Joana Silveira Soares

Effects of complex mixtures of antagonistic endocrine disrupting chemicals using the model fish *Danio rerio*

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Orientador: Doutor Miguel Machado Santos
Investigador Auxiliar,
Centro Interdisciplinar de Investigação Marinha e Ambiental.
Professor auxiliar convidado,
Departamento de Biologia, Faculdade de Ciências, Universidade do Porto.

Co-orientadores:
Professora Doutora Maria Armanda Reis Henriques
Professora Catedrática,
Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto.

Doutor Nuno Monteiro
Investigador auxiliar,
Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto.
Professor auxiliar,
Faculdade de Ciências da Saúde, Universidade Fernando Pessoa.

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Abstract

The number of anthropogenic chemicals that will ultimately reach aquatic ecosystems has increased dramatically with industrial development. Within all classes of contaminants, the presence of endocrine disrupting chemicals (EDCs) has assumed a growing concern among the international community. EDCs are known to interfere with receptor-mediated pathways and deregulate normal endocrine balance, thus affecting, among other processes, reproduction, embryonic development and behaviour. Available data indicates that fish are particularly sensitive to EDCs. From the wide variety of EDCs the most biologically active xenoestrogen in the aquatic environment is 17α-ethinylestradiol (EE_2) while among xenoandrogens, tributyltin (TBT) is known to have a ubiquitous distribution.

Although a variety of reproductive-related perturbations have been ascribed to compounds with estrogenic or androgenic properties, little attention has been directed at the potential consequences of such exposures to fish embryonic development and behaviour. Also, the mechanistic bases by which EDCs induces their disruptive effects are yet to be fully addressed. Recently, effects of EDCs on DNA repair processes have been suggested as a possible mechanism by which these compounds can modulate the incidence of DNA damage. Particularly, nucleotide excision repair (NER) and p53 pathways have been shown to be impacted by EDCs. NER is the primary DNA repair pathway responsible for removing a variety of DNA lesions while p53 protein has a fundamental role in maintaining the genomic stability. p53 target gene effectors, *p21*, *gadd45α* and *bax*, are involved in central cellular responses to damage, namely cell cycle arrest, DNA repair and/or apoptosis.

Although ecotoxicological risk assessment relies mostly on exposures to single compounds, most populations living in the vicinity of urban areas are exposed to complex mixtures of chemicals. Hence, the assessment of the toxicity of mixtures of environmental contaminants is becoming increasingly important, even though our understanding of the impact and the underlying mechanisms of chemicals acting through dissimilar modes of action is still on its infancy.

In order to fulfill some of these knowledge drawbacks, this work analyzed the effects of EE_2 and TBT, single and in combination, at different fish life-cycle

stages. A multidisciplinary approach that simultaneously integrated adverse effects endpoints with hypothetical underlying chemicals mechanisms of action was adopted. As a model species, we selected zebrafish (*Danio rerio*), which is widely used in toxicity testing of priority and emergent contaminants.

Initially, aimed at investigating the impact of EE_2 on reproductive capacity and embryo development, we exposed zebrafish to environmentally relevant concentrations of EE_2, 0.5, 1 and 2 ng/L (actual concentrations of 0.19, 0.24 and 1 ng/L, respectively). Life-cycle exposure to EE_2 in the F_1 generation did not affect reproductive capacity, although an increase in *vtg1* transcripts was observed at 1 ng/L EE_2, actual concentration. In contrast to the findings in the F_1 generation, a significant concentration-dependent increase in embryo mortality was observed at 24 hours post-fertilization (hpf) for all EE_2 treatments. Also, at the highest EE_2 exposure concentration, an increase in development abnormalities was observed at 8 hpf. Taken together, these results indicated that EE_2 impacted normal blastula, gastrula and early organogenesis. Also, embryo sensitivity to EE_2 matched the most sensitive phase of embryonic development under normal conditions.

In order to evaluate the impact of EDCs over different life traits, a parallel study addressed the effects of EE_2 on swimming behaviour of 40 days post-fertilization (dpf) juveniles. Exposure of zebrafish juveniles to EE_2 led to a significant alteration in the behaviour pattern, with a decrease in swimming activity even at the lowest actual concentration of 0.19 ng/L. Reduced locomotion may impact essential survival-related parameters with potential implications in the numbers of individuals that reach maturity and reproduce. Furthermore, the behavioural response followed the same pattern as embryo development (F_2), evidencing that behavioural endpoints are a useful non-invasive measure of EDCs exposure.

Since we did not observe any alteration in female and male gonads histology or in sperm quality parameters in our initial work, we hypothesized that alterations on parental DNA were an important causative mechanism for the disruption of embryo development. Hence, in a parallel work, we addressed the underlying mechanisms involved on embryonic disruption after low EE_2 level exposure. Life-cycle EE_2 exposure, in the range of 0.19-1 ng/L actual concentrations, significantly increased *p53* gene transcription, in male gonads, at all tested concentrations.

Female zebrafish did not show as great of a response in *p53* expression as their male counterparts. These results suggest that EE_2 exposure affected males more severely than females. Modulation of *p53* gene expression by EE_2 closely related with embryonic development data and further supported the hypothesis that embryo mortality after parental EE_2 exposure may be due to male decreased DNA repair capacity.

In order to evaluate the toxicological potential of binary mixtures of xenoestrogens and xenoandrogens, we then addressed the effects of EE_2 and TBT, single and in combination. We were particularly interested to investigate if exposure to mixtures of EE_2 and TBT would also affect DNA repair in zebrafish, impacting embryonic development. For that purpose, we have performed a zebrafish parental full life-cycle exposure to environmental relevant concentrations of EE_2 (0.75 and 1.75 ng/L), TBT (10 and 50 ng/L as Sn) and their binary mixtures (0.75 ng/L EE_2 + 50 ng/L TBT and 1.75 ng/L EE_2 + 50 ng/L TBT), nominal concentrations. In this work, exposure to 50 ng/L TBT significantly decreased fertilization success while exposure to both EE_2 concentrations and mixtures treatments significantly altered sex ratio towards females. Also, EE_2 (1.75 ng/L) single and combined with TBT, at 50 ng/L, significantly increased embryo mortality. Molecular analysis revealed that the exposure to EE_2 and TBT, single and combined, significantly impacted NER and p53 pathways genes expression.

Collectively, the results suggested that increased embryo mortality at the highest EE_2 concentration was related to a decreased DNA repair capacity, supported by the significant decreases in *xpc* and *p53* transcripts levels at this treatment. When EE_2 and TBT were combined, at the highest concentration, embryo mortality was enhanced. An increased damage could lead to induction of several DNA repair processes, which is consistent with the observed significant increases of all p53 target genes (*p21*, *gadd45α* and *bax*) at the highest mixture concentration. However, if damage was too harsh, apoptosis mechanism may have predominated, which is also in agreement with the observed increases in *xpd*, *gadd45α* and *bax* transcripts, allowing to partially explain the significant increase in embryo mortality in this treatment.

Overall, our findings raise major concerns on the population-level impacts for wildlife of long-term exposure to low concentrations of xenoestrogens and xenoandrogens. We demonstrate here that EE_2 and TBT, single and combined,

affect ecological-relevant endpoints such as embryo development and fish behaviour and, concomitantly, the expression pattern of key genes involved in organism homeostasis, unveiling possible mechanisms of action of these compounds. Furthermore, the results indicate that embryo development, behaviour patterns as well as gene expression assays are sensitive mechanistic-based endpoints that have the potential to point out long-term adverse effects and can be used as good early indicators of environmental risk in the screening of EDCs in wild fish populations.

Resumo

O número de compostos de origem antrópica, que terão como destino final os ecossistemas aquáticos, aumentou dramaticamente com o desenvolvimento industrial. Considerando todas as classes de contaminantes, a presença de químicos disruptores endócrinos (EDCs) assumiu uma preocupação crescente entre a comunidade internacional. Os EDCs são conhecidos por interferirem com vias mediadas por recetores e por desregularem o funcionamento normal do sistema endócrino, afetando, entre outros processos, a reprodução, o desenvolvimento embrionário e o comportamento. A informação disponível indica que os peixes são particularmente sensíveis a EDCs. Entre a grande variedade de EDCs, o 17α-etinilestradiol (EE_2) é o xenoestrogénio biologicamente mais ativo no ambiente aquático enquanto, entre os compostos xenoandrogénicos, o tributilestanho (TBT) é conhecido por ter uma distribuição ubíqua.

Nos ecossistemas aquáticos a maioria dos exemplos de disrupção endócrina estão associados com a exposição a compostos com propriedades estrogénicas ou androgénicas. Apesar de diversas perturbações relacionadas com a reprodução terem sido atribuídas a estes compostos pouca atenção tem sido direcionada para possíveis consequências no desenvolvimento embrionário e comportamento dos peixes. Por outro lado, os mecanismos de ação dos EDCs não são ainda totalmente conhecidos. Recentemente, os efeitos dos EDCs em processos de reparação do ADN têm sido sugeridos como um possível mecanismo através do qual estes compostos são capazes de modular a incidência de dano no ADN. Em particular, foi reportado que a reparação por

excisão de nucleótidos (NER) e as vias de sinalização da proteína p53 são alvos de EDCs. O sistema NER constitui a via primária de reparação do ADN, sendo responsável por remover uma variedade de lesões, enquanto a proteína p53 tem um papel preponderante na manutenção da estabilidade genómica. Os genes alvo da proteína p53 (*p21*, *bax* e *gadd45α*) estão envolvidos nas principais respostas celulares, na presença de dano: paragem do ciclo celular, reparação do ADN e/ou apoptose.

Embora a avaliação de risco ecotoxicológico se baseie principalmente em exposições individuais, a maioria das populações que vivem nas proximidades de áreas urbanas estão expostas a misturas complexas de compostos químicos. Por este motivo a avaliação da toxicidade de misturas de contaminantes ambientais está a assumir uma preponderância crescente, apesar do conhecimento sobre o impacto e os mecanismos subjacentes de compostos químicos que operam por diferentes modos de ação ser ainda inicial.

De modo a preencher estas lacunas no conhecimento, este trabalho analisou os efeitos do EE_2 e do TBT, isolados e em misturas, em diferentes fases do ciclo de vida. Adotou-se uma abordagem multidisciplinar, que integrou, simultaneamente, efeitos adversos em parâmetros relevantes do ponto de vista ecológico com hipotéticos mecanismos de ação destes químicos. O peixe-zebra (*Danio rerio*) foi selecionado como espécie modelo uma vez que é amplamente utilizado em ensaios de toxicidade de contaminantes prioritários e emergentes.

Inicialmente, tendo por objetivo investigar os efeitos do EE_2 na capacidade reprodutiva e desenvolvimento embrionário, expôs-se o peixe-zebra a concentrações relevantes, do ponto de vista ambiental, de EE_2, 0,5, 1 e 2 ng/L (concentrações reais de 0,19, 0,24 e 1 ng/L, respetivamente). Na geração F_1, a exposição ao EE_2 durante todo o ciclo de vida, não afetou a capacidade reprodutiva, apesar de se ter verificado um aumento da transcrição do gene *vtg1* para 1 ng/L EE_2. Em contraste com os resultados da geração F_1, foi observado um aumento significativo na mortalidade dos embriões às 24 horas pós-fertilização (hpf), para todos os tratamentos. Além disso, a exposição à concentração mais elevada de EE_2 provocou um aumento do número de anomalias no desenvolvimento às 8 hpf. Em conjunto, estes resultados indicam que o EE_2 teve uma repercussão negativa na fase de blástula, gástrula e

organogénese inicial. A sensibilidade do embrião ao EE_2 correspondeu, também, à fase mais sensível do desenvolvimento embrionário sob condições normais.

Paralelamente avaliaram-se os efeitos do EE_2 no comportamento natatório de juvenis de peixe-zebra expostos durante 40 dias pós-fertilização. A exposição dos juvenis ao EE_2 levou a uma alteração significativa do padrão de comportamento, com uma diminuição da atividade natatória, mesmo para concentração mais baixa de EE_2 (concentração real de 0,19 ng/L). Uma diminuição da atividade de locomoção pode interferir com vários parâmetros essenciais à sobrevivência, tendo potenciais implicações no número de adultos que atinge a maturidade e se reproduz. Adicionalmente, a resposta comportamental seguiu o mesmo padrão que o desenvolvimento embrionário (F_2), evidenciando que o comportamento é um parâmetro útil e não invasivo da exposição a EDCs.

Dado que no nosso estudo inicial não foram observadas alterações histológicas nas gónadas das fêmeas e dos machos, nem nos parâmetros de qualidade do esperma, colocou-se a hipótese de que alterações no ADN parental seriam um mecanismo importante na disrupção do desenvolvimento embrionário. Assim, num trabalho paralelo, estudaram-se possíveis mecanismos subjacentes às alterações no desenvolvimento embrionário, resultantes da exposição a baixas concentrações de EE_2. A exposição de ciclo de vida completo ao EE_2, na gama de concentrações testadas (0,19-1 ng/L), aumentou significativamente a transcrição do gene *p53* nas gónadas dos machos, em todas as concentrações. Nas fêmeas a magnitude da expressão génica do *p53* não foi tão pronunciada quanto nos machos. Estes resultados sugerem que a exposição a EE_2 afetou mais severamente os machos que as fêmeas. A expressão do gene *p53*, em machos expostos a EE_2, apresentou uma boa correlação com os dados do desenvolvimento embrionário, apoiando a hipótese de que a mortalidade dos embriões após a exposição a EE_2 se deve a uma diminuição da capacidade de reparação do ADN pelos machos.

De modo a avaliar o potencial toxicológico de misturas binárias de xenoestrogénios e xenoandrogénios, avaliaram-se os efeitos do EE_2 e do TBT, isolados e em mistura. Havia um interesse particular em investigar se a exposição a misturas de EE_2 e TBT iria afetar a capacidade de reparação do ADN no peixe-zebra, provocando uma repercussão no desenvolvimento embrionário. Com este propósito realizou-se um ensaio de ciclo de vida, expondo o peixe-zebra a

concentrações ambientais relevantes de EE_2 (0.75 e 1.75 ng/L), TBT (10 e 50 ng/L Sn/L) e misturas binárias dos mesmos compostos (0.75 ng/L EE_2 + 50 ng/L TBT e 1.75 ng/L EE_2 + 50 ng/L TBT). Neste trabalho, a exposição a 50 ng/L TBT diminuiu significativamente o sucesso de fertilização enquanto que a exposição a EE_2 e às misturas de ambos os compostos alterou significativamente o rácio sexual, a favor das fêmeas. Adicionalmente, o EE_2 e a mistura de EE_2 e TBT (1.75 ng/L EE_2 + 50 ng/L TBT) aumentaram significativamente a mortalidade dos embriões. O estudo de expressão génica revelou que a exposição a EE_2 e TBT, tanto isolados como em mistura, levou a uma alteração significativa no padrão de expressão de genes das vias de sinalização do NER e p53.

Coletivamente, os resultados sugerem que o aumento da mortalidade dos embriões, na concentração mais elevada de EE_2, se deve a uma diminuição da capacidade de reparação do ADN, o que é apoiado pela diminuição significativa do nível de transcritos do gene *xpc* e *p53*. Quando o EE_2 e o TBT foram combinados, nas concentrações mais elevadas, a mortalidade dos embriões foi aumentada. Um aumento de dano pode levar à indução de vários processos de reparação do ADN, o que é consistente com o aumento significativo da expressão de todos os genes alvo do p53 (*p21*, *gadd45α* e *bax*) na mistura com concentrações mais elevadas dos compostos isolados. Contudo, se o dano foi demasiado severo pode ter predominado o mecanismo de apoptose, o que é apoiado pelo aumento do número de transcritos dos genes *xpd*, *gadd45α* e *bax*. Tal fato permitiria explicar, parcialmente, o aumento da mortalidade e anomalias nos embriões deste tratamento.

De forma global, os resultados do presente trabalho levantam preocupações acerca da repercussão, a nível populacional, da exposição prolongada a baixas concentrações de xenoestrogénios e xenoandrogénios. O presente estudo demonstra que o EE_2 e o TBT, isolados e em misturas, afetam parâmetros relevantes do ponto de vista ecológico, como o desenvolvimento embrionário e o comportamento, e, simultaneamente, a expressão de genes essenciais na manutenção da homeostasia do organismo, revelando possíveis mecanismos de ação destes compostos. Adicionalmente, os resultados indicam que o desenvolvimento embrionário, o padrão de comportamento e a análise da expressão génica são parâmetros sensíveis, que poderão ser utilizados como

indicadores precoces de risco ambiental de EDCs em populações de peixes selvagens.

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Acronyms list

AE	Androstenedione
11-KT	11-ketotestosterone
17α,20βP	17α,20β-dihydroxyprogesterone
Bax	Bcl2-associated X protein
bax	Bcl2-associated X gene
Bcl-2	B-cell lymphoma 2 protein
cdks	Cyclin-dependent kinases
DDT	Dichlorodiphenyltrichloroethane
dpf	Days post-fertilization
E_1	Estrone
E_2	17β-estradiol
ECs	Estrogenic chemicals
EDCs	Endocrine Disrupting Chemicals
EE_2	17α-ethinylestradiol
ER	Estrogen receptor
F_0	Parental generation
F_1	Parental exposed generation
F_2	Descendents of F_1 generation
FSH	Follicle stimulating hormone
G1	Gap1 cell cycle phase
G2	Gap2 cell cycle phase
Gadd45α	Growth arrest and DNA damage induced 45 alpha protein
gadd45α	Growth arrest and DNA damage induced 45 alpha gene
GGR	Global genome repair
GnRH	Gonadotropin-releasing homone

GSI	Gonadosomatic index
h	Hours
hpf	Hours post-fertilization
HPG	Hypothalamus-pituitary-gonadal axis
HR23B	Ultraviolet excision repair protein RAD23 homolog B
K	Condition factor
LH	Luteinizing hormone
M	Mitosis cell cycle phase
Mdm2	Murine double minute 2 protein
mdm2	Murine double minute 2 gene
min	Minutes
mpf	Months post-fertilization
NER	Nucleotide excision repair
p21	Cyclin-dependent kinase 1A inhibitor protein
p21	Cyclin-dependent kinase 1A inhibitor gene
p53	Tumor suppressor protein p53
p53	Tumor suppressor gene p53
PCBs	Polychlorinated biphenyls
PCNA	Proliferating cell nuclear antigen
pec-fin	Pectoral fins
PFOS	Perfluorooctanesulfonate
pRB	Retinoblastoma protein
prim-6	Lateral line primordium over myotome 6
prim-16	Lateral line primordium over myotome 16
RPA	Replication protein A
s	Seconds

S	Synthesis cell cycle phase
T	Testosterone
TBT	Tributyltin
TCR	Transcription-coupled repair
TFIIH	Transcription repair factor IIH
Vtg	Vitellogenin protein
vtg	Vitellogenin gene
XPA-G	*Xeroderma pigmentosum* proteins A-G
xpa-f	*Xeroderma pigmentosum* genes a-f

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CHAPTER 1

Introduction

1. Introduction

1.1 Endocrine disruption – Background overview

Since the last decades we have witnessed a growing environmental contamination. In the 1960s, the evidence that common pesticides, such as DDT (dichlorodiphenyltrichloroethane), were able to interfere with the endocrine system raised an increasing awareness to this topic (Carson, 1962). Studies beginning in the mid-1960s in herring gulls (*Larus argentatus*) in Lake Michigan suggested that environmental contaminants were adversely affecting hatching success (Keith, 1966). The use of diethylstilbestrol (DES) by pregnant women from the late 1940s until 1971, to help prevent miscarriages, resulted in infertility (Colborn et al., 1993). However, it was not until the early 1990s that endocrine disruption received the much deserved attention, becoming a concern to the scientific community and governments (Colborn et al., 1996).

Several studies reported cases of endocrine disruption, ranging from wildlife to humans (e.g.: gastropods, Smith, 1971; crustaceans, Rodriguez et al., 2007; reptiles, Guillette et al., 1994; fishes, Bortone and David, 1994; birds, Quaglino et al., 2002 and humans, Caserta et al., 2008). Endocrine disruption is translated in a wide variety of negative effects on reproduction (vitellogenin induction, bias in the sex ratio, decreased fertility and fecundity), embryonic development (increased mortality and developmental defects), behaviour (disturbed mating and activity performance), steroidogenesis (altered/abnormal hormone levels), stress response, immune and nervous system function, thyroid metabolism and also increased incidence of cancer (WHO/IPCS, 2002).

The compounds that are able to interfere with the endocrine system producing these adverse effects are known as endocrine disruptors, commonly referred to as Endocrine Disrupting Chemicals (EDCs) (WHO/IPCS, 2002). EDCs exert their action in the endocrine system by mimicking or antagonizing endogenous hormones (estrogens/androgens), altering the natural pattern of hormone synthesis or metabolism and/or by modifying hormone receptor levels (Sonnenschein and Soto, 1998).

Substances suspected to act as EDCs are used in industry, agriculture and consumer products, and can also be derived as by-products of industrial processes (Sonnenschein and Soto, 1998). Some known EDCs include industrial intermediates (4-nonylphenol, bisphenol A, phthalates), pesticides (DDT, atrazine, endosulfan), organic compounds (organotins), polychlorinated biphenyls (PCBs), phytoestrogens, pharmaceuticals, natural estrogens and heavy metals (Colborn et al., 1993; Matthiessen and Sumpter, 1998; Sumpter, 2003).

There is extensive scientific literature on the effects of EDCs on animals, both in the wild and under laboratory conditions. It is of critical importance to study and confirm the biological mode of action of these compounds as well as their ability to induce relevant ecological consequences in order to improve risk assessment of the chemical under study and also anticipate the effects of other chemicals that act through a similar mode of action. Additional compounds will likely be identified as more work is completed and better structure/activity relationships are developed.

1.2 Endocrine disrupting chemicals (EDCs) – Estrogenic and androgenic compounds

In the environment, chemicals with different effects on the physiology of invertebrates and vertebrates have been described. Those that interfere with reproductive capacity are often grouped according to their estrogenic and/or androgenic properties. Estrogenic chemicals (ECs) have received most attention due to their wide environmental occurrence and well-documented adverse effects (Sumpter, 2005). Among the several ECs, some products stand out, such as surfactants (e.g. alkylphenols), used in the synthesis of detergents and as antioxidants; persistent organic pollutants (e.g. PCBs and pesticides), applied in electric equipment and agriculture; plasticizers (e.g. bisphenol A and phthalates), used to reduce the stiffness of certain polymers; polycyclic aromatic hydrocarbons (PAHs), found in petroleum; phytoestrogens and natural estrogens (e.g. 17β-estradiol E_2 and estrone E_1) as well as synthetic estrogens (e.g. ethinylestradiol EE_2) (Sonnenschein and Soto, 1998; WHO/IPCS, 2002; Sumpter, 2005).

Although ECs have received most of the attention, chemicals with androgenic activity have also been detected in the environment. For instance, in a survey in seven different United Kingdom rivers, using a yeast androgen screen assay, it

was demonstrated the presence of diverse chemicals with androgenic activity (Thomas et al., 2002). In fish, androgens are the male reproductive hormones that are produced in the testes and contribute to the expression of secondary sexual characters. They include testosterone (T), androstenedione (AE), 11-ketotestosterone (11-KT) and 11-hydroxy testosterone. Among xenoandrogens, organotin compounds like tributyltin (TBT) and triphenyltin (TPT), gained special importance due to their commercial relevance. In particular, TBT was used as an effective biocide in antifouling paints to prevent the settlement of organisms to the hull of ships (responsible for ship diminished performance and increased fuel consumption) and other submerged structures. TBT cost-effective properties led to its widespread application.

1.2.1 Xenoestrogens – Ethinylestradiol (EE_2)

ECs are among the most widely studied EDCs mostly because field studies have shown the presence of fish feminization in many aquatic ecosystems (Sumpter, 2005). Fish can be exposed to EDCs through a variety of sources, but waste waters are the primary source of ECs (Folmar et al., 2000; Metcalfe et al., 2001; Kolpin et al., 2002). Studies on natural and anthropogenic ECs from waste water treatment plants are particularly important in assays of risk assessment since they reveal an endocrine disruption potential far more elevated than that of industrial pollutants (Harries et al., 1996; Klotz et al., 1996; Jobling et al., 1996; Tyler, 1998). Although the behaviour of ECs in the environment is not yet fully understood, available data show that xenoestrogens interfere with endogenous hormone receptors or exert their effects through the mediator system of specific receptors (Gillesby and Zacharewski, 1998; Matthiessen and Sumpter, 1998; Andersen et al., 2000).

The most biologically active xenoestrogen in the aquatic environment is 17α-ethinylestradiol (EE_2) (Folmar et al., 2000; Metcalfe et al., 2001; Kolpin et al., 2002), the active ingredient of contraceptive pills. EE_2 is excreted in urine in an inactive conjugated form but is readily activated through bacterial activity in the sewage (Guengerich, 1990). After entering the aquatic environment, EE_2 is relatively persistent. For instance, although EE_2 concentrations in surface waters are generally lower than those of natural estrogens, EE_2 biological activity in fishes

is about 10 to 50 times higher than that of E_1 and E_2 *in vivo*, due to its longer half-life time and ability to bioconcentrate (Segner et al., 2003b; Thorpe et al., 2003). EE_2 is not only more effective in eliciting estrogenic responses but also more stable than the natural estrogen E_2 (Länge et al., 2001; Lai et al., 2002; Thorpe et al., 2003). The structural similarity of EE_2 with the natural estrogen E_2 is presented in Figure 1.1.

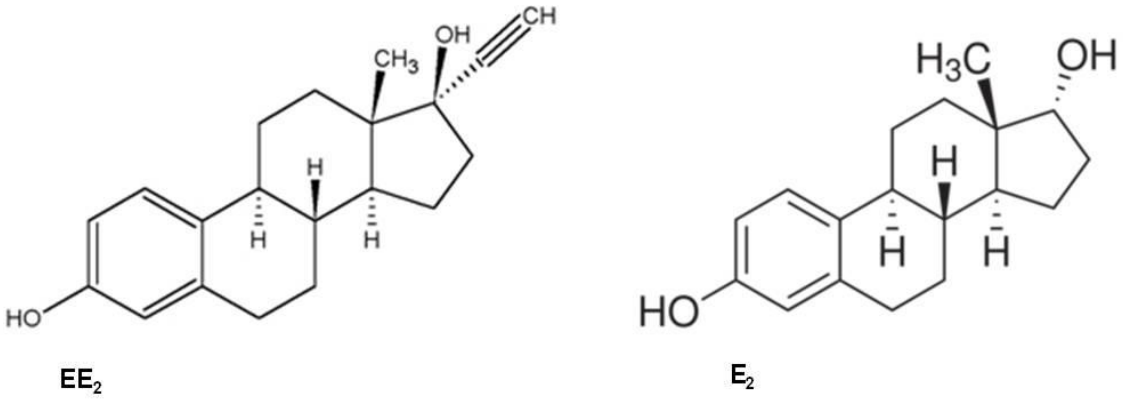

Figure 1.1 Structural similarity of the synthetic estrogen ethinylestradiol (EE_2) with that of the natural estrogen 17β-estradiol (E_2).

Environmental EE_2 concentrations in water are highly variable, from non-detectable to a maximum concentration of 830 ng/L in certain U.S.A. rivers (Kolpin et al., 2002). In Europe, the majority of the observations were below 5 ng/L (Desbrow et al., 1998; Belfroid et al., 1999; Ternes et al., 1999; Johnson et al., 2000; Svenson et al., 2002). Nevertheless, even at low concentrations, this compound is still able to cause endocrine disruption in fish (Tyler et al*.,* 1998). In several fish species, concentrations of 0.1 ng/L EE_2 induced vitellogenin (Vtg) production (Purdom et al., 1994; Sumpter and Jobling, 1995); concentrations of 0.1-15 ng/L affected development and normal sexual differentiation (Metcalfe et al., 2001; van Aerle et al., 2002; Andersen et al., 2003; Van den Belt et al., 2003; Weber et al., 2003); at 2-10 ng/L reduced fecundity and reflected in the gonadosomatic index (GSI) (Scholz and Gutzeit, 2000; Länge et al., 2001; Van den Belt et al., 2002; Nash et al., 2004); at 10 ng/L affected reproductive behaviour (Balch et al., 2004) and concentrations of 1-10 ng/L diminished fertilization success or viability of eggs from exposed adults (Länge et al., 2001; Hill and Janz, 2003; Segner et al., 2003a; Nash et al., 2004). EE_2 can also reduce

testicular growth and inhibit spermatogenesis at concentrations lower than 10 ng/L (Routledge et al., 1998; Larsson et al., 1999; Christiansen and Helweg, 2002) and induce complete feminization of males (Papoulias et al., 1999; Scholz and Gutzeit, 2000). However, the ecological impact of xenoestrogens to wild fish populations is still not fully understood.

1.2.2 Xenoandrogens – Tributyltin (TBT)

The best documented example of endocrine disruption in wildlife is the masculinization of female neogastropods by TBT, a phenomenon called imposex (Smith, 1971; Matthiessen and Gibbs, 1998; Barroso et al., 2000; Santos et al., 2005) or pseudohermaphroditism (Jenner, 1979) that is characterized by the superimposition of male secondary sexual characteristics in females of gonochoristic gastropods, namely a penis and a *vas deferens*. Imposex is irreversible and in advanced stages can lead to female reproductive failure leaving populations close to extinction. Among the affected snail species, the dogwhelk *Nucella lapillus* is one of the most sensitive to TBT with a water threshold level for imposex induction below 1 ng TBT Sn/L (Gibbs et al., 1988). Figure 1.2 presents TBT chemical structure.

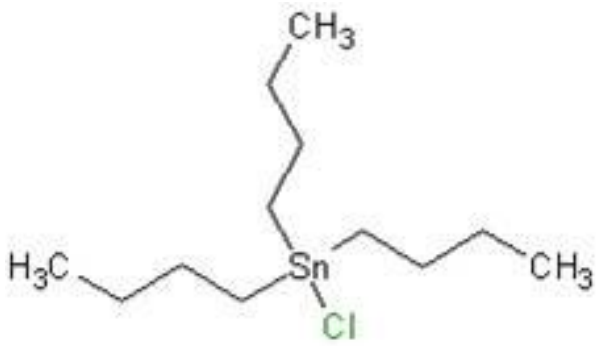

Figure 1.2 Molecular structure of tributyltin chloride.

Other examples of TBT exposure in molluscs are shell deformations and reproductive failure (Alzieu, 1991), reduced rates of fertilization and development (Fent, 1996) and alterations in steroid and lipid metabolism (Janer et al., 2006; Santos et al., 2012). However, only recently it was demonstrated that TBT also induces masculinization in vertebrates, namely in fish. Exposure of Japanese

flounder *(Paralichthys olivaceus*) and zebrafish (*Danio rerio*) to TBT resulted in a male biased sex ratio (McAllister and Kime, 2003; Shimasaki et al., 2003) and low quality sperm lacking flagella (McAllister and Kime, 2003). Male fish exposed to TBT also showed reduced gonadal development and histological changes in the testis (Zhang et al., 2009). In fish, TBT is also known to diminish female fertility, affect sexual behaviour and disrupt embryonic development (Nakayama et al., 2004, 2005; Zhang et al., 2011). Other adverse effects reported in TBT exposed females include alterations in follicular development and decreased GSI (Zhang et al., 2007). Other studies have reported adverse effects in annelids and crustaceans (Fent, 1996).

One of the hypothetical mechanisms of action proposed for TBT is the inhibition of aromatase cytochrome P450 enzyme, responsible for the aromatization of T to E_2 and the conversion of AE to E_1, thus interfering in the synthesis of sex steroids (Santos et al., 2005). Recently, several reports suggest that TBT action in reproductive-related endpoints, such as imposex in snails and altered sex ratio in fish, may occur through its interaction with nuclear receptors e.g., the retinoid x receptors (RXRs) and the peroxisome proliferated-activated receptors (PPARs) (Nishikawa et al., 2004; Castro et al., 2007; Nakanishi, 2007; Pascoal et al., 2013). Despite the large number of species affected by TBT, the involved signaling pathways have yet to be fully deciphered.

TBT ubiquity in aquatic ecosystems in conjunction with its elevated and broad spectrum toxicity, with well documented adverse effects, led to its total ban, as an antifouling agent on ship hulls, in 2008 by the International Maritime Organization (IMO) (Anon, 2001; Santos et al., 2009; Barroso et al., 2011). Although its use has been forbidden, the levels of TBT in the aquatic environment are still a cause of great concern (Rüdel et al., 2007).

1.3 Complex mixtures of EDCs with a dissimilar mode of action

In the aquatic environment, EDCs rarely occur isolated. However, the combined effects of EDCs mixtures seem to be a rather unexplored ground. In fact, 95% of all toxicological studies evaluate the effects of single chemicals, with only a low percentage of studies addressing the issue of mixtures. Among these works, literature focuses mostly on combined effects of EDCs from the same

toxicology category. Particular emphasis is made in the study of ECs, using Vtg production as the relevant endpoint (e.g. Thorpe et al., 2001, Thorpe et al., 2003; Brian et al., 2005). Having a similar mode of action, mixtures of ECs will operate in an additive manner (Brian et al., 2007). However, combinatory studies with dissimilarly acting EDCs are limited and few studies have been published (Spurgeon et al., 2010).

EDCs with a distinct mode of action have the potential for interactions which may result in responses that are difficult to predict base upon single chemical toxicological data. Single ecotoxicological evaluations may not reflect the multitude of antagonistic or synergic stimulus that wildlife and humans may face. In addition, the effects of blends were studied mainly on parameters related to reproduction (Kortenkamp, 2007). Studies addressing a full life-cycle perspective and other endpoints with an ecological relevance are scarce. These facts highlight the need to implement more robust approaches that take these uncertainties into consideration.

These major knowledge drawbacks will be addressed in this thesis. We will analyze the effects of EE_2 and TBT, single and in combination, at different fish life-cycle stages while relating the observed effects to the molecular mode of action. As a model species, we selected *Danio rerio*, which has long been used in toxicological studies, and in which biological impacts have been reported after EE_2 and TBT exposure.

1.4 Endocrine control mechanisms in fish: reproduction, embryonic development and behaviour

Many EDCs modulate sex steroid signalling and the hypothalamic-pituitary-gonadal (HPG) axis (Ankley and Johnson, 2004; Segner et al, 2006), interfering with reproduction, development and behaviour (Guillette et al; 1995; Bigsby et al., 1999, Nakayama et al., 2004). Any alteration of these processes may lead to dysfunctions that are often life-threatening. Teleost fish seem to be particularly sensitive to chemicals impacting the HPG axis, which is most likely related with the high reproductive plasticity found in many species.

1.4.1 Fish reproduction

The endocrine system of vertebrates consists of various glands located throughout the body which synthesize and secrete hormones to regulate an array of biological processes, including reproduction.

Signals from the brain control the hypothalamic secretion of gonadotropin-releasing homone (GnRH) which stimulate de pituitary gland (hypophysis) to release gonadotropins. Gonadotropins stimulate gonadal development and production of steroid hormones: E_2 in females, T and 11-KT in males. Maturation inducing hormones, mainly 17α,20β-dihydroxyprogesterone (17,20βP) are produced in both sexes (Hachfi et al., 2012). Although estrogens are typically associated with females and androgens with males, evidence indicates that both female and male vertebrates produce and use estrogens and androgens. Figure 1.3 presents a schematic illustration of the HPG axis in fish.

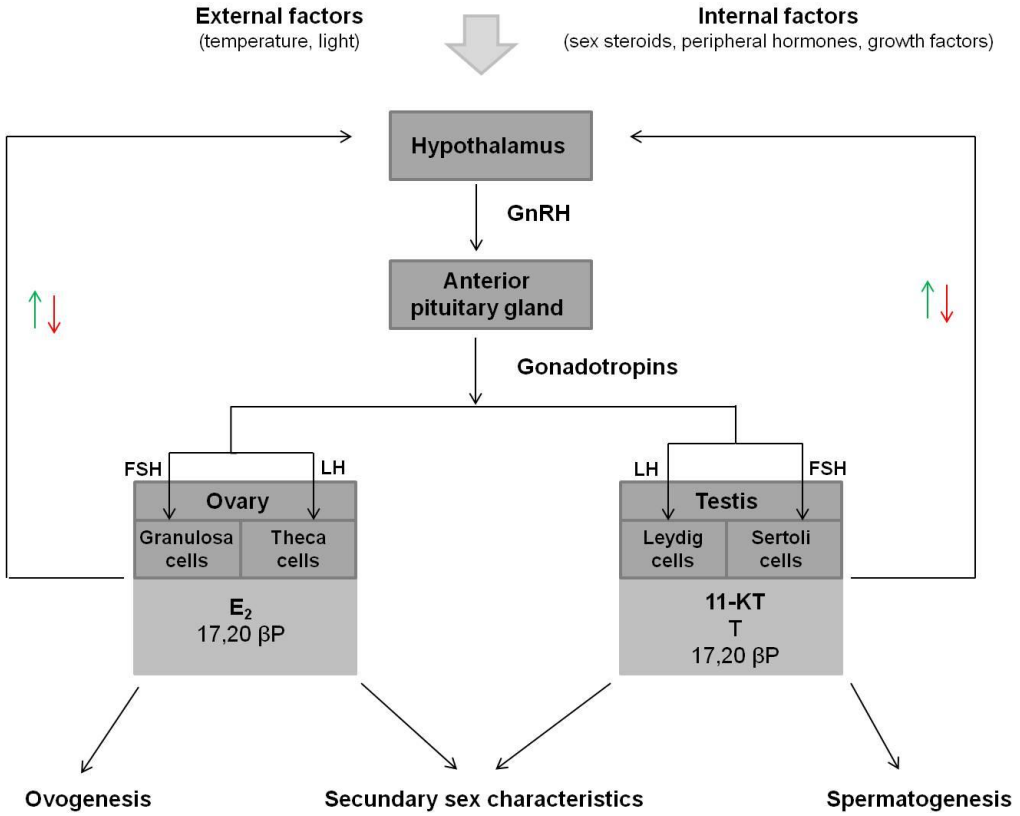

Figure 1.3 Schematic illustration of the hypothalamus-pituitary-gonadal axis in fish (GnRH - gonadotropin releasing hormone, LH - luteinizing hormone, FSH - follicle stimulating hormone, E_2 - 17β-estradiol, T - testosterone, 17,20βP --17α,20β-dihydroxy-4-pregnen-3-one, 11-KT - 11-ketotestosterone, ↑ - activation, ↓ - inhibition) (modified from Hachfi et al., 2012).

The gonadotropins are found in two distinct forms, follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH is responsible for gonadal growth and gametogenesis while LH is responsible for gonadal maturation and spawning (Kawauchi et al., 1989).

Fish gonads are structurally similar to those of other vertebrates, with germ cells intermixed with supporting somatic cells. Oocytes are surrounded by an inner granulosa, where FSH initiates follicular growth, and an outer theca layer. In the theca layer, T and other precursor androgens are produced, by stimulation of LH. In the granulosa layer, aromatization of T into estrogen takes place, with E_2 being the main female estrogen. E_2 is responsible for Vtg synthesis, through interaction with estrogen receptors (ER), leading to induction and transcription of *vtg* genes. Vtg is a female protein which is released by the liver and incorporated in oocytes, where it serves as a food reserve for embryos and larvae. Vtg is normally present at high levels in females undergoing oogenesis while it is only marginally expressed in males and immature females (Tyler et al., 1999). Therefore, abnormal changes in *vtg* levels in males are viewed as an indicator of estrogenic contamination (Hachfi et al., 2012).

In the testis, the Leydig cells are the main site of androgen synthesis, under stimulation of LH. In fish, 11-KT is the major testicular androgen. FSH targets sertoli cells which support the development of the spermatozoa and secrete androgen binding proteins.

Sexual differentiation is dependent on a delicate balance between androgens and estrogens within the body. Steroids initiate changes in secondary sex characters, development and maturation of gametes (Miller, 1988; Stocco and Clark, 1996). Steroid biosynthesis requires the action of several oxidative enzymes responsible for the conversion of cholesterol into steroids. The cytochrome P450 11β-hydroxylase and the cytochrome P450 aromatase are involved in the final steps of the synthesis of 11-KT and the conversion of T to E_2, respectively (Sreenivasulu, 2009). Final plasma concentrations of steroids depend on their level of synthesis and the rate of degradation by the liver. Initial phases of gonadal recrudescence are under the control of E_2 and 11-KT. 17α,20βP secretion induces the final maturation of oocytes or testis (Hachfi, 2012).

All of the above mentioned coordinated processes are controlled by positive and negative feedback mechanisms to maintain homeostasis and avoid extreme

alterations in hormone levels. The mechanisms mediating feedback control are complex and can be caused by direct effects of steroids at the pituitary and hypothalamic levels and/or by steroid modulation of the activity of the neuronal systems, affecting neuropeptides and neurotransmitters release which may influence the reproductive axis (Zohar et al., 2010). External factors, such as light and water temperature, can also act on the brain to control the timing of gonadal development and maturation in fishes.

Sexual steroids, produced by the gonads, are essential components of the hormonal communication along the HPG axis. Therefore, reproduction requires sexual steroids inductive actions. Any disruption of the normal hormonal function could lead to a reproductive disturbance. Therefore, exposure to EDCs, with hormonal effects, can disrupt the reproductive endocrine system. Indeed, such xenobiotics are known to alter reproductive capacity (fecundity and fertilization success) as well as other parameters related to reproduction, namely *vtg* induction, sexual differentiation of the gonad, timing of sexual maturation, GSI, reproductive tract and gonad morphology (Milnes et al., 2006).

1.4.2 Embryonic development

One of the most promising alternatives in toxicity testing is the use of embryo bioassays. These biological tools require minimum sacrificing of adult animals, and several thousands of embryos can be easily obtained. Importantly, available toxicological data dealing with biologically active compounds and priority chemicals indicates that early life stages of aquatic animals seem to be highly sensitive to a vast array of pollutants (Braunbeck et al., 2005).

Fish embryonic development broadly consists of seven periods. These periods are the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and finally hatching. Currently, the zebrafish is one of the favored models for studies of vertebrate development. A complete series of normal stages of zebrafish embryonic development can be viewed in Kimmel et al. (1995).

In brief, the zygote period is a 1-cell stage in which the corion (egg shell) swells and detaches from the newly fertilized embryo. The cytoplasm streams away from the vegetal pole and towards the animal pole to form a blastodisc. As cleavage occurs, the cells will divide allowing the accumulation of new cells and giving rise

to 3 tiers of blastomeres. During the blastula period, the embryo will continue to accumulate cells and the marginal blastomeres form rows of yolk syncytial layer. Then, epiboly begins. Epiboly is a process in which the yolk of the syncytial layer and blastodisc thins and spreads over the yolk cell, originating the blastoderm. During the gastrula period the blastoderm continues to cover a large fraction of the yolk (approximately 90%-epiboly), forming a germ ring and an embryonic shield. By the end of this period the embryo develops brain and notochord rudiments. The next stage is marked by the segmentation of somites (1-26^{+}) which leads to the development of primordial regions, such as trunk, tail and lens. In the pharyngula period, the embryo will begin to exhibit fully formed organs. During hatching, the external indicators of fish develop: gills, jaw and pectoral fins grow. Once the development is complete, the embryo is ready to hatch.

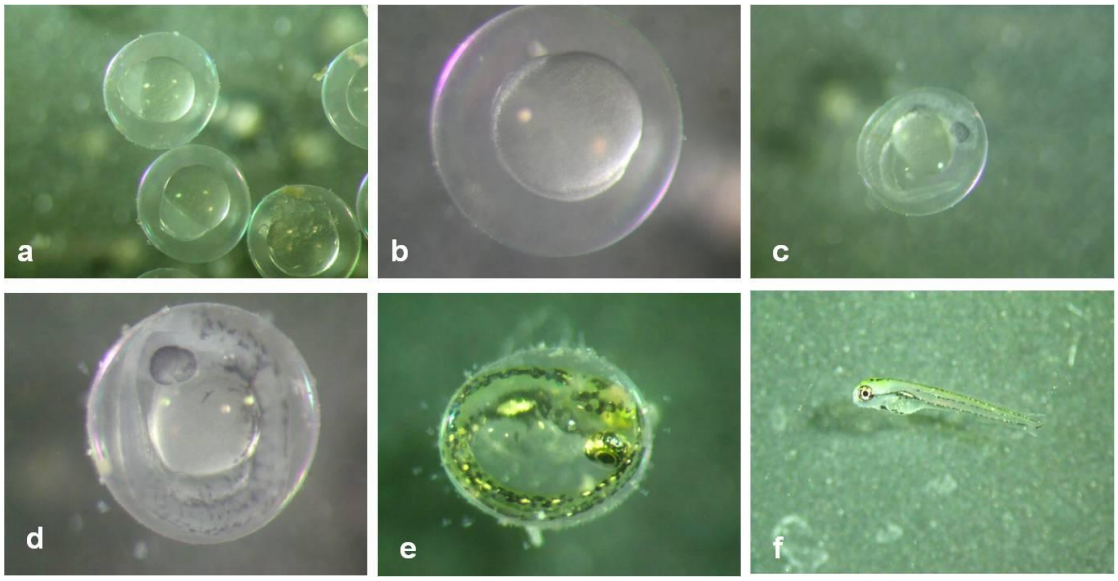

Figure 1.4 Zebrafish embryonic development, followed in a preliminary assay: evaluation steps and corresponding developmental stages (a) late high blastula, 4 hpf; b) 75%-epiboly, 8 hpf; c) prim-6, 24 hpf; d) prim-16, 32 hpf; e) pec-fin, 55 hpf; f) protruding mouth, 80 hpf).

Figure 1.4 shows important stages of zebrafish embryonic development, namely late high blastula, 4 hours post-fertilization, hpf (cleavage period); 75%-epiboly, 8 hpf (gastrula period); prim-6 (lateral line primordium over myotome 6), 24 hpf and prim-16, 32 hpf, (pharyngula period with early and late organogenesis); pectoral-fins (pec-fin), 55 hpf and protruding mouth, 80 hpf (hatching period). The

development stages with their characteristic features and the corresponding endpoints of evaluation at each stage are presented in table 1.1.

Table 1.1 Zebrafish embryonic development: important developmental stages and characteristic features, and corresponding specific endpoints for evaluation (see Fig. 1.5 for corresponding photographs) (modified from Oberemm, 2000).

Age (hpf)	Normal stage	Features	Specifc endpoints
4	high blastula Fig.1.5/a	elevated cap of regular small cells on top of the yolk	start of exposure
8	75%-epiboly Fig.1.5/b	late gastrula, epiboly in progression, embryo still spherical, keel-like embryonic shield visible	progression of ectodermal front, homogeneity of front and tissue
24	prim-6 Fig.1.5/c	Basic vertebrate body organization, beginning of heart beat, melanophores in the eye, spontaneous movements	Head and tail differentiation, length of tail, spontaneous movements, presence of heart beat
32	prim-16 Fig.1.5/d	Blood circulatory system, melanophores spread on the trunk	Heart rate, malformations of the blood circulatory system, edema, distribution of melanophores
55	pec-fin Fig.1.5/e	Hatching begins, melanophores pronounced, blood circulatory system fully developed	Heart rate, hatching rate, malformations of the blood circulatory system, edema, state of melanophores
80	protruding mouth Fig.1.5/f	eleutheroembryo: resting on side, yolk still present, xantophores present (embryos gleaming yellowish)	Heart rate, expression of pigmentation, edema, finfold lesions

These endpoints enable the determination of any alteration on the embryonic development associated with EDCs exposure.

1.4.3 Behaviour

As in other vertebrates, fish behaviour is under endocrine system control, particularly by steroid hormones. Any modification on hormone regulation may lead to severe behavioural changes with potential impacts on fish population dynamics. In this context, EDCs play an important role.

Among the internal factors that interfere with behaviour, it is primarily the hormone levels that determine the intensity and also, to some extent, the course of some behaviours. This hormonal role has been supported by an abundance of studies demonstrating that by administering hormones behaviours may be modified (e.g. androgens elevate the expression of aggressive behaviours in fishes; Trainor et al., 2004). In addition, steroid hormones may organize the brain in ways that enhance synaptic strengths or modify cellular constituents (neuromodulators, neurotransmitters, hormone receptors) in behaviourally relevant brain areas that modulate behaviour responses, allowing the integration of the nervous and the endocrine systems (Hsu et al., 2006).

Neuroendocrinology helps explaining the mechanisms underlying behaviour. For example, cichlid (*Astatotilapia burtoni*) territorial males have higher forebrain messenger (m)RNA expression of several specific androgen and oestrogen receptors compared to non-territorial males, suggesting that their brains are more sensitive to circulating androgens, possibly amplifying the behavioural effects of hormones (Burmeister et al., 2007).

Fish specialized behaviours are primarily concerned with the three most important activities in the fish's life: reproduction, feeding and escape from enemies. Physical interaction, visual and species-specific olfactory cues (pheromones) are hypothesized to trigger a cascade of hormonal responses that influence maturity and successful reproduction. The reproductive success in most fish species depends, partly, on their ability to perform correct sexual behaviour. Therefore, altered behavioural patterns can impair reproductive output. In fact, exposure of breeding zebrafish to 10 ng/L EE_2 was shown to disrupt reproductive hierarchies, with a decrease in aggressiveness of exposed males and diminished reproductive output (Coe et al., 2008). Several studies report that EE_2 and TBT exposure affect fish reproductive behaviour (Robinson et al., 2003; Balch et al., 2004; Nakayama et al., 2004; Larsen et al., 2008; Partridge et al., 2010).

Imbalances in non-reproductive patterns of behaviour have also been reported after EDCs exposure. Perturbation in aggression, vertical distribution, predation, escape, anxiety and schooling behaviours have been documented (Bell, 2004; Coe et al., 2009; Reyhanian et al., 2011; Sárria et al., 2011a,b).

Behavioural endpoints have been slow to be integrated in aquatic toxicology because, until recently, there was a poor understanding of how alterations in behaviour may be related to ecologically-relevant issues such as growth, stress resistance, reproduction and longevity (Kane et al., 2005). Since behaviour is a highly structured sequence of activities designed to ensure maximal fitness and survival of the individual (and species), behavioural endpoints should serve as a valuable tool to discern and evaluate effects of exposure to environmental stressors (Kane et al., 2005). In addition, behavioural assays provide biologically relevant endpoints to evaluate sub-lethal exposure effects and may compliment traditional toxicity testing. In fact, analysis of behavioural responses can provide quantitative measures of neural and mechanical disruption, reflecting biochemical and physiological alterations (Brewer et al., 2001).

Generally, toxicological studies that monitor behavioural endpoints are focused in mature individuals, targeting reproductive behaviour. Studies on EDCs effects on fish larvae/juveniles are scarce and should be considered when addressing the effects of these contaminants, given the implications on population connectivity, stability and persistence (Sárria et al., 2011b). An alteration in larval dispersal or juvenile recruitment can lead to inappropriate aggregation events, affecting the number of young fish that will reach adulthood (Hinckley et al., 1996), thereby disrupting population demography and community dynamics over time.

Although behavioural studies are scarcely explored grounds they are important from an ecological standpoint and can be applied during early life as reliable indicators of EDCs exposure, helping to predict and prevent effects exerted upon individual viability and population stability.

1.5 Disruption of embryonic development

1.5.1 DNA damage

From an ecological point of view, genetic damage may lead to heritable mutations which could compromise the fitness of wild fish populations (Anderson and Wild, 1994; Kirsch-Volders et al., 2002). DNA damage in a variety of aquatic animals has been associated with abnormal development and reduced survival of embryos (Lee and Steinert, 2003). These findings support the hypothesis that embryo mortality after EDCs exposure may be due to DNA damage. Therefore, it is particularly important to evaluate EDCs impact on DNA repair pathways. However, there are just a few studies describing the potential genotoxic effects of EDCs on fish, when administered single and, specially, in combination.

Evidence shows that EE_2 is genotoxic in fish (Cooke and Hinton, 1999; Tilton et al., 2006) and mammals (Siddique et al., 2005). Similarly, strong evidences indicate that TBT is genotoxic on molluscs (Hagger et al., 2002), fish (Tiano et al., 2001; Wang et al., 2009) and mammals (Liu et al., 2006).

1.5.2 Nucleotide excision DNA repair mechanism (NER)

One biological process that circumvents DNA lesions is DNA repair. The nucleotide excision repair (NER) pathway is responsible for the repair of a wide range of DNA damage, including bulky DNA lesions formed by exposure to radiation or chemicals.

NER consists of a general pathway termed global genome repair (GGR) that acts on the elimination of lesions from non-transcribed strands from the entire genome and a pathway referred to as transcription-coupled repair (TCR), confined to DNA lesions in the strand of transcriptionally active genes (Thoma and Vasquez, 2003). For the purpose of this thesis, only GGR will be approached.

Excision repair is a multistep process involving the assembly of several proteins, the XP-A through XP-G repair factors. This designation results from defects in excision repair in humans that cause a photosensitivity syndrome called *xeroderma pigmentosum* (XP), which is characterized by a very high incidence of light-induced skin cancer (Cleaver, 1968). Among the various complementation

groups, XP-C is unique, as only GGR is compromised in this group (Venema et al., 1991).

The basic steps of nucleotide excision repair are damage recognition, damage verification and open complex formation, incision on either side of the lesion, excision of the damaged DNA template, repair synthesis and ligation (Sancar et al., 2004).

Initial damage recognition and verification are performed by XPC and XPA. XPC is the principal DNA damage binding protein essential for the recruitment of NER components to the site of DNA damage (Volker et al., 2001). Then XPC and XPA proteins work in conjunction and initiate open complex formation.

Once the XPC-HR23B complex has recognized damaged DNA, the formation of the open complex begins with association of the damage verification heterodimer XPA-RPA and recruitment of the TFIIH complex (de Laat et al., 1999). TFIIH is a transcription/repair factor with 3´ to 5´ and 5´ to 3´ activities, imparted by the helicases XPB and XPD, respectively, responsible for DNA unwind at the vicinity of the lesion (Volker et al., 2001). The nuclease XPG has a high affinity for unwound DNA. At this step, a 3´ incision takes place. Finally, the nuclease XPF is responsible for the 5´ incision, which results in the irreversible dual incisions and release of the excised oligomer (Sancar et al., 2004). Figure 1.5 presents formed NER incision complex. Once the damaged oligonucleotide is removed, a patch is synthesized by a polymerase, anchored to the replication site by proliferating cell nuclear antigen (PCNA), and sealed by ligases (Moser et al., 2007).

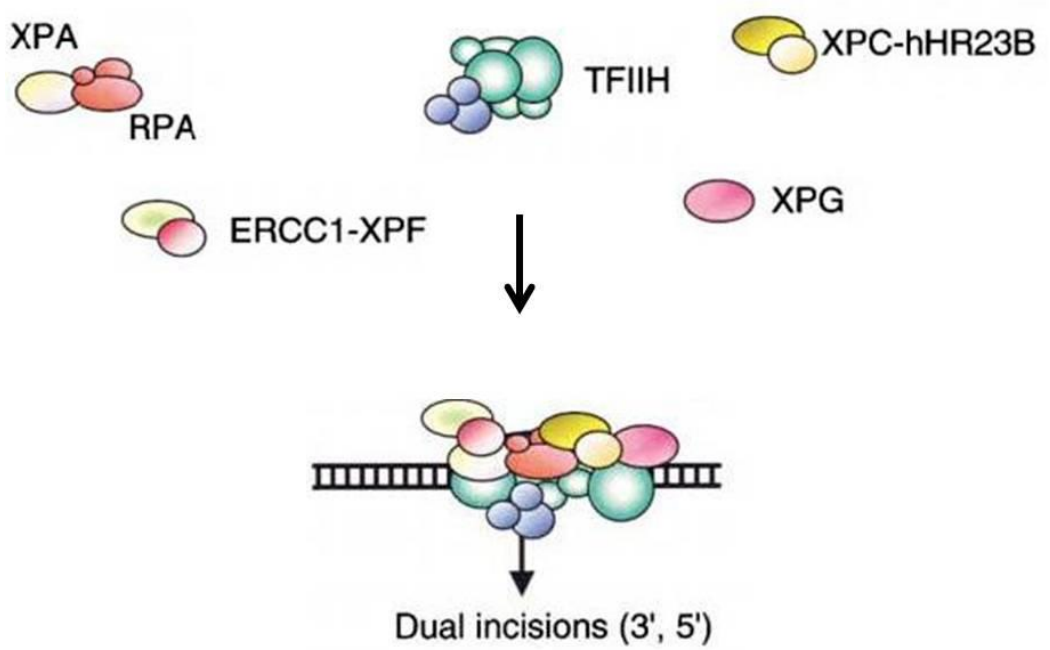

Figure 1.5 Complete formation of NER incision complex (modified from Volker et al., 2001).

Loss or decrease of any NER associated protein may lead to an increased incidence of uncorrected DNA lesions, leading to genomic instability of individuals.

It has been reported that exposure to EE_2 can alter mRNA abundance of NER genes in liver of adult zebrafish and the rate and magnitude of NER processes in zebrafish liver cells (Notch et al., 2007; Notch and Mayer, 2009a,b). Also, Zuo et al. (2012) reported that exposure to TBT induced DNA damage in cuvier (*Sebasticus marmoratus*) liver by altering the transcription levels of the NER genes. However, to date, there are no available studies analyzing the combined effects of these compounds on NER mechanism.

1.5.3 p53 pathway

Damage to DNA induces several cellular responses that enable the cell either to eliminate or cope with the damage or activate a programmed cell death process (Sancar et al., 2004). In addition to DNA repair mechanisms, other DNA damage responses include DNA damage checkpoints and apoptosis.

The tumor suppressor p53 is a multifunctional protein that has a fundamental role in maintaining the genomic stability and the cellular response to DNA damage. In response to a variety of genotoxic stimuli, damage sensor proteins detect DNA damage and initiate signal transduction cascades that activate p53. The induction

of p53 is achieved through inhibition of its negative regulators and by several post-translational mechanisms that allow rapid activation of p53 and avoid relying on transcription from potentially damaged DNA templates. Post-translational alterations include extensive covalent modifications, such as phosphorylation, acetylation and sumoylation events (Woods and Vousden, 2001). Activated p53 protein can act through direct protein-protein interactions and, mainly, as a transcription factor, increasing the expression of downstream genes such as *p21*, *gadd45α* or *bax*, all involved in the three major p53 signaling pathways in response to damage, namely cell cycle arrest, DNA repair and apoptosis (Adimoolam and Ford, 2003).

DNA damage checkpoints are biochemical pathways that delay or arrest cell cycle progression in response to DNA damage in order to allow for repair and prevention of the transmission of DNA damage. In an unperturbed cell cycle, the transition points G1/S (Gap1/synthesis) and G2/M (Gap2/mitosis) are tightly controlled (Sancar et al., 2004). The decision to enter the different phases of cell cycle is governed by cyclin-dependent kinases (cdks), which assemble with cyclins to form functional holoenzymes whose phosphorylations of key substrates facilitate these transitions. Progression through the cell cycle is controlled by different cyclins-cdks complexes.

D-type cyclins and cyclin E are synthesized sequentially during G1 (Sherr and Roberts, 1995). Cyclin D assembles with cdk4 and cdk6. Cyclin E is expressed at maximum levels near G1/S transition and forms complexes with cdk2 (Dulic et al., 1992). These cyclins-cdks are shown to phosphorylate pRB (retinoblastoma protein) sequentially, releasing bound transcription factors such as E2F, which activates genes required for S-phase entry (Sherr and Roberts, 1995). Specifically, the activity of the Cyclin B-cdc2 (cdk1) complex is pivotal in regulating the G2/M phase transition (Elledge, 1996).

p21, the p53-regulated cyclin-dependent kinase 1A inhibitor, is a primary mediator of the p53-dependent G1 cell cycle arrest following DNA damage (Harper et al., 1993). After damage, p53 levels increase, resulting in the induction of p21. Subsequently, induced p21 forms complexes with cdks-cyclins and inhibits the activity of cdk4-cyclin D, cdk6-cyclin D, cdk2-cyclin E, resulting in hypophosphorylated pRB and G1 arrest (Slebos et al., 1994), thus preventing cells from entering the S phase in the presence of DNA damage. It has been

demonstrated that the disruption of endogenous p21 abrogates the G1/S checkpoint after cell exposure to DNA damage, leading to genomic instability (Waldman et al., 1995).

GADD45α (growth arrest and DNA damage induced 45, alpha) was found to be the first stress gene that is transcriptionally activated by p53 (Kastan et al., 1992). Gadd45α has been shown to associate with cyclin B and to dissociate it from cdc2, thereby inhibiting cdc2 cyclin B kinase activity (Zhang et al., 1999). A G2 checkpoint, with inhibition of cdc2-cyclin B kinase is probably the key mechanism for preventing premature entry into mitosis and propagation of genetic errors (Zhang et al., 1999).

Beside its role in cell cycle checkpoint, Gadd45α is also directly involved in DNA repair. Maeda et al. (2002) reported that Gadd45-deficient keratinocytes have a slower rate of nucleotide excision repair, following ultraviolet radiation. It was shown that all three Gadd45 proteins interact with PCNA, a nuclear protein that plays a central role in DNA repair and replication (Vairapandi et al, 1996). Gadd45α has also been implicated in DNA repair through DNA demethylation and apoptosis, by modulating JNK pathway (c-Jun NH(2)-terminal kinase) (Harkin et al., 1999; Rai et al., 2008).

As an alternative and decidedly more dramatic defense against DNA damage, cells can undergo programmed cell death. Induction of apoptosis, under these circumstances, depends at least in part on p53. p53 can induce expression of a wide array of genes involved in the apoptotic pathway. *bax* is a key gene in p53 mediated apoptosis. Recently, both Bcl-2 (B-cell lymphoma 2), which promotes cell survival, and Bax (Bcl2-associated X protein), which promotes cell death, have been implicated as major players in the control of apoptotic pathways, and it has been suggested that the ratio of Bcl-2 and Bax proteins controls the relative susceptibility of cells to death stimuli. p53 has been shown to down-regulate endogenous Bcl-2 expression and up-regulate Bax expression, inducing apoptosis (Selvakumaran et al., 1994). The exact mechanism of p53-induced apoptosis is unknown. Apoptosis seems to predominate as DNA damage is more severe, eliminating heavily damaged or seriously deregulated cells, preventing genetic transformation (Sun et al., 2009). There is evidence that when DNA damage is not repaired, activation of p53 can lead to apoptosis (Liu and Kulesz-Martin, 2001).

p53 is a short-lived protein that is maintained at low, often undetectable, levels in normal cells. One mechanism by which p53 function is controlled is through interaction with the Mdm2 protein (murine double minute 2 protein), the principal p53 regulator. Activation of p53 is limited by a short feedback loop involving Mdm2. p53 stimulates transcription of the *mdm2* gene, and Mdm2 protein inhibits p53 function by direct binding, hindering the p53 transactivation domain and targeting p53 protein for proteosome-mediated degradation in the cytoplasm (Molinari et al., 2000).

Several recent studies have shown that p53 is involved in nucleotide excision repair, especially in GGR (Zhu et al., 2000). Figure 1.6 presents hypothetical mechanisms of p53-dependent DNA repair.

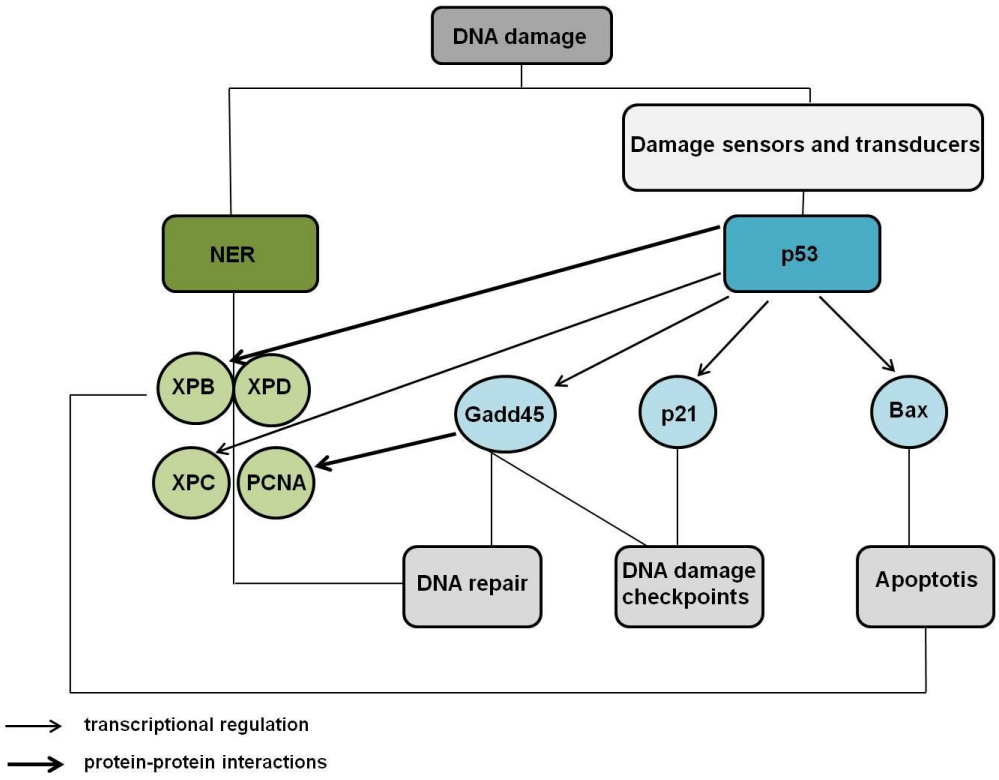

Figure 1.6 Hypothetical mechanisms of p53-dependent DNA repair.

Recent evidence points to a transcriptional regulatory role for p53 in NER, mediating expression of the damage recognition gene XPC (Adimoolam and Ford, 2003). Interestingly, it has been shown that XPC protein also plays a role in chromatin unfolding, modulating accessibility of damaged DNA (Baxter and Smerdon, 1998). p53 may also be directly involved in repair or repair related

processes through protein-protein interactions. For example, p53 has been found to both physically and functionally interact with XPB and XPD NER proteins inhibiting their helicases activities (Frit et al., 1999). Physiological significance of this alteration still has to be determined but recent evidences suggest the participation of XPB and XPD in p53-induced apoptosis (Wang et al., 1995). Conceivably, p53 can be involved in NER by regulating its own downstream genes, which proteins are either related to or actively participate in NER. For example, Gadd45 was found to bind to PCNA and stimulated DNA excision repair *in vitro* (Smith et al., 1994). Also, it has recently been shown that PCNA accumulation in the nucleus, post UV, is defective in Gadd45 null mouse embryo fibroblasts (Smith et al., 2000). Gadd45α has also been implicated in chromatin accessibility (Sun et al., 2009). Regulation of chromatin structure is clearly necessary for lesion accessibility by NER factors. Defects in any of these responses, resulting in uncorrected DNA damage, can lead to genomic instability that potentially could be transmitted to offspring affecting their development and survival and impacting population dynamics and community structure.

Genomic instability has been a recognized feature of many tumours and *p53* is one of the most commonly mutated genes in human cancer (Hollstein et al., 1991). In recent years it became evident that both the molecular basis for the initial susceptibility of cancer cells to anticancer genotoxic drugs, and the development of treatment resistance originate from genetic lesions that alter the function of genes playing roles in negative growth control, notably in determining cell cycle arrest and apoptotic setpoints (Muschel et al., 1998). Thus, understanding the molecular-genetic pathways that mediate the response of cells to genotoxic stress as well as other types of stress is of high priority from both basic science and clinical points of view.

The majority of the studies addressing p53 pathways are focused on exposure to DNA damaging agents, including estrogens, alterations of p53 mechanisms and cancer development. For example, Molinari et al. (2000) presented evidence that E_2 was able to induce functional inactivation of p53 protein as evident from the reduced expression of p21, preventing growth inhibition that could lead to estrogen-dependent tumorigenesis. However, we are not aware of any study analysing the effects of exposure to mixtures of xenoestrogens plus xenoandrogens in p53 pathways.

1.6 Animal model – *Danio rerio* (zebrafish)

Fish are recommended and accepted as representative models of higher trophic levels in determining the effects of chemicals in the aquatic environment. The close phylogenetic relationship between teleosts and mammals facilitate the extrapolation of the acquired knowledge among these groups. Other characteristics convert fish into good models for toxicological studies, namely their sexual plasticity, the good knowledge of their endocrinology, the ease of exposure to contaminants and the good response of fishes to experimental manipulation (Matthiessen, 2003).

The zebrafish (*Danio rerio*) is recommended as a test species in a number of ecotoxicological test protocols for risk assessment of EDCs (Maack and Segner, 2003; Ankley and Johnson, 2004; OECD, 2004). The species size, robustness and easy maintenance and reproduction under laboratory conditions are advantages for its use as bioassay organism. Furthermore, a completely sequenced genome (http://www.ensembl.org/index.html) and a gonads histological atlas (http://www.rivm.nl/fishtoxpat) are available.

Belonging to the family Cyprinidae, *Danio rerio* (F. Hamilton, 1822) is commonly called zebrafish because of its striped integument. The zebrafish is native of northeastern India (Ganges and Brahmaputra rivers) and extends to Pakistan and northern Burma (Engeszer et al., 2007)

Zebrafish is a tropical freshwater fish that can be induced to breed all year round under laboratory conditions. Its life-cycle is short, of approximately 10 to 12 weeks. The development is external and the eggs are translucent and non-adherent, allowing the following of embryonic development, which lasts for 3 days at 28.5 ºC (Kimmel et al., 1995). Zebrafish spawns at dawn, with a frequency of about three days, and the daily number of eggs laid by a female is quite variable, ranging from 100-500 eggs in each occasion. Zebrafish are oviparous and fertilization is external. Spawning is synchronized by light, so large numbers of eggs at similar stages of development are available and easily collected. Sexes are easily distinguished, as the males are more slender and their belly coloration and caudal fins are yellow, females are more silvery and the abdomen has a more spherical form (Oberemm, 2000) (Figure 1.7).

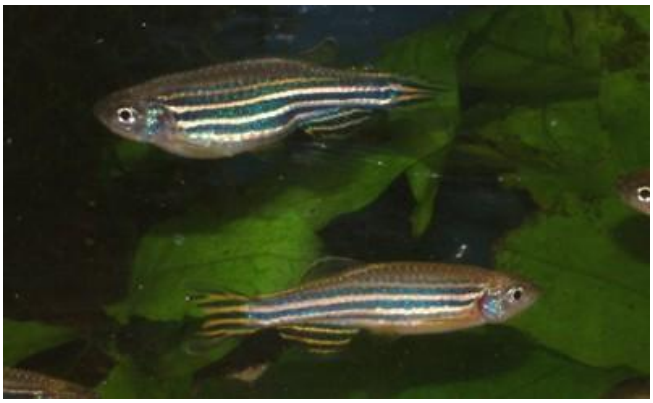

Figure 1.7 Female and male zebrafish (http://www.freewebs.com).

As a gonochoristic undifferentiated species, both sexes undergo a phase of immature non functional ovaries before phenotypic sexual differentiation. In males, this includes a type of juvenile hermaphroditism, as they pass through a female phase before the final development of the gonads (Örn et al., 2003). Gonad differentiation as ovaries begins approximately 10 days after egg laying (Laale, 1977; Maack and Segner, 2003). Around day 23, ovaries of about 50% of the juveniles begin to degenerate occurring, subsequently, the proliferation of the testicles (Hill and Janz, 2003). The process of sex differentiation is mostly completed approximately at 40 days post-hatch and the final maturation of the gonads will generally be finished at an age of 60 – 75 days (Takahashi, 1977; Uchida et al., 2002; Anderson et al., 2003; Hill and Janz, 2003).

The zebrafish has no sex chromosomes and the sex may be determined exclusively by sexual genes located on autosomes, but also, or in combination with environmental factors determining of sex (e.g. pH, stock density, temperature) (Örn et al., 2003). The phenotypic sex determination in fish is a dynamic process influenced by genes and environment. Therefore, if influenced by the environment, zebrafish can be a very sensitive model for the screening of EDCs effects.

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Chapter 2

Objectives

2. Objectives

The overall aim of this thesis is to investigate the impact of life-cycle chronic low-level exposure of prioritary EDCs; the xenoestrogen ethinylestradiol and the xenoandrogen tributyltin, single and in binary mixtures. In order to accomplish this, ecologically relevant endpoints such as reproduction, embryonic development and behaviour were screened and then linked with the chemicals mode of action, using the model teleost, zebrafish (*Danio rerio*) (Figure 2.1).

Four specific objectives were established:

- To address the toxicological effects of xenoestrogens and xenoandrogens, single and combined, in the physiology and reproductive traits of a model fish species (*Danio rerio*);
- To evaluate the impact of xenoestrogens and xenoandrogens, single and combined, at different stages of the life-cycle of the selected species;
- To study the effects of xenoestrogens in the behaviour of *Danio rerio*;
- To contribute to a better understanding of the mechanisms of toxicity of xenoestrogens and xenoandrogens, single and in binary mixtures, using molecular and biochemical techniques.

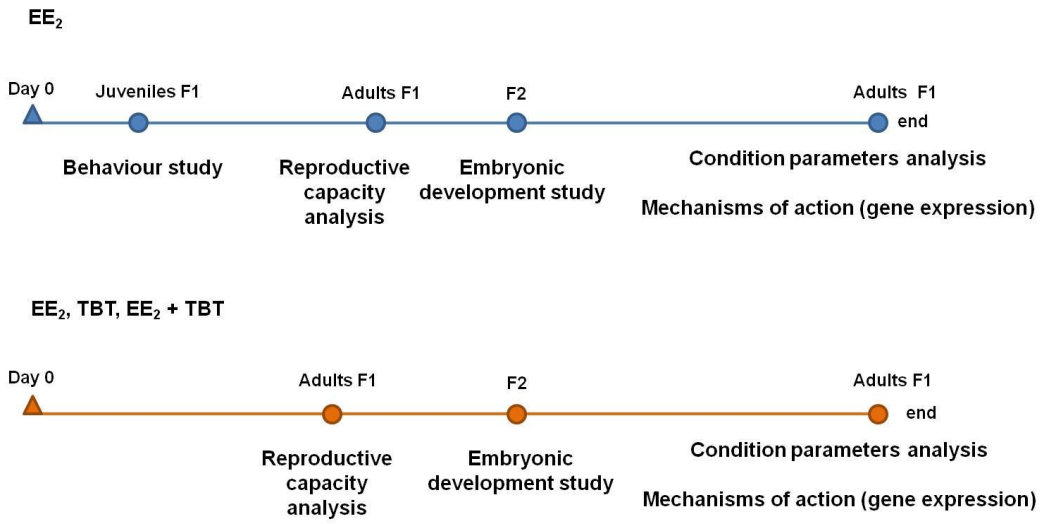

Figure 2.1 Simplified schematic diagram illustrating the main topics studied in this thesis.

In more detail, the established objectives are addressed in Chapters 3-6:

Chapter 3 – This chapter analyses the effects of chronic full life-cycle zebrafish exposure to low levels of EE_2 in reproductive capacity (e.g., fecundity and fertilization success) and offspring embryonic development. Other reproductive-related parameters are also determined, namely induction of *vtg*, gonad morphology, GSI and sex ratio. The effects of EE_2 at the second generation are evaluated by analyzing the embryonic development, identifying the most sensitivity phases. The population-level implications are discussed;

Chapter 4 - This chapter examines the use of a behaviour pattern of zebrafish juveniles as an effective early indicator of sub-lethal EE_2 exposure. The behavioural responses are compared with established endpoints used in the screening of EE_2 effects at adulthood (e.g., *vtg* induction and reproductive parameters);

Chapter 5 - This chapter addresses the mechanisms by which EE_2 disrupts zebrafish embryonic development. The hypothesis that EE_2 affect the repair of damaged DNA, leading to lesions that could be transmitted to progeny, is assessed. EE_2 impact on the transcription levels of key genes involved in homeostasis maintenance, namely NER repair factors (*xpc*, *xpa*, *xpd*, *xpf*), *p53* and selected downstream effectors *p21*, *gadd45α* and *bax*, as well as its negative regulator *mdm2* are analyzed;

Chapter 6 – This chapter investigates single and combined effects of mixtures of EE_2 and TBT upon full life-cycle zebrafish exposure. This section assesses the reproductive and embryonic development effects caused by exposure to EE_2, TBT, and their binary mixtures, with special emphasis on the underlying mechanisms. A thorough evaluation of reproductive-related endpoints in the parental generation at adulthood is performed and zebrafish embryonic development is analyzed. In order to get further insight into the mechanism of action of these compounds the transcriptional levels of *xpc*, *xpa* and *xpd* NER genes as well as *p53*, its downstream target genes (*p21*, *gadd45α* and *bax*) and principal regulator (*mdm2*),

in male adult gonads, are assessed. The adverse effects endpoints are integrated with the underlying molecular mechanisms.

Chapter 3

Disruption of zebrafish (*Danio rerio*) embryonic development after full life-cycle parental exposure to low levels of ethinylestradiol

Soares, J., Coimbra, A.M., Reis-Henriques, M.A., Monteiro, N.M., Vieira, M.N., Oliveira, J.M.A., Guedes-Dias, P., Fontaínhas-Fernandes, A., Parra, S.S., Carvalho, A.P., Santos, M.M., 2009. Aquatic Toxicology 95, 330-338.

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"Primo15 – 15th. International Symposium on Pollutant responses in Marine Organisms." May 2009 Bordeaux, France. Oral presentation with the title "Disruption of zebrafish (*Danio rerio*) embryonic development after full life-cycle parental exposure to low levels of ethinylestradiol." Santos, M.M.

3. Disruption of zebrafish embryonic development after full life-cycle exposure to low levels of ethinylestradiol

3.1 Abstract

Exposure of fish to the synthetic estrogen ethinylestradiol (EE_2) has been shown to induce a large set of deleterious effects. In addition to the negative impact of EE_2 in reproductive endpoints, concern has recently increased on the potential effects of EE_2 in fish embryonic development. Therefore, the present study aimed at examining the effects of EE_2 on the full embryonic development of zebrafish in order to identify the actual phases where EE_2 disrupts this process. Hence, zebrafish were exposed to environmentally relevant low levels of EE_2 (actual concentrations of 0.19, 0.24 and 1 ng/L) from egg up to eight months of age (F_1), and the survival as well as the occurrence of abnormalities in their offspring (F_2), per stage of embryonic development, was investigated. A thorough evaluation of reproductive endpoints and *vtg1* gene expression in the parental generation (F_1), at adulthood, was performed.

No significant differences could be observed for the two lowest treatments, in comparison with controls, whereas *vtg1* transcripts were significantly elevated (40-fold) in the 2 ng/L EE_2 treatment. In contrast to the findings in the F_1 generation, a significant concentration-dependent increase in egg mortality between 8 and 24 hours post-fertilization (hpf) was observed for all EE_2 treatments, when compared with controls. The screening of egg and embryo development showed a significant increase in the percentage of abnormalities at 8 hpf for the highest EE_2 concentration, a fact that might explain the increased embryo mortality at the 24 hpf time-point observation. Taken together, these findings indicate that the two lowest tested EE_2 concentrations impact late gastrulation and/or early organogenesis, whereas exposure to 2 ng/L EE_2 also disrupts development in the blastula phase. After early organogenesis has been completed (24 hpf), no further mortality was observed.

These results show that increased embryo mortality occurs at EE_2 levels below those inducing reproductive impairment and *vtg1* gene induction in the male parental generation, thus suggesting that EE_2 may impact some fish populations at levels below those inducing an increase in *vtg1* transcripts. Hence, these findings

have important implications for environmental risk assessment, strongly supporting the inclusion of embryonic development studies in the screening of endocrine disruption in wild fish populations.

3.2 Introduction

The presence, in the aquatic ecosystems, of chemicals able to interfere with the normal endocrine function of animals, generally named endocrine disrupting chemicals (EDCs), is a matter of great concern (Ashby et al., 1997; Mills and Chichester, 2005; Iguchi et al., 2006; Sanderson, 2006). Many EDCs modulate sex steroid signalling and the hypothalamic-pituitary-gonad axis (Ankley and Johnson, 2004; Segner et al, 2006), interfering with both development and reproduction (Guillette et al; 1995; Bigsby et al., 1999). Estrogenic chemicals (ECs) are among the most widely studied EDCs mostly because field studies have shown the presence of fish feminization in many aquatic ecosystems (Sumpter, 2005). Fish can be exposed to EDCs through a variety of sources, but waste waters are the primary source of ECs such as the active ingredient of contraceptive pills, 17α-ethinylestradiol (EE_2) (Folmar et al., 2000; Metcalfe et al., 2001; Kolpin et al., 2002).

Environmental EE_2 concentrations in water are highly variable, from non-detectable to a maximum concentration of 830 ng/L in U.S.A. rivers (Kolpin et al., 2002). In Europe, the majority of the measured concentrations are below 5 ng/L (Desbrow et al., 1998; Belfroid et al., 1999; Ternes et al., 1999; Johnson et al., 2000; Svenson et al., 2002). EE_2 has the ability to bioconcentrate in fish tissue (650 fold in whole-body tissues), and is not only more effective in eliciting estrogenic responses but also more stable than the natural estrogen 17β-estradiol (Länge et al., 2001; Lai et al., 2002; Thorpe et al., 2003). Under laboratory conditions, EE_2 has been reported to cause a wide variety of negative effects in fish reproduction, such as a bias in the sex ratio toward females, decrease fertility and fecundity and vitellogenin induction in males (Nash et al., 2004). In the model teleost, zebrafish (*Danio rerio*), several studies involving EE_2 exposure have been performed. Only few, however, covered the full life-cycle, and have tested the impact on reproductive parameters. Segner et al. (2003) tested EE_2 exposure from fertilization to adult stage and observed a decline in the egg number per female

and the fertilization success at concentrations above 1.67 ng/L; Fenske et al. (2005) found an inhibition on egg production when fish were exposed to 3 ng/L EE_2 from 0 to 180 days post-fertilization (dpf). More recently, Schäfers et al. (2007) observed a decrease in the number of eggs and also a decline on the number of fertilized eggs of zebrafish exposed to concentrations of 1.1 ng/L, from 0 to 177 dpf; Larsen et al. (2008) observed a decline in the number of fertilized eggs at 0.05 and 0.5 ng/L EE_2 from 1 to 124 dpf.

In addition to the negative effects of EE_2 in reproductive endpoints, it has recently been pointed out that embryo development may also be a target of EE_2 (Länge et al., 2001; Nash et al., 2004; Brown et al., 2007). The effects of EE_2 on embryo fish survival have been reported after chronic parental EE_2 exposure, thus raising considerable concern as it occurs at environmentally relevant EE_2 concentrations. While these early studies have determined embryo mortality at specific time-points over the embryonic period, none has investigated in detail the effects of EE_2 on the full embryonic development. Hence, in order to understand the mechanism(s) by which EE_2 disrupts embryonic development, it is essential to identify the actual phases where EE_2 acts.

Therefore, the main aim of the present study was to examine the concentration-dependent effects of parental zebrafish full life-cycle exposure to low levels of EE_2 on the different stages of the offspring embryonic development. In order to achieve this, zebrafish were exposed to low levels of EE_2 (nominal concentrations of 0.5, 1 and 2 ng/L), from egg up to eight months of age, and the survival and the occurrence of abnormalities in their offspring, per stage of embryonic development, was evaluated. The results of embryo mortality patterns were integrated with the data of the reproductive parameters and vitellogenin gene induction (*vtg1*) in the parental generation and discussed in relation to the potential population-level impact of EE_2 for wildlife fish populations.

3.3 Materials and methods

3.3.1 Species selection

Zebrafish (*Danio rerio*) is recommended as a test species in a number of ecotoxicological test protocols (Oberemm, 2000). This species size, robustness, short life-cycle and the fact that it can be induced to breed all year round under laboratory conditions, are advantages for its use as bioassay organism. Furthermore, particularly important for the present study, zebrafish eggs are translucent and non-sticky thus allowing an easy screening of embryonic development under a stereo-microscope.

3.3.2 Parental animals (F_0)

Adult wild-type zebrafish, obtained from local suppliers in Singapore, were used as breeding stock. The stock was kept at a water temperature of 28 ± 1 ºC and under a photoperiod 14:10 hours (h) (light:dark), in 250 L aquaria with dechlorinated and aerated water in a recirculation system with both mechanical and biological filters. The fish were fed *ad libitum* twice a day with a commercial fish diet Tetramin (Tetra, Melle, Germany) supplemented with live brine shrimp (*Artemia spp.*).

3.3.3 Egg production of F_0 generation

In the afternoon before breeding, two groups of 4-6 males and 10-12 females were independently housed in cages with a net bottom cover with glass marbles within a 30 L aquarium under the same water and photoperiod conditions as the stock and fed with live brine shrimp. At the following day, breeding fish were removed 1.5 h after the beginning of the light period and the eggs were collected and cleaned. Fertilized eggs were randomly allocated to experimental aquaria.

3.3.4 Exposure of F_1 generation

Exposures were performed using a flow through system with slight modifications from the experimental setup described in Santos et al. (2006). Before entering the system, the water used was heated and charcoal filtered. The water flow (50 ml/min) was maintained by a peristaltic pump (ISM 144, ISMATEC) whereas EE_2 and DMSO solutions (0.018 ml/min flow) were administered by a second peristaltic pump (205U Watson-Marlow). The water and the solutions were mixed together, in a mixing chamber, before entering, by gravity, in the continuously aerated aquaria. The utilization of peristaltic pumps to control water and contaminants fluxes increases the accuracy of dosage over time, which was confirmed by weekly measurements. Throughout the experimental period, zebrafish were under a 14:10 h (light:dark) photoperiod and water physical-chemical parameters were measured weekly with exception of temperature that was checked on a daily basis (28 ± 1 ºC of temperature; pH 7.7 ± 0.2; 6 ± 1 mg/L of dissolved oxygen; 376 µS/cm of conductivity; 0.08 ± 0.06 mg/L of ammonium and 0.01 ± 0.01 mg/L of nitrite).

Five exposure conditions, in duplicate, were set up: an experimental control, a solvent control (DMSO) and three EE_2 concentrations (nominal concentration: 0.5; 1 and 2 ng/L).

17α-Ethinylestradiol (EE_2 98%, Sigma) (stock solution: 1 mg/ml) was diluted in dimethylsulfoxide (DMSO 99.5%, Sigma). From this solution, aliquots of the working solutions were prepared and kept at -20 ºC until use. Working EE_2 solutions were diluted in MilliQ water and renewed three times a week; all solutions were prepared in order to have a final DMSO volume of 0.000002%.

In order to test EE_2 effects when zebrafish reach their maximal fecundity, the study lasted for 8 months, with continuous exposure of the F_1 generation (Westerfield, 2000). Before the beginning of the exposure, the aquaria were equilibrated during 15 days. The experiment was initiated by randomly allocating 450 eggs ≈2 hours post-fertilization (hpf) in 5 L aquaria, placed within each 30 L aquarium. At 20 dpf, zebrafish were allocated to 30 L aquaria and their number adjusted to fit 100 juveniles per aquarium. The number of zebrafish in each aquarium was again reduced to 70, at 40 dpf, and later to 30 at 60 dpf, a density which was maintained up to the end of the experiment (8 months). Feeding was

initiated at day 6 dpf with two meals of Tetramin supplemented with one brine shrimp meal per day on a slight modification of Carvalho et al. (2006); at 9 dpf this was changed to two brine shrimp meals and one Tetramin meal per day, which was maintained up to the end of exposure.

Mortality was assessed by daily recordings during the entire exposure period, and dead eggs/individuals removed. At the end of the experiment, the remaining zebrafish were immobilized in ice-cold water, immediately decapitated, and their weight and length recorded for body condition factor calculation (K = weight x $lenght^{-3}$ x 100). Liver was removed and stored in RNAlater (Sigma) for molecular biology determinations. Gonads were also excised and weighed for gonad somatic index calculation (GSI = gonad weight as percentage of total weight), and observed under a stereo-microscope for sex determination, complemented with histology.

3.3.5 EE_2 concentration in water

In order to determine actual EE_2 concentrations, water samples were collected twice during the exposure of F_1 generation (at 40 and 210 dpf) and pre-treated according to the "Sample Pre-treatment Protocol for Female Steroid Hormones" (Japan Envirochemicals). Briefly, steroids were extracted in a solid phase C18 column (Sep-Pak Plus C18 cartridges, Waters Corporation), 80% methanol eluted, concentrated under nitrogen gas flow and frozen at -20 ºC until analysis. EE_2 concentration was determined using the "Jec ELISA kit" (Japan Envirochemicals) according to the manufacturer´s protocol. Concentrations of EE_2 were found to be below the detection limit in the reference aquaria and to be 1.00 ± 0.12, 0.24 ± 0.02 and 0.19 ± 0.02 ng/L, respectively, for 2, 1 and 0.5 ng/L nominal concentrations. The extraction procedure rendered a recovery of over 90%, and therefore no correction was performed to the obtained EE_2 concentrations.

3.3.6 Reproductive capability of F_1 generation

Reproductive capability studies were performed after 8 mpf (months post-fertilization) in all experimental groups. Reproductive success was evaluated as fecundity (number of eggs per female per day) and % of viable eggs per female

per day after 1-1.5 hpf. For reproductive studies, the 30 L aquaria were divided in two compartments and female and male zebrafish were randomly distributed in cages, with a net bottom covered with glass marbles, in a manner that matched the sex ratio of each treatment (4 replicates per treatment) (Figure 3.1). Subsequently, during three consecutive days, 1-1.5 h after the beginning of the light period, eggs were collected, counted and the percentage of viable eggs recorded. The sex ratio per replicate was confirmed at the end of the reproductive trial.

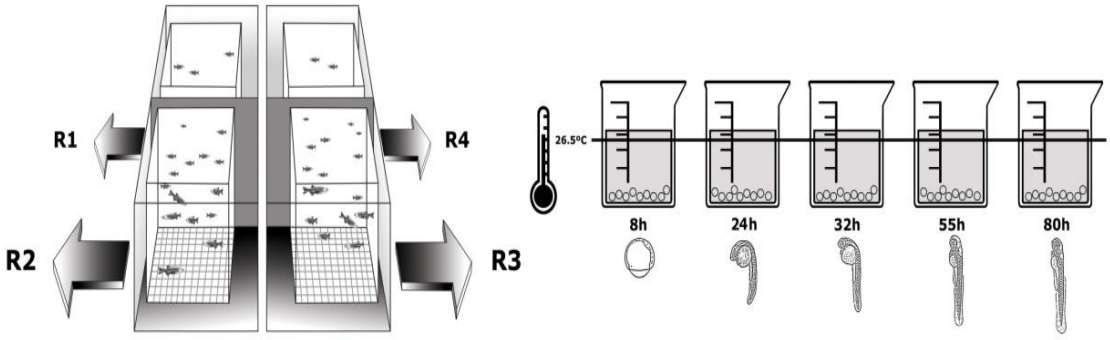

Figure 3.1 Schematic representation of the experimental set up used for breeding trials of the F_1 generation and the embryonic assay with their offspring (F_2). After 8 mpf, breeding trials were performed using four replicates per treatment (R1, R2, R3, and R4). Viable eggs from each replicate were randomly distributed to 100 ml beakers (40 eggs per beaker). From each replicate, one beaker per stage (8, 24, 32, 55, and 80 hpf) was set, which means that during the five studied embryonic stages, a total of 20 beakers per treatment were used.

3.3.7 Histology and stereological analysis of F_1 generation

In order to perform a histological sex determination, approximately 50 fish per treatment (30 fish for the 0.5 ng/L EE_2 treatment) were collected at the end of exposure (8 months). Animals were fixated in Bouin solution (Panreac) for 48 h, paraffin-embedded, sectioned into 5 µm sections and mounted on slides. Slides were stained with hematoxylin-eosin and mounted with Entellan®. Identification of gonad cell populations was conducted according to Weber et al. (2003). Briefly, ovarian follicles were staged as: oogonia (Oo - small in size and eosinophilic ooplasm with relatively large nucleus); previtellogenic follicles (PreV - small in size with basophilic ooplasm, large nucleus with visible chromatin and single somatic cell layer); vitellogenic follicles (Vit - basophilic ooplasm that is enlarging relative to

nucleus and as one or more layers of somatic cells) and preovulatory follicles (PreO - distinct zona radiata, multiple differentiated somatic cell layers and vacuolated ooplasm). Testicular populations were identified as follows: spermatogonia (Sg - eosinophilic cytoplasm with relatively large nucleus); spermatocytes (Sc - thread-like or condensed chromatin, with relatively smaller cytoplasm) and spermatids or mature sperm (M - tightly packaged nuclear material lacking surrounding cytoplasm).

To determine the relative volume density (VV) of spermatogonia, spermatocytes, spermatids and spermatozoa in testes as well as oogonia, previtellogenic follicles, vitellogenic follicles and preovulatory follicles in ovaries (n = 6), a stereological approach was designed based on point counting (Freere and Weibel, 1967), using a microscope (Olympus IX 51, Japan) equipped with a CCD camera (Olympus U CMDA3, Color View soft imaging system). A detail description of the method can be found in Monteiro et al. (2009).

3.3.8 Sperm quality parameters in F_1 males

Functional imaging of the spermatozoids was performed with a system composed of an inverted microscope (Eclipse TE300, Nikon, Tokyo, Japan) equipped with a 40x air objective and a CCD camera (C6790; Hamamatsu Photonics, Hamamatsu, Japan). Sperm motility analysis was performed as previously described by Wilson-Leedy and Ingermann (2007), with minor modifications, namely a 53 Hz capture rate and a resolution of 329 x 247 pixels (2 x 2 binning). Sperm was collected from one testicle of 6 males exposed to the above-described treatments. Sperm motility videos were acquired during 44 seconds (s) and sub-sequentially split into 22 videos of 2 s. Image processing was performed with Aquacosmos 2.5 (Hamamatsu Photonics) and ImageJ (National Institutes of Health; available at http://rsb.info.nih.gov/ij/). Video and numerical data calculations were automated with ImageJ.

Several parameters were collected from the video analysis (see Wilson-Leedy and Ingermann, 2007), namely sperm motility (MOT), curvilinear velocity (VCL), velocity over a straight line (VSL) and progression (PROG).

3.3.9 Embryogenesis studies of F_2 generation

When reproductive trials of F_1 generation reached the third day, 200 viable eggs per replicate were kept for the embryogenesis studies. Eggs that had passed the 4 cell stage (1-1.5 hpf), were randomly distributed to 100 ml beakers (40 eggs per beaker) with daily replacement of water obtained from the flow through system where dosing of F_1 generation was taking place. Thus, embryos were exposed to the same treatments as the parental F_1 generation, mimicking a scenario where fish are exposed to ECs through the entire life-cycle. The beakers were randomly maintained on a water bath at 26.5 ± 0.5 ºC and kept under the same photoperiod conditions as the adults. For each treatment, four beakers per stage were set (four replicates), which means that during the five studied embryonic stages, a total of 20 beakers per treatment were used (Figure 3.1).

For embryogenesis studies embryo tests and endpoints were performed with slight modifications of the protocol proposed by Oberemm (2000). The following endpoints were evaluated during zebrafish egg/embryo development stages, briefly: at 8 hpf (65-75%-epiboly), progression of ectodermal front and homogeneity of front and tissue; at 24 hpf (prim 5-6), head and tail differentiation, length of tail, spontaneous movements and presence of heart beat; at 32 hpf (prim 15-16) and 55 hpf (pec-fin), heart rate, malformations of the blood circulatory system, oedema and melanophores distribution; and at 80 hpf (protruding mouth), heart rate, expression of pigmentation, oedema and fin fold lesions; the mortality rate was assessed at all stages. Different embryos, even within a single clutch, develop at slightly different rates (Kimmel et al., 1995). Therefore, our time-point observations include eggs/embryos in slight different phases, which have been indicated above. However, for simplicity, in the figures we have adopted the classification of Oberemm (2000), with a single stage per time-point observation. Inspection started by checking mortality and discharging dead eggs/embryos to avoid water quality decay. Then, 15 eggs/embryos, from each beaker (60 per treatment/developmental stage), were randomly collected and placed on a Petri dish for observation with a stereo-microscope (SMZ1000, Nikon, magnification up to 80 times). Any modification from normal development and/or morphological anomalies were recorded by digital photography (Nikon Coolpix 5400) and scored as presence/absence. A detailed description and categorization of the different

anomalies will be performed in the future. After each time-point observation, the beakers were removed, thus assuring the independence of data between developmental stages. The heart beat and spontaneous mobility were evaluated at 24 hpf in three embryos per replicate and the cardiac frequency was determined in one embryo per replicate at 32, 55 and 80 hpf, using a stop-watch, during two 15 s periods, restarting the counting if the embryo moved.

3.3.10 *vtg1* gene expression in F_1 zebrafish males after 8 months of EE_2 exposure

For *vtg1* transcription in adult male zebrafish, after 8 months of exposure to EE_2, total RNA was isolated from liver using "illustra RNAspin Mini kit" (GE Healthcare kit) according to the manufacturer´s protocol, with on column DNase digestion during the extraction procedure. After column elution with 30 µl of RNase-free water, RNA concentration was determined by fluorescence (Fluoroskan Ascent, Labsystems) using the "Quant-iT^{TM} RiboGreen® RNA Assay Kit" (Invitrogen) and RNA quality was checked in a 1% agarose gel. All RNA samples were stored at -80 ºC until further use. For cDNA synthesis, the iScript cDNA synthesis kit (Bio-Rad) was used with 0.5 µg of total RNA.

For amplification and mRNA expression analysis through real-time PCR (RT-PCR) of *vtg1* (NM_170767), primers were designed in a region outflanking an intron using Beacons Design^{TM} software (Premier Biosoft International) and synthesised by SigmaProligo:

Forward primer 5'-CTT ACG ACA CAG GAT TCA G-3';
Reverse primer 5'-GTC TTC ATA GGT CTC AAT GG-3'.

The PCR reaction was prepared to a final volume of 25 µl with primers final concentration of 200 nM and using 2 µl of cDNA.

RT-PCR was initiated at 95 ºC for 3 minutes (min). Thereafter, 35 cycles of amplification were carried out with denaturation at 95 ºC for 10 s, annealing at 53 ºC for 30 s and extension at 72 ºC for 30 s (data collection), followed by a melting curve analysis to determine the specificity of the reaction. A standard curve consisted of eight 10-fold serial dilutions of positive control cDNA prepared from a

total RNA sample isolated from a spawning female liver and used as reference sample for construction of a standard curve (reaction efficiency close to 100%, and r^2 = 0.994) and for *vtg1* relative concentration determination (ΔCt method), together with the male test samples and negative control (template-free). The RT-PCR products were analysed by agarose gel electrophoresis to confirm the presence of a single band with the expected size (200 bp) (data not shown). The identity of the PCR product amplified by the real time primers was confirmed by cloning and subsequent sequencing. For sequencing, the PCR product was excised from the gel using the QIAquick Gel Extraction kit (Qiagen) and cloned into pGEM®-T Easy Vector (Promega). The ligation product was transformed into Novablue Singles cells I (Novagen) and grown over-night on IPTG/X-gal-coated LB-plates with ampicillin (50 µg/ml) at 37 ºC. Plasmid preparations were prepared using QUIprep Spin Miniprep Kit (Qiagen).

3.3.11 Statistics

Data were analysed using Statistica 6.0 software. After testing for ANOVA assumptions (homogeneity of variances and normality of data), statistical differences in embryo mortality rate and abnormal eggs/embryos among treatments and stages were evaluated by a two-way factorial ANOVA, followed by Fisher LSD multiple comparison test. Mortality data were Arcsine-square root transformed before ANOVA analysis. Significant differences in the reproductive parameters, *vtg1* gene expression and stereological analysis were evaluated by a one-way ANOVA, followed by Fisher LSD multiple comparison test. Sex ratio differences were tested by the chi-square (χ^2) test, using the solvent control group as expected values. For sperm quality assessment, a preliminary analysis of the data showed a great degree of homogeneity among the analysed videos and, thus, values were averaged among each treatment (control, solvent control, and the three tested EE_2 concentrations) only to determine the breakpoint of the curve that indicated the onset of the decay. A piecewise regression was used and the estimated breakpoint (38 s) was similar in all tested parameters (MOT, VCL, VSL and PROG) and treatments (data not shown). Thus, the values comprised between the onset of analysis and the calculated breakpoint were averaged (for

each tested fish) and used in a MANOVA (4 parameters: MOT, VCL, VSL and PROG; 5 factors: control, solvent control group, three tested EE_2 concentrations).

3.4 Results

3.4.1 Mortality of F_1 generation

The mortality rates, from embryo up to 20 dpf, varied between 36 and 55% and no differences were observed between control and EE_2-exposed zebrafish groups (data not shown). No mortality occurred after this period. These mortality rates are within the normal expected values for zebrafish (Hill and Janz, 2003; Santos et al., 2006).

3.4.2 Weight, length, K and GSI of F_1 generation

With the exception of male total length, which was significantly increased ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test) in all groups of zebrafish exposed to EE_2 if compared with solvent control (4.37 ± 0.06 and 4.37 ± 0.04 cm for control and solvent control, and 4.60 ± 0.04, 4.52 ± 0.05 and 4.57 ± 0.05 cm for, EE_2, 0.5, 1 and 2 ng/L, respectively), the remaining parameters tested were not affected by EE_2 exposure (data not shown).

3.4.3 Sex ratio of F_1 generation

At the end of the experiment, with the exception of EE_2 0.5 ng/L, all groups showed a percentage of females between 49 and 61%, which is within the normal range in zebrafish raised in captivity (Hill and Janz, 2003) (Table 3.1). Despite this fact, control showed a percentage of females that was significantly higher than that of solvent control. Animals exposed to the lowest EE_2 concentration showed a significant increase ($p < 0.05$, χ^2 test) in the percentage of males in comparison to solvent control.

Table 3.1 Reproductive parameters for all EE_2 treatments groups (F_1 generation) at 8 mpf.

Group	**Control**	**DMSO**	**EE_2**		
			0.5 ng/L	**1 ng/L**	**2 ng/L**
Reproductive parameters (8 mpf)					
Female					
% females	60.9 *	49.2	34.6 *	57.1	55.8
Fecundity	183 ±79	114 ±24	75 ±30	78 ±33	140 ±50
Fertility success (%)	96 ±1.0	99 ±0.4	96 ±0.3	94 ±2.2	94 ±2.8
% Previtelogenic follicles	5.4 ±1.1	5.5 ±0.3	6.8 ±0.6	8.6 ±1.4	6.2 ±0.6
% Vitelogenic follicles	6.8 ±0.6	7.8 ±0.9	11.4 ±1.7	11.2 ±1.5	7.0±0.9
% Preovulatoryfollicles	62.6 ±2.8	62.8 ±1.8	58.5 ±2.4	53.2 ±5.3	62.4 ±3.8
Male					
% males	39.1 *	50.8	65.4 *	42.9	44.2
% Spermatogonia	8.5 ±1.0	8.4 ±2.1	7.6 ±1.2	7.0 ±0.8	8.4 ±1.6
% Spermatocyst	9.3 ±0.9	12.6 ±1.2	12.1 ±1.7	12.0 ±1.5	12.7 ±1.5
% Spermatid	13.9 ±1.4	11.9 ±1.2	17.8 ±1.4	18.1 ±2.1	12.4 ±1.5
% Spermatozoa	34.1 ±2.7	40.7±28.2	28.2 ±1.4	32.4 ±3.8	36.5 ±2.6

*Significantly different from solvent control (DMSO). Values are presented as mean ± standard error. % of females and males tested by χ^2 test, $p < 0.05$.

3.4.4 Stereological analysis of gonads and sperm quality in F_1 generation

The statistical analysis of the data dealing with the relative volume percentage of the ovaries, occupied by each of the oocyte stages, showed no differences between treatments. Also, the testicular tissue occupied by the different spermatogenic stages, in 8 months old zebrafish, did not show significant differences between treatments ($p > 0.05$; one-way ANOVA) (Table 3.1). Likewise, no significant differences were observed in the sperm quality assessment (Table 3.2) MANOVA ($p = 0.55$) nor in any of the univariate results obtained for each of the selected parameters when comparing the different treatment groups ($p > 0.05$).

Table 3.2 Sperm quality parameters registered for F_1 males (motility, curvilinear velocity, velocity over and average path and progression) in all EE_2 tested treatments (mean ± standard deviation).

Treatment	Motility (MOT; %)	Curvilinear velocity (VCL; µm/s)	Velocity over straight line (VSL; µm/s)	Progression (PROG; µm)	Average number of analyzed spermatozoa per fish
Control	58.02±17.24	88.92±8.49	56.12±6.37	2952.88±357.51	100
DMSO	48.59±22.04	80.42±5.59	46.79±5.73	2506.29±267.45	127
EE_2 0.5 ng/L	52.96±17.37	86.67±14.11	49.30±9.44	2648.79±528.10	144
EE_2 1 ng/L	37.56±16.12	79.07±9.79	48.98±7.90	2558.38±388.43	88
EE_2 2 ng/L	47.03±13.73	87.60±13.12	51.48±6.22	2733.30±309.58	150

For simplicity purposes, presented values result from the averaged values between the onset of video recording, second 17 post sperm activation, and second 38, the calculated breakpoint common to all treatments.

3.4.5 mRNA expression of vtg1 gene in F1 zebrafish males after 8 months of EE2 exposure

The mRNA expression of *vtg1* in male liver, at the age of 8 months, was significantly induced in the highest concentration of EE_2 (up to 40-fold) in comparison with all other treatments ($p < 0.01$; one-way ANOVA, followed by Fisher LSD multiple comparison test). A trend towards an increase in *vtg1* transcripts was already evident at nominal EE_2 concentrations of 1 ng/L, although it did not differ significantly from solvent control. The lowest EE_2 concentration had no effect on the levels of *vtg1* transcripts (Figure 3.2).

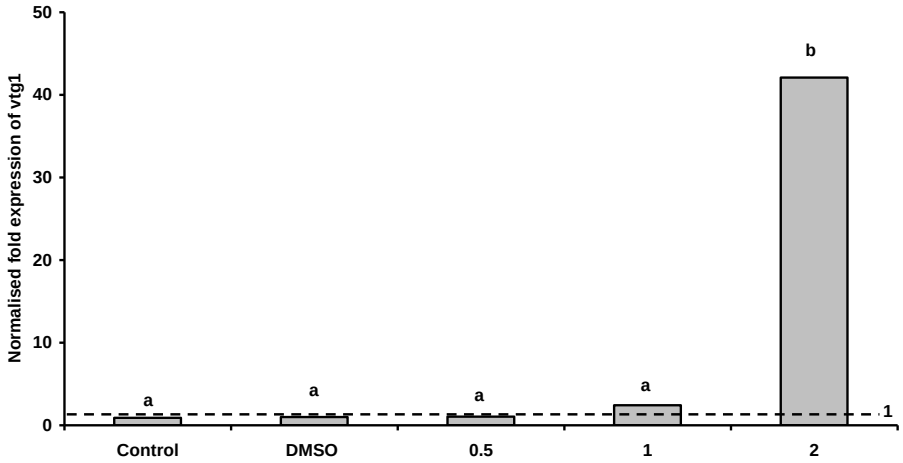

Figure 3.2 Normalised fold expression of *vtg1* transcripts in 8 months zebrafish males (n =5) after exposure to different EE_2 concentrations (ng/L), solvent control (DMSO) group was used as the reference group (= 1 fold). Values are presented as mean ± standard error. Different letters indicate significant differences among treatments ($p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test).

3.4.6 Reproductive capacity of F_1 generation

At 8 mpf, fecundity and % viable eggs (1-1.5 hpf) in females exposed to EE_2 did not differ significantly from solvent control ($p > 0.05$; one-way ANOVA). However, a trend towards a decrease in fertility was observed in females of the groups exposed to EE_2 at 0.5 and 1 ng/L when compared with solvent control females (Table 3.1).

3.4.7 Embryogenesis

3.4.7.1 Mortality

Cumulative mortality rates of embryos at the different phases of embryonic development are displayed in Figure 3.3. Between 8 and 80 hpf, mortality rate ranged from 10% (solvent control group) to 60% (2 ng/L EE_2 parental exposure). Even though the mortality rate from EE_2 parental exposed embryos at 8 hpf did not differ significantly from solvent control, a trend towards an increase in mortality was evident in the highest EE_2 treatment group. A significant increase ($p < 0.05$; two-way ANOVA, followed by Fisher LSD multiple comparison test) in the mortality

rate of embryos from all EE_2 parental exposed groups occurred between 8 hpf and 24 hpf, in a concentration-dependent manner. At 24 hpf, solvent control treatments displayed approximately 10% egg mortality (out of the initial fertilized eggs), whereas 0.5, 1 and 2 ng/L EE_2 treatment groups showed, respectively, 25%, 30% and 60% egg mortality. After 24 hpf, cumulative mortality rates remained unchanged up to the end of the embryonic development. The mortality rate in control beakers at 24 hpf was slightly (but significantly) increased if compared to the same treatment at 55 and 80 hpf. This probably reflects the fact that different beakers were used for each time-point.

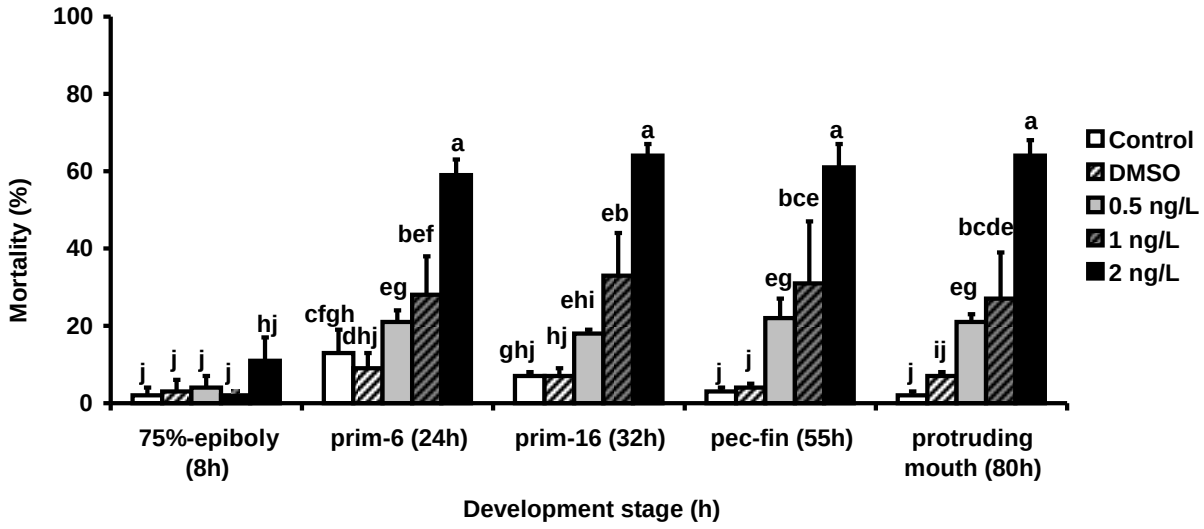

Figure 3.3 Percentage of cumulative mortality, at several developmental stages: 75%-epiboly (8 hpf); prim-6 (24 hpf); prim-16 (32 hpf); pec-fin (55 hpf) and protruding mouth (80 hpf) of embryos (F_2), descendents of zebrafish exposed to different EE_2 treatments for 8 mpf (F_1). Values are presented as mean ± standard error. Different letters indicate significant differences among treatments ($p < 0.05$, two-way ANOVA, followed by Fisher LSD multiple comparison test).

3.4.7.2 Abnormal development

During the different embryogenesis stages (8, 24, 32, 55 and 80 hpf) the presence of eggs/embryos showing anomalies were recorded within each group (Figure 3.4). At 8 hpf, only the group exposed to the highest EE_2 concentration showed a significant increase ($p < 0.05$; two-way ANOVA, followed by Fisher LSD multiple comparison test) in the percentage of eggs (approximately 43%) with

abnormal development (Figure 3.4). Most of these abnormal eggs had not yet entered gastrulation at the 8 hpf and showed an disorganization of cells at the animal pole, which contrasted with the other treatments where most eggs had reached 65-75%-epiboly at 8 hpf. At 8 hpf, the solvent control group showed slightly higher percentage of abnormal egg development if compared with control (not significant, $p > 0.05$). Rather than an effect of the solvent, this pattern seems to reflect an increase in the percentage of abnormal eggs in one out of the four replicates, which explains the high standard error. After 8 hpf, the rate of abnormal eggs/embryos was similar in all treatments and was kept at low levels (usually below 10%). Nevertheless, the percentage of abnormal eggs was increased in EE_2 2 ng/L exposures if compared with solvent control at 32 hpf and both control and EE_2 1 ng/L groups displayed an increase at 80 hpf if compared with solvent control.

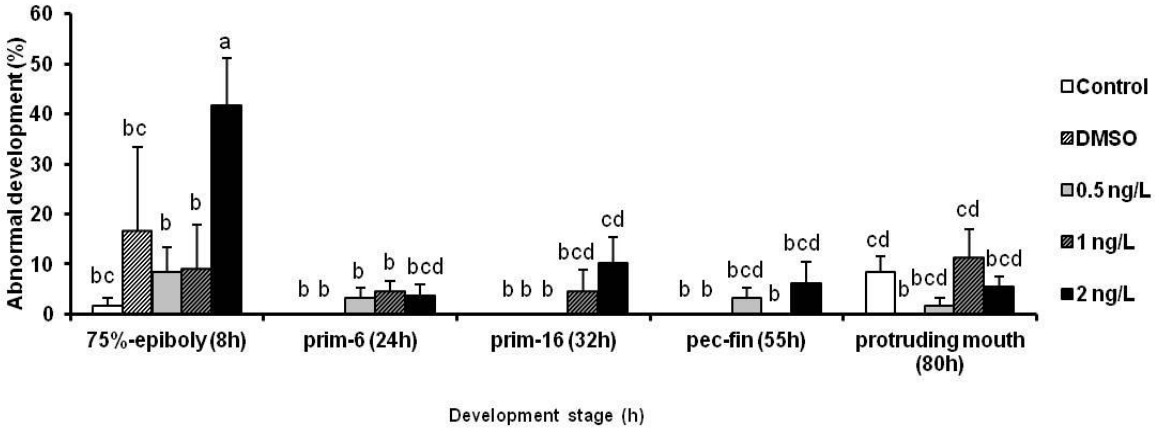

Figure 3.4 Percentage of abnormal eggs/embryos (F_2), at several developmental stages: 75%-epiboly (8 hpf); prim-6 (24 hpf); prim-16 (32 hpf); pec-fin (55 hpf) and protruding mouth (80 hpf) of offspring of zebrafish exposed to different EE_2 treatments for 8 mpf (F_1). Values are presented as mean ± standard error. Different letters indicate significant differences between treatments ($p < 0.05$, two-way ANOVA, followed by Fisher LSD multiple comparison test).

3.4.8 Heart beat rate

The cardiac frequency at 32, 55 and 80 hpf was similar in all groups ($p > 0.05$, one-way ANOVA) (data not shown), which indicates that EE_2 exposure did not have an effect in heart rate in zebrafish embryos at the tested concentrations.

3.5 Discussion

The presence of ECs in the aquatic environment has raised considerable concern during the last decade (Sumpter, 2005). In an attempt to complement field data, and better anticipate the negative impact of ECs under controlled conditions, several laboratory studies have been performed using model fish species (Sumpter, 2005). The most noticeable effects reported after EE_2 exposures were the decrease in fertility and fecundity after partial or full life-cycle exposure (Nash et al., 2004). In zebrafish, most full life-cycle studies on the effects of EE_2 report a decrease in the number and percentage of fertilized eggs, at concentrations ranging from 1.1 to 3 ng/L (Hill and Janz, 2003; Segner et al., 2003; Fenske et al., 2005; Schäfers et al., 2007), which is in agreement with our observations. Similarly, our data in sperm quality parameters, stereological analysis of female gonads and *vtg1* induction corroborates previous findings for the range of EE_2 concentrations used in the present study (Hill and Janz, 2003; Xu et al., 2008). However, two recent studies with full life-cycle zebrafish exposure to EE_2 have observed a decline in the fecundity and fertilization success at concentrations below the 1 ng/L (Larsen et al., 2008; Xu et al., 2008) These differences among studies may reflect the use of different zebrafish strains and/or exposure periods.

Recent studies indicated that the embryonic development of offspring of EE_2-exposed fishes may also be a target of EE_2 (Nash et al., 2004; Brown et al., 2007). Yet, none of the previous studies have evaluated EE_2 effects through the entire embryonic development. Hence, identifying the most sensitive embryonic development stage to EE_2 is essential when trying to understand the mechanisms of action of this EC. In the present study, parental full life-cycle exposure to EE_2 resulted in a concentration-dependent increase in egg mortality between 8 and 24 hpf. During zebrafish development this period includes late gastrulation and early organogenesis (Kimmel et al., 1995). After early organogenesis has been completed (24 hpf), no further mortality was observed (Figure 3.3). This demonstrates that EE_2 impact in embryo development occurs only up to early for the range of concentrations tested in the course of the present study. In accordance with the results of egg mortality, an increase in abnormal egg development in the highest EE_2 exposure concentration was observed at 8 hpf. These findings indicate that the abnormal development recorded at 8 hpf, together

with the egg mortality observed up to 8 hpf, might explain the increased mortality at the 24 hpf time-point observation. Taken together, the data indicate that the highest EE_2 level (actual concentration of 1 ng/L) impacts normal blastula, affects gastrulation and perhaps early organogenesis. In contrast to the highest EE_2 exposure group, no increase in abnormal egg development could be recorded at 8 hpf (65-75%-epiboly) in the other EE_2 exposure groups. This might indicate that the two lowest concentrations of EE_2 impact late gastrulation and/or early organogenesis only. In the present study, embryo tests were performed under similar EE_2 concentrations as adults, thus mimicking a field scenario where full life-cycle takes place in EE_2 contaminated ecosystems. Thus, it could be hypothesized that embryo mortality may also be related to exposure of embryos to EE_2 in water. However, this is unlikely to be the case as a previous study with exposure of zebrafish embryos to EE_2 concentrations ranging from 1 to 100 ng/L, led to no increase in mortality at 120 hpf (Versonnen and Janssen, 2004). The phase where EE_2 disrupts the embryonic development of zebrafish matches the period where the development of zebrafish embryo is most sensitive under normal conditions. In our assay, the overall embryo mortality recorded in the control treatments was approximately 10%, which is within the lowest reported in the literature (Kishida et al., 2001), thus demonstrating the adequacy of the experimental set up. Similar to EE_2-exposed embryos, mortality in control treatments was observed only up to 24 hpf, the period where the most important changes in embryo development take place. In a recent study, Brown et al. (2007) reported an increase in rainbow trout (*Onchorynchus mykiss*) embryo mortality at 19 dpf after male parental exposure for 56 days to EE_2 at environmentally relevant concentrations (0.8 and 8.3 ng/L). The embryonic development of rainbow trout is longer than that of zebrafish, taking approximately 30 days at 11 ºC. At 19 dpf, rainbow trout embryos have already gone through early organogenesis. However, since in the Brown et al. (2007) study no screening in embryo mortality was done between 9 and 19 dpf, the possibility that rainbow trout embryo survival was also impacted at early organogenesis cannot be excluded. This would agree with our findings for the two lowest concentrations of EE_2, where an increase in embryonic mortality was observed at 24 hpf. Taken together, these findings indicate that EE_2, at actual concentrations below 1 ng/L range, impact the embryonic development of two fish species with different life-strategies; while zebrafish is a short-lived species and

fractional spawner, the rainbow trout is a long-lived species and a periodic annual spawner. This indicates that the effects of EE_2 on embryonic development may affect fish species with different life-strategies, thus suggesting that several wild fish populations may respond in a similar manner.

In a recent male and female zebrafish replacing experiment, Xu et al. (2008) found that male but not female replacement rescued embryo mortality at 12 hpf if the parental population were exposed to EE_2 from egg to 3-month-old fish. This led us to conduct a more detailed screening of male testis histology and spermatozoa quality at adulthood (8 months) of F_1 generation. At the end of the exposure period, no apparent differences could be observed in gonad histology between treatments. Furthermore, both stereological analysis of the testis and the tested sperm quality parameters did not differ between treatments. Similarly, no differences could be observed in female F_1 gonads at 8 mpf. Hence, the reduced embryo survival observed in their offspring is probably related with changes on the genetic information carried by male spermatozoa and/or alterations on the expression patterns of certain genes, rather than with the measured functional parameters. We have recently observed that zebrafish EE_2 exposure at 3.5 ng/L from 5 dpf up to 4 months induced an increase in genotoxic damage in erythrocytes both in adult males and females (Micael et al., 2007). This is consistent with the observation that EE_2 hinders nucleotide excision repair in zebrafish liver cells (Notch and Mayer, 2009). Likewise, Contractor et al. (2004) have shown that EE_2 induces methylation changes in the estrogen receptor and aromatase genes of medaka (*Oryzias latipes*). More recently, Brown et al. (2008) showed that when male rainbow trout were exposed to EE_2 for 50 days during sexual maturation an increase of aneuploidy levels in sperm cells as well as in embryos could be observed. These findings further support the hypothesis that embryo mortality after parental EE_2 exposure may be due to DNA damage. Thus, the question of whether this damage may be heritable through genetic or epigenetic mechanisms is of major importance. In mammals, the EC methoxychlor induces reproductive effects that can be inherited by future generations through an epigenetic mechanism (Anway et al., 2005; Crews et al., 2007). In an attempt to evaluate if EE_2 effects are heritable in fish, Brown et al. (2009) tested if the offspring produce by male rainbow trout, exposed to EE_2 during sexual maturation, showed the same decrease of progeny survival as the parental generation, when

raised in water free from EE_2. No heritable defects were detected. Whether the same applies to other fish species, particularly when the parental generation is exposed during the entire life-cycle, remains to be investigated.

The screening of Vtg levels in plasma or *vtg* gene induction in male fish is the most common approach to evaluate the exposure of wild fish populations to ECs (Hutchinson et al., 2006; Ferreira et al., 2009). The current findings of increased embryo mortality at EE_2 levels below those inducing reproductive impairment and *vtg1* gene induction in the male parental generation show that environmental risk assessment based on *vtg* induction only may not be sufficient to protect wild fish populations from EE_2 exposure. Conversely, if our data is extrapolated to wild fish populations, an elevation of *vtg* induction could be an indication of a negative ecological impact. In the present study, a “no observed effect concentration” (NOEC) of EE_2 on embryonic survival could not be determined, as the lowest concentration tested (actual EE_2 levels of 0.19 ng/L) still significantly increased the mortality of embryos at 24 hpf (150% over the controls). This EE_2 level is at present found in several rivers and estuaries in the vicinity of urban areas, thus raising considerable concern. In wild fish populations, the screening of ecological level effects of ECs is not an easy task if there is not a massive decline in population over a few generations (Sumpter, 2005). However, in most cases, only gonad histology and *vtg* induction were evaluated. As the field studies progress, an increasing body of literature indicates the presence of a population-level impact of ECs (Jobling et al., 2002; Kidd et al., 2007). Considering the present study, despite a thorough evaluation of reproductive endpoints and *vtg1* induction in the parental generation, no effects could be observed for the two lowest EE_2 exposure levels. Hence, if our study reflected a real case scenario, and embryonic survival of the offspring would not have been screened, we could easily have missed the negative effects that could lead to population-level impacts. In the wild, many factors, such as predation and food availability, may interfere with embryo survival. Thus, future studies should investigate the population-level impact of reduced embryo survival after parental EE_2 exposure, in comparison to other important causes of embryo mortality. Nevertheless, our results strongly support the inclusion of embryonic development studies in the screening of EDCs in wild fish populations.

Overall, the present study shows that life-cycle exposure of zebrafish to very low levels of EE_2 severely impacts embryo survival at concentrations below those inducing a significant increase in *vtg1* gene transcripts in parental males. The window of embryo sensitivity to EE_2 matches the most sensitive phase of zebrafish embryonic development under normal conditions, with the two lowest EE_2 concentrations impacting late gastrulation and/or early organogenesis, whereas exposure to nominal 2 ng/L EE_2 impact blastula and gastrula phases. Future studies should focus on the molecular changes upon EE_2 exposure during embryonic blastula, gastrula and early organogenenis phases, as well as identifying the period within the 8 and 24 hpf where zebrafish embryos are more sensitive to very low levels (below 0.5 ng/L) of EE_2. Likewise, the screening of male gonad DNA at adulthood may help to understand the mechanisms of EE_2 disruption and reveal more sensitive biomarkers of EE_2 exposure based on adverse effect endpoints which can be directly related with population dynamics.

3.6 Acknowledgements

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Chapter 4

Rapid-behaviour responses as a reliable indicator of estrogenic toxicity in zebrafish juveniles

Sárria, M.P.[1], Soares, J.[1], Vieira, M.N., Castro, L.F.C., Santos, M.M., Monteiro, N.M., 2011. Chemosphere 85, 1543-1547.

[1] Authors contributed equitably for the article.

4. Rapid-behaviour responses as a reliable indicator of estrogenic chemical toxicity in zebrafish juveniles

4.1 Abstract

Whereas biochemical and molecular parameters have been well recognized as important “signposts” of individual disturbance to endocrine disrupting chemicals (EDCs) exposure, behavioural endpoints are yet greatly overlooked as a routine tool in environmental risk assessment of EDCs. However, life histories are intimately associated with numerous inter- and intra-specific interactions, which invariably depend on the performance of effective behaviours. Within fish species, one of the most important factors influencing energy turnover earlier in the development is locomotor activity. This essential trait reflects the organism’s ability to generate and coordinate the metabolic energy required for both reproductive and non-reproductive behaviours. Inappropriate movement responses due to toxic effects of contaminants may ultimately impact important ecological variables.

Therefore, in the present study, the swimming bursts of zebrafish juveniles exposed for 40 days post-fertilization (dpf) to the synthetic estrogen ethinylestradiol (EE_2), tested at environmentally relevant concentrations (nominal concentrations of 0.5, 1 and 2 ng/L), were investigated in order to address the potential of rapid-behaviour patterns as an effective response indicator of estrogenic chemicals (ECs) exposure. This synthetic estrogen was selected due to its high prevalence in aquatic ecosystems, ability to mimic natural estrogens and proven record of causing negative effects in fish reproduction. The behavioural responses were compared with established endpoints used in the screening of EE_2 effects at adulthood. Results indicate that zebrafish juveniles swimming activity was significantly decreased upon EE_2 exposure. Since reduced locomotion of zebrafish may impact foraging, predator avoidance, drift and transport, and even interfere with social and reproductive behaviours, a fitness decline of wild fish populations can ultimately be hypothesized. Furthermore, behavioural endpoints were found to display higher sensitivity to EE_2 than either vitellogenin gene induction or reproductive parameters determined at adulthood.

Overall, the findings of this work not only demonstrate the power of high-throughput behavioural responses, able to act as sensitive early warning signals of EC exposure, but also highlight the potential of behavioural endpoints in providing a more comprehensive and non-invasive measure of EDCs exposure.

4.2 Introduction

The detrimental impact of several groups of chemicals in different dimensions of the environment is now well documented (USEPA, 2008 ROE; AMAP, 2009; EEA, 2010 NECD). Given the ubiquity and persistent nature of numerous organic pollutants exhibiting endocrine disrupting activity (endocrine disrupting chemicals - EDCs), a large number of studies have been focusing on whether and how EDCs interfere with the normal endocrine system of animals as well as their impact in fundamental processes such as reproduction (WHO, 2002). Evidence suggests that both invertebrates and vertebrates have been impacted by EDCs exposure, although most reports derive from aquatic animals (Fent et al., 1998; Santos et al., 2002; Thibaut and Porte, 2004; Rodrigues et al., 2006; Rempel and Schlenk, 2008; Ankley et al., 2009). This is a predictable outcome considering that a large group of chemicals are released to the aquatic ecosystems, both from point and non-point sources (e.g. waste water plant discharges and silt-laden runoffs, respectively).

Chemicals with the ability to mimic estrogens, estrogenic disrupting chemicals (ECs), have been shown to be prevalent in most aquatic ecosystems in the vicinity of urban areas. Most of these chemicals act as agonists of the estrogen receptors (ERs), thus eliciting biological responses similar to those of the natural estrogens (Kime, 1998). The most bioactive EC, at the receptor level, is the 17α-ethinylestradiol (EE_2), used in oral contraceptives and hormone replacement therapies (Gutendorf and Westendorf, 2001). EE_2, at concentrations below 2 ng/L, has been reported to disturb several crucial processes in fish such as gonad development, egg production, embryo mortality or courtship behaviour (Länge et al., 2001; Robinson et al., 2003; Balch et al., 2004; Soares et al., 2009) and, at 5 ng/L it has been reported to lead to population collapse of fathead minnow, *Pimephales promelas* (Kidd et al., 2007).

The most common approach to evaluate the exposure to ECs consists in determining the induction of the egg-yolk protein precursor, vitellogenin (Vtg), in adult male fish liver (Sumpter and Jobling, 1995; Kime et al., 1999). Although Vtg induction in male fish has been largely used both in the field and under laboratory conditions to monitor the exposure to ECs, it also shows some constraints mostly related with the ecological relevance of the response, species and gender differences in response, life-cycle period of exposure (adult Vtg is more inducible than larvae and juvenile) (Tyler et al., 1996, 1999; Navas and Segner, 2006; Shi et al., 2006; Liao et al., 2009). In addition, seasonal-dependent regulation of gene expression reported for some annual cycle species can also induce difficulties in data interpretation (Moussavi et al., 2009).

Within fish species, one of the most important factors influencing energy turnover earlier in the development is locomotor behaviour (Brett and Groves, 1979). The 'free-swimming' stage (after yolk depletion and swim-up) is a crucial phase for survival, since it reflects the juveniles ability to generate and coordinate the metabolic energy required for both reproductive and non-reproductive behaviours. Therefore, unorthodox movement responses due to the toxic effects of contaminants may ultimately impact population sustainability. Previous studies on vertebrates have demonstrated that natural estrogens can exert neuromodulatory effects, by directly affecting neuron functioning, which influences vertebrate locomotor activity (Mermelstein et al., 1996; Kelly et al., 2003; Dickinson, 2006). Yet, despite the well-conserved basic structure and function of vertebrate endocrine axis, data regarding effects of teleost fish movement patterns under EC exposure remains scarce, especially when long-term exposures to contaminants are considered (e.g. Sárria et al., 2011a). In fact, whereas endpoints at the cellular, molecular and biochemical level have been well recognized as important "signposts" of individual disturbance to EDCs exposure (Hutchinson et al., 2006), behavioural endpoints are yet greatly overlooked as a routine tool for detecting environmental pollution effects. Importantly, recent studies show a good agreement between selected behavioural endpoints in fish and EDCs and pharmaceuticals toxicological action. The observed behavioural effects reported in these non-invasive toxicity tests ranged from marked changes in locomotion patterns and predation-escape, to more subtle disorders in courtship and aggression responses (Airhart et al., 2007; Speedie and Gerlai, 2008; McGee et

al., 2009; Kokel et al., 2010; Saaristo et al., 2010; Sária et al., 2011a). The application of rapid-behaviour indicators on EDCs risk assessment may potentially contribute in providing very expedite responses, both ecologically relevant and cost-effective, and thus overcome the often less immediate response which is commonly provided by other types of approaches (Clotfelter et al., 2004).

Zebrafish, *Danio rerio* Hamilton (1822), has been well recognized as a model vertebrate species for developmental studies, an essential work basis to several scientific areas, such as behavioural neuroscience and ecotoxicology. As an example, zebrafish locomotor activity has already allowed for a specific high-throughput behavioural profiling that ultimately led to the discovery and characterization of psychotropic drugs (Kokel et al., 2010; Rihel et al., 2010). In contrast, there is virtually no information available on zebrafish swimming behaviour under EDCs exposure. Thus, in the present study, taking into account the already anticipated estrogenic potency of EE_2, the swimming bursts performed by juvenile zebrafish, exposed to environmental relevant concentrations of this EC, was investigated. Since *Danio rerio* locomotor network development and kinematics are well described (Drapeau et al., 2002; Müller and van Leeuwen, 2004), and considering that the motor-sensory endocrine axis is transversal through the generality of the vertebrate species, we discuss not only the potential use of this rapid-behaviour pattern as an effective responsive indicator of sub-lethal EDC toxicity, but also the applicability of this approach in other teleost species.

4.3 Materials and Methods

4.3.1 Chemicals

17α-ethinylestradiol [$C_{20}H_{24}O_2$] (EE_2, purity ≥ 98%, Sigma - stock solution: 1mg/mL); dimethylsulfoxide [$(CH_3)_2SO$] (DMSO, purity 99.5%, Sigma).

4.3.2. Experimental fish

Zebrafish, *Danio rerio* (Teleostei, Cyprinidae) is a small benthopelagic tropical freshwater fish, native from India and Southeast Asia (Engeszer et al., 2007). The

species size, robustness, short life-cycle, high fecundity and potential to easily breed in captivity are advantages for experimental bioassays. Zebrafish has been widely recognized as a model vertebrate species for developmental biology and gerontology assessment (Grunwald and Eisen, 2002), behavioural ecology, genetics research (Eisen, 1996; Engeszer et al., 2004), and ecotoxicology (Hill and Janz, 2003; Micael et al., 2007; Segner, 2009).

4.3.3 17α-ethinylestradiol (EE_2) exposure

Wild-type parental zebrafish (F_0) were obtained from local suppliers in Singapore, and used as breeding stock according to Soares et al. (2009). In the afternoon before breeding, two groups of 4-6 males and 10-12 females were housed in cages with a net bottom cover with glass marbles within a 30 L aquarium under the same water and photoperiod conditions as the stock and fed with live brine shrimp (*Artemia spp.*). At the following day, breeding fish were removed 1.5 hours after the beginning of the light period and the eggs were collected and cleaned. Fertilized eggs were randomly allocated to experimental aquaria. A flow through system was used to administer EE_2 with slight modifications of Santos et al. (2006). The experimental setup, including details on fish basic monitoring, exposure conditions, physico-chemical parameters are described in detail in Soares et al. (2009). Briefly, after 15 days of aquaria system equilibration, 450 eggs collected ≈2 hours post-fertilization (hpf) were randomly allocated to 5 L aquaria (each placed within 30 L aquarium) and exposed to EE_2 (nominal concentrations: 0.5; 1 and 2 ng/L). Each treatment was replicated twice, including experimental and solvent controls. DMSO was used as solvent in all treatments, except experimental control, at a volume of 0.000002%. At 20 days post-fertilization (dpf), zebrafish were allocated to 30 L aquaria and their number adjusted to fit 100 juveniles per aquarium. Feeding was initiated at 6 dpf with two meals of Tetramin supplemented with one brine shrimp meal per day; at 9 dpf this was changed to two brine shrimp meals and one Tetramin meal per day, which was maintained up to the end of exposure. The feeding regime of larvae was based on a slight modification of Carvalho et al. (2006), which maximizes survival and growth.

4.3.4 Water chemical analysis

Concentrations of EE_2 were found to be below the detection limit in the reference aquaria and to be 0.19 ± 0.02, 0.24 ± 0.02 and 1.00 ± 0.12 ng/L, respectively, for the 0.5, 1 and 2 ng/L nominal concentrations. Briefly, the extraction procedures consisted of a pre-treatment according to the "Sample Pre-treatment Protocol for Female Steroid Hormones" (Japan Envirochemicals), extraction in a solid phase C18 column (Sep- Pak Plus C18 cartridges, Waters Corporation), 80% methanol elution and concentration under nitrogen gas flow. EE_2 concentration was then determined using the "Jec ELISA kit" (Japan Envirochemicals) according to the manufacturer's protocol.

4.3.5 Collected data

4.3.5.1 Swimming behaviour

During the entire exposure period, mortality was assessed daily and dead individuals were removed. At 40 dpf, 12 juveniles from each treatment (six per replicate) were randomly selected and the number of movement bursts was counted during sixty seconds. Basically, each time a fish actively propelled itself in either direction, a movement was scored. In order to avoid bias, "blind" observations were performed by a single person.

4.3.5.2 Additional endpoints measured at 8 mpf

In order to allow for a comparison between behavioural parameters determined at 40 dpf, and more established endpoints used in the screening of EE_2 impact in fish, the exposure was extended up to eight months post-fertilization (mpf), when zebrafish females reach their maximum fecundity. A vast array of endpoints were determined at 8 mpf, such as fecundity and fertility rate, male and female gonad histology coupled with a stereological analyses, sperm quality parameters, *vtg1* induction in male liver, as well as embryonic development of the F_2 generation. A detailed description of these endpoints has been reported in Soares et al. (2009).

4.3.6 Statistical analysis

In order to assess any hypothetical bias in mortality patterns, a Wilcoxon matched pair test was applied to detect if the replicates were indeed homogenous. Then, using the average mortality of the control as the expected value, a chi-square (χ^2) test was conducted where the observed values derived from the average mortalities observed in the solvent control and EE_2 treatments. Furthermore, in order to investigate the influence of EE_2 on zebrafish juveniles swimming behaviour (number of movement bursts) a one-way ANOVA was conducted, with five levels: experimental control, solvent control (DMSO), lowest, intermediate and highest EE_2 concentrations. All analyses were conducted in STATISTICA (v7).

4.4 Results

4.4.1 Mortality

The mortality rates, from embryo up to 20 dpf, varied between 36 and 55%. These values are well within the ranges reported by several other zebrafish partial life-cycle studies (Hill and Janz, 2003, Santos et al., 2006). No differences among replicates were observed (Wilcoxon matched pairs test: N = 5; Z = 0.944; P = 0.345). Also, there were no significant differences in mortality between controls and EE_2-exposed groups (χ^2= 3.043; DF = 3; P = 0.385).

4.4.2 Swimming behaviour

EE_2 concentration directly affected zebrafish juveniles swimming activity [one-way ANOVA: F(4,55) = 7.985, P < 0.01] causing a significant reduction in the number of juvenile fish swimming bursts. No differences were recorded between the controls (SNK, data not shown), which were significantly different from the EE_2-exposed groups. The increase in EE_2 concentration, although not significant, was indeed translated into a decrease in the number of movement bursts (Figure 4.1): experimental control = 102.33 ± 1.80 (average number of bursts ± S.E.);

solvent control = 98.25 ± 1.83; $[EE_2]_{0.5ng/L}$ = 91.42 ± 2.82; $[EE_2]_{1ng/L}$ = 89.00 ± 2.36; $[EE_2]_{2ng/L}$ = 87.92 ± 2.08.

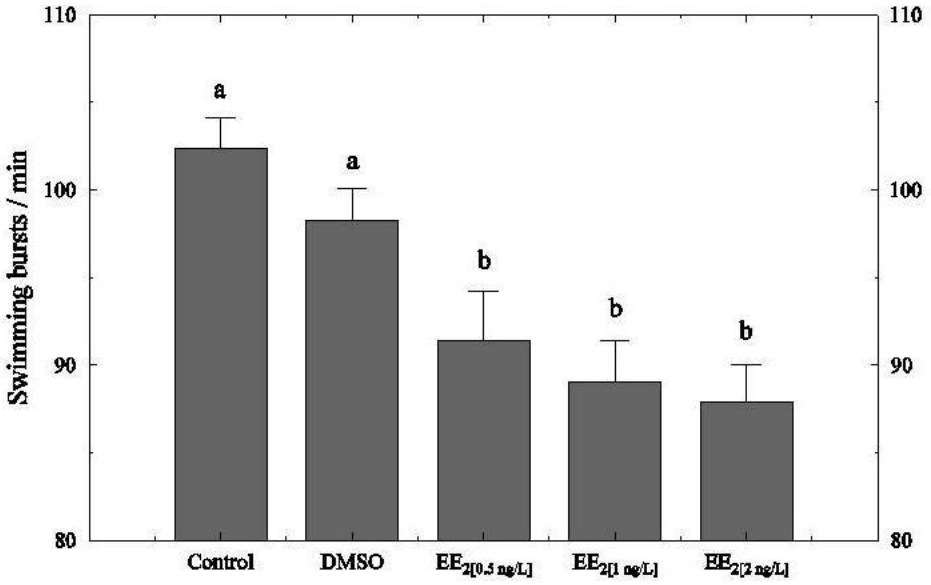

Figure 4.1 The effects of EE_2 exposure on zebrafish (*Danio rerio*) juveniles swimming behaviour (bars depict standard errors and different letters indicate differences among treatments).

4.4.3 Additional endpoints measured at 8 mpf

With the exception of *vtg*1 gene expression in males that was induced at the highest EE_2 exposure level (2 ng/L EE_2, nominal concentration) none of the other monitored parameters was altered at adulthood at the selected environmentally low EE_2 levels (i.e. fecundity, fertility, male sperm quality parameters and male and female gonad stereological analysis; Soares et al., 2009). In addition to the effects of EE_2 in the F_1 generation, the impact in the embryonic development of the F_2 generation was also tested, with significant changes observed in zebrafish embryos for all tested EE_2 concentrations (Soares et al., 2009).

4.5 Discussion

In contrast to biochemical and molecular endpoints, the use of fish behavioural parameters in environmental risk assessment is still not a common approach.

However, recent ecotoxicological and drug discovery studies support the sensitivity of fish behaviour endpoints in the hazard assessment of chemicals and in the characterization of pharmaceuticals action mechanisms (e.g. Kokel et al., 2010; Rihel et al., 2010). Therefore, if one wishes to integrate this approach in environmental risk assessment of EDCs, validation under laboratory control conditions is still required. In the present study, exposure of zebrafish larvae to the ubiquitous EDC, EE_2, led to a significant alteration in juvenile zebrafish behaviour patterns, with a decrease in swimming activity even at the lowest actual concentration of 0.19 ng/L. Even though it is out of the scope of the present study to determine the mechanisms of EE_2 disruption, it could be hypothesized that the observed increase in lethargy of the EE_2-exposed zebrafish juveniles might be caused by estrogenic neurotoxicity. In fact, not only the motor-sensory endocrine axis of vertebrates tends to be well conserved, but also estrogens have already been shown to be neurotoxic in mammals, causing changes on locomotor activity (Mermelstein et al., 1996; Kelly et al., 2003).

Exposure to EDCs, including EE_2, has been demonstrated to induce a set of negative effects in fish, including decreased reproductive capacity, male feminization, decreased embryonic survival, altered reproductive hierarchies, disrupted sexual selection in group-spawning fish, alterations in parentage outcomes and population collapse (e.g. Kidd et al., 2007; Coe et al., 2009; Colman et al., 2009; Saaristo et al., 2009; Soares et al., 2009; Soyano et al., 2010). However, most studies have focused on reproductive-related parameters at adulthood. Nevertheless, from an ecological standpoint, juvenile development is also a crucial phase since any alteration in parameters such as feeding and predator avoidance may result in lower survival rate, with clear implications in the number of individuals that reach maturity and reproduce. The findings of the present study indicate that EE_2, even at remarkably low levels, decrease juvenile swimming activity which may impact foraging, predator avoidance, drift and transport, and even interfere with social behaviours (e.g. aggression). Interestingly, our recent findings with a pipefish species indicate also that EE_2 exposure alters larvae behaviour when facing a potential predator (see Sária et al., 2011a) while altering the natural patterns of vertical distribution (Sária et al., 2011b). Taken together, these data indicate that EE_2 may impact fish populations

through subtle changes in the behavioural patterns expressed during the juvenile stage.

In comparison with established parameters used in the screening of EE_2 effects in fish, behavioural endpoints were found to display higher sensitivity than the measured endpoints at adulthood (*vtg1* expression, fecundity, fertility, male sperm quality parameters and male and female gonad stereological analysis). Furthermore, the behavioural endpoints displayed the same response pattern as embryonic development in the F_2 generation, thus demonstrating the promising role of this behaviour-based approach in anticipating the exposure effects of EE_2, and perhaps those of other EDCs. Hence, our results on zebrafish swimming behaviour after EE_2 exposure further demonstrate the power of high-throughput rapid behavioural responses as sensitive early warning signals of endocrine disruption.

The ecological level effects of EDCs are difficult to detect in the absence of a massive populational decline over a few generations (Sumpter, 2005), and therefore subtle changes as the one reported in the present study can easily be overlooked. In most field studies addressing the impact of ECs, only gonad histology and *vtg* induction are evaluated and no data is available on behavioural changes. Given the results of the present study, it can be argued that behavioural endpoints could be of great value in the risk assessment of EDCs in wild fish populations. Nevertheless, we have to take into account that field animals are exposed to mixtures of chemicals, which may lead to behavioural responses that differ from those of single exposures. In fact, this seems to be true for mixtures of EE_2 and the androgenic chemical tributyltin (Sárria et al., 2011a). Therefore, additional studies under controlled laboratory conditions are required to address the emergent issue of mixture effects of chemicals, as well as their impact in behavioural endpoints.

4.6 Conclusion

The present study highlights the promising role of cost-effective rapid fish behavioural analyses in EDCs environmental risk assessment, which eases the detection of EE_2 effects, a process usually pursued with the use of more time-consuming and methodologically demanding approaches. Locomotion is indeed a

relevant ecologically parameter, hence, behavioural endpoints not only contribute as an early warning sign of EE_2 effects, as they are also informative from an ecological point of view, a limitation of many biochemical and molecular approaches. Given the findings presented in the present study, additional behavioural studies with other EDCs should be performed to allow the integration of behavioural endpoints in the hazard assessment of EDCs.

4.7 Acknowledgements

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Chapter 5

Zebrafish (*Danio rerio*) life-cycle exposure to chronic low doses of ethinylestradiol modulates *p53* gene transcription within the gonads, but not NER pathways

Soares, J., Castro, L.F.C., Reis-Henriques, M.A., Monteiro, N.M., Santos, M.M., 2012. Ecotoxicology 21, 1513-1522.

5. Zebrafish (*Danio rerio*) life-cycle exposure to chronic low doses of ethinylestradiol modulates *p53* gene transcription within the gonads, but not NER pathways

5.1 Abstract

Parental full life-cycle exposure to ethinylestradiol (EE_2) significantly affects embryo development and survival. One of the possible mechanisms of action of EE_2 may involve the impairment of an organism's ability to repair DNA damage.

DNA repair mechanisms have sophistically evolved to overcome DNA damaging hazards that threaten the integrity of the genome. In the present study, changes in the transcription levels of key genes involved in two of the most thoroughly studied DNA repair systems in mammals were evaluated in adult zebrafish (*Danio rerio*) gonad upon full life-cycle exposure to chronic environmentally low levels of EE_2 (i.e., 0.5, 1 and 2 ng/L EE_2). Real time PCR was used to analyse the expression levels of nucleotide excision repair genes (NER) as well as the tumor suppressor *p53* and downstream selected effectors, i.e., *p21* (cyclin-dependent kinase inhibitor 1A), *gadd45α* (growth arrest and DNA damage induced 45, alpha), *bax* (bcl2-associated X protein) and p53 key regulator *mdm2* (murine double minute 2 protein).

NER genes transcription levels in gonads did not differ significantly among treatments. In contrast, the number of transcripts of *p53* gene was significantly increased in male gonads at all EE_2 exposure concentrations and in females at 1 ng/L EE_2. Despite the increase in *p53* transcripts, transcription levels of *p21*, *gadd45α* and *bax* genes were not affected upon EE_2 treatment, whereas *mdm2* gene expression significantly increased in females at the intermediate EE_2 dose (1 ng/L).

Overall, the present study indicate that chronic low levels of EE_2 significantly modulates the expression of *p53*, a key gene involved in DNA repair, particularly in male zebrafish gonads, which supports the hypothesis of an impact of EE_2 in male gonad DNA repair pathways.

5.2 Introduction

The aquatic environment is the ultimate recipient of an increasing range of anthropogenic contaminants. Among these, endocrine disrupting chemicals (EDCs) are of major concern since they can interfere with the endocrine system of vertebrates and thereby modulate or disrupt developmental and reproductive processes (Colborn and Clement, 1992). Among the broad group of EDCs, estrogenic chemicals (ECs) have received increasing attention because of their wide environmental occurrence and well-documented adverse effects (Sumpter, 2005). The most biologically active xenoestrogen in the aquatic environment is 17α-ethinylestradiol (EE_2) (Folmar et al., 2000; Metcalfe et al., 2001; Kolpin et al., 2002), the active ingredient of contraceptive pills.

ECs have been reported to increase plasma vitellogenin (Vtg) in male and female fish, increase the levels of intersex fish, decrease egg and sperm production, reduce gamete quality and induce complete feminization of male fish (Nash et al., 2004; Panter et al., 1998; Rodgers-Gray et al., 2001; Sohoni et al., 2001; Van den Belt et al., 2002; Solé et al., 2003). In contrast to the wealth of information dealing with the negative impact of ECs in reproduction, studies on non-reproductive processes are scarce. However, embryo development was shown to be affected following EE_2 exposure (Länge et al., 2001; Nash et al., 2004; Brown et al., 2007; Soares et al., 2009). In fact, an increase in zebrafish embryo mortality after chronic parental EE_2 exposure has recently been reported, in the range of 0.19-1ng/L, actual concentrations (Soares et al., 2009). Since embryo mortality, after parental full life-cycle EE_2 exposure, was observed at concentrations below those inducing reproductive impairment and *vtg1* induction in adults, we hypothesize that alterations on parental DNA are likely to be an important causative mechanism for the embryo mortalities observed. Supporting this hypothesis, estrogens, including EE_2, are known to promote mutagen-induced hepatic neoplasia in medaka (*Oryzias latipes*) and rainbow trout (*Oncorhynchus mykiss*) (Cooke and Hinton, 1999). Additionally, chronic zebrafish (*Danio rerio*) exposure to ethinylestradiol (EE_2) induces an increase in the formation of erythrocytic nuclear abnormalities that are associated with clastogenic and/or aneugenic DNA damage (Micael et al., 2007).

Recently, effects of EE_2 on DNA repair processes, in zebrafish, have been suggested as a possible mechanism by which estrogens can modulate incidence of DNA mutations (Notch and Mayer, 2009a). These compounds can alter mRNA abundance of nucleotide excision repair (NER) genes in liver of adult zebrafish and the rate and magnitude of NER processes in zebrafish liver cells (Notch et al., 2007; Notch and Mayer, 2009a,b). These findings suggest that EE_2 may impact the genomic stability of individuals, which could result in a decreased fitness of population. NER is the primary DNA repair pathway responsible for removing a variety of DNA lesions caused by bulky adduct forming mutagens (de Laat et al., 1999, Sancar et al., 2004), through two subpathways: global genome repair (GGR) and transcription coupled repair (TCR) (Thoma and Vasquez, 2003). This system has evolved early in evolution and appears to be highly conserved from prokaryotes to eukaryotes. Seven XP, *xeroderma pigmentosum* groups, have been identified representing different repair genes XPA-G (de Laat et al., 1999). XPC is responsible for initial damage recognition (Sugasawa et al., 1998) and XPA for damage verification and initiation of repair complex assembly (de Laat et al. 1999). TFIIH complex contains the helicases XPB and XPD (de Laat et al., 1999), responsible for DNA unwind and the nucleases XPF and XPG, that cleave the open complex (Sancar et al., 2004). After the removal of the damaged DNA segments, DNA synthesis and ligation completes the process. Loss or decrease of any NER associated protein may lead to an increased incidence of uncorrected DNA lesions.

In addition to NER, other proteins are known to play a central role in DNA damage protection mechanisms. Of particular importance is the tumor suppressor p53, a multifunctional protein that has an essential role in the cellular response to a variety of genetic insults. DNA damage hazards that threaten the integrity of the genome induce an activation of p53. Initially, p53 protein levels are increased via the inhibition of its interaction with negative regulators such as the murine double minute 2 protein (Mdm2). Then, induced p53 functions through protein-protein interactions and/or as a transcription factor increasing the expression of downstream genes, such as *p21* (cyclin-dependent kinase inhibitor 1A), *gadd45α* (growth arrest and DNA damage induced 45, alpha) and *bax* (Bcl2-associated X protein), all involved in cell cycle regulation pathways, namely cell cycle arrest, DNA repair and/or apoptosis (Adimoolam and Ford, 2003) (Figure 5.1).

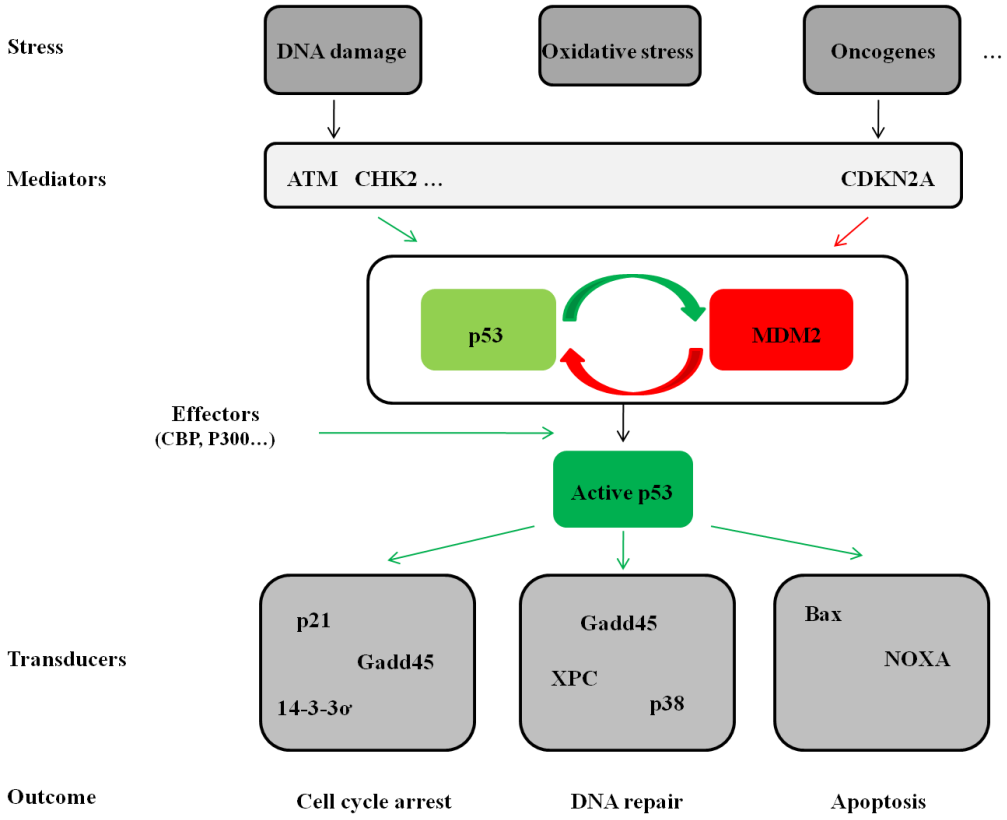

Figure 5.1 Simplified scheme of p53 network. Several types of stress responses activate p53 pathway. Upstream mediators detect and interpret this signals leading to an increase in p53 protein levels, through translation and activation of p53 and via inhibition of its negative regulator Mdm2. Induced p53 activates several transducers within three major pathways: cell cycle arrest, DNA repair and apoptosis. Green arrows indicate activation and red arrows inhibition. Modified from http://*p53*.free.fr/.

Since previous research has shown that EE_2 alters hepatic mRNA abundance of key NER genes in adult zebrafish (Notch et al., 2007; Notch and Mayer, 2009a,b), we hypothesize that the alteration of DNA repair processes by EE_2 in gonads may be associated with the embryonic disruption of zebrafish development as observed in a previous study (Soares et al., 2009). Hence, the aim of the present work was to examine the effects of chronic low levels of EE_2 on adult zebrafish gonads through the analysis of key DNA repair genes, i.e., NER, *p53* and selected downstream effectors genes as well as its negative regulator *mdm2*.

5.3 Material and Methods

5.3.1 Species selection

Adult wild-type zebrafish (*Danio rerio*) were used as breeding stock. The stock was kept at a water temperature of 28 ± 1 ºC and under a photoperiod of 14:10 h (light:dark), in 250 L aquaria with dechlorinated and aerated water, in a recirculation system with both mechanical and biological filters. The fish were fed *ad libitum* twice a day with a commercial fish diet Tetramin (Tetra, Melle, Germany) supplemented with live brine shrimp (*Artemia spp.*).

5.3.2 Exposure of F_1 generation

Exposures were performed using a flow through system. Before entering the system the water was heated and charcoal filtered. The water flow (50 ml/min) and treatment solutions (0.018 ml/min flow) were administered by two peristaltic pumps. The water and the solutions were combined, in a mixing chamber, before entering, by gravity, in the continuously aerated aquaria. Throughout the experimental period, zebrafish were maintained in the same temperature and photoperiod regime as the breeding stock. Water physical-chemical parameters were measured weekly, with the exception of temperature that was monitored on a daily basis. Four exposure conditions, in duplicate, were set up: a solvent control (DMSO) and three EE_2 concentrations (nominal concentration: 0.5; 1 and 2 ng/L).

17α-Ethinylestradiol (EE_2 98%, Sigma) (stock solution: 1mg/ml) was diluted in dimethylsulfoxide (DMSO 99.5%, Sigma). From this solution aliquots were prepared and kept at -20 ºC until use. Working EE_2 solutions were diluted in MilliQ water and renewed three times a week. DMSO volume in aquaria was 0.000002%.

In order to test EE_2 effects when zebrafish reach their maximal fecundity (Westerfield, 2000), the study lasted for 8 months, with continuous exposure. After a period of accommodation, the breeding stock was induced to reproduce. The experiment was initiated by randomly allocating 450 eggs ≈2 hpf (hours post-fertilization) in 5 L aquaria. Each of the 5 L aquaria was placed inside of one 30 L aquaria (where larvae were subsequently allocated when they reached 20 dpf, days post-fertilization) to keep the water temperature constant. Throughout the

experiment, the number of zebrafish in each aquarium, as well as the amount of food distributed, was adjusted according to fish development, number and size. Feeds consisted of commercial fish diet Tetramin (Tetra, Melle, Germany) supplemented with live brine shrimp (*Artemia spp.*). For further detail see Soares et al. (2009). Mortality was assessed on a daily basis, throughout the entire exposure period, and dead eggs/individuals removed. At the end of the experiment (8 months), adult zebrafish were immobilized in ice-cold water, weighed, measured, and immediately decapitated. All biological material excised (gonad and liver) was conserved in appropriate medium for further analysis. Gonads, from part of the adult animals, were stored in RNAlater (Sigma) (-80 ºC) and observed under a stereo-microscope for sex determination, complemented with histology (data not shown). Additional parameters where also determined and reported in a parallel study (Soares et al., 2009) such as male and female gonad stereological analyses, functional spermatozoa parameters, *vtg1* liver gene transcription, reproductive and embryonic development trials.

5.3.3 EE_2 concentration in water

In order to determine actual EE_2 concentrations, water samples were collected twice during the exposure (at 40 and 210 dpf) and pre-treated according to the “Sample Pre-treatment Protocol for Female Steroid Hormones” (Japan Envirochemicals). EE_2 concentration was determined using the “Jec ELISA kit” (Japan Envirochemicals) according to the manufacturer´s protocol (Soares et al., 2009). Concentrations of EE_2 were found to be below the detection limit in the reference aquaria and to be 1.00 ± 0.12, 0.24 ± 0.02 and 0.19 ± 0.02 ng/L, respectively, for 2, 1 and 0.5 ng/L nominal concentrations. The extraction procedure rendered a recovery of over 90% and therefore no correction was performed to the obtained EE_2 concentrations.

5.3.4 RNA extraction and cDNA synthesis

Total RNA was extracted from gonads of adult individuals (five males and five females per treatment) in the end of the exposure period (8 months), using the “illustra RNAspin Mini Kit” (GE Healthcare) following manufacturer´s protocol. On

column DNase digestion was used during the extraction procedure. RNA quality was verified in a 1% agarose gels. Total RNA concentration was estimated using the "Quant-it™ RiboGreen® RNA Assay Kit" (Invitrogen). cDNA was synthesized from 180 ng total RNA using "iscript cDNA synthesis kit" (Bio-Rad).

5.3.5 Primer design

Sequences for zebrafish *β-actin*, NER genes (*xpc*, *xpa*, *xpd*, *xpf*), *p53*, *p21*, *gadd45α*, *bax* and *mdm2* genes were obtained from GenBank and Ensembl (Table 5.1). Specific primers were designed with the Beacons Design™. software (Premium Biosoft International) in a region outflanking an intron (18-25 nucleotide length and GC content 40-60%). NCBIs basic local alignment search tool (BLAST) was used to verify primer specificity. Primers were synthesised by STABVIDA (Portugal).

Table 5.1 Primers used for quantitative PCR for specific gene amplification.

Gene Product	Accession number	Forward primer	Reverse primer
β-actin	NM_181601.3	ACTGTATTGTCTGGTGGTAC	TACTCCTGCTTGCTAATCC
xpc	NM_001045210	GCCAACATCCGTCTCAGAAT	GAACGGTTGGAAAAACCAAG
xpa	NM_200471	CCTGATTATCTGATGTGCGAAG	CGGTGGCTCCCTCTTATCC
xpd	NM_200926	CAGACAGGTGCCCATTCC	GCGTGAAGAAGTGCGTAAC
xpf	NM_199785	TGAATGCCTGCCTGAAAGAG	CTGAGTGAGGTAGAGAAGAAGG
p53	NM_131327	CCTCACAATCATCACTCTGG	TTCTTGAAGTTGCTCTCCTC
p21	XM_001923789	GACCAACATCACAGATTTCTAC	TGTCAATAACGCTGCTACG
gadd45α	NM_200576	ATGACTTTTGAAGAACCGTGTGG	GATCTGGAGGGCCACAT
bax	AF231015	GGCTATTTCAACCAGGGTTCC	TGCGAATCACCAATGCTGT
mdm2	NM_131364.2	AGAGAACGCAGGAGAAGGCATAAG	ACCAATCACGCACCAAGACAGG

NCBI accession numbers for sequences used in primer design.

5.3.6 Real-time PCR

Fluorescence-base quantitative PCR was performed with the iQ5 optical system (Bio-Rad). Each sample was amplified in duplicate using 96-well optical plates in a 20 µL reaction volume using 2 µL of cDNA, 10 µL of iQ™ SYBR Green (Bio-Rad), 200 nM of the appropriate forward and reverse primers. In each plate, a "no template control" was included.

PCR profile had the following conditions: 95 ºC of initial denaturation for 3 min; thereafter, 38 cycles of amplification were carried out with a denaturation at 95 ºC

for 10 s, annealing at 55 ºC for 30 s (except for *β-actin*, annealing was 56 ºC) and extension at 72 ºC for 30 s (data collection). A melting curve was generated in every run to confirm the specificity of the assays. The PCR products were also analysed by agarose gel electrophoresis to confirm the presence of single bands (data not shown). Finally, PCR products were sequenced to confirm the sequence identity.

In order to determine reaction efficiency, a standard curve that consisted of five 5-fold serial dilutions of cDNA, from a mix of all female samples, was also performed in each run (reaction efficiency close to 100% for all reactions). Relative change in transcript abundance was normalized to *β-actin* and calculated utilizing the $2^{-\Delta\Delta Ct}$ analysis method (Livak and Schmittgen, 2001). Control expression levels (DMSO) were normalized to a value of 1.

5.3.7 Data analysis

Data were analysed using Statistica 8.0 software. After testing for ANOVA assumptions (homogeneity of variances and normality of data), one-way ANOVA was performed on $\Delta\Delta$Ct values for a given gene for all treatments. Some data were $\log_{10}(n+1)$ transformed before ANOVA analysis. When statistically significant differences were found between treatment groups ($p < 0.05$), Fisher's Least Significant Difference (LSD) multiple comparison test was performed in order to determine differences among treatments. Solvent control was used as the reference group (= 1-fold). To validate that EE_2 exposure did not alter *β-actin* expression, ΔCt values were analyzed by one-way ANOVA and $p > 0.05$ was used to determine no significant differences among treatments. Regression analyses were performed in transcription data.

5.4 Results

5.4.1 Transcription levels of nucleotide excision repair genes (NER)

Transcription levels of *xpc*, *xpa*, *xpd*, *xpf* in male and female gonads did not differ significantly between control and EE_2 exposed groups ($p > 0.05$, one-way ANOVA) (Figure 5.2). However, a concentration related trend towards an increase

in transcription was observed for some NER genes (i.e., *xpc* in ovary, r = 0.52, p = 0.018; *xpa* in testis, r = 0.36, p = 0.12; *xpd* in ovary, r = 0.40, p = 0.08). *xpc* gene transcription in male's liver followed a concentration related increased (r = 0.74; p = 0.0015), that reached significance for the highest EE_2 concentration ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test) (Figure 5.3).

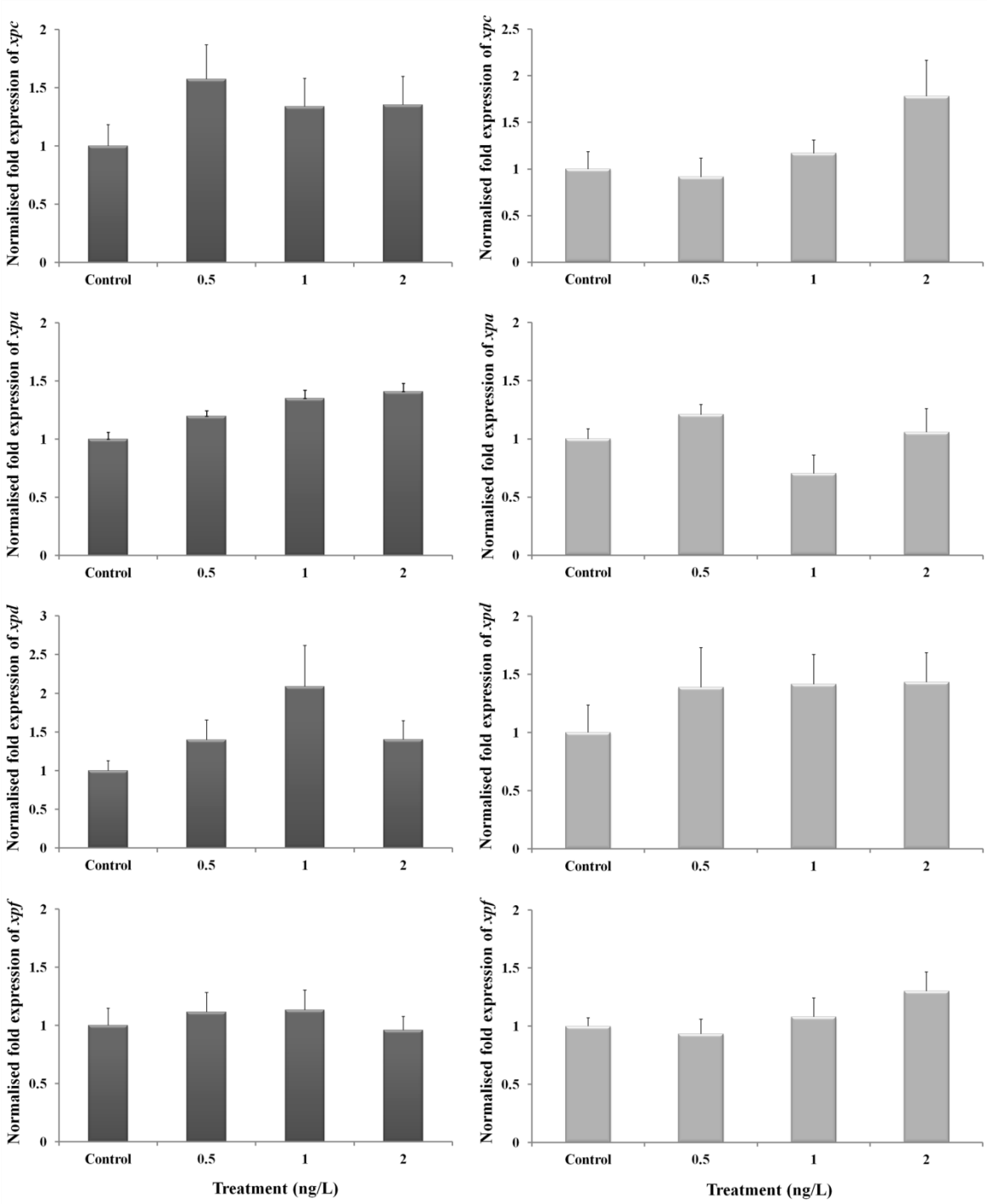

Figure 5.2 Normalised fold expression of *xpc*, *xpa*, *xpd* and *xpf* NER genes in 8 months zebrafish male (dark grey) and female (light grey) gonads after exposure to different EE_2 concentrations (ng/L). Solvent control (DMSO) was used as the reference group (= 1-fold). Values are presented as mean ± standard error.

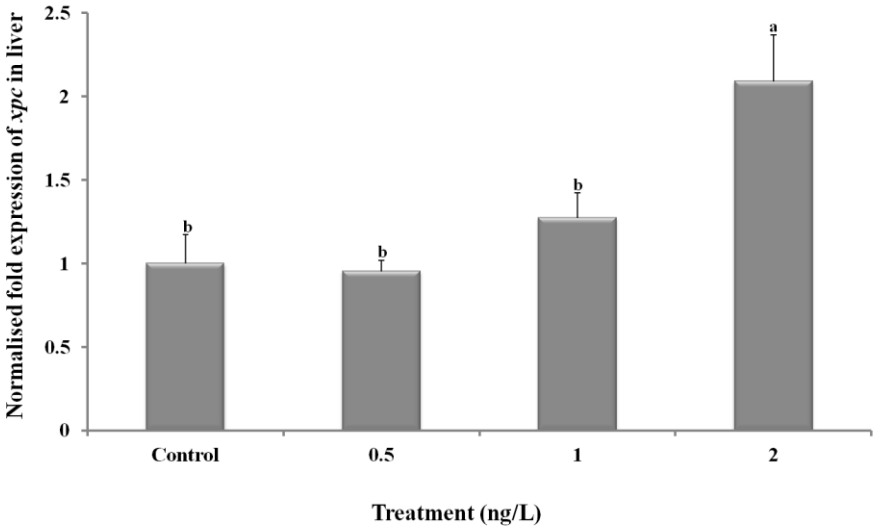

Figure 5.3 Normalised fold expression of *xpc* NER gene in 8 months zebrafish male livers after exposure to different EE_2 concentrations (ng/L). Solvent control (DMSO) was used as the reference group (= 1-fold). Values are presented as mean ± standard error. Different letters indicate significant differences among treatments ($p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test).

5.4.2 Transcription levels of *p53* and *mdm2*

Figure 5.4 displays *p53* and *mdm2* gene transcription in male and female gonads. In testis, EE_2 exposure led to a significant ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test) 2-fold increase in *p53* gene transcription at all EE_2 exposure concentrations in comparison with control animals. Female gonads displayed a significant increase of *p53* gene expression at the intermediate dose (1 ng/L) ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test), whereas no changes were observed in females raised at 0.5 and 2 ng/L of EE_2 ($p > 0.05$, one-way ANOVA). *mdm2* gene transcription levels in EE_2 exposed males did not differ significantly from the control group ($p > 0.05$, one-way ANOVA). Female gonads displayed an expression pattern similar to that of *p53* with a significant increase of *mdm2* gene transcription at the intermediate dose (1 ng/L) ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test).

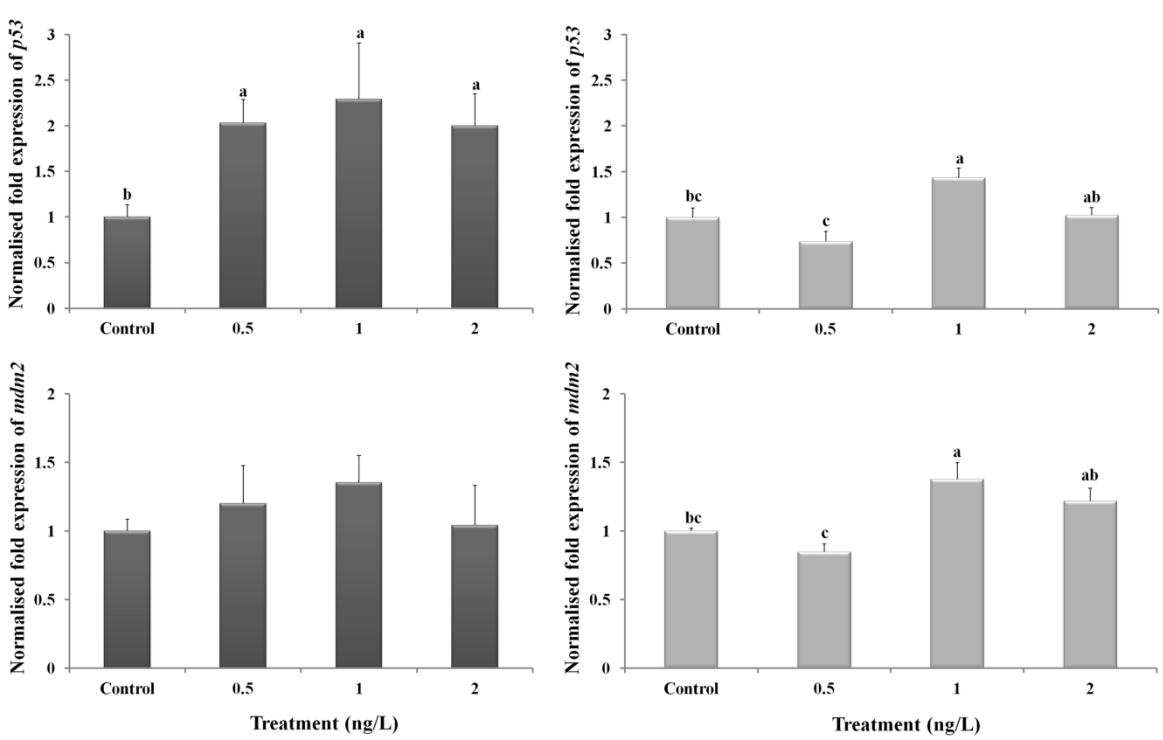

Figure 5.4 Normalised fold expression of *p53* and *mdm2* in 8 months zebrafish male (dark grey) and female (light grey) gonads after exposure to different EE_2 concentrations (ng/L). Solvent control (DMSO) was used as the reference group (= 1-fold). Values are presented as mean ± standard error. Different letters indicate significant differences among treatments ($p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test).

5.4.3 Transcription levels of p53 downstream pathways (p21, gadd45α and bax) genes

Figure 5.5 displays *p21*, *gadd45α* and *bax* genes transcription in male and female gonads. In both sexes, there were no statistically significant differences in *p21*, *gadd45α* and *bax* gene transcription levels between EE_2 exposed and control groups ($p > 0.05$, one-way ANOVA). Nevertheless, transcription patterns differed between sexes. In male gonads, *p21* and *gadd45α* transcription levels were similar between treatments with a relative trend toward a decrease for the highest EE_2 dose (2 ng/L). Female gonads displayed a concentration-dependent trend toward an increase in gene transcription for *p21* ($r = 0.51$, $p = 0.022$) and, less pronounced, for *gadd45α* ($r = 0.32$, $p = 0.16$) and *bax* ($r = 0.29$, $p = 0.21$).

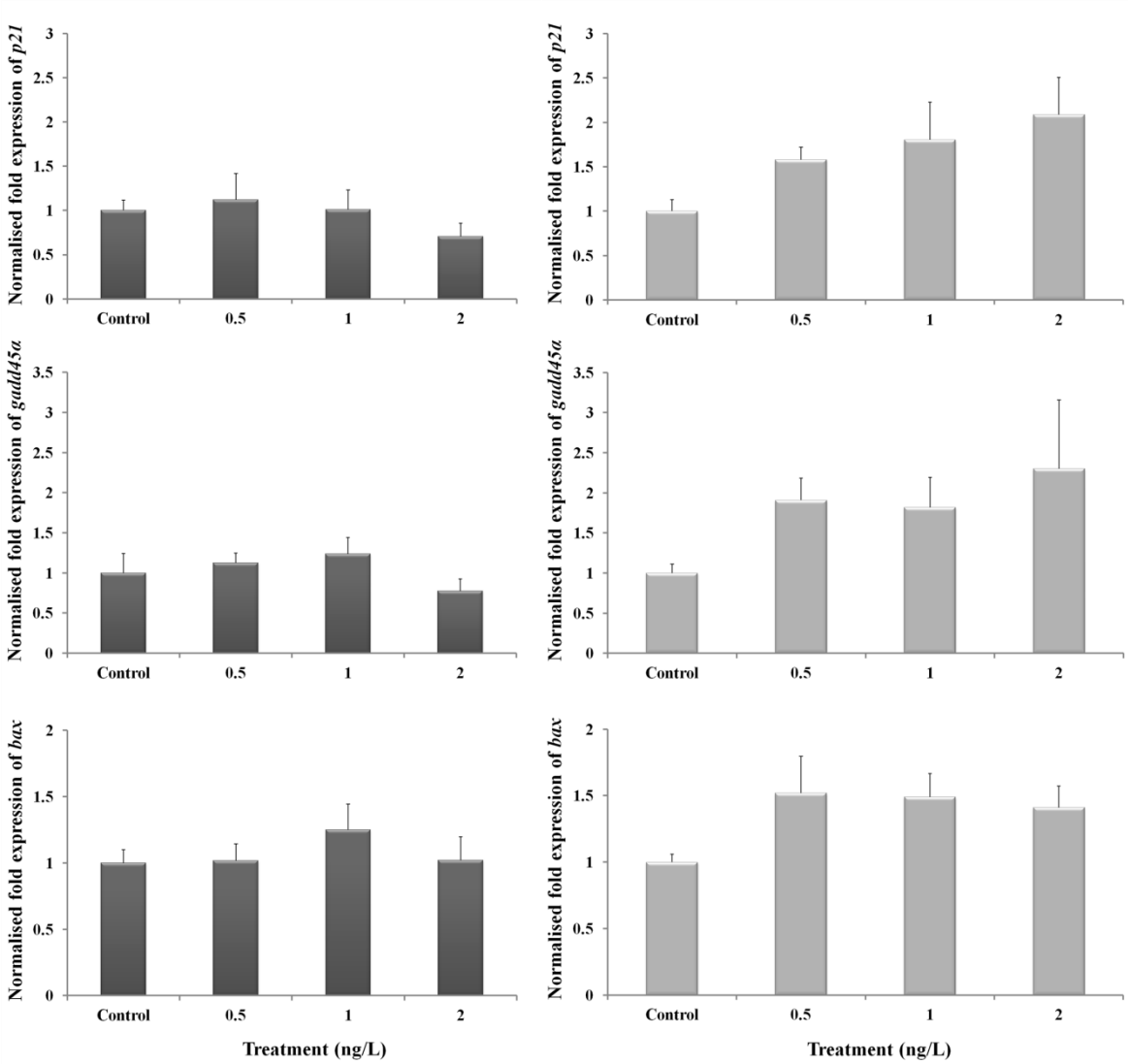

Figure 5.5 Normalised fold expression of *p21*, *gadd45α* and *bax* in 8 months zebrafish male (dark grey) and female (light grey) gonads after exposure to different EE_2 concentrations (ng/L). Solvent control (DMSO) was used as the reference group (= 1-fold). Values are presented as mean ± standard error ($p > 0.05$, one-way ANOVA).

5.5 Discussion

Recent data indicates that exposure of fish to environmental estrogens impacts progeny survival (Nash et al., 2004; Brown et al., 2007; Soares et al., 2009). When morphologically normal zebrafish males, exposed to EE_2 from egg to adulthood (3 months), were replaced by unexposed males, embryo mortality was rescued (Xu et al., 2008). On the contrary, female replacement did not improve progeny survival, thus suggesting that it can be related with male spermatozoa. Interestingly, Filby et al. (2007) found that in fathead minnows (*Pimephales*

promelas), short-term exposure to EE_2 (21 days at 10 ng/L) induces DNA damage (single-strand DNA breaks) in testis, but no effects were recorded in the ovary. This observation integrates well with the suggested impact of EE_2 on the reproductive physiology of male fish, and gives support to the results of the present study where altered *p53* gene transcription levels in EE_2 exposed groups were male gonad's specific. Hence, to understand the mechanistic action of EE_2 on zebrafish reproductive physiology and embryonic development it seems particularly important to evaluate EE_2 impact on the transcription levels of key genes involved in DNA repair in gonads.

In the present study, exposure to EE_2, in the range of 0.19-1 ng/L (actual concentrations), did not significantly affected NER genes expression in both male and female gonads. However, a concentration related trend towards an increase in several NER genes was observed. As a positive control, based on results by Notch et al. (2007), we determined the expression level of *xpc* in liver of EE_2 exposed males and a significant increase in expression was observed at 1 ng/L, actual concentration. Similarly, Notch et al. (2007) reported an increase in NER transcripts levels in zebrafish liver at exposure levels of 1 ng/L EE_2, while for higher, less environmentally relevant EE_2 exposure levels (i.e., 10 and 100 ng/L), an opposite effect was recorded. These findings suggest that NER impairment in gonads by EE_2 exposure might occur primarily at concentrations above those tested in our study.

Although, EE_2 did not impact the transcription levels of NER genes in gonads, exposure to all EE_2 tested concentrations significantly increased *p53* gene transcription in male gonads. Female gonads displayed a different pattern, with a significant increase at the intermediate exposure level (0.24 ng/L EE_2, actual concentration), whereas no changes were recorded in the other treatment groups. This finding clearly indicates that EE_2 exposure, even at the lowest exposure level (0.19 ng/L), modulates *p53* gene transcription, particularly in males. If p53 function in males is impaired in some way, cell cycle checkpoint function may also be impaired, which may lead to genomic instability (Molinari et al., 2000).

In order to elucidate the impact of EE_2 on p53 signaling pathways, we analyzed the transcription levels of several downstream genes (i.e., *p21*, *gadd45α* and *bax*) that are key players in the three major p53 signaling pathways involved in the cellular response to damage (cell cycle regulation, DNA repair and

apoptosis) as well as the transcription of its principal regulator, *mdm2*. p21, a primary mediator of the p53-dependent G1 cell cycle arrest following DNA damage, is an inhibitor of cyclin dependent kinase 1A (CDKN1A). Progression through the cell cycle checkpoints is positively controlled by different cdk´s that require cyclin binding for activity. Activation of p53 is typically associated with an increased expression of p21 which is translated into a delayed transit in cell division from G1 to S, thus preventing the effects of DNA lesions on vital cellular functions (Zhu et al., 2000). These delayed transits allow cells to repair DNA damage before replication or segregation of defective chromosomes and propagation of heritable genetic errors (Liu and Kulesz-Martin, 2001). Koroxenidou et al. (2005) reported that long-term EE_2 treatment decrease cyclin E and cdk2 expression, reduces cdk2 kinase activity and inhibits S phase cell entry in regenerating rat liver, suggesting a block in late G1 or G1/S transition. Furthermore, an increase in p53 and p21 expression levels was observed, indicating a possible role for the p53/p21 pathway in the cell cycle block. Despite these observations in rat liver, in the present study transcription levels of *p21* were not significantly affected by EE_2. Interestingly, a different expression pattern between sexes was observed. Whereas females displayed a concentration-dependent trend towards an increase in *p21* expression levels, males exhibited an inverse response with a decreased expression at the highest EE_2 treatment (1 ng/L EE_2, actual concentration). Similarly, *gadd45α*, the member of the Gadd45 family best associated with DNA damage (Rai et al., 2008; Sun et al., 2009), followed the same expression pattern than that of the *p21* gene, for both sexes.

Another key gene involved in p53 mediated responses is *bax*, which is a p53-apoptosis induced gene. Apoptosis seems to predominate as DNA damage is more severe or p53 protein is more highly induced (Liu and Kulesz-Martin, 2001; Sun et al., 2009). Similar to *p21* and *gadd45α*, *bax* expression levels did not differ among treatments, for both sexes, although basal *bax* expression in female gonads was increased, when compared with male gonads.

The finding that increased transcription levels of *p53* after EE_2 exposure did not induce an up regulation of pivotal downstream effectors is somehow unexpected. The possibility exists that differences in p53 post-translational modifications and interactions with its cooperating factors could contribute to a differential regulation of p53-mediated responses. A disruption in p53 signaling

pathways could also explain the lack of up regulation of their key downstream genes involved in cell cycle arrest, DNA repair and apoptosis. Interestingly, it has recently been demonstrated, in mammalian cells, that ERα binds to p53, resulting in inhibition of transcriptional regulation by p53 (Liu et al., 2006). The molecular mechanism by which ERα suppresses p53-mediated transcriptional activation involves the recruiting of nuclear receptor corepressors (NCoR and SMRT) and histone deacetylase 1 (HDAC1), resulting in p53-mediated transcriptional inhibition of p21. Consistently, 17β-estradiol (E_2) enhanced ERα binding to p53 and inhibited p21 transcription (Konduri et al., 2010). Future studies at molecular level should clarify if a similar disruption of p53 signalling pathways in fish is observed under EE_2 exposure.

In normal cells p53 protein is tightly controlled at negligible levels. One of the central components in the regulation of p53 is Mdm2. Activation of p53 protein is limited by a short feedback loop, involving the *mdm2* gene product. p53 stimulates transcription of the *mdm2* gene and Mdm2 protein binds to activated p53 protein. This interaction inhibits p53 activity through relocalization to the cytoplasm for proteasome-mediated degradation (Molinari et al., 2000). In our study, male EE_2 exposure did not significantly changed transcription levels of *mdm2*. Female gonads displayed a different pattern, with a significant increase in *mdm2* gene transcription at the intermediate exposure level, whereas no changes were recorded in the other treatment groups. This observation of a similar pattern of expression between *p53* and *mdm2* upon EE_2 exposure (particularly in females) is consistent with the autoregulatory feedback loop described in mammals for this complex of proteins. Future studies should confirm if changes in *p53* gene transcription parallel that of the p53 protein levels.

Recent studies indicate that p53 participate in the modulation of mitotic/meiotic checkpoint regulatory activities and cell death (Feitsma et al., 2007; Tomasini et al., 2008). Hence, inappropriate regulation of p53 could play a role in mitotic/meiotic arrest failure, chromosomal instability and aneuploidy. It has been identified that aberrant meiotic recombination is an important factor causing meiotic non-disjunction, and consequent improper chromosome segregation. Absent or reduced levels of meiotic recombination or even suboptimally positioned recombination events, results in genetically imbalanced sperm or oocytes that if fertilized may lead to an aneuploid embryo (Hassold and Hunt, 2001). A role for

p53 in meiotic recombination has been postulated from the observation that *p53* mRNA levels in testis of mice is high and specific for spermatocytes in zygotene to pachytene, the meiotic stages at which homologous chromosomes synapse for genetic exchange (Schwartz et al., 1993) and that p53 impairment results in germ cell degeneration during meiotic prophase (giant cell syndrome) (Rotter et al., 1993). Although it remains to be tested if the observed zebrafish embryo mortality, after parental life-cycle exposure to EE_2 (Soares et al., 2009), is linked with an increase in aneuploidy in male and/or female gonad, the findings of Brown et al. (2008), who reported an increase in aneuploidy in male rainbow trout (*Onchorynchus mykiss*) gonads and their offspring only when the period of EE_2 encompassed male sexual maturation (late spermatogenesis/spermiation), suggests that meiosis may be affected and support the idea of some genomic defect of the sperm DNA.

Taken together, the data of the present study and the findings of the decreased embryo survival after chronic parental low EE_2 exposure (Nash et al., 2004; Brown et al., 2007,2008; Soares et al., 2009), suggest that adverse population-level consequences are likely to occur in aquatic ecosystems in the vicinity of urban areas, where EE_2 is present at the concentration levels tested. In a parallel study (Soares et al., 2009), we determined the transcription levels of *vtg1* in male zebrafish liver, a marker of estrogenic exposure. An increment of *vtg1* gene transcription was observed only at the highest concentration tested (1 ng/L EE_2, actual concentration), whereas embryonic development was impaired at all EE_2 exposed groups (i.e., 0.19; 0.24; 1 ng/L, actual concentrations). Hence, the pattern of *p53* gene transcription in male gonads observed in the present study, under EE_2 exposure, closely relates ($r = 0.5$; $p = 0.045$) with embryonic development data from Soares et al. (2009). Although other xenobiotics have been reported to modulate *p53* gene transcription (Shi et al., 2008), the findings of the present study suggests that the screening of *p53* gene transcription in male gonads from wild fish population may reveal useful since it could be related with adverse effect endpoints. Future studies should address this hypothesis.

Overall, our study shows that full life-cycle exposure to EE_2 did not affect the NER repair system in gonads. In contrast, the significant induction of *p53* transcripts in male gonads at all EE_2 exposure levels supports the hypothesis of an impact of EE_2 in male gonad DNA repair pathways. The precise mechanism

linking *p53* gene transcription induction in male fish gonad, aneuploidy formation and embryo mortality should be the focus of additional studies.

5.6 Acknowledgements

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Chapter 6

Zebrafish (*Danio rerio*) life-cycle exposure to mixtures of estrogenic and androgenic chemicals disrupts embryonic development and modulates the expression of genes involved in NER and p53 DNA repair pathways

Soares, J., Neuparth, T., Lyssimachou, A., Lima, D., André, A.I.D., Reis-Henriques, M.A., Castro, L.F.C., Carvalho, A.P., Monteiro, N.M., Santos, M.M. (in preparation)

6. Zebrafish (*Danio rerio*) life-cycle exposure to mixtures of estrogenic and androgenic chemicals disrupts embryonic development and modulates the expression of genes involved in NER and p53 DNA repair pathways

6.1 Abstract

Although chemicals are present in the environment in complex mixtures, toxicological risk assessment relies mostly on data from single exposures. Therefore, the present study aimed at examining the effects of two ubiquitous endocrine disrupting chemicals (EDCs), ethinylestradiol (EE_2) and tributyltin (TBT), single and combined, in zebrafish (*Danio rerio*) reproductive-related parameters and embryonic development, and to correlate these effects with their molecular mode of action, focusing particularly in DNA repair pathways.

Chronic life-cycle exposure to environmental relevant concentrations of both EDCs led to significant effects in reproduction and embryonic development. TBT exposure at 50 ng/L impacted the percentage of fertilized eggs. EE_2 exposure at 0.75 and 1.75 ng/L led to a bias of the sex towards females and at 1.75 ng/L impacted embryonic development. Mixtures of EE_2 (1.75 ng/L) and TBT (50 ng/L) induced an impairment of embryonic development which was more severe than single chemical exposures. Mixture treatments (0.75 and 1.75 ng/L EE_2 plus 50 ng/L TBT) also biased sex ratio towards females. The study of DNA repair pathways indicated that EE_2 and TBT, single and combined, significantly modulated the expression levels of key genes in male gonads, e.g., *xpc* and *xpd* NER genes as well as the tumor suppressor *p53*, its target effectors, i.e. *p21*, *gadd45α* and *bax*, and key regulator (*mdm2*). The observed impact in the transcription of these genes supports the hypothesis of an effect of EE_2, TBT and their combined mixtures on DNA repair pathways, thus suggesting that impairment of embryonic development of F_2 generation could be linked to disruption of DNA repair processes in gonads from the parental generation. This study evidences the importance of examining non-reproductive effects and highlights the need of addressing the effects of mixtures of EDCs. Hence, these findings have important implications for environmental risk assessment, strongly supporting the inclusion

of embryonic development and gene expression studies in the screening of endocrine disruption in wildlife.

6.2 Introduction

In aquatic ecosystems, the presence of endocrine disrupting chemicals (EDCs) is a matter of great concern. EDCs are able to interfere with hormone controlled-processes, altering normal endocrine functions, which affect both development and reproduction (Guillette, 1995; Bigsby et al., 1999). Estrogenic chemicals (ECs) are among the most studied EDCs (Sumpter, 2005), and their effects include increased plasma vitellogenin in male and female fish, increased number of intersex individuals, decreased fertility and fecundity and even complete feminization of male fish (Panter et al., 1998; Rodgers-Gray et al,. 2001; Sohoni et al., 2001; Jobling et al., 2002; Van den Belt et al., 2002; Solé et al., 2003; Nash et al., 2004). One of the most ubiquitous and biologically active xenoestrogen in the aquatic environment is 17α-ethinylestradiol (EE_2) (Folmar et al., 2000; Metcalfe et al., 2001; Kolpin et al., 2002), the active ingredient of contraceptive pills.

Despite the fact that several types of EDCs are continuously arriving and concentrating in the aquatic ecosystems, rendering the evaluation of their effects increasingly difficult, one of the clearest examples of endocrine disruption in wildlife is still the masculinization of female neogastropods (imposex) by the antifouling compound tributyltin (TBT) (Matthiessen and Gibbs, 1998; Barroso et al., 2000; Santos et al., 2005). TBT has also been reported to affect fish sex differentiation, biasing the sex ratio toward males, even at extremely low levels (Shimasaki et al., 2003; McAllister and Kime, 2003). Although the use of TBT has been completely banned by the International Maritime Organization (Anon, 2001; Santos et al., 2009) due to its persistence in the environment, the levels of TBT are still a cause of concern (Rüdel et al., 2007).

In contrast to the wealth of information dealing with the negative impacts of EDCs in reproduction, studies specifically focusing on non-reproductive processes remain scarce. However, it has been demonstrated that TBT affects, for instance, embryo development in several aquatic organisms such as echinoderms, molluscs, fish and amphibians (Roepke et al., 2005; Inoue et al., 2006; Hano et al., 2007; Guo et al., 2010). EE_2 has also been shown to possess embryotoxic

properties (Länge et al., 2001; Nash et al., 2004; Brown et al., 2007; Soares et al., 2009). In a recent study, we reported an increase in zebrafish (*Danio rerio*) abnormal development and mortality after chronic parental EE_2 exposure in the range of 0.5-2 ng/L, nominal concentrations (Soares et al., 2009). Since these effects occurred at concentrations below those able to induce reproductive impairment in adults, we hypothesized that alterations on parental DNA are likely to be an important causative mechanism for the observed embryo mortalities. This is not a farfetched scenario as, estrogens, including EE_2, are known to promote mutagen-induced hepatic neoplasia in medaka (*Oryzias latipes*) and rainbow trout (*Oncorhynchus mykiss*) (Cooke and Hinton, 1999). Similar evidences derived from studies with molluscs (Jha et al, 2000; Hagger et al., 2002), fish (Tiano et al., 2001; Ferraro et al., 2004) and mammals (Liu et al., 2006) show that TBT is also genotoxic. Additionally, chronic zebrafish exposure to EE_2, TBT and their mixtures induces an increase in the formation of erythrocytic nuclear abnormalities that are associated with clastogenic and/or aneugenic DNA damage (Micael et al., 2007). Increased DNA mutations can lead to genomic instability and eventually promote a decreased fitness within populations (Wirgin and Waldman, 1998).

One biological process that circumvents DNA lesions is DNA repair. NER is the primary DNA repair pathway responsible for removing a variety of DNA lesions caused by bulky adduct forming mutagens (de Laat et al., 1999, Sancar et al., 2004), through two subpathways: global genome repair (GGR) and transcription coupled repair (TCR) (Thoma and Vasquez, 2003). These occur via a multiple step process involving the assembly of numerous proteins at the site of DNA damage. Seven XP, *xeroderma pigmentosum* groups, have been identified representing different repair factors, XPA-G (de Laat et al., 1999). XPC is responsible for initial damage recognition and XPA for damage verification (Sugasawa et al., 1998). Then, both work in conjunction to initiate the repair complex assembly (de Laat et al., 1999). TFIIH complex contains the helicases XPB and XPD, responsible for DNA unwind (de Laat et al., 1999). Two nucleases, XPF and XPG, then cleave the open complex (Sancar et al., 2004). After the damaged DNA segment is removed, DNA synthesis and ligation complete the process.

Other proteins are known to play a central role in DNA damage protection mechanisms. Of particular importance is the tumor suppressor p53, a

multifunctional protein that has an essential role in the cellular response to a variety of potential insults. p53 is also known to interact with several DNA repair pathways in order to maintain homeostasis. Several types of stress, including genetic damage, activate p53 pathways. Upstream mediators detect and interpret these signals leading to an increase in p53 protein levels, first through inhibition of its negative regulators. Then, p53 is activated by several post-translational mechanisms that regulate the specificity of its activity. Induced p53 functions via protein-protein interactions and, mainly, as a transcriptional factor, activating the expression of several downstream genes, such as *p21* (cyclin-dependent kinase inhibitor 1A), *gadd45α* (growth arrest and DNA damage induced 45, alpha) and *bax* (Bcl2-associated X protein), all involved in cell cycle regulation pathways, namely cell cycle arrest, DNA repair and apoptosis (Adimoolam and Ford, 2003). In normal cells p53 protein is tightly controlled at negligible levels. One of the central components in the regulation of p53 is Mdm2, the murine double minute 2 protein. Mdm2 interacts with p53 in the nucleus where it blocks p53 activation-transcription and shuttles it into the cytoplasm to be degraded by the cytoplasmic proteasomes (Freedman et al., 1999). Additionally, Mdm2 is a transcriptional target of p53 and so establishes an autoregulatory feedback loop in which p53 drives expression of its own negative regulator, keeping these proteins in balance (Wu et al., 1993).

Recently, the effects of EDCs on DNA repair processes have been suggested as possible mechanisms by which these compounds can modulate the incidence of DNA mutations. Zuo et al. (2012) reported that exposure to TBT induced DNA damage in cuvier (*Sebasticus marmoratus*) liver by altering the transcription levels of the NER genes. Also, EE_2 can alter mRNA abundance of NER genes in liver of adult zebrafish as well as the rate and magnitude of NER processes in zebrafish liver cells (Notch et al., 2007; Notch and Mayer, 2009a,b). In a previous study we reported that exposure to low levels of EE_2 modulated *p53* gene transcription within adult zebrafish gonads (Soares et al., 2012). From the above mentioned studies it became evident that zebrafish males seem to be more affected by low EE_2 levels than their female counterparts.

Despite the ubiquitous coexistence of both estrogenic and androgenic contaminants on aquatic ecosystems, the combined effects of EDCs mixtures with a different mode of action seem to be a rather unexplored field, with only a few

studies addressing this issue. For example, Santos et al. (2006) reported that EE_2, at environmental relevant concentrations, could block the masculinizing effects of TBT on fish and, more recently, Sárria et al. (2011) demonstrated that combined mixtures of EE_2 and TBT have an impact on pipefish (*Syngnathus abaster*) larvae behaviour in the presence of a predator. Clearly, studying the effects of single chemicals may not allow to understand what is actually occurring in the environment, considering that animals are recurrently exposed to mixtures of xenoestrogens and xenoandrogens.

Since previous research has shown that EE_2 and TBT exposure affects embryonic development and survival as well as the regulation of key genes involved in DNA repair mechanisms, the main aim of this study was to investigate the impact of co-exposures to these two EDCs in ecologically relevant endpoints and link them with the chemicals mode of action. To this extent, we have performed a zebrafish parental full life-cycle exposure to environmentally relevant concentrations of EE_2, TBT and their binary mixtures. The fertility and fecundity of the parental generation and the survival and occurrence of abnormalities in their offspring, per stage of embryonic development, was evaluated. Since we, as well as others, have previously reported that zebrafish males are more affected than females to low EE_2 and TBT levels, the transcription of key DNA repair genes (i.e., NER, *p53* and selected downstream effectors genes and its negative regulator *mdm2*) were analyzed in male gonads in order to address the mode of action of these compounds.

6.3 Material and Methods

6.3.1 Parental animals (F_0)

Adult wild-type zebrafish were used as breeding stock. The stock was kept at a water temperature of 28 ± 1 ºC and under a photoperiod of 14:10 h (light:dark), in 250 L aquaria with dechlorinated and aerated water in a recirculation system with both mechanical and biological filters. The fish were fed *ad libitum* twice a day with a commercial fish diet Tetramin (Tetra, Melle, Germany) and live brine shrimp (*Artemia spp.*).

6.3.2 Egg production of F_0 generation

In the afternoon before breeding, two groups of 4-6 males and 10-12 females were independently housed in cages with a net bottom covered with glass marbles within a 30 L aquarium under the same water and photoperiod conditions as the stock and fed with live brine shrimp. At the following day, breeding fish were removed 1.5 h after the beginning of the light period and the eggs were collected and cleaned. Fertilized eggs were randomly allocated to experimental aquaria.

6.3.3 Exposure of F_1 generation

Exposures were performed using a flow through system. Before entering the system, the water was heated and charcoal filtered. The water flow (33 L/day) was maintained by a peristaltic pump (ISM 144, ISMATEC). Working solutions were administered by direct injection into the water, in the morning and in the afternoon, in a volume that was equivalent to the water renewal during that time, in order to maintain exposure concentrations. Eight exposure conditions, in duplicate, were set up: an experimental control, a solvent control (acetone), two EE_2 concentrations (nominal concentrations: 0.75 and 1.75 ng/L), two TBT concentrations (nominal concentrations: 10 and 50 ng/L) and their binary mixtures (nominal concentrations: 0.75 ng/L EE_2 + 50 ng/L TBT and 1.75 ng/L EE_2 + 50 ng/L TBT). The selection of mixtures concentrations were based in previous studies, considering environmental relevant concentrations (McAllister and Kime, 2003; Soares et al., 2009).

17α-Ethinylestradiol (EE_2 98%, Sigma; stock solution: 1 mg/ml) and tributyltin (TBTCl 96%, Aldrich; stock solution: 1 mg/ml TBT as Sn) were diluted in acetone (Merck). From these solutions, aliquots of the working solutions were prepared and kept at -20 ºC until use. All solutions were prepared in order to have a final acetone volume of 0.0002%.

Throughout the experimental period, zebrafish were under a 14:10 h (light:dark) photoperiod and water physical-chemical parameters were measured weekly with exception of temperature that was checked on a daily basis (28 ± 1 ºC of temperature; pH 7.7 ± 0.2; 6 ± 1 mg/L of dissolved oxygen; 376 µS/cm of conductivity; 0.08 ± 0.06 mg/L of ammonium and 0.01 ± 0.01 mg/L of nitrite).

The study lasted for 9.5 months, with continuous exposure of the F_1 generation. Before the beginning of the exposure, the aquaria were equilibrated during 15 days. The experiment was initiated by randomly allocating 300 eggs ≈2 hours post-fertilization (hpf) in 5 L aquaria that had been placed within each 30 L aquarium. At 15 days post-fertilization (dpf), zebrafish were allocated to 30 L aquaria. Between 1-14 dpf exposure time, in 5 L aquaria, a semi-static system was implemented. Water was renewed during 7 h, and contaminants were directly administered in water afterwards.

Throughout the experiment, the number of zebrafish in each aquarium, as well as the amount of food distributed was adjusted according to fish development, number and size. Feeds consisted mainly of live brine shrimp (*Artemia spp.*), supplemented with commercial fish diet Tetramin (Tetra, Melle, Germany). Mortality was assessed by daily recordings, during the entire exposure period, and dead eggs/individuals removed. At the end of the experiment, the remaining zebrafish were anesthetized with 300 mg/L of buffered tricaine methanesulfonate (MS-222) and their weight and length recorded for body condition factor calculation ($K = \text{weight} \times \text{lenght}^{-3} \times 100$). Gonads were excised for additional determinations. Gonadosomatic index (GSI = gonad weight / total weight x100) was also calculated.

6.3.4 TBT analysis

Butyltin compounds analysis was performed by headspace solid-phase micro-extraction (SPME) in a fiber of poly(dimethylsiloxane) (PDMS) with 100 µm and posterior desorption in a Varian CP 3900 gas chromatograph with detection by Varian Saturn 2000 Tandem mass spectrometer (GC-MS/MS) (Carvalho et al., 2007). The described analytical method enables the simultaneous analysis of TBT and its breakdown products, dibutyltin (DBT) and monobutyltin (MBT), which are also reported in this work.

Concentrations of TBT were found to be below the detection limit in the reference aquaria. In the exposure tanks, after dosing, TBT concentrations were close to nominal concentrations and then decayed over time, as expected. For that reason, the aquaria were dosed twice a day. The concentrations of TBT in the water, from the 50 ng/L TBT (as Sn) exposure tanks, at 0 h (15 minutes after the

first dosing), 8 h (before the second dosing) and 24 h (before the first next-day dosing) were 37.2, 7.1 and 2.1 ng/L, respectively. Dibutyltin (DBT) was not detected and monobutyltin (MBT) was detected at concentrations of 5.2 and 25.1 ng/L at 8 and 24 h, respectively.

6.3.5 Reproductive capability of F_1 generation

Reproductive capability studies were performed after 6.5 mpf (months post-fertilization) in all experimental groups. Reproductive success was evaluated as fecundity (number of eggs per female per day) and percentage of fertilization (% of viable eggs per female per day, after 1-1.5 hpf). For reproductive studies, the 30 L aquaria were divided in two compartments and female and male zebrafish were randomly distributed in cages, with a net bottom covered with glass marbles, in a manner that matched the sex ratio of each treatment (4 replicates per treatment). Subsequently, during five consecutive days, 1-1.5 h after the beginning of the light period, eggs were collected and preserved in 70% ethanol, at room temperature in dark conditions, for posterior counting and determination of percentage of fertilized eggs.

6.3.6 Embryogenesis studies of F_2 generation

When reproductive trials reached the fifth day, 120 viable eggs per replicate were kept for the embryogenesis studies. Eggs that had passed the 4 cell stage (1-1.5 hpf), were randomly distributed to 100 ml beakers (40 eggs per beaker) with daily replacement of clean water obtained from the flow through system. The beakers were randomly maintained on a water bath at 26.5 ± 0.5 ºC and kept under the same photoperiod conditions as the adults. For each treatment, four beakers per embryonic stage were set, which means that we had four replicates per treatment with a total of 480 eggs.

For embryogenesis studies embryo tests and endpoints were performed with slight modifications of the protocol proposed by Oberemm (2000). The following endpoints were evaluated during zebrafish egg/embryo development stages, briefly: at 8 hpf (65-75%-epiboly) progression of ectodermal front and homogeneity of front and tissue; at 32 hpf (prim 15-16) malformations of the blood circulatory

system, oedema and melanophores distribution; and at 80 hpf (protruding mouth) expression of pigmentation, oedema and fin fold lesions; the mortality rate was assessed at all stages. Different embryos, even within a single clutch, develop at slightly different rates (Kimmel et al., 1995). Therefore, our time-point observations include eggs/embryos in slight different phases, which have been indicated above. However, for simplicity, in the figures we have adopted the classification of Oberemm (2000), with a single stage per time-point observation. Inspection started by checking mortality and discharging dead eggs/embryos to avoid water quality decay. Then, 15 eggs/embryos, from each beaker (60 per treatment/developmental stage), were randomly collected and placed on a Petri dish for observation with a stereo-microscope (SMZ1000, Nikon, magnification up to 80 times) to establish: the developing phase and development anomalies. Any modification from normal development and/or morphological anomalies were recorded by digital photography (Nikon Coolpix 5400) and scored as presence/absence. After each time-point observation, the beakers were eliminated, thus assuring the independence of data among development stages.

6.3.7 RNA extraction and cDNA synthesis

Total RNA was extracted from the gonads of adult males at the end of the exposure period (9.5 months), using the “illustra RNAspin Mini Kit” (GE Healthcare) following the manufacturer´s protocol. On column DNase digestion was used during the extraction procedure. RNA quality was verified in a 1% agarose gels. Total RNA concentration was estimated using the “Quant-it^{TM} RiboGreen® RNA Assay Kit” (Invitrogen). cDNA was synthesized from 800 ng total RNA using “iscript cDNA synthesis kit” (Bio-Rad) following the manufacturer´s protocol.

6.3.8 Primer design

Sequences for zebrafish NER genes (*xpc*, *xpa* and *xpd*), *p53*, *p21*, *gadd45α*, *bax* and *mdm2* genes were obtained from GenBank and Ensembl (for further detail see Soares et al., 2012). Specific primers were designed with the Beacons Design$^{TM.}$ software (Premium Biosoft International) in a region outflanking an intron

(18-25 nucleotide length and GC content 40-60%). NCBIs basic local alignment search tool (BLAST) was used to verify primer specificity. Primers were synthesised by STABVIDA (Portugal).

6.3.9 Real-time PCR

Fluorescence-base quantitative PCR was performed with the iQ5 optical system (Bio-Rad). Each sample was amplified in duplicate using 96- well optical plates in a 20 µL reaction volume using 4 µL of cDNA, 4 µL of 5x Hot FirePol EvaGreen qPCR Mix Plus (Solis BioDyne), 200 nM of the appropriate forward and reverse primers. In each plate, a “no template control” was included.

PCR profile had the following conditions: 95 ºC of initial denaturation for 3 min; thereafter, 38 cycles of amplification were carried out with a denaturation at 95 ºC for 10 s, annealing at 60 ºC for 30 s and extension at 72 ºC for 30 s (data collection). A melting curve was generated in every run to confirm the specificity of the assays. The PCR products were also analysed by agarose gel electrophoresis to confirm the presence of single bands (data not shown). Finally, PCR products were sequenced to confirm the sequence identity.

In order to determine reaction efficiency, a standard curve that consisted of five 5-fold serial dilutions of cDNA, from a mix of all samples, was also performed in each run (reaction efficiency close to 100% for all reactions). Relative change in transcripts abundance was calculated utilizing the $2^{-\Delta Ct}$ analysis method (Livak and Schmittgen, 2001). Control expression levels (acetone) were normalized to a value of 1.

6.3.10 Comet assay analysis

The alkaline single-cell gel electrophoresis assay (comet assay) was used to determine the level of DNA damage (DNA strand breaks) in gonad cells according to Singh et al. (1988) and as previously described by Neuparth et al. (2013). Analysis was run immediately after gonad cells collection to ensure maximum cell viability.

Gonads were dissected and placed in 300 µl of cold homogenizing buffer (75 mM NaCl, 24 mM Na_2EDTA, pH 7.5). Gonads were homogenized gently and the

cells filtered through a 60 µm mesh to produce a single cell suspension. 30 µl of gonad cells were then diluted in liquid (37 ºC) 1% w/v low-melting point agarose and placed (2 x 75 ml) on microscopy slides previously coated with dry high melting point agarose. All steps onward were performed under dim light and at 4 ºC to avoid addition of DNA strand breakage and agarose lifting from the slides. After the gel had set, the slides were placed into a freshly prepared lysing solution for 1 h (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, pH 10, 1% Triton X-100, 10% DMSO). Slides were then placed in cold alkaline electrophoresis solution (0.1 mM Na_2-EDTA, 0.3 M NaOH), for 40 min. Electrophoresis was then run for 30 min at 25 V using a horizontal gel electrophoresis tank. Slides were afterwards neutralized in Tris-HCl buffer (pH 7.5), and then dehydrated with ice-cold absolute methanol to be preserved until analysis. Before the examination, the slides were rehydrated and then stained with 20 µL of 5 µg/mL ethidium bromide solution and viewed under ultraviolet fluorescence light. A total of 100 randomly chosen cells were scored per slide, the % of DNA in the comets tail were used as a direct measure of total (single and double strand) DNA strand-breakage (Lee and Steinert, 2003). Image analysis was performed with the software CometScore 1.5 (TriTek).

6.3.11 Statistics

Data were analysed using Statistica 8.0 software. ANOVAs were performed after testing for assumptions (homogeneity of variances and normality of data). Whenever necessary, data was log transformed. When statistically significant differences were found between treatment groups ($p < 0.05$), Fisher's Least Significant Difference (LSD) multiple comparison test was performed. For embryonic development data, ANOVA analysis did not show significant differences between experimental and solvent control exposed groups. For that reason, these treatments were grouped together in order to empower statistical analysis. Statistical differences in embryo mortality rate and abnormal eggs/embryos among treatments and stages were evaluated by a two-way factorial ANOVA. Significant differences in condition and reproductive parameters, and percentage of DNA damage were evaluated by a one-way ANOVA. Some condition parameters did not conform to ANOVA assumptions and data were analyzed by non-parametric

Kruskal-Wallis Anova and median test, followed by multiple comparisons of mean ranks for all groups ($p < 0.05$). A one-way ANOVA was also performed on ΔCt values for a given gene. Solvent control was used as the reference group (= 1-fold).

Mortality and sex ratio differences were tested by the χ^2 test, using the solvent control group results as expected values.

6.4 Results

6.4.1 Mortality of F_1 generation

The mortality rates, from embryo up to 20 dpf (a critical period for zebrafish survival), varied between 29 and 41% and no differences were observed between control and exposed zebrafish groups (data not shown). These mortality rates are within the normal expected values for zebrafish (Hill and Janz, 2003; Santos et al., 2006; Soares et al., 2009).

6.4.2 Weight, length, K and GSI of F_1 generation

Table 6.1 displays total length, total weight, K and GSI on female and male zebrafish at the end of 9.5 months of exposure. When analyzing exposure effects of EE_2 and TBT on female total length, single and in combination, a significant decrease was observed at 0.75 ng/L EE_2 + 50 ng/L TBT and 1.75 ng/L EE_2 + 50 ng/L TBT treatments ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test) in comparison to solvent control. Also, females from the 50 ng/L TBT group are significantly smaller from the ones of the two mixtures ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test). Female total weight was not affected by contaminant exposure ($p > 0.05$; one-way ANOVA). However, a 10% decrease in total weight was observed at 0.75 ng/L EE_2, 10 ng/L TBT and 0.75 ng/L EE_2 + 50 ng/L TBT treatments when compared with solvent control ($p = 0.12$). Female K from 1.75 ng/L EE_2 and 1.75 ng/L EE_2 + 50 ng/L TBT was significantly increased if compared with 0.75 ng/L EE_2, 10 ng/L TBT and 50 ng/L TBT ($p < 0.05$, Kruskal-Wallis Anova and median test). Nevertheless, exposure treatments did not differ from solvent control ($p >0.05$). Female GSI was

significantly decreased at 0.75 and 1.75 ng/L EE_2, 10 and 50 ng/L TBT and 0.75 ng/L EE_2 + 50 ng/L TBT treatments when compared with solvent control ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test).

In males, a decrease in total length was observed at 1.75 ng/L EE_2 and 0.75 ng/L EE_2 + 50 ng/L TBT treatments if compared with 50 ng/L TBT group ($p < 0.05$, Kruskal-Wallis Anova and median test, followed by multiple comparisons of mean ranks for all groups). However, no differences were observed between treatments and solvent control ($p > 0.05$). Male total weight was significantly increased ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test) in experimental control, 10 ng/L TBT and 1.75 ng/L EE_2 + 50 ng/L TBT when compared with solvent control. In addition, 10 ng/L TBT and 1.75 ng/L EE_2 + 50 ng/L TBT were significantly different (increased) in comparison with the 1.75 ng/L EE_2 group. Male K was significantly increased in experimental control and 10 ng/L TBT when compared with solvent control ($p < 0.05$, Kruskal-Wallis Anova and median test, followed by multiple comparisons of mean ranks for all groups). Also, there was a 10% K increase at 1.75 ng/L EE_2, that did not reach significance, partially, because of a relatively high standard error (S.E.). Differences between experimental control and control solvent might indicate that acetone has an effect on male K and male weight. Male GSI was not affected by contaminant exposure ($p > 0.05$, Kruskal-Wallis Anova and median test). Nevertheless, a 20% decrease in GSI was observed at 0.75 ng/L EE_2 and 0.75 ng/L EE_2 + 50 ng/L TBT, a 30% decrease at 1.75 ng/L EE_2 and 50 ng/L TBT and a 40% decrease at 10 ng/L TBT ($p = 0.25$).

6.4.3 Sex ratio of F_1 generation

At 6.5 mpf, no differences in sex ratio were observed between zebrafish TBT exposed groups and solvent control ($p > 0.05$, χ^2 test) (Table 6.2). The percentage of males in both controls was approximately 70% which is slightly higher than that recorded in zebrafish reared under laboratory conditions (usually between 50-65%) (Hill and Janz, 2003). On the other hand, EE_2 and both mixtures exposed groups showed a significant increase in the percentage of females, in a dose dependent manner (46% for 0.75 ng/L EE_2, 61% for 1.75 ng/L EE_2, 42% for 0.75

ng/L EE_2 + 50 ng/L TBT and 55% for 1.75 ng/L EE_2 + 50 ng/L TBT), in comparison to solvent control (Table 6.2) ($p < 0.05$, χ^2 test).

6.4.4 Reproductive capacity of F_1 generation

At 6.5 mpf, fecundity in females from exposed groups did not differ significantly from the solvent control (Table 6.2) ($p > 0.05$; one-way ANOVA). However, a 2.5-fold decrease in fecundity was observed in females exposed to 1.75 ng/L EE_2 + 50 ng/L TBT when compared with solvent control females ($p = 0.2$). Exposure to 50 ng/L TBT significantly decreased the % of fertilized eggs in comparison to solvent control (Table 6.2) ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test). % of fertilization at 0.75 ng/L EE_2 was higher than that of females from the 10 and 50 ng/L TBT and 1.75 ng/L EE_2 + 50 ng/L TBT treatment groups.

Table 6.1 Condition parameters (total length, total weight, body condition factor (K %) and gonadosomatic index (GSI %) for EE_2, TBT and their combined mixtures groups (F_1 generation) at 9.5 mpf

Group			EE_2		TBT		Mixtures	
	Control	Acetone	0.75 ng/L	1.75 ng/L	10 ng/L	50 ng/L	EE_2 L + TBT H	EE_2 H + TBT H
Condition parameters (9.5 mpf)								
Female								
Lenght (cm)	4.43 ± 0.07	4.49 ± 0.04	4.45 ± 0.04	4.40 ± 0.04	4.43 ± 0.05	4.49 ± 0.07	4.29 ± 0.04 *	4.34 ± 0.03 *
Weight (g)	1.13 ± 0.06	1.14 ± 0.04	1.03 ± 0.04	1.11 ± 0.03	1.01 ± 0.04	1.05 ± 0.06	1.01 ± 0.04	1.09 ± 0.03
K (%)	1.29 ± 0.04	1.26 ± 0.04	1.15 ± 0.03	1.30 ± 0.04	1.15 ± 0.03	1.15 ± 0.03	1.27 ± 0.03	1.33 ± 0.04
GSI (%)	23.85 ± 1.64	26.88 ± 1.23	18.76 ± 1.05 *	22.79 ± 1.40 *	18.51 ± 1.29 *	19.54 ± 1.60 *	22.69 ± 1.09 *	24.67 ± 1.48
Male								
Lenght (cm)	4.24 ± 0.04	4.26 ± 0.03	4.21 ± 0.04	4.02 ± 0.09	4.23 ± 0.04	4.29 ± 0.03	4.11 ± 0.03	4.24 ± 0.04
Weight (g)	0.73 ± 0.02 *	0.69 ± 0.01	0.69 ± 0.01	0.64 ± 0.03	0.73 ± 0.02 *	0.71 ± 0.02	0.65 ± 0.02	0.73 ± 0.02 *
K (%)	0.96 ± 0.02 *	0.88 ± 0.02	0.93 ± 0.02	1.01 ± 0.08	0.96 ± 0.01 *	0.90 ± 0.02	0.93 ± 0.01	0.96 ± 0.02
GSI (%)	2.31 ± 0.37	2.15 ± 0.45	1.78 ± 0.19	1.61 ± 0.21	1.27 ± 0.11	1.53 ± 0.14	1.69 ± 0.24	2.43 ± 0.85

Table 6.2 Reproductive parameters for EE_2, TBT and their combined mixtures groups (F_1 generation) at 6.5 mpf.

Group			EE_2		TBT		Mixtures	
	Control	Acetone	0.75 ng/L	1.75 ng/L	10 ng/L	50 ng/L	EE_2 L + TBT H	EE_2 H + TBT H
Reproductive parameters (6.5 mpf)								
Female								
% Females	28	28	46 *	61 *	23	26	42 *	55 *
Fecundity	49 ± 9.6	44 ± 11.6	39 ± 4.6	50 ± 19.9	48 ± 15.7	53 ± 13.4	42 ± 6.6	17 ± 3.1
% Fertilization	80 ± 6.3	87 ± 2.6	94 ± 0.8	91 ± 2.4	80 ± 4.9	63 ± 1.8 *	93 ± 3.3	85 ± 5.5
Male								
% Males	72	72	54 *	39 *	77	74	58 *	45 *

(L, lowest concentration; H, highest concentration). *Significantly different from solvent control (acetone). Values are presented as mean ± standard error; $p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test.

6.4.5 Embryogenesis

6.4.5.1 Mortality

Cumulative mortality rates of embryos at the different phases of embryonic development are displayed in Figure 6.1. Between 8 and 80 hpf, mortality rates ranged from 8% (control groups) to 64% (1.75 ng/L EE_2 + 50 ng/L TBT, parental exposure). At 8 hpf mortality rates of exposed groups did not differ significantly from controls. A significant increase ($p < 0.05$; two-way ANOVA, followed by Fisher LSD multiple comparison test) in the mortality rate of embryos from 1.75 ng/L EE_2 and 1.75 ng/L EE_2 + 50 ng/L TBT parental exposed groups occurred between 8 hpf and 32 hpf. Even though it did not reach significance, the 0.75 ng/L EE_2 group showed a lower mortality rate in comparison with the other treatments. At 32 hpf, control treatments displayed approximately 27% embryo mortality (out of the initial fertilized eggs), whereas 0.75 ng/L EE_2, 1.75 ng/L EE_2 and 1.75 ng/L EE_2 + 50 ng/L TBT treatment groups showed, respectively, 16%, 45% and 59% embryo mortality. After 32 hpf, mortality rates increased slightly up to the end of the embryonic development and maintained the same pattern observed at 32 hpf. In addition, a significant decrease in mortality at 0.75 ng/L EE_2 was observed, confirming the trend towards a decrease observed at 32 hpf time-point observation. At 80 hpf, controls exhibited approximately 30% embryo mortality whereas 0.75 ng/L EE_2, 1.75 ng/L EE_2 and 1.75 ng/L EE_2 + 50 ng/L TBT treatment groups showed, respectively, 16%, 47% and 64% egg mortality. Although it did not reach significance, a 40% increase in embryo mortality was observed at 50 ng/L TBT exposure.

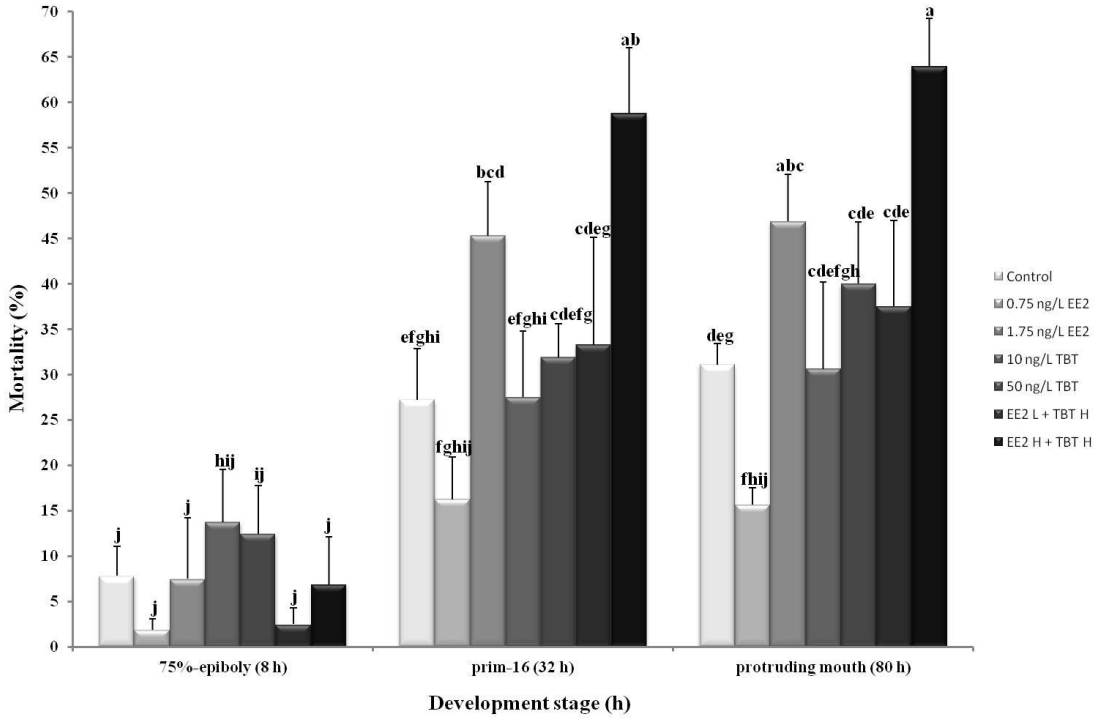

Figure 6.1 Percentage of cumulative mortality, at several developmental stages: 75%-epiboly (8 hpf); prim-16 (32 hpf) and protruding mouth (80 hpf) of embryos (F_2), descendents of zebrafish exposed to EE_2, TBT and their combined mixtures for 6.5 mpf (F_1) (L, lowest concentration; H, highest concentration). Values are presented as mean ± standard error. Different letters indicate significant differences among treatments ($p < 0.05$, two-way ANOVA, followed by Fisher LSD multiple comparison test).

6.4.5.2 Abnormal development

During the different embryogenesis stages (8, 32, and 80 hpf) the presence of eggs/embryos showing anomalies were recorded within each group. No significant differences were observed in zebrafish exposed groups in comparison to controls ($p > 0.05$; two-way ANOVA) throughout the experiment (data not shown). Nevertheless, at 8 hpf there was an increase in the percentage of abnormal eggs for the two mixtures (11.25% for 0.75 ng/L EE_2 + 50 ng/L TBT and 16.25% for 1.75 ng/L EE_2 + 50 ng/L TBT) in comparison to controls (5.6%). Most of these abnormal eggs had not yet entered gastrulation at the 8 hpf and showed an abnormal development, which contrasted with the other treatments where most embryos had reached 65-75%-epiboly. After 8 hpf, the rate of abnormal embryos was similar among treatments and kept at low levels (below 7%).

6.4.6 DNA repair genes expression in male gonads

6.4.6.1 Transcription levels of nucleotide excision repair genes (NER)

Male fish exposed to 0.75 ng/L EE_2 exhibited a 2.5-fold increase in *xpc* transcripts levels ($p < 0.01$; one-way ANOVA, followed by Fisher LSD multiple comparison test) in comparison with solvent control. Although it did not reach significance, a mean increase was evident for 10 ng/L TBT and 1.75 ng/L EE_2 + 50 ng/L TBT treatments. In contrast, at 1.75 ng/L EE_2 and 50 ng/L TBT a significant decrease in *xpc* gene transcription was observed ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test) (Figure 6.2). No differences were observed in *xpa* transcripts among treatments ($p > 0.05$; one-way ANOVA). The *xpd* transcription levels increased in the two mixtures in a dose-dependent manner (2.87 and 3.30-fold increases) ($p < 0.01$; one-way ANOVA, followed by Fisher LSD multiple comparison test) (Figure 6.2).

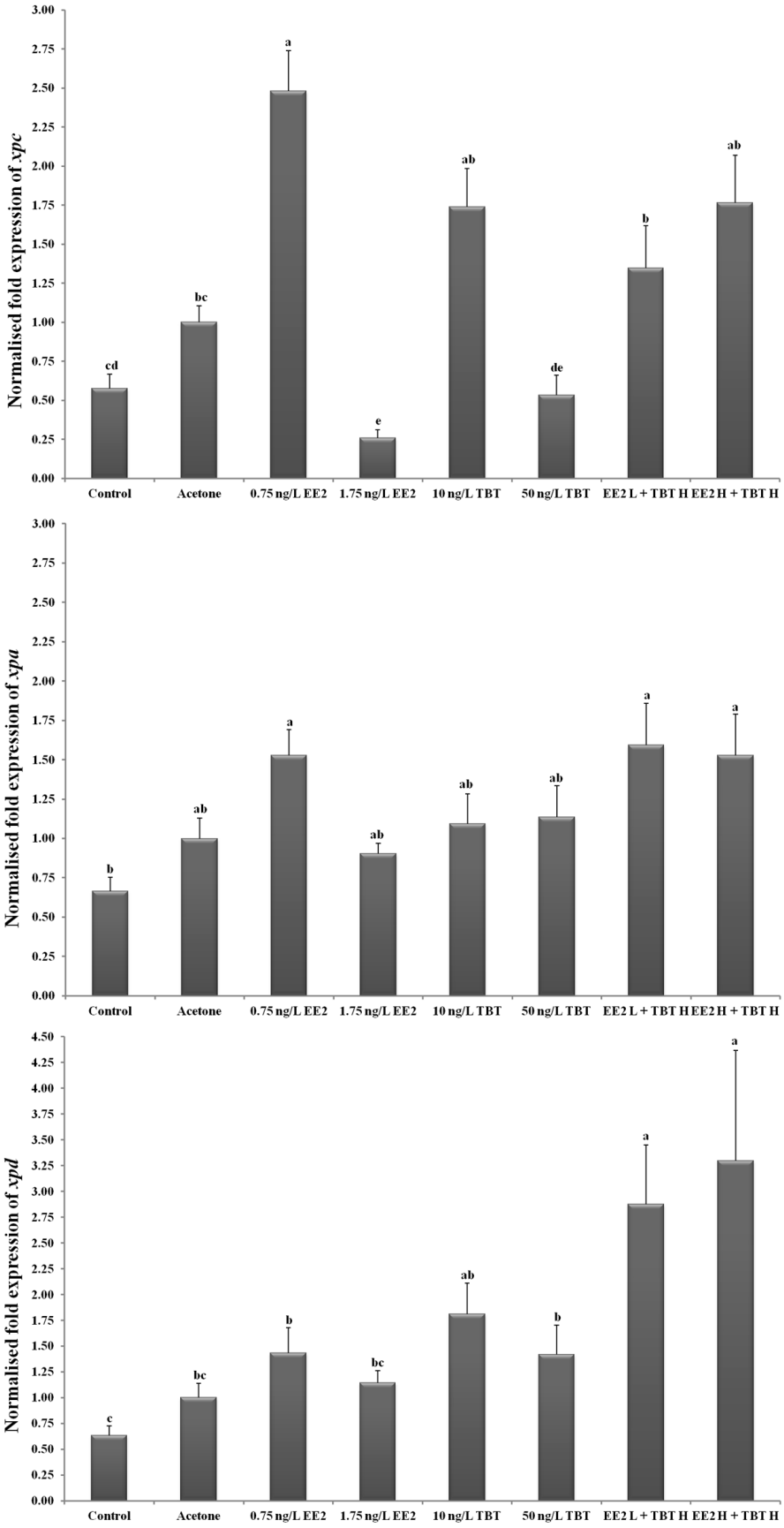

Figure 6.2 Normalised fold expression of *xpc*, *xpa* and *xpd* NER genes in 9.5 months zebrafish male gonads after exposure to EE_2, TBT and their combined mixtures (ng/L) (L, lowest

concentration; H, highest concentration). Solvent control (acetone) was used as the reference group (= 1-fold). Values are presented as mean ± standard error. Different letters indicate significant differences among treatments ($p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test).

6.4.6.2 Transcription levels of *p53* and *mdm2*

Figure 6.3 displays *p53* and *mdm2* gene transcription. 0.75 ng/L EE_2 exposure led to a significant 1.6-fold increase in *p53* gene transcription while the highest EE_2 dose (1.75 ng/L) caused an opposite effect with a significant decrease in comparison to solvent control animals ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test). No significant differences were observed for the other treatments ($p > 0.05$; one way ANOVA). *mdm2* followed a similar expression pattern to that of *p53* with a significant increase of *mdm2* gene transcription at 0.75 ng/L EE_2 (1.4-fold) and a significant 0.56-fold decrease at 1.75 ng/L EE_2 ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test). Additionally, a significant decrease in *mdm2* transcription levels was observed at 50 ng/L TBT (1.54-fold) and, to a lesser extent, at 0.75 ng/L EE_2 + 50 ng/L TBT.

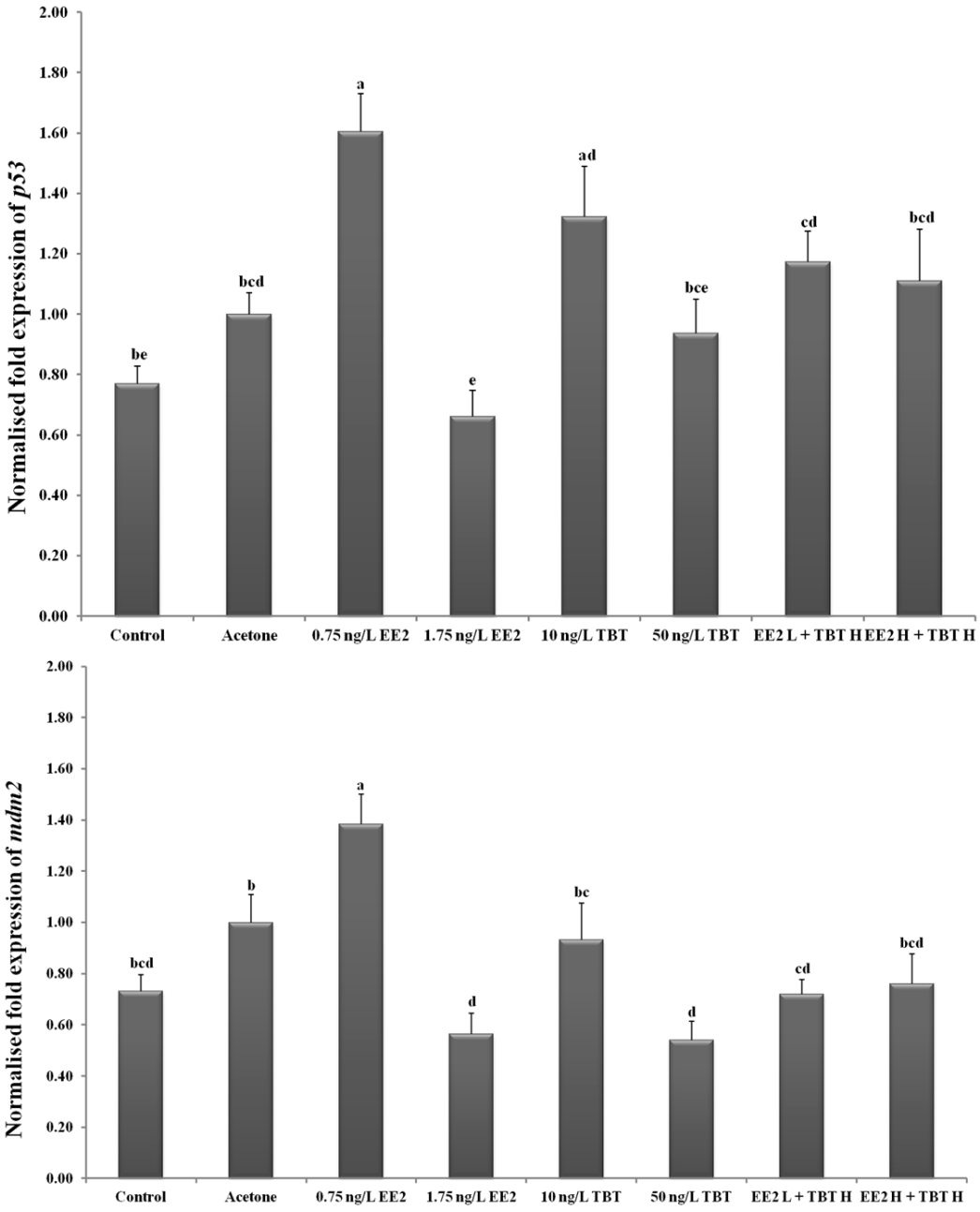

Figure 6.3 Normalised fold expression of *p53* and *mdm2* in 9.5 months zebrafish male gonads after exposure to EE_2, TBT and their combined mixtures (ng/L) (L, lowest concentration; H, highest concentration). Solvent control (acetone) was used as the reference group (= 1-fold). Values are presented as mean ± standard error. Different letters indicate significant differences among treatments ($p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test).

6.4.6.3 Transcription levels of p53 downstream pathways genes (*p21*, *gadd45α* and *bax*)

Figure 6.4 displays p53 downstream effectors genes transcription. *p21* gene transcription was significantly increased at 0.75 ng/L EE_2 (2.4-fold), 10 and 50 ng/L TBT (approximately 2.0-folds) and 1.75 ng/L EE_2 + 50 ng/L TBT (1.70-fold) in comparison with solvent control ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test). Although it did not reach significance a mean increase was evident at 0.75 ng/L EE_2 + 50 ng/L TBT treatment. *gadd45α* transcripts levels were significantly increased for all treatment exposures in comparison to solvent control ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test). Single exposures led to a more pronounced increase in *gadd45α* transcripts levels at the lowest doses (2.11-fold at 0.75 ng/L EE_2 and 2.83-fold at 10 ng/L TBT) in comparison with the highest concentrations (1.77-fold at 1.75 ng/L EE_2 and 2.37-fold at 50 ng/L TBT). In the performed EDC mixtures, *gadd45α* increased transcription levels were dose-dependent with a 5-fold increase at 0.75 ng/L EE_2 + 50 ng/L TBT and a 6-fold increase at 1.75 ng/L EE_2 + 50 ng/L TBT ($p < 0.01$). *bax* gene transcription was significantly increased at the lowest concentrations of the compounds and at the two mixtures in comparison with solvent control ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test). *bax* gene transcription in male gonads from the 0.75 ng/L EE_2 and 10 ng/L TBT treatments displayed a 2 and 3-fold increase, respectively. In the mixture exposures, *bax* transcripts levels increased in a dose-dependent manner with a 1.5 fold-increase at 0.75 ng/L EE_2 + 50 ng/L TBT and 2.36-fold increase at 1.75 ng/L EE_2 + 50 ng/L TBT. *bax* gene transcription was significantly decreased at experimental control and 1.75 ng/L EE_2 treatment groups ($p < 0.01$).

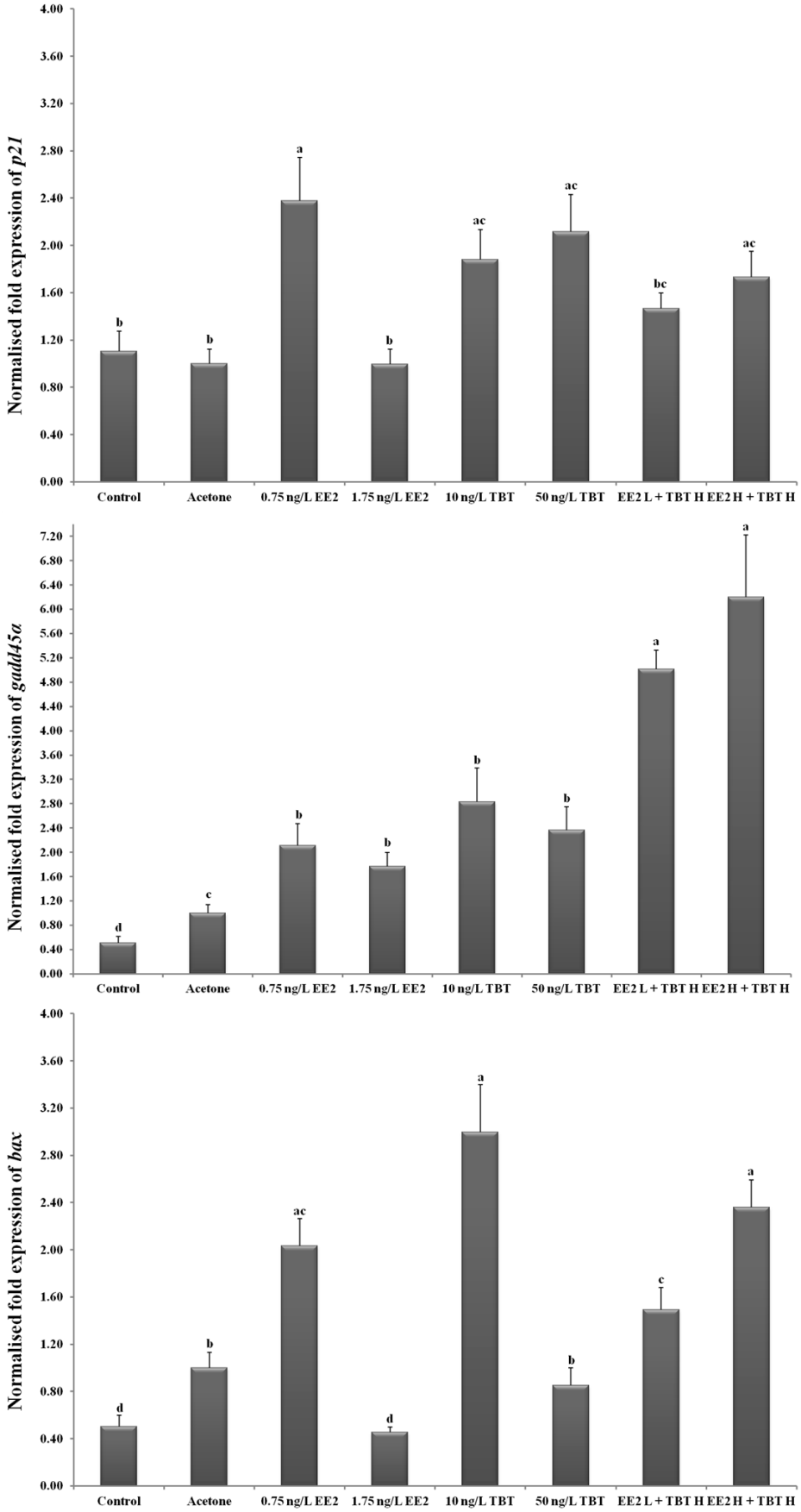

Figure 6.4 Normalised fold expression of *p21*, *gadd45α* and *bax* in 9.5 months zebrafish male gonads after exposure to EE_2, TBT and their combined mixtures (ng/L) (L, lowest concentration; H,

highest concentration). Solvent control (acetone) was used as the reference group (= 1-fold). Values are presented as mean ± standard error. Different letters indicate significant differences among treatments ($p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test).

6.4.7 Percentage of DNA damage

Figure 6.5 shows the DNA damage results obtained by comet assay analysis of zebrafish male gonads of specific selected groups. The % of DNA damage was significantly increased at 50 ng/L TBT in comparison with solvent control ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test). A 2-fold increase in the % of DNA damage was observed for the highest EE_2 concentration in comparison with control males, although differences were just above significance ($p = 0.09$). This might be related to an analysis of a lower number of males ($n = 5$), in comparison to the other treatments ($n = 8$), since at this concentration the number of males was significantly reduced due to the significant alteration in sex ratio towards females.

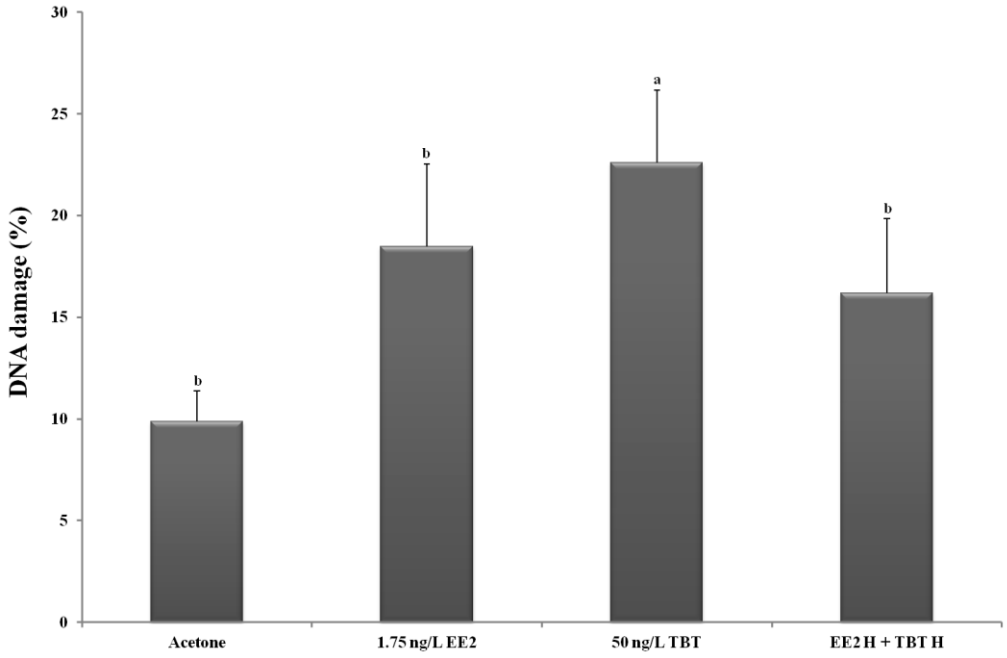

Figure 6.5 Percentage of DNA damage in zebrafish male gonads after exposure to EE_2, TBT and their combined mixture, at the highest concentrations (ng/L) (H, highest concentration). Solvent control (acetone) was used as the reference group (= 1-fold). Values are presented as mean ± standard error. Different letters indicate significant differences among treatments ($p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test).

6.5 Discussion

The presence of EDCs in the aquatic environment has raised considerable concern during the last decade (Sumpter, 2005). Although xenoestrogens and xenoandrogens exist in combination in the aquatic environment, there is still limited information regarding their combined effects (Lyssimachou et al., 2006; Santos et al., 2006; Sária et al., 2011). Nevertheless, previous findings indicate that further studies should address the effects of combined exposures in key endpoints such as reproductive-related parameters and embryonic development. Here, we addressed the effects of EE_2, TBT and their combined exposure in ecological relevant endpoints and link it with the chemicals mode of action, focusing in DNA repair mechanisms.

When analysing the effects of EE_2 and TBT, single and in combination, in zebrafish condition parameters, a significant impact was observed. Whereas single exposures did not affect female total length, both mixtures rendered smaller females in comparison with the control treatments. Male condition factor was significantly increased at 10 ng/L TBT and male total weight was significantly increased at 10 ng/L TBT and at the highest concentration mixture (1.75 ng/L EE_2 + 50 ng/L TBT). Similarly the black-striped pipefish (*Syngnathus abaster*) larvae exposed during seven days to EE_2 (3 and 9 ng/L), TBTCl (10 and 50 ng/L Sn/L) and their mixtures exhibited behavioural and development alterations (Sária et al., 2011). While EE_2 depressed pipefish growth, TBT increased it. When exposed to a mixture of the highest concentrations of EE_2 and TBT pipefish growth was depressed, suggesting an impact of the mixture in pipefish development. Female GSI was significantly decreased at both EE_2 and TBT concentrations as well as at the lowest mixture treatment. In previous studies, it has been reported that females exposed to EE_2, from 0.5 to 100 ng/L, show a decrease in ovary size, a delay or suppression of ovarian development and an increase in the number of atretic oocytes (Papoulias et al., 1999; Scholz and Gutzeit, 2000; Metcalfe et al., 2001), which is in accordance with the decrease in GSI observed in our study. Similarly, a TBT induced decrease in female GSI was reported in cuvier after exposure for 50 days (Zhang et al., 2007). Interestingly, exposure to the highest concentration mixture did not impact GSI, which contrasts with the effects of single exposures.

Recently, it has been demonstrated that EDCs can interfere with fish sex differentiation. It has previously been reported that TBT can lead to a sex bias towards males in some species (McAllister and Kime, 2003; Shimasaki et al., 2003) while estrogenic compounds can induce fish feminization (Papoulias et al., 1999; Scholz and Gutzeit, 2000; Länge et al., 2001). However, it has been reported that several parameters such as chemical exposure, temperature, diet and stock origin, might interfere with the overall percentage of sexes in the population (Andersen et al., 2003, 2006; Örn et al., 2003). In the present study, the percentage of males in controls was slightly higher than the expected in zebrafish reared under normal laboratory conditions (usually between 50-65%) (Hill and Janz, 2003). We hypothesized that this might be associated with a diet that consisted mainly of *Artemia spp.* during the first two months. EE_2 and mixture exposed groups showed a significant increase in the percentage of females, in a dose-dependent manner, which is in agreement with previous studies (Papoulias et al., 1999; Scholz and Gutzeit, 2000; Länge et al., 2001; Santos et al., 2006). In the present work, the lack of a significant effect of TBT in the sex ratio might be related with the already mentioned slightly high percentage of males in the control group. Nevertheless, the effects of TBT in fish sex ratio are far from being conserved across species or even within the same species. In the Japanese medaka, full-life cycle exposure to a range of TBT concentrations failed to induce a bias of sex towards males (Kuhl and Brouwer, 2006). Interestingly, we have recently observed a feminizing effect of TBT in zebrafish full-life cycle exposure to 1 µg TBT/g in the diet (Lima et al., in prep.), which contrasts with previous findings with the same species (McAllister and Kime, 2003; Santos et al., 2006).

It has been demonstrated that TBT-exposed fish have decreased reproductive success. Nakayama et al. (2004) assessed the reproductive success, during week 3, of male medaka exposure to TBT (1 µg/g body weight daily) and reported that fertilization success was significantly decreased while fecundity was unaffected. Similarly, in the present study, full-life cycle exposure to TBT, at the highest concentration, led to a significant decrease in fertilization success while fecundity was unaltered. Interestingly, co-exposure to EE_2 completely rescued TBT effects in the fertilization success, suggesting that this event could be associated with depressed estrogens levels. A clear trend towards a decrease in fecundity was observed at the highest concentration mixture. This indicates that while single

contaminant exposure did not affect fecundity, when EE_2 and TBT are combined an interaction is produced.

Recent studies indicate that the embryonic development of offspring of EE_2 and TBT-exposed fishes may also be a target of these xenobiotics (Nash et al., 2004; Brown et al., 2007; Hano et al., 2007; Soares et al., 2009). Identifying the most sensitive embryonic development stage to contaminant exposure is essential when trying to understand the mechanisms of action of these EDCs. In the present study, parental full life-cycle exposure to 1.75 ng/L EE_2 and 1.75 ng/L EE_2 + 50 ng/L TBT resulted in an increase in embryo mortality between 8 and 32 hpf. During zebrafish development this period includes late gastrulation and organogenesis (Kimmel et al., 1995). After 32 hpf, mortality rates increased slightly up to the end of the embryonic development. However, at 80 hpf, a significant decrease in embryo mortality was observed at 0.75 ng/L EE_2 treatment. Although not significant, at 8 hpf there was an increase in the percentage of abnormal eggs for the two mixtures. After this period, the rate of abnormal embryos was similar among treatments and kept at low levels. In agreement with these findings, in a recent study we reported an increase in zebrafish embryo mortality after chronic parental EE_2 exposure, in the range of 0.5-2 ng/L, nominal concentrations (Soares et al. 2009). Furthermore, EE_2 impact in embryo development occurred only up to early. In the present study, the higher mortality rates observed at the highest EE_2 + TBT mixture indicate than when these compounds are combined their single effects seem to be potentiated. Similarly, Nakayama et al. (2005) reported that mixtures of TBT and PCBs produced more adverse effects on developmental stage of embryo than did each chemical given alone.

In a previous study, we hypothesized that the observed disruption of embryonic development could be related to alterations on parental DNA and/or changes on the expression patterns of key genes rather than with functional changes (Soares et al., 2009). Hence, to understand the mechanism of action of EE_2, TBT and their binary mixtures on zebrafish reproductive physiology and embryonic development, the impacts on the transcription levels of key genes involved in DNA repair in gonads were evaluated.

In the present study, single contaminant exposure at the lowest EE_2 concentration significantly increased *xpc* transcripts levels. On the other hand, EE_2 and TBT exposure, at the highest concentrations, significantly decreased *xpc*

mRNA levels. Combination of EE_2 and TBT resulted in an up regulation of *xpd* transcripts levels, in a dose-dependent manner, while no changes were observed for the other treatments. Similar to our study, Notch et al. (2007) reported an increase in NER transcripts levels in zebrafish liver at 1 ng/L EE_2, while for higher, less environmentally relevant EE_2 exposure concentrations (i.e., 10 and 100 ng/L), an opposite effect was recorded. Zuo et al. (2012) demonstrated that cuvier exposure, during 48 days, to TBT (1, 10, 100 ng/L as Sn) significantly decreased liver DNA integrity and affected the expression of most NER genes. Taken together, these results indicate that EE_2 and TBT could indeed impact NER and alter DNA repair capacity, even in species with different habitats (marine cold water versus freshwater tropical fish). Decreased NER gene expression may, in part, explain increased rates of uncorrected DNA lesions that could present a risk of fixed mutations. Uncorrected DNA damage can lead to genomic instability that potentially could be transmitted to offspring affecting their development and survival. On the other hand, increased NER gene expression could potentially be linked with increased DNA repair capacity, preventing genetic transformation.

The tumor suppressor protein p53 plays a central role in modulating the cellular responses to DNA damage and is known to interact with several DNA repair mechanisms, including NER, in order to maintain homeostasis. Our previous study showed that exposure to EE_2 (in the range of 0.5-2 ng/L) was associated with a significant increase in the number of transcripts of *p53* gene in male gonads at all EE_2 exposure concentrations (Soares et al., 2012). In the present study, we extended our findings to the effects of EE_2, TBT and their combined mixtures on the expression mRNA levels of *p53*, several downstream genes as well as the transcription of its principal regulator, *mdm2*. A significant increase in *p53* transcripts was observed at 0.75 ng/L EE_2 while at the highest EE_2 concentration (1.75 ng/L) a significant repression in *p53* gene expression was observed. Upon exposure to EE_2, *mdm2* gene expression followed the same pattern as the observed for *p53*, which is consistent with the autoregulatory feedback loop described in mammals for this complex of proteins (Molinari et al., 2000). Additionally, a significant decrease in *mdm2* expression levels was observed at the highest TBT concentration and lowest mixture treatment. DNA damage can modulate the p53-Mdm2 feedback loop to allow appropriate p53 responses (Freedman et al., 1999; Sun et al., 2009). Hence, the observed

decrease in *mdm2* transcripts levels at the highest TBT concentration and lowest mixture treatment could possibly lead to a posterior increase in *p53* transcripts levels that was not registered in the time frame of analysis. Potentially, this could be partially corroborated by the observed increase in transcripts levels of p53 target genes at these treatments. When EE_2 and TBT were combined at the lowest EE_2 concentration mixture, TBT counteracted the EE_2 effect in *mdm2* transcripts and TBT action seemed to predominate. On the other hand, when TBT was combined with the highest EE_2 concentration single exposure effects of both contaminants were suppressed. The p53 protein is a sequence-specific transcriptional activator of target genes. In the present study, *p21* gene expression was significantly increased at the lowest EE_2 dose, both TBT concentrations and at the highest mixture treatment. Cell cycle arrest by p21 is one of the best understood mechanisms of p53 response to DNA damage. Activation of p53 is typically associated with an increased expression of p21 which is translated into delayed transit in cell division from G1 to S, thus preventing the effects of DNA lesions on vital cellular functions and propagation of heritable genetic errors (Zhu et al., 2000; Liu and Kulesz-Martin, 2001). *gadd45α* transcripts levels were significantly increased at all treatment exposures, suggesting that Gadd45α has a central role in the cellular response to damage. The higher increases in *gadd45α* gene expression were observed at the mixtures treatments, in a dose-dependent manner, which correlates well with embryonic development data. This indicates that when EE_2 and TBT are combined a significant increase on *gadd45α* transcripts levels is observed. Gadd45α acts in the control of cell cycle arrest at the G2/M phase transition but it is also directly involved in DNA repair. Indeed, Gadd45α is the member of the Gadd45 family best associated with DNA damage. It has been implicated in several events that include DNA demethylation, nucleotide excision repair, chromatin accessibility and apoptosis (Harkin et al., 1999; Rai et al., 2008; Sun et al., 2009). Another key gene involved in p53 mediated responses is *bax*, which is a p53-apoptosis induced gene. Apoptosis ensures the integrity of the organism by eliminating cells with irreparable DNA damage, preventing genetic transformation (Sun et al., 2009). *bax* gene expression was significantly reduced at the highest EE_2 treatment and significantly increased at the lowest concentrations of the single exposures. At the mixture treatments, *bax* transcripts levels significantly increased, in a dose-dependent

manner. Despite the identification of apoptosis-related genes as downstream transcriptional targets of p53, the mechanism of p53-mediated apoptosis is largely unknown. Apoptosis seems to predominate as DNA damage is more severe or p53 protein is more highly induced, depending on the capacity of the damaged cell to repair (cells less robust for DNA repair proteins) (Liu and Kulesz-Martin, 2001).

Strong evidence now point to a transcriptional regulatory role for p53 in NER. Adimoolam and Ford (2003) reported the regulation of XPC by p53 in response to DNA damage and showed that the mRNA and protein products of XPC increased in a p53-dependent manner (Adimoolam and Ford, 2002). Therefore, p53 appears to control the damage recognition step in NER global genome repair. p53 might also modulate NER through protein-protein interactions. DNA repair machinery is involved in p53-mediated apoptosis. p53-mediated apoptosis is abrogated in cells with either XPB or XPD mutations and can be restored by introduction of wild type XP genes (Wang et al., 1995).

Integrating the data of embryonic development, NER DNA repair mechanism and p53 pathways, we suggest that the significant increase in embryo mortality observed at the highest EE_2 concentration may be related to a decreased DNA repair capacity, which is in agreement with the significant repression in *xpc* and *p53* genes expression observed at this treatment. A trend towards an increased in embryo mortality was observed at the highest TBT concentration. Also, at this treatment *xpc* mRNA expression was significantly reduced. Results from comet assay analysis showed that TBT exposure, at the highest concentration, significantly increased the % of DNA damage in zebrafish male's gonads. Although it did not reach significance a 2-fold increase in the % of DNA damage was observed for EE_2 exposure, at the highest concentration. These results further corroborate the hypothesis that increased embryo mortality, upon single exposures, may be related to increased DNA damage. At the lowest EE_2 concentration the expression pattern of *xpc* and *p53* was the opposite of the observed at the highest EE_2 concentration. This increased gene expression could potentially be related to an increased DNA repair capacity. Interestingly, at the lowest concentration, EE_2 had a protective effect in embryo mortality, further corroborating our hypothesis. The higher embryo mortality rate was observed at the highest EE_2 and TBT co-exposure. At this treatment all p53 target genes were significantly induced. This suggests that when EE_2 and TBT are combined, at the

highest concentrations, DNA damage could be more severe, leading to the activation of several DNA repair processes. However, if damage level is too severe and DNA repair processes are unable to cope with it apoptosis mechanisms may predominate, which is also consistent with the increases in *xpd*, *gadd45α* and *bax* transcripts at the highest mixture concentration. This could partly explain the embryo malformations and ultimately increased mortality observed at this treatment. For instance, Shi et al. (2008) reported that exposure of zebrafish embryos to perfluorooctanesulfonate (PFOS) significantly decreased larval survivorship and induced development malformations that were mediated via apoptosis. The exact mechanisms leading to the choice between the p53 final major outputs need to be clearly identified. The regulation of p53 at multiple levels (mediators/inhibitors), the complexity of p53 post-translational modifications and the DNA sequence variation in the p53 binding sites are likely to determine the outcome in cellular response toward growth arrest, DNA repair or apoptosis, depending on cell type and stimulation signals (Liu and Kulesz-Martin, 2001).

Based on the genotoxic effects of single exposures, observed in the present study, we could expect to find a significant increase in DNA damage in the group exposed to the highest EE_2 and TBT co-exposure. However, exposure to this mixture did not increase the % of DNA damage. A similar finding was reported early by Micael et al. (2007) in zebrafish erythrocytes nuclear abnormalities. Cells employ distinct damage repair pathways to fix different kinds/levels of DNA damage. Also, as endocrine disruptors may act at different levels, when EE_2 and TBT are combined their mode of action may differ from single exposure and the mechanism(s) of genotoxicity targeted may not necessarily be the same. Nevertheless, these data clearly demonstrate that p53 signaling pathways are impacted by xenobiotics exposure.

It has been identified that aberrant meiotic recombination is an important factor causing meiotic non-disjunction, and consequent improper chromosome segregation. Absent or reduced levels of meiotic recombination or even suboptimally positioned recombination events, results in genetically imbalanced sperm or oocytes that if fertilized may lead to an aneuploid embryo (Hassold and Hunt 2001). A role for p53 in meiotic recombination has been postulated from the observation that changes in *p53* mRNA expression are associated with key phases regulating meiotic progression (Schwartz et al., 1993). Hence,

inappropriate regulation of p53 could play a role in mitotic/meiotic arrest failure, chromosomal instability and aneuploidy. We hypothesize that increased embryo mortality, at single contaminant exposure, may result from increased DNA damage. Although it remains to be tested if the observed zebrafish embryo mortality after parental life-cycle exposure to EE_2 and TBT is linked with an increase in aneuploidy, the findings from Brown et al. (2008), who reported an increase in aneuploidy in male rainbow trout gonads and their offspring's only when the period of EE_2 exposure encompassed male sexual maturation, suggests that meiosis may be affected and support the idea of some genomic defect of the sperm DNA. Further supporting our hypothesis, Perrin et al. (2009) have shown that DNA fragmentation rate depends on the presence of a chromosomal abnormality in spermatozoa. Also, Nili et al. (2011) reported a good correlation between DNA damage and chromosomal aneuploidy in subfertile men spermatozoa.

This work unveiled the effects of combined mixtures of EE_2 and TBT in endpoints not yet studied, namely embryonic development and DNA repair mechanisms. Overall, our study shows that parental full life-cycle exposure to EE_2, TBT and their combined mixtures significantly affected zebrafish embryo survival as well as the transcription levels of key genes involved in DNA repair systems- NER mechanism and p53 pathways- evidencing possible mechanisms of action of these compounds. Furthermore, these effects occurred at low and environmentally relevant concentrations and predicted long-term effects, strongly supporting the inclusion of embryonic development and gene expression studies in the screening of endocrine disruption in wild fish populations.

Modulation of *p53* and NER gene expression levels are currently used as important epidemiological markers of cancer risk and decreased DNA repair capacity in humans. Taken together, the findings of this study contribute to a better understanding of the impact of mixtures of the ubiquitous contaminants EE_2 and TBT, single and combined, in ecological relevant endpoints, and its link with the chemicals mode of action, while at the same time also suggest that p53 and NER pathways can become sensitive biomarkers of wild fish population health.

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Chapter 7

Discussion

7. Discussion

7.1 General remarks

The presence of EDCs in the environment is a growing problem, not only to wildlife but also to humans. Although the literature agrees on the association between the use of anthropogenic contaminants and the increased documentation of endocrine disruption in wild species, it is important to further unveil the population-level impacts. Furthermore, several studies have reported effects of EDCs in fish at individual and sub-cellular level but few have investigated potential effects at the entire life-cycle. Specifically, research has neither sufficiently explored the extent to which endocrine disruption influences population stability, neither related the observed effects with the contaminants mode of action.

Concern about EDCs on the environment generates a requirement for a coordinated effort from sub-disciplines of biology including ecology, ethology, toxicology, endocrinology, histology and molecular biology. The choice of zebrafish as a model organism in this work enables an integrated approach as it has previously been used in numerous studies in the aforementioned sub-disciplines. The screening of endpoints at various levels of biological organization allows the integration of the information to better evaluate the risk of EE_2 and TBT exposure for wild fish populations.

7.2 EE_2 effects on population relevant endpoints: reproduction, embryonic development and behaviour

Many EDCs can disrupt endocrine control mechanisms, interfering with reproduction, development and behaviour (Guillette et al; 1995; Bigsby et al., 1999, Nakayama et al., 2004). Evaluation of reproductive and development effects is extremely relevant in contaminant hazard assessment. Although EDCs can have long-term effects on reproduction and subsequent progeny development, most chemical safety assessments rely on short-term exposures.

In Chapter 3, it was investigated if full life-cycle zebrafish parental exposure to low levels of EE_2, 0.5, 1 and 2 ng/L (actual concentrations of 0.19, 0.24 and 1 ng/L, respectively) affected reproduction and embryonic development. The

interplay between reproductive endpoints and progeny survival was assessed, with special focus on embryonic development analysis.

In this work, reproductive capacity, translated in terms of fecundity and percentage of fertilized eggs, was not affected by EE_2 exposure. These results confirmed previous studies, which reported alterations in zebrafish reproduction at concentrations ranging from 1.1 to 3 ng/L EE_2 (Hill and Janz, 2003; Segner at al., 2003; Fenske et al., 2005; Schäfers et al., 2007), although in some works a decline in fecundity and fertilization success was observed at concentrations below 1 ng/L EE_2 (Larsen et al., 2008; Xu et al., 2008). Biomarkers directly associated with steroid hormone signaling pathways are the most widely used as molecular indicators of endocrine disruption by ECs. In our study, an induction in *vtg1* gene was verified at the highest EE_2 concentration. Likewise, Pawlowski et al. (2004) reported that in fathead minnow (*Pimephales promelas*) the lowest observed effective concentration of EE_2 for induction of *vtg* was 1 ng/L.

In order to assess EE_2 effects at the second generation, a full embryonic development analysis was performed. EE_2 exposure resulted in a concentration-dependent increase in egg mortality between 8 and 24 hpf, reaching 60% at the highest EE_2 concentration (1 ng/L), with no further mortality increases observed up to the end of the assay (Chapter 3, fig. 3.3). Also, at the highest EE_2 exposure level, an increase in development abnormalities (approximately 43%) was observed at 8 hpf (Chapter 3, fig. 3.4). Most of these abnormal eggs had not yet entered gastrulation and showed an abnormal accumulation of cells at the animal pole, which contrasted with the other treatments in which most eggs had reached 65-75%-epiboly. Appropriate DNA replication is an essential step for cell cycle progression and mitosis (Fujita, 2006). Santos et al. (2007) reported that DNA replication impairment is one of the target pathways by which EE_2 exposure resulted in the impairment of gametogenesis in female zebrafish. If a similar phenomenon occurs in embryo formation it could explain the abnormal cell divisions and impaired development observed in zebrafish embryos exposed to EE_2. After 8 hpf the rate of abnormal embryos was similar in all treatments and kept at low levels. Occasionally development anomalies included edemas, dorsal curvature, bent tails and finfold lesions. Figure 7.1 presents some of the observed EE_2 effects on embryonic development in comparison with normal development of control embryos.

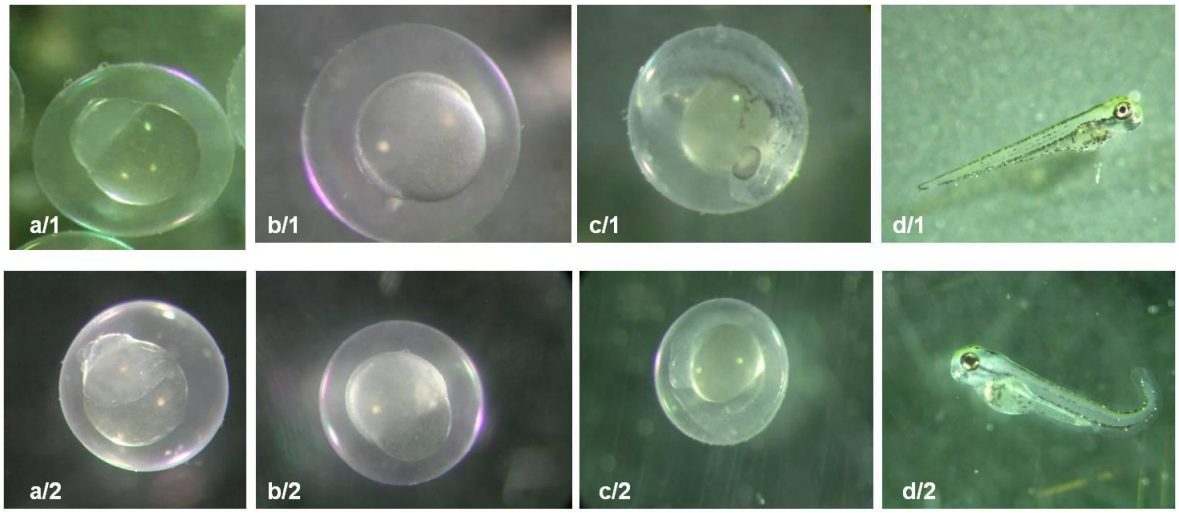

Figure 7.1 Zebrafish embryonic development disruption by EE_2 exposure (1-control embryo, 2-treatment; a/1) 4 hpf, late high blastula; a/2) abnormal accumulation of cells at the animal pole; b/1) 8 hpf, 75%-epiboly; b/2) protruding yolk, "exogastrulation"; c/1) 32 hpf, prim-16; c/2) severe retardation; d/1) 80 hpf, protruding mouth; d/2) bent tail.

Tail malformation in zebrafish embryo at 72 hpf is one of the toxicological endpoints for evaluating the teratogenicity of chemicals. Bent and hook-like tails have been observed in 72 hpf zebrafish embryos treated with 5 μM of curcumin (Wu et al., 2007). In the present study, this effect was also recorded in some embryos from the EE_2 treatments. Similarly, Boudreau et al. (2004) reported that in mumichog (*Fundulus heteroclitus*) EE_2 exposure increased the incidence of skeletal abnormalities (scoliosis and lordosis).

Integrating embryo mortality with abnormal development data we can conclude that EE_2 at the highest level impacted normal blastula, gastrula and, possibly, early organogenesis, while the two lowest EE_2 concentrations impacted late gastrulation and early organogenesis only. We have identified the window of embryo sensitivity to EE_2, which matched the most sensitive phase of embryonic development under normal conditions. In support to our early results, Tong et al. (2011) reported that the zebrafish embryonic stage sensitive to EE_2 mainly started at cleavage and lasted up to the organogenesis with time-accumulating effects.

As a member of the family Cyprinidae, the zebrafish is related to the most abundant cyprinid species in Europe. Therefore, results of zebrafish embryonic development may also have significance for other species (Woodland and Maly, 1997). In a recent study, Brown et al. (2007) reported an increase in rainbow trout

(*Onchorynchus mykiss*) embryo mortality after male parental exposure to 0.8 and 8.3 ng/L EE_2. Taken together with our results, these findings indicate that EE_2, at actual 1 ng/L range, impacted the embryonic development of two species with different life strategies, suggesting that several wild fish populations may respond in a similar manner.

Fish embryo tests have been shown to detect not only samples characterized by strong fish toxicity, but also samples that induce only minor toxicity in conventional acute *in vivo* fish tests (Braunbeck et al., 2005). This is in line with our results in which full life-cycle zebrafish exposure to EE_2 disrupted embryonic development at EE_2 concentrations below those that induced reproductive impairment and *vtg1* gene induction in the parental generation. Furthermore, our work contributed to identify the most sensitive phase of embryonic development to extremely low EE_2 exposure concentrations. Ecological level effects are difficult to screen in wild fish populations, especially if dramatic changes are not observed. This work shows that increased embryo mortality after EE_2 exposure, at concentrations found in many aquatic ecosystems in the vicinity of urban areas, may lead to ecological relevant impacts, highlighting the need for more detailed studies. Additionally, these data shows that environmental risk assessment based only on adult endpoints may not be sufficient to predict EE_2 effects and strongly support the inclusion of embryonic development studies in the toxicity screening of ECs and other priority and emergent pollutants.

A fundamental goal when addressing population dynamics is to understand the processes that influence individual fitness. An influencing key factor is recruitment, or the abundance of juveniles entering the population (Stearns and Koella, 1986). The increasing environmental contamination, with well documented adverse effects, plays a central role in this context. However, the processes involved are complex and not fully understood. Thus, in Chapter 4 it was investigated if a 40 dpf exposure of zebrafish juveniles to environmentally relevant concentrations of EE_2 could lead to detectable alterations in normal behaviour, such as locomotion patterns.

In this study, exposure of zebrafish juveniles to EE_2 led to a significant alteration in juvenile zebrafish behaviour patterns, with a decrease in swimming activity even at the lowest actual concentration of 0.19 ng/L (Chapter 4, fig. 4.1).

Activity level is a simple but critically important behaviour that can influence survivorship (Werner and Anholt, 1993). For instance, hypoactivity may disrupt feeding and increase vulnerability to predation (Laurence, 1972; Steele, 1983). Also, decreased locomotor activity may impact drift and transport, and even interfere with social behaviours (e.g. aggression), which may result in lower survival rate with a clear implication in the numbers of individuals that reach maturity and reproduce. For example, fish exposed to heavy metals displayed alterations in dominance, feeding behaviour, growth, and predator avoidance (Weis and Weis, 1998; Sloman et al., 2003). Therefore, the observed inappropriate behaviour responses, due to the toxic effects of EE_2, may ultimately impact important ecological variables leading to effects at population-level.

Recently, Sária et al. (2011b) reported that EE_2, at environmentally relevant concentrations, affected pipefish (*Syngnathus abaster*) larvae behaviour, namely the vertical distribution patterns of pipefish newborns. Together with our data, it seems that EE_2 may impact different fish populations through subtle changes in behavioural patterns, expressed during the early stages, and possibly our results can be extrapolated to other fish species.

Alterations on locomotor activity are frequently observed in alterations of the form, frequency, or posture of swimming movements, with changes often occurring much earlier than mortality (Little and Finger, 1990). For example, development of locomotory responses, frequency of swimming movements and duration of activity were significantly inhibited before effects on survival or growth were observed in brook trout (*Salvelinus fontinalis*) alevins exposed to aluminum (Cleveland et al., 1991). In our study, swimming behaviour was found to be more sensitive to EE_2 than either *vtg1* induction or reproductive parameters determined at adulthood. Similarly, Coe et al. (2008) reported that reproductive hierarchies in breeding colonies of zebrafish were disrupted by exposure to EE_2 at a concentration that did not affect the number of eggs produced.

Accumulating evidence show a good agreement between selected behavioural endpoints in fish and EDCs toxicological action (Airhart et al., 2007; Speedie and Gerlai, 2008; McGee et al., 2009; Kokel et al., 2010; Saaristo et al., 2010; Sária et al., 2011a). Our work contributes to evidence the relevance of ECs effects on fish behaviour, an ecologically relevant parameter, at extremely low EE_2 levels. Furthermore, the behavioural response followed the same pattern as embryo

development (F_2), evidencing that behavioural endpoints are a useful non-invasive measure of EDCs exposure.

Integrating embryonic development (Chapter 3) and behaviour (Chapter 4) data, it was shown that early life stages of fish were more sensitive to chemical stress than adults and exposure effects could be seen at much lower concentrations than the ones that affected mature individuals. Additionally, embryo tests and behaviour assay proved to be highly sensitive to detect estrogenic effects of the potent synthetic estrogen EE_2. These results strongly support that cases of long-term toxicity can be predicted by results from studies with early life-stages and supports the inclusion of embryonic development and behaviour studies in the screening of ECs in wild fish populations, as good early indicators of toxicological risk.

Kidd et al. (2007) found that in a lake environment, multigenerational exposure of a fish population to 5 ng/L EE_2 resulted in population collapse after three generations. There were a number of possible causes for this population collapse including disrupted sexual development, disrupted development of gametes, embryo death, and lowered gamete viability. We have conducted a detailed screening of female and male gonad histology, coupled with a stereological analysis, and also assessed several sperm quality parameters at adulthood (Chapter 3, table 3.1 and 3.2). However, we did not observed any alterations, which corroborated previous findings for the range of EE_2 concentrations used in this study (Hill and Janz, 2003; Xu et al., 2008). These led us to hypothesize that the reduced embryo survival was probably related with changes in the genetic information carried by gametes of the parental generation and/or with alterations on the expression levels of key genes.

Several xenobiotics are known to disrupt the reproductive endocrine system of fish (Hachfi et al., 2012) and to affect gamete development and viability, being genotoxic damage one important mechanism responsible for these effects (Brown et al., 2008, 2009). EE_2 genotoxicity has been reported by several studies (Cooke and Hinton, 1999; Contractor et al., 2004; Micael et al., 2007). These findings further support the hypothesis that embryo mortality after EE_2 exposure, observed in our study, may be due to DNA damage. Thus, the question of whether this damage may be heritable is of critical importance.

The heritability of EDCs-induced defects can be associated with two major processes: an epigenetic mechanism or a direct genetic mechanism. Epigenetic alterations are changes that affect gene function without modifying the DNA sequence, such as an altered methylation pattern. On the other side, direct genetic mechanisms, such as induced genetic mutations, alter DNA structure (Brown et al., 2009). In mammals, EDCs have been shown to induce heritable effects through both mechanisms. Studies on direct genetic mechanisms indicate that EE_2 and some oestrogens, progestins and androgens have genotoxic effects, impacting DNA repair mechanisms and inducing formation of aneuploid cells during mitosis (Notch et al., 2007; Kayani and Parry, 2008).

Recently, effects of EE_2 on DNA repair processes, in zebrafish, have been suggested as a possible mechanism by which estrogens can modulate the incidence of DNA damage (Notch and Mayer, 2009a). These findings suggest that EE_2 may impact the genomic stability of individuals, leading to decreased fitness of populations. However, the mechanisms by which this chemical produces its effects are not well understood. Therefore, baseline studies are required to establish the involved mechanisms.

Since endocrine disruption can be linked to molecular interactions, the variation of transcription levels of genes coding for key proteins involved in DNA repair could be used as an indication of DNA damage and would allow the identification of candidate pathways leading to the effects induced by EE_2. Therefore, Chapter 5 study was set out to determine the molecular mechanisms associated with embryonic development disruption following EE_2 exposure. This was achieved by analyzing gene expression in gonads of individual zebrafish exposed to EE_2 and by anchoring these against the associated effects on offspring embryonic development. Real time PCR was used to analyze the expression levels of NER damage recognition (*xpc*), damage verification (*xpa*), helicase (*xpd*), and endonuclease (*xpf*) genes, *p53* and downstream effectors as well as p53 principal regulator, *mdm2*.

In this work, exposure to EE_2, in the range of 0.19-1 ng/L, did not affect NER genes expression in both male and female gonads. However, a concentration related trend towards an increase in several NER genes was observed, especially in the ovaries (Chapter 5, fig. 5.2). In agreement with our results, in a previous study, Notch et al. (2007) reported an increase in NER transcripts levels in

zebrafish liver at an exposure concentration of 1 ng/L EE_2, while for higher EE_2 exposure doses (10 and 100 ng/L), a significant decrease was recorded. Taking in consideration both works, it can be suggested that NER impairment in gonads by EE_2 exposure might occur primarily at concentrations above those tested in our study. These authors also showed a sexually dimorphic alteration of NER gene expression, with males being more severely impacted than females.

In cells, a variety of cellular responses following genotoxic exposure may contribute to prevent DNA lesions from interfering with essential cellular functions. Of these cellular responses, activation of p53 pathways is of critical importance. In this work, exposure to EE_2 significantly increased *p53* gene transcription, in male gonads, at all tested concentrations. Female gonads depicted a different pattern, with a significant rise at the intermediate exposure level (0.24 ng/L). At this concentration, females also displayed a significant increase in *mdm2* gene transcription, which is consistent with the autoregulatory feedback loop described for this complex of proteins (Molinari et al., 2000) (Chapter 5, fig. 5.4). Altered *p53* gene expression occurred at lower concentrations than reproductive endpoints and *vtg1* induction analyzed in a parallel work (Chapter 3). In a previous report, Liney et al. (2006) also observed that genotoxic endpoints frequently occurred at lower levels than measurable reproductive effects. In accordance with our results, environmental pollutants have been reported to increase *p53* gene expression in fish. Brzuzan et al. (2006) demonstrated that *p53* is inducible by benzo[a]pyrene exposure in the whitefish (*Coregonus lavaretus*) and Hong et al. (2007) reported that Japanese medaka (*Oryzias latipes*) exposure to diclofenac significantly increased *p53* expression levels.

In order to elucidate EE_2 impact on p53 signaling pathways we evaluated if EE_2 exposure altered specific p53 target genes. Downstream effectors, *p21*, *gadd45α* and *bax*, were selected due to their crucial role in the major p53 cell responses to damage: cell cycle arrest, DNA repair and/or activation of a cell death program. In this study, EE_2 exposure did not affect the transcription levels of p53 target genes in both sexes. However, a distinct expression pattern was observed. While females showed a concentration-dependent trend towards an increase in *p21* and *gadd45α* transcripts levels, males presented an opposite response with a decreased expression at the highest EE_2 concentration (1 ng/L). Similarly, basal *bax* expression in female gonads was increased when compared

with male gonads (Chapter 5, fig. 5.5). The dichotomy of responses between male and female fish is an interesting aspect of this study. In contrast to males, female zebrafish exposed to EE_2 showed less alteration in *p53* gene expression. This seems to indicate that EE_2 exposure, at environmental relevant concentrations, affects males more severely than their female counterparts, which corroborates several previous reports (Filby et al., 2007; Notch et al., 2007; Xu et al., 2008).

In this study, the fact that increased transcription levels of *p53* in males gonads were not translated into transcriptional regulation of target genes by p53 was unexpected. A disruption in p53 signaling pathways by EE_2 exposure could result in impairment of DNA repair processes, leading to an increased incidence of DNA lesions and genomic instability that could be transmitted to progeny. For example, Waldman et al. (1995) reported that disruption of endogenous p21 abrogates the G1/S checkpoint after cell exposure to DNA damage, leading to genomic instability (Waldman et al., 1995). Gadd45α has been shown to be involved in cell cycle arrest at G2/M phase (Zhang et al., 1999). A G2 checkpoint is probably a key mechanism for preventing premature entry into mitosis and propagation of genetic errors. Additionally, Brown et al. (2008) showed that when male rainbow trout were exposed to EE_2 an increase of aneuploidy levels in sperm cells as well as in embryos could be observed. These findings add backing to the hypothesis of an impact of EE_2 in male gonad DNA damage.

In a parallel study (Chapter 3) we showed that EE_2 exposure at all tested concentrations, (0.19, 0.24, 1 ng/L) disrupted zebrafish embryonic development. Therefore the results of the present work, in which EE_2, at the same concentrations, modulated the transcription of *p53* gene, particularly in male zebrafish gonads, closely relates with embryonic development data ($r = 0.5$; $p = 0.045$) and further support the hypothesis that embryo mortality after parental EE_2 exposure may be due to male DNA decreased repair capacity. Additionally, these data suggest that screening *p53* gene transcription in male gonads from wild fish populations can be a sensitive indicator of population health, since it can be linked with detrimental endocrine disruption effects.

7.3 Chronic life-cycle effects of mixtures of EE_2 and TBT on reproductive traits and embryonic development and their link with the chemicals molecular mode of action

In the aquatic environment ECs rarely exist individually. Instead, most often there is a complex mixture of compounds. While it is difficult to attribute specific biological effects to individual components of mixtures, hypotheses regarding toxicity can be derived based upon endpoints generated from tests utilizing individual chemicals (Notch and Mayer, 2009b). Nevertheless, in mixtures, biological interaction of two or more compounds can result in toxic effects not observed in single chemical exposures (Eaton and Gilbert, 2008). At the same time, many unanswered questions still persist, such as the effects of mixtures of compounds with a distinct mode of action simultaneously on adult reproductive performance and progeny survival. Likewise, the mechanisms through which many EDCs induce and promote endocrine disruption are still poorly understood.

In this context, in Chapter 6, we evaluated the effects of EE_2 and TBT, single and combined, on reproduction and embryonic development, and integrated the observed effects with the hypothetical molecular mechanisms of action of these compounds. For that purpose, we have performed a zebrafish parental full life-cycle exposure to environmental relevant concentrations of EE_2 (0.75 and 1.75 ng/L), TBT (10 and 50 ng/L) and their binary mixtures (0.75 ng/L EE_2 + 50 ng/L TBT and 1.75 ng/L EE_2 + 50 ng/L TBT).

In this study, EE_2 and co-exposure of EE_2 and TBT did not affect adult reproductive capacity. Nevertheless, TBT single exposure, at 50 ng/L, significantly reduced fertilization success. The fact that EE_2 did not impact fecundity and fertilization success at the tested concentrations is supported by several studies (Hill and Janz, 2003; Segner et al., 2003; Fenske et al., 2005), including our earlier results (Chapter 3). Decreased fertility after TBT exposure is in agreement with a previous work which reported that 3 week exposure to TBT (1 µg/g body weight daily) significantly decreased male medaka (*Oryzias latipes*) fertilization success (Nakayama et al., 2004).

Similarly to EE_2, TBT has also been shown to be embryotoxic in fish (Nakayama et al., 2004; Hano et al., 2007; Zhang et al., 2011). Therefore, in this work we aimed to investigate the impact of co-exposures of these two compounds

at specific stages of embryonic development. Between 8 and 32 hpf, embryo mortality rates significantly increased at the highest EE_2 (45%) and mixture (59%) treatments, 1.75 ng/L and 1.75 ng/L EE_2 + 50 ng/L TBT, respectively (Chapter 6, fig. 6.1). After this time-point observation mortality rates only marginally incremented up to the end of embryonic development. However, at 80 hpf, a significant decrease in embryo mortality was observed at 0.75 ng/L EE_2 treatment.

Taken together with our previous findings (Chapter 3), these results further support that EE_2 impact in embryo development occurs only up to early. Furthermore, embryo development was more sensitive to EE_2 and mixture exposure than reproductive endpoints, suggesting that embryonic development can be an adequate indicator for ECs and EE_2 and TBT combined mixture effects. Studies analyzing the effects of combined mixtures of compounds with a distinct mode of action are scarce. A novel aspect of this work was the verification that when EE_2 was co-exposed with TBT, both at environmental relevant concentrations, their single effects on embryonic development were enhanced, with higher embryo mortality rates. Similarly, Nakayama et al. (2005) reported that mixtures of TBT and PCBs produced more adverse effects on embryo development than did each chemical given alone. These data highlights the importance of addressing the toxicological risk of chemical mixtures considering that in field conditions wild populations are exposed to a cocktail of pollutants.

In Chapter 5, we observed that EE_2 exposure altered *p53* transcripts levels, particularly in zebrafish males, and that there was a close correlation between *p53* gene expression and embryonic development data. Additionally, it has been reported that EE_2 can alter mRNA abundance of NER genes in liver of adult zebrafish (Notch et al. 2007). In a follow up study, decreased NER expression was correlated with a reduced ability of zebrafish liver cells to repair damaged DNA through NER processes (Notch and Mayer 2009a,b). Similarly, TBT has also been shown to be genotoxic (Tiano et al., 2001; Ferraro et al., 2004; Micael et al., 2007). Zuo et al. (2012) reported that exposure to TBT induced DNA damage in cuvier (*Sebasticus marmoratus*) liver and concomitantly altered the transcription levels of NER genes. Therefore, considering the relevance of chemical mixtures, we hypothesized that mixtures of EE_2 and TBT could also affect DNA repair pathways in zebrafish, impacting offspring embryonic development. Hence, the

transcription levels of key DNA repair genes, namely NER and p53 pathways involved genes were analyzed in zebrafish male gonads.

In the present study, *xpc* transcripts levels were significantly increased at the lowest EE_2 level (0.75 ng/L) and significantly depressed at the highest EE_2 and TBT concentrations (1.75 ng/L and 50 ng/L, respectively) (Chapter 6, fig. 6.2). Consistent with our results, significant decreases in *xpc* mRNA abundance have been reported in adult zebrafish after laboratory exposure to high concentrations of EE_2 (Notch et al., 2007). *xpd* transcripts levels were significantly increased at both mixtures in a dose-dependent manner (0.75 ng/L EE_2 + 50 ng/L TBT and 1.75 ng/L EE_2 + 50 ng/L TBT) (Chapter 6, fig. 6.2). XPD factor is part of the TFIIH complex that also plays a role in normal transcription and cell cycle regulation (Notch et al., 2007). Therefore, following treatment exposure, different mechanisms may be activated in order to cope with DNA damage. Taken together, these results indicate that EE_2 and TBT, single and combined, modulate NER genes expression potentially leading to alterations in DNA repair capacity. If EE_2 and TBT do in fact alter DNA repair capacity, this may potentiate effects of environmental mutagens leading to higher rates of DNA damage and genetic alterations in individuals from contaminated environments. For example, Kang and Lee (2005) reported that co-exposure of E_2 and benzo[a]pyrene in human breast cancer cells resulted in increased DNA adducts as compared with benzo[a]pyrene exposure alone. In addition, other compounds, including arsenic, cadmium and nickel have been shown to alter NER processes (Hartwig et al., 2002; Hu et al., 2004).

At the cellular level, the principal function of p53 is to promote survival or deletion of cells exposed to agents that cause DNA damage, preventing genomic instability. In the present work, exposure to EE_2 and TBT, single and combined, induced a wide range of alterations in p53 genes pathways. EE_2 exposure significantly modulated *p53* gene transcription, with increased *p53* transcripts levels at the lowest EE_2 dose (0.75 ng/L) and depressed at the highest EE_2 concentration (1.75 ng/L) (Chapter 6, fig. 6.3). Supporting our results, field as well as laboratory studies have demonstrated modulation of p53 in response to exposure to environmental chemicals (Min et al., 2003; Brzuzan et al., 2006; Hong et al., 2007). *mdm2* gene expression followed the same expression pattern of *p53*, which is in agreement with the autoregulatory feedback control mechanism

described for this complex of proteins (Molinari et al., 2000). Additionally, a significant decrease in *mdm2* transcripts levels was observed for the highest TBT concentration (50 ng/L) and lowest mixture treatment (0.75 ng/L EE_2 + 50 ng/L TBT) (Chapter 6, fig. 6.3). It has been reported that initial reduction of Mdm2 appears to be sufficient to disrupt the equilibrium of the negative feedback loop between Mdm2 and p53 and allow activation of a p53 response (Sun et al., 2009). Potentially, *mdm2* decreased gene expression at these treatments would lead to an increase in *p53* transcripts, which was not registered in the time frame analysis. This could be partially corroborated by the observed increase in transcripts levels of p53 target genes at these treatments.

Table 7.1 summarizes alterations on NER and p53 pathways genes expression after chemicals exposure.

Table 7.1 Overview of EE_2, TBT, EE_2 + TBT impact on gene transcription of NER and p53 pathways (↑ induction, ↓ repression, − unaffected, ↗ trend towards an increase).

Effects on gene expression						
Treatments	**EE_2**		**TBT**		**EE_2 + TBT**	
(ng/L)	**0.75**	**1.75**	**10**	**50**	**0.75 + 50**	**1.75 + 50**
xpc	↑	↓	↗	↓	−	↗
xpa	−	−	−	−	−	−
xpd	−	−	−	−	↑	↑
p53	↑	↓	−	−	−	−
mdm2	↑	↓	−	↓	↓	−
p21	↑	−	↑	↑	↗	↑
bax	↑	↓	↑	−	↑	↑
gadd45 α	↑	↑	↑	↑	↑	↑

All treatments impacted p53 target genes. In general, single contaminant exposure, at the lowest concentrations, as well as the combined mixtures, increased *p21* and *bax* transcripts levels. Also, EE_2 at the highest concentration significantly decreased *bax* mRNA expression (Chapter 6, fig. 6.4). Activation of p53 is commonly associated with an increased expression of p21 which is translated in a G1/S cell cycle block. Long-term exposure to EE_2 has been reported to result in a block in late G1 or G1/S transition simultaneously increasing p53 and p21 levels (Koroxenidou et al., 2005). This delayed transit would allow cells to repair DNA damage before proliferation of inborn genetic failures (Liu and

Kulesz-Martin, 2001). Also, p53 has been shown to up-regulate Bax expression, inducing apoptosis (Selvakumaran et al., 1994). Nevertheless, the mechanism of p53-mediated apoptosis is largely unknown. Recent evidences suggest the participation of XPD in p53-induced apoptosis (Wang et al., 1995). Apoptosis seems to predominate as DNA damage is more severe, preventing genetic transformation (Sun et al., 2009). Also, damaged cells that fail to finish a complete DNA repair process due to overwhelming damage or the malfunction of examination and repair steps undergo cell death via apoptosis.

In the present study *gadd45α* transcripts levels were significantly increased at all treatments, especially in the mixtures, in a dose-dependent manner, suggesting that Gadd45α has a central role in the cellular response to damage (Chapter 6, fig. 6.4). In fact, the more highly induced gene was *gadd45α* in the highest mixture concentration. Gadd45α participates in several DNA repair events, including nucleotide excision repair, chromatin accessibility, cell cycle regulation and apoptosis (Harkin et al., 1999; Hollander and Fornace, 2002; Sun et al., 2009). Previous evidence indicated that Gadd45α may contribute to the repair of benzene induced DNA lesions (Boley et al., 2002). Gadd45α has also been implicated in apoptosis of UV-irradiated keratinocytes via p53 (Hildesheim et al., 2002).

Recent observations on the role of p53 in NER indicate that alterations in p53 may lead to increased genomic instability due to a reduced efficiency of DNA repair. Adimoolam and Ford (2003) reported the regulation of XPC by p53 in response to DNA damage and noted that mRNA and protein products of XPC increase in a p53-dependent manner. In our study, the expression pattern of *xpc* followed the same pattern of *p53*, with a significant decrease at the highest EE_2 concentration. Taken together, these results seem to indicate that the increased embryo mortality at the highest EE_2 concentration could be associated with a decreased DNA repair capacity that would lead to an increased accumulation of DNA lesions and ultimately embryo death. *xpc* and *p53* transcripts levels were significantly increased at the lowest EE_2 concentration, which could be translated in an increased DNA repair capacity. Interestingly, this treatment had a protective effect in embryo mortality, further corroborating our hypothesis. A trend towards an increased in embryo mortality was observed at the highest TBT concentration. Also, at this treatment *xpc* mRNA expression was significantly reduced. Comet assay analysis showed that TBT exposure (50 ng/L) significantly increased the %

of DNA damage in zebrafish male's gonad (Chapter 6, fig. 6.5). The genotoxicity effect of TBT in fishes is supported by several reports (Tiano et al., 2001; Ferraro et al., 2004; Micael et al., 2007). EE_2 exposure, at the highest concentration, resulted in a 2-fold increase in the % of DNA damage, although it did not reach significance (Chapter 6, fig. 6.5). This probably reflects the low number of males analyzed, in comparison with the other treatments, since at this concentration EE_2 significantly biased sex ratio towards females. Several reports support EE_2 genotoxic effects (Siddique et al., 2005; Micael et al., 2007). Taken together, these results further corroborate the hypothesis that embryo mortality might be associated with increased DNA damage.

Conceivably, impairment of NER and p53 pathway may result in activation of other repair mechanisms in an attempt to repair DNA damage. Interestingly, at the highest EE_2 concentration *gadd45α* gene expression was increased. *gadd45* genes have been found to be induced under a wide variety of stress conditions. Further investigation has indicated that Gadd45 is widely involved in genotoxic agent-induced responses in either p53-dependent or -independent manners (Vairapandi et al, 1996; Marhin et al., 1997; Maeda et al., 2003). Also, many endogenous and exogenous factors were reported to directly bind with Gadd45α promoter region and activate Gadd45α protein formation in a p53-independent manner. Therefore, we cannot exclude that EE_2 and TBT may impact DNA repair through mechanisms other then NER and p53 pathways.

When EE_2 and TBT were combined, an increase in embryo mortality rate was observed. However, despite the approximately 2-fold increase in the % DNA damage in the highest mixture treatment in comparison to controls, differences did not reach significance, and were less severe than single chemical exposure (Chapter 6, fig. 6.5). These results are in agreement with the ones reported by Micael et al. (2007). As EDCs may act at different levels, when these compounds are combined their mode of action may differ from single exposure and the mechanism(s) of genotoxicity may differ as well. Also, the fact that all p53 target genes were significantly increased at the highest mixture treatment suggests the activation of several DNA repair processes. However, if damage level is too severe and DNA repair processes are unable to cope with it, apoptosis mechanisms may be induced leading to embryo malformations and death, which is also consistent with the increases in *xpd*, *gadd45α* and *bax* transcripts at the

highest mixture concentration. This could partly explain the significant increase in embryo mortality at this treatment. For example, it has been reported that zebrafish embryos exposed to PFOS display significantly diminished larval survivorship and induced development anomalies that were induced via apoptosis (Shi et al., 2008). Nevertheless, we have to take in consideration that high levels of gene expression do not necessarily mean high protein levels. In fact, an induction of gene transcripts may be an attempt to counteract low protein concentrations. If an increase in expression of p53 target effectors in zebrafish correlates to an increase in DNA repair capacity needs to be further investigated. However, these data clearly demonstrate that p53 signaling pathways are affected by EE_2 and TBT exposure.

Increased DNA damage results in higher rates of DNA mutations, leading to genomic instability which could ultimately promote karyotype changes. Aberrant meiotic recombination is a crucial component causing improper chromosome segregation which results in genetically imbalanced sperm or oocytes that if fertilized may lead to an aneuploid embryo (Hassold and Hunt, 2001). A role for p53 in meiotic recombination has been postulated from the observation that p53 participates in the modulation of mitotic/meiotic regulatory activities (Feitsma et al., 2007; Tomasini et al., 2008). This leads to the hypothesis that p53 could be an important component between mitotic/meiotic arrest failure and chromosomal instability.

It has been hypothesized that loss of p53 function can lead both to an increase in probability of chromosome non-disjunction and to abrogation of negative control of proliferation of cells with chromosome segregation errors (Agapova et al., 1996). Therefore, it could be suggested that the observed decreased in *p53* gene expression in male gonads, at the highest EE_2 concentration, could lead to genetic instability and, in particular, to accumulation of aneuploid cells that could be inherited by offspring. Despite it lacks to be demonstrated if the observed zebrafish embryo mortality, after EE_2 and TBT exposure, is correlated with an increase in aneuploidy in male gonads, the findings from Brown et al. (2008) support the idea of male sperm DNA damage. These authors reported an increase in aneuploidy in male rainbow trout gonads as well as on their progeny after EE_2 exposure during male sexual maturation, also suggesting that meiosis can be affected. Nili et al. (2011) reported that in subfertile men the frequencies of sperm

aneuploidy and the extent of DNA damage (measured by comet assay) were significantly higher than normal. These authors suggested that DNA damage might contribute to chromosomal aneuploidy in spermatozoa and may be an indicator of aneuploidy. Taken together with our results these data further support the hypothesis that increased embryo mortality may result from DNA damage.

Figure 7.2 summarizes the DNA repair pathways impacted by EE_2, TBT and EE_2 + TBT based on the findings of the present work.

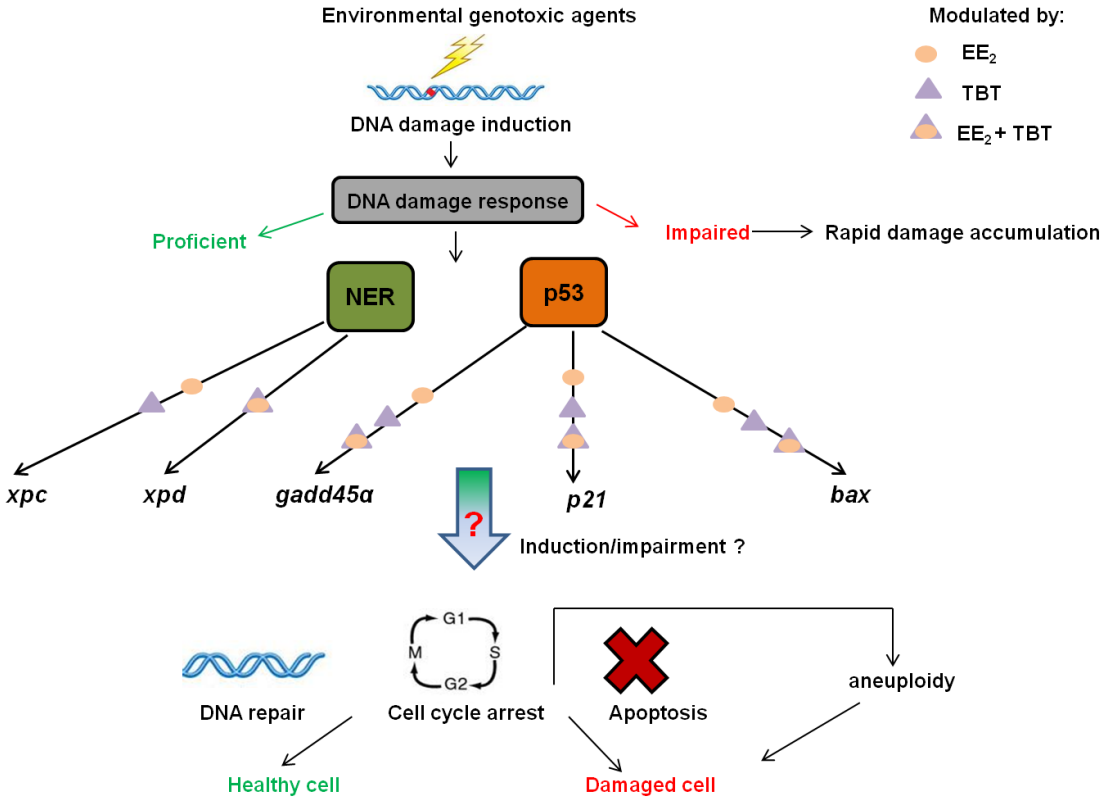

Figure 7.2 Hypothetical mechanisms for EE_2, TBT and EE_2 + TBT –modulated DNA repair capacity.

This work highlights the effects of complex mixtures of EE_2 and TBT in key endpoints not yet studied, namely embryonic development and DNA repair mechanisms. We report here that combined exposures affects embryo development, an important parameter from an ecological point of view, and concomitantly the expression pattern of key genes involved in organism homeostasis, unveiling possible mechanisms of action of these compounds. Furthermore, this study contributes to demonstrate that, in addition to embryo

tests, gene expression data can be essential to clarify the mechanism of action of pollutants, improving risk assessment, and be potentially used as early warning signals of disruption at higher levels of biological organization.

In addition to implications for aquatic organisms, data from this work may have potential human health implications, considering the phylogenetic proximity between zebrafish and mammals. In fact, previous studies suggest that several chemicals or drugs have similar toxic effects in zebrafish embryos and humans (Milan et al., 2003; Zhang et al., 2003; Lam et al., 2005, 2006). Also, alterations on transcription levels of NER genes have been shown to be an important epidemiological marker for increased cancer risk and decreased DNA repair capacity in humans. The critical role of *p53* gene in maintaining the integrity of the genome is evident in that *p53* is the most commonly altered gene in human cancer (Liu and Kulesz-Martin, 2001). In this context, results from this study are innovative. For the first time it is demonstrated that, in addition to estrogenic chemicals, xenoandrogens as well as mixtures of these compounds modulate NER and p53 pathways.

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Chapter 8

Final remarks

8. Final remarks

8.1 Conclusions

The present thesis investigated the exposure effects of two of the most ubiquitously EDCs present in the environment, EE_2 and TBT, single and combined, using the model teleost zebrafish (*Danio rerio).*

The use of a multiparametric approach, combining the screening of ecological relevant parameters such as reproduction, embryo development and behaviour, with molecular endpoints, demonstrated that EE_2 and TBT, single and combined, are able to disrupt fish endocrine control mechanisms at rather low concentrations, potentially leading to impacts at the population-level.

Disruption of embryonic development and behaviour patterns at concentrations below those affecting adult reproductive capabilities and *vtg1* induction, indicate that these parameters can be used as early signs of organisms exposure to EDCs and should be routinely included in environmental risk assessment. Additionally, new molecular targets of EE_2 and TBT have been identified providing further information on the mode of action of these compounds. The findings of altered transcription levels of NER, p53 and downstream effectors genes after chemical insult, and its correlation with disruption of embryonic development, supports the integration of both signaling pathways in the screening of EDCs in wild fish populations.

Considering that wild populations are exposed to complex mixtures of environmental chemicals, the verification that parental full life-cycle exposure to EE_2, TBT and their combined mixtures, at environmentally relevant concentrations, significantly enhanced zebrafish embryo mortality as well as the transcription levels of key genes involved in DNA repair, evidences the importance of addressing the toxicological risk of chemical mixtures in EDCs hazard assessment.

This study demonstrated that combining full life-cycle studies, embryo bioassays, fish behaviour and gene transcription analysis can deliver abundant information on the impact of EDCs and the underlying mechanisms, rendering this strategy an informative approach to test for endocrine disruption in a whole organism system. Such tools enable the regulatory authorities to evaluate risk

assessment with improved accuracy. Given the expected increase in the use of bioactive compounds, mostly related with human health, the findings of the present thesis further highlight the risk of pharmaceuticals and other organic pollutants to non-target organisms.

8.2 Future perspectives

One of the important drawbacks in the hazard assessment of EDCs is the lack of a clear link between laboratory studies and impacts in wild fish populations. The present work evidences that exposure to EDCs at low, environmental relevant concentrations, can disrupt embryonic development and behaviour. In the wild, in addition to EDCs, many factors may interfere with embryo survival and other ecological relevant endpoints. Future studies should address the population-level impact of increased embryo mortality and disrupted juvenile behaviour after EDCs insult, in comparison to other factors affecting the fitness of individuals, such as predation and food availability.

Information on the effects of EDCs in fish behaviour is scarce. Since it was demonstrated that EE_2 disturbs zebrafish behaviour patterns, future works should contemplate the effects of other chemicals in fish behaviour in order to allow the integration of behavioural endpoints in the toxicity assessment of EDCs.

This work established a link between EE_2- and TBT-induced adverse effects and hypothetical molecular mechanisms of action. In particular, molecular assays should be further validated to foster its application in environmental monitoring of priority and emergent contaminants. Data from these studies will contribute to improve the understanding of mechanisms of toxicity, as examining effects on the molecular level is likely to provide highly sensitive, mechanism-based and potentially predictive molecular biomarkers.

Literature studies have shown the genotoxic effects of EE_2 and TBT in somatic cells. However, very little attention has been given to genetic damage in the gametes and/or embryos. To be reproductively viable, sperm cells must possess intact genomic DNA. If the DNA integrity is compromised, then reproduction and ultimately population viability could be at risk. In our study, comet assay analysis revealed an increase in the percentage of DNA damage in gonads of exposed

males. Future research should focus on karyotype studies in order to confirm the occurrence of aneuploidy as a result of DNA damage.

A major gap on EDCs hazard assessment is the lack of information regarding the effects of complex mixtures of chemicals. Single ecotoxicological evaluations may not reflect the multitude of stimulus that wild populations may face. This study highlights the effects of combined contaminants exposure in embryo development and DNA repair pathways, further supporting the emergent issue of mixtures toxicity in risk assessment.