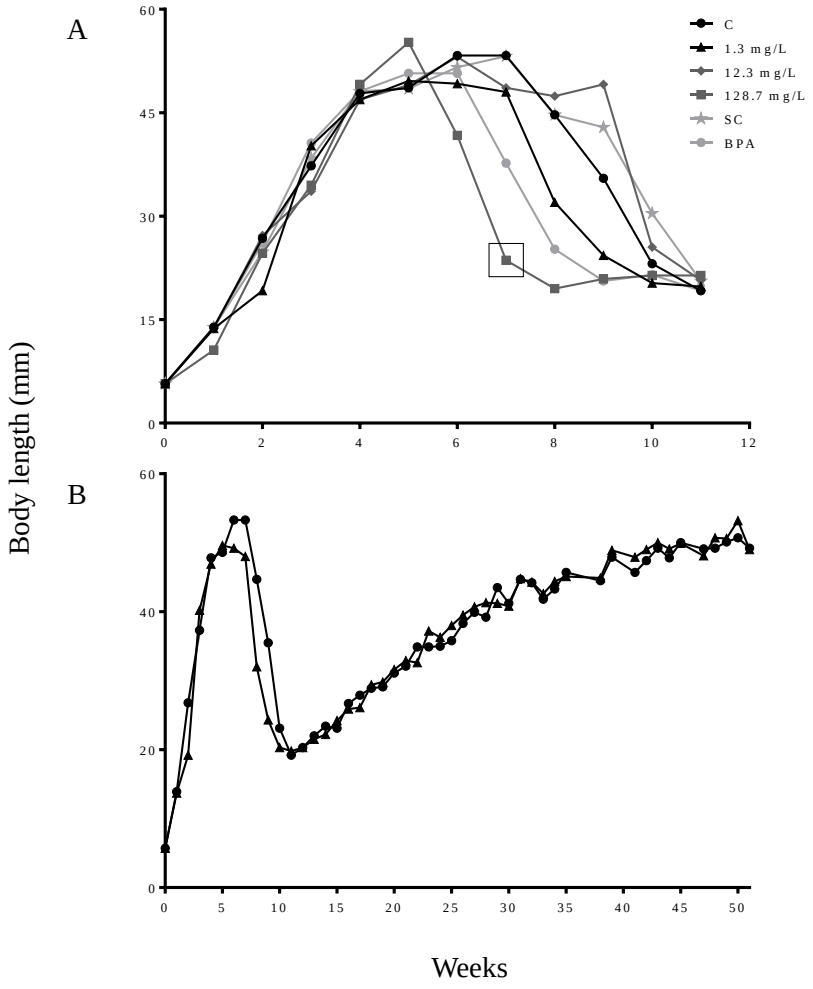

FIGURE 3.4 Effect of monomethyl phthalate (MMP) spiked water on Western clawed frog body length after (A) an 11 w and (B) a 51 w exposure. Data are expressed as mean. Every week, between 6 and 10 animals were measured from mouth to tail (NF46-NF66) and or from snout-to-vent (NF66 and on). Each week was analysed separately using (A) Kruskal-Wallis test and two-tailed Dunn's procedures with the Bonferroni corrections or (B) with F-tests and case two (equal variance) two-tailed t-tests. The box indicates a statistically significant difference between the highest MMP concentration and the water-only control. BPA: 3.0 mg/L bisphenol A as positive control, C: water-only control, SC: solvent control for BPA (0.01% v/v DMSO).

TABLE 3.2 Effect of monomethyl phthalate (MMP) spiked water on Western clawed frog morphological measurements after an 11 w exposure. Data are expressed as mean ± SD. The measurements were obtained from 26 to 76 animals per treatment. Body weight and liver weight were Log_{10} transformed. Snout-to-vent length (SVL), body weight and liver weight data were analysed using one-way ANOVA, Tukey's test and two-sided Dunnet's tests ($p < 0.05$). Hind limb length and interocular distance were analysed with Kruskal-Wallis tests and two-tailed Dunn's procedures with the Bonferroni corrections. Asterisks indicate statistically significant differences between treatments and the water-only control. BPA: 3.0 mg/L bisphenol A as positive control, C: water-only control, SC: solvent control for BPA (0.01% v/v DMSO).

Treatments (mg/L)		**Mean SVL (mm ± SD)**	**Mean hind limb length (mm ± SD)**	**Mean interocular distance (mm ± SD)**	**Mean body weight (mg ± SD)**	**Mean liver weight (mg ± SD)**
BPA	SC	21.4 ± 2.1	17.6 ± 4.8	5.8 ± 3.0	1266.3 ± 295.5	35.8 ± 9.6 *
	3.0	22.1 ± 2.2	19.9 ± 2.7	4.5 ± 0.4	1266.6 ± 410.8	37.4 ± 14.1 *
MMP	C	21.0 ± 1.9	18.4 ± 2.9	5.0 ± 1.9	1137.4 ± 322.3	27.1 ± 11.4
	1.3	21.6 ± 1.9	18.9 ± 2.0	4.5 ± 0.2	1184.5 ± 297.3	27.5 ± 10.8
	12.3	21.5 ± 2.1	19.3 ± 3.3	4.7 ± 1.5	1205.8 ± 337.9	30.4 ± 11.5
	128.7	22.2 ± 1.9 *	20.2 ± 2.0 *	4.4 ± 0.4	1233.1 ± 333.1	27.6 ± 10.4

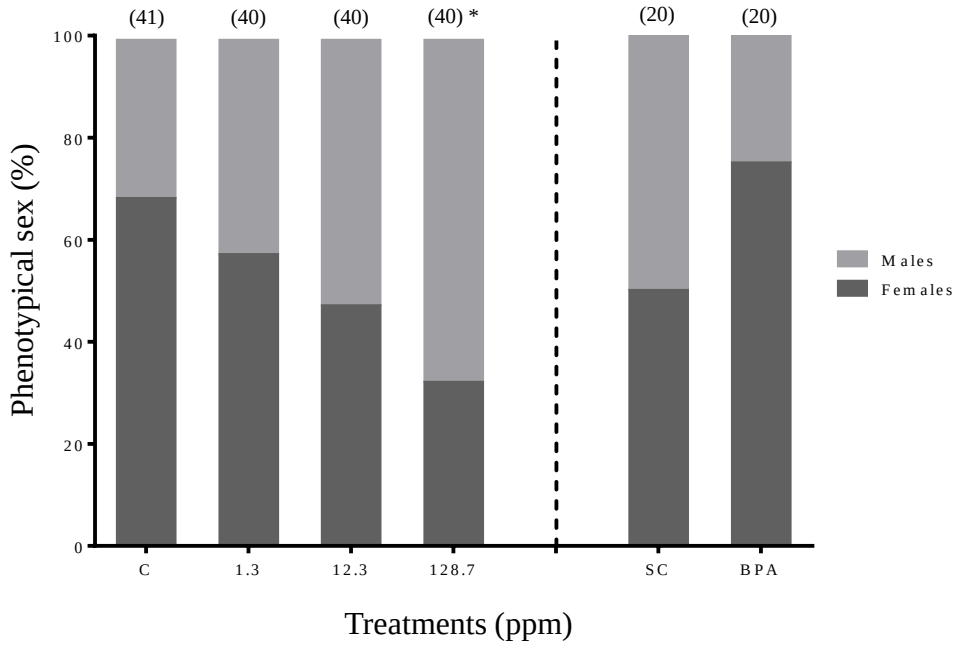

FIGURE 3.5 Effect of monomethyl phthalate (MMP) spiked water on Western clawed frog sex ratios after an 11 w exposure. Data are expressed as percentage of male and female per treatment. Sex ratios were determined from 20 to 41 animals per treatment. Data were analysed with Pearson's chi squared test ($p < 0.05$). The asterisk indicates a statistically significant difference between 128.7 mg/L MMP and the water-only control. BPA: 3.0 mg/L bisphenol A as positive control, C: water-only control, SC: solvent control for BPA (0.01% v/v DMSO).

TABLE 3.3 Summary of the effect of monomethyl phthalate (MMP) spiked water on the expression of thyroid hormone, sex steroid, cellular stress and transcription regulation-related genes in Western clawed frog livers after juvenile and adult exposures. The significant result is presented below with the fold change and an arrow representing the direction of the change. Each treatment was tested in 4 to 10 replicates of one liver. Square root, Log_{10} and natural Log transformations were performed on certain genes. Data were analysed using one-way ANOVAs, two-sided Dunnet's tests (11 w), Tukey's tests (51 w), or Kruskal-Wallis tests and two-tailed Dunn's procedures with the Bonferonni correction ($p < 0.05$). Ornithine decarboxylase (*odc*) and ribosomal protein L8 (*rpl8*) were used as housekeeping genes to normalise the expression of target genes. *ar*: androgen receptor, BPA: 3.0 mg/L bisphenol A as positive control, *dio2*: deiodinase type 2, *dio3*: deiodinase type 3, *hsp70*: heat shock protein 70, *hsp90*: heat shock protein 90, *p53*: tumor protein 53, *pparα*: peroxisome proliferator-activated receptor alpha, *pparγ*: peroxisome proliferator-activated receptor gama, SC: solvent control for BPA (0.01% v/v DMSO), *star*: steroidogenic acute regulatory protein, *trβ*: thyroid hormone receptor beta.

Treatments (mg/L)		Thyroid hormone			Sex steroid		Cellular stress			Transcription regulation	
		trβ	*dio2*	*dio3*	*ar*	*star*	*hsp70*	*hsp90*	*p53*	*pparα*	*pparγ*
BPA	SC	-	-	-	-	-	-	-	-	-	-
11 w	3.0	-	-	-	-	-	-	-	-	-	-
	1.3	-	-	-	-	-	-	-	-	-	-
MMP	1.5	-	-	-	-	-	-	-	-	-	-
11 w	12.3	-	-	-	-	-	-	-	-	-	-
	128.7	-	-	-	-	-	-	-	-	-	-
MMP	1.3 Male	-	-	-	-	-	∨ 0.4	-	-	-	-
51 w	1.3 Female	-	-	-	-	-	-	-	-	-	-

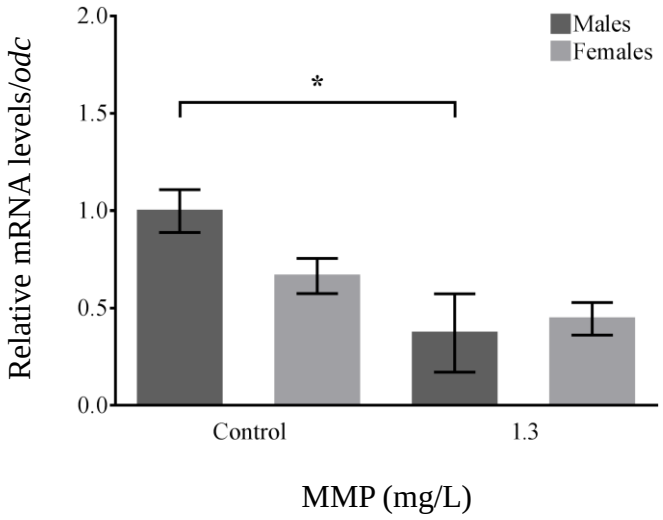

FIGURE 3.6 Effect of monomethyl phthalate (MMP) spiked water on the expression of heat shock protein 70 (*hsp70*) in Western clawed frogs after a 51 w exposure. Data are represented as mean ± SEM. Each treatment was tested in 4 to 10 replicates of one liver. Data were analysed using one-way ANOVAs and Tukey's tests ($p < 0.05$). Ornithine decarboxylase (*odc*) was used as control gene to normalise the expression of target genes. The asterisk indicates a statistically significant differences between treated and water-only control males.

3.4 Discussion

Phthalates have been shown to affect later life events such as reproduction in mammals (Higuchi et al., 2003; Tyl et al., 2004; Noriega et al., 2009; Yamasaki et al., 2009; Frederiksen et al., 2012; Giribabu et al., 2012). However, there is a critical lack of data on the chronic effects of phthalates in amphibians. In this study, Western clawed frog tadpoles were chronically exposed to MMP until metamorphosis completion or until sexual maturity in an attempt to investigate the lethal and sublethal effects of the monoester. The entire range of MMP concentrations reduced survival in juveniles. Similarly, African clawed frog juvenile survival was affected in 5 week and 12 week exposures to DBP (Higuchi et al., 2001; Lee and Veermachaneni, 2005). In order to decipher the mechanistic behind the observed lethality, an investigation of cellular stress-related gene expression was carried out. Although mRNA levels for the genes investigated in this study did not significantly change among treatments, other genes from the cellular stress axis could be involved in the decreased survival. Oxidative stress, for example, could result from the activation of PPARα by MMP (Hurst and Waxman, 2003). The expression of *pparα*, however, was not disrupted by MMP in our study. In rat testes, phthalate-induced oxidative stress has been observed and has led to apoptosis (Kasahara et al., 2002). In addition, Craig and collaborators (2013) reported that DBP disrupted the expression of apoptosis-related genes such as BH3-interacting domain death agonist (*bid*) and B cell leukemia/lymphoma 2 (*bcl2*) in mouse ovarian follicles. Investigating the effects of MMP on genes from the apoptotic pathway and from the oxidative stress axis such as, *gpx*, *sod* and *cat* could allow for a better understanding of the decreased survival. In adult Western clawed frogs, survival was not affected by MMP and a slight decrease in *hsp70* mRNA levels was observed. This change in gene expression may have affected endpoints that were not investigated here.

Amphibian metamorphosis disturbance is a sensitive means of screening for thyroid axis disruption (Degitz et al., 2005). Early development in amphibians is divided into three periods according to the functional state of the thyroid system. Premetamorphosis ends when the thyroid gland becomes functional and is followed by the prometamorphosis. A series of TH-dependent morphological changes occur during this second period, the most obvious being the formation of hind and fore limbs (Yaoita and Brown, 1990; Nieuwkoop and Faber, 1992; reviewed in Brown and Cai, 2007). Thirdly, metamorphosis corresponds to the TH climax and is marked by TH-dependent resorption of the tail and gills. Metamorphosis is complete at NF66, when the animals resemble a small frog (Yaoita and Brown, 1990; Nieuwkoop and Faber, 1992; Veldhoen et al., 2002; reviewed in Brown and Cai, 2007). Literature suggests that phthalates delay metamorphosis in aquatic species (Dumpert and Zietz, 1984; Shen et al., 2011;

Zhou et al., 2011a) possibly by inducing epigenetic alterations in *trβ* (Shen et al., 2011). Contrarily, MMP urged metamorphosis in Western clawed frogs as seen by the hastening of developmental stages corresponding to prometamorphosis and metamorphosis. The latter suggest that the monoester acts on the TH axis. Interestingly, the expression of TH-related genes was not disrupted by MMP in juveniles. Perhaps MMP affected other aspects of the TH axis, such as iodide uptake (Wenzel et al., 2005). In addition to THs, the timing of metamorphosis has been suggested to be controlled by glucocorticoids. Among others, corticoids potentiate the actions of THs by increasing the conversion of T4 to T3 (reviewed in Hayes, 1997) and by enhancing the binding of T3 to TR in African clawed frogs and bullfrogs (Suzuki and Kikuyama, 1983; Gray and Janssens, 1990). In metamorphosing frogs, corticosterone levels raise concurrent with the THs increase (Glennemeier and Denver, 2002) and tail resorption results from the synergism between corticoids and THs (Frieden and Naile, 1955; Kikuyama et al., 1983; Krain and Denver, 2004; Bonett et al., 2010). These lines of evidence suggest that glucocorticoids, along with TH, can regulate the timing of metamorphosis in anurans. Glucocorticoid-induced hastened metamorphosis may be an attempt to help frogs escape the phthalate-contaminated pond and avoid lethality (Denver, 2009). Altogether, our data suggests that MMP may have accelerated amphibian metamorphosis by affecting the stress and TH axes. This opens new research directions for MOAs of phthalates in amphibians.

During the postmetamorphic period, GH synthesis is enhanced and is regulating amphibian growth (Takahashi et al., 1992). As a result, the animals grow in size, but morphology is not affected (Kikuyama et al., 1984). Genes from the GH axis were shown to be disrupted in rats treated with DBP (Bowman et al., 2005). Although the effects of MMP on GH-related genes were not investigated in our study, the monoester is not believed to have affected the GH axis. Indeed, morphological measurements were not affected in adults. Similarly, the disturbances of juvenile development are believed to be the results of accelerated metamorphosis, as opposed to an increase in GH. The decrease in body length observed at week 7 and the increases in snout-to-vent length and hind limb length at exposure completion are likely due to the hastened metamorphosis. Animals treated with phthalate reached NF66 prematurely, lost their tail and saw their body length decrease earlier than water control animals. After being fully metamorphosed, these animals started the GH-regulated developmental period before controls, leading to greater morphological measurements. It is speculated that MMP did not affect the GH axis.

Our results show that Western clawed frog metamorphosis was also disrupted by the positive control. According to a study by Heimeier et al. (2009), BPA inhibited the T3-induced gut remodeling and downregulated T3-response genes in NF54 African clawed frog tadpoles. Interestingly, the present study shows that the positive control enhanced amphibian metamorphosis without disrupting the expression of TH-related genes. Therefore, BPA might have affected amphibian development through the disruption of another axis. Sex steroids for example, have the capacity to regulate anuran metamorphosis (reviewed in Hayes, 1997). Interestingly, the apogee of amphibian sex steroid binding protein synthesis coincides with metamorphosis in salamanders (Martin and Collenot, 1975), thus suggesting that bioavailability of sex steroids changes during this important period of development. In the common toad, E1 enhanced metamorphosis, as seen by a reduction in body length (Frieden and Naile, 1955). In addition, BPA was shown to increase E1 concentrations in human cell cultures (Zhang et al., 2011) and urinary BPA levels were positively correlated with E1 levels in humans (Kim et al., 2014). Therefore, BPA is thought to have accelerated Western clawed frog metamorphosis via a putative disruption of the sex steroid axis. Such disarray of the reproductive axis could have repercussions at the organismal level, by affecting sexual differentiation for example.

Gonadal development in anurans has been proposed as a valuable model to evaluate the physiological outcome of exposure to (anti)estrogenic and (anti)androgenic compounds (Kloas and Lutz, 2006). Unlike mammals, amphibian sex differentiation does not depend solely on the genome. The influence of hormones, temperature and food availability can prevail over genotypical sex determination (reviewed in Hayes, 1998; and in Eggert, 2004). Sex differentiation takes place between NF49 and NF52 (Nieuwkoop and Faber, 1992; Miyata et al., 1999; Hu et al., 2008). When Western clawed frogs were exposed to MMP during this sensitive time window, the phthalate irreversibly skewed sex ratios towards males. To the best of the author's knowledge, only two studies investigated the effects of phthalates on sex ratios in vertebrates. In Chikae et al. (2004b), 0.01 µg/L DEHP induced more male phenotypes in a non-significant manner in medaka fry, whereas 0.01 µg/L DEHP significantly skewed sex ratios towards females in medaka embryos (Chikae et al., 2004a). Such feminisation was proposed to be mediated by CYP19 (Andrade et al., 2006). Literature suggests that phthalates decrease the activity of this enzyme (Andrade et al., 2006; Noda et al., 2007) and that other nonsteroidal CYP19 inhibitors induce male sex determination in lizards, turtles, chickens and salmon (Elbrecht and Smith, 1992; Piferrer et al., 1994; Wibbels and Crews, 1994). The CYP19 inhibitor fadrozole also masculinised treated Western clawed frogs (Duarte-Guterman et al., 2009; Olmstead et al., 2009). The effect of MMP on *cyp19* transcription could not be investigated, as *cyp19* mRNA is not detected in African clawed frog liver. The effect of MMP on CYP19 activity remains to be elucidated.

At the tested concentration, BPA was not expected to induce mortality nor malformations (Mathieu-Denoncourt et al., in preparation b). However, BPA reduced survival by 27% compared to its solvent control. BPA was chosen as a positive control of endocrine disruption and was expected to disrupt sex ratios towards females, as observed in medaka (1.8 mg/L; Yokota et al., 2000) and African clawed frogs (0.2 mg/L; Kloas et al., 1999;Levy et al., 2004). The plasticiser did not significantly affect sex ratios nor disrupt expression of sex steroid-related genes. Perhaps a higher BPA concentration or E2 would be more appropriate positive controls when working with Western clawed frogs. Although BPA's solvent increased liver weight possibly due to enzyme induction (reviewed in Maronpot et al., 2010) and delayed metamorphosis, DMSO seemed suitable for chronic amphibian assays since gene expression was not altered.

3.5 Conclusion

Since acute assays with amphibians did not assess the consequences of toxicants on later life events, the aim of this chapter was to investigate the chronic effects of MMP on Western clawed frogs throughout the life cycle. Taken altogether, our results showed that MMP induced slight mortality and that all surviving animals metamorphosed. Frogs exhibited normal physiology and were healthy, as indicated by the lack of malformations and transcriptional disruption. Considering that sex ratios were skewed at a high concentration, MMP may be a good candidate for replacement of other toxic phthalates such as BzBP, DBP and DEHP. Our study demonstrates that life cycle analyses are highly important in ecotoxicology. Sensitivity seemed to vary with age, as indicated by the survival results. In addition, if morphological measurements had been taken at exposure completion only, the acceleration of metamorphosis by MMP may have gone undetected. Selecting endpoints throughout the development allowed us to detect subtle effects on metamorphosis. This study was the first to explore the chronic effects and MOAs of phthalates in amphibians.

Chapter 4

General discussion and conclusions

4.1 Ecological significance of the results

The CMP has highlighted the paucity of data pertaining to compounds such as DMP and DCHP. In addition, although the effects of phthalates on mammals have been characterised, more research efforts are needed to determine their consequences on aquatic species. In the previous chapters, Western clawed frogs were exposed to phthalates from embryogenesis until organogenesis. Tadpoles were then chronically exposed to a monoester until metamorphosis or sexual maturity. This chapter attempts to elucidate the ecological significance of our study by comparing the results from the acute and chronic exposures. Recommendations pertaining to current regulations, prevailing animal protocols and research directions are also presented.

In our study, phthalate diesters were found to be toxic to tadpoles. Acute exposures to DMP and DCHP resulted in significant mortality. Surviving animals were often malformed, underdeveloped, or experienced altered gene expression. A principle of ecology states that four characteristics determine population size: age structure, survival rate, fecundity and sex ratio (Nunney, 1991). We have demonstrated that acute exposures to phthalate diesters reduced survival rate and decelerated tadpole development, which could thereby alter age structure. Consequently, our results suggest that phthalate diesters could be harmful to amphibian populations. The LOECs for DMP and DCHP, however, are two to three orders of magnitude greater than the highest reported concentrations in water bodies (Teil et al., 2007; Zhang and Shen, 2010). The tested diesters are therefore not believed to pose a hazard to early life stages of Western clawed frog in current environmental settings.

Contrarily to the diesters, MMP exposures had minute impacts in tadpoles. In juveniles, the monoester induced moderate mortality and prompted metamorphosis at concentrations four orders of magnitude higher than reported environmental concentrations (Suzuki et al., 2001). At the end of the life cycle, surviving Western clawed frogs had normal physiology and did not exhibit organismal or molecular signs of stress. In addition, sex ratios were skewed at a concentration well beyond reported environmental contamination. It is therefore unlikely that MMP would threaten amphibian populations in the environment.

4.2 Recommendations for Environment Canada and Health Canada

The use of DEHP, DBP, BzBP, DINP, DIDP and DNOP in soft vinyl toys or child care articles has been regulated in the European Union since 2005 and in the United States since 2008. Such consumer products cannot contain more than 1000 mg/kg of phthalate (European Union, 2006; United States, 2008). In 2009, Canada harmonised its phthalate legislation with those in effect in the United States and the European Union (Canadian Ministry of Justice, 2010), but Canada's policies are outdated when it comes to other consumer products. For example, DEHP is the only phthalate to have been prohibited in cosmetics (Canadian Ministry of Justice, 1999), whereas DBP, DEHP, BzBP, DPeP, diisopentyl phthalate, n-pentyl-isopentyl phthalate and bis(2-methoxyethyl) phthalate are banned from European cosmetics (European Union, 1976). In Canada, phthalates are not proscribed from food-contact plastics unlike in Europe (European Union, 2011). Similarly, there is no limit as to how much phthalate can be found in Canadian drinking water while American drinking water cannot contain more than 0.006 mg/L DEHP (United States, 2002). Canada could offer the same level of safety as foreign countries by adopting similar legislations and by continuing to be a leader in toxicant risk assessment (i.e. the CMP; Canada Gazette, 2013). The Canadian government could also encourage the replacement of phthalates by adipates, since the latter were shown to be much less toxic in rats and fish (Singh et al., 1973; Felder et al., 1986; Dalgaard et al., 2003). In addition, citric acid esters were found not to leach as easily as phthalates and are relatively nontoxic to rats and cats (Finkelstein and Gold, 1959; Nara et al., 2009). Implementing new legislation and employing alternatives would protect Canadians and ultimately the environment.

4.3 Recommendations pertaining to current protocols

On top of varying among chemicals and within doses, the pattern of responses observed in our study varied amongst ages. We found that MMP-induced mortality was highly dependent on age; the monoester was not toxic to embryos nor mature frogs, but significantly decreased survival in juveniles. Other toxicological studies found that juveniles were more sensitive to phthalates than adults. In male rats exposed to DEHP, weights of androgen-dependent organs and immune parameters were affected at lower doses in juveniles than in adults (Tonk et al., 2012). Likewise, in a soil-dwelling collembolan study, juvenile mortality has been shown to occur at DBP concentrations 10 times lower than concentrations inducing mortality in adults. The authors suggested that this effect was due to the

larger surface to volume ratio of juveniles, leading to higher phthalate uptake (Jensen et al., 2001). In our study, Western clawed frogs were exposed to MMP through different routes: direct exposure via the gills, diffusion through the skin and ingestion of MMP dissolved in water or sorbed to particulates. The hypothesis put forward by Jensen et al. (2001) could apply to our study as well; Western clawed frog juveniles could absorb higher amounts of MMP than adults since they have gills and a higher surface to volume ratio. Comparing MMP bioaccumulation in juvenile and adult tissues could help elucidate the increased mortality observed in juveniles. The influence of age on our mortality results demonstrates the importance of life cycle analyses. In addition, investigating the expression of sex steroid-related genes in tadpoles did not predict the effects on sexual differentiation in juveniles. It is therefore important to conduct chronic exposures and to evaluate the effects of a chemical at every stage of development.

Another noteworthy aspect of our survival results is that lower concentrations induced lower survival rates in juveniles, creating a non-monotonic dose-response curve. DCHP also induced non-monotonic response curves for *star*, *cyp19* and *pparγ* mRNA levels in tadpoles. For example, animals from the 1.5 mg/L DCHP treatment showed higher transcripts levels than animals from the higher concentration group. The latter is the concentration at which mortality, malformations and development began to be significantly affected. Surviving tadpoles that were sampled for gene expression analysis therefore had a different physiology and seemed to be more resistant to DCHP, which could potentially explain the lower mRNA levels. Although this type of non-monotonic curve corroborates with effects induced by endogenous hormones (reviewed in Calabrese, 2001) and other EDCs (Giesy et al., 2000; Oehlmann et al., 2000; Gray et al., 2001; Andrade et al., 2006), current protocols are not designed for this sort of response. Thresholds such as LC_{50}s, for example, are calculated with linear regressions. Reviewing the methods for calculating thresholds and widening the range of tested concentrations would be desirable in order to properly assess the effects of a toxicant.

Finally, gene expression analysis showed that DMSO induced cellular stress at concentrations below the recommended threshold (American Society for Testing and Materials, 1998). In the FETAX protocol, the solvent recommendations are based on only one study which assessed tadpole toxicity via mortality, malformation, growth inhibition and microsomal aminopyrine demethylase activity (Fort et al., 1989). The use of DMSO in our study showed the importance of assessing biomolecular endpoints in toxicology. Therefore, the current protocols should be revised in order to suggest more suitable solvents and to recommend the investigation of sensitive molecular endpoints in future toxicological studies.

4.4 Future research directions

Future research directions include the investigation of the chronic effects of DMP and DCHP on Western clawed frogs. In addition, assessing the effects of phthalates on oocyte and sperm production would be interesting considering that sexual differentiation was altered by MMP. Although we analysed the expression of genes belonging to several axes, clear MOAs remain to be elucidated. Hence, the binding and activation of amphibian PPARs by phthalates could be studied. Measuring the expression of genes from the oxidative stress, apoptosis and endocrine stress pathways may also enable a better understanding of the MOAs of phthalates. The latter will be accomplished shortly by microarray analysis (Mathieu-Denoncourt et al., in preparation b).

4.5 Conclusion

Overall, our results indicate that larvae, juveniles and adult frogs respond differently to phthalates. At environmentally relevant concentrations however, phthalates are not likely to induce adverse health effects in amphibian populations. Replacing phthalates by other plasticisers such as adipates or citric acid esters and limiting the use of phthalates would reduce the risk of amphibian toxicity encounter.

5. Summary

In summary, polymer flexibility and elasticity is enhanced by plasticisers. However, plasticisers are often not covalently bound to plastics and thus leach from products into the environment. A lot of research efforts focused on their effects in mammals, but data on aquatic species is lacking. In chapter 2, Western clawed frog larvae were acutely exposed to water spiked with MMP, DMP and DCHP for 72 h. The diesters induced toxicity and cellular stress, as seen by larval mortality, malformation and the upregulation of a cellular stress-related gene. DMP and DCHP also hindered tadpole development and altered the expression of *dio1*. MMP had a slight effect on development at 1,595.5 mg/L. In chapter 3, premetamorphic tadpoles were exposed to MMP for 11 or 51 weeks. The monoester induced mortality at all concentrations in juveniles. MMP also hastened metamorphosis and skewed sex ratios. Adult exposure to MMP did not have significant adverse health effects. This is the first complete investigation of the effects of phthalates in a frog species which encompasses the entire life cycle of the organisms. This study provided valuable data to the CMP that will help better assess and manage the risk of the production and use of phthalates.

References

Abbott BD, Wolf CJ, Schmid JE, Das KP, Zehr RD, Helfant L, Nakayama S, Lindstrom AB, Strynar MJ and C Lau. *Perfluorooctanoic acid-induced developmental toxicity in the mouse is dependent on expression of peroxisome proliferator-activated receptor-alpha*. Toxicological sciences, **98**, 571-581 (2007).

Abdellatif AG, Préat V, Taper HS and M Roberfroid. *The modulation of rat liver carcinogenesis by perfluorooctanoic acid, a peroxisome proliferator*. Toxicology and applied pharmacology, **111**, 530-537 (1991).

Adams WJ, Biddinger GR and KA Robillard. *A summary of the acute toxicity of 14 phthalate esters to representative aquatic organisms*. Environmental toxicology and chemistry, **14**, 1569-1574 (1995).

Akingbemi BT, Ge R, Klinefelter GR, Zirkin BR and MP Hardy. *Phthalate-induced Leydig cell hyperplasia is associated with multiple endocrine disturbances*. Proceedings of the National Academy of Sciences, **101**, 775-780 (2004a).

Akingbemi BT, Sottas CM, Koulova AI, Klinefelter GR and MP Hardy. *Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells.* Endocrinology, **145**, 592-603 (2004b).

Albrechtsen N, Dornreiter I, Grosse F, Kim E, Wiesmüller L and W Deppert. *Maintenance of genomic integrity by p53: complementary roles for activated and non-activated p53*. Oncogene, **18**, 7706-7717 (1999).

Albro PW and B Moore. *Identification of the metabolites of simple phthalate diesters in rat urine*. Journal of chromatography, **94**, 209-218 (1974).

Alonso-Magdalena P, Morimoto S, Ripoll C, Fuentes E and A Nadal. *The estrogenic effect of bisphenol A disrupts pancreatic β-cell function in vivo and induces insulin resistance*. Environmental health perspectives, **114**, 106-112 (2006).

Aluru N, Leatherland JF and MM Vijayan. *Bisphenol A in oocytes leads to growth suppression and altered stress performance in juvenile rainbow trout*. Plos one, **5**, e10741 (2010).

American Society for Testing and Materials. 1998. Standard guide for conducting the frog embryo teratogenesis assay—*Xenopus* (FETAX). E 1439-98. Philadelphia.

Andersen HR, Halling-Sørensen B and KO Kusk. *A parameter for detecting estrogenic exposure in the copepod Acartia tonsa.* Ecotoxicology and environmental safety, **44**, 56-61 (1999).

Andrade AJM, Grande SW, Talsness CE, Grote K and I Chahoud. *A dose-response study following in utero and lactational exposure to di-(2-ethylhexyl)-phthalate (DEHP): non-monotonic dose-response and low dose effects on rat brain aromatase activity.* Toxicology, **227**, 185-192 (2006).

Ankley GT, Degitz SJ, Diamond SA and JE Tietge. *Assessment of environmental stressors potentially responsible for malformations in North American anuran amphibians*. Ecotoxicology and environmental safety, **58**, 7-16 (2004).

Arase S, Ishii K, Igarashi K, Aisaki K, Yoshio Y, Matsushima A, Shimohigashi Y, Arima K, Kanno J and Y Sugimura. *Endocrine disrupter bisphenol A increases in situ estrogen production in the mouse urogenital sinus*. Biology of reproduction, **84**, 734-742 (2011).

Ashby J and H Tinwell. *Uterotrophic activity of bisphenol A in the immature rat*. Environmental health perspectives, **106**, 719-720 (1998).

Aso S, Ehara H, Miyata K, Hosyuyama S, Shiraishi K, Umano T and Y Minobe. *A two-generation reproductive toxicity study of butyl benzyl phthalate in rats.* Journal of toxicological sciences, **30**, 39-58 (2005).

Aylward LL, Hays SM, Gagné M and K Krishnan. *Derivation of biomonitoring equivalents for di-n-butyl phthalate (DBP), benzylbutyl phthalate (BzBP), and diethyl phthalate (DEP).* Regulatory toxicology and pharmacology, **55**, 259-267 (2009).

Bantle JA, Dumont JN, Finch RA, Linder G and DJ Fort. 1998. Atlas of abnormalities: a guide for the performance of FETAX – second edition. Printing services, Oklahoma State University.

Bantle JA, Finch RA, Fort DJ, Stover EL, Hull M, Kumsher-King M and AM Gaudet-Hull. *Phase III Interlaboratory study of FETAX Part 3. FETAX validation using 12 compounds with and without an exogenous metabolic activation system.* Journal of applied toxicology, **19**, 447-472 (1999).

Barber ED and DC Topping. *Subchronic 90-day oral toxicology of di(2-ethylhexyl) terephthalate in the rat*. Food and chemical toxicology, **33**, 971-978 (1995).

Barlow NJ, Phillips SL, Wallace DG, Sar M, Gaido KW and PMD Foster. *Quantitative changes in gene expression in fetal rat testes following exposure to di(n-butyl) phthalat*e. Toxicological sciences, **73**, 431-451 (2003).

Barnabé S, Beauchesne I, Cooper DG and JA Nicell. *Plasticizers and their degradation products in the process streams of a large urban physicochemical sewage treatment plant.* Water research, **42**, 153-162 (2008).

Barse AV, Chakrabarti T, Ghosh TK, Pal AK and SB Jadhao. *Endocrine disruption and metabolic changes following exposure of Cyprinus carpio to diethyl phthalate*. Pesticide biochemistry and physiology, **88**, 36-42 (2007).

Biles JE, McNeal TP, Begley TH and HC Hollifield. *Determination of bisphenol-A in reusable polycarbonate food-contact plastics and migration to food-simulating liquids*. Journal of agricultural and food chemistry, **45**, 3541-3544 (1997).

Blair JD, Ikonomou MG, Kelly BC, Surridge B and FAPC Goba. *Ultra-trace determination of phthalate ester metabolites in seawater, sediments and biota from an urbanized marine inlet by LC/ESI-MS/MS.* Environmental science and technology, **43**, 6262-6268 (2009).

Blitz IL and KWY Cho. *Anterior neurectoderm is progressively induced during gastrulation: the role of the Xenopus homeobox gene orthodenticle*. Development, **121**, 993-1004 (1995).

Blount BC, Silva MJ, Caudill SP, Needham LL, Pirkle JL, Sampson EJ, Lucier GW, Jackson RJ and JW Brock. *Levels of seven urinary phthalate metabolites in a human reference population.* Environmental health perspectives, **108**, 979-982 (2000).

Bonett RM, Hoopfer ED and RJ Denver. *Molecular mechanisms of corticosteroid synergy with thyroid hormone during tadpole metamorphosis*. General and comparative endocrinology, **168**, 209-219 (2010).

Borch J, Metzdorff SB, Vinggaard AM, Brokken L and M Dalgaard. *Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis.* Toxicology, **223**, 144-155 (2006).

Bowman CJ, Turner KJ, Sar M, Barlow NJ, Gaido KW and PMD Foster. *Altered gene expression during rat Wolffian duct development following di(n-butyl) phthalate exposure*. Toxicological sciences, **86**, 161-174 (2005).

Bradley EL, Burden RA, Leon I, Mortimer DN, Speck DR and L Castle. *Determination of phthalate diesters in foods*. Food additives and contaminants part A, **30**, 722-734 (2013).

Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V and N Olea. *Xenoestrogens released from lacquer coatings in food cans*. Environmental health perspectives, **103**, 608-612 (1995).

Brown DD and L Cai. *Amphibian metamorphosis*. Developmental biology, **306**, 20-33 (2007).

Burridge LE and K Haya. *A review of di-n-butylphthalate in the aquatic environment: concerns regarding its use in salmonid aquaculture.* Journal of the world aquaculture society, **26,** 1-13 (1995).

Burýšková B, Hilscherová K, Bláha L, Maršálek B and I Holoubek. *Toxicity and modulations of biomarkers in Xenopus laevis embryos exposed to polycyclic aromatic hydrocarbons and their N-heterocyclic derivatives*. Environmental toxicology, **21**, 590-598 (2006).

Calabrese EJ. *Estrogen and related compounds: biphasic dose responses.* Critical reviews in toxicology, **31**, 503-515 (2001).

Calafat AM, Kuklenyik Z, Reidy JA, Caudill SP, Ekong J and LL Needham. *Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population.* Environmental health perspectives, **113**, 391-395 (2005).

Call DJ, Markee TP, Geiger DL, Brooke LT, VandeVenter FA, Cox DA, Genisot KI, Robillard KA, Gorsuch JW, Parkerton TF, Reiley MC, Ankley GT and DR Mount. *An assessment of the toxicity of phthalate esters to freshwater benthos. 1. Aquatic exposures.* Environmental toxicology and chemistry, **20**, 1798-1804 (2001).

Campioli E, Batarseh A, Li J and V Papadopoulos. *The endocrine disruptor mono-(2-ethylhexyl) phthalate affects the differentiation of human liposarcoma cells (SW 872)*. Plos one, **6**, e28750 (2011).

Canada Gazette, Part I: Vol.147, No. 29 - July 13, 2013.

Canadian Ministry of Justice. 1999. Canadian environmental protection act, 1999 S.C. 1999, c. 33.

Canadian Ministry of Justice. 2010. Phthalates regulations SOR/2010-298.

Cao XL. *Phthalate esters in foods: sources, occurrence, and analytical method*s. Comprehensive reviews in food science and food safety, **9**, 21-43 (2010).

Carlsen E, Giwercman A, Keiding N and NE Skakkebæk. *Evidence for decreasing quality of semen during past 50 years*. British medical journal, **305**, 609-613 (1992).

Carlstedt F, Jönsson BAG and CG Bornehag. *PVC flooring is related to human uptake of phthalates in infants*. Indoor air, **23**, 32-39 (2012).

Carnevali O, Tosti L, Speciale C, Peng C, Zhu Y and F Maradonna. *DEHP impairs zebrafish reproduction by affecting critical factors in oogenesis*. Plos one, **5**, e10201 (2010).

Changez M, Koul V, Krishna B, Dinda AK and V Choudhary. *Studies on biodegradation and release of gentamycin sulphate from interpenetrating network hydrogels based on poly(acrylic acid) and gelatin: in vitro and in vivo*. Biomaterials, **25**, 139-146 (2004).

Cherradi N, Rossier MF, Vallotton MB, Timberg R, Friedberg I, Orly J, Wang XJ, Stocco DM and AM Capponi. *Submitochondrial distribution of three key steroidogenic proteins (steroidogenic acute regulatory protein and cytochrome P450scc and 3β-hydroxysteroid dehydrogeniase isomerise enzymes) upon stimulation by intracellular calcium in adrenal glomerulosa cells.* Journal of biological chemistry, **272**, 7899-7907 (1997).

Chikae M, Hatano Y, Ikeda R, Morita Y, Hasan Q and E Tamiya. *Effects of bis(2-ethylhexyl) phthalate and benzo[a]pyrene on the embryos of Japanese medaka (Oryzias latipes)*. Environmental toxicology and pharmacology, **16**, 141-145 (2004a).

Chikae M, Ikeda R, Hatano Y, Hasan Q, Morita Y and E Tamiya. *Effects of bis(2-ethylhexyl) phthalate, γ-hexachlorocyclohexane, and 17β-estradiol on the fry stage of medaka (Oryzias latipes)*. Environmental toxicology and pharmacology, **18**, 9-12 (2004b).

Cho WS, Han BS, Ahn B, Nam KT, Choi M, Oh SY, Kim SH, Jeong J and DD Jang. *Peroxisome proliferator di-isodecyl phthalate has no carcinogenic potential in Fischer 344 rats*. Toxicology letters, **178**, 110-116 (2008).

Christiansen LB, Pedersen KL, Korsgaard B and P Bjerregaard. *Estrogenicity of xenobiotics in rainbow trout (Oncorhynchus mykiss) using in vivo synthesis of vitellogenin as a biomarker*. Marine environmental research, **46**, 137-140 (1998).

Clara M, Windhofer G, Hartl W, Braun K, Simon M, Gans O, Scheffknecht C and A Chovanec. *Occurrence of phthalates in surface runoff, untreated and treated wastewater and fate during wastewater treatment*. Chemosphere, **78**, 1078-1084 (2010).

Crago J and R Klaper. *A mixture of an environmentally realistic concentration of a phthalate and herbicide reduces testosterone n male fathead minnow (Pimephales promelas) through a novel mechanism of action.* Aquatic toxicology, **110**, 74-83 (2012).

Craig ZR, Hannon PR, Wang W, Ziv-Gal A and JA Flaws. *Di-n-butyl phthalate disrupts the expression of genes involved in cell cycle and apoptotic pathways in mouse ovarian antral follicles*. Biology of reproduction, **88**, 1-10 (2013).

Crain DA, Eriksen M, Iguchi T, Jobling S, Laufer H, LeBlanc GA and LJJ Guillette. *An ecological assessment of bisphenol-A: evidence from comparative biology*. Reproductive toxicology, **24**, 225-239 (2007).

Dalgaard M, Hass U, Vinggaard AM, Jarfelt K, Lam HR, Sørensen IK, Sommer HM and O Ladefoged. *Di(2-ethylhexyl) adipate (DEHA) induced developmental toxicity but not antiandrogenic effects in pre- and postnatally exposed Wistar rats*. Reproductive toxicology, **17**, 163-170 (2003).

Daniels PH. *A brief overview of theories of PVC plasticization and methods used to evaluate PVC-plasticizer interaction*. Journal of vinyl and additive technology, **15**, 219-223 (2009).

Dawson DA, Fort DJ, Newell DL and JA Bantle. *Developmental toxicity testing with FETAX : evaluation of five compounds*. Drug and chemical toxicology, **12**, 67-75 (1989).

Déchaud H, Ravard C, Claustrat F, Brac de la Perrière A and M Pugeat. *Xenoestrogen interaction with human sex hormone-binding globulin (hSHBG)*. Steroids, **64**, 328-334 (1999).

DeFoe DL, Holcombe GW, Hammermeister DE and KE Biesinger. *Solubility and toxicity of eight phthalate esters to four aquatic organisms.* Environmental toxicology and chemistry, **9**, 623-636 (1990).

Degitz SJ, Holcombe GW, Flynn KM, Kosian PA, Korte JJ and JE Tietge. *Progress towards development of an amphibian-based thyroid screening assay using Xenopus laevis. Organismal and thyroidal responses to the model compounds 6-propylthiouracil, methimazole, and thyroxine.* Toxicological sciences, **87**, 353-364 (2005).

Demir APT and S Ulutan. *Migration of phthalate and non-phthalate plasticizers out of plasticized PVC films into air*. Journal of applied polymer science, **128**, 1948-1961 (2013).

Denver RJ. *Stress hormones mediate environment-genotype interactions during amphibian development.* General and comparative endocrinology, **164**, 20-31(2009).

Deyo JA. *Carcinogenecity and chronic toxicity of di-2-ethyhexyl terephthalate (DEHT) following a 2-year dietary exposure in Fischer 344 rats*. Food and chemical toxicology, **46**, 990-1005 (2008).

Duan Z, Zhu L, Zhu L, Yao K and X Zhu. *Individual and joint toxic effects of pentachlorophenol and bisphenol A on the development of zebrafish (Danio rerio) embryo*. Ecotoxicology and environmental safety, **71**, 774-780 (2008).

Duarte-Guterman P, Langlois VS, Hodgkinson K, Pauli BD and GM Cooke. *The aromatase inhibitor fadrozole and the 5-reductase inhibitor finasteride affect gonadal differentiation and gene expression in the frog Silurana tropicalis.* Sexual development, **3**, 333-341 (2009).

Duarte-Guterman P, Langlois VS, Pauli BD and VL Trudeau. *Expression and T3 regulation of thyroid hormone- and sex steroid-related genes during*

Silurana (Xenopus) tropicalis early development. General and comparative endocrinology, **166**, 428-435 (2010).

Dufour JM, Vo MN, Bhattacharya N, Okita J, Okita R and KH Kim. *Peroxisome proliferators disrupt retinoic acid receptor alpha signaling in the testis.* Biology of reproduction, **68**, 1215-1224 (2003).

Dumpert K and E Zietz. *Platanna (Xenopus laevis) as a test organism for determining the embryotoxic effects of environmental chemicals.* Ecotoxicology and environmental safety, **8**, 55-74 (1984).

Duty SM, Singh NP, Silva MJ, Barr DB, Brock JW, Ryan L, Herrick RF, Christiani DC and R Hausser. *The relationship between environmental exposures to phthalates and DNA damage in human sperm using the neutral comet assay.* Environmental health perspectives, **111**, 1164-1169 (2003).

Eertmans F, Dhooge W, Stuyvaert S and F Comhaire. *Endocrine disruptors: effects on male fertility and screening tools for their assessment.* Toxicology in vitro, **17**, 515-524 (2003).

Eggert C. *Sex determination: the amphibian models*. Reproduction nutrition development, **44**, 539-549 (2004).

Eisenberg ML, Hsieh MH, Walters RC, Krasnow R and LI Lipshultz. *The relationship between anogenital distance, fatherhood, and fertility in adult men.* Plos one, **6**, e18973 (2011).

Elbrecht A and RG Smith. *Aromatase enzyme activity and sex determination in chickens*. Science, **255**, 467-470 (1992).

Ema M, Amano H and Y Ogawa. *Characterization of the developmental toxicity of di-n-butyl phthalate in rats.* Toxicology, **86**, 163-174 (1994).

Ema M, Harazono A, Miyawaki E and Y Ogawa. *Characterization of developmental toxicity of mono-n-benzyl phthalate in rats.* Reproductive toxicology, **10**, 365-372 (1996).

Ema M, Miyawaki E and K Kawashima. *Developmental effects of plasticizer butyl benzyl phthalate after a single administration in rats*. Journal of applied toxicology, **19**, 357-365 (1999).

Ema M, Miyawaki E, Harazono A and K Kawashima. *Developmental toxicity evaluation of phthalic acid, one of the metabolites of phthalic acid esters, in rats*. Toxicology letters, **93**, 109-115 (1997).

Ema M, Miyawaki E, Hirose A and E Kamata. *Decreased anogenital distance and increased incidence of undescended testes in fetuses of rats given monobenzyl phthalate, a major metabolite of butyl benzyl phthalate.* Reproductive toxicology, **17**, 407-412 (2003).

Ema M. *Antiandrogenic effects of dibutyl phthalate and its metabolite, monobutyl phthalate, in rats*. Congenital anomalies, **42**, 297-308 (2002).

Eriksen MS, Bakken M, Espmark Å, Braastad BO and R Salte. *Prespawning stress in farmed Atlantic salmon Salmo salar: maternal cortisol exposure and hyperthermia during embryonic development affect offspring survival, growth and incidence of malformations*. Journal of fish biology, **69**, 114-129 (2006).

Erkekoglu P, Giray BK, Kizilgün M, Rachidi W, Hininger-Favier I, Roussel AM, Favier A and F Hincal. *Di(2-ethylhexyl)phthalate-induced renal oxidative stress in rats and protective effect of selenium*. Toxicology mechanisms and methods, **22**, 415-423 (2012).

Euling SY, White LD, Kim AS, Sen B, Wilson VS, Keshava C, Keshava N, Hester S, Ovacik MA, Ierapetritou MG, Androulakis IP and KW Gaido. *Use of genomic data in risk assessment case study: II. Evaluation of the dibutyl phthalate toxicogenomic data set*. Toxicology and applied pharmacology, **271**, 349-362 (2013).

European Union. 1976. Council directive of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products (76/768/EEC).

European Union. 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the registration, evaluation, authorisation and restriction of chemicals (REACH), establishing a European Chemical Agency, amending Directive 1999/45/EC and repealing Council regulation (EEC) No 793/93 and Commission regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC.

European Union. 2011. Commission regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food.

Evans NP, North T, Dye S and T Sweeney. *Differential effects of the endocrine-disrupting compounds bisphenol-A and octylphenol on gonadotropin secretion, in prepubertal ewe lambs*. Domestic animal endocrinology, **26**, 61-73 (2004).

Faber WD, Deyo JA, Stump DG and K Ruble. *Two-generation reproduction study of di-2-ethylhexyl terephthalate in Crl:CD rats*. Birth defects research part B, **80**, 69-81 (2007a).

Faber WD, Deyo JA, Stump DG, Navarro L, Ruble K and J Knapp. *Developmental toxicity and uterotrophic studies with di-2-ethylhexyl terephthalate*. Birth defects research part B, **80**, 396-405 (2007b).

Fasano E, Bono-Blay F, Cirillo T, Montuori P and S Lacorte. *Migration of phthalates, alkylphenols, bisphenol A and di(2-ethylhexyl)adipate from food packaging*. Food control, **27**, 132-138 (2012).

Feige JN, Gelman L, Rossi D, Zoete V, Métivier R, Tudor C, Anghel SI, Grosdidier A, Lathion C, Engelborghs Y, Michielin O, Wahli W and B Desvergne. *The endocrine disruptor monoethyl-hexyl-phthalate is a selective peroxisome proliferator-activated receptor γ modulator that promotes adipogenesis.* Journal of biological chemistry, **282**, 19152-19166 (2007).

Feinstein RN, Fry RJM and EF Staffeldt. *Carcinogenic and antitumor effects of aminotriazole on acatalasemic and normal catalase mice*. Journal of the national cancer institute, **60**, 1113-1116 (1978).

Felder JD, Adams WJ and VW Saeger. *Assessment of the safety of dioctyl adipate in freshwater environments*. Environmental toxicology and chemistry, **5**, 777-784 (1986).

Fernandez MF, Arrebola JP, Taoufiki J, Navalón A, Ballesteros O, Pulgar R, Vilchez JL and N Olea. *Bisphenol-A and chlorinated derivatives in adipose tissue of women.* Reproductive toxicology, **24**, 259-264 (2007).

Field EA, Price CJ, Sleet RB, George JD, Marr MC, Myers CB, Schwetz BA and RE Morrissey. *Developmental toxicity evaluation of diethyl and dimethyl phthalate in rats.* Teratology, **48**, 33-44 (1993).

Finkelstein M and H Gold. *Toxicology of the citric acid esters: tributyl citrate, acetyl tributyl citrate, triethyl citrate, and acetyl triethyl citrate*. Toxicology and applied pharmacology, **1**, 283-298 (1959).

Flood DEK, Fernandino JI and VS Langlois. *Thyroid hormones in male reproductive development: evidence for direct crosstalk between the androgen and thyroid hormone axes*. General and comparative endocrinology, **192**, 2-14 (2013).

Fort DJ, James BL and JA Bantle. *Evaluation of the developmental toxicity of five compounds with the Frog Embryo Teratogenesis Assay: Xenopus (FETAX) and a metabolic activation system*. Journal of applied toxicology, **9**, 377-388 (1989).

Fort DJ, Rogers RL, Thomas JH, Buzzard BO, Noll AM and CD Spaulding. *Comparative sensitivity of Xenopus tropicalis and Xenopus laevis as test species for the FETAX model.* Journal of Applied Toxicology, **24**, 443-457 (2004).

Foster PMD, Lake BG, Thomas LV, Cook MW and SD Gangolli. *Studies on the testicular effects and zinc excretion produced by various isomers of monobutyl-o-phthalate in the rat*. Chemico-biological interactions, **34**, 233-238 (1981).

Foster PMD. *Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters.* International journal of andrology, **29**, 140-147 (2006).

Frederiksen H, Sørensen K, Mouritsen A, Aksglaede L, Hagen CP, Petersen JH, Skakkebaek NE, Andersson AM and A Juul. *High urinary phthalate concentration associated with delayed pubarche in girls.* International journal of andrology, **35**, 216-226 (2012).

Frieden E and B Naile. *Biochemistry of amphibian metamorphosis: I. Enhancement of induced metamorphosis by gluco-corticoids.* Science, **121**, 37-39 (1955).

Fromme H, Küchler T, Otto T, Pilz K, Müller J and A Wenzel. *Occurrence of phthalates and bisphenol A and F in the environment*. Water research, **36**, 1429-1438 (2002).

Fujii S, Yabe K, Furukawa M, Hirata M, Kiguchi M and T Ikka. *A two-generation reproductive toxicity study on diethyl phthalate (DEP) in rats.* Journal of toxicological sciences, **30**, 97-116 (2005).

Fukuoka M, Tanimoto T, Zhou Y, Kawasaki N and A Tanaka. *Mechanism of testicular atrophy induced by di-n-butyl phthalate in rats. Part 1*. Journal of applied toxicology, **9**, 277-283 (1989).

Gayathri NS, Dhanya CR, Indu AR and PA Kurup. *Changes in some hormones by low doses of di (2-ethyl hexyl) phthalate (DEHP), a commonly used plasticizer in PVC blood storage bags & medical tubing.* The Indian journal of medical research, **119**, 139-144 (2004).

Gazouli M, Yao ZX, Boujrad N, Corton JC, Culty M and V Papadopoulos. *Effect of peroxisome proliferators on leydig cell peripheral-type benzodiazepine receptor gene expression, hormone-stimulated cholesterol transport, and steroidogenesis: role of the peroxisome proliferator-activator receptor α.* Endocrinology, **143**, 2571-2583 (2002).

Ghisari M and EC Bonefeld-Jorgensen. *Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions.* Toxicology letters, **189**, 67-77 (2009).

Ghorpade N, Mehta V, Khare M, Sinkar P, Krishnan S and CV Rao. *Toxicity study of diethyl phthalate on freshwater Cirrhina mrigala.* Ecotoxicology and environmental safety, **53**, 255-258 (2002).

Gibert Y, Sassi-Messai A, Fini JB, Bernard L, Zalko D, Cravedi JP, Balaguer P, Andersson-Lendahl M, Demeneix B and V Laudet. *Bisphenol A induces otolith malformations during vertebrate embryogenesis.* BioMed central developmental biology, **11**, 1-17 (2011).

Giesy JP, Pierens SL, Snyder EM, Miles-Richardson S, Kramer VJ, Snyder SA, Nichols KM and DA Villeneuve. *Effects of 4-nonylphenol on fecundity and biomarkers of estrogenicity in fathead minnows (Pimephales promelas).* Environmental toxicology and chemistry, **19**, 1368-1377 (2000).

Gillardin V, Silvestre F, Dieu M, Delaive E, Raes M, Thomé JP and P Kestemont. *Protein expression profiling in the African clawed frog Xenopus laevis tadpoles exposed to the polychlorinated biphenyl mixture aroclor 1254.* Molecular and cellular proteomics, **8**, 596-611 (2009).

Giribabu N, Sainath SB and PS Reddy. *Prenatal di-n-butyl phthalate exposure alters reproductive functions at adulthood in male rats*. Environmental toxicology, **29**, 534-544 (2014).

Glennemeier KA and RJ Denver. *Developmental changes in interrenal responsiveness in anuran amphibians.* Integrative and comparative biology, **42**, 565-573 (2002).

Gray KM and PA Janssens. *Gonadal hormones inhibit the induction of metamorphosis by thyroid hormones in Xenopus laevis tadpoles in vivo, but not in vitro.* General and comparative endocrinology, **77**, 202-211 (1990).

Gray LEJ, Barlow NJ, Howdeshell KL, Ostby JS, Furr JR and CL Gray. *Transgenerational effects of di (2-ethylhexyl) phthalate in the male CRL:CD(SD) rat: Added value of assessing multiple offspring per litter*. Toxicological sciences, **110**, 411-425 (2009).

Gray LEJ, Ostby J, Furr J, Price M, Veeramachaneni DNR and L Parks. *Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat.* Toxicological sciences, **58**, 350-365 (2000).

Gray LEJ, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, Veeramachaneni DN, Wilson V, Price M, Hotchkiss A, Orlando E and L Guillette. *Effects of environmental antiandrogens on reproductive development in experimental animals.* Human reproductive update, **7**, 248-264 (2001).

Gray TJB, Lake BG, Beamand JA, Foster JR and SD Gangolli. *Peroxisomal effects of phthalate esters in primary cultures of rat hepatocytes*. Toxicology, **28**, 167-179 (1983).

Gupta RK, Singh JM, Leslie TC, Meachum S, Flaws JA and HHC Yao. *Di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate inhibit growth and reduce estradiol levels of antral follicles in vitro.* Toxicology and applied pharmacology, **242**, 224-230 (2010a).

Gupta SC, Sharma A, Mishra M, Mishra R and DK Chowdhuri. *Heat shock proteins in toxicology: how close and how far?* Life sciences, **86**, 377-384 (2010b).

Hanioka N, Jinno H, Nishimura T and M Ando. *Suppression of male-specific cytochrome P450 isoforms by bisphenol A in rat liver*. Archives of toxicology, **72**, 387-394 (1998).

Hannas BR, Furr J, Lambright CS, Wilson VS, Foster PMD and LEJ Gray. *Dipentyl phthalate dosing during sexual differentiation disrupts fetal testis function and postnatal development of the male Sprague-Dawley rat with greater relative potency than other phthalates*. Toxicological sciences, **120**, 184-193 (2011).

Hauser R, Meeker JD, Singh NP, Silva MJ, Ryan L, Duty S and AM Calafat. *DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites.* Human reproduction, **22**, 688-695 (2007).

Hayes TB. *Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms.* Journal of experimental zoology, **281**, 373-399 (1998).

Hayes TB. *Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis*. American zoologist, **37**, 185-194 (1997).

Heimeier RA, Das B, Buchholz DR and YB Shi. *The xenoestrogen bisphenol A inhibits postembryonic vertebrate development by antagonizing gene regulation by thyroid hormone*. Endocrinology, **150**, 2964-2973 (2009).

Hendriks AJ, Mass-Diepeveen JL, Noordsij A and MA Van der Gaag. *Monitoring response of XAD-concentrated water in the Rhine delta: a major part of the toxic compounds remains unidentified*. Water research, **28**, 581-598 (1994).

Hernández-Díaz S, Mitchell AA, Kelley KE, Calafat AM and R Hauser. *Medications as a potential source of exposure to phthalates in the U.S. population*. Environmental health perspectives, **117**, 185-189 (2009).

Higuchi TT, Gray LE, Sawyer HR and DNR Veeramachaneni. *Chronic exposure to di-n-butyl phthalate causes high mortality and adversely affects metamorphosis in Xenopus laevis*. Biology of reproduction, **64**, 348-349 (2001).

Higuchi TT, Palmer JS, Gray LEJ and DNR Veeramachaneni. *Effects of dibutyl phthalate in male rabbits following in utero, adolescent, or postpubertal exposure*. Toxicological sciences, **72**, 301-313 (2003).

Hirano M, Ishibashi H, Matsumura N, Nagao Y, Watanabe N, Watanabe A, Onikura N, Kishi K and K Arizono. *Acute toxicity responses of two crustaceans, Americamysis bahia and Daphnia magna, to endocrine disrupters*. Journal of health science, **50**, 97-100 (2004).

Hofmann PJ, Schomburg L and J Köhrle. *Interference of endocrine disrupters with thyroid hormone receptor – dependent transactivation.* Toxicological sciences, **110**, 125-137 (2009).

Horn O, Nalli S, Cooper D and J Nicell. *Plasticizer metabolites in the environment*. Water research, **38**, 3693-3698 (2004).

Hoshino N, Iwai M and Y Okazaki. *A two-generation reproductive toxicity study of dicyclohexyl phthalate in rats*. Journal of toxicological sciences, **30**, 79-96 (2005).

Hotchkiss AK, Rider CV, Blystone CR, Wilson VS, Hartig PC, Ankley GT, Foster PM, Gray CL and LE Gray. *Fifteen years after «wingspread» - environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go*. Toxicological sciences, **105**, 235-259 (2008).

Howdeshell KL, Wilson VS, Furr J, Lambright CR, Rider CV, Blystone CR, Hotchkiss AK and LEJ Gray. *A mixture of five phthalate esters inhibits fetal testicular testosterone production in the Sprague-Dawley rat in a cumulative, dose-additive manner*. Toxicological sciences, **105**, 153-165 (2008).

Hu F, Smith EE and JA Carr. *Effects of larval exposure to estradiol on spermatogenesis and in vitro gonadal steroid secretion in African clawed frogs, Xenopus laevis.* General and comparative endocrinology, **155**, 190-200 (2008).

Hu Y, Dong C, Chen M, Lu J, Han X, Qiu L, Chen Y, Qin J, Li X, Gu A, Xia Y, Sun H, Li Z and Y Wang. *Low-dose monobutyl phthalate stimulates steroidogenesis through steroidogenic acute regulatory protein regulated by SF-1, GATA-4 and C/EBP-beta in mouse Leydig tumor cells.* Reproductive biology and endocrinology, **11**, 72 (2013).

Huang PC, Kuo PL, Guo YL, Liao PC and CC Lee. *Associations between urinary phthalate monoesters and thyroid hormones in pregnant women*. Human reproduction, **22**, 2715-2722 (2007).

Hurst CH and DJ Waxman. *Activation of PPARα and PPARγ by environmental phthalate monoesters.* Toxicological sciences, **74**, 297-308 (2003).

Hushka LJ, Waterman SJ, Keller LH, Trimmer GW, Freeman JJ, Ambroso JL, Nicolich M and RH McKee. *Two-generation reproduction studies in rats fed di-isodecyl phthalate*. Reproductive toxicology, **15**, 153-169 (2001).

Ike M, Chen M-Y, Jin C-S and M Fujita. *Acute toxicity, mutagenicity, and estrogenicity of biodegradation products of bisphenol-A.* Environmental toxicology, **17**, 457-461 (2002).

Imaoka S, Mori T and T Kinoshita. *Bisphenol A causes malformation of the head region in embryos of Xenopus laevis and decreases the expression of the ESR-1 gene mediated by Notch signaling.* Biological and pharmacological bulletin, **30**, 371-374 (2007).

Isenberg JS, Kamendulis LM, Ackley DC, Smith JH, Pugh GJ, Lington AW, McKee RH and JE Klaunig. *Reversibility and persistence of di-2-ethylhexyl phthalate (DEHP)- and phenobartial-induced hepatocellular changes in rodents.* Toxicological sciences, **64**, 192-199 (2001).

Isenberg JS, Kamendulis LM, Smith JH, Ackley DC, Pugh GJ, Lington AW and JE Klaunig. *Effects of di-2-ethylhexyl phthalate (DEHP) on gap-junctional intercellular communication (GJIC), DNA synthesis, and peroxisomal beta oxidation (PBOX) in rat, mouse, and hamster liver*. Toxicological sciences, **56**, 73-85 (2000).

Ishihara A, Sawatsubashi S and K Yamauchi. *Endocrine disrupting chemicals: interference of thyroid hormone binding to transthyretins and to thyroid hormone receptors.* Molecular and cellular endocrinology, **199**, 105-117 (2003).

Ito Y, Yoshimura M, Tanaka T and K Tanaka. *Effect of lipophilicity on the bioavailability of drugs after percutaneous administration by dissolving microneedles*. Journal of pharmaceutical sciences, **101**, 1145-1156 (2012).

Iwamuro S, Sakakibara M, Terao M, Ozawa A, Kurobe C, Shigeura T, Kato M and S Kikuyama. *Teratogenic and anti-metamorphic effects of bisphenol A on embryonic and larval Xenopus laevis.* General and comparative endocrinology, **133**, 189-198 (2003).

Iwamuro S, Yamada M, Kato M and S Kikuyama. *Effects of bisphenol A on thyroid hormone-dependent up-regulation of thyroid hormone receptor α*

and β and down-regulation of retinoid X receptor γ in Xenopus tail culture. Life sciences, **79**, 2165-2171 (2006).

Jagnytsch O, Opitz R, Lutz I and W Kloas. *Effects of tetrabromobisphenol A on larval development and thyroid hormone-regulated biomarkers of the amphibian Xenopus laevis.* Environmental research, **101**, 340-348 (2006).

Jarfelt K, Dalgaard M, Hass U, Borch J, Jacobsen H and O Ladefoged. *Antiandrogenic effects in male rats perinatally exposed to a mixture of di(2-ethyhexyl) phthalate and di(2-ethyhexyl) adipate*. Reproductive toxicology, **19**, 505-515 (2005).

Jensen J, van Langevelde J, Pritzl G and PH Krogh. *Effects of di(2-ethylhexyl) phthalate and dibutyl phthalate on the collembolan Folsomia fimetaria*. Environmental toxicology and chemistry, **20**, 1085-1091 (2001).

Jobling S, Casey D, Rodgers-Gray T, Oehlmann J, Schulte-Oehlmann U, Pawlowski A, Baunbeck T, Turner AP and CR Tyler. *Comparative responses of mulluscs and fish to environmental estrogens and an estrogenic effluent*. Aquatic toxicology, **65**, 205-220 (2003).

Jones HB, Garside DA, Liu R and JC Roberts. *The influence of phthalate esters on Leydig cell structure and function in vitro and in vivo*. Experimental and molecular pathology, **58**, 179-193 (1993).

Jonsson S and A Baun. *Toxicity of mono- and diesters of o-phthalic esters to a crustacean, a green alga, and a bacterium*. Environmental toxicology and chemistry, **22**, 3037-3043 (2003).

Jurgella GF, Marwah A, Malison JA, Peterson R and TP Barry. *Effects of xenobiotics and steroids on renal and hepatic estrogen metabolism in lake trout.* General and comparative endocrinology, **148**, 273-281 (2006).

Kang SC and BM Lee. *DNA methylation of estrogen receptor α gene by phthalates*. Journal of toxicology and environmental health part A, **68**, 1995-2003 (2005).

Kasahara E, Sato EF, Miyoshi M, Konaka R, Hiramoto K, Sasaki J, Tokuda M, Nakano Y and M Inoue. *Role of oxidative stress in germ cell apoptosis induced by di(2-ethylhexyl)phthalate*. Biochemical journal, **365**, 849-856 (2002).

Kawai K, Murakami S, Senba E, Yamanaka T, Fujiwara Y, Arimura C, Nozaki T, Takii M and C Kubo. *Changes in estrogen receptors α and β expression in the brain of mice exposed prenatally to bisphenol A.* Regulatory toxicology and pharmacology, **47**, 166-170 (2007).

Keith Y, Cornu MC, Canning PM, Foster J, Lhuguenot JC and CR Elcombe. *Peroxisome proliferation due to di (2-ethylhexyl) adipate, 2-ehtylhexanol and 2-ethylhexanoic acid.* Archives of toxicology, **66**, 321-326 (1992).

Kelley KE, Hernández-Díaz S, Chaplin EL, Hauser R and AA Mitchell. *Identification of phthalates in medications and dietary supplement formulations in the United States and Canada*. Environmental health perspectives, **120**, 379-394 (2012).

Kikuyama S, Ishii T, Kobayashi T and K Yamamoto. *Effect of growth hormone-containing fraction obtained from bullfrog hypophyses on growth of Xenopus juveniles*. Proceedings of the Japan Academy series B, **60**, 69-72 (1984).

Kikuyama S, Niki K, Mayumi M, Shibayama R, Nishikawa M and N Shintake. *Studies on corticoid action on the toad tadpole tail in vitro.* General and comparative endocrinology, **52**, 395-399 (1983).

Kim EJ, Lee D, Chung BC, Pyo H and J Lee. *Association between urinary levels of bisphenol-A and estrogen metabolism in Korean adults*. Science of the total environment, **470-471**, 1401-1407 (2014).

Kim SW, Petersen RV and ES Lee. *Effect of phthalate plasticizer on blood compatibility of polyvinyl chloride*. Journal of pharmaceutical sciences, **65**, 670-673 (1976).

Kitamura S, Jinno N, Ohta S, Kuroki H and N Fujimoto. *Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A.* Biochemical and biophysical research communications, **293**, 554-559 (2002).

Kitamura S, Kato T, Iida M, Jinno N, Suzuki T, Ohta S, Fujimoto N, Hanada H, Kashiwagi K and A Kashiwagi. *Anti-thyroid hormonal activity of tetrabromobisphenol A, a flame retardant, and related compounds: affinity to the mammalian thyroid hormone receptor, and effect on tadpole metamorphosis.* Life sciences, **76**, 1589-1601 (2005a).

Kitamura S, Suzuki T, Sanoh S, Kohta R, Jinno N, Sugihara K, Yoshihara S, Fujimoto N, Watanabe H and S Ohta. *Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds*. Toxicological sciences, **84**, 249-259 (2005b).

Kloas W and I Lutz. *Amphibians as model to study endocrine disrupters*. Journal of chromatography A, **1130**, 16-27 (2006).

Kloas W, I Lutz and R Einspanier. *Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals in vitro and in vivo*. The science of total environment, **225**, 59-68 (1999).

Klonisch T, Fowler PA and S Hombach-Klonisch. *Molecular and genetic regulation of testis descent and external genitalia development.* Developmental biology, **270**, 1-18 (2004).

Knudsen FR and TG Pottinger. *Interaction of endocrine disrupting chemicals, singly and in combination, with estrogen-, androgen-, and corticosteroid-binding sites in rainbow trout (Oncorhynchus mykiss).* Aquatic toxicology, **44**, 159-170 (1999).

Koch HM, Müller J and J Angerer. *Determination of secondary, oxidised di-iso-nonylphthalate (DINP) metabolites in human urine representative for the exposure to commercial DINP plasticizers.* Journal of chromatography B, **847**, 114-125 (2007).

Kowalewski MP, Dyson MT, Manna PR and DM Stocco. *Involvement of peroxisome proliferator-activated receptor γ in gonadal steroidogenesis and steroidogenic acute regulatory protein expression.* Reproduction, fertility and development, **21**, 909-922 (2009).

Krain LP and RJ Denver. *Developmental expression and hormonal regulation of glucocorticoid and thyroid hormone receptors during metamorphosis in Xenopus laevis.* Journal of endocrinology, **181**, 91-104 (2004).

Kuiper GGJM, Lemmen JG, Carlsson BO, Corton JC, Safe SH, Van der Saag PT, Van der Burg B and JA Gustafsson. *Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β*. Endocrinology, **139**, 4252-4263 (1998).

Kurata Y, Kidachi F, Yokoyama M, Toyota N, Tsuchitani M and M Katoh. *Subchronic toxicity of di(2-ethylhexyl)phthalate in common marmosets:*

lack of hepatic peroxisome proliferation, testicular atrophy, or pancreatic acinar cell hyperplasia. Toxicological sciences, **42**, 49-56 (1998).

Kusk KO, Krüger T, Long M, Taxvig C, Lykkesfeldt AE, Frederiksen H, Andersson AM, Andersen HR and KMS Hansen. *Endocrine potency of wastewater: contents of endocrine disrupting chemicals and effects measured by in vivo and in vitro assays*. Environmental toxicology and chemistry, **30**, 413-426 (2011).

Kwack SJ, Kim KB, Kim HS and BM Lee. *Comparative toxicological evaluation of phthalate diesters and metabolites in Sprague-Dawley male rats for risk assessment.* Journal of toxicology and environmental health part A, **72**, 1446-1454 (2009).

Kwak HI, Bae MO, Lee MH, Lee YS, Lee BJ, Kang KS, Chae CH, Sung HJ, Shin JS, Kim JH and WC Mar. *Effects of nonylphenol, bisphenol A, and their mixture on the viviparous swordtail fish (Xiphophorus helleri)*. Environmental toxicology and chemistry, **20**, 787-795 (2001).

Kwintkiewicz J, Nishi Y, Yanase T and LC Giudice. *Peroxisome proliferator-activated receptor-γ mediates bisphenol A inhibition of FSH-stimulated IGF-1, aromatase, and estradiol in human granulosa cells.* Environmental health perspectives, **118**, 400-406 (2010).

Lahnsteiner F, Berger B, Kletzl M and T Weismann. *Effect of bisphenol A on maturation and quality of semen and eggs in the brown trout, Salmo trutta f. fario.* Aquatic toxicology, **75**, 213-224 (2005).

Lahousse SA, Wallace DG, Liu D, Gaido KW and KJ Johnson. *Testicular gene expression profiling following prepubertal rat mono-(2-ethylhexyl) phthalate exposure suggests a common initial genetic response at fetal and prepubertal ages.* Toxicological sciences, **93**, 369-381 (2006).

Lake BG, Foster JR, Collins MA, Stubberfield CR, Gangolli SD and SP Srivastava. *Studies on the effects of orally administered dicyclohexyl phthalate in the rat.* Acta pharmacologica et toxicologica, **51**, 217-226 (1982).

Langlois VS, Duarte-Guterman P, Ing S, Pauli BD, Cooke GM and VL Trudeau. *Fadrozole and finasteride exposures modulate sex steroid- and thyroid hormone-related gene expression in Silurana (Xenopus) tropicalis early larval development.* General and comparative endocrinology, **166**, 417-427 (2010).

Lapinskas PJ, Brown S, Leesnitzer LM, Blanchard S, Swanson C, Cattley RC and JC Corton. *Role of PPARα in mediating the effects of phthalates and metabolites in the liver*. Toxicology, **207**, 149-163 (2005).

Larsson P and A Thurén. *Di-2-ethylhexylphthalate inhibits the hatching of frog eggs and is bioaccumulated by tadpoles*. Environmental toxicology and chemistry, **6**, 417-422 (1987).

Latini G, Scoditto E, Verrotti A, De Felice C and M Massaro. *Peroxisome proliferator-activated receptors as mediators of phthalate-induced effects in the male and female reproductive tract: epidemiological and experimental evidence*. PPAR research, **2008**, 359267 (2008).

Latini G. *Monitoring phthalate exposure in humans*. Clinical chimica acta, **361**, 20-29 (2005).

Lee E, Ahn MY, Kim HJ, Kim IY, Han SY, Kang TS, Hong JH, Park KL, Lee BM and HS Kim. *Effect of di(n-butyl) phthalate on testicular oxidative damage and antioxidant enzymes in hyperthyroid rats*. Environmental toxicology, **22**, 245-255 (2007).

Lee HJ, Chattopadhyay S, Gong E-Y, Ahn RS and K Lee. *Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor*. Toxicological sciences, **75**, 40-46 (2003).

Lee SK and DNR Veeramachaneni. *Subchronic exposure to low concentrations of di-n-butyl phthalate disrupts spermatogenesis in Xenopus laevis frogs*. Toxicological sciences, **84**, 394-407 (2005).

Lee SK, Owens GA and DNR Veermachaneni. *Exposure to low concentrations of di-n-butyl phthalate during embryogenesis reduces survivability and impairs development of Xenopus laevis frogs*. Journal of toxicology and environmental health part A, **68**, 763-772 (2005).

Lee YJ, Lee E, Kim TH, Choi JS, Lee J, Jung KK, Kwack SJ, Kim KB, Kang TS, Han SY, Lee BM and HS Kim. *Effects of di(2-ethylhexyl) phthalate on regulation of steroidogenesis or spermatogenesis in testes of Sprague-Dawley rats*. Journal of health science, **55**, 380-388 (2009).

Lehmann KP, Phillips S, Sar M, Foster PMD and KW Gaido. *Dose-dependent alterations in gene expression and testosterone synthesis in the fetal testes of male rats exposed to di (n-butyl) phthalate*. Toxicological sciences, **81**, 60-68 (2004).

Leitz J, Kuballa T, Rehm J and DW Lachenmeier. *Chemical analysis and risk assessment of diethyl phthalate in alcoholic beverages with special regard to unrecorded alcohol*. Plos one, **4**, e8127 (2009).

Levy G, Lutz I, Krüger A and W Kloas. *Bisphenol A induces feminization in Xenopus laevis tadpoles.* Environmental research, **94**, 102-111 (2004).

Liang DW, Zhang T, Fang HHP and J He. *Phthalates biodegradation in the environment*. Applied microbiology and biotechnology, **80**, 183-198 (2008).

Lington AW, Bird MG, Plutnick RT, Stubblefield WA and RA Scala. *Chronic toxicity and carcinogenic evaluation of diisononyl phthalate in rats.* Fundamental and applied toxicology, **36**, 79-89 (1997).

Lopéz-Carillo L, Hernández-Ramírez RU, Calafat AM, Torres-Sánchez L, Galván-Portillo M, Needham LL, Ruiz-Ramos R and ME Cebrián. *Exposure to phthalates and breast cancer risk in northern Mexico*. Environmental health perspectives, **118**, 539-544 (2010).

Lopéz-Cervantes J and P Paseiro-Losada. *Determination of bisphenol A in, and its migration from, PVC stretch film used for food packaging.* Food additives and contaminants, **20**, 596-606 (2003).

Lovekamp TN and BJ Davis. *Mono-(2-ethylhexyl) phthalate suppresses aromatase transcript levels and estradiol production in cultured rat granulosa cells*. Toxicology and applied pharmacology, **172**, 217-224 (2001).

Lovekamp-Swan T, Jetten AM and BJ Davis. *Dual activation of PPARα and PPARγ by mono-(2-ethylhexyl) phthalate in rat ovarian granulosa cells.* Molecular and cellular endocrinology, **201**, 133-141 (2003).

Lutz I and W Kloas. *Amphibians as a model to study endocrine disruptors: I. Environmental pollution and estrogen receptor binding*. The science of the total environment, **225**, 49-57 (1999).

Lutz I, Kloas W, Springer TA, Holden LR, Wolf JC, Krueger HO and AJ Hosmer. *Development, standardization and refinement of procedures for evaluating effects of endocrine active compounds on development and sexual differentiation of Xenopus laevis*. Analytical and bioanalytical chemistry, **390**, 2031-2048 (2008).

Mackay D. 2001. Multimedia environmental models: The fugacity approach – Second edition. Lewis publishers, Boca Raton.

Mahood IK, Scott HM, Brown R, Hallmark N, Walker M and RM Sharpe. *In utero exposure to di(n-butyl) phthalate and testicular dysgenesis: comparison of fetal and adult end points and their dose sensitivity*. Environmental health perspectives, **115**, 55-61 (2007).

Main KM, Mortensen GK, Kaleva MM, Boisen KA, Damgaard IN, Chellakooty M, Schmidt IM, Suomi AM, Virtanen HE, Petersen JH, Andersson AM, Toppari J and NE Skakkebæk. *Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age*. Environmental health perspectives, **114**, 270-276 (2006).

Makris SL, Euling SY, Gray LEJ, Bensin R and PMD Foster. *Use of genomic data in risk assessment case study: I. Evaluation of the dibutyl phthalate male reproductive development toxicity data set.* Toxicology and applied pharmacology, **271**, 336-348 (2013).

Maloney EK and DJ Waxman. *trans-Activation of PPARα and PPARγ by structurally diverse environmental chemicals.* Toxicology and applied pharmacology, **161**, 209-218 (1999).

Mann AH, Price SC, Mitchell FE, Grasse P, Hinton RH and JW Bridges. *Comparison of the short-term effects of di(2-ethylhexyl) phthalate, di(n-hexyl) phthalate, and di(n-octyl) phthalate in rats.* Toxicology and applied pharmacology, **77**, 116-132 (1985).

Maronpot RR, Yoshizawa K, Nyska A, Harada T, Flake G, Mueller G, Singh B and JM Ward. *Hepatic enzyme induction: histoapathology*. Toxicologic pathology, **38**, 776-795 (2010).

Martin B and A Collenot. *Appearance of the ''amphibian sex steroid binding protein'' (A.SBP) during metamorphosis in female Pleurodeles waltlii serum*. General and comparative endocrinology, **27**, 548-552 (1975).

Martin JW, Smithwick MM, Braune BM, Hoekstra PF, Muir DCG and SA Mabury. *Identification of long-chain perfluorinated acids in biota from the Canadian Arctic*. Environmental science and technology, **38**, 373-380 (2004).

Mathieu-Denoncourt J, de Solla SR and VS Langlois. *Review of the sublethal and lethal effects of plasticizers*. In preparation a.

Mathieu-Denoncourt J, de Solla SR, Martyniuk CJ, Yargeau V and VS Langlois. *Lethal and sublethal effects of acute exposures to phthalates in Western clawed frog larvae*. In preparation b.

Mathieu-Denoncourt J, de Solla SR, Yargeau V and VS Langlois. *Lethal and sublethal effects of chronic exposures to monomethyl phthalate in Western clawed frogs*. In preparation c.

Mathieu-Denoncourt J, Martyniuk CJ, de Solla SR, Balakrishnan VK and VS Langlois. *Sediment contaminated with the azo dye disperse yellow 7 alters cellular stress- and androgen-related transcription in Silurana tropicalis larvae*. Environmental science and technology, **48**, 2952-2961 (2014).

Mayer FLJ and HO Sanders. *Toxicology of phthalic acid esters in aquatic organisms*. Environmental health perspectives, **3**, 153-157 (1973).

Mazzeo P, Di Pasquale D, Ruggieri F, Fanelli M, D'Archivio AA and G Carlucci. *HPLC with diode-array detection for the simultaneous determination of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate in seminal plasma*. Biomedical chromatography, **21**, 1166-1171 (2007).

McCarthy JF and DK Whitmore. *Chronic toxicity of di-n-butyl and di-n-octyl phthalate to Daphnia magna and the fathead minnow*. Environmental toxicology and chemistry, **4**, 167-179 (1985).

McCormick JM, Paiva MS, Häggblom MM, Cooper KR and LA White. *Embryonic exposure to tetrabromobisphenol A and its metabolites, bisphenol A and tetrabromobisphenol A dimethyl ether disrupts normal zebrafish (Danio rerio) development and matrix metalloproteinase expression*. Aquatic toxicology, **100**, 255-262 (2010).

Meeker JD, Calafat AM and R Hauser. *Di(2-ethylhexyl) phthalate metabolites may alter thyroid hormone levels in men.* Environmental health perspectives, **115**, 1029-1034 (2007).

Metcalfe CD, Metcalfe TL, Kiparissis Y, Koenig BG, Khan C, Hughes RJ, Croley TR, March RE and T Potter. *Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (Oryzias latipes)*. Environmental toxicology and chemistry, **20**, 297-308 (2001).

Michael PR, Adams WJ, Werner AF and O Hicks. *Surveillance of phthalate esters in surface waters and sediments in the United States*. Environmental toxicology and chemistry, **3**, 377-389 (1984).

Mieritz MG, Frederiksen H, Sørensen K, Aksglaede L, Mouritsen A, Hagen CP, Skakkebaek NE, Andersson A-M and A Juul. *Urinary phthalate excretion in 555 healthy Danish boys with and without pubertal gynaecomastia*. International journal of andrology, **35**, 227-235 (2012).

Mihaich EM, Friederich U, Caspers N, Hall AT, Klecka GM, Dimond SS, Staples CA, Ortego LS and SG Hentges. *Acute and chronic toxicity testing of bisphenol A with aquatic invertebrates and plants*. Ecotoxicology and environmental safety, **72**, 1392-1399 (2009).

Minucci S, Leid M, Toyama R, Saint-Jeannet JP, Peterson VJ, Horn V, Ishmael JE, Bhattacharyya N, Dey A, Dawid IB and K Ozato. *Retinoid X receptor (RXR) within the RXR-retinoic acid acid receptor heterodimer binds its ligand and enhances retinoid-dependent gene expression*. Molecular and cellular biology, **17**, 644-655 (1997).

Minucci S, Saint-Jeannet JP, Toyama R, Scita G, DeLuca LM, Taira M, Levin AA, Ozato K and IB Dawid. *Retinoid X receptor-selective ligands produce malformations in Xenopus embryos.* Proceedings of the National Academy of Sciences, **93**, 1803-1807 (1996).

Mitsui N, Fujii T, Miyahara M, Oka T, Kashiwagi A, Kashiwaki K, Hanada H, Urushitani H, Santo N, Tooi O and T Iguchi. *Development of metamorphosis assay using Silurana tropicalis for the detection of thyroid system-disrupting chemicals.* Endocrinology and environmental safety, **64**, 281-287 (2006).

Miyata K and K Ose. *Thyroid hormone-disrupting effects and the amphibian metamorphosis assay*. Journal of toxicologic pathology, **25**, 1-9 (2012).

Miyata S, Koike S and T Kubo. *Hormonal reversal and the genetic control of sex differentiation in Xenopus*. Zoological science, **16**, 335-340 (1999).

Monfort N, Ventura R, Platen P, Hinrichs T, Brixius K, Schänzer W, Thevis M, Geyer H and J Segura. *Plasticizers excreted in urine: indication of autologous blood transfusion in sports*. Transfusion, **52**, 647-657 (2012).

Moral R, Wang R, Russo IH, Mailo DA, Lamartiniere CA and J Russo. *The plasticizer butyl benzyl phthalate induces genomic changes in rat*

mammary gland after neonatal/prepubertal exposure. BioMed central genomics, **8**, 453 (2007).

Moriyama K, Tagami T, Akamizu T, Usui T, Saijo M, Kanamoto N, Hataya Y, Shimatsu A, Kuzuya H and K Nakao. *Thyroid hormone action is disrupted by bisphenol A as an antagonist.* Journal of clinical endocrinology and metabolism, **87**, 5185-5190 (2002).

Morvan Dubois G, Sebillot A, Kuiper GGJM, Verhoelst CHJ, Darras VM, Visser TJ and BA Demeneix. *Deiodinase activity is present in Xenopus laevis during early embryogenesis.* Endocrinology, **147**, 4941-4949 (2006).

Munksgaard EC. *Leaching of plasticizers from temporary denture soft lining materials.* European journal of oral sciences, **112**, 101-104 (2004).

Mussi MA and NB Calcaterra. *Paraquat-induced oxidative stress response during amphibian early embryonic development.* Comparative biochemistry and physiology part C, **151**, 240-247 (2010).

Mylchreest E, Cattley RC and PMD Foster. *Male reproductive tract malformations in rats following gestational and lactational exposure to di(n-butyl) phthalate: an antiandrogenic mechanism?*. Toxicological sciences, **43**, 47-60 (1998).

Mylchreest E, Sar M, Cattley RC and PMD Foster. *Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide*. Toxicology and applied pharmacology, **156**, 81-95 (1999).

Mylchreest E, Wallace DG, Cattley RC and PMD Foster. *Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation*. Toxicological sciences, **55**, 143-151 (2000).

Nagy L, Kao HY, Chakravarti D, Lin RJ, Hassig CA, Ayer DE, Schreiber SL and RM Evans. *Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase*. Cell, **89**, 373-380 (1997).

Nara K, Nishiyama K, Natsugari H, Takeshita A and H Takahashi. *Leaching of the palsticizer, acetyl tributyl citrate: (ATBC) from plastic kitchen wrap*. Journal of health science, **55**, 281-284 (2009).

Nieuwkoop P. and J Faber. 1994. Normal Table of Xenopus laevis (Daudin). Garland Publishing, Inc., New York.

Noda M, Ohno S and S Nakajin. *Mono-(2-ethylhexyl) phthalate (MEHP) induces nuclear receptor 4A subfamily in NCI-H295R cells: a possible mechanism of aromatase suppression by MEHP*. Molecular and cellular endocrinology, **274**, 8-18 (2007).

Nomura Y, Mitsui N, Bhawal UK, Sawajiri M, Tooi O, Takahashi T and M Okazaki. *Estrogenic activity of phthalate esters by In vitro VTG assay using primary-cultured Xenopus hepatocytes*. Dental materials journal, **25**, 533-537 (2006).

Noriega NC, Howdeshell KL, Furr J, Lambright CR, Wilson VS and LEJ Gray. *Pubertal administration of DEHP delays puberty, suppresses testosterone production, and inhibits reproductive tract development in male Sprague-Dawley and Long-Evans rats*. Toxicological sciences, **111**, 163-178 (2009).

Nunney L. *The influence of age structure and fecundity on effective population size.* Biological sciences, **246**, 71-76 (1991).

O'Connor JC, Frame SR and GS Ladics. *Evaluation of a 15-day screening assay using intact male rats for identifying antiandrogens*. Toxicological sciences, **69**, 92-108 (2002).

Oehlmann J, Oetken M and U Schulte-Oehlmann. *A critical evaluation of the environmental risk assessment for plasticizers in the freshwater environment in Europe, with special emphasis on bisphenol A and endocrine disruption*. Environmental research, **108**, 140-149 (2008).

Oehlmann J, Schulte-Oehlmann U, Bachmann J, Oetken M, Lutz I, Kloas W and TA Ternes. *Bisphenol A induces superfeminization in the ramshorn snail Marisa cornuarietis (gastropoda: prosobranchia) at environmentally relevant concentrations*. Environmental health perspectives, **114**, 127-133 (2006).

Oehlmann J, Schulte-Oehlmann U, Kloas W, Jagnytsch O, Lutz I, Kusk KO, Wollenberger L, Santos EM, Paull GC, Van Look KJW and CR Tyler. *A critical analysis of the biological impacts of plasticizers on wildlife*. Philosophical transactions of the Royal Society, **364**, 2047-2062 (2009).

Oehlmann J, Schulte-Oehlmann U, Tillmann M and B Markert. *Effects of endocrine disruptors on prosobranch snails (mollusca: gastropoda) in the laboratory. Part I : bisphenol A and octylphenol as xeno-estrogens.* Ecotoxicology, **9**, 383-397 (2000).

Ohashi A, Kotera H, Hori H, Hibiya M, Watanabe K, Murakami K, Hasegawa M, Tomita M, Hiki Y and S Sugiyama. *Evaluation of endocrine disrupting activity of palsticizers in polyvinyl chloride tubes by estrogen receptor alpha binding assay*. Journal of artificial organs, **8**, 252-256 (2005).

Ohtani H, Miura I and Y Ichikawa. *Effects of dibutyl phthalate as an environmental endocrine disruptor on gonadal sex differentiation of genetic males of the frog Rana rugosa*. Environmental health perspectives, **108**, 1189-1193 (2000).

Oishi S and K Hiraga. *Effects of phthalic acid monoesters on mouse testes*. Toxicology letters, **6**, 239-242 (1980).

Oka T, Adati N, Shinkai T, Sakuma K, Nishimura T and K Kurose. *Bisphenol A induces apoptosis in central neural cells during early development of Xenopus laevis.* Biochemical and biophysical research communications, **312**, 877-882 (2003).

Olea N, Pulgar R, Pérez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A, Pedraza V, Soto AM and C Sonnenschein. *Estrogenicity of resin-based composites and sealants used in dentistry*. Environmental health perspectives, **104**, 298-305 (1996).

Olmstead AW, Kosian PA, Korte JJ, Holcombe GW, Woodis KK and SJ Degitz. *Sex reversal of the amphibian, Xenopus tropicalis, following larval exposure to an aromatase inhibitor*. Aquatic toxicology, **91**, 143-150 (2009).

Opitz R, Braunbeck T, Bögi C, Pickford DB, Nentwig G, Oehlmann J, Tooi O, Lutz I and W Kloas. *Description and initial evaluation of a Xenopus metamorphosis assay for detection of thyroid system-disrupting activities of environmental compounds.* Environmental toxicology and chemistry, 24, 653-664 (2005).

Osano O, Oladimeji AA, Kraak MHS and W Admiraal. *Teratogenic effects of amitraz, 2,4-dimethylaniline, and paraquat on developing frog (Xenopus) embryos*. Archives of environmental contamination and toxicology, **43**, 42-49 (2002).

Otake T, Yoshinaga J and Y Yanagisawa. *Exposure to phthalate esters from indoor environment.* Journal of exposure analysis and environmental epidemiology, **14**, 524-528 (2004).

Ozretich RJ, Randall RC, Boese BL, Schroeder WP and JR Smith. *Acute toxicity of butylbenzyl phthalate to shiner perch (Cymatogaster aggregate)*. Archives of environmental contamination and toxicology, **12**, 655-660 (1983).

Pannese M, Polo C, Andreazzoli M, Vignali R, Kablar B, Barsacchi G and E Boncinelli. *The Xenopus homologue of Otx2 is a maternal homeobox gene that demarcates and specifies anterior body regions*. Development, **121**, 707-720 (1995).

Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ and LEJ Gray. *The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat.* Toxicological sciences, **58**, 339-349 (2000).

Pathirana IN, Kawate N, Tsuji M, Takahashi M, Hatoya S, Inaba T amd H Tamada. *In vitro effects of estradiol-17β, monobutyl phthalate and mono-(2-ethylhexyl) phthalate on the secretion of testosterone and insulin-like peptide 3 by interstitial cells of scrotal and retained testes in dogs*. Theriogenology, **76**, 1227-1233 (2011).

Patyna P and KR Cooper. *Multigeneration reproductive effects of three phthalate esters in Japanese medaka (Oryzias latipes)*. Marine environmental research, **50**, 191-199 (2000).

Patyna PJ, Brown RP, Davi RA, Letinski DJ, Thomas PE, Cooper KR and TF Parkerton. *Hazard evaluation of diisononyl phthalate and diisodecyl phthalate in a Japanese medaka multigenerational assay*. Ecotoxicology and environmental safety, **65**, 36-47 (2006).

Pavlikova N, Kortner TM and A Arukwe. *Peroxisome proliferator-activated receptors, estrogenic responses and biotransformation system in the liver of salmon exposed to tributyltin and second messenger activator*. Aquatic toxicology, **99**, 176-185 (2010).

Peakall DB. *Effects of di-n-butyl and di-2-ethylhexyl phthalate on the eggs of Ring Doves*. Bulletin of environmental contamination and toxicology, **12**, 698-702 (1974).

Pereira C, Mapuskar K and CV Rao. *Chronic toxicity of diethyl phthalate in male Wistar rats – a dose-response study.* Regulatory toxicology and pharmacology, **45**, 169-177 (2006).

Pettersson I and C Berg. *Environmentally relevant concentrations of ethynylestradiol cause female-biased sex ratios in Xenopus tropicalis and Rana temporaria.* Environmental toxicology and chemistry, **26**, 1005-1009 (2007).

Pettersson I, Arukwe A, Lundstedt-Enkel K, Mortensen AS and C Berg. *Persistent sex-reversal and oviducal agenesis in adult Xenopus (Silurana) tropicalis frogs following larval exposure to the environmental pollutant ethynylestradiol.* Aquatic toxicology, **79**, 356-365 (2006).

Phrakonkham P, Viengchareun S, Belloir C, Lombès M, Artur Y and MC Canivenc-Lavier. *Dietary xenoestrogens differentially impair 3T3-L1 preadipocyte differentiation and persistently affect leptin synthesis.* Journal of steroid biochemistry and molecular biology, **110**, 95-103 (2008).

Pickford DB, Hetheridge MJ, Caunter JE, Hall AT and TH Hutchinson. *Assessing chronic toxicity of bisphenol A to larvae of the african clawed frog (Xenopus laevis) in a flow-through exposure system.* Chemosphere, **53**, 223-235 (2003).

Piferrer F, Zanuy S, Carillo M, Solar II, Devlin RH and EM Donaldson. *Brief treatment with an aromatase inhibitor during sex differentiation causes chromosomally female salmon to develop as normal, functional males*. Journal of experimental zoology, **270**, 255-262 (1994).

Planelló R, Herrero O, Martínez-Guitarte JL and G Morcillo. *Comparative effects of butyl benzyl phthalate (BBP) and di(2-ethylhexyl) phthalate (DEHP) on the aquatic larvae of Chironomus riparius based on gene expression assays related to the endocrine system, the stress response and ribosomes.* Aquatic toxicology, **105**, 62-70 (2011).

Prahlad V and RI Morimoto. *Integrating the stress response: lessons for neurodegenerative diseases from C. elegans*. Trends in cell biology, **19**, 52-61 (2008).

Quaranta A, Bellantuono V, Cassano G and C Lippe. *Why amphibians are more sensitive than mammals to xenobiotics*. Plos one, **4**, e7699 (2009).

Rayburn JR, Fort DJ, McNew R and JA Bantle. *Synergism and antagonism induced by three carrier solvents with t-retinoic acid and 6-aminonicotinamide using FETAX*. Bulletin of environmental contamination and toxicology, **46**, 625-632 (1991).

Rhodes JE, Adams WJ, Biddinger GR, Robillard KA and JW Gorsuch. *Chronic toxicity of 14 phthalate esters to Daphnia magna an rainbow trout (Oncorhynchus mykiss)*. Environmental toxicology and chemistry, **14**, 1967-1976 (1995).

Richter CA, Birnbaum LS, Farabollini F, Newbold RR, Rubin BS, Talsness CE, Vandenbergh JG, Walser-Kuntz DR and FS vom Saal. *In vivo effects of bisphenol A in laboratory rodent studies*. Reproductive toxicology, **24,** 199-224 (2007).

Rubin BS, Murray MK, Damassa DA, King JC and AM Soto. *Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels*. Environmental health perspectives, **109**, 675-680 (2001).

Rusyn I, Peters JM and ML Cunningham. *Modes of action and species-specific effects of di-(2-ethylhexyl) phthalate in the liver*. Critical review in toxicology, **36**, 459-479 (2006).

Rykowska I and W Wasiak. *Properties, threats, and methods of analysis of bisphenol A and its derivatives*. Acta chromatographica, **16**, 7-27 (2006).

Ryu JY, Lee E, Kim TH, Lee YJ, Lee J, Lee BM, Kwack SJ, Jung KK, Han SY, Kim SH, Kacew S and HS Kim. *Time-response effects of testicular gene expression profiles in Sprague-Dawley male rats treated with di(n-butyl) phthalate.* Journal of toxicology and environmental health part A, **71**, 1542-1549 (2008).

Saillenfait AM, Gallissot F and JP Sabaté. *Differential developmental toxicities of di-n-hexyl phthalate and dicyclohexyl phthalate administered orally to rats.* Journal of applied toxicology, **29**, 510-521 (2009).

Saillenfait AM, Lagonné I and B Leheup. *Effects of mono-n-butyl phthalate on the development of rat embryos: in vivo and in vitro observations*. Pharmacology and toxicology, **89**, 104-112 (2001).

Saillenfait AM, Roudot AC, Gallissot F, Sabaté JP and MC Chagnon. *Developmental toxic potential of di-n-propyl phthalate administered orally to rats*. Journal of applied toxicology, **31**, 36-44 (2011).

Saillenfait AM, Sabaté JP and F Gallissot. *Developmental toxic effects of diisobutyl phthalate, the methyl-branched analogue of di-n-butyl phthalate, administered by gavage to rats*. Toxicology letters, **165**, 39-46 (2006).

Sakaue M, Ohsako S, Ishimura R, Kurosawa S, Kurohmaru M, Hayashi Y, Aoki Y, Yonemoto J and C Tohyama. *Bisphenol-A affects spermatogenesis in the adult rat even at low dose.* Journal of occupational health, **43**, 185-190 (2001).

Salian S, Doshi T and G Vanage. *Impairment in protein expression profile of testicular steroid receptor coregulators in male rat offspring perinatally exposed to bisphenol A*. Life sciences, **85**, 11-18 (2009).

Sandhoff TW, Hales DB, Hales KH and MP McLean. *Transcriptional regulation of the rat steroidogenic acute regulatory protein gene by steroidogenic factor 1*. Endocrinology, **139**, 4820-4831 (1998).

Sathyanarayana S, Karr CJ, Lozano P, Brown E, Calafat AM, Liu F and SH Swan. *Baby care products: possible sources of infant phthalate exposure*. Pediatrics, **121**, e260-e268 (2008).

Savabieasfahani M, Kannan K, Astapova O, Evans NP and V Padmanabhan. *Developmental programming: differential effects of prenatal exposure to bisphenol-A or methoxychlor on reproductive function.* Endocrinology, **147**, 5956-5966 (2006).

Schoonjans K, Staels B and J Auwerx. *The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation.* Biochimical et biophysica acta, **1302**, 93-109 (1996).

Segner H, Caroll K, Fenske M, Janssen CR, Maack G, Pascoe D, Schäfers C, Vandenbergh GF, Watts M and A Wenzel. *Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project*. Ecotoxicology and environmental safety, **54**, 302-314 (2003).

Shen HY, Jiang HL, Mao HL, Pan G, Zhou L and YF Cao. *Simultaneous determination of seven phthalates and four parabens in cosmetic products*

using HPLC-DAD and GC-MS methods. Journal of separation science, **30**, 48-54 (2007).

Shen O, Wu W, Du G, Liu R, Yu L, Sun H, Han X, Jiang Y, Shi W, Hu W, Song L, Xia Y, Wang S and X Wang. *Thyroid disruption by d-n-butyl phthalate (DBP) and mono-n-butyl phthalate (MBP) in Xenopus laevis*. Plos one, **6**, e19159 (2011).

Shibata N, Matsumoto J, Nakada K, Yuasa A and H Yokota. *Male-specific suppression of hepatic microsomal UDP-glucoronosyltransferase activities toward sex hormones in the adult male rat administered bisphenol A*. Biochemical journal, **368**, 783-788 (2002).

Shono T, H Kai, S Suita and H Nawata. *Time-specific effects of mono-n-butyl phthalate on the transabdominal descent of the testis in rat fetuses*. British journal of urology international, **86**, 121-125 (2000).

Shultz VD, Phillips S, Sar M, Foster PMD and KW Gaido. *Altered gene profiles in fetal rat testes after in utero exposure to di(n-butyl) phthalate*. Toxicological sciences, **64**, 233-242 (2001).

Singh AR, Lawrence WH and J Autian. *Embryonic-fetal toxicity and teratogenic effects of adipic acid esters in rats.* Journal of pharmaceutical sciences, **62**, 1596-1600 (1973).

Sive H and L Bradley. *A sticky problem: the Xenopus cement gland as a paradigm for anteroposterior patterning*. Developmental dynamics, **205**, 265-280 (1996).

Sohoni P and JP Sumpter. *Several environmental oestrogens are also anti-androgens*. Journal of endocrinology, **158**, 327-339 (1998).

Sohoni P, Tyler CR, Hurd K, Caunter J, Hetheridge M, Williams T, Woods C, Evans M, Toy R, Gargas M and JP Sumpter. *Reproductive effects of long-term exposure to bisphenol A in the fathead minnow (Pimephales promelas)*. Environmental science and technology, **35**, 2917-2925 (2001).

Soliman MA, Pedersen JA, Park H, Castaneda-Jimenez A, Stenstrom MK and IH Suffet. *Human pharmaceuticals, antioxidants, and plasticizers in wastewater treatment plant and water reclamation plant effluents.* Water environment research, **79**, 156-167 (2007).

Sone K, Hinago M, Kitayama A, Morokuma J, Ueno N, Watanabe H and T Iguchi. *Effects of 17β-estradiol, nonylphenol, and bisphenol-A on developing Xenopus laevis embryos.* General and comparative endocrinology, **138**, 228-236 (2004).

Soriano JJ, Mathieu-Denoncourt J, Norman G, de Solla SR and VS Langlois. *Toxicity of the azo dyes acid red 97 and bismarck brown y to Western clawed frog (Silurana tropicalis).* Environmental science and pollution research, **21**, 3582-3591 (2014).

Soriano S, Alonso-Magdalena P, García-Arévalo M, Novials A, Muhammed SJ, Salehi A, Gustafsson J-A, Quesada I and A Nadal. *Rapid insulinotropic action of low doses of bisphenol-A on mouse and human islets of Langerhans: role of estrogen receptor β*. Plos one, **7**, e31109 (2012).

Srivastava SP, Srivastava S, Saxena DK, Chandra SV and PK Seth. *Testicular effects of di-n-butyl phthalate (DBP): biochemical and histopathological alterations.* Archives of toxicology, **64**, 148-152 (1990).

Staples CA, Adams WJ, Parkerton TF, Gorsuch JW, Biddinger GR and KH Reinert. *Aquatic toxicity of eighteen phthalate esters.* Environmental toxicology and chemistry, **16**, 875-891 (1997a).

Staples CA, Dorn PB, Klecka GM, O'Block ST and LR Harris. *A review of the environmental fate, effects, and exposures of bisphenol A*. Chemosphere, **36**, 2149-2173 (1998).

Staples CA, Guinn R, Kramarz K and M Lampi. *Assessing the chronic aquatic toxicity of phthalate ester plasticizers.* Human and Ecological Risk Assessment, **17**, 1057–1076 (2011).

Staples CA, Peterson DR, Parkerton TF and WJ Adams. *The environmental fate of phthalate esters: a literature review.* Chemosphere, **35**, 667-749 (1997b).

Stoker TE, Laws SC, Crofton KM, Hedge JM, Ferrell JM and RL Cooper. *Assessment of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture, in the EDSP male and female pubertal protocols.* Toxicological sciences, **78**, 144-155 (2004).

Štrac IV, Pušić M, Gajski G and V Garaj-Vrhovac. *Presence of phthalate esters in intravenous solution evaluated using gas chromatography-mass spectrometry method*. Journal of applied toxicology, **33**, 214-219 (2013).

Stroheker T, Picard K, Lhuguenot JC, Canivenc-Lavier MC and MC Chagnon. *Steroid activities comparison of natural and food wrap compounds in human breast cancer cell lines*. Food and chemical toxicology, **42**, 887-897 (2004).

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL and RW Waller. *Status and trends of amphibian declines and extinctions worldwide*. Science, **306**, 1783-1786 (2004).

Sugiyama S-I, Shimada N, Miyoshi H and K Yamauchi. *Detection of thyroid system-disrupting chemicals using in vitro and in vivo screening assays in Xenopus laevis.* Toxicological sciences, **88**, 367-374 (2005).

Sumida K, Igarashi Y, Toritsuka N, Matsushita T, Abe-Tomizawa K, Aoki M, Urushidani T, Yamada H and Y Ohno. *Effects of DMSO on gene expression in human and rat hepatocytes*. Human and experimental toxicology, **30**, 1701-1709 (2011).

Sumpter JP and S Jobling. *Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment.* Environmental health perspectives, **103**, 173-178 (1995).

Sun Y, Irie M, Kishikawa N, Wada M, Kuroda N and K Nakashima. *Determination of bisphenol A in human breast milk by HPLC with column-switching and fluorescence detection*. Biomedical chromatography, **18**, 501-507 (2004).

Suzuki E, Kunimoto M, Nishizuka M and M Imagawa. *Evaluation of ability of chemicals to bind frog (Xenopus laevis) estrogen receptor by in vitro binding assay*. Journal of health science, **50**, 685-688 (2004).

Suzuki MR and S Kikuyama. *Corticoids augment nuclear binding capacity for triiodothyronine in bullfrog tadpole tail fins.* General and comparative endocrinology, **52**, 272-278 (1983).

Suzuki T, Yaguchi K and S Suzuki. *Monitoring of phthalic acid monoesters in river water by solid-phase extraction and GC-MS determination*. Environmental science and technology, **35**, 3757-3763 (2001).

Takahashi N, Kikuyama S, Gen K, Maruyama O and Y Kato. *Cloning of a bullfrog growth hormone cDNA: expression of growth hormone mRNA in larval and adult bullfrog pituitaries*. Journal of molecular endocrinology, **9**, 283-289 (1992).

Talsness CE, Andrade AJM, Kuriyama SN, Taylor JA and FS vom Saal. *Components of plastic: experimental studies in animals and relevance for human health.* Philosophical transactions of the royal society B, **364**, 2079-2096 (2009).

Tan BLL and AM Mustafa. *Leaching of bisphenol A from new and old babies' bottles, and new babies' feeding teats.* Asia-Pacific journal of public health, **15**, 118-123 (2003).

Teil MJ, Blanchard M, Dargnat C, Larcher-Tiphagne K and M Chevreuil. *Occurrence of phthalate diesters in rivers of the Paris district (France)*. Hydrological processes, **21**, 2515-2125 (2007).

Thibaut R and C Porte. *Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish*. Journal of steroid biochemistry and molecular biology, **92**, 485-494 (2004).

Thomas P and J Dong. *Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption*. Journal of steroid biochemistry and molecular biology, **102**, 175-179 (2006).

Thompson CJ, Ross SM and KW Gaido. *Di(n-butyl) phthalate impairs cholesterol transport and steroidogenesis in the fetal rat testis through a rapid and reversible mechanism*. Endocrinology, **145**, 1227-1237 (2004).

Tonk ECM, Verhoef A, Gremmer ER, van Loveren H and AH Piersma. *Relative sensitivity of developmental and immune parameters in juvenile versus adult male rats after exposure to di(2-ethylhexyl) phthalate*. Toxicology and applied pharmacology, **260**, 48-57 (2012).

Topping DC, Ford GP, Evans JG, Lake BG, O'Donoghue JL and HBJr Lockhart. *Peroxisome induction studies on di(2-ethylhexyl)terephthalate.* Toxicology and industrial health, **3**, 63-78 (1987).

Tsujishita Y and JH Hurley. *Structure and lipid transport mechanism of a StAR-related domain*. Nature structural biology, **7**, 408-414 (2000).

Tyl RW, Myers CB, Marr MC, Fail PA, Seely JC, Brine DR, Barter RA and JH Butala. *Reproductive toxicity evaluation of dietary butyl benzyl phthalate (BBP) in rats*. Reproductive toxicology, **18**, 241-264 (2004).

United States. 2002. Title XIV of the public health service act safety of public water systems (Safe drinking water act).

United States. 2008. Consumer product safety improvement act of 2008.

United States Environmental Protection Agency. 2009a. Endocrine disruptor screening program test guidelines OPPTS 890.1100: Amphibian metamorphosis (Frog).

United States Environmental Protection Agency. 2009b. Phhtalates action plan.

Uren-Webster TM, Lewis C, Filby AL, Paull GC and EM Santos. *Mechanisms of toxicity of di(2-ethylhexyl)phthalate on the reproductive health of male zebrafish*. Aquatic toxicology, **99**, 360-369 (2010).

Vandenberg LN, Hauser R, Marcus M, Olea N and WV Welshons. *Human exposure to bisphenol A (BPA).* Reproductive toxicology, **24**, 139-177 (2007).

Veldhoen N, Crump D, Werry K and CC Helbing. *Distinctive gene profiles occur at key points during natural metamorphosis in the Xenopus laevis tadpole tail*. Developmental dynamics, **225**, 457-468 (2002).

Vitali M, Guidotti M, Macilenti G and C Cremisini. *Phthalate esters in freshwaters as markers of contamination sources – a site study in Italy*. Environment international, **23**, 337-347 (1997).

Ward JM, Peter JM, Perella CM and FJ Gonzalez. *Receptor and nonreceptor-mediated organ-specific toxicity of di(2-ethylhexyl) phthalate (DEHP) in peroxisome proliferator-activated receptorα-null mice*. Toxicologic pathology, **26**, 240-246 (1998).

Warren JR, Lalwani ND and JK Reddy. *Phthalate esters as peroxisome proliferator carcinogens.* Environmental health perspectives, **45**, 35-40 (1982).

Watson CS, Bulayeva NN, Wozniak AL and RA Alyea. *Xenoestrogens are potent activators of nongenomic estrogenic responses*. Steroids, **72**, 124-134 (2007).

Wenzel A, Franz C, Breous E and U Loos. *Modulation of iodide uptake by dialkyl phthalate plasticisers in FRTL-5 rat thyroid follicular cells.* Molecular and cellular endocrinology, **244**, 63-71 (2005).

Weschler CJ and WW Nazaroff. *Semivolatile organic compounds in indoor environments*. Atmospheric environment, **42**, 9018-9040 (2008).

Wibbels T and D Crews. *Putative aromatase inhibitor induces male sex determination in a female unisexual lizard and in a turtle with temperature-dependent sex determination*. Journal of endocrinology, **141**, 295-299 (1994).

Wilson VS, Lambright C, Furr J, Ostby J, Wood C, Held G and LEJ Gray. *Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis*. Toxicology letters, **146**, 207-215 (2004).

Wirnitzer U, Rickenbacher U, Katerkamp A and A Schachtrupp. *Systemic toxicity of di-2-ethylhexyl terephthalate (DEHT) in rodents following four weeks of intravenous exposure.* Toxicology letters, **205**, 8-14 (2011).

Wittassek M, Koch HM, Angerer J and T Brüning. *Assessing exposure to phthalates- the human biomonitoring approach*. Molecular nutrition and food research, **55**, 7-31 (2011).

Wright ML, Cykowski LJ, Lundrigan L, Hemond KL, Kochan DM, Faszewski EE and CM Anuszewski. *Anterior pituitary and adrenal cortical hormones accelerate or inhibit tadpole hindlimb growth and development depending on stage of spontaneous development or thyroxine concentration in induced metamorphosis*. Journal of experimental zoology, **270**, 175-188 (1994).

Xu C, Chen JA, Qiu Z, Zhao Q, Luo J, Yang L, Zeng H, Huang Y, Zhang L, Cao J and W Shu. *Ovotoxicity and PPAR-mediated aromatase downregulation in female Sprague-Dawley rats following combined oral exposure to benzo[a]pyrene and di-(2-ethylhexyl) phthalate*. Toxicology letters, *199*, 323-332 (2010).

Xu SY, Zhang H, He PJ and LM Shao. *Leaching behaviour of bisphenol A from municipal solid waste under landfill environment*. Environmental technology, **32**, 1269-1277 (2011).

Yamamoto T and A Yasuhara. *Quantities of bisphenol A leached from plastic waste samples*. Chemosphere, **38**, 2569-2576 (1999).

Yamamoto T, Yasuhara A, Shiraishi H and O Nakasugi. *Bisphenol A in hazardous waste landfill leachates*. Chemosphere, **42**, 415-418 (2001).

Yamasaki K, Okuda H, Takeuchi T and Y Minobe. *Effects of in utero through lactational exposure to dicyclohexyl phthalate and p,p'-DDE in Sprague-Dawley rats*. Toxicology letters, **189**, 14-20 (2009).

Yaoita Y and DD Brown. *A correlation of thyroid hormone receptor gene expression with amphibian metamorphosis.* Genes and development, **4**, 1917-1924 (1990).

Yokota H, Tsuruda Y, Maeda M, Oshima Y, Tadokoro H, Nakazono A, Honjo T and K Kobayashi. *Effects of bisphenol A on the early life stage in Japanese medaka (Oryzias latipes).* Environmental toxicology and chemistry, **19**, 1925-1930 (2000).

Zacharewski TR, Meek MD, Clemons JH, Wu ZF, Fielden MR and JB Matthews. *Examination of the in vitro and in vivo estrogenic activities of eight commercial phthalate esters.* Toxicological sciences, **46**, 282-293 (1998).

Zhang M and Y Sheng. *An indirect competitive fluorescence immunoassay for determination of dicyclohexyl phthalate in water samples*. Journal of fluorescence, **20**, 1167-1173 (2010).

Zhang X, Chang H, Wiseman S, He Y, Higley E, Jones P, Wong CKC, Al-Khedhairy A, Giesy JP and M Hecker. *Bisphenol A disrupts steroidogenesis in human H295R cells.* Toxicological sciences, **121**, 320-327 (2011).

Zheng Z, He PJ, Shao LM and DJ Lee. *Phthalic acid esters in dissolved fractions of landfill leachates*. Water research, **41**, 4696-4702 (2007).

Zhou J, Cai ZH and KZ Xing. *Potential mechanisms of phthalate ester embryotoxicity in the abalone Haliotis diversicolor supertexta*. Environmental pollution, **159**, 1114-1122 (2011a).

Zhou J, Zhu XS and ZH Cai. *Influences of DMP on the fertilization process and subsequent embryogenesis of abalone (Haliotis diversicolor supertexta) by gametes exposure*. Plos one, **6**, e25951 (2011b).

Zilfou JT and SW Lowe. *Tumor suppressive functions of p53*. Cold Spring Harbor perspectives in biology, **1**, a001883 (2009).

Zoeller RT. *Environmental chemicals as thyroid hormone analogues: new studies indicate that thyroid hormone receptors are targets of industrial chemicals?* Molecular and cellular endocrinology, **242**, 10-15 (2005).

Appendix 1 – Genes that were not disrupted by MMP acute exposures in a biologically significant manner

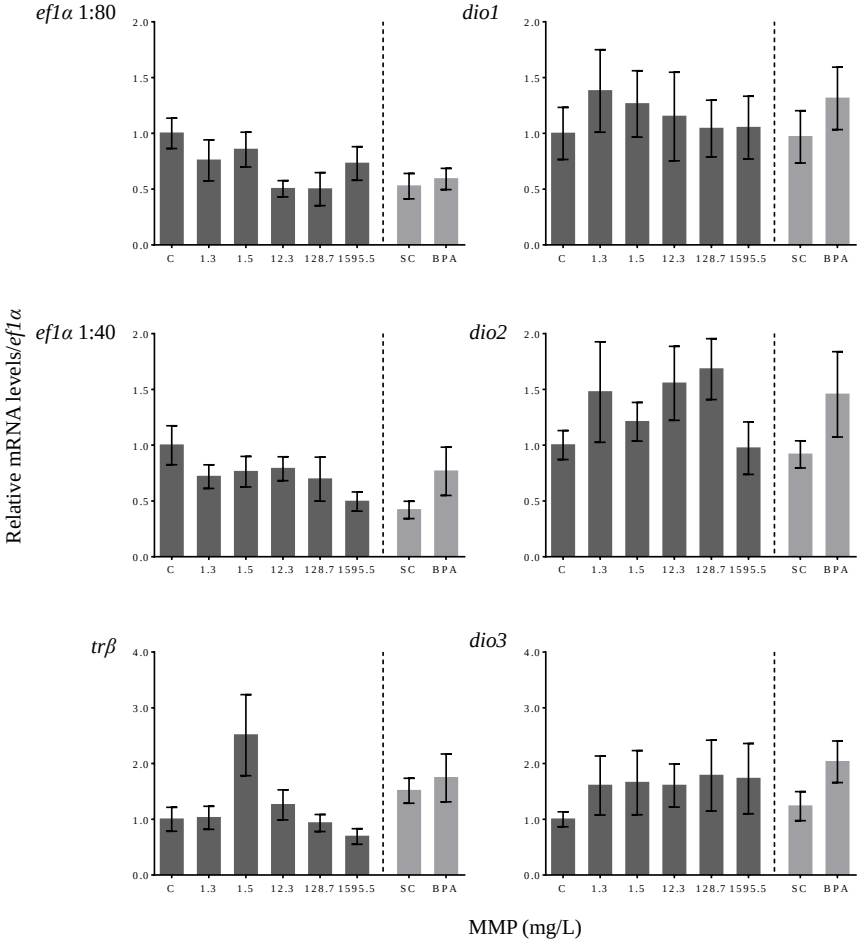

FIGURE A1.1 Effect of monomethyl phthalate (MMP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures. Data are presented as mean ± SEM. Each treatment was tested in 4 to 9 replicates of 10 whole embryos. Data were analysed using one-way ANOVAs and two-sided Dunnet's tests or Kruskal-Wallis tests and two-tailed Dunn's procedures with the Bonferonni correction ($p < 0.05$). Elongation factor 1 alpha (*ef1α*) was used as control gene to normalise the expression of target genes. The scale of the Y axis varies among genes. Asterisks indicate statistically significant differences between treatments and the water-only controls, while boxes indicate biologically significant results. C: water-only control, SC: solvent control for BPA (0.01% v/v DMSO).

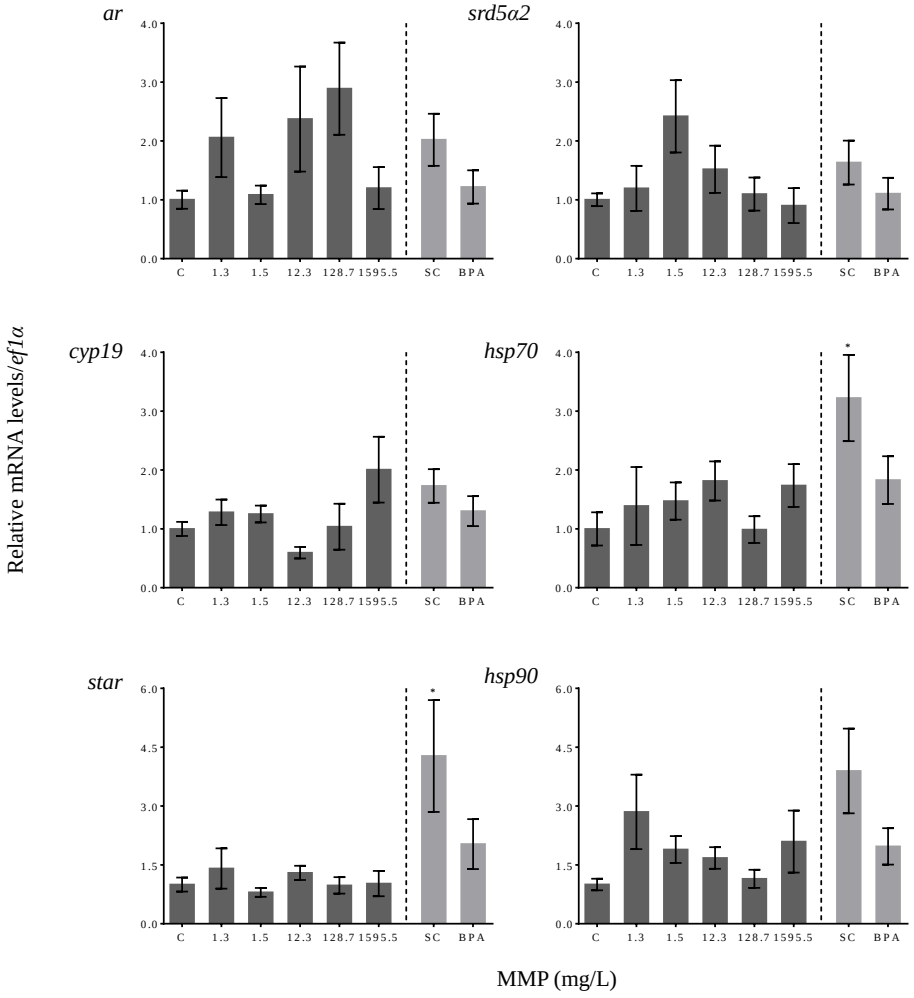

FIGURE A1.2 Effect of monomethyl phthalate (MMP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures. Data are presented as mean ± SEM. Each treatment was tested in 4 to 9 replicates of 10 whole embryos. Data were analysed using one-way ANOVAs and two-sided Dunnet's tests or Kruskal-Wallis tests and two-tailed Dunn's procedures with the Bonferonni correction ($p < 0.05$). Elongation factor 1 alpha (*ef1α*) was used as control gene to normalise the expression of target genes. The scale of the Y axis varies among genes. Asterisks indicate statistically significant differences between treatments and the water-only controls, while boxes indicate biologically significant results. C: water-only control, SC: solvent control for BPA (0.01% v/v DMSO).

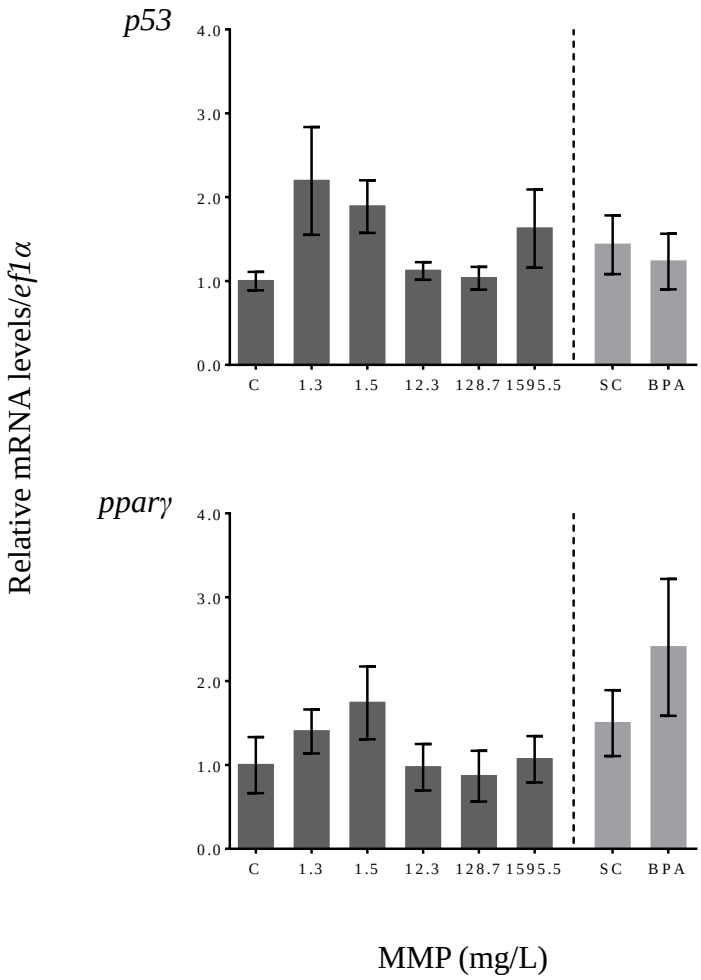

FIGURE A1.3 Effect of monomethyl phthalate (MMP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures. Data are presented as mean ± SEM. Each treatment was tested in 4 to 9 replicates of 10 whole embryos. Data were analysed using one-way ANOVAs and two-sided Dunnet's tests or Kruskal-Wallis tests and two-tailed Dunn's procedures with the Bonferonni correction ($p < 0.05$). Elongation factor 1 alpha (*ef1α*) was used as control gene to normalise the expression of target genes. The scale of the Y axis varies among genes. Asterisks indicate statistically significant differences between treatments and the water-only controls, while boxes indicate biologically significant results. C: water-only control, SC: solvent control for BPA (0.01% v/v DMSO).

Appendix 2 – Genes that were not disrupted by DMP acute exposures in a biologically significant manner

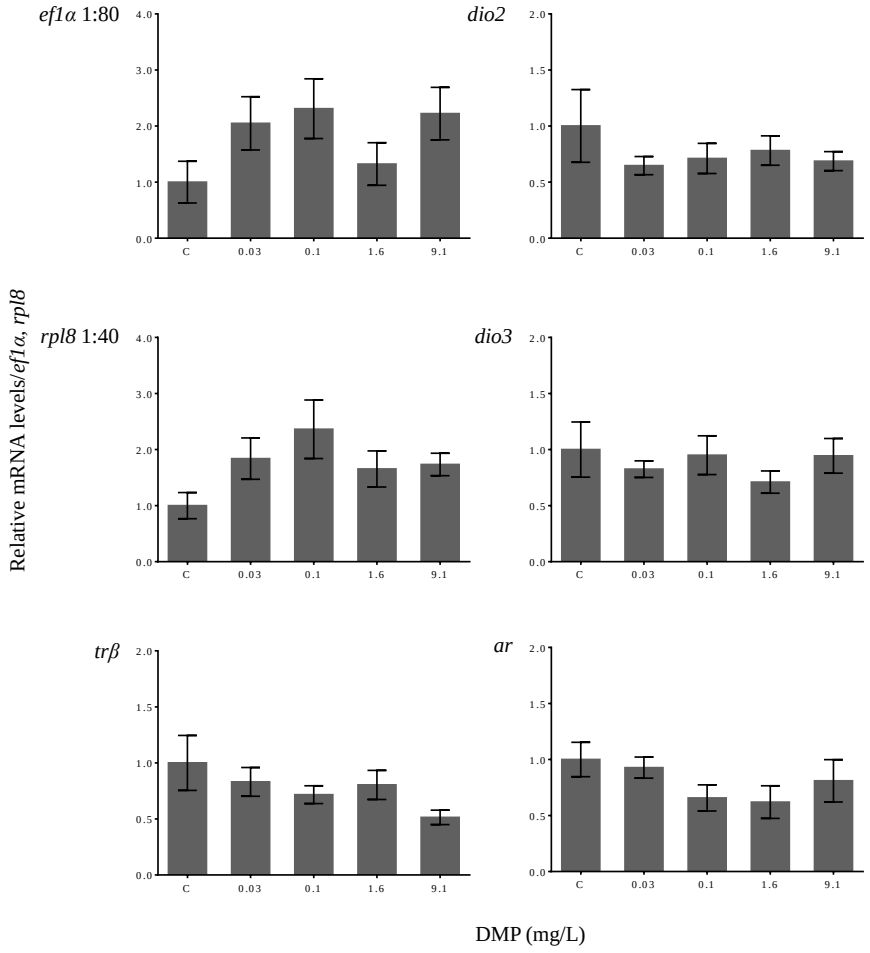

FIGURE A2.1 Effect of dimethyl phthalate (DMP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures. Data are presented as mean ± SEM. Each treatment was tested in 4 to 9 replicates of 10 whole embryos. Data were analysed using one-way ANOVAs and two-sided Dunnet's tests or Kruskal-Wallis tests and two-tailed Dunn's procedures with the Bonferonni correction ($p < 0.05$). Elongation factor 1 alpha (*ef1α*) and ribosomal protein L8 (*rpl8*) were used as control genes to normalise the expression of target genes. The scale of the Y axis varies among genes. Asterisks indicate statistically significant differences between treatments and the water-only controls. C: water-only control.

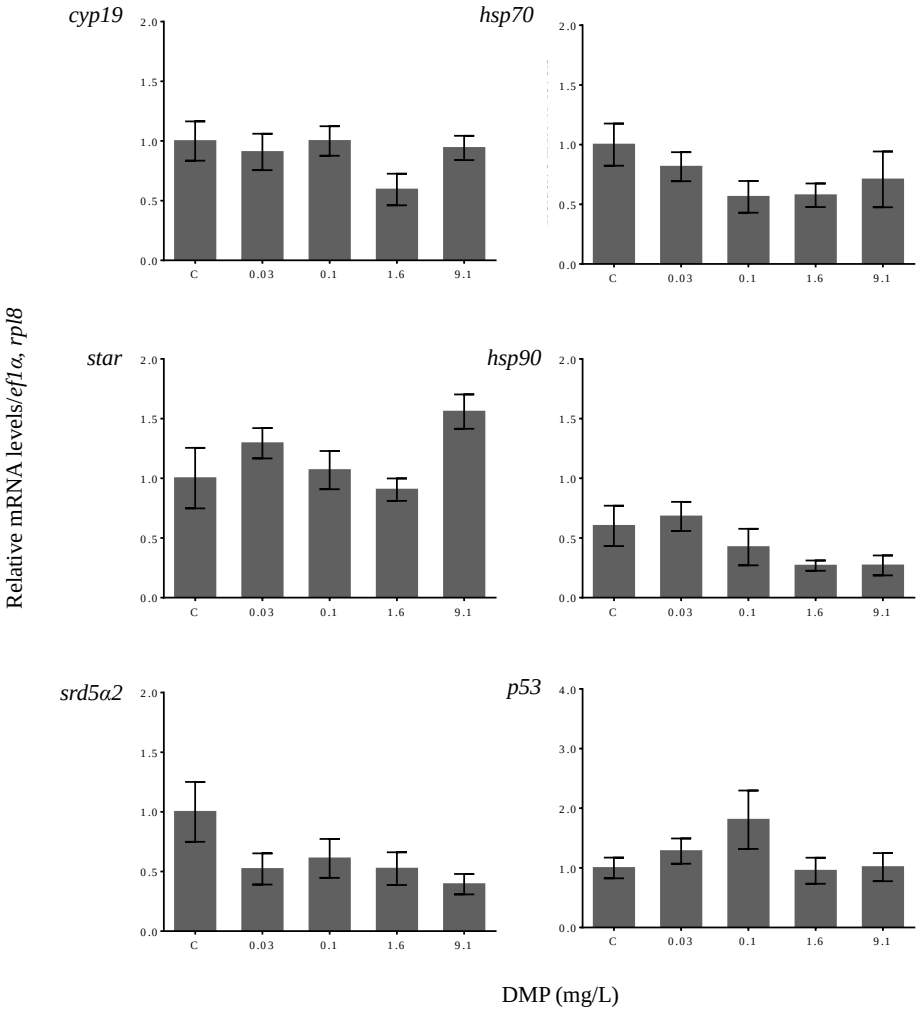

FIGURE A2.2 Effect of dimethyl phthalate (DMP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures. Data are presented as mean ± SEM. Each treatment was tested in 4 to 9 replicates of 10 whole embryos. Data were analysed using one-way ANOVAs and two-sided Dunnet's tests or Kruskal-Wallis tests and two-tailed Dunn's procedures with the Bonferonni correction ($p < 0.05$). Elongation factor 1 alpha (*ef1α*) and ribosomal protein L8 (*rpl8*) were used as control genes to normalise the expression of target genes. The scale of the Y axis varies among genes. Asterisks indicate statistically significant differences between treatments and the water-only controls. C: water-only control.

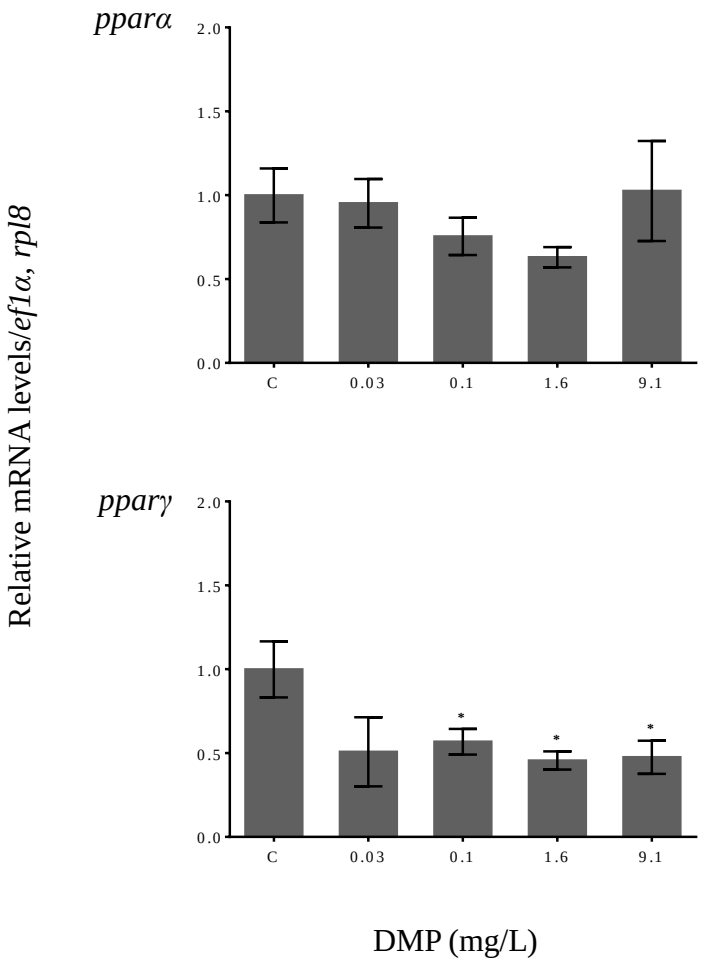

FIGURE A2.3 Effect of dimethyl phthalate (DMP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures. Data are presented as mean ± SEM. Each treatment was tested in 4 to 9 replicates of 10 whole embryos. Data were analysed using one-way ANOVAs and two-sided Dunnet's tests or Kruskal-Wallis tests and two-tailed Dunn's procedures with the Bonferonni correction ($p < 0.05$). Elongation factor 1 alpha (*ef1α*) and ribosomal protein L8 (*rpl8*) were used as control genes to normalise the expression of target genes. The scale of the Y axis varies among genes. Asterisks indicate statistically significant differences between treatments and the water-only controls. C: water-only control.

Appendix 3 – Genes that were not disrupted by DCHP acute exposures in a biologically significant manner

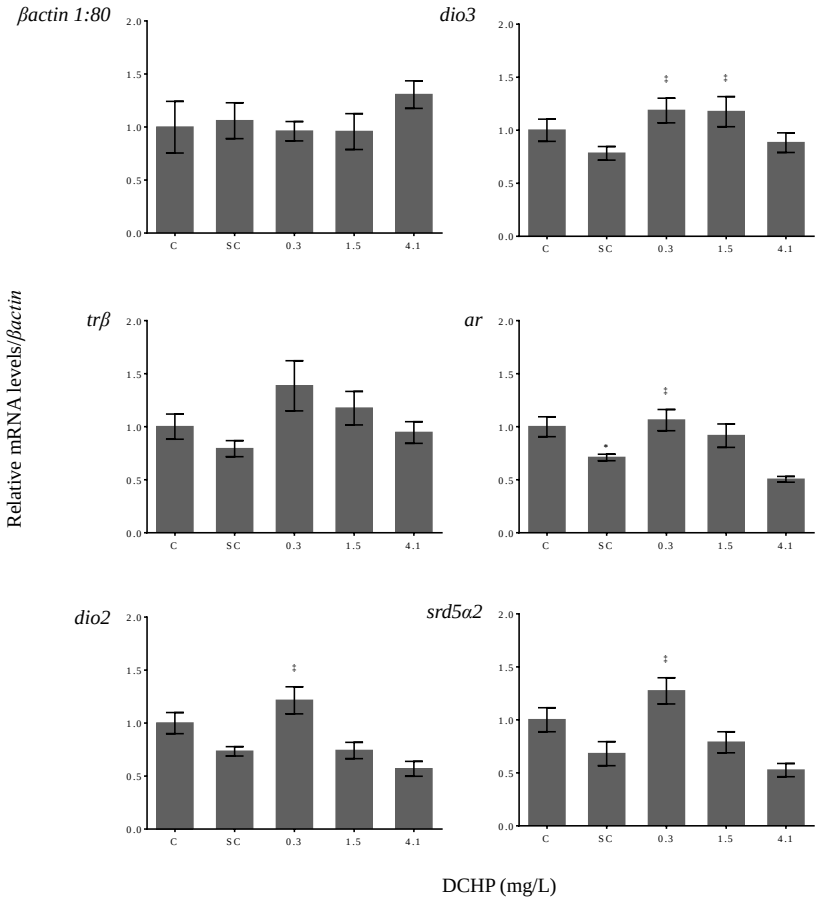

FIGURE A3.1 Effect of dicyclohexyl phthalate (DCHP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures. Data are presented as mean ± SEM. Each treatment was tested in 4 to 9 replicates of 10 whole embryos. Data were analysed using one-way ANOVAs and two-sided Dunnet's tests or Kruskal-Wallis tests and two-tailed Dunn's procedures with the Bonferonni correction ($p < 0.05$). Beta actin (*βact*) was used as control gene to normalise the expression of target genes. The scale of the Y axis varies among genes. Asterisks indicate statistically significant differences between the solvent control and the water-only control, while the symbol ‡ indicates statistically significant differences between treatments and the solvent control. C: water-only control, SC: solvent control (0.82% v/v DMSO).

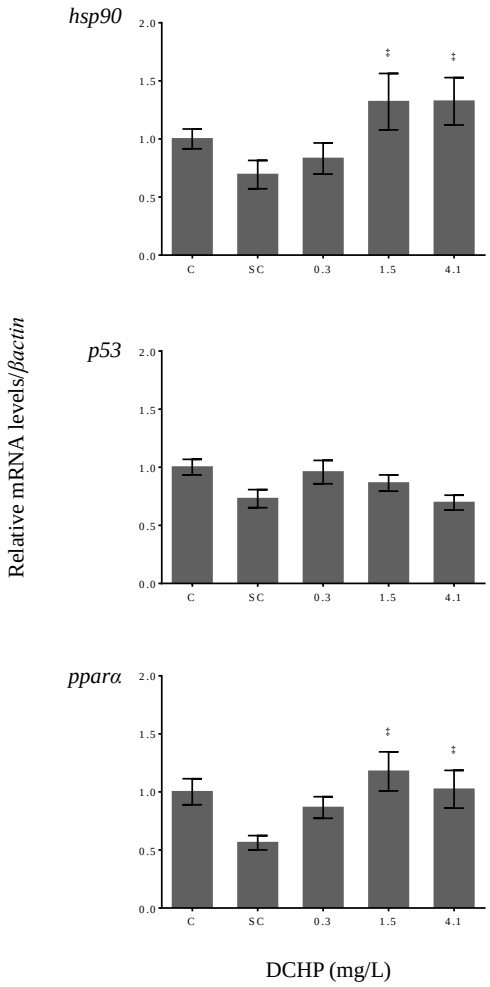

FIGURE A3.2 Effect of dicyclohexyl phthalate (DCHP) spiked water on the gene expression in Western clawed frog larvae after 72 h exposures. Data are presented as mean ± SEM. Each treatment was tested in 4 to 9 replicates of 10 whole embryos. Data were analysed using one-way ANOVAs and two-sided Dunnet's tests or Kruskal-Wallis tests and two-tailed Dunn's procedures with the Bonferonni correction ($p < 0.05$). Beta actin (*βact*) was used as control gene to normalise the expression of target genes. The scale of the Y axis varies among genes. Asterisks indicate statistically significant differences between the solvent control and the water-only control, while the symbol ‡ indicates statistically significant differences between treatments and the solvent control. C: water-only control, SC: solvent control (0.82% v/v DMSO).

Appendix 4 – Genes that were not disrupted by the juvenile exposure to MMP

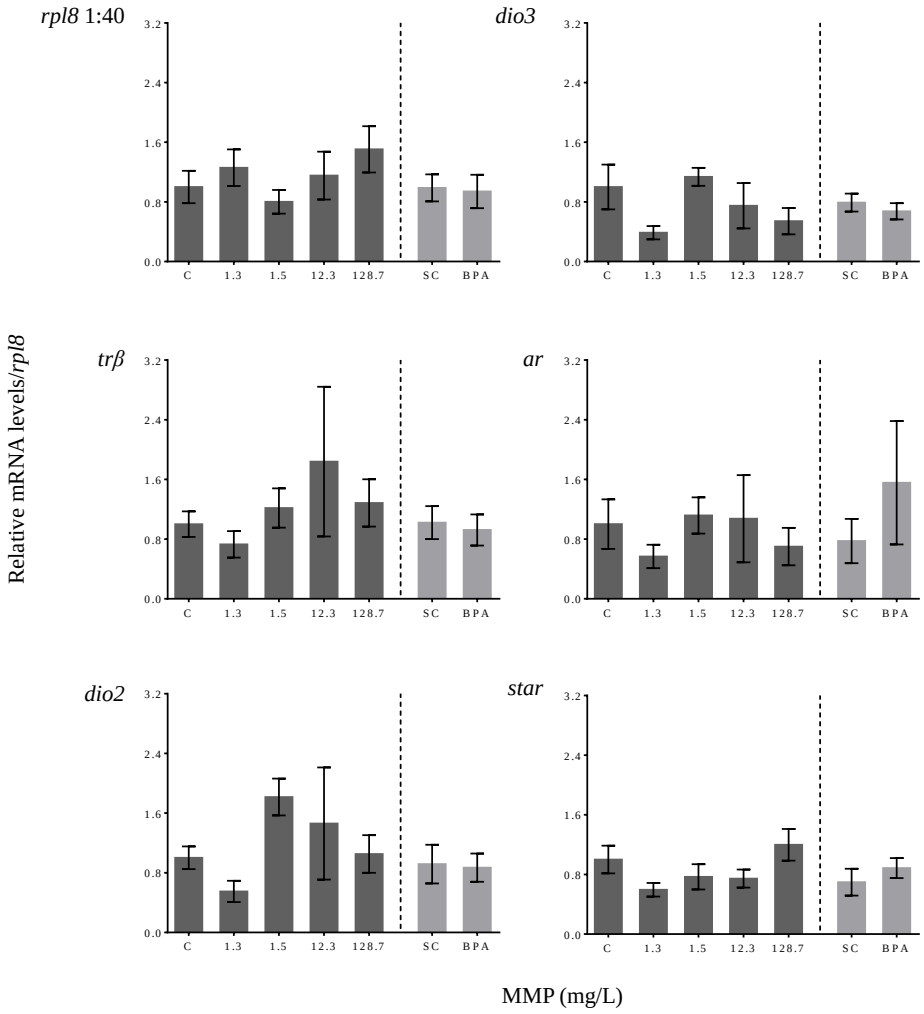

FIGURE A4.1 Effect of monomethyl phthalate (MMP) spiked water on the gene expression in Western clawed frogs after 11 w exposures. Data are presented as mean ± SEM. Each treatment was tested in 4 to 10 replicates of one liver. Data were analysed using one-way ANOVAs and Tukey's tests ($p < 0.05$). Ribosomal protein L8 (*rpl8*) was used as control gene to normalise the expression of target genes.

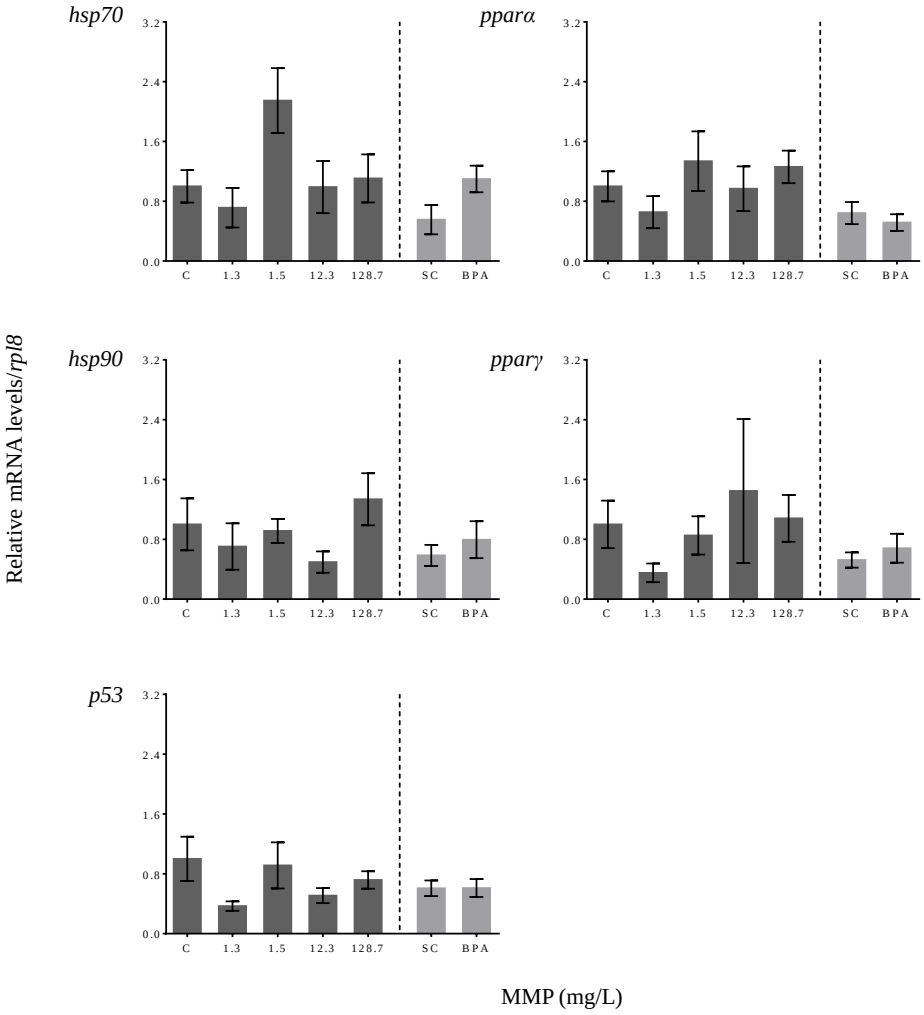

FIGURE A4.2 Effect of monomethyl phthalate (MMP) spiked water on the gene expression in Western clawed frogs after 11 w exposures. Data are presented as mean ± SEM. Each treatment was tested in 4 to 10 replicates of one liver. Data were analysed using one-way ANOVAs and Tukey's tests ($p < 0.05$). Ribosomal protein L8 (*rpl8*) was used as control gene to normalise the expression of target genes.

Appendix 5 – Genes that were not disrupted by the adult exposure to MMP

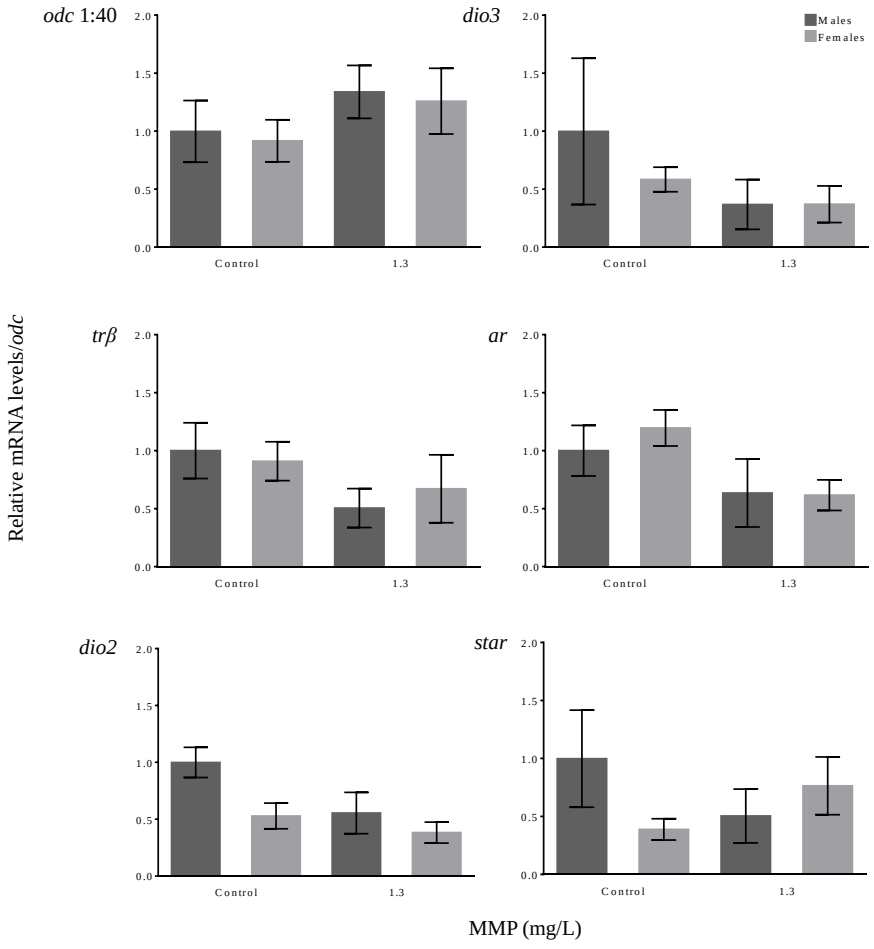

FIGURE A5.1 Effect of monomethyl phthalate (MMP) spiked water on the gene expression in Western clawed frogs after 51 w exposures. Data are presented as mean ± SEM. Each treatment was tested in 4 to 10 replicates of one liver. Data were analysed using one-way ANOVAs and Tukey's tests ($p < 0.05$). Ornithine decarboxylase (*odc*) was used as control gene to normalise the expression of target genes.

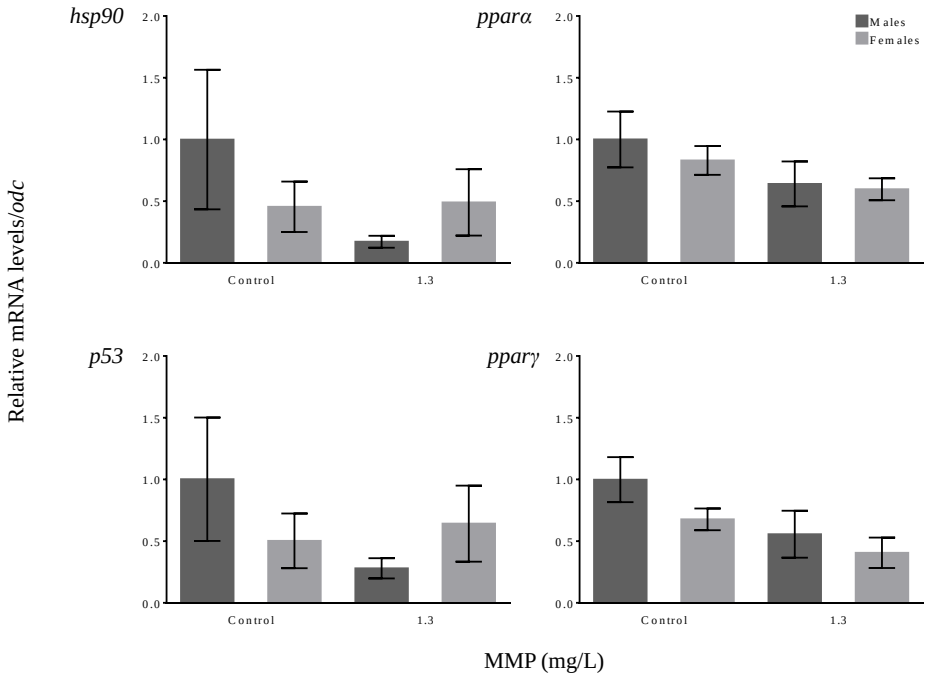

FIGURE A5.2 Effect of monomethyl phthalate (MMP) spiked water on the gene expression in Western clawed frogs after 51 w exposures. Data are presented as mean ± SEM. Each treatment was tested in 4 to 10 replicates of one liver. Data were analysed using one-way ANOVAs and Tukey's tests ($p < 0.05$). Ornithine decarboxylase (*odc*) was used as control gene to normalise the expression of target genes.