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LEAE

Impacts and mechanisms of action of endocrine disrupting chemicals on the hermaphroditic freshwater gastropod Lymnaea stagnalis (Linnaeus, 1758)

A Thesis submitted for the degree of PhD in Sciences
by
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Cover page photography: *Lymnaea stagnalis* by Maël Dugué ©

Résumé

Des molécules connues pour leur capacité d'interférer avec le système endocrinien des vertébrés peuvent engendrer, à de faibles concentrations, des effets néfastes sur la reproduction des invertébrés. Ces produits, appelés perturbateurs endocriniens, peuvent à terme avoir des conséquences préjudiciables sur la croissance et la survie des populations exposées. Parmi les espèces d'invertébrés touchées par les perturbateurs endocriniens, les mollusques voient leur reproduction affectée par de nombreuses molécules à des concentrations inférieures que celles atteignant d'autres phyla. Les mollusques ont donc été proposés comme espèce-modèle pour l'évaluation de la reprotoxicité des produits chimiques, dont font partie les perturbateurs endocriniens, dans les guidelines développées par l'OCDE. *Lymnaea stagnalis*, une espèce de gastéropode dulçaquicole hermaphrodite, a été proposée comme espèce de référence pour le développement de ce type de test. Le premier objectif de cette thèse consiste à définir les impacts de différentes molécules, choisies sur base de leurs interactions avec le système endocrinien des vertébrés, sur la reproduction de *L. stagnalis*. Son deuxième objectif vise à apporter une meilleure compréhension des mécanismes d'action de ces perturbateurs endocriniens. Dans ce travail, nous avons testé plusieurs produits choisis sur base de leurs interactions avec le système endocrinien des vertébrés. La testostérone est le ligand naturel du récepteur aux androgènes. Trois autres molécules sont des antagonistes avérés de ce récepteur : l'acétate de cyprotérone qui est un stéroïde synthétique, la vinclozoline qui est un fongicide dicarbamide, et le fénitrothion qui est un insecticide organophosphoré. La chlordécone, un pesticide organochloré, peut se lier au récepteur aux oestrogènes. Enfin, les deux organoétains, triphenyltin (TPT) and tributyltin (TBT), peuvent induire une masculinisation des femelles, consistant en la formation additionnelle de caractères masculins (l'imposexe) chez les gastéropodes prosobranches.

Les résultats de ce travail sont divisés en deux volets. Le premier regroupe les résultats obtenus sur les impacts de ces substances sur la reproduction de *L. stagnalis*. Le nombre total d'œufs (fécondité) et de pontes (oviposition) produits par individu sur toute la période d'exposition à ces molécules nous a permis d'évaluer l'impact des différentes substances. De plus, l'analyse de la qualité des œufs produits a montré que toutes les molécules testées, excepté le TPT, peuvent augmenter la fréquence des œufs malformés produits par les individus exposés. Les œufs polyembryonés (contenant plusieurs embryons par œuf) sont les plus fréquemment produits parmi tous les œufs malformés. Etant donné que ces effets sur la qualité des œufs sont observés à des concentrations plus faibles que celles affectant l'oviposition et la fécondité, l'inclusion de ce paramètre lors de l'analyse des impacts de reprotoxicité sur *L. stagnalis* pourrait améliorer la sensibilité des tests, particulièrement pour l'étude des perturbateurs endocriniens.

Le second volet des résultats, présentés dans les chapitres 3 et 4, nous a permis de mieux comprendre les mécanismes d'action des molécules induisant des effets sur la reproduction de *L. stagnalis* suite à des interactions probables avec le système endocrinien de cette espèce. Dans le chapitre 5, nous avons analysé les impacts de la testostérone, de l'acétate de cyprotérone, de la chlordécone et du tributylétain sur les concentrations en testostérone sous forme libre et estérifiée présentes dans les tissus d'individus exposés pendant 21 jours. La testostérone est une hormone stéroïdienne de « type vertébré » qui a été isolée dans les tissus de nombreuses espèces de mollusques. Nos résultats ont confirmé que les lymnées peuvent incorporer ce stéroïde depuis le

milieu environnant. De plus, chez cette espèce comme chez d'autres espèces de mollusques, une régulation homéostatique des concentrations en testostérone permet de stocker, dans les tissus adipeux, les excès de testostérone en forme libre sous une forme estérifiée. Cependant, dans le premier volet des résultats, les impacts de ces molécules sur la reproduction n'ont pu être mis en relation avec les modifications des concentrations en testostérone. Dès lors, le rôle joué par cette hormone stéroïde dans la reproduction de la lymnée n'a pu être élucidé. Dans le chapitre 6, des analyses protéomiques (2D-DIGE, western blot) ont été réalisées afin d'identifier les impacts de la testostérone, de l'acétate de cyprotérone, de la chlordécone et du tributylétain sur l'expression de protéines extraites des tissus reproducteurs des individus exposés pendant 21 jours. Ces techniques d'analyse nous ont permis d'identifier des protéines qui jouent un rôle dans la reproduction de cette espèce (yolk ferritin, ovipostatin et PIWI). Les altérations d'expression de ces protéines pourraient expliquer les effets de ces molécules sur la reproduction de *L. stagnalis* observés dans les chapitres précédents.

En intégrant les résultats obtenus au cours de cette thèse, il apparaît que les produits testés peuvent induire des impacts néfastes sur la reproduction en interagissant avec le système endocrinien de *Lymnaea stagnalis*. De plus, l'étude de la qualité des œufs, ainsi que l'analyse de protéines impliquées dans la reproduction, constituent indéniablement des outils utiles pour évaluer la reprotoxicité de ces molécules chez *Lymnaea stagnalis*.

Summary

Adverse impacts exerted by molecules called endocrine disruptors on reproduction have been extensively described in vertebrates. However, endocrine-disrupting chemicals might alter reproduction of invertebrate species, at lower concentrations or through different modes of action, with consequences on population growth and stability. Among invertebrates, molluscs have been shown to be very sensitive to endocrine disruptors and were therefore proposed as model species for the development of OECD guideline for the testing and the assessment of reprotoxic effect of chemicals, including EDCs. The hermaphrodite gastropod species *Lymnaea stagnalis* is a candidate species for the development of such guidelines. In this context, the aim of this thesis is to provide a better understanding of the impacts and the mechanisms of action of endocrine disrupting chemicals on the reproduction of *L. stagnalis*. The chemicals tested in the present work were chosen based on their interactions with the endocrine system of vertebrates. Testosterone is the natural ligand of the androgen receptor in vertebrates. Three different molecules were selected for their antagonism of the androgen receptor in vertebrates: the synthetic steroid cyproterone acetate, the dicarbamide fungicide vinclozolin and the organophosphate insecticide fenitrothion. Chlordecone is an organochlorinated pesticide, which binds oestrogen receptors in vertebrates. Finally, two organotin compounds, triphenyltin and tributyltin, are able to induce the development of imposex, imposition of male sex organs in females, in gonochoric gastropod molluscs.

Results of the present work are divided into two main sections. Firstly, we investigated the impacts of the selected chemicals on the reproduction of *L. stagnalis*. Our results displayed that TBT, TPT, and chlordecone are able to induce adverse impacts on the reproduction of *L. stagnalis* through a reduction of the cumulative number of eggs and clutches produced per individual. The assessment of the quality of eggs produced showed that exposure to the chemicals, except TPT, increases the frequency of abnormal eggs laid. The impacts on egg quality were observed at lower concentrations than those affecting the classical reproductive endpoints assessed in reprotoxicity tests in this species. Our results highlight that the implementation of egg quality as an endpoint should increase the sensitivity of the reprotoxicity test with *L. stagnalis*.

The second section of results aims at providing new insights on the mechanisms of action of putative endocrine disruptors in *L. stagnalis*. Results from chapter 3 and 4 allowed us to select chemical treatments (testosterone, tributyltin, cyproterone acetate, and chlordecone) that induced adverse effects, by interacting with the endocrine system, on the reproduction of *L. stagnalis*. In Chapter 5, we analysed endogenous concentrations of testosterone, in its free and esterified forms, in the reproductive organs of *L. stagnalis*. Exposure to waterborne testosterone has confirmed that molluscs can take up this steroid from environmental media and that a homeostatic regulation of endogenous concentrations occurs through the esterification of steroids to fatty acid esters. Moreover, our results support that testosterone is endogenously synthesised in *L. stagnalis*, as it has been suggested in other mollusc species. However, the impacts on the reproduction and the modifications of endogenous concentrations of testosterone reported in this thesis could not be associated. In chapter 6, proteomic experiments were used to identify proteins differently expressed in reproductive organs of *L. stagnalis* exposed to testosterone, tributyltin, cyproterone acetate, and chlordecone.

Modifications in the expression of three proteins involved in *L. stagnalis* reproduction (i.e., ovipostatin, yolk ferritin and PIWI) could be associated with the alterations of reproductive endpoints reported in chapters 3 and 4, therefore suggesting possible endocrine disruption mechanisms.

Altogether, the results obtained in the present work provide evidences of interactions between chemicals and the endocrine system of this hermaphrodite species. Moreover, the additional sensitive reproductive endpoints described in this thesis (i.e., egg quality) as well as the identification of particular proteins (i.e., ovipostatin, yolk ferritin and PIWI) and their differential expression patterns following chemical exposure, provide reliable tools to screen potency of toxicants to be endocrine disruptors in a partial life cycle test on the freshwater gastropod *Lymnaea stagnalis*.

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Acronyms List

2D-DIGE: two-dimensional differential in-gel electrophoresis
AEAT: fatty acid acyl CoA:estradiol acyltransferase
AEC: time-weighted average exposure concentrations
AR: androgen receptor
ATAT: fatty acid acyl-CoA:testosterone acyltransferase
BCF: bioconcentration factor
BPA: bisphenol A
CAS: Chemical Abstract Service
CDCs: caudo-dorsal cells
CDNB: 1-chloro-2, 4 dinitrobenzene
CHCH: caudo-dorsal cell hormone
CLD: chlordecone
CMR: carcinogenic, mutagenic and reprotoxic
CPA: cyproterone acetate
CYPs: cytochrome P450 enzymes
DB: dorsal body
DBH: dorsal boby hormone
DBT: dibutyltin
DDE: dichlorodiphenylchloroethane
DDD: dichlorodiphenyldichloroethane
DDT: dichlorodiphenyltrichloroethane
DES: diethylstilboestrol
DHEA: dehydroepiandrosterone
DPT: diphenyltin
ECD: electron capture detector
ECx: effect concentration
EDs: endocrine disruptors
EDCs: endocrine disrupting chemicals
EDTA: endocrine disrupter testing and assessment
E2: 17ß-oestradiol
EE2: ethyniloestradiol
ER: oestrogen receptor
EROD: ethoxyresorufin-O-deethylase
FEN: fenitrothion
GC: gas chromatography
GSH: glutathione
GSTs: glutathione S-transferases
HSDs: hydroxysteroid dehydrogenases
HPLC: High-performance liquid chromatography
IPCS: International Programme on Chemical Safety
IUPAC: International Union of Pure and Applied Chemistry
LBD: ligand binding domain
LCx: lethal concentration
LOEC: lowest-observed effect concentration
MBT: monobutyltin
MPT: monophenyltin
MS: mass spectrometry
NAD(P)H: nicotinamide adenine dinucleotide (phosphate)
NCBI: National Center for Biotechnology Information
NOEC: no-observed effect concentration
NP: nonylphenol
OECD: Organisation for Economic Co-operation and Development
OP: octylphenol
ORFs: open reading frames
PAHs: polycyclic aromatic hydrocarbons
PCBs: polychlorinated biphenyls
PCDDs: polychlorinated dibenzodioxins
PPAR: peroxisome proliferator-activated receptors
PROD: penthoxyresorufin-O-deethylase
RIA: radio immuno assay
RPSI: relative penis size index
RXR: retinoid X receptor
SD: standard deviation
SEM: standard-error of the mean
SPE: solid phase extraction
SULT: sulfotransferases
T: testosterone
TBT: tributylin
TPT: triphenyltin
UGT: UDP-glucuronosyl transferase
US-EPA: US Evironmental Protection Agency
VDSI: *vas deferens* sequence index
Vtg: vitellogenin
VZ: vinclozolin
WHO: World Health Organisation
YES: yeast estrogen screen

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Foreword

The impact of molecules called endocrine disruptors has been extensively described. These impacts have primarily been reported in vertebrates and their modes of action are, in general, well understood in mammals and fishes. The recognition of environmental threats exerted by these molecules, either natural or synthetic, is recent and the authorities are still trying to improve the legislation about their production and the use of chemicals that may affect growth and survival of natural populations organisms. Up to now, the European legislation has included the requirement of specific authorisations for of the use of three types of molecules that are: substances which are carcinogenic, mutagenic or reprotoxic (CMRs); persistent, bioaccumulative and toxic (PBTs) or very persistent and very bioaccumulative (vPvBs). Substances, such as those having endocrine disrupting properties (EDCs), for which there is scientific evidence of probable serious effects to human health or the environment have recently been added to this list. However, the ED potential assessment of substances is limited due to the low number of standardised tests available.

Therefore Organisation for Economic Co-operation and Development (OECD) aims to establish standardised method guidelines for the testing and the assessment of chemicals, which may induce adverse impacts on the environment. Since 1996, and the first definition of endocrine disruptors, the OECD has developed and improved guidelines for the testing of endocrine disruption potency of chemicals. *In vitro* and *in vivo* screening and testing guidelines were first released in 2002. *In vitro* or *in vivo* screening tests help to assess the potency of a molecule to be an endocrine disruptor. Test guidelines for the assessment of endocrine disruptors are well developed for vertebrate species (rodents, amphibians and birds). However, endocrine disrupting chemicals might induce adverse impacts on invertebrate species at lower concentrations or through different modes of action with consequences on population growth and stability. Among invertebrates, molluscs were shown to be very sensitive to endocrine disruptors and were therefore proposed as model species for the development of a guideline for the testing and the assessment of reprotoxic effect of chemicals. A guideline for a partial life cycle test on mollusc is currently under development by a consortium of European laboratories.

The aim of this thesis is to provide a better understanding of the impacts and the mechanisms of action of endocrine disrupting chemicals (EDCs) on the hermaphrodite pulmonate gastropod species, *Lymnaea stagnalis*.

Part 1:

Introduction

1. Endocrine disrupting chemicals

The term endocrine disruptor was first defined in 1996 as "an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary (or consequent) to changes in endocrine function (Weybridge 1996)". Later, in 2002, the World Health Organisation (WHO) appended the term "(sub)population" to this definition (IPCS 2002):

"An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations."

This definition implies that exposure to chemicals results in adverse effects on individuals and/or populations and these effects are due to interactions of the chemicals with the endocrine system. This system consists in glands that are responsible for the production and release of hormones in the organism. Hormones are chemical substances that present specific effects on the activity of cells and organs at low concentrations, hence allowing the regulation of the development, growth, reproduction, and homeostatic mechanisms in the organism (US-EPA 1997). Environmental and physiological stimuli induce the secretion of neuropeptides by the major neuroendocrine glands (e.g., hypothalamus, pituitary), then hormonal cascades act through different endocrine glands to finally exert important effects on target organs (deFur et al. 1999; US-EPA 1997).

Endocrine disruptors induce adverse impacts by interacting with hormone synthesis, hormone storage and/or release, hormone transport or clearance, hormone receptor binding or the post-receptor activation (IPCS 2002). A wide variety of chemicals can interact with the endocrine system in organisms, including natural and synthetic hormones, pesticides, plasticisers, and industrial by-products (Table 1.1). Some chemicals were purposely designed to interfere with the human endocrine system, such as pharmaceutical designed for hormonal treatment (e.g., anti-thyroid drugs, contraceptive pills, cancer therapy), or with the endocrine system of other organisms, such as insecticides, fungicides, and herbicides used in pest control (e.g., alteration of insect growth and moulting, alteration of steroid synthesis in fungi).

Moreover, some industrial products and by-products have shown endocrine disrupting potency with endocrine systems as side effects (i.e., phthalates, organotins, alkylphenols).

Action/ Hormone Target	Chemicals	Organisms impacted
Hormone agonist		
Oestrogen receptor (ER)	Ethyniloestradiol (EE2), alkylphenols, organochlorides	Mammals, amphibian, reptile, Fish, mollusc
Androgen receptor (AR)	17ß-trembolone, Pulp Mill Effluents	Fish
Glucocorticoid receptor	Synthetic glucocorticoids (e.g., dexamethasone)	Fish
Retinoid X receptor (RXR)	Organotins (e.g., TBT)	Molluscs
Ecdysteroid receptor	Pesticides	Invertebrates
Hormone antagonist		
Oestrogen receptor (ER)	DDT	Amphibians
Androgen receptor (AR)	Phthalates, vinclozolin, cyproterone acetate	Mammals, fish, molluscs
Glucocorticoid receptor	Bisphenol A	Mammals
Thyroid receptor	$MeSO_2$-PCBs	Mammals
Enzyme induction/inhibition		
Cytochrome P450 enzymes	Atrazine, azole fungicides	Amphibians, reptiles, fish
Hormone transport/metabolism		
Oestrogens	PCBs, nonylphenols	Mammals, fish
Thyroids	PBDEs	Mammals
Progesterone	DDT, dieldrin	Birds, fish
Testosterone	DDT, dieldrin	Birds

Table 1.1: Interaction of chemicals with the endocrine systems of animals. (From (Matthiessen 2013))

Environmental release of these endocrine disrupting chemicals (EDCs) may be intentional (e.g., pesticides) or may be due to unintentional release during the different stages of the chemical’s life cycle, from the manufacturing process to the use of the compounds (e.g., polychlorinated biphenyls (PCBs), alkylphenols). Depending on the physicochemical properties of the EDCs, the molecules degrade and behave differently in the environment and are submitted to different metabolic pathways in animals. Therefore, exposure to EDCs may occur through air, water, sediment, soil, and food. The latter route of exposure is considered as the major route of exposure for wildlife, especially for persistent, lipophilic organic molecules such as organochlorines (e.g., PCBs) and organotins (e.g., tributyltin (TBT)), which may bioaccumulate and biomagnify in organisms throughout the trophic chain (IPCS 2002). Aquatic ecosystems are usually

the more sensitive and the more exposed compartments to EDCs, as water and sediment constitute the final sinks for chemicals that can be dissolved and/or bind to organic matter. Therefore, aquatic vertebrates and invertebrates, as well as terrestrial animals that feed on aquatic organisms, are particularly susceptible to be affected by EDCs.

Field observations have highlighted that some deleterious effects induced on wildlife could be attributed to endocrine disruption phenomena such as abnormal thyroid function in birds and fish, alterations of immune function in birds and mammals, decreased hatching success in fish, birds and reptiles (Colborn et al. 1994). Adverse impacts on the reproduction of vertebrates, such as defeminisation and masculinisation of fish, reptiles, birds and mammals, have been attributed to interactions of chemicals with steroid hormone pathways (Fry 1995; Gray et al. 1994; Guillette et al. 1994; Jobling et al. 1998). Sex steroid hormones (i.e., testosterone, oestradiol, progesterone) synthesis and release are under the hypothalamic-pituitary-gonadal control. These hormones act by binding specific receptors thereby activating gene transcription which, in turn, leads to the development and growth of reproductive organs (IPCS 2002).

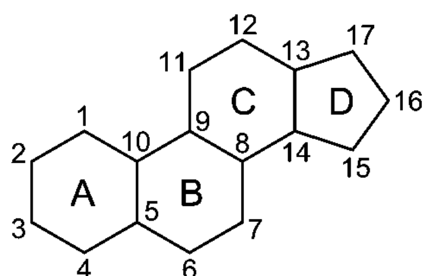

Fig.1.1: cyclopenta[*a*]phenanthrene skeleton of steroids consists of three cyclohexane cycles (A,B and C) and one cyclopentane cycle (D). Numbers correspond to the IUPAC numerations of carbon atoms of the steroid ring.

All steroids share a common structure characterised by the cyclopenta[*a*]phenanthrene skeleton, which consists in three cyclohexane cycles (A, B and C rings) and a cyclopentane cycle (D ring) (Fig.1.1) (IUPAC 1989). The properties of the different steroid molecules are therefore due to the different side chains adjoined to this skeleton. In vertebrates, steroidogenesis is the process of biotransformation of the main steroid precursor, cholesterol, in different steroid hormones such as glucocorticoids, mineralocorticoids and sex steroid hormones (Sanderson and van den Berg 2003). The biosynthesis of sex steroid hormones (i.e., testosterone, oestradiol and progesterone) occurs in gonads and is mediated through the action of different key enzymes (Fig. 1.2). In vertebrates, these hormones play an important role in gender

differentiation during embryonic development and further control the development and maturation of gonads and gametes. Therefore endocrine disrupting chemicals can induce adverse effects on the reproduction of organisms, either by direct binding to steroid receptors, or by altering hormone synthesis (e.g., interaction with enzymes involved in steroidogenesis). Thus, EDCs typically alter the reproduction of organisms by more than one particular mechanism (IPCS 2002; Lintelmann et al. 2003; US-EPA 1997) (Table 1.1). Generally, the endocrine disrupting potency of molecules is assessed by *in vitro* tests, providing insight on the receptor binding affinity of the molecule and its agonist or antagonist activity (e.g., receptor binding assay, yeast estrogen screen (YES)). Even though synthetic steroids are purposely designed for steroid binding and used in medical treatments, chemicals with totally different structures are able to bind and regulate receptor activation with a lower affinity than their natural ligand and synthetic steroids. Yet, due to the complexity of endocrine systems and the differences in the metabolisation of molecules among taxa, and even among species, and due to the multiple modes of action of these molecules, analysing the impacts of EDCs *in vivo* is essential to provide a more comprehensive understanding of the impact and mode of action of these compounds on organisms (IPCS 2002).

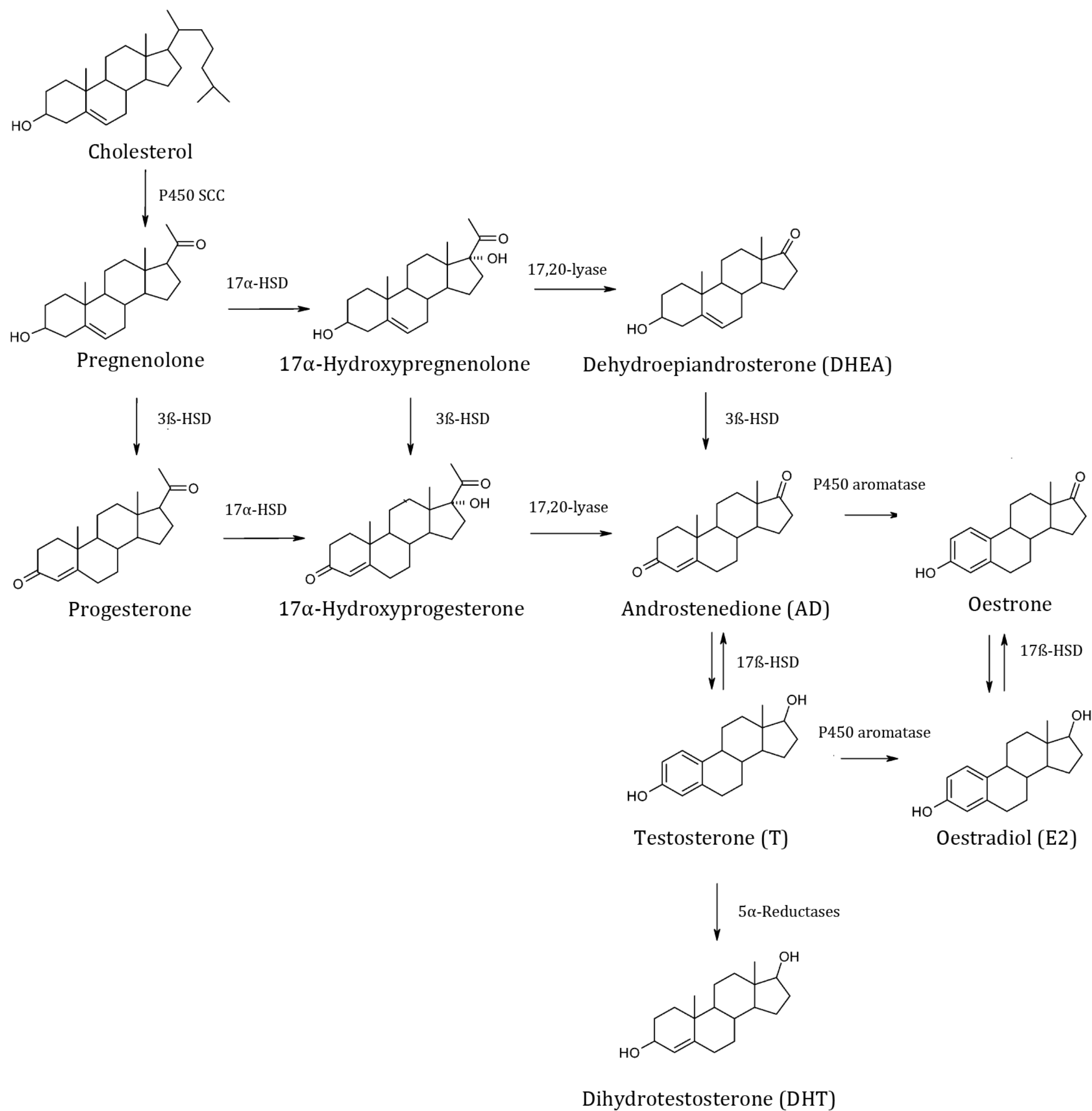

Fig. 1.2: Steroidogenesis of sex steroids in vertebrates. P450scc: Cytochrome P450 side cleavage chain enzyme, 3α/ß-HSD: 3α/ß-hydroxysteroid dehydrogenases, 17α/ß-HSD: 17α/ß-hydroxysteroid dehydrogenases.

1.1. Impacts of EDCs on vertebrates

Many pesticides were identified as potent binders of the vertebrate sex steroid receptors (i.e., androgen and oestrogen receptors) in *in vitro* studies, resulting in the activation or repression of the steroid receptor mediated responses (Fang et al. 2003; Fang et al. 2000). Therefore, EDCs can induce either androgen (AR) or oestrogen receptor (ER) mediated impacts on the reproduction of organisms through either agonist or antagonist action. However, all the compounds that showed *in vitro* steroid receptor binding did not necessarily induce adverse impacts *in vivo* (Daxenberger 2002; IPCS 2002).

The oestrogenic activity of synthetic chemicals was first reported in 1936, and oral administration of such chemicals, e.g., diethylstilboestrol (DES), was shown to restore vaginal oestrus in ovariectomised rats or to interrupt early pregnancy in rodents (Dodds and Lawson 1936; Dodds and Lawson 1938; Parkes et al. 1938). Therefore, these molecules were proposed as oral contraceptives or as medical treatments (e.g., ovarian insufficiency, amenorrhea) in humans. Another molecule, bisphenol A, was synthesised in the 1890s and is now found ubiquitously in food and beverage plastic storage, heating containers and in line metal cans (vom Saal et al. 2007). This molecule has affinity for the oestrogen receptors and was shown to be as potent as oestradiol for membrane oestrogen receptors binding and activation in mammals (Quesada et al. 2002). Organochlorinated compounds such as dichlorodiphenyltrichloroethane (DDT), extensively used as an insecticide in the 1940s and 1950s, and polychlorinated biphenyls (PCBs), mainly used to insulate fluids in transformers and capacitors, have shown oestrogenic activity in rats. These molecules are now known to bind human oestrogen receptors (i.e., ERα and ERß), however with relatively low affinity (from 20 to 10000 times less potent than the natural ligand, 17ß-oestradiol) (Kuiper et al. 1998).

Exposure to 17α-methyltestosterone, a synthetic steroid chemical used to cure androgen insufficiency in men, induces the formation of breeding tubercles and masculinised females in fish species (Ankley et al. 2001; Smith 1974). Androgen mediated impacts were observed in male rats exposed *in utero* and *in vivo* to vinclozolin and to DDE (a metabolite of DDT) (Gray et al. 1994; Kelce et al. 1997; Ostby et al. 1999). Vinclozolin (VZ) is a dicarbamide fungicide, and its butenoic acid (M1) and enanilide

(M2) metabolites are able to compete with androgens for AR bindings (Kelce et al. 1997; Ostby et al. 1999; Wong et al. 1995). Laboratory exposure of vertebrates to vinclozolin have shown to induce anti-androgenic alterations (e.g., agenesis of the prostate in male rats (Ostby et al. 1999), intersex in fish (Kiparissis et al. 2003)).

Furthermore, some chemicals are able to interact with the activity of enzymes involved in steroidogenesis pathways. For example, azole chemicals are anti-fungal agents that have been developed to inhibit the biosynthesis of ergosterol, an essential component of membranes of fungi and yeast. These pesticides act through the inhibition of a cytochrome P450 enzyme, the 14α-demethylase, which is involved in the demethylation of lanosterol to ergosterol (Whitehead and Rice 2006; Zarn et al. 2003). Other cytochrome P450 enzymes (CYPs) are involved in vertebrate steroidogenesis and it was observed that several azoles were able to bind and to potently inhibit the activity of cytochrome P450 aromatase (CYP19), the enzyme that catalyses the conversion of androgens to oestrogens (Ronis et al. 1994; Trosken et al. 2004; Whitehead and Rice 2006). Exposure of organisms to aromatase inhibitors may decrease the levels of oestradiol, which alter the development of female organs, therefore leading to reproductive impairments (Zarn et al. 2003), e.g., exposure of the fish species *Pimephales promelas* to the ketoconazole fungicide reduces the number of spawning and the number of eggs produced per female (Ankley et al. 2007).

Interestingly, it was reported that molecules that are strong ER binders, such as natural oestrogens (i.e., oestradiol and oestriol), phytoestrogens (e.g., genistein, zearalenone), and synthetic oestrogens (e.g., DES), can also be potent binders to AR, however with different behaviour. Synthetic chemicals that were not designed for steroid receptor binding, such as DDT and DDE, were also reported to be potent steroid receptor binders (Fang et al. 2003; Fang et al. 2000). Similarly, some CYPs inhibitors (e.g., imidazoles fungicides) can bind to mammalian oestrogen receptors (Fink et al. 1999). These observations highlight the difficulty to predict the impacts of chemicals on the endocrine system of organisms *in vivo* based solely on *in vitro* assays and emphasise that these impacts are mediated through different mechanisms of action.

Because of the massive worldwide use of molecules known for their oestrogenic and androgenic properties in vertebrates, either natural (e.g., natural steroids, phytoestrogens or mycotoxins) or synthetic (e.g., DES, DDT or vinclozolin), their presence in the environment has been reported for nearly 30 years (McLachlan et al. 1984). Synthetic oestrogens, as well as androgens, (i.e., hormones, veterinary drugs and pharmaceuticals) are found in wastewater treatment plant effluents, sewage sludge, pulp- and paper-mill effluents, and agricultural runoff at concentrations ranging from ng/L to μg/L (Andrew-Priestley et al. 2012; Bellet et al. 2012; Chen et al. 2010; Harries et al. 1999; Jeannot et al. 2002; Kolodziej et al. 2003; Kusk et al. 2011; Matthiessen et al. 2006; Nakada et al. 2004; Nieto et al. 2010; Orlando et al. 2004; Salste et al. 2007; Shappell 2006; Tashiro et al. 2003). Therefore aquatic ecosystems are exposed to a mixture of chemicals that induce adverse impacts on the reproduction of exposed organisms.

One of the most striking examples of the impacts of EDCs on wildlife is probably the demasculinisation of male alligators in lake Apopka, Florida, following a chemical spill. Male alligators were exposed to a mixture of DDT (and its metabolites: DDD and DDE), dicofol, chlorbenzilate and dichlorobenzophenone, and exhibited reduced penis length, abnormal gonadal development and lowered steroid levels, however the ratio of oestrogen/androgen steroid hormones was increased. Moreover, females of the Apopka population exhibited higher oestradiol levels together with polynuclear oocytes and polyovular follicles suggesting super feminisation (Guillette et al. 1994). Furthermore, a reduction of the hatching success of the eggs was linked to the reduction of juvenile alligator population observed in lake Apopka (Guillette et al. 1994; Woodward et al. 1993). Similar demasculinising impacts were observed on the red-eared turtle population of this lake. Even though the impacts of exposure of lake Apopka's reptiles following the chemical spill have been well documented over the last decades, the association with exposure to specific chemicals as well as the modes of action of the molecules are still not fully understood.

In the United Kingdom, field observations have reported the occurrence of hermaphrodite fishes downstream of sewage treatment plants (Purdom et al. 1994). These intersex fishes were characterised by the presence of both male and female

reproductive ducts and by the presence of female germ cells within male testis (Jobling et al. 1998). Furthermore, expression of vitellogenin, the precursor protein of egg yolk, which production is under the control of oestrogens in females, was shown to increase in juveniles and male rainbow trout, *Oncorhynchus mykiss*, exposed to these effluents (Purdom et al. 1994). It was postulated that these alterations were due to the oestrogenic compounds present in the sewage treatment plant effluents. Laboratory studies have confirmed that exposure to oestrogens lead to similar alterations to those reported in field experiments (i.e., intersex and vitellogenin induction in males) in several fish species (Jobling et al. 1998; Purdom et al. 1994).

Moreover, it was reported that exposure to agricultural run off could also induce adverse impacts on wildlife by either initiating or repressing the androgenic signal transduction (Daxenberger 2002). Downstream of a paper mill effluent in Florida, female poeciliid fish exhibited male gonopodium (i.e., a modified anal fin used by males during mating to transfer sperm to female genitalium) (Bortone and Cody 1999; Howell et al. 1980). This masculinisation of fish was linked to the androgen chemicals found in these waters based on *in vivo* assessment (Parks et al. 2001).

The impacts and the mechanisms of action of EDCs were mainly investigated on vertebrate species. However, invertebrates represent almost 95 % of the animal kingdom and are key species in aquatic ecosystems (deFur et al. 1999). Therefore, it is necessary to identify and understand in more depth the impact of endocrine disrupting chemicals on invertebrates. For over 30 years, field and laboratory studies have reported adverse impacts of different chemicals on mollusc reproduction. Exposure to low environmental concentrations (ng/L range) of EDCs was shown to induce the feminisation of bivalve molluscs as well as the androgenisation of gastropods (Lagadic et al. 2007; Matthiessen 2008). In the most adverse cases, reproduction was totally inhibited which resulted in a considerable reduction of mollusc populations in the most contaminated areas.

2. EDCs on molluscs

More than 130 000 mollusc species have been described and are classified in seven classes (i.e., Aplacophora, Polyplacophora, Monoplacophora, Gastropoda, Bivalvia, Scaphopoda and cephalopoda). They are ubiquitously distributed and represent key species in ecosystems, especially in aquatic ecosystems, in which they occupy different trophic levels (from primary consumers to top predator) (deFur et al. 1999; Lafont and Mathieu 2007).

2.1. Impacts on reproduction

Sexual abnormalities were first observed in the neogastropod mollusc, *Nucella lapillus*, on the coast of the United Kingdom in the early 1970s. Neogastropods are gonochoric species (sexes are separated), however a penis-like outgrowth was observed behind the right tentacle of females (Blaber 1970). Similarly, females of the mud snail *Ilyanassa obsoleta* (previously named *Nassarius obsoletus*) exhibiting a penis and a sperm duct (i.e., *vas deferens*) were observed on the east coast of the United States. These abnormalities of sex organs were named *imposex*, which was defined as the superimposition of male sex organs on female (Smith 1971) (Fig. 1.3). This phenomenon was already observed in 39 gastropod species in Europe and the US when field surveys have highlighted that imposex incidence in *I. obsoleta* was associated with the proximity of harbours and marinas and therefore to chemicals leaking from antifouling paints, and more precisely to tributyltin (TBT), an organotin compound (Smith 1981a; Smith 1981b). Since then, the masculinising impact of TBT was observed in several gastropod species during field surveys all over the world (e.g., the European *Littorina littorea*, *Nassarius reticulatus*, the Japanese rock shells *Thais clavigera* and *Thais bronni* and the South American *Buccinanops cochlidium* (Barroso et al. 2005; Bauer et al. 1997; Bigatti et al. 2009; Horiguchi et al. 1994)). Following these studies, a progressive ban on the utilisation of organotin-based antifouling paints was initiated in France and soon after in the UK, the US, and Canada until a complete prohibition of its use was adopted in 2008 (IMO 2005).

Laboratory experiments have shown a concentration-dependent development of imposex in *I. obsoleta* exposed to TBT (Smith 1981b). Six different stages of imposex intensity were described depending on the sensitivity of species and on TBT concentrations (Fig 1.4) (Bettin et al. 1996; Stroben et al. 1992). In the most adverse cases, sterilisation of females was observed and associated with the modification of the oviduct, which leads to the inhibition of copulation or of the egg capsule deposition (Matthiessen and Gibbs 1998; Stroben et al. 1992). Nowadays, imposex development caused by TBT exposure has been reported in over 200 mesogastropod and neogastropod species worldwide (Horiguchi et al. 2012). In the periwinkle, *Littorina littorea*, adverse impacts of TBT (i.e., reduction in egg production and few juveniles) have been reported in Germany and the United Kingdom, however imposex has never been observed. Thus, in this species, transformations of the pallial and genital organs into male morphological structures were described in females. In the most adverse cases, females developed a prostate gland and a sperm duct, which inhibited their reproductive capabilities. This phenomenon was named *intersex* (Bauer et al. 1997; Matthiessen et al. 1995; Oehlmann et al. 1998).

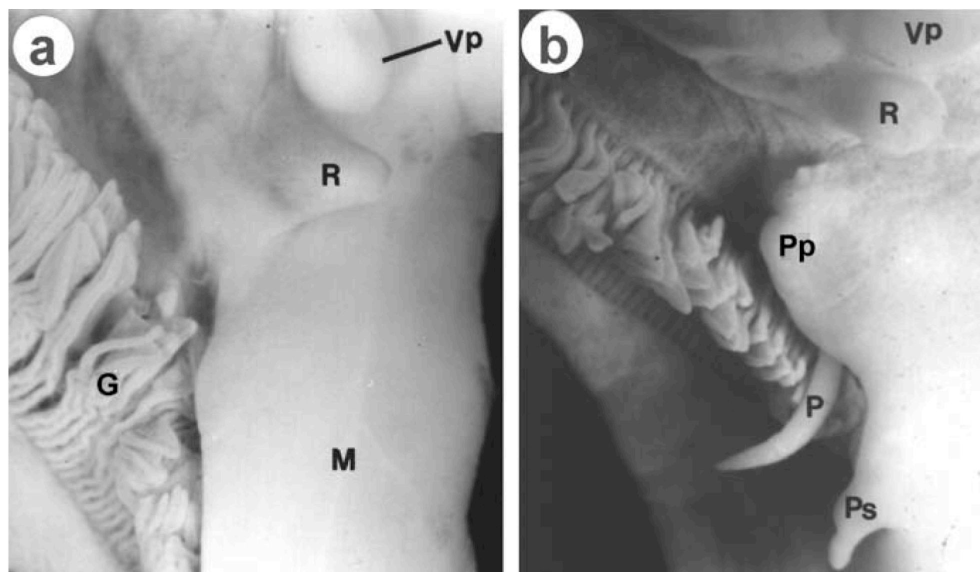

Fig. 1.3: Mantle cavity of *Marisa cornuarietis* females (a) without imposex, and (b) affected by imposex (stage 3). G: gills, M: mantle, P: penis, Pp: penis pouch, Ps: penis sheath, R: rectum, Vp: vaginal papilla. (From Shulte-Oehlmann et al. 2004).

Experimental studies have shown that gastropods were particularly responsive to TBT as concentrations as low as 1 ng Sn/L were sufficient to induce the development of the first stages of imposex (Matthiessen and Gibbs 1998). In some gastropod species, higher concentrations can also induce adverse impacts on gametogenesis (e.g., suppression of oogenesis and induction of spermatogenesis) (Gibbs and Bryan 1996).

The relative penis size index (RPSI) and the *vas deferens* sequence index (VDSI) were used to assess the severity of the development of imposex in laboratory experiments as well as in field surveys (Bryan et al. 1986; Gibbs and Bryan 1986). Because of the correlation between TBT concentrations and the stages of imposex in prosobranch gastropods, imposex was proposed as a biomonitoring tool for the evaluation of the pollution of coastal marine ecosystems (Oehlmann et al. 1996).

In bivalve molluscs, TBT is mostly known for its adverse impacts on the shell thickening of the oyster *Crassostrea gigas* cultured in Arcachon Bay, France (Alzieu et al. 1982). However, these impacts are not due to endocrine disruption. Nevertheless, the larval production decreased in the contaminated areas, suggesting this organotin can also induce adverse impacts on oyster populations through mechanisms that are probably linked to endocrine pathways (Alzieu 2000). In Canada, the zebra mussel *Dreissena polymorpha* and the clam *Mya arenaria* sampled in the Saint-Laurent River exhibited delayed gametogenesis that was correlated with the concentrations of organotins in sediments (Regoli et al. 2001; Siah et al. 2003). Furthermore, skewed sex ratio towards males were reported in clam populations exposed to organotin molecules (Gagné et al. 2003). These observations suggest that reproduction of bivalve molluscs could also be affected by TBT through endocrine disruption. Experiments confirmed that exposure to TBT reduced the production and release of embryos in bivalve species, but at higher concentrations (approximately 250 ng Sn/L) than those reported to affect larval development and shell growth (Ruiz et al. 1995; Thain et al. 1986).

The impacts of endocrine disrupting chemicals on bivalve molluscs are mainly reported following exposure to oestrogenic compounds. Intersex, described as the development of ovotestis in males, was reported in bivalves from field and experimental studies (Andrew et al. 2010; Andrew-Priestley et al. 2012; Chesman and Langston 2006; Langston et al. 2007; Nice et al. 2003; Tankoua et al. 2012). Injection of 17ß-oestradiol (E2) in *C. gigas* oysters during early stages of maturation induced sex reversal from male to female (Mori et al. 1969) whereas spermatogenesis, oogenesis as well as gonad

development and spawning were stimulated following E2 injection in *Placopecten magellanicus* (Wang and Croll 2004; Wang and Croll 2006).

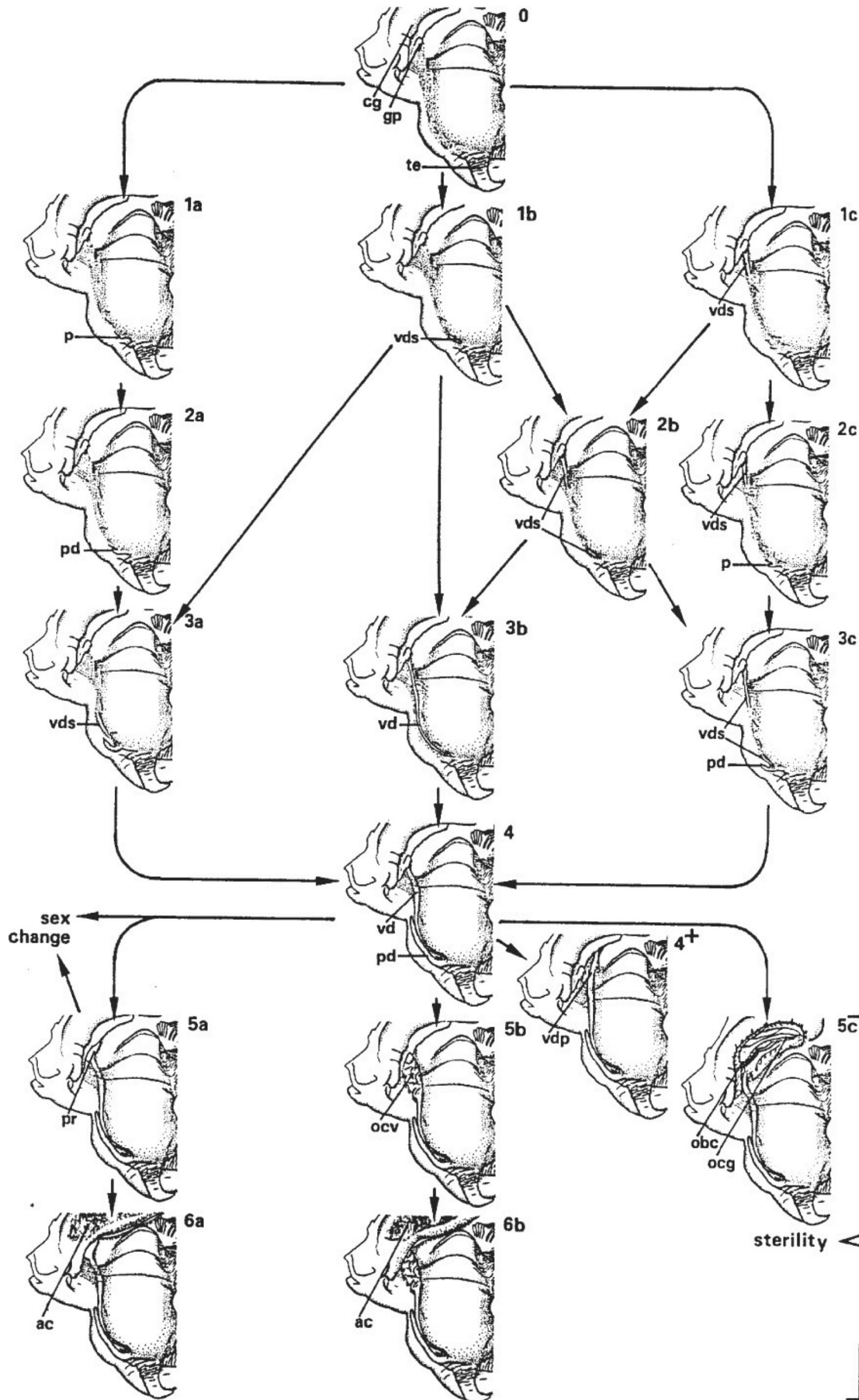

Fig. 1.4: Different imposex stages described in prosobranch molluscs. Stage 0: normal females. ac: abortive capsules, cg: capsule glands; gp: genital papilla, obc: open *bursa copulatrix*, ocg: open capsule gland, ocv: occlusion of the vulva, p: penis without duct, pd: penis with duct, pr: prostate, te: tentacle, vd: *vas deferens*, vdp: *vas deferens* passage into the capsule gland, vds: *vas deferens* section. (From Oehlmann et al. 1996).

Similarly to TBT, studies on the impacts of oestrogenic molecules were predominantly conducted on gastropod species. Exposure of the freshwater gonochoric prosobranch *Marisa cornuarietis* to bisphenol A (BPA) and to octylphenol (OP), at concentrations ranging from 0.1 to 100 µg/L, increased the production of eggs and clutches (Oehlmann et al. 2000). Exposure of the parthenogenetic prosobranch *Potamopyrgus antipodarum* to BPA, OP and nonylphenols (NP) through sediments induced a significant increase in embryo production after 8 weeks of exposure to 1 µg/Kg of BPA and OP and to 10 µg/Kg of NP (Duft et al. 2003). In this species, 28 days of exposure to waterborne BPA in the µg/L range increased the number of embryos produced and reared in the brood pouch of females (Sieratowicz et al. 2011). However, a study conducted by Forbes and colleagues reported that exposure of *M. cornuarietis* to 0.1, 1, 25 and 640 µg/L of BPA during 26 weeks had no impact on egg production, whereas female growth was reduced at 640µg/L and male growth was stimulated in the 1 µg/L treatment (Forbes et al. 2008).

The hermaphroditic pulmonate species *Biomphalaria taenogophila* when exposed to 0.1 and 1 mg/L of nonylphenol ethoxylate, an oestrogenic chemical, featured a decrease in oviposition and fecundity after 8 weeks of exposure (Oliveira-Filho et al. 2009). Ethinyloestradiol (EE2), a potent oestrogenic contraceptive pharmaceutical, was investigated for its potential impact on the reproduction of *M. cornuarietis*. During the main spawning season, which extends from October to March, the number of eggs laid was reduced at a concentration of 1 ng/L of EE2 whereas 10 ng/L increased fecundity before the onset of the main spawning period. These results suggest that seasonal differences impact the effects of EE2 in this species (Schulte-Oehlmann 2004). Exposure of the parthenogenetic snail *P. antipodarum* to 50 ng/L of EE2 increased its reproduction (Sieratowicz et al. 2011). A non-significant decrease in the number of eggs produced per individual was observed from 0.5 ng/L EE2 in the prosobranch species *Bithynia tentaculata,* whereas reproduction was stimulated in the simultaneous hermaphrodite *Radix balthica* (Hallgren et al. 2012). These results indicate species-specific responses to the synthetic steroid. The mode of reproduction is not the only difference between the species as they exhibit different feeding habits. Thus, it remains to be investigated if the differences in the reproductive endpoints observed following exposure to oestrogens could be linked to different reproductive pathways (Hallgren et al. 2012).

Overall, studies on the impacts of synthetic oestrogens on gastropod reproduction suggest that the responses and sensibility to EDCs concentrations are species-specific. Furthermore, as reported by Schulte-Oehlmann (2004), Oehlmann et al. (2006) and Hallgren et al. (2012), low concentrations tend to stimulate egg production whereas higher concentrations have a negative impact on the cumulated reproduction, highlighting the difficulty to assess the impacts of EDCs on mollusc reproduction.

The impacts of chemicals that bind androgen receptors in vertebrates, either as agonists or antagonists, have scarcely been described in molluscs. A five months exposure of *Nucella lapillus* and *Nassarius reticulatus* to 1.25 mg/L of cyproterone acetate (CPA), an AR antagonist pharmaceutical, induced a reduction of the penis size and of spermatogenesis in males. Comparable adverse impacts were observed after exposure to vinclozolin, an anti-androgenic fungicide, in the ng/L range (30 and 100 ng/L) (Tillmann et al. 2001). The AR agonist methyltestosterone induced imposex in *M. cornuarietis* females. Furthermore, spermatogenesis and egg production were adversely affected in the ng/L range in this species (Albanis et al. 2006; Schulte-Oehlmann 2004).

Even though impacts are generally well described (e.g., imposex formation in gonochoric gastropod species exposed to TBT), the mechanisms of action of molecules known for their androgenic and oestrogenic properties in vertebrates are still poorly understood in molluscs.

2.2. Impacts of EDCs on steroid metabolism in molluscs

Although endocrine systems of invertebrates regulate development, growth, and reproduction as in vertebrates, a wide range of different hormonal controls are found among the different invertebrate taxa, some being unique to specific phyla (deFur et al. 1999; Oehlmann and Schulte-Oehlmann 2003). This high variability is due to the complexity/diversity of reproductive systems (i.e., gonochorism, hermaphroditism and parthenogenesis) and of the life cycle (i.e., metamorphosis, diapause) of invertebrates (deFur et al. 1999; Lafont and Mathieu 2007). Endocrine controls in invertebrates are mainly mediated through neuropeptides, however invertebrate-specific steroids (i.e., ecdysteroids) and vertebrate-like steroids (i.e., testosterone, progesterone and

oestradiol) were found in different invertebrate taxa (Lafont and Mathieu 2007; Segner et al. 2003).

The molluscan endocrine system is under the control of neurosecretory glands of the central nervous system, which produce neuropeptide hormones (Geraerts 1976b; Geraerts and Algera 1976). However, vertebrate-like steroid hormones have been measured in cephalopods, bivalves and gastropods (Lafont and Mathieu 2007). Even though it has been suggested that molluscs accumulate steroids from the environment (Scott 2012), there is evidence suggesting that these steroid hormones are also synthesised *de novo*, as most of the enzymes involved in steroidogenesis have been observed in molluscs and have been shown to biosynthesise androgen precursors *in vitro* and *in vivo* (Gottfried and Dorfman 1970; Wootton et al. 1995). *In vitro* incubations of tissues (e.g., ovotestis, digestive gland) or microsomal fraction with radiolabelled vertebrate-like steroid precursors have helped to identify the major metabolites involved in steroidogenesis. Studies on the steroidogenesis in molluscs has been investigated in different species of gastropods (e.g., *Ariolimax californicus*, *M. cornuarietis*) (Gottfried and Dorfman 1970; Janer et al. 2005b) and bivalves (e.g., *Mytilus edulis*, *C. gigas*) (De Longcamp et al. 1974; Le Curieux-Belfond et al. 2001), show that most steps involved in steroid synthesis in mollusc are similar to vertebrate sex steroid synthesis reviewed in Fernandes et al. (2011) (Fig. 1.5).

Biosynthesis of cholesterol, the main sex steroid precursor in vertebrate, from acetate was reported to occur in molluscs (Gottfried and Dorfman 1969; Lupo Di Prisco et al. 1973). Similarly to vertebrates, in mussels pregnenolone is synthesised from cholesterol through the activity of P450 side cleavage chain enzymes, which are located in the basophilic cells in the digestive gland (Martínez et al. 2008). The following steps of steroid biosynthesis involve the activity of 3α/β- and 17ß-hydroxyteroid dehydrogenases (HSDs), which catalyse the reduction/oxidation of the keto/hydroxyl group located on the carbon 3 or 17 of the cyclopenta[*a*]phenanthrene backbone of steroids (cf. Fig. 1.1). In molluscs, as in vertebrates, 3α-HSD is involved in the synthesis of progesterone from pregnenolone, and of androstenedione from dehydroepiandrosterone (DHEA). Another enzyme, 5-α reductase, prevents the aromatisation of androgens to oestrogens and therefore reduces the levels of metabolites that are able to bind vertebrate androgen receptors (Wilson 2001). The presence of these enzymes was reported and their activities were observed in

microsomal fractions and in cytosol of different mollusc species (D'Aniello et al. 1996; Gottfried and Dorfman 1970; Janer et al. 2005b; Lupo Di Prisco et al. 1973). Finally, oestrogenic steroids are formed from androgen precursors by the activity of cytochrome P450 enzymes (CYPs). In vertebrates, aromatisation of a C19 steroid (androgen) to a C18 steroid (oestrogen) is catalysed by the cytochrome P450 aromatase (CYP19). In molluscs, few studies have reported the presence of this enzyme in gonads (Matsumoto et al. 1997; Osada et al. 2004) but the aromatase activity was observed in bivalves (Le Curieux-Belfond et al. 2001; Morcillo et al. 1998). Interestingly, it was reported that a sexual dimorphism in androgen metabolism occurs in the gastropod *M. cornuarietis*. In this species, males metabolise androstenedione to dihydrotestosterone and testosterone at a higher rate than females (Janer et al. 2005b).

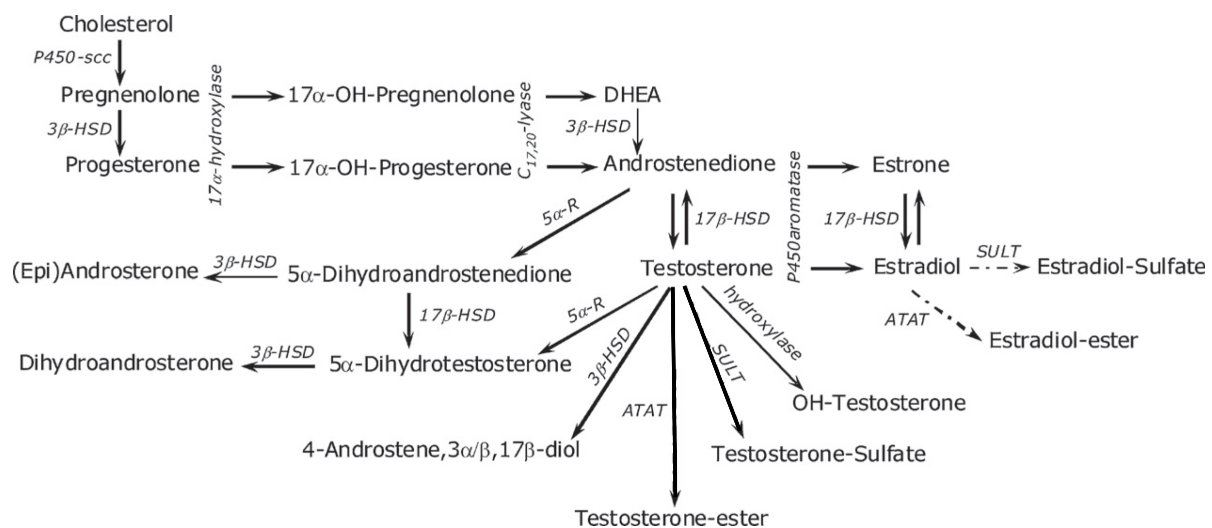

Fig. 1.5: Steroidogenesis and metabolic pathways described in molluscs through the measurement of radiolabelled steroids. P450scc: cytochrome P450 side cleavage chain enzyme, 17α/ß-HSD: 17α/ß-hydroxysteroid dehydrogenases, 3α/ß-HSD: 3α/ß-hydroxysteroid dehydrogenases, 5 α-R: 5 α-reductase, ATAT: fatty acid acyl-CoA:testosterone acyltransferase, SULT: sulfotransferases, DHEA: dehydroepiandrosterone. (From Fernandes et al. 2010).

Even though steroidogenesis occurs in molluscs, the biological role of these hormones remains to be discovered. Evidence of endogenous modifications of steroid concentrations in field and laboratory experiments according to the reproductive status of animals and gender differences in steroid titres highlight the possibility that testosterone and oestradiol might play a physiological role in mollusc reproduction. Furthermore, oestrogen receptors (ERs) with high homology with vertebrate ERs were found in different mollusc species (e.g., *Aplysia californica, Nucella lapillus, Lymnaea*

ollula, Octopus vulgaris, Mytilus edulis) (Castro et al. 2007b; Keay et al. 2006; Kumkate et al. 2009; Raingeard et al. 2013; Thornton et al. 2003). However, it was shown that the receptors identified were not able to bind oestradiol and that mollusc ERs is constitutively active and thus binds to DNA and activate the transcription of the classic oestrogen pathway without the need to be activated by an endogenous binding hormone (Keay et al. 2006; Thornton et al. 2003). Androgen receptors have never been identified in mollusc species. These observations contrast with the endogenous synthesis of vertebrate-like steroids, as the lack of receptors suggests that vertebrate-like hormones do not feature a biological activity in molluscs.

Retinoid X receptor (RXR) was recently identified in several mollusc species (Castro et al. 2007a; Nishikawa 2006; Nishikawa et al. 2004; Raingeard et al. 2013) and was shown to be highly similar to the vertebrate RXR. This steroid/thyroid hormone nuclear receptor needs to dimerise, either with itself (homodimer) or with another nuclear receptor (heterodimer) to activate DNA transcription (Dawson and Xia 2012). Retinoid signalling has been shown to be crucial for spermatogenesis in vertebrates (Chung and Wolgemuth 2004) whereas in the gastropod *I. obsoleta*, a recent study has highlighted that RXR signalling may regulate the male reproductive differentiation (Sternberg et al. 2008b). Recent studies have suggested that imposex induction by TBT could be mediated through the retinoid X receptor (RXR) as the natural ligand of this receptor, 9-*cis* retinoid acid, was also able to induce imposex. Moreover, organotin compounds (tributyltin and triphenyltin) can efficiently bind to the RXR of molluscs (Castro et al. 2007a; Nishikawa 2006; Nishikawa et al. 2004). Interestingly, the ligand-binding domain (LBD) of the RXR of molluscs is highly similar to vertebrate AR and ER LBD suggesting that vertebrate-like sex steroids may bind to RXR and, therefore, activate retinoid-signalling pathways. However, the binding potency of testosterone and oestradiol to RXR has never been investigated in either vertebrates or invertebrates.

In vertebrates, steroid hormone concentrations are controlled through glucuronidation and sulfonation pathways. The formation of glucuronic and sulphate steroid conjugates, catalysed by UDP-glucuronosyl transferase (UGT) and sulfotransferase (SULT) enzymes, reduces the biological activity of the hormones and facilitate their excretion from cells as these metabolites are more polar (James 2011). In molluscs, regulation of testosterone and oestradiol endogenous concentrations, through

sulfonation and glucuronidation, has been shown to be rather low, whereas steroid esterification with fatty acids was observed to be the major steroid metabolic pathway (cf. Fig. 1.5) (Gooding and LeBlanc 2001; Janer et al. 2005a). The apolar steroid fatty acid esters formed are retained within the fat and, therefore, the bioactivity and bioavailability of the free forms of steroids are reduced. Specific enzymes, fatty acid acyl coenzyme A acyltransferases, catalyse fatty acid esterification and are highly specific for the steroid substrate. Indeed, it was observed that the acyl-CoA:cholesterol acyltransferase was not able to catalyse the esterification of other steroids than cholesterol (Hochberg 1998). Studies conducted on molluscs showed that esterification might act as a homeostatic process that helps regulate endogenous concentrations of the free forms of steroids.

Many studies have reported that chemicals, which have endocrine disrupting properties in vertebrates, are able to interfere with endogenous steroid concentrations, either free or esterified (Abidli et al. 2012; Gooding et al. 2003; Janer et al. 2006). In the following paper we will review the information concerning the metabolisation of vertebrate-like sex steroid hormones in molluscs and the impacts of EDCs on endogenous concentrations of these hormones.

Publication 1:

Esterification of vertebrate-like steroids in molluscs: A target of endocrine disruptors in molluscs?

Submitted for publication in Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology

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Abstract

Alterations of the reproduction in natural populations of gastropod molluscs exposed to pollutants have been reported for more than 40 years. In some cases these impacts have been linked to exposure to endocrine disrupting chemicals (EDCs), which are known to interfere with the endocrine system of vertebrates mainly by interacting with steroid hormone metabolism and signalling processes. Investigations on the mechanisms of action of endocrine disruptors in molluscs show that EDCs induce modifications of endogenous titres of androgens (e.g., testosterone, androstenedione) and oestrogens (e.g., 17ß-estradiol, progesterone). Alterations of the activity of enzymes related to steroid metabolism (i.e., Cytochrome P450 aromatase, acyltransferases) are also often observed. In bivalves and gastropods, fatty acid conjugation of steroids might constitute the major regulation of androgens and oestrogens homeostasis. The present review indicates that metabolisation of steroid hormones to fatty acid esters might be a target of synthetic EDCs that would be involved in the impacts observed on the concentrations of free, potentially bioactive forms of steroids.

Keywords: Endocrine disrupting chemicals; Molluscs; Steroid esterification; Steroid metabolism; Androgen; Oestrogen; Organotin

1. Introduction

Alterations of the reproduction in natural populations of gastropod molluscs exposed to pollutants have been reported for more than 40 years (Blaber 1970). The development of male sex organs in female is now described in more than 200 mesogastropods and neogastropods species (Horiguchi et al. 2012). This phenomenon called *imposex* can lead, in the most adverse cases, to the sterilisation of females, which may thus affect population survival (Bauer et al. 1997). The formation of male characteristics in females was linked to the exposure to tributyltin (TBT), the main constitutive compound of boat antifouling paints, which was found in harbours and marinas at high concentrations (in the µg/L range (IPCS 1990)). Other xenobiotics (e.g., organotins, pharmaceuticals, organochlorines) were also shown to alter reproduction in many gastropod species (Gust et al. 2009; Oehlmann et al. 2006; Oehlmann et al. 2000; Tillmann et al. 2001). Sex alterations such as masculinisation and feminisation of bivalve species were also observed in estuaries and rivers contaminated by anthropogenic wastes (Gagné et al. 2002; Gagné et al. 2011; Ortiz-Zarragoitia and Cajaraville 2010; Tankoua et al. 2012).

These contaminants, called endocrine disrupting chemicals (EDCs), are known to interfere with the endocrine system of vertebrates mainly by disrupting normal steroid hormone signalling processes. Over the last decade the knowledge on mollusc endocrinology has increased and evidence of synthesis and biological activity of vertebrate-like steroid hormones has been observed in at least 3 gastropod classes: bivalvia, gastropoda and cephalopoda (Gottfried and Dorfman 1970; Köhler et al. 2007; Lafont and Mathieu 2007; Naokuni 1979; Porte et al. 2006; Santos et al. 2002; Sternberg et al. 2010). Furthermore, unlike vertebrates which excrete steroids as polar glucuronic acids of sulphate conjugates (James 2011), molluscs can store steroids as fatty acid esters in lipid rich tissues. This fatty acid esterification of steroids may constitute the major regulation of androgens and oestrogens homeostasis in molluscs (Gooding and LeBlanc 2001; Janer et al. 2006; Janer et al. 2004; Labadie et al. 2007; Sternberg and LeBlanc 2006).

Investigations on impacts of putative endocrine disruptors in molluscs have shown that modifications of endogenous titres of androgens (testosterone, androstenedione) and oestrogens (17ß-estradiol, progesterone) occur in molluscs exposed to EDCs.

Moreover, these chemicals have been shown to alter the activity of enzymes related to steroid metabolism (e.g., Cytochrome P450 aromatase, acyltransferases) (Abidli et al. ; Gooding et al. 2003; Ronis and Mason 1996; Spooner et al. 1991). Exposure of molluscs to natural steroids and to androgenic and oestrogenic xenobiotics has been reported to disturb normal hormonal balance of free versus esterified form of both androgen and oestrogen hormones (Gust et al. 2009; Janer et al. 2006; Janer et al. 2004). Alteration of the esterification of steroids to fatty acid esters through enzymatic pathways interference is proposed amongst hypotheses on the mechanism of imposex induction of TBT (Gooding et al. 2003; Janer et al. 2005c; Sternberg and LeBlanc 2006).

In this review, we discuss the impacts of natural and anthropogenic compounds on endogenous concentrations of vertebrate-like steroids in their free and esterified forms in molluscs as well as metabolic processes by which vertebrate-like steroids are esterified and the possibility that alteration of hormone homeostasis is a specific target of endocrine disruptors in molluscs.

2. Steroid metabolism in molluscs and seasonal variation of endogenous levels

As shown in *in vivo* studies on cholesterol biosynthesis, oestrogens (e.g., pregnenolone, progesterone) and androgens (e.g., androstenedione, androsterone) are synthesised *de novo* in molluscs (De Longcamp et al. 1974; Gottfried and Dorfman 1970). The different steps of cholesterol metabolism in gastropods molluscs were investigated through the injection of radiolabelled steroid precursors, involved in vertebrate steroidogenesis. Metabolites produced were analysed and the rate of biotransformation of each precursor was assessed. Additionally, cytochrome P450 aromatase and 5α-reductase, responsible for conversion of androgens to oestrogens, 3α/ß- and 17ß-hydroxydehydrogenases, which catalyse the reduction of ketosteroids or the oxidation of hydroxysteroids, sulfotransferases, involved in sulphate conjugation of steroids to polar metabolites, and fatty acid acyl coenzyme A acyltransferases, which convert steroids to apolar fatty steroids, were found in molluscs (reviewed by Fernandes et al. (2011)). The presence and the activity of these enzymes provide further evidence that the endogenous synthesis of vertebrate-like sex steroids occurs in molluscs. However, (Scott 2012) recently argued that molluscs only pick up steroids from their environment and therefore questioned the capacity of molluscs to

biosynthesise vertebrate-like steroids. Hormone concentrations in molluscs vary between species, gender and tissues. Furthermore, endogenous levels of androgens (e.g., androstenedione, testosterone) and oestrogens (e.g., 17ß-estradiol, progesterone) have been shown to vary according to the reproductive status of the animals. In the clam *Ruditapes decussatus*, highest concentrations of testosterone and progesterone were observed after gametogenesis whereas oestradiol levels were found to increase at the beginning of vitellogenesis (Ketata et al. 2007). In the soft-shell clam *Mya arenaria*, oestradiol, progesterone and testosterone concentrations were the highest at previtellogenic and spawning stages (Gauthier-Clerc et al. 2006; Siah et al. 2002). Testosterone and oestradiol concentrations in gonads of the cockle *Fulvia mutica* were highest at the onset of gametogenesis and during spawning (Liu et al. 2008). Those field studies showed that concentration peaks are observed at different times of the year in males and females according to their reproductive cycle (Gauthier-Clerc et al. 2006; Ketata et al. 2007). In gastropod species, clear seasonal patterns were also observed in sex steroid concentrations. In *Ilyanassa obsoleta,* the highest testosterone concentrations in tissues were observed during the reproductive season in both sexes with no notable differences between genders. Oestradiol levels were not modulated during the reproductive cycle in males while higher concentrations were found during the dormant stages in females (Barroso et al. 2005; Gooding and LeBlanc 2004; Sternberg et al. 2008a). In *Potamopyrgus antipodarum*, females exhibited the highest oestradiol levels during the reproductive period while testosterone concentrations were lower during this period (Gust et al. 2011). In the cephalopod *Octopus vulgaris*, concentrations of 17ß-estradiol and progesterone increased significantly just before the ovarian maturation in females (Di Cosmo et al. 2001).

Laboratory experiments have provided evidence that exposure of bivalve and gastropod species to sex steroid hormones leads to modifications of sex characteristics such as superfemales and imposex females in gastropods and skewed sex ratios or alteration in oocytes in bivalves (Schulte-Oehlmann 2004; Wang and Croll 2004) or induces impacts on spawning, gametogenesis and embryogenesis (Benstead et al. 2011; Schulte-Oehlmann 2004; Wang and Croll 2006).

Seasonal variations linked to reproductive cycle and laboratory experiments suggest that vertebrate-like sex steroids in molluscs likely play physiological roles in reproduction. Nevertheless steroid concentrations used in laboratory experiments are

high compared to physiological levels of the molecules (in the pg/g wet weight range in mollusc tissues). Further research is thus needed to elucidate the potential biological involvement of steroids in molluscs.

3. Esterification of steroids

Metabolism of steroids in molluscs is different from the vertebrate classical pathway in which stable concentrations of the bioactive forms of steroid hormones are predominantly maintained by excretion via glucuronic acid and sulphate conjugates (James 2011). Indeed, steroid excretion through polar metabolites is low in bivalves and gastropods whereas fatty acid conjugation of steroids has been reported in many studies and seems to constitute the major regulation process of androgens and oestrogens homeostasis (Gooding and LeBlanc 2001; Janer et al. 2006; Janer et al. 2004; Labadie et al. 2007; Sternberg and LeBlanc 2006).

Indeed, early studies on steroid metabolism in molluscs identified fatty acid esterification of sterols in gastropods (De Souza and De Oliveira 1976; van der Horst and Voogt 1972). Further studies showed that testosterone esterification occurs in *Ilyanassa obsoleta* (Gooding and LeBlanc 2001). Oestradiol and testosterone fatty acid esters have now been reported in bivalve (Janer et al. 2004; Labadie et al. 2007; Lazzara et al. 2012; Peck et al. 2007; Riva et al. 2010) and in gastropod species (Abidli et al. 2012; Gust et al. 2011; Janer et al. 2006; Lyssimachou et al. 2008). Furthermore, enzymes known to be involved in testosterone and oestradiol esterification (e.g., acyl coenzyme A:steroid acyltransferases) and in steroid ester hydrolysis processes in vertebrates have been found in molluscs (De Souza and De Oliveira 1976; Gooding and LeBlanc 2001; Sternberg and LeBlanc 2006). The concentrations of esterified and free forms of steroids were monitored in environmental samples during the whole reproductive cycle in male and female tissues of different mollusc species. It was observed that concentrations of the free forms of steroid hormones during the reproductive period are higher than levels found during the reproductive dormancy suggesting a biological activity of non-esterified hormones in reproduction (Gooding and LeBlanc 2004; Gust et al. 2010; Gust et al. 2011; Sternberg et al. 2008a). Moreover, a sharp decrease in testosterone ester concentrations was concomitant to the increase in free testosterone levels observed during the breeding seasons in *Ilyanassa obsoleta* (Gooding and LeBlanc 2004).

Species	Exposure: Chemical	Exposure: Concentration Range	Tissues	Changes in steroid concentrations: Total	Free	Esterified	Reference
Mytilus galloprovincialis (Mediterranean mussel, Bivalvia)	17ß-oestradiol	20; 200; 2000 ng/L	Total soft body	Dose dependent ↗ [E2]	↗ [E2] at 2000 ng/L	Dose dependent ↗ [E2]	(Janer et al. 2005a)
					No modification in [T]		
	Testosterone	20; 200; 2000 ng/L		Dose dependent ↗ [T]	↗ [T] at 2000 ng/L	Dose dependent ↗ [T]	(Fernandes et al. 2010)
					No modification in [E2]		
Mytilus edulis (Blue mussel, Bivalvia)	[4-^{14}C]-17ß-oestradiol	10 ng/L	Total soft body	Steady ↗ [E2] during exposition duration	-	Steady ↗ [E2] during exposition duration	(Labadie et al. 2007)
	[4-^{14}C]-oestrone	70 ng/L		-	-	Steady ↗ [E2] during exposition duration	
	North Sea crude Oil (0 group) Mixture North Sea crude oil + alkylated phenols + PAHs (OAP group)	O group: Crude Oil 0.5 ppm OAP group: Crude Oil 0,5 ppm + AP 0,1 ppm + PAHs 0,1 ppm	Gonad Tissues	-	No modification in [E2]	↗ [E2] in OAP group	(Lavado et al. 2006)
				-	No modification in [T]	↗ [T] in OAP group	
			Peripheral tissues	-	↗ [E2] in O group	No modification in [E2]	
				-	No modification in [T]	↗ [T] in OAP group	
Dreissena polymorpha (Zebra mussel, Bivalvia)	[4-^{14}C]-17ß-oestradiol	10 ng/L	Total soft body	-	-	↗ [E2]	(Peck et al. 2007)
	4-n-nonylphenol	1; 10; 50 µg/L		Non significant ↘ [E2] at all concentrations	No modification in [E2]	-	(Riva et al. 2010)
				↘ [T]	No modification in [T]	-	
	Fluoxetine	20; 200 ng/L		-	No modification in [E2]	↗ [E2] at 200 ng/L	(Lazzara et al. 2012)
					No modification in [T]		
Meretrix meretrix (Clam, Bivalvia)	Tributyltin chloride	0.1; 1; 10 ng Sn/L	Gonad Tissues	No modification in [E2] during exposure	-	-	(Wang et al. 2005)
				↗ [E2] at 1 and 10 ng Sn/L following transfer to clean tank	-	-	
				↗ [T] at all concentrations	-	-	

Table 1.2: Steroid concentration in mollusc tissues following exposure to steroids and to endocrine disrupting chemicals.

Species	Exposure: Chemical	Exposure: Concentration Range	Tissues	Changes in steroid concentrations: Total	Changes in steroid concentrations: Free	Changes in steroid concentrations: Esterified	Reference
Ilyanassa obsoleta (Eastern Mudsnail, gastropoda) Females	[^{14}C]-Testosterone	1.0 µM	Total soft body	↗ [T]	-	↗ [T]	(Gooding and LeBlanc 2001)
	Testosterone	2.8 and 5.6 mg/L		↗ [T]	No impact on [T]	↗ [T]	
	4-n-nonylphenol	2.5µg		↘ [T]	No impact on [T]	↘ [T]	
	Tributyltin chloride	0.1; 1; 10 ng Sn/L		No impact on [T]	↗ [T]	↘ [T]	(Gooding et al. 2003)
Potamopyrgus antipodarum (New Zealand mud snail, Gastropoda)	Fluoxetine	3.; 11.1; 33.3; 100 µg/L	Total soft body	No impact on [E2]			(Gust et al. 2009)
				Dose dependent ↗ [T]	-	-	
				↗ [Progesterone] at 33.3 µg/L	-	-	
Marisa cornuarietis (giant ramshorn snail, Gastropoda)	Triphenyltin	30; 125; 500 ng Sn/L	Digestive gland/gonad complex	-	-	↘ fatty acid both in chain length and saturation degree	(Lyssimachou et al. 2009a)
	Triphenyltin	30; 125; 500 ng Sn/L		- -	No impact on [E2] No impact on [T]	↘ [E2] in females at 500 ng Sn/L ↗ [T] in females at 30 and 125 ng Sn/L	(Lyssimachou et al. 2008)
	Tributyltin	0; 30; 125; 500 ng Sn/L		- No impact on [T]	- ↗ [T] in females at 125 and 500 ng Sn/L	↘ [E2] in females at 125 and 500 ng Sn/L ↘ [T] in females at 30;125 and 500 ng Sn/L	(Janer et al. 2006)
	Methyltestosterone	0; 30; 300 ng/L		-	-	No impact on [T] nor in [E2]	
	Fenarimol	0; 300; 3000 ng/L		-	-	No impact on [T] nor in [E2]	
Littorina littorea (Periwinkle, Gastropoda)	Tributyltin	0; 0.5; 5 µM	Digestive gland/Kidney/ gills	Dose dependent ↗ [T] in tissues	-	-	(Ronis and Mason 1996)
Bolinus brandaris (Purple dye murex, Gastropoda)	Tibutyltin	0; 5; 50 ng TBT/L	Digestive gland/gonad complex	- -	No impact on [E2] in males and females ↗ [T] in females No impact on [T] in males	No impact on [E2] in males and females ↘ [T] in females No impact on [T] in males	(Abidli et al. 2012)

Table 1.2: Steroid concentration in mollusc tissues following exposure to steroids and to endocrine disrupting chemicals. (Continued)

Species	Exposure		Tissues	Changes in steroid concentrations			Reference
	Chemical	Concentration Range		Total	Free	Esterified	
Hexaplex trunculus (Banded-dye murex, Gastropoda)	Tibutyltin	0; 5; 50 ng TBT/L	Digestive gland/gonad complex	-	No impact on [E2] in males and females	No impact on [E2] in males and females	(Abidli et al. 2012)
				-	↗ [T] in females No impact on [T] in males	↗ [T] in females No impact on [T] in males	
Nucella lapillus (dog whelk, Gastropoda)	Tributyltin	5; 50; 100 ng Sn/L	Total soft body	Dose, time and imposex stages dependent ↗ [T] in females	-	-	(Bettin et al. 1996)
				No impact on [E2]	-	-	
				↗ [T]/[E2] ratio after 6 months as 100 ng Sn/L	-	-	
Nassarius reticulatus (netted dog whelk, Gastropoda)	Tributyltin	5; 50; 100 ng Sn/L	Total soft body	Dose, time and imposex stages dependent ↗ [T] in females	-	-	
				↗ [E2] after 4 months	-	-	
				No impact on [T]/[E2] ratio	-	-	
Lymnaea stagnalis (Giant pond snail, Gastropoda)	Testosterone	2; 10; 50 ng/L	Total soft body	Dose dependent ↗ [T]	No impact on [T]	Dose dependent ↗ [T]	(Giusti et al. In Press; Part 4: chapter 5)
	Tributyltin	45, 220 ng Sn/L		No impact on [T]			
	Cyproterone acetate	2, 50 µg/L		↗ [T] at 50 µg/L	↗ [T] at 50 µg/L	No impact on [T]	
	Chlordecone	4.5, 50 µg/L		No impact on [T]			

Table 1.2: Steroid concentration in mollusc tissues following exposure to steroids and to endocrine disrupting chemicals. (Continued)

4. Impacts of steroid hormones on endogenous steroid titres

To assess the impact of steroids on molluscs, studies where animals were exposed to steroid hormones (i.e., testosterone, oestradiol and progesterone) through water (Fernandes et al. 2010; Janer et al. 2005a; Labadie et al. 2007; Peck et al. 2007) or through direct injections (Gooding and LeBlanc 2004; Wang and Croll 2004; Wang and Croll 2006) showed that endogenous sex steroid hormones concentrations are modified by such exposures (Table 1.2).

4.1. Oestradiol

Esterification of oestrogens in molluscs exposed to spiked media or through direct injection might act as homeostatic mechanism to maintain endogenous level of free oestradiol in molluscs. In the mussel *Mytilus galloprovincialis,* exposure to 17ß-oestradiol at 20, 200 and 2000 ng/L increased total oestradiol titres (free + esterified) while only the highest concentration induced an increase in free oestradiol (Janer et al. 2005a). Moreover, the activity of palmitoyl-CoA:estradiol acyltransferase, an acyl CoA:estradiol acyltransferase enzyme (AEAT), was shown to increase in a dose dependent manner (Table 1.3). This enzyme is responsible of the esterification of oestradiol with a C16:0 fatty acid. Another bivalve species, *Dreissena polymorpha*, stored [^{14}C]-oestradiol as a lipophilic metabolite by esterification. Oestrone was either rapidly metabolised or not synthesised as this hormone was not detected in this study (Peck et al. 2007). Similar results were obtained on the blue mussel, *Mytilus edulis*, exposed to [4-^{14}C]-oestradiol. A steady increase in estradiol ester concentrations was observed over the 13 days of the experiment duration. Similarly exposure of the mussel to 70 ng/L of [4-^{14}C]-oestrone for 8 days induced a significant increase in estradiol fatty esters levels. In this study, radio-NPHPLC and GC-MS analysis of tissues samples revealed that all the oestrone taken up by the mussel was rapidly metabolised as [4-^{14}C]-oestradiol concentrations were increased after one day of exposure (Labadie et al. 2007). Radiolabelling allowed determining that the levels of esterified oestradiol in tissue samples were not only due to the uptake from the water but that half of the oestradiol was biosynthesised. These results suggest that oestradiol esterification helps to maintain stable free oestradiol concentrations when exposed to low concentrations

(ng/L range) of the hormone in water while at high concentrations (µg/L range) the accumulation process overwhelms its metabolisation. The administration of oestradiol had no impact on testosterone concentration either free or esterified and the androgen metabolisation of androstenedione to testosterone was not affected in *Mytilus galloprovincialis* (Janer et al. 2005a). However, exposure of the mud snail *Ilyanassa obsoleta* to 17ß-oestradiol inhibited fatty acid esterification of testosterone by direct inhibition of acyl coenzymeA:testosterone acyltransferase (ATAT) (Sternberg and LeBlanc 2006).

4.2. Testosterone

Uptake and biotransformation of testosterone was assessed in the mud snail *Ilyanassa obsoleta* through exposure to 1µM of the radiolabelled [^{14}C]-testosterone. Testosterone and its metabolites were not readily excreted but converted to less polar molecules, which can be stored in lipid rich tissues (Gooding and LeBlanc 2001). Total testosterone levels following exposure to testosterone were significantly increased compared to the controls. In this species, injection or exposure to testosterone (500 ng and 2.8 ng/L, respectively) led to an increase in the storage of testosterone as fatty acid ester while free testosterone concentrations were maintained to a normal level (Gooding and LeBlanc 2004). In the hermaphroditic gastropod *Lymnaea stagnalis*, exposure to testosterone concentrations (1.4 and 6.8 ng/L) led to a significant dose dependent increase in esterified testosterone concentrations while levels of the free form of the hormone were not different from controls (Giusti et al. In Press; Part 4: chapter 5).

Exposure of the mussel *Mytilus galloprovincialis* to testosterone through water had no impact on free testosterone titres at 20 and 200 ng/L whereas the highest tested concentration, 2000 ng/L, induced a 5-fold increase in free testosterone levels compared to controls. Esterified testosterone concentrations increased significantly at all concentrations tested (Fernandes et al. 2010). These results suggest that testosterone esterification helps to maintain stable free testosterone concentrations when exposed to low concentrations (ng/L range) of the hormone in water while at high concentrations (µg/L range) the accumulation process overwhelms the metabolisation.

Total and free oestradiol concentrations were not altered by testosterone exposure (Table 1.2).

Authors also assessed the activities of enzymes involved in the steroid metabolism in molluscs. Addition of palmitoyl- and oleoyl-coenzymeA in the water containing 1µM of [^{14}C]-testosterone increased the concentration of apolar metabolites in *Ilyanassa obsoleta* suggesting the role of acyl CoA acyltransferases in testosterone metabolism in molluscs (Gooding and LeBlanc 2001). An increase in the acyl CoA:testosterone acyltransferase (ATAT) activity was observed *in vitro* in digestive gland microsomal proteins dissected from *Mytilus galloprovincialis* exposed to 2000 ng/L of testosterone. Androgen precursors, androstenedione and 5 α-dihydroandrostenedione, titres were not modified while 5 α-dihydrotestosterone synthesis by digestive gland microsomes was significantly increased following exposure to the highest concentration (Table 1.3).

Similarly to oestradiol metabolism, the esterification of testosterone apparently acts as homeostatic mechanism to maintain endogenous level of free testosterone stable following spiking of environmental media or injection (Table 1.2).

5. Impacts of Endocrine Disruptors on endogenous steroid titres

The presence of endocrine disrupting compounds in the environment is now well established, especially in aquatic environments that constitute the final sink for environmental contaminants. Wastewater treatment plant effluents and sewage sludge were shown to contain mainly oestrogenic compounds such as hormones and pharmaceuticals coming from anthropogenic sources (Andrew-Priestley et al. 2012; Bellet et al. 2012; Jeannot et al. 2002; Kolodziej et al. 2003; Kusk et al. 2011; Nakada et al. 2004; Nieto et al. 2010; Salste et al. 2007; Shappell 2006). Surface waters near livestock farms were also shown to present oestrogenic activity due to veterinary drugs, which can cause endocrine disruption in aquatic organisms (Chen et al. 2010; Matthiessen et al. 2006; Orlando et al. 2004; Tashiro et al. 2003). The impacts of endocrine disruptors on the ratio of free, bioactive, versus esterified, stored, steroids (Table 1.3) and on the activity of enzymes involved in the esterification process (Table 1.4) were investigated as possible mechanisms of action of those compounds in molluscs (Abidli et al. 2012; Fernandes et al. 2010; Gooding et al. 2003; Janer et al. 2006; Sternberg and LeBlanc 2006).

Species	Exposure: Chemical	Exposure: Concentration Range	Tissues	Steroid metabolism	Enzymes activity	Reference
Mytilus galloprovincialis (Mediterranean mussel, Bivalvia)	17ß-oestradiol	20; 200; 2000 ng/L	Digestive gland microsomal proteins	Dose dependent ↗ [DHT] and [DHA] synthesis from androstenedione precursor No modification in [T] synthesis from androstenedione precursor	Dose dependent ↗ Palmitoyl-CoA:estradiol acyltransferase No modifications of estradiol-sulfotransferase activity ↘ P450-aromatase activity at 20 ng/L ↗ P450-aromatase activity at 2000 ng/L	(Janer et al. 2005a)
	Testosterone	20; 200; 2000 ng/L		↘ [DHT] synthesis from androstenedione precursor at 2000 ng/L No modification in [T] synthesis from androstenedione precursor Dose dependent ↘ BFC-**O**-debenzyloxylase activity	Dose dependent ↗ Palmitoyl-CoA:testosterone acyltransferase	(Fernandes et al. 2010)
Mytilus edulis (Blue mussel, Bivalvia)	17ß-oestradiol	200 ng/L	Total soft body	↗ [E2-$C_{16:0}$], [E2-$C_{16:1}$] and [E2-$C_{16:2}$]	-	(Labadie et al. 2007)
	North Sea crude Oil (0 group) Mixture North Sea crude oil + alkylated phenols + PAHs (OAP group)	O group: Crude Oil 0.5 ppm OAP group: Crude Oil 0,5 ppm + AP 0,1 ppm + PAHs 0,1 ppm	Gonad Tissues	-	↗ P450-aromatase activity in OAP	(Lavado et al. 2006)
			Digestive gland microsomal proteins	-	↗ P450-aromatase activity in OAP ↗ Estradiol sulfotransferase activity in O and OAP	
Ilyanassa obsoleta (Eastern mudsnail, Gastropoda) Females	Tributyltin chloride	2.5; 5; 10; 20; 200 ng Sn/L	Total soft body	↗ Androstenediol production rate at 2.5, 5 and 10 ng Sn/L		(Oberdörster et al. 1998)
	Tributyltin chloride	0.1; 1; 10 ng Sn/L	Gonad-viscera complex microsomal proteins	-	No impacts on ATAT activity	(Gooding et al. 2003)
	Tributyltin chloride	2 to 100 µM		-	Inhibition of ATAT activity *in vitro* Competition with Testosterone for steroid binding site of ATAT Inhibition of AEAT activity *in vitro* No competition with 17ß-oestradiol for steroid binding site of AEAT	(Sternberg and LeBlanc 2006)

Table 1.3: Steroid metabolism and enzyme activity modification following exposure of molluscs to steroids and to endocrine disrupting chemicals.

Species	Exposure		Tissues	Steroid metabolism	Enzymes activity	Reference
	Chemical	Concentration Range				
Marisa cornuarietis (giant ramshorn snail, Gastropoda)	Triphenyltin	30; 125; 500 ng Sn/L	Gonad-viscera complex peroxisomal fraction	-	↗ Peroxisomal acyl-CoA oxidase activity at 30 and 500 ng Sn/L in females and at 30 ng Sn/L in males	(Lyssimachou et al. 2009a)
	Triphenyltin	30; 125; 500 ng Sn/L	Digestive gland/gonad complex microsomal proteins	Sexually dimorphic High 17ß-HSD activity in males High 5α-reductases activity in females No impacts of TPT	No modification of P450 aromatases activity in females	(Lyssimachou et al. 2008)
		0; 30; 60; 125; 250; 500 ng Sn/L		-	↗ ATAT activity in males at 500 ng Sn/L	
	Methyltestosterone	0; 30; 300 ng/L		-	Dose dependent ↗ ATAT activity in males and females	
	Fenarimol	0; 300; 3000 ng/L		-	No impacts on ATAT activity	
Littorina littorea (Periwinkle, Gastropoda)	Tributyltin	0; 0.5; 5 μM	Digestive gland/Kidney/ gills	↗ [DHT]; [A]; [DHT-diols]; in the 3 tissues ↗ [DHA] in Kidney	-	(Ronis and Mason 1996)
		0; 1; 10; 100 μM	Gonad-viscera complex microsomal proteins	-	Small reduction in cytochrome P450 aromatase activity	
Bolinus brandaris (Purple dye murex, Gastropoda)	Tributyltin	0; 0.1; 1; 10 μM		Inhibition of A metabolisation to DHA in females at 10 μM; No impacts on males	-	(Lyssimachou et al. 2009b)
	Triphenyltin	0; 0.1; 1; 10 μM		Inhibition of A metabolisation to DHA in females at 0.1; 1; 10 μM; No impacts on males	-	
Hexaplex trunculus (Banded-dye murex, Gastropoda)	Tributyltin	0; 0.1; 1; 10 μM		↗ T formation rate in females a 10μM; No impacts on males	-	(Lyssimachou et al. 2009b)
	Triphenyltin	0; 0.1; 1; 10 μM		No impacts on T formation rate in either males or females	-	
Buccinum undatum (common whelk, Gastropoda)	Different shipping density (TBT contaminated environment)	-		-	↘ cytochrome P450 aromatase activity with ↗ imposex stages and with ↗ [TBT] in tissues ↗ NADH cytochrome *c* reductase activity with ↗ imposex stages and with ↗ [TBT] in tissues No impact on NADPH cytochrome *c* reductase activity	(Santos et al. 2002)

Table 1.3: Steroid metabolism and enzyme activity modification following exposure of molluscs to steroids and to endocrine disrupting chemicals. (Continued)

5.1. Oestrogens

Oestrogenic compounds are able to interfere with the mollusc reproduction leading to the alteration of sex specific characters (e.g., superfemales in gastropods (Oehlmann et al. 2006)) or to the modification of the fertility (e.g., stimulated egg and embryo production in bivalves and gastropods (Duft et al. 2003; Wang and Croll 2006)). It appears that chemicals known for their oestrogenic or anti-oestrogenic properties in vertebrates can alter endogenous steroid titres in molluscs. Depending on the molecules, free, esterified or both forms of oestradiol, progesterone and testosterone concentrations can be modified suggesting an interaction of those chemicals with the steroid metabolism in molluscs (Table 1.2).

Exposure of the parthenogenic prosobranch snail *Potamopyrgus antipodarum* to fluoxetine (3.7; 11.1; 33.3 and 100 µg/L), an antidepressant pharmaceutical known to reduce oestradiol concentrations and oestrogen receptors in goldfish (Mennigen et al. 2008), induced a concentration-dependent increase in total testosterone titres. Progesterone levels were significantly higher in snails exposed to 33.3 µg/L whereas the concentration of oestradiol was not modified (Gust et al. 2009). Exposition of zebra mussel *Dreissena polymorpha* to 200 ng/L of fluoxetine induced a significant increase in esterified oestradiol while no effect was observed on free oestradiol and testosterone (either free or esterified) (Lazzara et al. 2012).

In the gastropod *Ilyanassa obsoleta*, injection or exposure to 4-nonylphenol (2.5 µg and 50 µg/L, respectively) had no impact on free testosterone levels while the concentration of the esterified form of the steroid was significantly lower (Gooding and LeBlanc 2004). Those results were confirmed on the bivalve species *Dreissena polymorpha* with exposure from 1 to 50 µg/L of 4-nonylphenol. Even if non significant, a tendency to a reduction of total oestradiol levels was observed in this species while titres of the free forms of both testosterone and oestradiol were not altered (Riva et al. 2010). Exposure of the mussel *Mytilus edulis* to crude oil increased free oestradiol levels only in peripheral tissues. Exposure to oil in addition with alkylphenol increased significantly the esterified steroids levels in the bivalve tissues (Lavado et al. 2006). This latter exposure also increased the activity of cytochrome P450 aromatase in all tissues.

Wastewater treatment plant effluents are known for their oestrogenic activities due mainly to nonylphenols, alkylphenols and octylphenols, derived from industrial, agriculture and household applications, to bisphenol A, largely used as plasticizer, and to other sterols and hormones, mainly synthetics, with pharmaceutical uses (Gust et al. 2010; Jeannot et al. 2002). Concentrations of those endocrine disruptors are found usually in the ng/L up to the μg/L range in water and sludge in the most polluted areas (Jeannot et al. 2002; Kusk et al. 2011; Santos et al. 2008) and oestrogenic activity can be monitored using methods such as Yeast Estrogen Screen (YES) or E-Screen (Kusk et al. 2011; Soto et al. 1995; Thorpe et al. 2006). *In situ* monitoring of the impacts of those effluents on molluscs provides interesting results on the alterations environmental mixtures of chemicals can induce in molluscs. In *Potamopyrgus antipodarum* caged downstream of wastewater treatment plants, levels of total oestradiol were different amongst the rivers analysed. The concentrations of the hormone were either lower or higher in downstream individuals than in molluscs caged in upstream sites. Those modifications were linked to the compounds found in waters, which were different depending on the studied sites. Testosterone concentrations in all downstream effluents were higher than in upstream stations (Gust et al. 2010).

These changes in hormone titres observed following exposure to chemicals exhibiting oestrogenic or anti-oestrogenic activity could be explained by alterations of the esterification of steroids, as is likely the case with oestradiol exposure. It is to be noted that exposure of the hermaphrodite prosobranch *Valvata piscinalis* to fluoxetine did not induce any change in steroids titres (Gust et al. 2009).

5.2. Androgens

Even though androgenic and anti-androgenic molecules were shown to interfere with mollusc reproduction (Schulte-Oehlmann et al. 2000; Tillmann et al. 2001; Wang and Croll 2003), only few studies investigated the impacts of androgenic or anti-androgenic molecules on steroid concentrations in molluscs. Methyltestosterone, a synthetic agonist of the androgen receptors in vertebrates, did not altered steroid levels in *Marisa cornuarietis* (Janer et al. 2006) but induced imposex in this species (Schulte-Oehlmann 2004). In this study, exposure to methyltestosterone led to an increase in ATAT activity in both male and females *M. cornuarietis* and was concentration-

dependent. The anti-androgenic pharmaceutical, cyproterone acetate, only increased free oestradiol levels in this species. Authors investigated the impacts of the co-administration of the two compounds, cyproterone acetate and methyltestosterone, exhibiting antagonist mechanisms of action in vertebrates. A significant increase in free oestradiol was observed but in a higher degree than it was observed following exposure to cyproterone acetate alone (Santos et al. 2005). Free testosterone concentrations were significantly increased in the freshwater gastropod *Lymnaea stagnalis* exposed to 28.7 μg/L of cyproterone acetate during 21 days (Giusti et al. In Press; Part 4: chapter 5).

5.3. Organotins

The mechanisms underlying the development of male sex characteristics in female gastropods, known as imposex, following exposure to organotins (i.e., tributyltin and triphenyltin) is still not fully understood. Many authors have investigated the alteration of steroid concentrations in molluscs following exposure to those molecules. They found modifications in testosterone concentrations (free or esterified) (Table 1.2) and also in the activity of enzymes involved in steroid esterification (Table 1.3) following exposure to androgenic and anti-androgenic chemicals.

Ronis and Mason (1996) showed that cytochrome P450 aromatase activity was reduced by 30-40% when *Littorina littorea* was exposed to TBT. Moreover, they observed a dose-dependent increase in androstenedione titres, which is probably a consequence of the decrease in the activity of the cytochrome P450 aromatase involved in the metabolism of androgens to oestrogens. TBT concentrations of 0.5 and 5 μM reduced sulphate conjugates and increased free testosterone and 5α-reduced metabolites in water. In *Nucella lapillus* and *Hinia reticulata*, testosterone concentrations were significantly increased by TBT in females (Bettin et al. 1996).

Studies on the impacts of TBT on steroid esterification in molluscs showed that 3 months of exposure of the gonochoric gastropod *Ilyanassa obsoleta* to the organotin led to a significant increase in free testosterone body burdens while no effect was observed on total testosterone titres. Concentrations of free testosterone were reported to increase with TBT levels (from 0.1 to 10 ng/L) (Gooding et al. 2003). The ramshorn snail *Marisa cornuarietis* showed a reduction in esterified steroid titres of both testosterone

and oestradiol after 100 days of exposure to TBT in females (Janer et al. 2006). Even if concentrations of free testosterone were not significantly different from controls, a significant increase in the ratio of free/esterified testosterone was observed from 125 ng/L. In the muricids *Hexaplex trunculus* and *Bolinus brandaris*, TBT significantly increased free testosterone concentrations in females while males of both species were not affected. Furthermore, esterified testosterone titres in males of those species were not affected by TBT. Esterified testosterone concentrations decreased in *Bolinus brandaris* females while it increased in *Hexaplex trunculus* females (Abidli et al. 2012). Esterified testosterone levels were increased in *Nucella lapillus* females exposed to TBT (50 ng Sn/L) but free and total concentrations were not altered (Santos et al. 2005). In the hermaphroditic snail *Lymnaea stagnalis*, exposure to 19 and 94 ng Sn/L of TBT for 21 days had no effect on either free or esterified testosterone concentrations (Giusti et al. In Press; Part 4: chapter 5). Moreover, the incubation of *Ilyanassa obsoleta* with [^{14}C]-testosterone indicated that accumulation of [^{14}C]-testosterone fatty acid was reduced with increasing TBT concentrations (Gooding and LeBlanc 2001). So it appears that TBT generally increases free testosterone concentrations and/or decreases fatty acid esters in females of most of the species studied although in some species, it is the esterified testosterone levels that are increased. Furthermore, (Sternberg and LeBlanc 2006) showed in *Ilyanassa obsoleta* that TBT directly inhibits the activities of microsomal acyl CoA:testosterone acyltransferases (ATAT) and acyl CoA:estradiol acyltransferases (AEAT), the enzymes which catalyse steroid fatty acid esterification, in a concentration dependent manner from 9 µM (Sternberg and LeBlanc 2006), which indicates that TBT inhibits fatty acid esterification of steroids. Moreover, they showed that TBT is a competitive inhibitor of ATAT by competition with testosterone for the binding site but is not competitive with 17ß-estradiol for AEAT (Sternberg and LeBlanc 2006). However, ATAT activity in microsomal fractions isolated from digestive gland/gonad complex in *Marisa cornuarietis* was significantly increased in males following exposure to 500 ng/L TBT during 50 days (Janer et al. 2006).

In the muricid gastropods *Bolinus brandaris* and *Hexaplex trunculus*, fatty acid oestradiol concentrations were not affected by TBT. Only females of *H. trunculus* showed an increase in free oestradiol levels following exposure to TBT concentrations from 5 ng/L (Abidli et al. 2012). Exposure of *Nucella lapillus* females to 50ng Sn/L increased esterified oestradiol concentrations while free oestradiol levels were not altered (Santos

et al. 2005). TBT increased testosterone concentrations in the clam *Meretrix meretrix* with no reduction in oestradiol levels (Wang et al. 2005).

Triphenyltin, another organotin known for its androgenic activity in vertebrates, induced a significant increase in testosterone ester concentrations in the gastropod *Marisa cornuarietis* while esterified oestradiol levels were lowered after only 1 week of exposure (Lyssimachou et al. 2008; Lyssimachou et al. 2009a). In this species, percentage of lipids, total fatty acid content, carbon chain length and unsaturation degree decreased in exposed females while no differences were observed in males (Lyssimachou et al. 2009a). An increase in activity of palmitoyl CoA oxidase, an enzyme responsible for the break down of C14-C18 and C>20 fatty acids, was also observed in males exposed to 30 ng Sn/L and in females exposed to 30 and 500 ng Sn/L (Lyssimachou et al. 2009a), which could explain the decrease in fatty acid concentrations. *In vitro*, microsomal fraction of the gastropods species *Hexaplex trunculus* and *Bolinus brandaris* were exposed to TBT and TPT to assess androstenedione metabolism. TPT had no impact on the formation rate of testosterone in either male or females *H. trunculus* while TBT increased the formation rate in females in a dose-dependent manner. The two organotins decreased the metabolisation to dihydroandrostenedione in *B. brandaris* females while they had no effect on males (Lyssimachou et al. 2009b). Significant differences were observed in androstenedione metabolisms between the two species. Androstenedione metabolism in *Marisa cornuarietis* exposed to TPT was not significantly modified even if testosterone and dihydrotestosterone formation rate tended to increase in males. Likewise, cytochrome P450 aromatase activity tended to increase in females exposed to 500 ng Sn/L of TPT, but the effect was not significant (Lyssimachou et al. 2008).

In environmental samples of *Ilyanassa obsoleta,* free testosterone levels were higher in snails from a marina compared to a less organotin polluted creek while esterified testosterone concentrations were lower (Gooding et al. 2003). Similarly, imposexed mud snails presented higher concentration of unmetabolised testosterone. Laboratory exposure of normal and imposexed individuals from the field to [^{14}C]-testosterone showed that in androgenised females the rate of reduced/dehydrogenated metabolites (androstenedione, androstenediol, 5α-dihydrotestosterone and androstanediol) production was significantly reduced (Oberdörster et al. 1998). In laboratory

experiment exposure to TBT led to an increase in androstanediol and androstenedione formation rate. Cytochrome P450 aromatase activity in imposexed population of *Buccinun undatum* was significantly lower than in normal females populations (Santos et al. 2002).

From the results of those studies it was hypothesised that organotins induce imposex in gastropod molluscs through the modification of free testosterone concentrations in tissues even though the biological role of testosterone in molluscs remains to be elucidated. Recently the involvement of retinoid signalling through the binding of TBT to the retinoid X receptors (RXR) is cited among other hypothesis on the mode of actions of this organotin in the development of imposex in neogastropods species (reviewed in (Sternberg et al. 2010)). It was reported that TBT binds this receptor found in different mollusc species (Castro et al. 2007a; Nishikawa 2006; Sternberg et al. 2008b) and studies have shown that the natural ligand of the receptor, 9-*cis* retinoid acid, could induce the development of imposex (Castro et al. 2007a; Nishikawa 2006). Protein sequence alignment of the oestrogen and androgen receptors of vertebrates with retinoid X receptor identified in *Biomphalaria glabrata* showed high homology of the ligand-binding domain (LBD), suggesting that RXR might bind vertebrate-like steroids in molluscs. (Sternberg et al. 2008b) recently reported that RXR signalling pathways may regulate male reproductive differentiation therefore steroid interaction with this receptor would play a physiological role in mollusc reproduction. Further investigations on the interaction of steroid hormones with the retinoid X receptor would be of great interest to elucidate their impacts on mollusc reproduction.

6. Conclusions and Perspectives

This review shows that steroid hormones are present and are metabolised in molluscs. Moreover, evidence of endogenous synthesis of steroid hormones exists in at least three mollusc classes. Although their biological role has not been elucidated yet, studies on natural populations show that androgens and oestrogens concentrations in molluscs vary according to the gender and the reproductive status of animals suggesting a biological activity of these hormones. Exposure of bivalves, gastropods and cephalopods to different EDCs in laboratory or in field experiments induced alterations of steroid hormones levels, which are species and sex dependent. Parallel to these

changes in testosterone and oestradiol titres, adverse impacts on reproduction have been observed which suggests these hormones play a role or interfere with this physiological phenomenon. The nature of the interference of these hormones and the reproduction in molluscs has not been established yet. It is also possible that these hormones interfere with the metabolisation of endogenous molecules involved in the reproduction of molluscs.

It has been established that molluscs metabolise steroids into more apolar products by esterification with fatty acids, which allows their storage in lipid rich tissues. This process likely acts as homeostatic regulation of the free, potentially bioactive form of steroids. However, endocrine disrupting compounds are known to alter steroid hormones levels. The review of the literature of studies concerning this topic indicates that natural and synthetic molecules known for their endocrine activity in vertebrates have impacts on the esterification process in bivalves, gastropods and cephalopods species. Fatty acid testosterone ester and fatty acid oestradiol ester concentrations were shown to increase following exposure to testosterone and oestradiol respectively suggesting the homeostatic regulation of steroids through esterification. In the case of exposure to high concentrations of hormones (µg/l range) it seems that the homeostasis through esterification is overwhelmed which causes an increase in the free steroid concentrations. Studies discussed in this review suggest that EDCs could target esterification of steroids to fatty acids, as they alter endogenous levels of free and/or esterified steroids. Moreover, these chemicals can have impacts on the activity of enzymes involved in this metabolic pathway. Furthermore, the more obvious hypothesis that comes to mind on the basis of these observations is that the free form of steroids are bioactive molecules involved in mollusc reproduction and that EDCs impacts reproduction through alterations of their levels.

It is also possible that EDCs interfere with other metabolic pathways involved in the reproduction. For example, retinoid signalling pathway was recently associated to the male reproductive differentiation in neogastropods species. Alignment of protein sequences of the vertebrate androgen receptor and oestrogen receptor with mollusc retinoid X receptor showed high homology of the ligand-binding domain therefore suggesting that vertebrate steroids might bind and act on retinoid signalling pathways.

Further analyses of the interactions of steroids with the RXR are needed to elucidate the biological activity of the free form of the hormones on mollusc reproduction.

This review highlights the need to elucidate whether vertebrate-like sex steroids have a biological activity and if these hormones play a role in mollusc reproduction. However, it is difficult to analyse the impacts of the free, putatively bioactive, forms of steroids on mollusc reproduction as homeostasis through esterification prevents changes in endogenous concentrations. Therefore, studies involving specific inhibition of the enzymes involved in the esterification or the stimulation of enzymes involved in ester hydrolysis could help to better understand the impacts of modulations of steroids levels on mollusc reproduction. Furthermore, It is important to understand how the EDCs act on steroidogenesis. It remains to be determined whether these chemicals act exclusively on the esterification of steroids or if they can also act directly through receptor activation, on the activity of enzymes or on other metabolic pathways. These observations raise the need to better describe and to better understand the endocrine system in molluscs, especially the control of the reproduction, to provide new insight on the impacts and the mechanisms of action of EDCs in molluscs and to allow a better assessment of the risk they represent for aquatic ecosystems.

Acknowledgement

This work was financially supported by Belgium FRS-FNRS funds under a F.R.I.A. doctoral grant and a F.R.F.C. grant n° 2.4.585.10.F

3. *Lymnaea stagnalis*

Lymnaea stagnalis (Linnaeus, 1758) (Fig. 1.6) is a pulmonate gastropod mollusc that belongs to the Basommatophora order. Within this monophyletic order, three families regroup the majority of the species, i.e., *Lymnaeaidae*, *Physidae* and *Planorbidae*, all freshwater species (Fig. 1.7). The great pond snail, *L. stagnalis*, is found in slow running and standing water bodies rich in vegetation throughout the northern hemisphere. This species feeds on organic constituents of plant and animal origins, but cannibalism occasionally occurs (OECD 2010). Adult shell length varies from 20 to 60 mm according to environmental condition (e.g., water volume, population density). As the rearing and the breeding of *L. stagnalis* under laboratory conditions is easy and well documented, it has been used as a model species for neurological and endocrinological studies for more than 40 years (Geraerts and Joosse 1975; Joosse 1964). The mating behaviour and the neurobiological control of the reproduction are particularly well described in this species, therefore *L. stagnalis* constitutes a suitable model species for the analysis of the impacts of putative endocrine disrupting chemicals on gastropods.

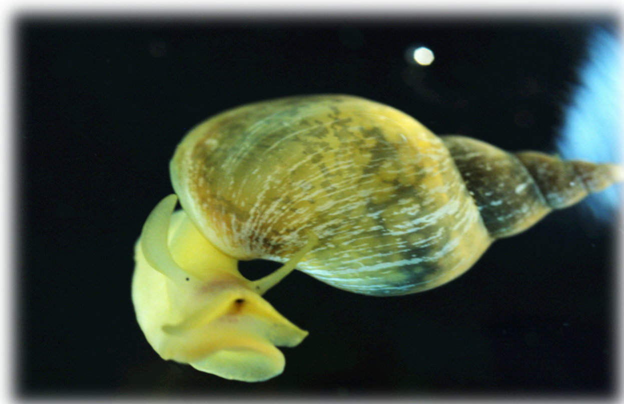

Fig. 1.6: *Lymnaea stagnalis* (photo M. Dugué).

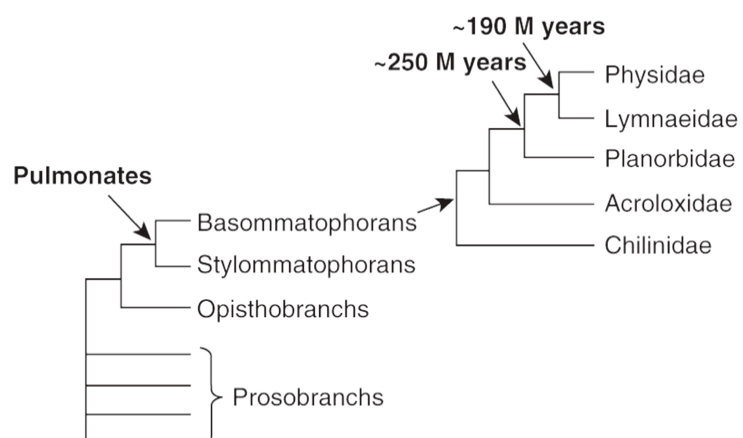

Fig. 1.7: Phylogenetic relationship among basommatophoran families based on molecular analysis.
(From Jarne et al. 2010)

3.1. Reproduction

The anatomy of the reproductive organs of *L. stagnalis* was first described in 1806 by Georges Cuvier (Cuvier 1806) who was astonished by the peculiarity of these organs. The complex morphology of the reproductive organs in *L. stagnalis*, along with other basommatophorans, is due to their particular mode of reproduction, the simultaneous hermaphroditism, which implies that both male and female organs are present at the same time, conversely to sequential hermaphroditism.

Fertilisation occurs in reproductive organs and, therefore, individuals can reproduce both by self- and cross-fertilisation (Cain 1956). *L. stagnalis* exhibits a low self-fertilisation rate (Puurtinen et al. 2007). However, fertilisation might not occur directly after insemination since allosperm can be stored in the *bursa copulatrix* (Fig. 1.9) in order to limit selfing, even when mates are lacking (Cain 1956). Male or female behaviour has to be determined between the mating partners to avoid sexual conflicts. Young sexually mature snails tend to act as males, whereas senior animals prefer mating as females (Hermann et al. 2009). Alternation of sexual roles between two partners, reciprocal copulation, appears to be inversely related to the age, thus, mating as males does not necessarily follow female copulation (Boer et al. 1997; Hermann et al. 2009). Social isolation and prostate gland size can also trigger male functions (de Boer et al. 1996; Koene and Ter Maat 2004). Male courtship and reproductive behaviours have been largely investigated (Koene 2006; van Duivenboden et al. 1985) (Fig 1.8).

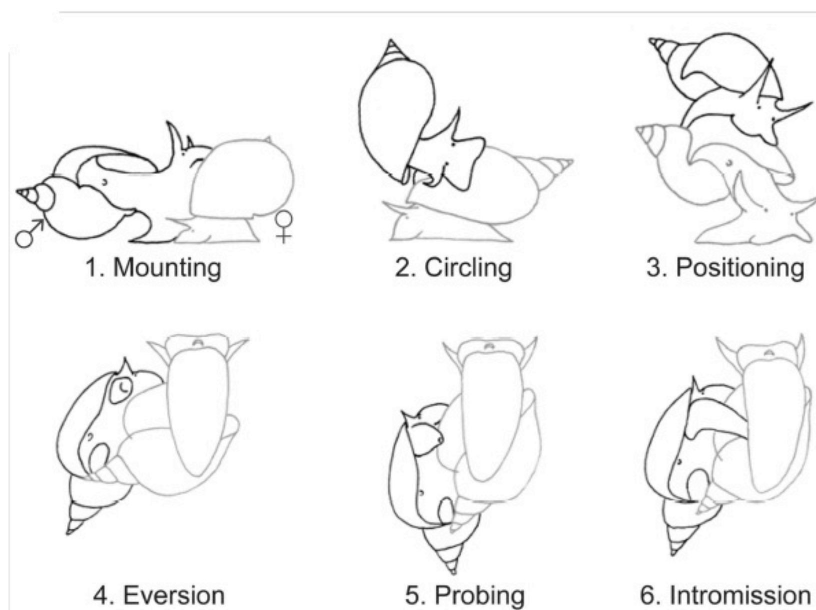

Fig. 1.8: Male courtship in *L. stagnalis*. (From Koene 2010).

In Basommatophora, the reproductive tract is divided in a hermaphrodite, male and female part (Fig 1.9). The hermaphrodite part is constituted by the ovotestis, which is itself divided into acini (responsible for gamete production) and the deferent duct. Spermatocytes and oocytes are produced in specialised regions of each acinus until their maturation. Mature oocytes are stored in the lumen of the ovotestis waiting ovulation, whereas ripe spermatocytes are stored in the seminal vesicle, located within the ovotestis. Fertilisation occurs in the carrefour, or fertilisation pouch, which marks the separation between the male and female reproductive tracts. The male reproductive tract starts with the sperm duct that passes through the prostate gland. This gland adds the seminal fluid and the *vas deferens* further transports the semen to the penis for insemination of the mating partner. Female organs are responsible for egg packaging. The albumen gland adds the perivitellin fluid to the fertilised eggs. *Membrana interna* and *externa*, which envelop each egg, are secreted by the *pars contorta*. Basommatophoran snails lay clutches that contain many eggs fused together in mucus and surrounded by a *tunica interna* and *tunica capsulis*. The muciparous gland secretes the mucus whereas the clutch membrane is produced in the oothecal gland. Finally, *L. stagnalis* lays clutches of 50 to 150 eggs, depending on the individual's age, shell length and environmental conditions, via the female gonopore. The clutches are stuck to the substrate, usually on aquatic plants (Jarne et al. 2010; Koene 2010).

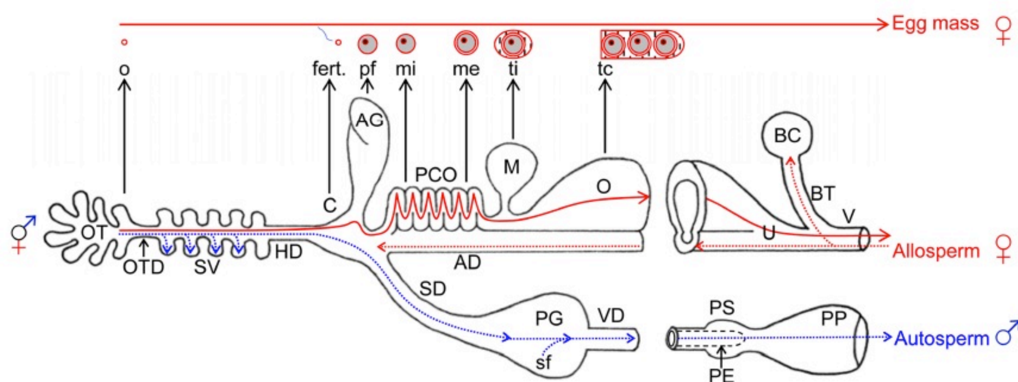

Fig. 1.9: Reproductive tract of *L. stagnalis*. Organs are indicated with capital letters. The different component of the egg masses are indicated in small letters, and their origin is indicated with black arrows. The red solid lines indicate the course of eggs. AD: allosperm duct; AG: albumen gland; BC: *bursa copulatrix*; C: carrefour/fertilisation pouch; fert.: fertilisation; HD: hermaphroditic duct; M: muciparous gland; me: *membrana externa*; mi: *membrana interna*; o: oocyte; O: oothecal gland; OT: ovotestis; P: penis; PCO: *pars contorta*; PE: preputium; pf: perivitellin fluid; PG: prostate gland; SD: sperm duct; sf : seminal fluid; SV: seminal vesicles; tc: *tunica capsulis*; ti: *tunica interna*; V: vaginal duct; VD: *vas deferens*.
(From Koene 2010).

The neuro-endocrine control of the reproduction is particularly well described for male and female functions. A gender laterality of the central nervous system has been observed (Fig. 1.9). The functions of the different regions of the cerebral ganglia have largely been investigated by cauterisation and implantation experiments (Geraerts 1976a; Geraerts and Algera 1976; Geraerts and Joosse 1975), whereas the neuropeptides secreted were identified and localised using immunocytochemistry and *in situ* hybridisation (Croll and Van Minnen 1992; de Lange et al. 1998). The right side of the cerebral ganglia is the main centre for the control of male reproduction. However, it has been observed that, depending on the chirality of the shell, the left side of the central nervous system can be involved in the control of male reproduction (Davison et al. 2009; Koene 2010). The anterior lobe, ventral lobe and pedal lobe clusters secrete the neuropeptides (e.g., APGWamide, conopressin, FMRF, serotonin) that play an essential role in male reproduction, notably through the regulation of the contraction of penis muscles, preputium eversion, and semen transport (reviewed in (Koene 2010)) (Table 1.4).

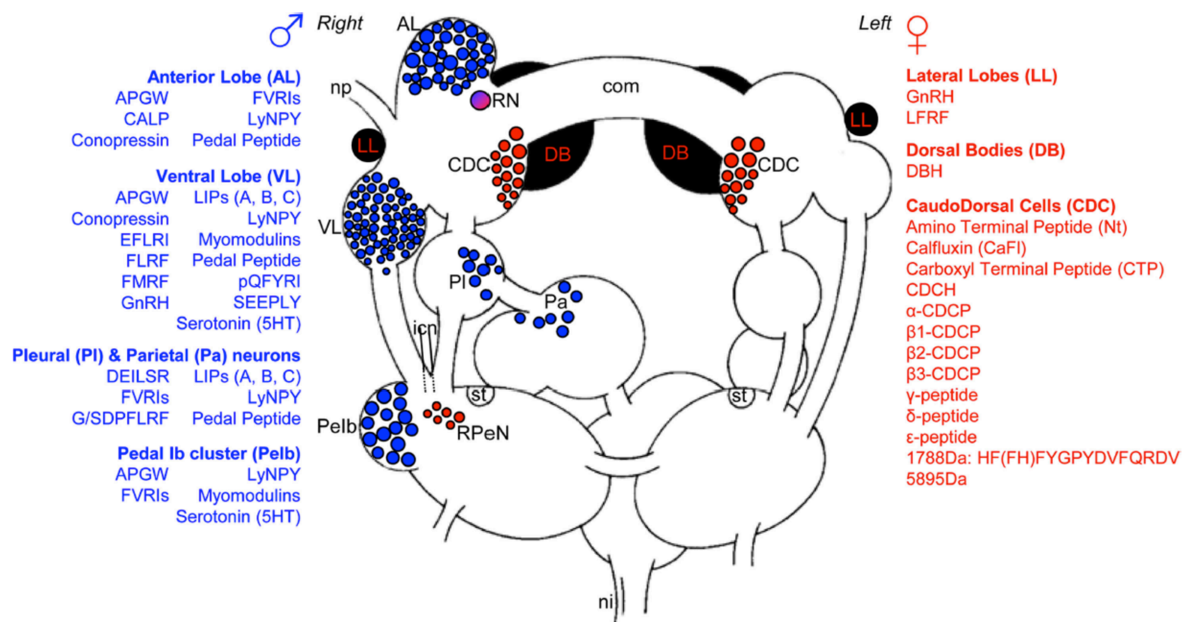

Fig. 1.10: Schematic drawing of the ganglia of the central nervous system. The blue and red areas show, respectively, the neuronal clusters that are involved in male and female reproduction. The substances involved in male reproduction are also indicated in blue; the female peptides are listed in red.
com: cerebral commissure; icn: inferior cervical nerve; ni: *nervus intestinalis*; np: *nervus penis*; RN: ring neuron; RPeN: right pedal neurons; st: statocyst. (From Koene 2010).

The control of female behaviour during reproduction is mainly related to egg production and to oviposition behaviour, as during mating, the female's role is limited to sperm reception. Oviposition is under the control of neuropeptides (i.e., CDCHs) that are secreted by the caudo-dorsal cells (CDCs) of the central nervous system (Geraerts 1976a), whereas vitellogenesis and the development of female reproductive organs are under the control of the dorsal bodies that release dorsal body hormones (DBH) (Geraerts and Joosse 1975) (Table 1.5).

Sex	Transmitter	Effect	Reference
Male	APGW	Eversion of preputium	(de Boer et al. 1997a; de Boer et al. 1997b)
		Relaxation of preputium retractor muscles Hyperpolarisation of Caudo-dorsal Cells (CDCs) and Light Green Cells (LGCs)	(Croll et al. 1991)
		Inhibitory of excitatory effect of conopressin	(van Golen et al. 1995a,b; van Kesteren et al. 1995a, b, c,;1996)
	Conopressin	Stimulation of *vas deferens* contractions	(Van Golen et al. 1995a, b)
		Inhibition of CDCs activity	(van Golen et al. 1995a,b; van Kesteren et al. 1995a, b, c)
	C-terminally located Anterior lobe Peptide (CALP)	Unknown	
	DEILSR	Unknown	
	EFLRI	Unknown	
	FLRF	Contraction of preputium retractor muscles	(van Golen et al. 1995)
	FMRF	Eversion of the preputium	(Fong et al. 2005; Muschamp and Fong 2001)
		Contraction of preputium retractor muscles	(van Golen et al. 1995)
		Inhibition of CDCs activity	(Brussaard et al. 1990)
	FVRIs	Inhibition of *vas deferens* contractions	(El Filali et al. 2006)
	G/SDPFRLRF	Relaxation of preputium retractor muscles	(van Golen et al. 1995)
	Gonadotropin releasing hormone (GnRH)	Unknown	
	Lymnaea inhibitory peptides (LIP A, B, C)	Relaxation of preputium retractor muscles	(Li et al. 1992)
		Reduction of amplitude of preputium retractor muscles contractions	(van Golen et al. 1995b)
	Lymnaea neuropeptide Y (LyNPY)	Relaxation of preputium retractor muscles	(Li et al. 1992; Smit et al. 2003)
		Inhibition of egg-laying and reduction of growth	
	Myomodulins	Relaxation of preputium retractor muscles	(Li et al. 1992)
		Modulation of amplitude of preputium retractor muscles contractions	(van Golen et al. 1996)
	Pedal peptide (PP)	Unknown	
	pQFYRI	Unknown	
	SEEPLY	Unknown	
	Serotonin (5HT)	Contraction of preputium retractor muscles	(Croll and Chiasson 1989; Croll et al. 1991) (Fong et al. 2005; Li et al. 1992)
		Increase in egg-laying	(Manger et al. 1996)

Table 1.4: Effects of the neuro-endocrine substances involved in male reproduction of basommatophoran gastropods. (From Koene 2010).

Sex	Transmitter	Effect	Reference
Females	1788Da:HF(FH)FYGPYDVFQRVD	Unknown	
	5895Da	Unknown	
	Amino terminal peptide (Nt)	Unknown	
	Calfluxin	Influx of calcium into mitochondria of albumen gland	(Dictus et al. 1987)
		Antagonised by schistosomin	(de Jong-Brink et al. 1988a; de Jong-Brink et al. 1988b; Zhang et al. 2009)
	Carboxy terminal peptide (CTP)	Unknown	
	Caudo-dorsal cell hormone (CDCH)	Initiation of ovulation (and thus egg-laying)	(Ter Maat et al. 1986; Ter Maat et al. 1988)
		Inhibition of right pedal motor neurons (RPeN)	(Ter Maat et al. 1988)
		Local autoexcitation of CDCs	(Ter Maat et al. 1988)(Brussaard et al. 1990)
		Stimulation of perivitellin fluid production	(Wijdenes et al. 1983)
		Inhibition of Ring neuron	(Jansen and Ter Maat 1985)
	Caudo-dorsal cell peptide α (α-CDCP)	Local autoexcitation of CDCs	(Ter Maat et al. 1988)(Brussaard et al. 1990)
		Excitation of motor neurons involved in shell movements and Buccal rasping	(Hermann et al. 1997)
	Caudo-dorsal cell peptide ß1	Unknown	
	Caudo-dorsal cell peptide ß2	Unknown	
	Caudo-dorsal cell peptide ß3	Excitation of motor neurons involved in shell movements and Buccal rasping	
	Dorsal body hormone (DBH)	Inhibition of vitellogenesis	(Geraerts and Joosse 1975)
		Stimulation of growth and development of female accessory sex organs	(Geraerts and Algera 1976; Geraerts and Joosse 1975)
		Stimulation of perivitellin fluid production	(Wijdenes et al. 1983)
	Gonadotropin releasing hormone (GnRH)	Unknown	
	LFRF	Inhibition of CDCs and LGCs activity	(Hoek et al. 2005)
	γ-peptide	Unknown	
	δ-peptide	Unknown	
	ε-peptide	Unknown	

Table 1.5: Effects of the neuro-endocrine substances involved in female reproduction of basommatophoran gastropods. (From Koene 2010).

3.2. Impacts of EDCs on Lymnaea stagnalis

Only a handful of studies have investigated the impacts of EDCs in partial or full life cycle tests in *Lymnaea stagnalis*. Exposure of *L. stagnalis* to 100 ng Sn/L of TBT for 49 days has shown to reduce oviposition (Czech et al. 2001). Exposure of clutches to TBT totally inhibited the development of eggs at 10µg/L whereas 1 µg/L induced an abnormal development of embryos (Leung et al. 2007). Furthermore, in the same study, adults that were exposed to 1 µg/L of TBT since their embryonic life stage displayed a reduction of fecundity. Similarly, exposure of clutches to the synthetic oestrogen, EE2, decreased the development of embryos from a concentration of 100 ng/L while exposure of juveniles for 10 weeks to concentrations of EE2 in the ng/L range increased the number of eggs produced per clutch and also increased oviposition (Segner et al. 2003). Exposure to the oestrogenic nonylphenols had adverse impacts on embryo genesis in the µg/L range (Lalah et al. 2007), whereas exposure to 100 µg/L of 4-nonylphenol (4-NP) during 8 weeks had no impact on fecundity, whereas a 12 week exposure significantly decreased the number of clutches laid by individuals (Czech et al. 2001). In the same study, the phyto-oestrogen, ß-sitosterol, induced a non-significant increase in oviposition. Exposure of young adults to the anti-androgenic vinclozolin decreased the number of eggs laid per individuals from 25 ng/L to 2500 µg/L (Ducrot et al. 2010). Finally, the herbicide fomesafen had no effects on clutch production in laboratory experiment, however this molecule has never been reported to interact with steroid hormones (Jumel et al. 2002).

3.3. Lymnaea stagnalis: *model species for OECD test Guideline*

The standardisation of tests used to assess the impact of chemicals released in the environment is of great concern, as it is needed to protect all organisms from potential harmful effects (OECD 2010). Currently, guidelines mainly assess the general toxicity (i.e., carcinogenicity, mutagenicity and reprotoxicity (CMR)) of molecules. However, the need to improve the testing and the evaluation of the endocrine disrupting potency of molecules is essential. Therefore, the development and the validation of OECD guidelines for potential endocrine disruptors were encouraged and an OECD Endocrine

Disrupter Testing and Assessment (EDTA) conceptual framework has been proposed (Fig. 1.11).

OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

Note: Document prepared by the Secretariat of the Test Guidelines Programme based on the agreement reached at the 6th Meeting of the EDTA Task Force

Level	Content
Level 1 Sorting & prioritization based upon existing information	•Physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability •Human & environmental exposure, e.g., production volume, release, use patterns •Hazard, e.g., available toxicological data
Level 2 *In vitro* assays providing mechanistic data	•ER, AR, TR receptor binding affinity •Transcriptional activation •Aromatase & Steroidogenesis *in vitro* •Aryl hydrocarbon receptor recognition/binding •High Through Put Prescreens •Thyroid function •Fish hepatocyte VTG assay •QSARs; Others (as appropriate)
Level 3 *In vivo* assays providing data about single endocrine Mechanisms and effects	•Uterotrophic Assay (estrogenic related) •Hershberger Assay (androgenic related) •Non-receptor mediated hormone function •Fish VTG assay (estrogenic related) •Others (e.g. thyroid)
Level 4 *In vivo* assays providing data about multiple endocrine mechanisms and effects	•Enhanced OECD 407 (endpoints based on endocrine mechanisms) •Male and female pubertal assays •Adult intact male assay •Fish gonadal histopathology assay •Frog metamorphosis assay
Level 5 *In vivo* assays providing data on effects from endocrine & other mechanisms	•1-generation assay (TG415 enhanced) •2-generation assay (TG416 enhanced) •Reproductive screening (TG421 enhanced) •Combined 28 day/reproduction screening test (TG 422 enhanced) •Partial and full life cycle assays in fish, birds, amphibians & invertebrates (development & reproduction)

Fig. 1.11: Conceptual framework for the Testing and Assessment of Endocrine Disrupting Chemicals. (From OECD 2010).

This framework is divided into five levels starting with the sorting and the prioritisation of the putative endocrine disruptors (Level 1), whereas *in vitro* assays, e.g., androgen and oestrogen binding assays, provide mechanistic data (Level 2). *In vivo* tests were mainly developed using vertebrates (i.e., fish, amphibians and rodents) to investigate the impacts and the mechanisms of endocrine disrupters. Even though tests on crustacean exist, as invertebrates are ecologically crucial organisms, it is important to develop further suitable tests for the endocrine disruptors, especially on molluscs that are particularly affected by compounds such as TBT. Another interest for the development of invertebrate testing is the ethical concern about vertebrate organisms used in tests (Matthiessen 2008). Even though it is unlikely that vertebrate testing can be totally avoided, invertebrate testing would help to prevent the systematic involvement of vertebrates in the test guidelines. Endogenous sex steroids are present and are suggested to be synthesised *de novo* in some invertebrate species. Furthermore,

some EDCs can alter steroid concentrations in molluscs. Moreover, molluscs are ecologically and economically important, additionally different lifestyles and reproductive systems are found among phyla (deFur et al. 1999; Lafont and Mathieu 2007). Based on these observations, molluscs were proposed as a candidate phylum for the development of full and partial life cycle tests (Matthiessen 2008). The development and the validation of test guidelines on molluscs are needed in order to provide more comprehensive information on the effects of EDCs (OECD 2010).

Among the multitude of molluscs that were observed to be responsive to endocrine disrupting chemicals, only few species were proposed as candidates for OECD test guidelines. As wild caught animals present unknown life history that can influence the endocrine system (i.e., parasitism, exposure to pollutants), the candidate species must be easily reared and bred under laboratory conditions. For the development of full- or partial life cycle, the generation time and the number of offspring produced are important endpoints (OECD 2010). Furthermore, the apical reproductive endpoints followed during the chemical exposure should be sensitive to EDCs (e.g., oviposition, gamete production, hatching success). Based on these requirements, *Lymnaea stagnalis* was proposed as one of the three candidate species for the development of OECD test guidelines for the assessment of endocrine disruptors (OECD 2010).

4. General aims of the Thesis

The aim of this thesis is to provide a better understanding of the impacts and the mechanisms of action of endocrine disrupting chemicals (EDCs) on gastropod molluscs, especially on the hermaphrodite species *Lymnaea stagnalis*. This species is proposed as a candidate species for the development of OECD guidelines for the Testing and the Assessment of EDCs in molluscs. As the impacts of EDCs have mainly been investigated on gonochoric species of molluscs, it is important to determine the reproductive impacts of these chemicals on hermaphrodites. Furthermore, among the molecules known to interfere with the steroid metabolism and receptors in vertebrates, mollusc studies have mainly focussed on the impacts of organotin and of oestrogenic chemicals. Only few studies have investigated the impacts of androgenic and anti-androgenic molecules on mollusc reproduction. Moreover, the mechanisms of action of endocrine disrupting chemicals are still not fully understood, especially in molluscs.

Therefore, two general objectives are being addressed in this work:

- To identify the impacts of chemicals chosen according to their interactions with steroids in vertebrates, on the reproduction of *L. stagnalis* through the assessment of different reproductive endpoints (e.g., oviposition, fertility).
- To provide new insights on the mechanisms of action of the chemicals tested which disrupted reproductive functions in *L. stagnalis.*

In order to achieve the first objective (Part 1), six different molecules were chosen based on their different mechanisms of action described in vertebrates or for their reported endocrine disrupting impact on molluscs:

- An androgen receptor agonist, i.e., testosterone;
- Three androgen receptor antagonists, i.e., cyproterone acetate, vinclozolin and fenitrothion;
- An oestrogen receptor agonist, i.e., chlordecone;
- A proven endocrine disruptor in molluscs, i.e., tributyltin

Testosterone is a steroid hormone and is the natural ligand of the androgen receptor in vertebrates (Fig. 2.1). Even though this steroid is probably synthesised *de novo* in molluscs, its impacts on the reproduction of molluscs are poorly studied in mollusc species, either bivalves or gastropods.

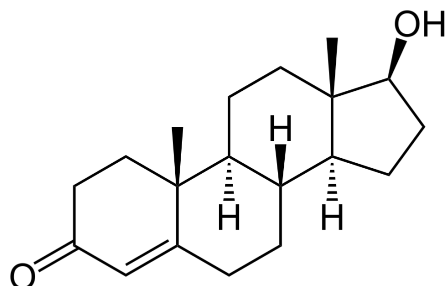

Fig. 1.12: Molecular structure of testosterone.

Cyproterone acetate (CPA) (Fig. 1.13) is a synthetic steroid that acts in vertebrates as an anti-androgen by competitively blocking androgen receptors (Bettin et al. 1996; Tillmann et al. 2001). This chemical is used, among others, as a treatment for prostate cancer, hirsutism and acne (Hammerstein et al. 1975; Tillmann et al. 2001). Furthermore, CPA is also a potent antagonist of the glucocorticoid receptor (Honer et al. 2003) and is known for its progestagenic properties in vertebrates (Raudrant and Rabe 2003). This molecule is considered as a model for anti-androgenic compounds (Kiparissis et al. 2003).

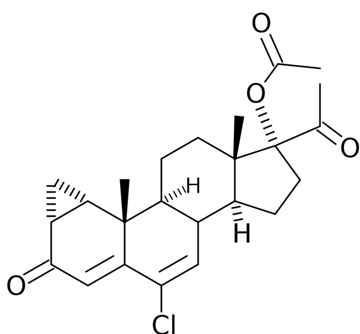

Fig. 1.13: Molecular structure of cyproterone acetate.

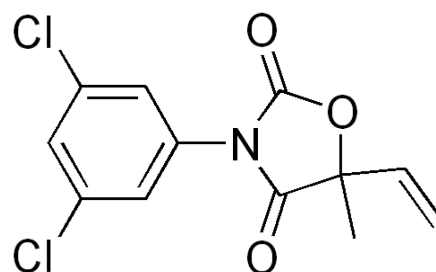

Fig. 1.14: Molecular structure of vinclozolin.

Vinclozolin (Fig. 1.14) is a dicarbamide fungicide used to control various blights and rots caused by fungal pathogens on fruits and vegetables. This molecule, as well as its butenoic acid (M1) and enanilide (M2) metabolites, is able to compete with androgens for androgen receptor binding sites (Kelce et al. 1997; Ostby et al. 1999; Wong et al. 1995). Vinclozolin is proposed as a model for anti-androgenic compounds (Kiparissis et al. 2003; Tillmann et al. 2001).

Fenitrothion (Fig. 1.15) is an organophosphate insecticide, which is a competitive antagonist of vertebrate androgen receptor *in vitro* (Freyberger and Ahr 2004; Tamura et al. 2001). The impacts of fenitrothion on mollusc reproduction have never been investigated.

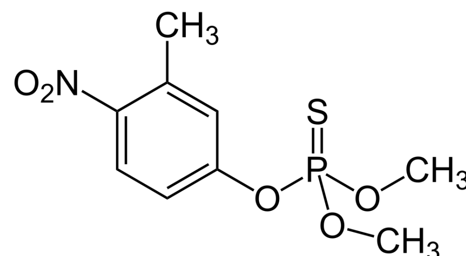

Fig. 1.15: Molecular structure of fenitrothion.

Chlordecone (CLD) is an organochlorinated pesticide (Fig. 1.16) that binds to the vertebrate oestrogen receptors (Hammond et al. 1979) albeit with relatively low affinity (Donohoe and Curtis 1996). This chemical was extensively used against banana roots borer in the French West Indies until its ban in 1993 (Beaugendre 2005; Dubuisson et al. 2007). Recently the Stockholm convention on Persistent Organic pollutants was extended to chlordecone (UNEP 2007; UNEP 2009). This oestrogenic chemical has never been investigated for its impacts on mollusc reproduction.

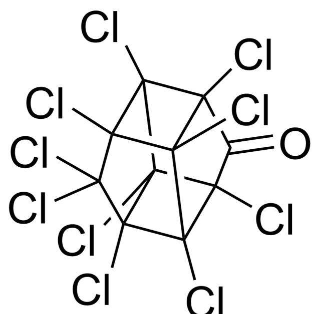

Fig. 1.16: Molecular structure of chlordecone.

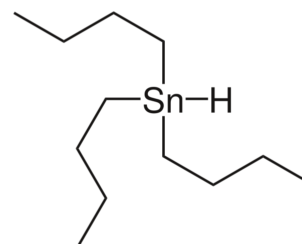

Fig. 1.17: Molecular structure of tributyltin.

Tributyltin (TBT) is an organotin chemical (Fig. 1.17) used as a stabiliser in the manufacturing of plastic products, a preservative for wood, textile, paper, leather and electrical equipment. Additionally, this compound was broadly used as a biocide agent in antifouling paint on the ship hulls until its prohibition in 2008 (IMO 2005). Even though this molecule is able to activate androgen receptor-mediated transcription (Yamabe et al. 2000), this chemical binds to the nuclear receptors, retinoid X receptor and PPARγ (Grün et al. 2006), and also inhibits the activity of aromatases *in vitro* in human cells (Saitoh et al. 2001). The impacts of TBT on mollusc reproduction have extensively been documented in laboratory and field experiments, however the mechanisms of action of this chemical are not fully understood.

The second objective of this thesis (Part 2) is to investigate and to provide new insights on the mechanisms of action of the chemicals that adversely impact the reproduction in *Lymnaea stagnalis*. This objective was subdivided in two parts:

- The impacts of chemical exposure on endogenous concentrations of the vertebrate-like steroid hormone testosterone, which is produced *de novo* in mollusc species, were investigated and the esterification metabolisation of testosterone was subsequently investigated.
- The analysis of the expression of proteins involved in the reproduction of *Lymnaea stagnalis* were performed using 2D-DIGE and Western Blot experiments in order to identify and quantify the alterations induced by the different chemicals.

These results will help to improve the knowledge on the impacts and the mechanisms of action of putative endocrine disrupting chemicals in the hermaphroditic gastropod *Lymnaea stagnalis*, which is necessary for the development of guidelines for the testing and the assessment of these chemicals on molluscs.

Part 2:

Identifying the impacts of EDCs on

Lymnaea stagnalis

Chapter 1:

Range Finding test

1. Introduction

Reprotoxicity tests were conducted in the laboratory according available methods developed to study endocrine disruptors in *Lymnaea stagnalis* gathered in the literature (Ducrot et al., 2010; OECD, 2010). Using these protocols, a range finding test was performed in order to select concentrations of the different chemicals of interest, which may alter the reproduction of *L. stagnalis* without inducing adverse effects on growth and survival. The chemicals were tested at 3 different concentrations with a factor 10 between low, medium and high concentrations. Nominal test concentrations were selected based on the literature specific to the reproductive impacts observed in gastropods (Leung et al., 2004; Lyssimachou et al., 2009b; Schulte-Oehlmann et al., 1995) and *L. stagnalis* (Czech et al., 2001; Leung et al., 2007).

Tributyltin (TBT) was shown to induce imposex in neogastropods species from 0.5 ng Sn/L. Exposure of *N. lapillus* to 1 ng Sn/L of TBT induced imposex while 5 ng Sn/L resulted in the sterilisation of females (Bryan et al., 1987). In *M. cornuarietis*, the calculated effective concentration that induced imposex in 10% of the organisms exposed (EC10) was 3.42 ng Sn/L of TBT and the median effect concentration (EC50) was 115 ng Sn/L (Albanis et al., 2006). The no-observed effect concentration (NOEC) for the development of imposex was lower than the lowest tested TBT concentration (5 ng Sn/L) in *N. reticulatus* (Stroben et al., 1992a). In *L. stagnalis*, exposure of clutches to 10 µg/L of TBT totally inhibited egg development and 1 µg/L was sufficient to induce abnormal embryonic development (Leung et al., 2007). Based on these results we have decided to test TBT concentrations of 1, 10 and 100 µg Sn/L in the range finding test.

The impacts of cyproterone acetate (CPA) on mollusc reproduction were mainly investigated in gastropod species through co-administration with TBT in order to elucidate the mechanisms of action of the imposex development. A concentration of 1.25 mg/L of this anti-androgenic pharmaceutical inhibited the development of imposex induced by 200 ng Sn/L of TBT in *M. cornuarietis* and by 50 ng Sn/L of TBT in *N. lapillus* and *N. reticulatus* (Tillmann et al., 2001). Used as a single contaminant, 1.25 mg/L of CPA led to a reduction in male sex organs and sperm production in males of the two prosobranch species, *N. lapillus* and *N. reticulatus*. The impacts of this molecule on *L. stagnalis* reproduction have never been investigated. The selected concentrations for the range finding test were set at 10, 100 and 1000 µg/L.

Vinclozolin (VZ) had no impact on immature *M. cornuarietis* females at concentrations ranging from 0.03 to 1 µg/L after 3 months of exposure. However, a small, but significant, reduction in the size of the reproductive organs was observed in juvenile males exposed to 0.03 and 0.1 µg/L (Tillmann et al., 2001). Similarly, exposure of sexually mature dogwhelks, *N. lapillus*, to 0.03 µg/L of VZ significantly reduced the penis length and the prostate size after only 1 month of exposure. Furthermore, impairments of spermatogenesis were also reported in this species. Concentrations selected for this experiment were set at 0.1, 1 and 10 µg/l.

The impacts of the organophosphate pesticide fenitrothion (FEN) on the reproduction have never been assessed in molluscs. Therefore the concentrations used in the range finding test were chosen based on the lethal concentrations (LC*x*) reported in two gastropod species, *Lymnaea natalensis* and *Biomphalaria glabrata* (LC10= 120 and 170 µg/L, respectively) (Tantawy, 2006) and were set at 0.2, 2 and 20 µg/L.

Similarly, impacts of chlordecone (CLD) on mollusc reproduction have never been examined. Thus, we have selected our concentration range based on the results of the effects of this pesticide on the reproduction and survival of another invertebrate species, the cladoceran *Daphnia magna* (Sanders et al., 1981). The reproduction of the water flea was significantly reduced after 21 days of exposure to 35 µg/L of CLD whereas a 100% mortality rate was observed after only 5 days of exposure to 15 µg/L. The CLD concentrations selected for the assessment of the impacts on the reproduction of *L. stagnalis* were set at 3, 30 and 300 µg/L.

2. Material and Methods

Lymnaea stagnalis (Linnaeus, 1758) (Mollusca, Gastropoda, Pulmonata, Basommatophora) was reared at the INRA Experimental Unit of Aquatic Ecology and Ecotoxicology (Rennes, France) under laboratory conditions as previously described (Coutellec and Lagadic 2006). The strain RENILYS® was used as this strain is the reference for the development of standard OECD test guidelines. Young adult snails, in which reproduction endpoints are more sensitive to some EDCs than in fully-grown snails (Ducrot et al. 2010), were used in this experiment. Young adults of homogenous size (22.5 ± 2.5 mm) and age (4 ± 0.5 months) were sampled from the culture and acclimatised to test conditions for 48h prior to chemical exposure. Culture and

exposure media consisted in dechlorinated, charcoal-filtered tap water with the following physico-chemical properties: pH = 7.7 ± 0.2, conductivity = 623 ± 60 µS/cm, dissolved oxygen = 7.3 ± 2 mg/L and water hardness = 254 ± 7 mg CaCo3/L. Tests were conducted at 20.5 ± 0.6°C and 14:10 Light:Dark photoperiod. Snails were fed every other day with approximately 2.5 g of organic lettuce (National AB–Agriculture Biologique and International Ecocert certifications) per beaker.

Analytical standards of chlordecone (CLD) (Pestanal®, CAS n° 143-50-0), fenitrothion (FEN) (Pestanal®, CAS n° 122-14-5), vinclozolin (VZ) (Pestanal®, CAS n° 50471-44-8), testosterone (T) (≥98 % purity, CAS n° 58-22-0), cyproterone acetate (CPA) (≥98 % purity, CAS n° 427-51-0) and tributyltin hydride (TBT) (97 % purity, CAS n° 688-73-3) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Due to the low solubility of these chemicals, acetone (99.9 % purity, Burdick & Jackson, Muskegon, MI, USA) was used as the vehicle solvent in which chemicals were dissolved to produce stock solutions that can be poured in the exposure medium, as recommended in the guidance document on aquatic toxicity testing of difficult substances provided by the OECD (2000). Therefore, in addition to water controls, solvent controls were added to assess the potential impact associated to acetone exposure (OECD 2000). It was previously reported that 0.1 % (1 mL/L) of acetone had adverse impacts on shell growth in young *L. stagnalis* whereas 0.4 % (4 mL/L) was the threshold limit for inhibitory effects on growth and for the reduction in the fecundity in adult snails (Bluzat et al. 1979). Therefore, in our study, the final solvent concentration was 100 µl/L (0.01 %) and was homogenous among treatments (except for water controls). Six replicates, consisting in 1L beakers containing 5 snails, per chemical concentration, controls and solvent controls were randomly distributed in the exposure room (Fig. 2.1).

Fig. 2.1: Exposure beakers randomly exposed in the experimental room for the range finding test. Colours of the stickers correspond to chemicals.

Snails were exposed to the chemicals for 21 days in a semi-static experiment. A regular water renewal was required in order to maintain exposure concentrations over the experiment duration. Based on the half-life of each toxicant in freshwater, test water was renewed with freshly contaminated medium every other day in T, FEN and CPA treatments whereas the contaminated water was changed weekly in VZ, CLD and TBT treatments as well as in controls and solvent controls. The contaminated media were sampled in triplicates. Each replicate (200 mL) consisted in the pooling of 100 mL of water sampled from two beakers, 15 min and 48 h after water renewal, and samples were frozen until chemical analysis. Briefly, T and CPA were extracted from water using C-18 solid phase extraction (SPE) and quantified by high-performance liquid chromatography–mass spectrometry (HPLC-MS-MS). CLD was extracted by handshaking 5 mL of exposure water with a volume of 5 mL of dichloromethane. TBT was derivatised and extracted as described in the ISO method for organotin analysis in water (ISO, 2004). CLD analysis and quantification were performed by capillary gas chromatography-electron capture detection (GC-MS-ECD), and TBT was analysed and quantified using capillary gas chromatography-mass spectrometry (GC-MS-MS). TBT concentrations were measured only in water sampled in the 1 µg Sn/L replicates. Actual exposure concentrations were calculated as the time-weighted average exposure concentrations (AEC) over the test period (Belgers et al., 2011). The analytical procedures for the extraction of FEN and VZ are not validated yet; therefore

nominal concentrations were used for these two chemicals (Table 2.1) in the thesis document.

Chemicals	Nominal Concentrations	AECs
Testosterone (T) (ng/L)	10 100 1000	1.4 13.5 125.4
Cyproterone acetate (CPA) (µg/L)	10 100 1000	5.5 59 833.7
Chlordecone (CLD) (µg/L)	30 30 300	1.8 11.3 190.3
Fenitrothion (FEN) (µg/L)	0.2 2 20	- - -
Vinclozolin (VZ) (µg/L)	0.1 1 10	- - -
Tributyltin (TBT) (µg Sn/L)	1	0.4

Table 2.1: Nominal water concentrations and time-weighted average exposure concentrations (AECs) of the endocrine disrupting chemicals tested over 21 days.

Dead snails were counted and removed daily. Shell length was measured to the nearest 0.1 mm using a digital calliper at days 0 and 21 to assess a possible impact of molecules on growth. Effects on *L. stagnalis* reproduction were estimated by monitoring the cumulated number of egg-clutches per snail (oviposition) and the cumulated number of eggs per snail (fecundity). Moreover, egg quality was assessed by determining the frequency of 4 types of abnormalities (Fig. 2.2 A–E): polyembryonic egg (the presence of several embryos per egg); unfertilised egg (the absence of embryo in the egg, which only consists of the eggshell and albumen); atrophied albumen (damaged eggshell containing an abnormally low albumen quantity); and single embryo (presence of a non-developing embryo, without an eggshell and without albumen). Polyembryonic and unfertilised eggs have been described before (Bandow and Weltje, 2012; Bluzat et al., 1979; Lanzer, 1999), and polyembryony has been shown to be a sensitive endpoint (Bluzat et al., 1979). In this work, the two other abnormalities are described for the first time in *L. stagnalis*. Every day, clutches were counted and removed using a sharp-edged spoon. The number and the quality of the eggs were determined using a dissecting microscope.

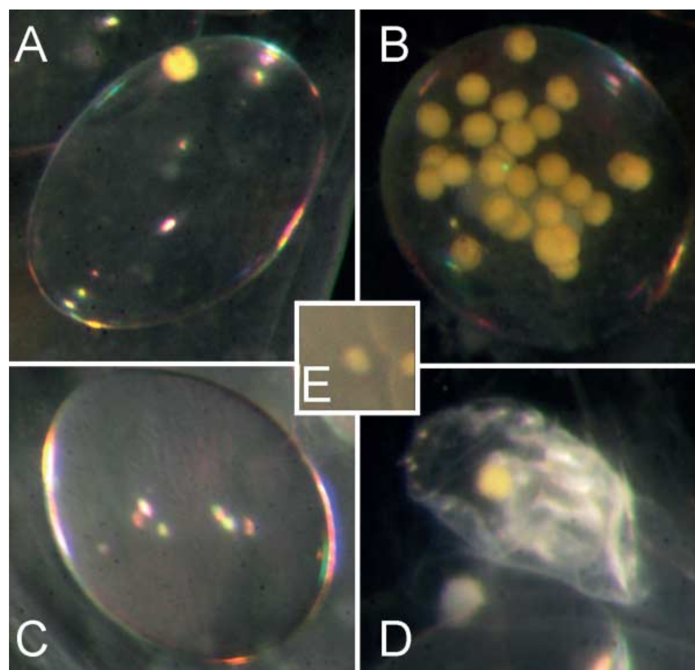

Fig. 2.2: Different egg abnormalities observed in *Lymnaea stagnalis*. (**A**) normal egg; (**B**) polyembryonic egg; (**C**) unfertilised egg; (**D**) egg with atrophied albumen; (**E**) single embryonic cell.

3. Results

3.1. Growth and survival

Survival was of 93 % in controls and 100% in solvent controls. Furthermore, acetone had no impacts on shell growth at the end of the experiment (p=0.48, Mann-Whitney *t*-test). These results suggest that 0.01 % of acetone has no toxic impacts on adult *L. stagnalis*, which is in agreement with previous results that showed a reduction in shell growth in adults from 0.4 % of acetone (Bluzat et al. 1979). Controls and solvent controls were pooled for subsequent statistical analyses.

Exposure of *L. stagnalis* to 10 and 100 µg Sn/L of TBT (nominal concentrations) induced 100 % of mortality after 14 and 3 days, respectively, whereas a 100 % survival rate was observed at the lowest TBT concentration tested (0.4 µg Sn/L, time-weighted average concentrations measured). In the latter TBT treatment, growth was significantly reduced compared to controls (p=0.0218, Mann-Whitney *t*-test). These results highlight that TBT exhibits both lethal and sub-lethal effects in *L. stagnalis* in the concentration range tested.

Exposure to 190.3 µg/L of CLD resulted in a 53.3 % mortality rate after 21 days of exposure. Therefore the corresponding mean lethal concentration (LC50) at 21 days was 183.2 µg/L (Confidence Interval: 143.9 – 403.9 µg/L). Growth, assessed by measuring shell length, was reduced in snails exposed 21 days to the highest CLD concentration tested (190.3 µg/L, $p<0.05$, Kruskal-Wallis, Dunn's *post hoc* test). Lower CLD concentrations had no impact on either growth or survival. None of the other chemicals tested induced adverse effects on shell growth or survival in *L. stagnalis* in the concentration range tested.

3.2. Oviposition and fecundity

No differences between water and solvent controls were observed on any of the reproductive endpoints analysed (i.e., oviposition, fecundity, egg quality) ($p>0.05$, Mann Whitney t-test). Therefore controls were pooled for subsequent statistical analyses. Exposure of *L. stagnalis* to CLD significantly reduced the number of egg-clutches (Fig. 2.3) and the number of eggs (Fig. 2.4) produced per snail from 11.3 µg/L ($p<0.05$, One-Way ANOVA, Dunnett's *post hoc* test). The lowest CLD concentration, 1.8 µg/L, significantly increased the number of eggs per clutch at the end of the experiment ($p<0.05$, Kruskal-Wallis, Dunn's *post hoc* test) (Fig. 2.5) while oviposition was not affected.

Exposure to 0.4 µg Sn/L of TBT reduced both oviposition and fecundity in *L. stagnalis* after 21 days ($p<0.001$, One-Way ANOVA, Dunnett's *post hoc* test) (Fig. 2.3 and 2.4). Moreover, a significant decrease in the number of eggs per clutch was observed ($p<0.001$, Kruskal-Wallis, Dunn's *post hoc* test) (Fig. 2.5). At higher TBT concentrations, oviposition was totally inhibited.

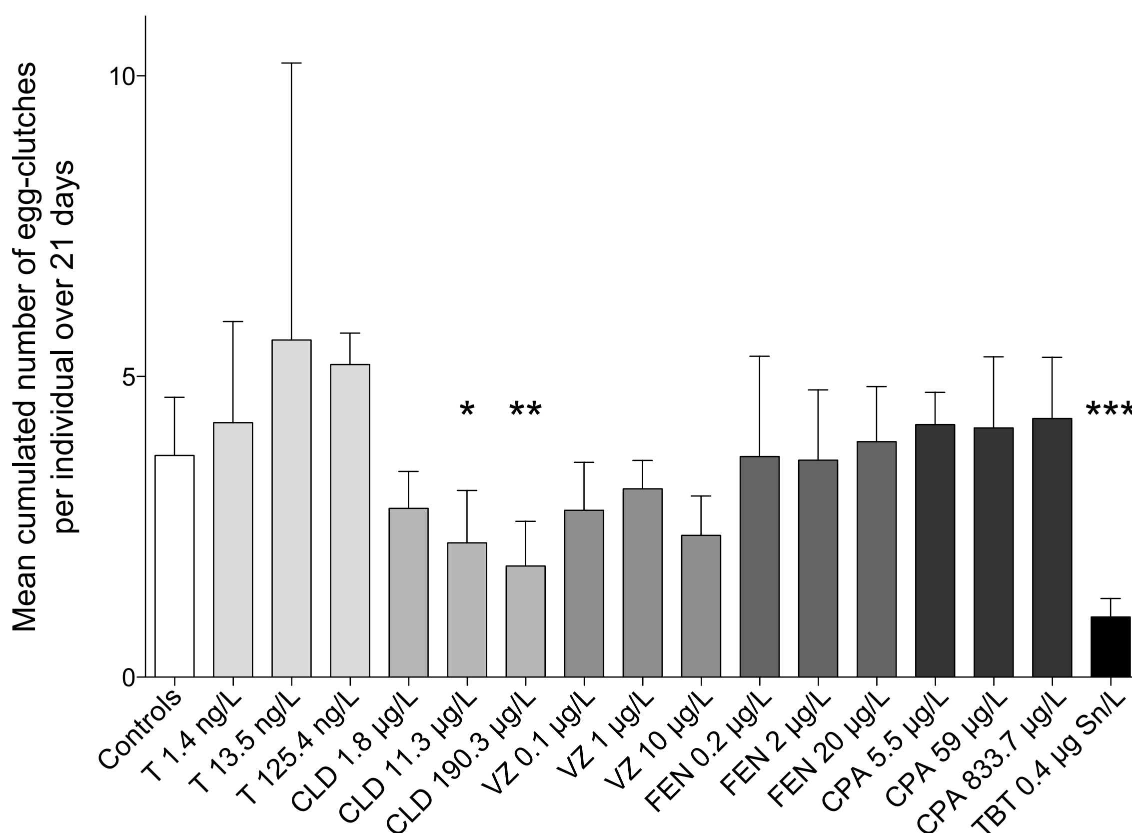

Fig. 2.3: Mean cumulated number of egg-clutches laid per individual over 21 days of exposure to testosterone (T), chlordecone (CLD), vinclozolin (VZ), fenitrothion (FEN) and tributyltin (TBT). Error bars are standard deviations (SD). (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Exposure to T, CPA, FEN and VZ had no impact on either the cumulated number of clutches or the cumulated number of eggs produced per individual over 21 days (Fig. 2.3 and 2.4). It has to be noted that water contaminated with T, FEN and CPA was renewed every other day whereas the exposure medium was changed every week in controls, CLD, VZ and TBT treatments. It was previously observed that water renewal stimulated oviposition in this species (clear water stimulus) (Ter Maat et al. 1983). Therefore, discrepancies in the number of water renewals between controls and treatments over the course of the experiment may induce misleading interpretations of the impacts of T, FEN and CPA on oviposition and fecundity of *L. stagnalis* over 21 days. VZ increased the mean number of eggs per clutch, however this increase was significant only at the lowest concentration tested (0.1 µg/L) ($p<0.05$, One-Way ANOVA, Dunnett's *post hoc* test) (Fig. 2.5). In contrast, in clutches laid by individuals exposed to 833.7 µg/L of CPA the number of eggs was significantly reduced ($p<0.05$, One-Way ANOVA, Dunnett's *post hoc* test) (Fig. 2.5). However, it is unlikely that the

effects reported on the number of eggs per clutch adversely impacted *L. stagnalis* reproduction, as the cumulated fecundity was not significantly altered in these treatments.

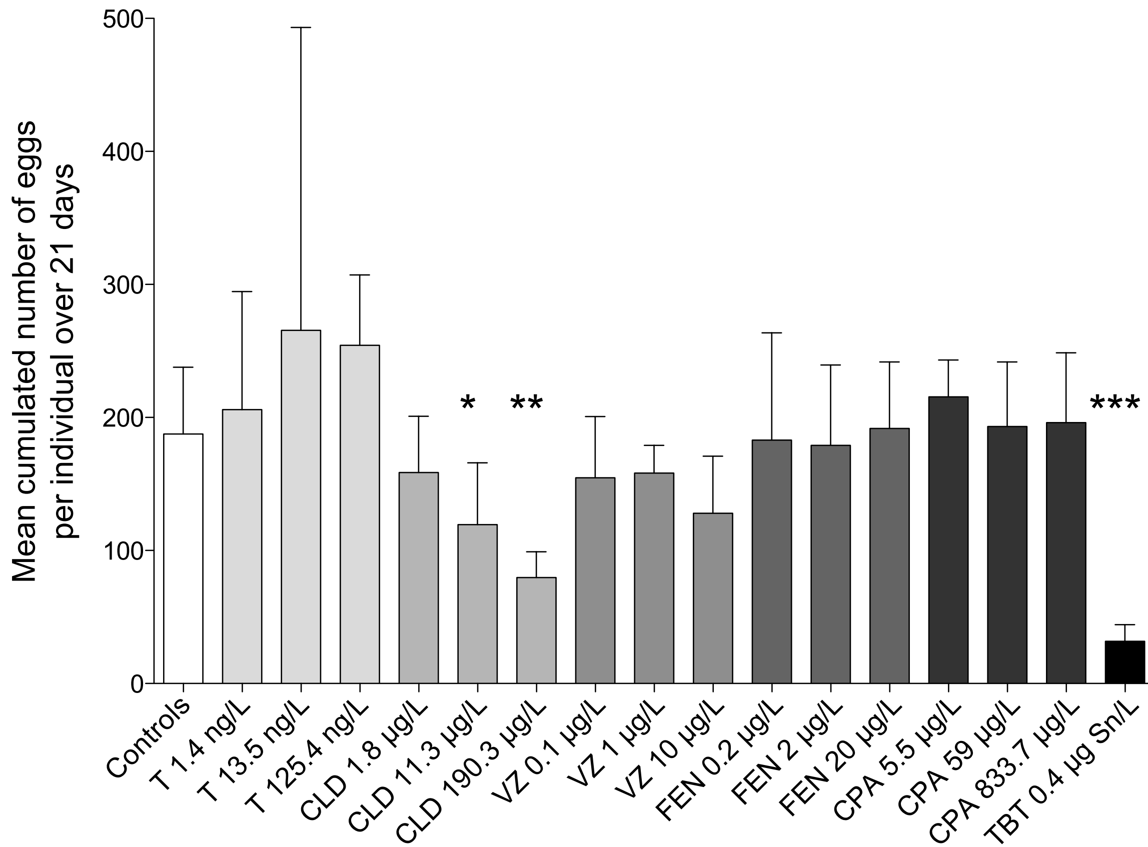

Fig. 2.4: Mean cumulated number of eggs laid per individual over 21 days of exposure to testosterone (T), chlordecone (CLD), vinclozolin (VZ), fenitrothion (FEN) and tributyltin (TBT). Error bars are SD. (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

3.3. Egg quality

The frequency of the occurrence of abnormal eggs was calculated as the ratio between the number of abnormal eggs and the total number of eggs produced per individual. Even though the frequency of abnormal eggs was not significantly altered, the cumulated number of abnormal eggs produced per snail over 21 days of exposure to CPA tended to increase (Fig. 4.6; the difference was statistically significant only when snails were exposed to 59 µg/L of CPA ($p<0.05$, One-Way ANOVA, Dunnett's *post hoc* test)). Exposure to T tended to increase the number of abnormal eggs produced per individual in a dose-response relationship (Fig. 4.6A), whereas 0.1 µg/L of VZ

induced a non-significant increase in abnormal egg frequency (Fig. 4.6B). Conversely, CLD and the 2 highest concentrations of VZ tended to reduce the production of abnormal eggs over 21 days of exposure (Fig. 4.6).

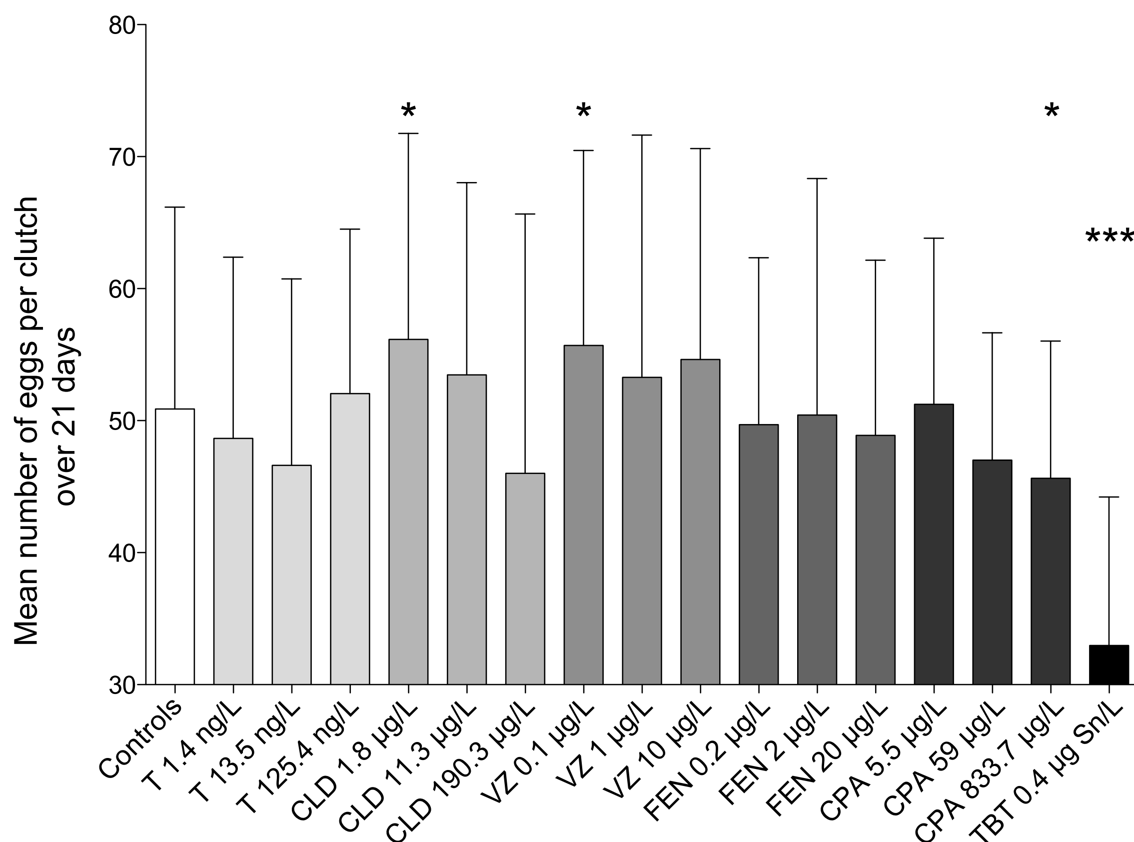

Fig. 2.5: Mean cumulated number of eggs per clutch over 21 days of exposure to testosterone (T), chlordecone (CLD), vinclozolin (VZ), fenitrothion (FEN) and tributyltin (TBT). Error bars are SD. (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Among the different egg abnormalities described, the proportion of albumen-atrophied eggs (over the total number of abnormal eggs) was reduced in *L. stagnalis* exposed to CLD (190.3 µg/L), VZ (10 µg/L) ($p<0.05$, Kruskal-Wallis, Dunn's *post hoc* test) and TBT (0.4 µg/L) ($p<0.01$, Kruskal-Wallis, Dunn's *post hoc* test). The frequency of the other egg abnormalities varied differently between concentrations and chemicals tested, albeit no statistical differences were observed (Table 2.2).

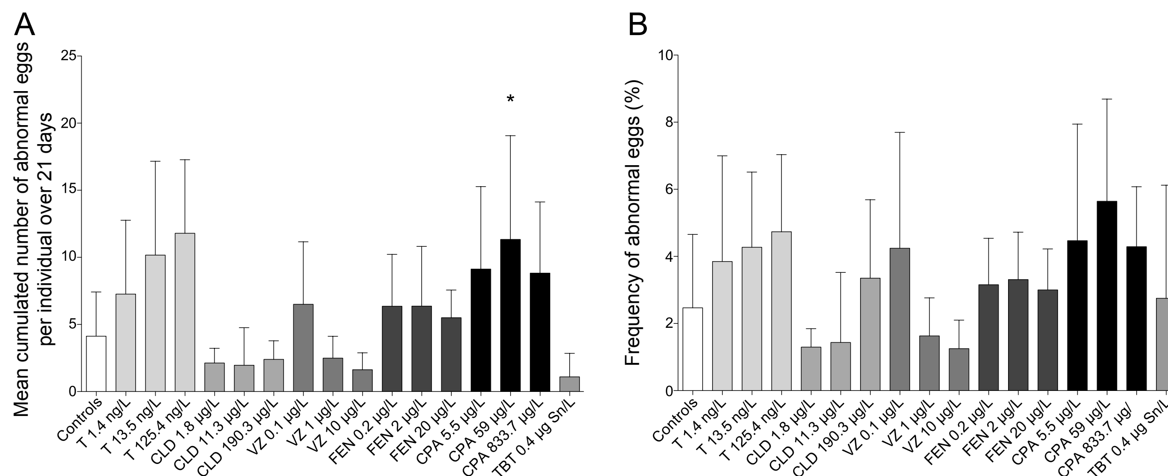

Fig. 2.6: (A) Mean cumulated number of abnormal eggs laid per individual and (B) frequency (%) of abnormal eggs over total number of eggs laid over 21 days of exposure to testosterone (T), chlordecone (CLD), vinclozolin (VZ), fenitrothion (FEN) and tributyltin (TBT). Error bars are SD. (*: p<0.05; **: p<0.01; ***: p<0.001).

Chemicals	Concentration	Frequency (%) (± SD) of unfertilised eggs	Frequency (%) (± SD) of polyembryonic eggs	Frequency (%) (± SD) of albumen atrophied eggs	Frequency (%) (± SD) of single embryo
Controls		24.0 ± 24.6	36.6 ± 21.9	50.7 ± 34.4	8.4 ± 8.4
Testosterone (T) (ng/L)	1.4	10.7 ±9.7	49.0 ± 21.9	30.3 ± 18.5	9.8 ± 9.3
	13.5	7.0 ± 4.8	33.2 ± 23.3	48.1 ± 20.2	12.9 ± 8.4
	125.4	17.0 ± 10.9	35.8 ± 8.3	36.5 ± 7.1	9.4 ± 6.0
Cyproterone acetate (CPA) (µg/L)	5.5	13.7 ± 12.3	36.5 ± 25.6	39.1 ± 27.5	10.3 ± 6.9
	59	34.8 ± 10.1	33.5 ± 7.6	24.7 ± 15.6	3.54 ± 3.4
	833.7	13.1 ± 11.0	51.5 ± 22.6	30.9 ± 12.1	3.8 ± 4.2
Chlordecone (CLD) (µg/L)	1.8	48.2 ± 36.2	33.4 ± 21.5	16.6 ± 13.7	1.8 ± 4.5
	11.3	21.1 ± 9.6	10.7 ± 8.8	60.7 ± 16.8	7.5 ± 9.2
	190.3	24.5 ± 31.6	70.0 ± 27.4	5.6 ± 7.9 *	0
Fenitrothion (FEN) (µg/L)	0.2	12.9 ± 8.5	48.8 ± 20.5	30.2 ± 20.9	2.6 ± 2.1
	2	24.5 ± 15.9	44.1 ± 11.7	24.5 ± 14.1	14.0 ± 24.1
	20	13.4 ± 5.1	33.0 ± 14.0	41.7 ± 21.4	7.0 ± 6.5
Vinclozolin (VZ) (µg/L)	0.1	47.9 ± 66.7	33.4 ± 8.9	32.7 ± 25.9	9.8 ± 13.3
	1	16.5 ± 13.4	48.9 ± 32.0	12.2 ± 14.7	18.3 ± 40.2
	10	18.4 ± 21.9	71.5 ± 24.3	10.1 ± 17.3 *	0
Tributyltin (TBT) (µg Sn/L)	0.4	12.0 ± 26.8	56.6 ± 42.0	4.0 ± 8.9 **	0

Table 2.2: Frequency of egg abnormalities (± SD) in the different treatments over 21 days of exposure to chemicals. Kruskal-Wallis with Dunn's *post hoc* test statistical significance *: p<0.05; **: p<0.01).

4. Discussion

The results of this range finding test showed that high TBT and CLD concentrations (µg/L range) induced toxic effects on growth and survival of *L. stagnalis*. However, at lower concentrations, which had no adverse impact on either growth or survival, both chemicals decreased oviposition and fecundity. The analysis of the number of eggs per clutch helped to investigate whether this decrease in fecundity was due to a side effect of an interaction of the chemicals with oviposition pathways, or if the tested compounds could also affect mechanisms involved in egg production. Our results suggest that CLD adversely affect *L. stagnalis* reproduction through the oviposition pathway. Indeed, we observed that the number of eggs per clutch was either increased or not affected compared to the controls. Exposure to 0.4 µg/L of TBT for 21 days negatively affected reproduction in *L. stagnalis*. These adverse impacts are due to a decrease in oviposition and egg production mechanisms. Because concentrations below 1 µg Sn/L adversely affected the reproduction of *L. stagnalis*, this hermaphroditic gastropod could be as sensitive to TBT as gonochoric species. T, CPA, FEN and VZ did not induce adverse impacts on growth and survival in the concentration range tested. The classical reproductive endpoints assessed, i.e., oviposition and fecundity, were not altered after 21 days of exposure to these chemicals. However, with the exception of VZ, the exposure medium was changed every other day conversely to the controls. It is therefore possible that the clean water stimulus could induce misleading interpretations on the impacts of these chemicals on oviposition (Ter Maat et al., 1983). Finally, the mean number of eggs per clutch was not different from controls except at the lowest concentration of VZ, albeit the total number of eggs produced per snail was not increased in this latter treatment.

Further investigations on the impacts of these molecules on egg quality have provided evidence that T (from nominal concentrations of 10 ng/L), CPA (from nominal concentration of 10 µg/L) and VZ (at a nominal concentration of 100 ng/L) contribute to an increase in the frequency of abnormal eggs. These results suggest that egg quality might constitute a more sensible endpoint than the classical reproductive endpoints (i.e., oviposition and fecundity) to assess adverse impacts of putative EDCs on *L. stagnalis* reproduction.

Based on the results of the range finding test, we have selected a concentration range for each chemical. The concentrations were selected based on their potential to induce alterations of the mollusc reproduction through interaction of the molecules with the endocrine system of *Lymnaea stagnalis*. A spacing factor of 2.2 between each concentration, and at least 5 concentrations were used for the selection of concentrations of chemicals in the subsequent experiments. This will allow to provide sufficient statistical power and an adequate calculation of effect concentrations (OECD 2010). Furthermore, in these further experiments, exposure medium will be renewed every other day in order to avoid misleading interpretations on the impact of the chemicals on the reproduction of *L. stagnalis* due to clean water stimulation of oviposition. In chapter 2, we will investigate the impacts on the reproduction of *Lymnaea stagnalis* of tributyltin (TBT) and triphenyltin (TPT), another organotin that is able to induce imposex development in other mollusc species. We will study their potential impact after 21 days of exposure through the assessment of reproductive endpoints, both classical (i.e., oviposition and fecundity) and egg quality, which we have highlighter in this chapter as very sensitive endpoint. In chapter 3, we will study the activity of enzymes, which are biomarkers of exposure to toxic compounds in vertebrates and invertebrates, in the gonado-reproductive organs of *L. stagnalis* exposed to TBT for 21 days. In chapter 4, we will investigate the impacts of testosterone, cyproterone acetate, chlordecone, fenitrothion, and vinclozolin on the reproduction of *Lymnaea stagnalis* as described in the chapter 2.

Chapter 2:

Impacts of the organotin chemicals tributyltin (TBT) and triphenyltin (TPT) on the reproduction of **Lymnaea stagnalis**

Publication 2:

Reproductive impacts of tributyltin (TBT) and triphenyltin (TPT) in the hermaphroditic freshwater gastropod *Lymnaea stagnalis*

Accepted for publication in Environmental Toxicology & Chemistry

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Abstract

Tributyltin (TBT) and triphenyltin (TPT) are emblematic endocrine disruptors, which have been mostly studied in gonochoric prosobranchs. Although both compounds can simultaneously occur in the environment, they have mainly been tested separately for their effects on snail reproduction. Because large discrepancies in experimental conditions occurred in these tests, the present study aimed to compare the relative toxicity of TBT and TPT under similar laboratory conditions in the range of 0 ng Sn/L to 600 ng Sn/L. Tests were performed on the simultaneous hermaphrodite *Lymnaea stagnalis*, a freshwater snail in which effects of TPT were unknown. Survival, shell length, and reproduction were monitored in a 21-day semi static test. Frequency of abnormal eggs was assessed as an additional endpoint. TPT hampered survival while TBT did not. Major effects on shell solidity and reproduction were observed for both compounds, reproductive outputs being more severely hampered by TBT than by TPT. Considering the frequency of abnormal eggs allowed increasing test sensitivity, because snail responses to TBT could be detected at concentrations as low as 19 ng Sn/L. However, the putative mode of action of the two compounds could not be deduced from the structure of the molecules or from the response of apical endpoints. Sensitivity *of L. stagnalis* to TBT and TPT was compared with the sensitivity of prosobranch molluscs with different habitats and different reproductive strategies.

Keywords: Reproductive toxicity; Endocrine Disruptors; Mollusc toxicology; Organotin; Toxicity mechanisms

1. Introduction

Organotins have been used for more than 50 years, mainly as pesticides, antifungal agents and, in the case of the widely used tributyltin (TBT) and triphenyltin (TPT), as the active biocides of antifouling paints (Hobler et al., 2010). Their use in antifouling paints was prohibited in 2008 (IMO, 2005), but residues can still be found both in marine environments and in freshwater ecosystems at concentrations up to, for example, 7.1 µg TBT/L (IPCS, 1990). Therefore, the assessment of toxic effects of these compounds to aquatic, freshwater wildlife is still relevant. To date, TBT and TPT have mostly been investigated separately as single compounds. These studies have pointed out some similarities between TPT and TBT regarding their physicochemical properties and biological effects. Indeed, these molecules have a closely related structure: a tetravalent tin core with either three butyls (TBT) or three phenyls (TPT). These compounds are fairly persistent in the environment, being retained in sediments (Antizar-Ladislao, 2008; Bigatti et al., 2009; De Oliveira et al., 2010). Furthermore, they penetrate biological systems and can be stored at high concentrations in lipid-rich tissues of aquatic organisms; for example, TBT levels up to 233 ng/g have been recorded in retail mollusc products (Forsyth and Casey, 2003). Both TBT and TPT accumulate preferentially in the hepatopancreas and kidneys and at lower levels in the heart and brain (Duft et al., 2003a; Harino et al., 2000; Strand and Jacobsen, 2005). Consequently, both TBT and TPT can induce adverse effects on wildlife, which have been studied mainly in marine molluscs.

As early as 1975, adverse impacts of TBT— shell calcification in adults leading to stunted growth—were observed in the oyster *Crassostrea gigas* (Alzieu, 1998). At low concentrations (nanograms per litre), TBT was shown to induce the imposition of male sex organs in female prosobranchs (Gibbs et al., 1988). This phenomenon, named *imposex*, has now been reported to occur in more than 200 mesogastropod and neogastropod marine and freshwater species (Horiguchi et al., 2012). At highly contaminated sites, females can be sterilised or even killed, which may affect population dynamics (Bauer et al., 1997). In addition, TPT was shown to induce reproductive failure through imposex in different gastropod species (Horiguchi et al., 1997; Lyssimachou et al., 2009a; Schulte-Oehlmann et al., 2000). A number of studies reported other types of reproductive alterations in freshwater species due to exposure

to organotins (Czech et al., 2001; Janer et al., 2006; Lagadic et al., 2007; Leung et al., 2007; Leung et al., 2004; Schulte-Oehlmann et al., 1995; Segner et al., 2003). For instance, exposure of *Lymnaea stagnalis* to TBT at 1 μg Sn/L was shown to induce abnormal development of embryos (absence of shell), and a decrease in egg hatchability; exposure to 10 μg Sn/L led to a complete hatching failure (Leung et al., 2007).

Organotins generally occur in the environment as mixtures of TBT and TPT and their derivatives (i.e., dibutyltin [DBT], monobutyltin [MBT], diphenyltin [DPT], and monophenyltin [MPT])(Ceulemans et al., 1998; Jadhav et al., 2011). Therefore, a growing number of studies are devoted to the study of the relative toxicity of these compounds, which constitutes the first step toward the study of mixture toxicity. Comparisons of bioaccumulation and biological responses to TBT and TPT have already been done in several gastropod species such as the muricids *Thais clavigera* and *Thais bronni* (Horiguchi et al., 1994), as well as *Bolinus brandaris* and *Hexaplex trunculus* (Lyssimachou et al., 2009b). These studies allow a straightforward comparison of TBT and TPT effects under similar test designs and experimental conditions and using similar test endpoints. This ensures both quantitative and qualitative reliability of effect comparisons through the avoidance of experimental confounding factors, which often occur when data from different studies are compared. To date, these comparative studies have dealt only with gonochoric species, in which toxic effects can be assessed through direct monitoring of the morphological changes in the sexual apparatus. Furthermore, impacts on endocrinology and especially on sexual steroids are more easily understood in species that exhibit separated genders, in which the male and female hormonal reproductive pathways can be distinguished.

Effects on the reproductive pathways and performances in hermaphroditic species are more subtle. Probably due to the complex sexual apparatus and the variety of reproduction strategies in simultaneous hermaphrodite gastropods (e.g., selfing vs. outcrossing) (Jarne et al., 2010), the effects of organotins in these animals have not yet been extensively investigated (Lagadic et al., 2007). In particular, it is important to investigate suitable endpoints that will allow the highlighting of reproductive effects in species in which sex differentiation cannot be used as an effect criterion. To date, most studies have been conducted using the great pond snail *L. stagnalis*. This holarctic freshwater snail lives in ponds and lakes. It is a simultaneous hermaphrodite, which

can outcross and self-fertilise (Coutellec and Lagadic, 2006). It has been identified as one of the most relevant mollusc species for assessing reprotoxic effects of chemicals (Jarne et al., 2010; Koene, 2010; Lagadic et al., 2007; Matthiessen, 2008; OECD, 1999). Because its neurohormonal control of reproduction is reasonably well understood compared with other mollusc species (Ducrot et al., 2010; Geraerts, 1976a; Koene, 2010; OECD, 1999), and also because it has been shown to be sensitive to endocrine disruptors (Czech et al., 2001; Ducrot et al., 2010; Leung et al., 2007; Matthiessen, 2008; Segner et al., 2003), standard Organisation for Economic Co-operation and Development (OECD) test guidelines for apical reprotoxicity tests (both partial and full life cycle tests) with *L. stagnalis* are currently under development.

In the present study, we aimed to investigate the reproductive effects of the emblematic organotin compounds TBT and TPT, known as endocrine disruptors in some mollusc species, in the hermaphroditic freshwater snail *L. stagnalis*. The effects of TBT and TPT were studied comparatively under the same controlled laboratory conditions. Reproductive effects were assessed through a set of complementary apical endpoints (i.e., number of egg clutches, number of eggs, and frequency and type of abnormal eggs). Adult survival and growth also were monitored. Effects of TBT and TPT were compared both qualitatively and quantitatively. The influence of the structure of the molecules on the biological responses and possible corresponding modes of toxic action are discussed. Moreover, biological responses of this hermaphroditic snail to TBT and TPT were compared with available data in other mollusc species.

2. Material and methods

2.1. Test organisms

Lymnaea stagnalis (Linnaeus, 1758; Mollusca, Gastropoda, Panpulmonata, Heterobranchia) were reared at the Experimental Unit of Aquatic Ecology and Ecotoxicology (National Institute for Agricultural Research, Rennes, France) under laboratory conditions as previously described (Coutellec and Lagadic 2006). The culture medium consisted of dechlorinated, charcoal-filtered tap water with the following physicochemical characteristics: pH 7.7 ± 0.2, conductivity 623 ± 60 µS/cm,

dissolved oxygen 7.3 ± 2 mg/L, and water hardness 254 ± 7 mg $CaCO_3$/L. Rearing conditions were as follow: temperature 20 ± 1 °C, photoperiod 14:10-h light:dark, and light intensity 155 ± 35 lux. Snails (RENILYS® strain) were fed three times per week with organic lettuce. Young adult snails - in which reproduction endpoints are more sensitive to chemicals, including some endocrine disrupting chemicals, than in fully grown snails (Ducrot et al. 2010) - were used. Snails of homogenous size (22.5 ± 2.5 mm) and age (4 ± 0.5 months) were sampled from the culture and acclimatised to test conditions (i.e., similar to culture conditions, but with a higher food quantity provided per individual) during the 48 h prior to chemical exposure.

2.2. Tested chemicals and concentrations

Tributyltin hydride (Chemical Abstract Service [CAS] number 688-73-3) and triphenyltin chloride (CAS number 639-58-7) were dissolved in analytical grade acetone (99.9% purity) to prepare stock solutions (10 µg/µL). Final solvent concentration (100 µL/L) was homogenous among treatments, as recommended by the OECD (2000) (except for the water controls). Exposure media consisted of culture water contaminated with stock solutions. Organotin concentrations were chosen based on literature data in gastropods (Leung et al. 2004; Lyssimachou et al. 2009a; Oehlmann et al. 1996; Schulte-Oehlmann et al. 1995) and *L. stagnalis* (Czech et al. 2001; Leung et al. 2007). Range-finding tests in *L. stagnalis* were also performed under similar tests conditions (Barsi et al. 2011; Giusti et al. 2010; Part 2: chapter 1). Based on this information, the chosen nominal concentrations were 45 ng Sn/L, 100 ng Sn/L, 220 ng Sn/L, 480 ng Sn/L, and 1065 ng Sn/L for TBT, and 100 ng Sn/L, 215 ng Sn/L, 755 ng Sn/L, 1000 ng Sn/L, and 2626 ng Sn/L for TPT. To facilitate the quantitative comparison of TBT versus TPT effects, all concentrations were expressed in tin equivalent; concentrations could thus be compared on a molar basis.

2.3. Test design and biological endpoints

Six replicates (each with 5 snails in 1-L glass beakers) per tested concentration, water control, and solvent control were randomly distributed in the exposure room. Snails were exposed to toxicant for only 21 days because this duration was sufficient to

provide evidence of effects and assess various effective concentration (EC*x*) values for the compounds tested in the proposed experimental conditions (as determined in pre-experiments). Test water was renewed with freshly contaminated medium to maintain exposure concentrations and adequate physicochemical properties of test water. Renewal rates resulted from a compromise between maintaining exposure concentration and avoiding too much stress to the snails. Independent studies showed that in our test conditions, TBT water concentration dropped rapidly (92% losses in 72 h), so that the TBT test medium was renewed every other day. The TPT was more stable (62% losses in 72 h), and thus the TPT test medium was renewed every 3 days. Tests were conducted at 20.5 ± 0.6°C and in a 14:10-h light:dark photoperiod as previously described (Ducrot et al. 2010). Snails were fed daily ad libitum with organic lettuce rinsed with culture water, which is an adequate food source to support adult snail growth and reproduction (Zimmer et al. 2012).

Dead snails were counted and removed daily. Individual shell length was measured using a digital calliper at days 0 and 21 to assess a possible impact on growth. Effects on reproduction were estimated by monitoring the cumulated number of egg clutches per snail and the cumulated number of eggs per snail. Every day, clutches were counted, removed using a sharp-edged spoon, and photographed. The number and quality of the eggs were determined by observation of the photographs. Egg quality was assessed by determining the frequency of 4 types of abnormalities (Fig. 3.1 A–E): polyembryonic egg (the presence of several embryos per egg); unfertilised egg (the absence of embryo in the egg, which only consists of the eggshell and albumen); atrophied albumen (damaged eggshell containing an abnormally low albumen quantity); and single embryo (presence of a nondeveloping embryo, without an eggshell and without albumen). Polyembryonic and unfertilised eggs have been described before (Bandow and Weltje 2012; Bluzat et al. 1979; Lanzer 1999), and polyembryony has been shown to be a sensitive endpoint (Bluzat et al. 1979). The 2 other abnormalities are described for the first time in *L. stagnalis*.

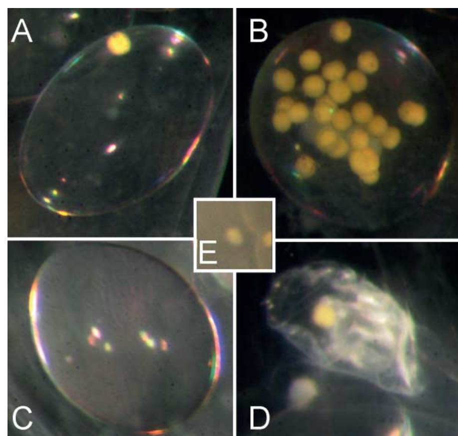

Fig. 3.1: Different egg abnormalities observed in *Lymnaea stagnalis*. (**A**) normal egg; (**B**) polyembryonic egg; (**C**) unfertilised egg; (**D**) egg with atrophied albumen; (**E**) single embryonic cell.

2.4. Chemical analysis

Water was sampled in controls and every tested concentration at the beginning, middle, and end of the tests. Water was collected both 15 min after stirring stock solutions in clean water (i.e., new exposure water) and just before water renewal (i.e., old exposure water) to allow calculation of time-weighted average exposure concentrations. For each concentration and sampling date, three samples of 1 L were collected, which consisted of a mixture of 165 mL of water extracted from each exposure replicate. In the TBT experiment, mucus that had accumulated on the walls of the test beakers was also collected for chemical analysis. Samples were frozen until analysis of their MBT, DBT, and TBT and TPT content. Analyses were performed by coupled capillary gas chromatography/mass spectrometry, with a limit of quantification of 10 ng Sn/L equivalent (ISO 2004).

2.5. Data analysis

Actual exposure concentrations were calculated as the time-weighted average of measured values over the test, using the formula proposed in Belgers et al. (2011). Biological data were analysed with standard statistical analysis procedures, as described by the OECD (2006), using Sigma-Stat (Jandel Scientific) and GraphPad Prism 5.0 software. Analyses of survival and reproduction data were based on observations for the six replicates, and analysis of growth data was based on individual length measurements. Analysis of abnormality frequency was based on the total number of clutches collected during the experiment. Statistical differences between

water and solvent controls were determined using *t* tests or Wilcoxon tests. When a significant difference with water control was shown for one of the compounds, solvent controls were used as the reference in subsequent statistical studies for both compounds. Indeed, using the same type of controls for the calculation of EC*x* values allows one to avoid confounding effects of the solvent. In other cases, water and solvent controls were combined.

Differences among treatments in survival, shell length, and cumulated number of clutches and eggs per individual were tested using Kruskal–Wallis tests, with Dunn's post hoc tests for survival and size and Dunnett's post hoc test for reproductive endpoints. Differences in the frequency of abnormal eggs were assessed using Mann–Whitney tests. All tests were performed with a $\alpha = 0.05$. In case of significant effects of TBT or TPT, different lethal concentrations (LC*x*) or EC*x* were calculated using a logistic regression model (Duggleby 1981). The 95% confidence intervals (CI) were simulated based on weighted residues to account for differences in variance across treatments and using 5000 bootstrap simulations. The Microsoft Excel macro REGTOX_EV7.0.6 xls was used for this purpose (Vindimian 2001).

3. Results

3.1. Actual exposure conditions

The chemical preparation and contamination method resulted in exposure concentrations corresponding to 62.7 ± 14.2 % and 87.5 ± 17.6 % of the nominal TBT and TPT concentrations, respectively, at day 0. Water concentrations dropped rapidly and with different kinetics for TBT and TPT. Before water renewal, exposure concentrations had dropped to 35.7 ± 4 % and 18.1 ± 3 % of the nominal TBT and TPT concentrations, respectively. This resulted in time-weighted average concentration ranges of 19 ng Sn/L, 43 ng Sn/L, 94 ng Sn/L, 197 ng Sn/L and 473 ng Sn/L for TBT and 45 ng Sn/L, 74 ng Sn/L, 187 ng Sn/L, 265 ng Sn/L and 590 ng Sn/L for TPT. High TBT concentrations were also found in the mucus sampled after 21 days (e.g., 1563 ± 638 ng Sn/L at the highest tested concentration). Both MBT and DBT were found in water, due to the degradation of TBT. Concentrations of MBT and DBT were not included in the calculation of TBT time-weighted average concentrations because their

concentration were generally lower than the quantification limit, so they could be neglected.

3.2. Survival

Survival was 100% in water controls from both tests and 93% and 88% in the solvent controls from TBT and TPT tests, respectively. No significant mortality was recorded following exposure to TBT over the test duration. Exposure to the highest tested concentration of TPT (590 ng Sn/L) led to 100% mortality after 3 days of exposure ($p < 0.05$, Dunn's *post hoc* test), while lower concentrations did not significantly affect survival. The corresponding median lethal concentration (LC50) at 21 days was estimated at 436.1 ng Sn/L (CI: 308.1 – 433.6 ng Sn/L).

3.3. Shell size and integrity

Shell length at 21 days was significantly reduced in snails exposed to TBT concentrations exceeding 94 ng Sn/L (Fig. 3.2 A). A non-significant decrease was also observed in snails exposed to 43 ng Sn/L. A careful inspection of snails showed that the apex of the shells was broken in some or all of the exposed individuals, leading a reduced shell length. Snails exposed to 0 ng Sn/L and 19 ng Sn/L generally did not suffer such injury. This finding suggests that broken shells were not experimental artefact but a consequence of exposure to TBT. Indeed, the frequency of harmed shells increased linearly with TBT concentration ($r^2 = 0.94$) and reached 100% at the highest tested concentration of 473 ng Sn/L (Fig. 3 A). Therefore, the reduction in shell length provides evidence for the effects of TBT on shell solidity. Analysis of size data only in snails that did not suffer shell injury highlighted a significant reduction in growth for TBT concentrations exceeding 94 ng Sn/L. As the growth effect was not very intense in the concentration range tested, no reliable EC*x* value could be calculated. Damaged shells were observed at all TPT concentrations tested (but not in controls), suggesting that TPT also had an impact on shell solidity (Fig. 3.3 B). Damage frequency was between 50 and 70%, regardless of exposure concentration. No significant effect on growth was found with TPT (Fig. 3.2 B).

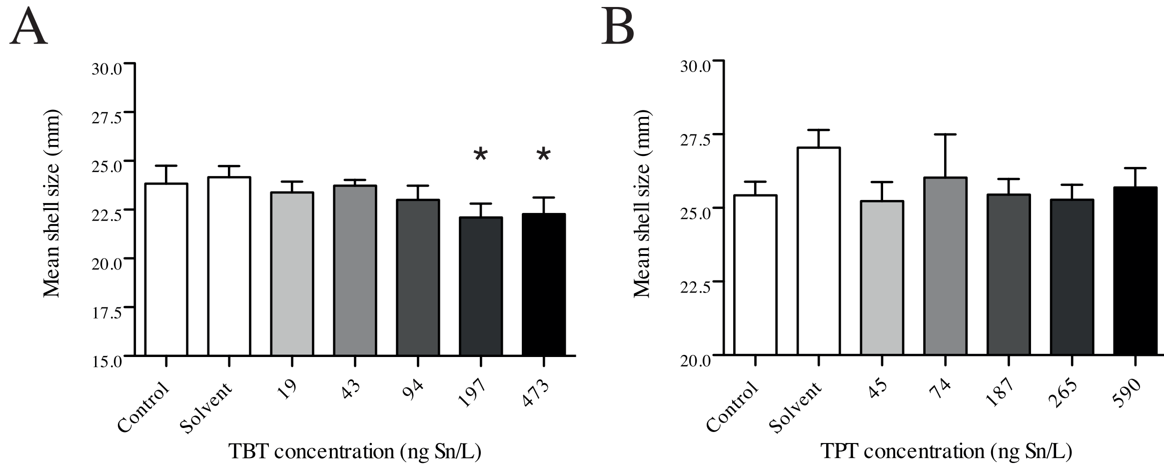

Fig. 3.2: Mean shell size after a 21-d exposure to (**A**) tributyltin (TBT) or (**B**) triphenyltin (TPT). Error bars represent standard deviation over six replicates (*****: p<0.05).

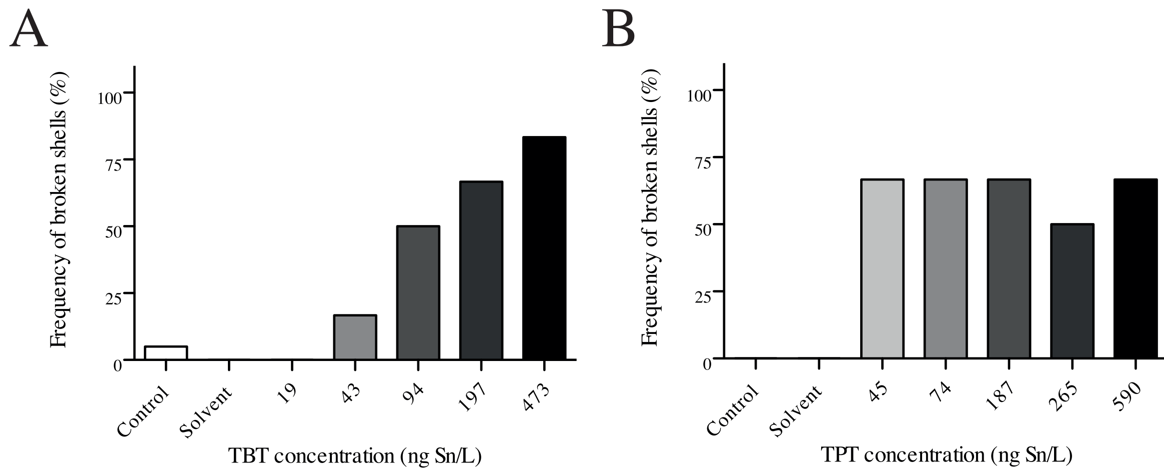

Fig. 3.3: Frequency of broken shells observed over six replicates after a 21-d exposure to (**A**) tributyltin (TBT) or (**B**) triphenyltin (TPT).

3.4. Egg-laying behaviour

The time-course of effect was different in snails' exposed TBT concentrations of 94 ng Sn/L or less: egg-laying occurred regularly but at a slower rate than in controls. For snails exposed to 197 ng Sn/L and 473 ng Sn/L, egg-laying ceased after 1 week of exposure. The cumulated number of clutches produced per individual over 21 days decreased in all snails exposed to TBT compared with the water controls, from -24% at 19 ng Sn/L to -96% at 473 ng Sn/L (Fig. 3.4 A). A significant difference from solvent controls in the number of produced clutches ($p < 0.05$, Dunnett's post hoc test) was

detected at 94 ng Sn/L. Exposure to the two highest TBT concentrations resulted in a severe reduction in egg-laying activity ($p < 0.001$, Dunnett's post hoc test). The corresponding median effective concentration (EC50) at 21 days was estimated to be 118.3 ng Sn/L (CI: 99.7 – 171.9 ng Sn/L).

In the TPT test, the time-course of effect was similar in snails exposed to all concentrations tested; snails regularly laid eggs, but the egg-laying rate was lower than in controls. Egg-laying activity was significantly lower in solvent control ($p < 0.05$, Wilcoxon rank sum test) than in water controls (Fig. 3.4B). Solvent control was thus used as a reference for statistical tests for both TPT and TBT. Exposures to 265 ng Sn/L and 590 ng Sn/L caused a significant decrease ($p < 0.01$ and $p < 0.001$, respectively, Dunnett's post hoc test) in the cumulated number of clutches produced (-38% and -81% of the solvent control value, respectively). Based on these results, the estimated EC50 21-d value was 264.1 ng Sn/L (CI: 258.5 – 280.5 ng Sn/L).

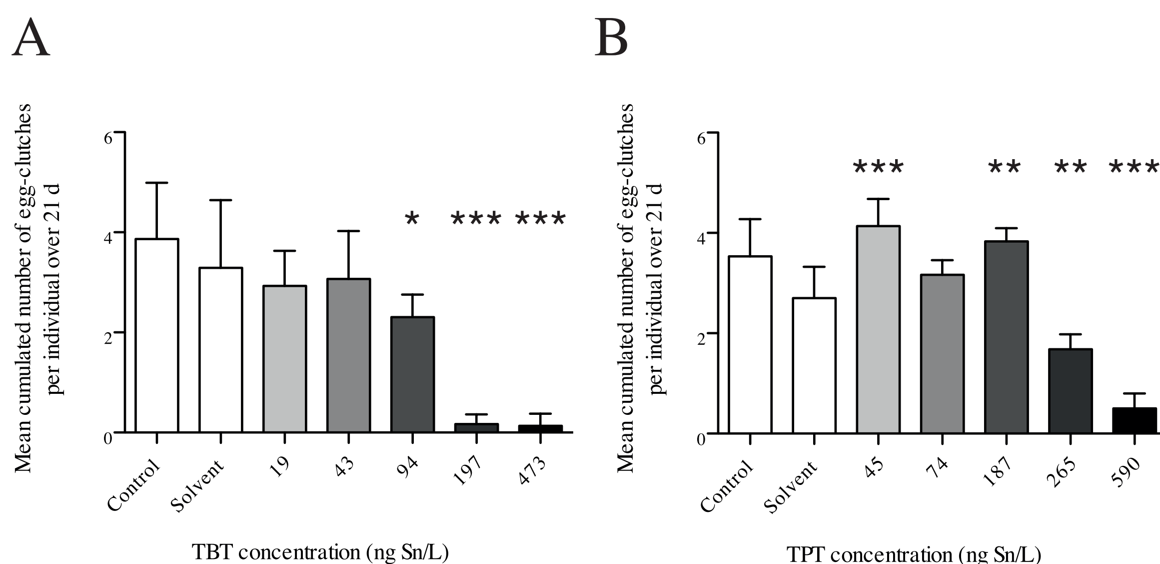

Fig. 3.4: Mean cumulated number of egg-clutches laid per individual after a 21-d exposure to (**A**) tributyltin (TBT) or (**B**) triphenyltin. Error bars represent standard deviation over six replicates. (*: p<0.05; **: p<0.01; ***: p<0.001).

3.5. Fecundity

The effect patterns and estimated no-observed effect concentration (NOEC) and lowest-observed effect concentration (LOEC) values of TBT and TPT for fecundity were similar to the patterns observed for egg-laying behaviour: the LOEC values were 94 ng Sn/L and 264 ng Sn/L ($p < 0.01$ and $p < 0.001$, respectively, Dunnett's post hoc test) for TBT and TPT, respectively.

Compared with water controls, a non-significant reduction in fecundity was already observed at the 2 lowest TBT concentrations and was particularly severe at the 2 highest tested concentrations (from -32% at 19 ng Sn/L to -97% at 473 ng Sn/L, as shown in Fig. 3.5 A). The corresponding EC50 21-d value was 106.2 ng Sn/L (CI: 84.4 – 125.6 ng Sn/L), which was not significantly different from the EC50 at 21 days found using oviposition as an endpoint.

A significant solvent effect on fecundity was again observed in the TPT test ($p < 0.01$, Wilcoxon rank sum test). Fecundity of the snails exposed to the two highest TPT concentrations (265 ng Sn/L and 590 ng Sn/L) was significantly reduced (-36% and -85% of the solvent control value, respectively) as shown in Fig. 3.5 B. The corresponding $EC_{50\text{-}21d}$ value was 263.9 [253.8 – 280.5] ng Sn/L, which was similar to the EC50 21-d found for oviposition.

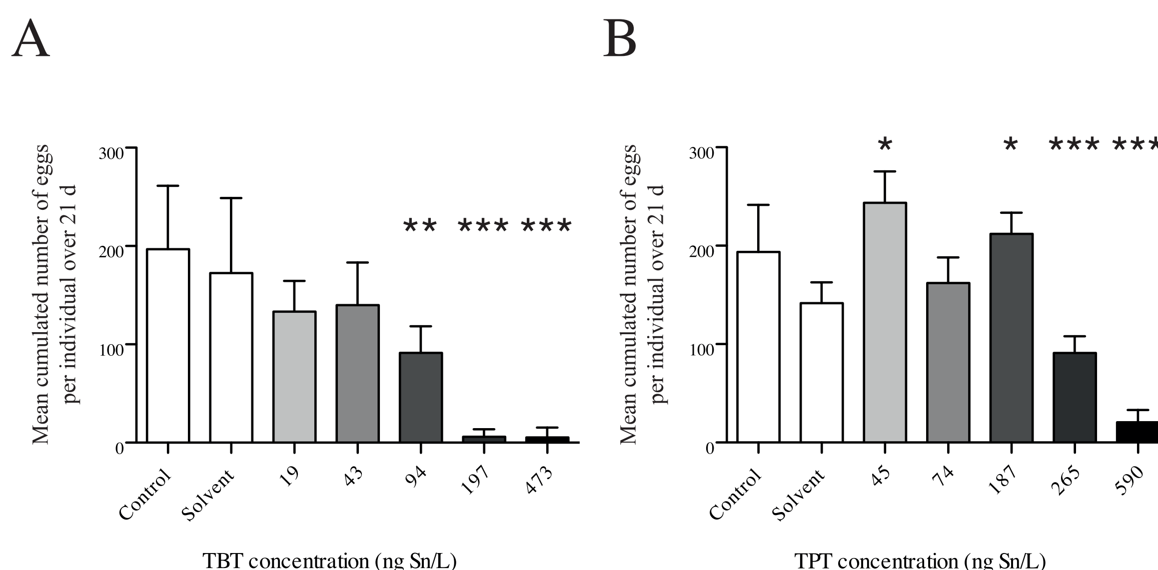

Fig. 3.5: Mean cumulated number of eggs laid per individual after a 21-d exposure to (**A**) tributyltin (TBT) or (**B**) triphenyltin. Error bars represent standard deviation over six replicates. (*: p<0.05; **: p<0.01; ***: p<0.001).

3.6. Egg-abnormalities

Frequency of abnormal eggs per clutch increased over the test duration. After 21 days, it was significantly higher in snails exposed to TBT than in controls even at the lowest tested concentration of 19 ng Sn/L (p < 0,05 Dunnett's post hoc test). The effects of TBT on the frequency of unfertilised eggs, eggs with atrophied albumen and single-embryo were not significant. Frequency of polyembryony was the most interesting endpoint: it represented 65 % to 100% of observed abnormalities in the lowest and highest tested concentrations, respectively. It increased from +80% at 19 ng Sn/L to +177% at 473 ng Sn/L compared with controls (Fig. 3.6). This increase was significant for all concentrations tested (p < 0.05, Dunnett's post hoc test). The corresponding EC50 at 21 days was 23.7 ng Sn/L (CI: 2.2 – 189.4 ng Sn/L). All eggs produced by parents exposed to 473 ng Sn/L exhibited polyembryony. In contrast, TPT induced no significant effects on the viability of eggs produced by exposed adults at the tested concentration range.

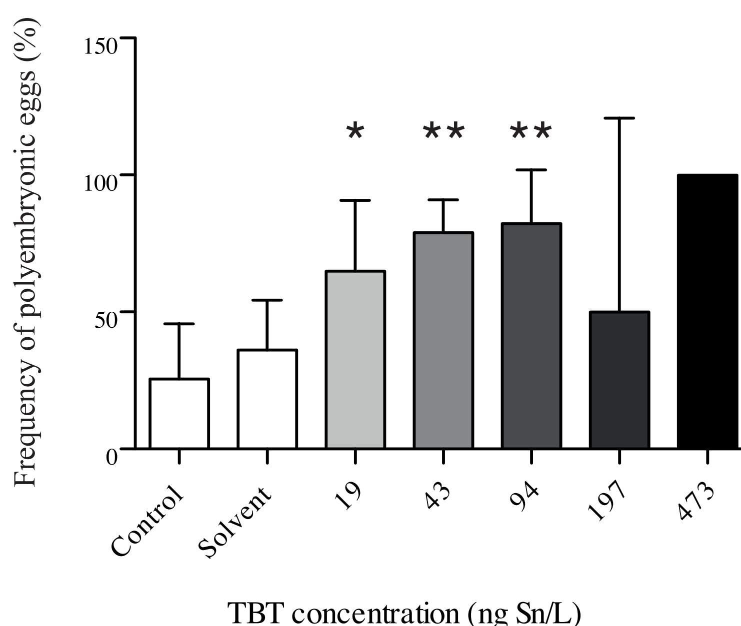

Fig. 3.6: Frequency of the polyembryonic eggs (among the total number of abnormal eggs) found during a 21-d exposure to tributyltin (TBT). Error bars represent standard deviation over all abnormal egg produced. (*: p<0.05; **: p<0.01; ***: p<0.001).

4. Discussion

4.1. Chronic effects of organotins in L. stagnalis

The TBT metabolite concentrations were very low (i.e., below the quantification limit), which suggests that degradation might not be the most significant process contributing to the decrease in TBT concentration in water. Losses were probably mostly due to adsorption in mucus, for example. Indeed, high TBT concentrations — 3 to 5-fold higher concentrations than the measured peak concentration in water — were found in mucus sampled after 21 days. The TBT content in mucus most likely resulted from direct adsorption from water after the mucus was released and possibly, to a lesser extent, from elimination of TBT by snails via mucus. This adsorption might partly explain why only 62.5% of the targeted nominal concentrations were found in the water samples. This highlights the need to wipe mucus off test beakers between water renewals to limit adsorption of TBT on the test chamber walls, which reduces its availability to the snails.

Exposure of *L. stagnalis* for 21 days to TPT in the concentration range of 45 ng Sn/L to 590 ng Sn/L induced mortality at the highest tested concentration (LC50 at 21 d, 436.1 ng Sn/L), and a variety of sublethal effects at lower concentrations, that is, decrease in shell solidity at all tested concentrations, decrease in egg-laying activity and fecundity, endpoints that led to an identical EC50 at 21-d value of 264 ng Sn/L. Egg quality at the concentration range tested was not affected by TPT.

A 21-day exposure of *L. stagnalis* to TBT in the range of 19 ng Sn/L to 473 ng Sn/L did not result in significant mortality in the present study. Alternatively, Segner et al. (2003) showed that adult survival was significantly reduced by TBT exposure, with an LC50 at 21 days of 290 ng Sn/L (nominal concentration), which is in contrast to both the present results and longer term studies from other authors. Indeed, no significant mortality was observed after 49 and 84 days of exposure to 100 ng Sn/L (nominal concentration (Czech et al., 2001)), as well as after 56 days of exposure to the concentration range of 7 ng Sn/L to 181 ng Sn/L (unpublished data from a round-robin test). At this concentration range, significant mortality was only observed in a 170-day study in which snails were exposed to 410 ng Sn/L (Leung et al., 2007).

Exposure to TBT induced a decrease in growth at concentrations exceeding 94 ng Sn/L. It also induced a decrease in shell solidity in snails exposed to concentrations exceeding 43 ng Sn/L, which was also observed by Segner et al. (2003) in 21-day tests for nominal concentrations exceeding 94 ng Sn/L. Exposure to TBT also induced a significant decrease in the egg-laying activity and fecundity of snails exposed to concentrations exceeding 45 ng Sn/L. This is consistent with previous results from Czech et al. (2001), who observed a significant decrease in egg-laying at 100 ng Sn/L (nominal concentration) in a 49-day experiment. Overall, these data confirm the possible occurrence of reproductive effects in *L. stagnalis* exposed to TBT at environmentally relevant concentrations. Results obtained in our partial life cycle tests are in accordance with results obtained in other partial life cycle tests (Czech et al., 2001; Segner et al., 2003) but differ from results obtained in the 170-day full life cycle test published by Leung et al. (2007), in which animals were exposed from embryonic stage to adulthood. In the study of Leung et al. (2007), adult fecundity was significantly modified at 41 ng Sn/L (NOEC), which was also the case in the present study (NOEC, 43 ng Sn/L). However, the effect pattern was quite different (S-shape dose–response curve in the present study vs. inverted U-shape in the study of Leung et al. (2007), with an increase in fecundity at 41 ng Sn/L compared with controls). The magnitude of effects at 410 ng Sn/L was also different. Indeed, the fecundity was reduced by a factor of 10 compared with control in the study of Leung et al. (2007), whereas it was reduced by a factor 100 in the present study. These results suggest that the test design might greatly influence the biological responses of *L. stagnalis*. Further studies are needed to assess the influence of test duration on the endpoint values in partial lifecycle tests and to compare the sensitivity of partial versus full life cycle tests with *L. stagnalis*.

Egg quality was altered by TBT, which was best evidenced through the frequency of polyembryonic eggs in clutches from exposed parents. The increase in polyembryony frequency was already significant at concentrations that were lower than those affecting egg production by adults. This finding indicates that polyembryony is more sensitive to TBT than the other reproductive endpoints tested (i.e., oviposition and fecundity, which are the common endpoints in reprotoxicity tests with snails), thus confirming previous findings with acetone (Bluzat et al., 1979). Test

endpoints/durations/protocols vary greatly among published studies on the effects of TBT in *L. stagnalis*, leading to a large variability in published NOEC values for some endpoints. This variability is also due to the lack of data on actual exposure concentrations in most published papers, which hampers a sound comparison of the published results. In this respect, the forthcoming standardisation of reproductive toxicity test protocols with *L. stagnalis* will help in deriving more reliable conclusions on the toxicity of TBT, TPT, and other types of chemicals.

4.2. Comparison of the responses of L. stagnalis to TBT vs. TPT

Differences in the sensitivity of *L. stagnalis* to similar concentration ranges of TBT and TPT were observed for most of the endpoints studied. The TBT concentrations tested (up to 473 ng Sn/L) had no significant effect on survival, whereas 100% mortality was observed at the highest tested TPT concentration (590 ng Sn/L), which suggests a higher toxicity of TPT than TBT to *L. stagnalis*. Results from a pre-test with TBT confirmed that TBT has no effect on survival at 590 ng Sn/L (NOEC in this pre-test was 627 ng Sn/L, while the LOEC was 6270 ng Sn/L, with 100% mortality occurring at the end of the test; Part 2: chapter 1). A significant effect on snail growth was found with TBT, while no effect occurred due to TPT exposure. Because both TBT and TPT led to shell injuries, shell size might not be the most reliable growth indicator when assessing the effects of organotins on *L. stagnalis*. Indeed, decreased shell size due to an injury can be wrongly interpreted as an effect on growth. To avoid such misinterpretation, we recommend not using length data from individuals with broken shells for the statistical analysis of growth effects. Discarding such individuals from the analysis might lead to loss of a number of data and thus to a reduced statistical power when effects on growth are tested (e.g., in the present study, 100% of snails were injured at the highest TBT concentration). Growth effects should rather be assessed through measurement of the soft body dry weight to avoid misinterpretation and maintain a sufficient statistical power. It is likely that the decrease in shell solidity was due to decalcification, which has already been observed for TBT in *L. stagnalis* (Segner et al., 2003) and other molluscs (e.g., oysters (Alzieu et al., 1982)). Similarly, trialkylated tin compounds have been shown to interfere with calcification processes in mammals (Alzieu et al., 1982), which might also apply to *L. stagnalis* exposed to TPT.

Both compounds significantly reduced egg-laying and fecundity, but effective concentrations were lower for TBT than for TPT, as reflected by the EC50 21-d values for reproductive endpoints. The EC50s at 21 days based on fecundity data were equivalent to values obtained from oviposition data for TBT (EC50 21-d values of 106 ng Sn/L and 118 ng Sn/L, respectively; no significant difference as assessed through the overlapping of 95% confidence intervals) and for TPT (with a similar EC50 21-d value of 264 ng Sn/L and no overlapping of confidence intervals). These results suggest that both reproductive endpoints have a similar sensitivity to TPT and TBT in this particular experimental setup, although oviposition and fecundity are known to be under the control of two different hormonal pathways in *L. stagnalis* (Koene, 2010). Exposure to TPT did not affect egg quality, whereas polyembryony often occurred in eggs produced by TBT-exposed snails. Polyembryony thus exhibited different responses to TBT and TPT and was the most sensitive endpoint in the TBT test. The use of acetone as the carrier solvent did not significantly influence snail reproduction or egg quality in the TBT test but did significantly hamper all reproductive performances in the TPT test. Previous in-house studies showed that reproductive effects due to acetone occur from time to time in our experimental conditions. The reasons remain unclear; this may be a result of, for example, differences in solvent purity or snail sensitivity from one experiment to another. This highlights the need for additional studies devoted to the assessment of solvent effects in juveniles and adults of *L. stagnalis* to complement the recently published results on solvent effects in embryos (Bandow and Weltje, 2012).

Reasons for differences in sensitivity of *L. stagnalis* to TBT versus TPT remain to be elucidated. Differences in molecular structure of these organotins might be a relevant explanation. Indeed, it is assumed that the toxicity of organotins is more influenced by the alkyl substitutes than by the anionic substitutes (Antizar-Ladislao, 2008). A recent study in *Mytilus edulis* confirmed that alkylation of organotins influences their toxicity to molluscs (Nesci et al., 2011). Therefore, differences in the responses of *L. stagnalis* to TPT versus TBT are likely to be related to differences in their alkylation. However, it is not known how such differences in molecular structure might lead to different modes of action of these compounds in *L. stagnalis*. Previous studies highlighted the possible endocrine effects of TBT and TPT in gastropods (Bettin et al., 1996; Castro et al., 2007a;

Gooding et al., 2003; Santos et al., 2005; Spooner et al., 1991). Hormonal pathways involved in the response of various gastropods to TBT were investigated, focusing on the mechanisms of imposex induction in gonochoric marine species (Gooding et al., 2003; LeBlanc et al., 2005; Ronis and Mason, 1996; Santos et al., 2005; Sternberg and LeBlanc, 2006). These studies highlighted the fact that imposex is related to an alteration in steroid homeostasis, mediated by inhibition of enzymes such as cytochrome-P450 aromatase or acyltransferase that are involved in biosynthetic steroid pathways (Ronis and Mason, 1996; Spooner et al., 1991; Sternberg et al., 2010). Other *in vivo* and *in vitro* studies showed that TBT and TPT are potent activators of nuclear receptors such as the retinoid X receptors, leading to transcription of genes involved in steroid homeostasis (Castro et al., 2007a; Kanayama et al., 2005; Nakanishi, 2008; Nishikawa, 2006; Nishikawa et al., 2004). Other studies highlighted the effects of TBT and TPT on different components of the microsomal monooxygenase system of the bivalves *Mytilus galloprovincialis* and *Ruditapes decussata* and of the gastropod *Thais haemastoma* (Morcillo and Porte, 1997). Finally, Lyssimachou et al. (2009a) showed that TPT was able to alter lipid metabolism in females of the ramshorn snail *Marisa cornuarietis*. However, it is not known to what extent these findings can be extrapolated to hermaphroditic species such as *L. stagnalis*. In the present study, reproductive effects of TPT occurred at quite high concentrations (i.e., the LC50 at 21 days was only 2-fold higher than the reproductive EC50 at 21 days). Values for EC50 at 21 days were similar when estimated using fecundity or egg-laying data, indicating no relationship between the hormonal pathways involved in the control of these processes and the biological response. Furthermore, no significant increase in the frequency of abnormalities occurred in the offspring of exposed snails. Therefore, results of the apical reproduction test suggest that the reproductive impacts observed are probably linked to the toxicity of TPT to the snails rather than being a consequence of endocrine disruption. Alternatively, TBT concentrations that induced reproductive effects in *L. stagnalis* were much lower than the lethal concentrations reported in other studies conducted with this species (Leung et al., 2007; Segner et al., 2003), and occurred at environmentally relevant concentrations. In addition, the frequency of polyembryony in the offspring increased in exposed snails. These results suggest that TBT might act as an endocrine disruptor in *L. stagnalis* as well. Even if apical endpoints might bring clues to possible modes of action of TBT and TPT in *L. stagnalis*, studies

should be implemented to determine to what extent and for which compounds the observed deleterious effects of organotins are actually due to endocrine disruption (Koene, 2010; Matthiessen, 2008).

4.3. Comparison of responses of the hermaphroditic snail vs. gonochoric species

Existing data, including the present results, have shown that TPT and TBT have different acute and chronic effects in various mollusc species. For instance, exposure of the parthenogenetic snail *Potamopyrgus antipodarum* to TBT and TPT in sediment biotests led to a significant reduction in reproductive output at environmentally relevant concentrations (Duft et al., 2003a; Schulte-Oehlmann, 1997). Effect concentrations were lower and the intensities of effects on reproduction were higher in mud snails exposed to TPT compared with those exposed to TBT. In contrast, acute toxicity occurred in snails exposed to TBT but not in those exposed to TPT. Based on these results, the effects of TPT and TBT in *P. antipodarum* are opposite to the effects we found in *L. stagnalis*. In another study, it was shown that females of the rock shell *T. clavigera* had a similar sensitivity to TBT and TPT (via direct injection in soft tissues), while males were more sensitive to TPT than to TBT (Horiguchi et al., 1997). Interestingly, it appears that three prosobranch snails with different habitats (freshwater, brackish water, and marine water) and different reproductive strategies (parthenogenesis, hermaphrodism, and gonochorism) were all responsive to organotin compounds, but their sensitivities were species- and sex-dependent, as reviewed by Ketata et al. (2008). Additional studies are required to provide explanations for differences in responsiveness and sensitivity to TBT and TPT in and between species.

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Chapter 3:

Impacts of tributyltin (TBT) on the activity of enzymatic biomarkers in **Lymnaea stagnalis**

1. Introduction

A biomarker is defined as any biological response to an environmental chemical at the below-individual level, which can be measured inside an organism or in its products (e.g., urine, faeces, hair, feathers), indicating a departure from the normal status that can not be detected from the intact organism (Gestel and Brummelen 1996). Biomarkers are divided in three classes: biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility. Biomarkers of exposure are biological responses specific to exposure to a particular substance, and which provide a link between external exposure and internal biological responses (i.e., physiological and molecular responses) (Henderson et al. 1987; van der Oost et al. 2003).

In organisms exposed to chemicals, biotransformation is an important metabolic process for the elimination of the toxic compounds through the formation of more hydrophilic molecules that will be excreted, therefore reducing the body burdens (Buhler and Williams 1988). Biotransformation of pesticides is generally subdivided in three reaction processes. Phase-I activities are localised in microsomes and consist in the enzymatic oxidation, reduction and hydrolysis, which result in the conversion of chemicals in more hydrophilic metabolites. These activities are catalysed by the cytochrome P450 superfamily of enzymes (CYPs). During phase-II, which occurs in both microsomes and cytosol, the functional groups produced in phase-I are conjugated with carbohydrates, glutathione (GSH), sulphate and amino acids. During phase-III, conjugated products are excreted from cells through efflux transporters, contributing to reduce body burdens (Buhler and Williams 1988; Katagi 2010; Xu et al. 2005).

It is now well documented that exposure of vertebrates and invertebrates to different pollutants (e.g., PAHs, PCBs, alkylphenols) induces the activity of enzymes involved in hepatic detoxification processes, the hepatic cytochrome P450-dependent monooxygenases (Baturo and Lagadic 1996; Gagnaire et al. 2010; van der Oost et al. 2003). Enzymatic activities of ethoxyresorufin-O-deethylase (EROD) and penthoxyresorufin-O-deethylase (PROD) were increased in fish exposed to organic pesticides (i.e., PAH, PCBs, PCDDs) and the measurement of the microsomal activity of these enzymes was proposed as a biomarker of exposure to certain classes of xenobiotic chemicals (reviewed in van der Oost et al. (2003)). Glutathione S-

transferases (GSTs) catalyse the conjugation of phase-I products with glutathione, a tripeptide mainly found under its reduced form within cells, to finally form hydrophilic metabolites that are excreted (Ketterer et al. 1983). These enzymes are, quantitatively, the most important enzymes of the phase-II detoxification process (Sheehan et al. 1995). The GSTs enzymatic activity was observed to respond to chemical exposure in vertebrates and invertebrate species (Ayoola et al. 2010; Baturo and Lagadic 1996; Förlin et al. 1995).

In this study, we have investigated the enzymatic activity of phase-I (i.e., EROD and PROD) and phase-II (i.e., GSTs) enzymes after exposure of *L. stagnalis* to TBT during 21 days. These results will help to elucidate whether the impacts of TBT observed on *L. stagnalis* reproduction are associated with enzymatic responses that could interfere with the metabolisation of hormones or putative endocrine disruptors.

2. Material and Methods

A *L. stagnalis* snail was sampled from each replicate of TBT treatments after 21 days of exposure (Part 2: chapter 2) and were frozen at -80°C until enzymatic activity measurements. The gonado-digestive complex was dissected, weighted and homogenised as previously described (Baturo and Lagadic 1996). Briefly tissues were ground in 100 mM potassium phosphate buffer (pH 7.4, 1 mM EDTA) with phenylmethyl-sulfonyl fluoride in isopropanol (final concentration of 0.5 mM) using a glass/Teflon potter. Cytosolic and microsomal fractions were separated by centrifugation at 10000 g for 20 min followed by ultracentrifugation at 150000 g during 90 minutes at 4°C.

Glutathione S-transferases (GSTs) activity was measured as previously described (Habig et al. 1974). Five microliters of a glutathione solution and 5 µL of 100 mM 1-chloro-2, 4 dinitrobenzene (CDNB) in ethanol were added to the cytosolic fraction. The change in absorbance was then recorded at 340 nm continuously during the first 80 s of reaction using a SpectraMax 340PC384 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, USA). Cytosolic activity of GST was expressed as micromoles of CDNB conjugated (GS-DNB) per minute per mg of cytosolic proteins (Baturo and Lagadic 1996). The microsomal fraction was used for EROD and PROD enzymatic activities that were measured at 37°C using a 10 min incubation time. The enzymatic

activity was determined following addition of 3.32 μM of either ethoxyresorufin or penthoxyresorufin and 0.20 mM of NADPH (Morcillo and Porte 1997). Dealkylation of the substrates results in the production of resorufin, which is measured by recording absorbance with 530 and 590 nm of excitation and emission wave length, respectively, using a SPECTRAmax GEMINI XS (Molecular Devices, Sunnyvale, USA) during 10 min. EROD and PROD activities were expressed as nanomoles of resorufin produced per mg of microsomal proteins. Total protein concentrations in microsomal and cytosolic fractions were measured by the Bradford method (Bradford 1976).

Data were analysed using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Statistical differences for enzymatic activities between water and solvent controls were determined using Mann-Whitney *t*-test. As no statistical differences were observed, control data were combined for subsequent statistical analyses. PROD and GST activities were tested for normality with Kolmogorov-Smirnov test and for homoscedasticity using a Bartlett and Kendall test. Differences among tested concentrations for each chemical and controls were then assessed using Kruskal-Wallis tests. All tests were performed with $\alpha = 0.05$.

3. Results and Discussion

In controls, EROD activity could not be measured in gonado-digestive microsomal extracts whereas low PROD activity was observed (Fig. 4.1 A). These results confirm previous observations, which showed that digestive gland homogenates of *L. stagnalis* could metabolise penthoxyresorufin but not ethoxyresorufin in presence of NADPH and NADH (Wilbrink et al. 1991a). Similarly, in bivalve species (i.e., *Mytilus galloprovincialis*, *Thais haemastoma* and *Tapes decussate*), EROD activity could not be measured (Morcillo and Porte 1997). Exposure of *L. stagnalis* to concentrations of 197 and 473 ng Sn/L of TBT during 21 days significantly increased PROD activity ($p<0.05$, Kruskal-Wallis, Dunn's post hoc test).

To date, no other study has investigated the responses of these biomarkers in molluscs to date. However, in freshwater fishes (i.e., *Oncorhynchus mykiss*, *Anguilla anguilla* and *Cottus gobio*), a concentration dependent decrease in EROD activity was observed in hepatic microsomes exposed *in vitro* to 0.1 mM of TBT and TPT. Moreover, the activity of the total microsomal cytochrome P450 enzymes decreased in a time-

and concentration relationship (Fent and Bucheli 1994). Similarly, in the marine fish *Mullus barbatus* exposed to 10 µM of TBT *in vitro*, the enzymatic activity of EROD was significantly inhibited (Morcillo and Porte 1997). Furthermore, *in vivo* exposure of *Stenotomus chrysop* decreased EROD activity following injection of a single dose of 3.3 mg/kg of TBT (liver concentrations after exposure was 8 µg/g) (Fent and Stegeman 1993). Water exposure of the salmon *Salmo salar* to TBT for 7 days had no impacts on EROD activity while 50 µg/L increased PROD activity. However, higher TBT concentrations had no impact on the enzyme activity (Mortensen and Arukwe 2007). As a conclusion, TBT could enhance PROD activity in fish whereas EROD activity was mainly reduced. Our results suggest that phase-I detoxification processes are triggered from 197 ng Sn/L, reflected by PROD activity, whereas lower concentrations did not induced metabolisation of TBT in *L. stagnalis*. However, as it is the case with other mollusc species, EROD activity could not be measured in *L. stagnalis*. Thus, it remains to be elucidated whether TBT specifically inhibits EROD, while activities of other phase-I enzymes are enhanced, or if TBT is able to inhibit other cytochrome P450 enzymes (CYP) as it was observed in mammals (Cooke 2002; Kim et al. 1998).

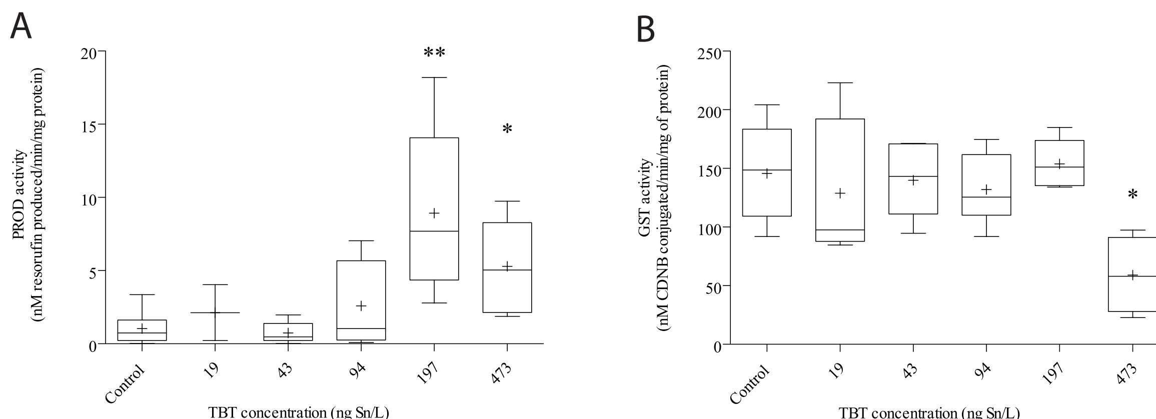

Fig. 4.1: Enzymatic activity of (A) PROD and (B) GST in gonado-digestive complex microsomal fraction of *Lymnaea stagnalis* exposed for 21 days to tributyltin (TBT). Boxes represent the 25 – 75 % interval whereas whiskers represent minimal and maximal measured values and (+) correspond to mean measured activity. (Kruskal-Wallis, Dunn's *post hoc* test: ∗: p<0.05).

The level of GSTs activity in cytosolic fraction of the gonado-digestive complex of control was measured at 145.8 ± 40.5 nM/min/mg (Fig 4.1 B). The results of this study suggest that the measured enzymatic activity of GSTs, in controls, was within the range of value reported by other authors for *L. stagnalis*. These authors have reported a GSTs

activity in digestive glands of *L. stagnalis* of 349 ± 74 nM/min/mg (Wilbrink et al., 1991b), whereas in whole tissue homogenates the GSTs activity ranged from 200 to 980 nM/min/mg (Dierickx, 1984; Stenersen et al., 1987). The variability of the GSTs activity within a single species was previously observed as changes in activity were observed in different tissues and seasons (Bebianno et al., 2007; Le Pennec and Le Pennec, 2003; Wilhelm Filho et al., 2001).

The activity of GSTs measured in *L. stagnalis* was only significantly reduced following exposure to the highest TBT concentration tested (473 ng Sn/L, $p< 0.05$, Kruskal-Wallis, Dunn's *post hoc* test) (Fig. 4.7 B). In the bivalve *Pecten maximus*, *in vitro* exposure of cytosolic fraction, extracted from the digestive gland, to concentrations ranging from 10^{-11} to 10^{-7} M of TBT significantly increased GSTs activity after 24h, whereas a significant decrease of the enzyme activity was observed after 4 days (Le Pennec and Le Pennec, 2003). Exposure of the clam *Meretrix meretrix* to 0.1 ng Sn/L increased GSTs activity after 2 days of exposure while after 20 days of exposure to 1 and 10 ng Sn/L the GSTs activity was decreased (Huang et al., 2005). This inhibitory effect of TBT was also reported in fish following *in vitro* exposure of cytosolic fraction, isolated from liver and kidney, to TBT concentrations in the µM range (Al-Ghais and Ali, 1999). *In vivo* exposure of *Sebasticus marmoratus* induced an increase of GSTs enzymatic activity at low concentrations (19.3 µg/kg), whereas an inhibition of activity was observed at higher concentrations (193 µg/kg) (Wang et al., 2005a). In the carp, exposure to 0.8 µg/L for 3 days led to an increase of GSTs activity in gills but not in liver. Furthermore, at higher concentrations, GSTs activity was not different from controls in this species (Schmidt et al., 2004). These results suggest that TBT could induce GSTs activity at low concentrations and during a short period of exposure, while exposure to high concentrations and for a longer period inhibits the activity of these phase-II enzymes.

The enzymatic activity of other enzymes involved in the phase-II of the detoxification, i.e., sulfotransferases (SULTs), a family of enzymes which catalyses the sulfation of endogenous chemicals but also synthetic chemicals, was strongly inhibited in the fish species *Lepidorhombus boscii* and *Cyprinus carpio* exposed *in vitro* to TBT, whereas the enzyme activity was not altered in *Mullus barbatus* (Martin-Skilton et al., 2006). In the prosobranch gastropod *Littorina littorea*, a reduction of sulphur-conjugated testosterone metabolites was observed following *in vivo* exposure to TBT.

This reduction was attributed to a decreased activity of SULT involved in testosterone sulfation (Ronis and Mason, 1996). Together, these results suggest that TBT can significantly alter the activity of phase-II enzymes as observed in the GSTs activity in *L. stagnalis*.

Even though the phase-I enzyme (i.e., PROD) activity was increased in *L. stagnalis*, the inhibition of phase-II enzymes (i.e., GST) induces a reduction of the metabolisation and, consequently, a reduction of the excretion of organotin. These observations are consistent with results obtained in the clam *Ruditapes decussatus* in which the bioconcentration factor was increased from 3800 to 7250 following a 7-day exposure to nominal concentrations of 91 and 454 ng Sn/L of TBT, respectively (Morcillo et al., 1998). The bioconcentration factor of TBT in molluscs generally varies from 1000 to 60000, depending on the species, concentration and exposure duration (US-EPA, 2003). Therefore, it remains to be determined if, in *L. stagnalis,* the inhibition of GSTs observed following exposure to 473 ng Sn/L increased the TBT body burdens in our experiment. Moreover, it was reported that mucus production was enhanced.

Chapter 4:

***Impacts of endocrine disrupting chemicals on the reproduction of* Lymnaea stagnalis**

Publication 3:

Effects of cyproterone-acetate, chlordecone, fenitrothion and vinclozolin on the reproduction of the hermaphroditic freshwater gastropod *Lymnaea stagnalis*

Prepared for submission in Ecotoxicology

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Abstract

The hermaphroditic gastropod *Lymnaea stagnalis* is proposed as a candidate species for the development of OECD guidelines for testing of the reprotoxicity of chemicals, including endocrine disrupting chemicals (EDCs). Up to now, only a few putative EDCs have been tested for their reproductive toxicity in this species. In this study, we investigate the effects of four chemicals with different affinities to the vertebrate oestrogen and androgen receptors (chlordecone as an oestrogen; cyproterone acetate, fenitrothion and vinclozolin as anti-androgens) on the reproduction of *L. stagnalis* in a 21-day semi-static test. Testosterone was used as reference compounds. The tested compounds had no significant impacts on growth and survival at the chosen concentration ranges. Chlordecone reduced the oviposition and fecundity of *L. stagnalis* from 19.6 µg/L. The frequency of abnormal eggs produced by chlordecone-exposed snails was significantly increased for a chemical concentration as low as 2.1 µg/L. Similarly, egg quality was negatively impacted by low concentrations of testosterone and cyproterone acetate although these compounds had no impact on oviposition and fecundity. Although the signalling pathways, which are involved in such reproduction impairments, remain unknown, our results suggest that egg quality is a more sensitive endpoint than the reproductive endpoints commonly assessed in mollusc toxicity tests.

Keywords: Endocrine Disruptors; Anti-androgens; Fecundity; Egg quality

1. Introduction

The majority of studies on the effects of endocrine disrupting chemicals (EDCs) in gastropods has been conducted on gonochoric species (e.g., *Nucella lapillus*, *Littorina littorea*, *Marisa cornuarietis*) (Oehlmann et al., 1998; Schulte-Oehlmann et al., 2000) both for historical reasons and because imposex and intersex can be easily assessed in these species. Yet, effects of EDCs are expected to differ in species with different mode of reproduction (OECD 2010). Therefore, studies have been conducted on the parthenogenetic mud snail *Potamopyrgus antipodarum* (Duft et al., 2003a; Duft et al., 2003b), as well as on the hermaphroditic pond snail *Lymnaea stagnalis* (Czech et al., 2001; Ducrot et al., 2010; Leung et al., 2007), which demonstrated effects of EDCs on reproduction. Both species have thus been proposed as candidate species for the development of OECD guidelines for the testing of the reprotoxicity of chemicals, including EDCs (Matthiessen, 2008; OECD, 2010). However, as compared to *P. antipodarum*, data on the effects of EDCs in *L. stagnalis* are scarce. This freshwater pulmonate species has been shown to be sensitive to organotins (i.e., triphenyltin and tributyltin) and to some other EDCs (e.g., vinclozolin, nonylphenol) (Czech et al., 2001; Ducrot et al., 2010; Giusti et al., 2013a; Part 2: chapter 2 Leung et al., 2007). Organotins significantly reduce both the oviposition and the number of eggs laid by individuals as a measure of the fecundity in individuals exposed to low concentrations (ng Sn/L range) (Czech et al., 2001; Giusti et al., 2013a; Part 2: chapter 2) whereas 4-nonylphenol (μg/L range) only significantly reduces the fecundity (Czech et al., 2001). These results suggest that classical reproductive endpoints (i.e., numbers of clutches and eggs produced per individual) are sensitive enough to allow the detection of different modes of action of EDCs in *L. stagnalis* since fecundity and oviposition are under different hormonal controls in this species (Koene, 2010). Nevertheless, only a few compounds have been tested for their effects on the reproduction of this snail and the tested concentrations were not always environmentally relevant (Lagadic et al., 2007; OECD, 2010). Furthermore, the test design (e.g., exposure duration, temperature, photoperiod, snail density) varied between these studies, which hampers comparison of the effects of different chemicals (OECD, 2010). In such conditions, establishing relationships between changes in snail reproduction and EDC modes of action, as described in vertebrates, is mostly difficult.

The present study was therefore designed to assess the effects of four chemicals, with different endocrine disrupting properties in vertebrates, on the reproduction of *L. stagnalis* in a semi-static test as designed for the OECD guideline under preparation. Chemicals were chosen on the basis of their affinities to the vertebrate oestrogen and androgen receptors, as assessed by *in vitro* screening tests. The synthetic steroid pharmaceutical cyproterone acetate (CPA) acts as an anti-androgen by competitively blocking androgen receptors in vertebrates (Fang et al., 2003). Chlordecone (CLD), an organochlorinated insecticide extensively used in the French West Indies against banana crop borers until its ban in 1993 (Beaugendre, 2005; Dubuisson et al., 2007), binds to the vertebrate oestrogen receptor (Hammond et al., 1979) albeit with relatively low affinity (Donohoe and Curtis, 1996). The organophosphate insecticide fenitrothion (FEN) is a competitive antagonist of the vertebrate androgen receptor *in vitro* (Freyberger and Ahr, 2004; Tamura et al., 2001). Finally, vinclozolin (VZ) is a dicarboximide fungicide, which metabolites are able to compete with androgens for the androgen receptor binding (Kelce et al., 1997; Ostby et al., 1999; Wong et al., 1995). Other modes of action, such as regulation of aromatase activity, which has been demonstrated or suggested for all the tested chemicals in mammals (Benachour et al., 2007; Karolczak and Beyer, 1998; Sanderson et al., 2002; Struve et al., 2007), may also be involved in potential alterations of the reproduction in *L. stagnalis*. In addition to these compounds, testosterone (T) was used as 'reference' compound. T is the natural ligand of the androgen receptor in vertebrates and is presumably synthesised *de novo* in molluscs (De Longcamp et al., 1974; Gottfried and Dorfman, 1970), including in *L. stagnalis* (Giusti et al., 2013b; Part 4: chapter 5).

Reproductive effects of the chemicals and 'reference' compound were assessed through the evaluation of oviposition and fecundity of young adult snails exposed to waterborne chemicals, as previously described for TBT and TPT (Giusti et al., 2013a; Part 2: chapter 2). In addition, effects of the chemicals on egg quality were investigated. In a previous study, we observed an increase in the frequency of egg abnormalities when adults were exposed to TBT, and these impacts were significant at lower concentrations than those affecting oviposition and fecundity (Giusti et al., 2013a; Part 2: chapter 2). This suggests that egg quality might be a relevant and sensitive additional endpoint when studying the effects of EDCs on *L. stagnalis*. Unlike embryonic toxicity studies, which aim at understanding the impacts of direct exposure

of early life stages on animal development (Bandow and Weltje, 2012; Buckley et al., 2006; Sanchez-Arguello et al., 2012; Zhou et al., 2011), monitoring egg quality does not require exposure of the offspring. Only adults are exposed (i.e., classical procedure for a reproduction test) and the consequences of adult exposure on their offspring is assessed in order to evidence impacts linked to the alteration of reproductive pathways (Giusti et al., 2013a; Part 2: chapter 2). We analysed the endocrine disrupting potency of the tested chemicals on *L. stagnalis* reproduction in the light of data available for this snail and other mollusc species. The relevance of the classification of EDCs on the basis of their steroid receptor-binding properties as described in vertebrates is discussed, the existence of functional steroid nuclear receptors in molluscs being still controversial (Scott, 2013) although oestrogen receptor genes have been found in *Lymnaeidae* (Kumkate et al., 2009).

2. Material and Method

2.1. Animals

L. stagnalis (Linnaeus, 1758) (Mollusca, Gastropoda, Pulmonata, Heterobranchia), RENILYS® strain, has been reared at the INRA Experimental Unit of Aquatic Ecology and Ecotoxicology (Rennes, France) under laboratory conditions as previously described (Coutellec and Lagadic 2006; Jumel et al. 2002). Young adults of homogenous size (22.5 ± 2.5 mm) and age (4 ± 0.5 months) were sampled from the culture and acclimatised to test conditions for 48 hours prior to chemical exposure.

2.2. Test substances

Analytical standards of CLD (CAS Nr 143-50-0), CPA (≥ 98% purity, CAS Nr 427-51-0) FEN (CAS Nr 122-14-5), VZ (CAS Nr 50471-44-8) and T (≥ 98% purity, CAS Nr 58-22-0) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Stock solutions were prepared in analytical grade acetone (99.9 % purity). Concentrations of stock solutions were chosen so as to ensure addition of the same amount of solvent among treatments (i.e., 100 µL/L, except for the water control) as recommended by (OECD 2000). Nominal concentrations (Table 1) were chosen according to results of previous

experiments with molluscs (Albanis et al. 2006; Janer et al. 2006; Tillmann et al. 2001) and of in-house range finding tests (Giusti et al. 2010; Part 2: chapter 1).

2.3. Test design

Exposure media consisted in dechlorinated, charcoal-filtered tap water (i.e., as for snail culture medium; pH = 7.7 ± 0.2, conductivity = 623 ± 60 µS/cm, dissolved oxygen = 7.3 ± 2 mg/L, water hardness = 254 ± 7 mg $CaCo_3$/L) contaminated with stock solutions. Six replicates (each with five snails in 1 L glass beaker) of each chemical concentration, water control and solvent control were randomly distributed in the exposure room. Tests were conducted at 20.5 ± 0.6°C and a 14:10 L:D photoperiod for 21 days. Test water and food supply were renewed every other day with freshly contaminated medium and approximately 2.5 g of organic lettuce (National AB–Agriculture Biologique and International Ecocert FR-BIO-01 certifications) per beaker, respectively.

2.4. Chemical analysis

Exposure water was sampled at the beginning, mid-term and end of the experiment. Three replicates were taken per concentration. Each replicate (200 mL) consists in a pool of 100 mL of water sampled from 2 beakers. Samples were taken fifteen minutes and 48 hours after water renewal and stored in glass flasks at -20°C until analysis. CLD was extracted by manually handshaking 5 mL of water with 5 mL dichloromethane. FEN and VZ extractions, using C-18 solid phase extraction (SPE), were based on the method described by (Martinez Vidal et al. 2000) followed by Florisil clean-up as recommended in the US-EPA method 622.1 (US-EPA 1992). CLD, FEN and VZ analysis and quantifications were performed by capillary gas chromatography - mass spectrometry (GC-MS-MS). CPA and T were extracted from water using C-18 solid phase extraction (SPE) and quantified by high-performance liquid chromatography - mass spectrometry (HPLC-MS-MS). Actual exposure concentrations were calculated as the time-weighted average exposure concentrations (AEC) over the test period (Belgers et al. 2011).

2.5. Endpoints

Dead snails were counted and removed daily. Shell length was measured at day 0 and 21 to the nearest 0.1 mm using a digital calliper. Egg-clutches were counted and collected every other day. The number of eggs per clutch and their quality were determined under a dissecting microscope (× 10 magnification). Egg quality was assessed by determining the frequency of four types of abnormalities: (i) “polyembryonic egg”, i.e., presence of multiple (developing or non viable) embryos per egg, (ii) “unfertilised egg”, i.e., absence of embryo in the egg, which only consists in the egg-shell and albumen, (iii) “atrophied albumen”, i.e., damaged egg-shell containing an abnormally low albumen quality/quantity, and (iv) “single embryo”, i.e., presence of a non-developing embryo, without an egg-shell and without albumen.

Based upon raw data, the following reproductive endpoints were assessed: cumulative number of clutches per individual (oviposition), number of eggs per clutch, cumulative number of eggs per individual (fecundity), frequency of abnormal eggs (calculated as the ratio of the number of abnormal eggs to the total number of eggs per replicates), and frequency of each abnormality type (e.g., frequency of polyembryony, calculated as the ratio of the number of polyembryonic eggs to the total number of abnormal eggs). Fecundity and oviposition were treated as separate endpoints because they are under different hormonal controls in *L. stagnalis* (Koene 2010). Analysis of the number of eggs per clutch allows distinguishing between cases where decrease in fecundity is a consequence of a decrease in oviposition *vs.* cases where oviposition is not affected but the number of eggs per individual is. Differentiating the types of egg abnormalities is another strategy to gain insight on possible alterations of reproductive pathways in adults (i.e., the different types of abnormalities can be viewed as “symptoms” of different alterations in the reproduction process).

2.6. Data Analysis

Biological data were analysed using standard statistical analysis procedures (OECD 2006) with the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Statistical differences between water and solvent controls were determined using

Mann-Whitney or Student tests. When no statistical differences were observed, both control data were pooled for further statistical analyses. Survival, shell length, cumulated numbers of clutches and eggs per individual and frequencies of egg abnormalities were tested for normality with Kolmogorov-Smirnov test and for homoscedasticity using a Bartlett and Kendall test. Differences among the tested concentrations of each chemical and controls were then assessed using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. Kruskal-Wallis tests followed by Dunn's *post-hoc* test were used when data did not meet the conditions of normality or homoscedasticity. All tests were performed with $\alpha = 0.05$. When the effects of chemicals were significant, LC_x or EC_x were calculated using a three-parameter logistic regression model (Duggleby 1981). The 95% confidence intervals were simulated based upon weighted residues in order to account for differences in variance across treatments and using 5000 bootstrap simulations. The Microsoft Excel macro REGTOX_EV7.0.6 was used for this purpose (Vindimian 2001).

3. Results

3.1. Concentrations of tested chemicals

Exposure concentrations are reported in Table 5.1. Peak concentrations of T reached 24.5 ± 9.1 % of the nominal concentration. After 2 days of exposure, T concentrations had dropped to 1.6 ± 1.3 % of the nominal concentrations. The average measured concentrations were 83.5 ± 21.8 % of the nominal concentration after 5 min and 23.7 ± 7.9 % after 2 days of exposure for chlordecone and were 98.8 ± 23.4 % of the nominal concentration after 5 min and 23.4 ± 2.8 % after 2 days of exposure for cyproterone acetate. Testosterone concentrations could not be maintained to ± 20 % of the nominal or measured initial concentrations throughout the test as recommended by (OECD 1998). Therefore AEC were used as proxies of the actual exposure concentrations of chemicals over time.

The average concentrations for fenitrothion and vinclozolin are not already measured; therefore nominal concentrations were used for these two chemicals.

Chemicals	Nominal water concentrations	Average Exposure Concentration
Testosterone (ng/L)	2	0,3
	4,5	0,7
	10	1,4
	22	3
	50	6,8
	100	14,8
Cyproterone acetate (µg/L)	2	1,1
	4,5	2,5
	10	5,6
	22	12,5
	50	28,7
Chlordecone (µg/L)	4,5	2,1
	10	3,9
	22	8,3
	50	19,6
	110	49,1
Fenitrothion (µg/L)	10	-
	22	-
	50	-
	110	-
	240	-
Vinclozolin (ng/L)	10	-
	22	-
	50	-
	110	-
	240	-

Table 5.1: Nominal water concentrations and time-weighted average water concentrations the tested endocrine disrupting chemicals.

3.2. Survival and shell length

Survival was 100% and 90% in the water and solvent controls, respectively. Tested chemicals did not induce more than 10% of mortality, except for the highest CLD concentration tested (49.1 µg/L), which led to 30% mortality after 21 days of

exposure. The corresponding median lethal concentration (LC50) at 21 days was 104.5 [98.9 – 11.7] µg/L. We observed no significant difference in the shell length of control and treated snails after 21 days of exposure to the other chemicals.

3.3. Impacts on oviposition and fecundity

From 19.6 µg/L, CLD induced a significant dose-dependent reduction of clutch production ($p < 0.01$, Kruskal-Wallis, Dunn's *post hoc* test) (Fig. 5.1 a). The corresponding EC50-21d value was 9.3 [4.1 – 18.3] µg/L. The two highest tested CLD concentrations (19.6 and 49.1 µg/L) also induced a significant reduction in the cumulative number of eggs produced per individual ($p < 0,001$, ANOVA, Dunnett's *post hoc* test) (Fig. 5.1 b). The EC50-21d value for cumulative fecundity was 11.3 [5 – 20.7] µg/L. CLD had no impact on the number of eggs per clutch (Table 5.2). This suggests that decrease in the cumulative fecundity of exposed snails exposed to CLD is a consequence of the decreased oviposition rate rather than an effect of CLD on egg-production, and explains why $EC_{50\text{-}21d}$ values are similar for both endpoints.

Effect patterns, related to egg and clutch production, were similar in snails exposed to T, CPA, FEN and VZ (Table 5.2). They do not affect oviposition, but they induced a decrease in the number of eggs per clutch as compared to the controls. However, effects were significant only for some of the tested concentrations, and no clear dose-response relationships were observed for this endpoint and for these compounds (Table 5.2). Furthermore, no significant effect of T, CPA, FEN and VZ was observed on the cumulative fecundity per individual.

Exposure condition	Average Exposure Concentration (AEC)	Mean number (± SD) of egg-clutches per individual (oviposition)	Mean number (± SD) of eggs per individual (fecundity)	Mean number (± SD) of eggs per clutch
Controls	0	3.6 ± 0.4	184.7 ± 19.8	51.4 ± 12.2
Testosterone (T; ng/L)	0.3	3.2 ± 0.5	153.2 ± 26.7	45.1 ± 15.3 *
	0.7	3.7 ± 0.5	175.9 ± 25.0	48.3 ± 14.2
	1.4	3.4 ± 0.3	159.3 ± 16.6	47.8 ± 14.2
	3	3.9 ± 0.5	177.1 ± 30.4	47.1 ± 14.4
	6.8	3.7 ± 0.6	170 ± 23.4	46.4 ± 15.9 *
	14.8	0.2 ± 0.3	155.8 ± 15.6	48.7 ± 14.8
Cyproterone acetate (CPA; µg/L)	1.1	3.3 ± 0.4	153.8 ± 21.6	46.8 ± 15.5
	2.5	4.2 ± 0.4	188.9 ± 10.6	45.0 ± 17.9 **
	5.6	3.1 ± 0.5	146.2 ± 25.8	47.4 ± 15.2
	12.5	3.9 ± 0.3	179.4 ± 12.7	45.6 ± 11.9 ***
	28.7	3.2 ± 0.4	159.3 ± 19.9	49.0 ± 14.1
Chlordecone (CLD; µg/L)	2.1	2.9 ± 0.3	151.4 ± 23.3	51.2 ± 13.0
	3.9	2.6 ± 0.3	129.9 ± 13.1	50.5 ± 12.6
	8.3	2.2 ± 0.3	124.6 ± 16.9	53.5 ± 12.7
	19.6	1.2 ± 0.2 **	55.8 ± 11.6 **	45.5 ± 17.2
	49.1	0.7 ± 0.1 ***	34.6 ± 5.2 ***	52.0 ± 12.1
Fenitrothion (FEN; µg/L)	-	3.1 ± 0.4	154.6 ± 18.3	50.0 ± 10.9
	-	3.2 ± 0.4	168.0 ± 27.4	52.4 ± 16.5
	-	2.9 ± 0.2	145.0 ± 10.5	49.4 ± 11.4
	-	3.0 ± 0.3	141.3 ± 15.4	46.6 ± 14.1 *
	-	5.5 ± 0.4	173.8 ± 18.9	49.2 ± 13.4
Vinclozolin (VZ; ng/L)	-	2.9 ± 0.4	141.5 ± 18.3	48.2 ± 12.2
	-	3.1 ± 0.3	149.6 ± 14.4	47.0 ± 12.2 *
	-	3.1 ± 0.1	139.0 ± 7.8	44.4 ± 12.4 ***
	-	3.3 ± 0.4	155.9 ± 20.8	46.8 ± 12.6 *
	-	3.2 ± 0.3	149.4 ± 13.6	47.2 ± 12.9 *

Table 5.2: Mean number of egg-clutches and of eggs produced per individuals (± SD) and mean number of eggs per clutch (± SD) produced by *L. stagnalis* over 21 days of exposure to chemicals. Kruskal-Wallis with Dunn's *post hoc* test statistical significance; ∗: p<0.05; ∗∗: p<0.01; ∗∗∗: p<0.001).

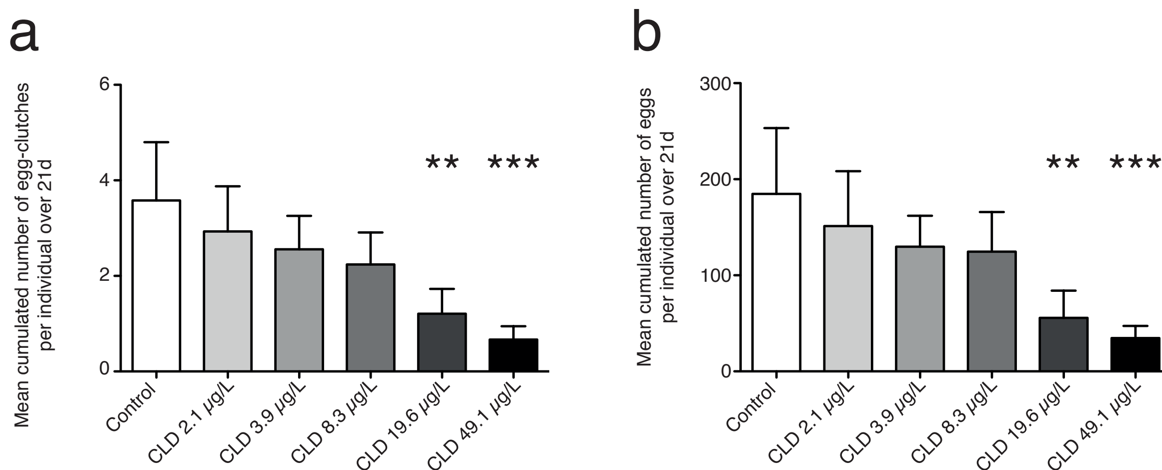

Fig. 5.1: Mean cumulated number of (a) egg-clutches and (b) eggs laid per individual after a 21 days exposure to chlordecone (CLD). Error bars stand for standard deviation over six replicates. (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

3.4. Egg quality

Overall, egg abnormalities were observed following the exposure of adult *L. stagnalis* to all the tested chemicals. With respect to the concentration-effect curve, distinct response patterns were observed for T, CLD and CPA on the one hand and for FEN and VZ on the other hand. Exposure to T, CLD and CPA resulted in U-shaped concentration-response curves (Fig. 5.2 a, b and c). The frequency of abnormal eggs was significantly higher than the control value when snails were exposed to the highest T concentrations (6.8 and 14.8 ng/L; $p < 0.05$, ANOVA, Dunnett's *post hoc* test). In contrast, as compared to the control, significant increases in the frequency of abnormal eggs were observed for the lowest tested concentrations of CLD (2.1 µg/L) and CPA (1.1 and 2.5 µg/L) ($p < 0.05$, ANOVA, Dunnett's *post hoc* test) (Fig. 5.2 b and c). Exposure to FEN and VZ resulted in increased frequency of abnormal eggs but, as compared to the control, difference was only significant when the snails were exposed to 22 µg/L FEN ($p < 0.05$, ANOVA, Dunnett's *post hoc* test), and no concentration-response relation was observed (Fig. 5.2 d and e).

Different types of egg abnormality were identified in exposed snails. Amongst the four types of egg abnormalities induced by the other chemicals, polyembryony was the most frequent (Fig. 5.3 a-e). Again, effects of the compounds on the frequency of egg polyembryony followed different dose-response patterns. Exposure to both CLD and

CPA resulted in linear concentration-dependent increases of the proportion of polyembryonic eggs (Fig. 5.4 a and c), with frequency values significantly different from the control value from 3.9 µg/L and 5.6 µg/L, respectively ($p < 0.05$, Kruskal-Wallis, Dunn's *post hoc* test). Effect patterns, as reflected by the absence of a concentration-response relation, were similar when considering the frequency of polyembryonic eggs from adults exposed to T, FEN and VZ (Fig. 5.4 b, d and e). T significantly increased the frequency of polyembryony at the lowest exposure concentration (0.3 ng/L) ($p < 0.05$, ANOVA, Dunnett's *post hoc* test), and amplitude of the effects remained similar for the other tested concentration (Fig. 5.4 b). In contrast, our results do not support the existence of effects of FEN and VZ on the frequency of egg polyembryony in *L. stagnalis*.

The relative sensitivity of the endpoints tested (as reflected by the NOEC values) varied depending on the tested compound. NOECs for the frequency of abnormal eggs were lower than those for the frequency of polyembryonic eggs for CLD and CPA but it was higher for T (Table 5.3).

Chemicals	Mortality	Growth	Fecundity	Oviposition	Frequency of abnormal eggs	Frequency of polyembryonic eggs
T; ng/L	NOEC: 14.8				LOEC: 6.8	LOEC: 0.3
CPA; µg/L	NOEC: 28.7				LOEC: 1.1	LOEC: 2.5
CLD; µg/L	LC 50: 104.5 [98.9 – 11.7]	NOEC: 49.1	EC50: 11.3 [5 – 20.7]	EC50: 9.3 [4.1 – 18.3]	LOEC 2.1	LOEC: 3.9
FEN; µg/L	NOEC: 240				LOEC: 22	LOEC: 10
VZ; ng/L	NOEC: 240					

Table 5.3: EC50, LC50, LOEC, and NOEC calculated for the reproductive endpoints in *Lymnaea stagnalis* after 21 days of exposure to testosterone (T), cyproterone acetate (CPA), chlordecone (CLD), fenitrothion (FEN), and vinclozolin (VZ). The confidence intervals are under brackets.

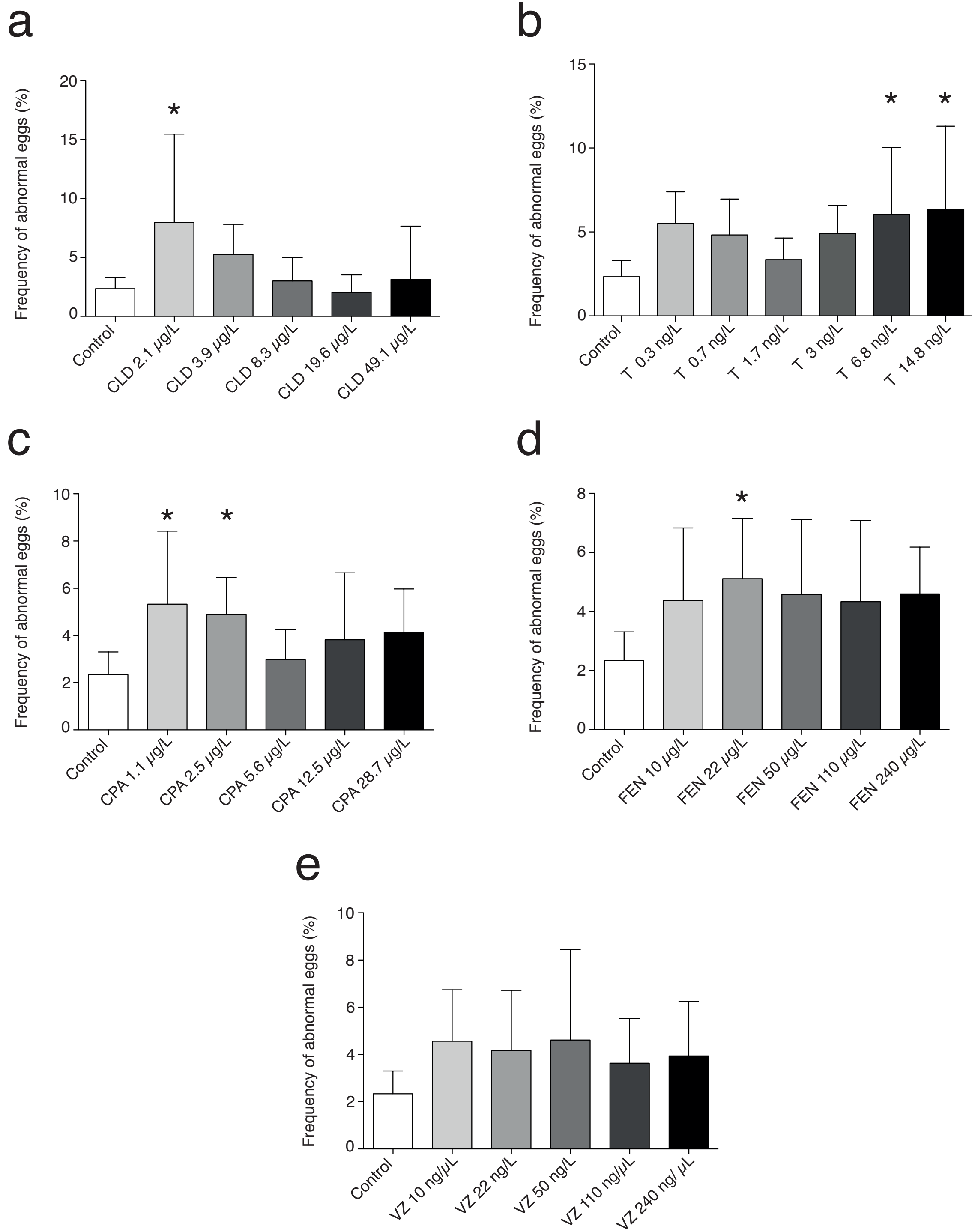

Fig. 5.2: frequency of abnormal eggs over total number of eggs laid per individual after a 21 days exposure to (a) chlordecone (CLD); (b) testosterone (T); (c) cyproterone acetate (CPA); (d) fenitrothion (FEN) and (e) vinclozolin (VZ). Error bars stand for standard deviation over six replicates (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

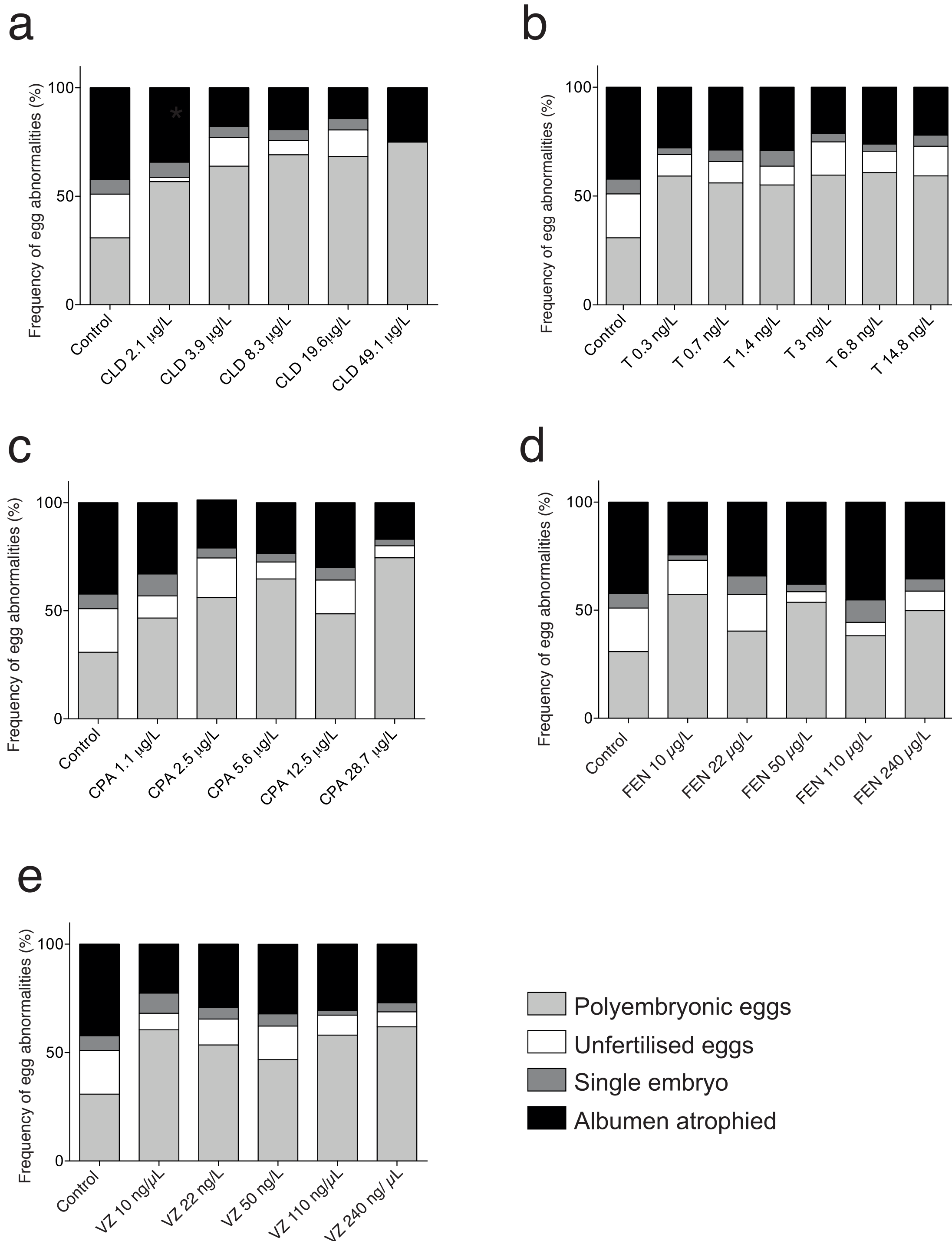

Fig. 5.3: frequency of egg abnormalities over total number of abnormal eggs laid per individual after a 21 days exposure to (a) chlordecone (CLD); (b) testosterone (T); (c) cyproterone acetate (CPA); (d) fenitrothion (FEN) and (e) vinclozolin (VZ). Error bars stand for standard deviation over six replicates. (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

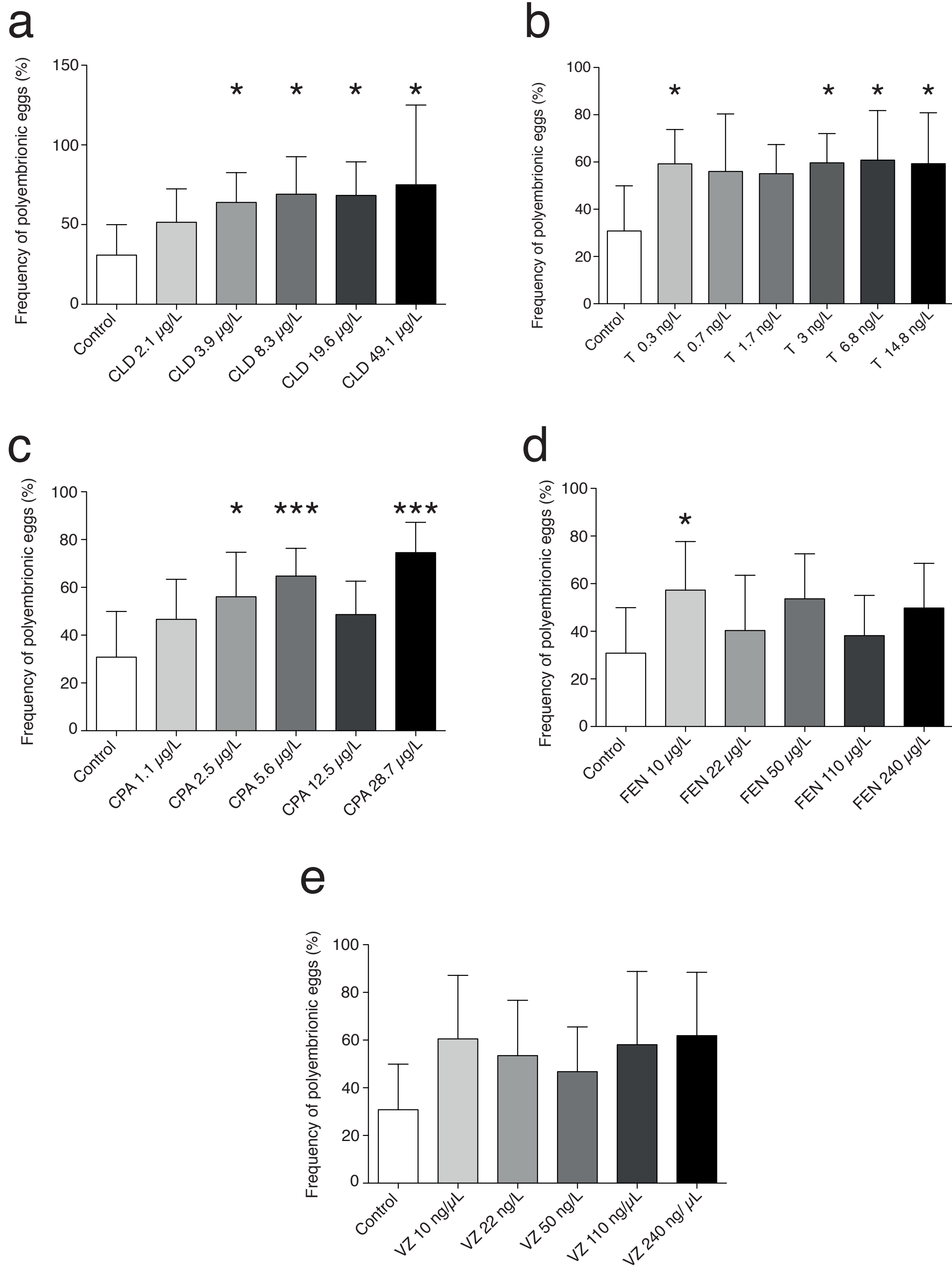

Fig. 5.4: frequency of polyembryonic eggs over total number of abnormal eggs laid per individual after a 21 d exposure to (a) chlordecone (CLD); (b) testosterone (T); (c) cyproterone acetate (CPA); (d) fenitrothion (FEN) and (e) vinclozolin (VZ). Error bars stand for standard deviation over six replicates (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

4. Discussion

4.1. Snail survival and growth

Exposure of *L. stagnalis* to T, CPA, FEN and VZ in the ng or µg/L range did not induced significant alterations of survival. This confirms previous findings, which showed that EDCs have a low acute toxicity and they generally do not exert lethal effects on molluscs at environmental concentrations (Lagadic et al. 2007; Matthiessen 2008; OECD 2010). Significant mortality was observed only in snails exposed to the highest CLD concentration tested (49.1 µg/L) which is 8-fold the maximal CLD concentrations found in surface waters (Bocquené and Franco 2005; Cabidoche et al. 2006; O.D.E 2009). The corresponding $LC_{50\text{-}21d}$ was 104.5 [98.9 – 111.7] µg/L. To our knowledge, this is the first study that provides a LC50 for CLD in a mollusc species although molluscs are potentially exposed to this compound in the field. This value is below LC_{50} values published for other invertebrates on the basis of short-term tests (e.g., $LC_{50\text{-}96h}$ of 210 µg/L and 180 µg/L for the blue crab, *Callinectes sapidus* and the amphipod *Gammarus pseudolimnaeus*, respectively; (Sanders et al. 1981; Schimmel and Wilson 1977)).

Growth was not affected by the exposure to any of the tested chemicals. This confirms previous finding with VZ (Ducrot et al. 2010) (no available literature data in *L. stagnalis* for the other tested compounds). These results suggest that the tested compounds did not significantly disturb the physiological processes that control growth in adults *L. stagnalis*, nor their feeding behaviour or the food quality.

4.2. Oviposition and fecundity

Oviposition and fecundity were not significantly altered when *L. stagnalis* was exposed to T, CPA, FEN and VZ. In contrast, exposure to CLD induced a significant decrease in the oviposition frequency, which resulted in a decrease in the cumulative fecundity per individual. Thus, the four compounds, which have androgenic or anti-androgenic properties in vertebrates (i.e., T, CPA, FEN and VZ), led to similar effect patterns in *L. stagnalis*. These effects were different from those induced by CLD, which has oestrogenic properties in vertebrates. This seems consistent with the existence of

oestrogen receptors (ERs) in *Lymnaeidae* (Kumkate et al., 2009), among other mollusc groups (Castro et al., 2007b; Kajiwara et al., 2006; Stange et al., 2012a), whereas the androgen receptor has never been found in any mollusc species to date (Reitzel and Tarrant, 2010), and the existence of an androgen signalling pathway has been recently questioned (Scott, 2012). In *Lymnaea ollula*, expression of the ER gene was transiently decreased when the snails were infected by *Fasciola gigantica*, and a decrease in fecundity was concomitantly observed (Kumkate et al., 2009). However, the co-occurrence of these effects is not sufficient to establish a functional link between an oestrogenic receptor signalling pathway and snail fecundity. Surprisingly, ER gene transcription level was not modified by the exposure of the *Lymnaeidae* species *Radix balthica* to EE2 (Börjesson, 2012). In other mollusc species, the ER is not activated by oestradiol nor by other steroids, suggesting that the constitutive transcriptional activity of this receptor (Bannister et al., 2007; Keay et al., 2006; Thornton, 2001). However, dose-dependent increase of the ER expression was reported to increase in *Nucella lapillus* exposed to oestrogenic effluents of sewage treatment plant (Castro et al., 2007c). In the parthenogenic *Potamopyrgus antipodarum* exposure to oestrogenic chemicals (i.e., bisphenol A and Ethinyloestradiol) significantly increased ER expression whereas the androgen, methyltestosterone, reduced the ER expression after 1 day of exposure (Stange et al., 2012a). These observations question the role of vertebrate-type oestrogens in controlling mollusc reproduction. Yet, the presence of ER has not been investigated in *L. stagnalis.* Nevertheless, pyrosequencing data analysis clearly showed the presence of a retinoid X receptor (RXR) in this species (Bouétard et al., 2013; Bouétard et al., 2012), and the ligand-binding domain of this receptor is close to that of vertebrate ER (A. Giusti, unpublished data).

Non ER-mediated interactions between CLD and hormonal processes, which control egg-laying in *L. stagnalis,* may have also occurred. In particular, as CLD was shown to inhibit aromatase in human (Benachour et al., 2007), interactions between this chlorinated compound and P450-dependent enzyme systems involved in steroid metabolism in *L. stagnalis* cannot be excluded (Giusti et al. 2013b; Part 4: chapter 5). Apart from endocrine disruption, CLD may have interfered with physiological processes that control energy allocation to reproduction, resulting in decreased egg production in the exposed snails. This cannot be defined as “endocrine disruption”,

although it might be linked with hormonal changes, as shown when *L. stagnalis* was exposed to the diphenyl ether herbicide fomesafen (Jumel et al., 2002).

As previously mentioned, the chemicals, which are known as androgens or anti-androgens in vertebrates, did not affect *L. stagnalis* oviposition and fecundity in the present experiment. For VZ, this may appear in contradiction with the data we already published (Ducrot et al. 2010). However, the present results were confirmed in two independent 28 days experiments under similar experimental conditions (V. Ducrot, unpublished data). Such a discrepancy may be due to the fact that the snails we used in the present study were larger (22.5 ± 2.5 mm) than the ones we used in our previous experiment (20.8 ± 0.8 mm). It has to be noted that the young adults used in the present experiment (and its two subsequent repetitions; V. Ducrot, unpublished data) were able to spawn from the first day of the exposure to VZ whereas, in the other experiment, smaller snails exposed to concentrations equal or below 25 µg/L VZ were not able to spawn before 13 days and those exposed to 2500 µg/L VZ did not spawn at all during the test (Ducrot et al., 2010). These results suggest that whether snails are exposed to EDCs before or after their sexual maturity might dramatically influence the outcome/sensitivity of the tests.

To our knowledge the effects of T on the reproduction of gastropods has never been investigated before, so that our results cannot easily be compared to or explained by literature data. The closest studies involved the pharmaceutical methyltestosterone, which also had no effect on *L. stagnalis* reproduction following 56 days of exposure to 1, 10 and 100 ng/L (Czech et al., 2001), or to 0.1 to 10000 ng/L (ten-fold steps) in our test conditions (A. Barsi, unpublished data). Measurements of internal T concentrations in *L. stagnalis* recently highlighted that total T concentration (i.e., free and esterified forms) increased in a concentration-dependent manner following exposure to 1.4 and 6.8 ng/L of T, but that the concentration of the free form of the steroid (i.e., putative active form) remained constant (Giusti et al., 2013b; Part 4: chapter 5). Although the mechanistic link between steroid internal concentrations and reproductive performances has not been established in molluscs (Scott, 2012), homeostatic regulation of T internal level may explain the absence of impacts of this steroid on oviposition and fecundity of *L. stagnalis*.

The present study is also the first attempt to investigate effects of CPA and FEN on offspring production in a mollusc reproduction test. Both compounds had no effect

on the oviposition and fecundity of *L. stagnalis.* No literature data were found on FEN for comparison. In contrast, the effects of CPA have been investigated in gonochoric prosobranch gastropods. CPA was able to reduce the penis length and to decrease spermatogenesis in *Hinia reticulata* and *Nucella lapillus* (Tillmann et al., 2001). Furthermore, CPA reduced or even prevented the development of imposex following exposure to TBT in those gastropod species (Bettin et al., 1996; Santos et al., 2005; Stange et al., 2012b; Tillmann et al., 2001). It has to be noted that a single dose of 1.25 mg/L was used in these studies, and effects on the fecundity of females were not investigated. Therefore, information about the impacts of CPA on molluscs is rather limited. It remains to be elucidated whether CPA is able to alter the gonochoric gastropod reproduction through egg production, or if the adverse effects of this compound on gastropods are limited to sex organogenesis.

4.3. Egg-quality

The tested EDCs significantly increased the frequency of abnormalities in eggs produced by exposed *L. stagnalis*. Endpoints related to egg-quality were more sensitive than oviposition and fecundity, as shown by the NOEC values we obtained for the tested compounds. This confirms the conclusions from a previous study on the effects of TBT in *L. stagnalis* (Giusti et al. 2013a; Part 2: chapter 2).

Exposure to T and CPA resulted in U-shaped concentration-response curves for the frequency of abnormal eggs. U-shaped dose-response relationships are observed in many studies on the impacts of EDCs, mainly following exposure to xeno-estrogens (e.g., Bisphenol A;(Weltje et al. 2005). Whether they are inverted or not, these non-monotonic concentration-response curves raise the importance of testing a wide range of concentrations to identify low concentrations of EDCs, which may have adverse effects (OECD 2010; Weltje et al. 2005).

A significant decrease in egg quality was observed for the snails exposed to 2.1 μg/L CLD whereas higher concentrations led to less intense effects. Similar observations were reported in a previous study on *L. stagnalis* exposed to TBT: exposure to concentrations ranging from 19 to 94 ng Sn/L during 21 days significantly increased the frequency of abnormal eggs while higher concentrations led to less pronounced effects on this endpoint. In both cases, we could not check whether

additional testing of a few concentrations above the maximum tested concentrations would have led to U-shape concentration-responses for the frequency of abnormal eggs, because oviposition and fecundity were strongly reduced at high TBT and CLD concentrations (from 197 ng Sn/L and 19.7 µg/L, respectively; (Giusti et al. 2013a; Part 2: chapter 2)).

Amongst the various types of egg-abnormalities we observed, polyembryony was the most frequent (i.e., in all treatments, the proportion of polyembryonic eggs was over 50% of the total abnormal eggs). Aside from polyembryony, the different other types of abnormalities were all detected, but their relative frequency varied depending on the tested compound and concentration. This result suggests that chemicals can disturb various physiological pathways involved in egg-production. Increased frequency of polyembryonic eggs is the most frequently reported alteration in *L. stagnalis* exposed to toxicants (Bandow and Weltje 2012; Bluzat et al. 1979; Giusti et al. 2013a; ; Part 2: chapter 3; Lanzer 1999). In this species, oogenesis is well documented (Koene 2010). Yet, the physiological mechanisms that explain polyembryony still need to be investigated.

5. Conclusion

The present study showed that endpoints commonly used in reproduction tests with *L. stagnalis* (i.e., oviposition and fecundity) were significantly impaired by CLD (oestrogen receptor agonist in vertebrates) but, in the concentration ranges tested, these endpoints were not responsive to T, CPA, VZ and FEN, known as androgens or anti-androgens in vertebrates. In contrast, CLD-induced reproduction impairment in *L. stagnalis* may plausibly be linked to the presence of oestrogen receptors in *Lymnaeidae* (Börjesson 2012; Kumkate et al. 2009). However, EE2 failed to activate ER gene transcription in *R. balthica* (Börjesson 2012) and did not affect *L. stagnalis* oviposition and fecundity in the present study. Other signalling pathways should therefore be considered to explain the effects of CLD on the reproduction of this snail. The retinoid signalling pathway might be a good candidate since an RXR has recently been found in *L. stagnalis* (Bouétard et al. 2012). Such an RXR could not bind T, or compounds that usually bind to the androgen receptor in vertebrates, and this may explain why such compounds do not affect the reproduction of *L. stagnalis*.

Implementation of egg quality as an endpoint may increase sensitivity of the reprotoxicity test with *L. stagnalis*. This was observed when snails were exposed to CLD, T and CPA. Along with results of a previous study with TBT (Giusti et al. 2013a; Part 2: chapter 2), present results suggest that the quality of eggs is an important parameter to consider when assessing the impacts of putative EDCs on *L. stagnalis* because (i) those results indicate that reproductive impairment in this species could occur at lower concentrations than those reported in literature based on classical endpoints like fecundity, and (ii) the type of abnormality induced in the offspring might provide additional indications on physiological pathways which were disturbed by chemicals in exposed adults, thus improving analysis of the mode of action of the tested compounds.

Acknowledgements

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Part 3:

Bioaccumulation of chlordecone and cyproterone acetate in **Lymnaea stagnalis** *tissue*

In this section of the thesis, we will investigate the bioaccumulation of cyproterone acetate and chlordecone in *Lymnaea stagnalis* tissues following a 21-day exposure. For this purpose, we have developed original extraction and analysis methods of the two chemicals. Bioconcentration factors were never reported for these molecules in molluscs and, therefore, these results will provide new insights on the putative metabolisation and excretion of the synthetic steroid, i.e., cyproterone acetate, and the organochlorinated pesticide, i.e., chlordecone.

Introduction

Chemical analysis of the exposure water have highlighted that concentrations of the organochlorine chlordecone and of the synthetic steroid cyproterone acetate fell to 20% of the nominal concentrations after 48 hours of exposure. These chemicals are hardly metabolisable and physical degradation is low (IPCS 1984). Furthermore, the physicochemical properties of these molecules suggest that it is highly probable that bioaccumulation occurs in lipid rich tissues of organisms. Bioaccumulation of chlordecone has mainly been studied along the trophic chain in the French West Indies, where chlordecone was extensively used until it was prohibited in 1993 (Coat et al. 2011; Kermarrec 1980). Conversely, bioaccumulation of the anti-androgenic pharmaceutical cyproterone acetate has never been reported in organisms. In this study, we have developed original protocols for the extraction and the quantification of these molecules in *L. stagnalis* tissues. Our results provide information on the bioaccumulation potential of these molecules after 21 days of exposure to concentrations in the µg/L range in a semi-static experiment.

1. Material and Methods

1.1. Sample conditioning

After 21 days of exposure, an individual was randomly sampled in each replicate per experimental condition. *L. stagnalis* samples were frozen and kept at -80°C until chemical extraction and analysis. After shell removal, wet weights of *L. stagnalis* tissue samples were obtained using a Mettler Toledo balance (AT261, Mettler Toledo, Spain). The samples were then freeze-dried with a Benchtop 3L Sentry Lyophilisator (VirTis, New-York, USA) overnight and dry tissue samples were weighted prior to chemical extraction.

1.2. Chlordecone extraction and analysis

Extraction of chlordecone was performed with 10 mL n-hexane:dichloromethane (90:10;V:V) (Riedel-de Haën, Seelze, Germany) using an Accelerated Solvent Extractor ASE (Dionex 200, Sunnyvale, USA). Prior to extraction, surrogate PCB congener 112 (Dr. Ehrenstorfer®, Augsburg, Germany) was added to the samples at a final concentration of 50 pg/µL in order to quantify possible loss of chlordecone during the extraction process. Extraction cells were heated 6 minutes before each extraction cycle using a pressure of 1500 psi at 125°C. Two cycles were performed for each sample, each cycle lasted 5 minutes. Whole extracts were used for lipid content determination: solvent was evaporated using a Turbovap LV (Zymarck, Hopkinton, Mass., USA) at 40°C until a constant lipid weight was obtained. Samples were then diluted in 3 mL *n*-hexane prior to the clean-up of the extracts, which was performed by adding 2 mL of sulphuric acid (Merck, Darmstadt, Germany). This step removed the organic matter extracted by the ASE method (lipids, lipoproteins, carbohydrates). The samples were then homogenised by vortexing with a Vibramax 110 (Heidolph, Germany) before being centrifuged for 3 min at 2160 G at 10°C with a JOUAN BR4i centrifuge (JOUAN, St-Nazaire, France). The organic phase was transferred to another tube and the acidic phase was extracted with 3 mL of n-hexane, vortexed and centrifuged for another 3 min. The organic layers of subsequent centrifugations were pooled and evaporated under a gentle stream of nitrogen to dryness using a Visidry evaporator (Supelco, Bellefonte, PA, USA). A final volume of 20 mL of hexane was added to each sample. For chemical analysis, 100 µl of PCB 209 (Dr. Ehrenstorfer®, Augsburg, Germany) (100 pg/µl diluted in hexane) were added to 100 µL of each sample, PCB 209 being used as internal standard at a final concentration of 50 pg/µl.

Chlordecone concentrations were determined by high-resolution gas chromatography (HRGC) using a Thermo Quest Trace 2000 gas chromatograph equipped with a Ni^{63} electron capture detector (ECD) (Thermo Quest, Milan, Italy) and an autosampler for liquids Thermo Quest AS 2000 (Thermo Quest, Milan, Italy). Two microliters of the purified extracts were injected through a cold "on column" injector. The carrier gas was hydrogen with a flow rate of 3.8 mL/min and a pressure of 130 kPa. The make up gas was $Ar:CH_4$ (95:5) at a flow rate of 30 mL/min. The injector was at ambient temperature and the detector was kept at 300°C. Chlordecone was

separated on a 30mX0.25mm (0.25 µm film) DB-XLB capillary column (J&W Scientific, Santa Clara, CA, USA). The temperature program was as follows: 2 min at 60°C, gradual heating from 60 to 180°C at the rate of 30°C/min, 3 min at 180°C, gradual heating from 180 to 270°C at the rate of 2.5°C/min and 12 min at 270°C. The post-run conditions were a pressure of 200 kPa and a temperature of 300°C during 5 min. Data were recorded using the Chromcard 2.2 software for Windows (Fisons Instruments, Italy). An integrator calculated the chemical peak areas and peaks were identified according to their retention time. Quantification was performed using the internal standard method. The linear calibration curve with at least six concentrations (Council 2002), which ranged from 10 to 250 pg/µL, was established with a certified chlordecone solution. This concentration range was defined by the expected concentration in samples.

1.3. Cyproterone acetate extraction and analysis

Extraction of cyproterone acetate was performed with 10 mL methanol (MeOH, Burdick & Jackson, Muskegon, MI, USA) using an Accelerated Solvent Extractor ASE (Dionex 200, Sunnyvale, USA). Before the extraction, medroxyprogesterone (Sigma-Aldrich, Schnelldorf, Germany) was added at a final concentration of 25 ng/mL to the samples as both internal standard and surrogate marker in order to assess possible loss of CPA during extraction and to quantify the molecule. Extraction cells were heated 6 min before each extraction cycle using a pressure of 1000 psi at 60°C. Two cycles were performed for each sample, each cycle lasted 5 minutes. Solvent was evaporated using a Turbovap LV (Zymarck, Hopkinton, Mass., USA) at 35°C to a volume of 1 mL of MeOH. Five millilitres of water were added to each sample. Further extraction of CPA from the samples was performed by Solid Phase Extraction (SPE) using Supelco Supelclean™ Envi-18 SPE Tubes 6 mL (1 g) (Supelco, Bellefonte, PA, USA). Cartridges were first conditioned with 10 mL of MeOH followed by 5 mL of MeOH/H_2O (50:50;V:V) and 5 mL of water using an elution device (Visiprep DL™ SPE Supelco) under 600 mbar vacuum. Samples were passed though columns and vials, which contained samples were rinsed with 3 mL of water, were added to the cartridges. Columns were then rinsed with 6 mL of water and with another 6 mL of H_2O/MeOH/NH_3 (90:10:0,2;V:V:V) and cartridges were then dried for at least 30 min.

CPA was eluted with 5 mL of MeOH and 5 mL of water were added. One millilitre of each sample was transferred to 2 mL vials, which were then stored at -20°C until analysis.

Cyproterone acetate was analysed and quantified by High-Resolution Liquid Chromatography coupled with mass spectrometry (HPLC/MS-MS) using a 2690 Waters Alliance Separation Modules integrated autosampler, solvent delivery system and column heater (Waters, Milford, MA, USA) coupled to a Quattro Ultima Platinum triple quadrupole mass spectrometer (Micromass, Manchester, UK). The mass spectrometer was equipped with an electrospray ionisation interface (ESI) operated with a positive ionisation mode. A volume of 20 µL of extracts was injected on an Intersil ODS-3 column (5µm, 2.1 X 150 mm) (Atas-GL International BV, Veldhoven, Netherland) held at 35°C. Gradient elution began with 50% MeOH /formic acid (100:0.1;V:V) and 50% H20/formic acid (100:0.1;V:V) for 1 min, and then MeOH was increased to 95% in 8 min and maintained isocratic for 2 min. Then solvent gradient was returned to the original condition in 2 min for the next elution. Capillary and cone voltage were set at 3 kV and 58 V, respectively and temperature source was kept at 125°C. Desolvation temperature was 250°C. Cone and desolvating gas were not used. Ions were monitored in the Multiple Reaction Monitoring (MRM) mode and data acquisition and quantification was performed with Quanlynx Software (Micromass, Manchester, UK). Table 6.1 summarises the mass spectrometer acquisition parameters for cyproterone acetate and the internal standard medroxyprogesterone. Quantification was performed using the internal standard method. The linear calibration curve with at least six concentrations (Council 2002) which ranged from 0 to 1000 ng/mL was established with a certified CPA solution. This concentration range was defined by the expected concentration in samples.

Channel	Compounds	Parent ion (Da)	Daughter ion (Da)	Dwell time (s)	Collision Energy (eV)	Inter channel Delay (s)	Elution time (min)
1	Medroxyprogesterone (I.S.)	387.4	123.0	0.1	20	0.02	8.3 -9.0
2	Medroxyprogesterone (I.S.)	387.4	327.4	0.1	10	0.02	8.3 -9.0
3	Cyproterone acetate	417.3	147.2	0.2	20	0.02	8.05-8.75
4	Cyproterone acetate	417.3	357.3	0.2	15	0.1	8.05-8.75

Table 6.1: Mass spectrometer parameters of the multiple reaction monitoring (MRM) operated from 6 to 11.6 minute of the HPLC run.

2. Results and Discussion

CPA concentrations ranged from 2.96 to 72.29 µg/g of wet weight after 21 days of exposure. The measured bioconcentration factor (BCF) ranged from 2300 to 3000 approximately (Table 6.1). These results suggest that CPA bioaccumulates in *L. stagnalis* exposed for 21 days and that metabolisation is low. Pharmacokinetics of CPA has only been investigated in humans. CPA is metabolised in 15ß-hydroxycyproterone acetate by a cytochrome P450 enzyme, CYP3A4, and excreted through bile (70%) and urine (30%) (Kuhl 2001). Even though CYP3-like genes were identified in molluscs (Wootton et al. 1995; Zanette et al. 2013), our observations highlight that *L. stagnalis* is not able to metabolise and excrete this synthetic steroid chemical efficiently, even at low concentrations, as the bioaccumulation factor was not different among the concentrations tested. We have previously reported that, in *L. stagnalis* exposed to 1.1 µg/L of CPA, the frequency of abnormal eggs was increased, mainly due to an increase in polyembryonic eggs (cf. publication 3). The reported body burdens in these individuals were 2.96 ± 0.90 µg/g. Therefore, molluscs exposed to low environmental concentrations (ng/L), for long periods, might accumulate synthetic steroid molecules at levels that might induce adverse impacts on mollusc reproduction. This is the first report of bioconcentration of cyproterone acetate in invertebrates.

Chemicals	Concentrations in water		Concentrations in *L. stagnalis* tissues		BCF (wet weight)
	Nominal (µg/L)	(AEC) (µg/L)	(µg/g wet weight)	(µg/g dry weight)	
Cyproterone acetate (CPA)	2	1.1	2.96 ± 0.90	26.77 ± 14.79	2692.99 ± 822.66
	4.5	2.5	7.37 ± 1.83	52.94 ± 11.51	2949.06 ± 730.30
	10	5.6	16.97 ± 2.72	116.82 ± 19.31	2995.13 ± 486.27
	22	12.5	28.83 ± 8.87	204.76 ± 63.24	2306.78 ± 709.78
	50	28.7	72.29 ± 29.46	555.67 ± 258.67	2518.98 ± 1026.47
Chlordecone (CLD)	4.5	2.1	0.51 ± 0.17	3.86 ± 1.82	242.02 ± 80.72
	10	3.9	0.20 ± 0.18	1.57 ± 1.42	51.06 ± 47.35
	22	8.3	0.65 ± 0.24	4.44 ± 1.29	78.47 ± 29.48
	50	19.6	1.33 ± 0.87	14.04 ± 9.38	67.99 ± 44.36
	110	49.1	4.51 ± 1.55	48.65 ± 27.90	91.80 ± 31.63

Table 6.2: Nominal and time-weighted average water concentrations (µg/L) and concentrations in *Lymnaea stagnalis* tissues expressed in µg/l of wet and dry weight. Bioconcentration factor (BCF) over 21 days of exposure was expressed based on *L. stagnalis* concentrations expressed on the wet weight.

Chlordecone concentrations in *L. stagnalis* tissues ranged from 0.2 to 1.33 µg/g of wet weight, therefore, the calculated BCF ranged from 50 to 250. A study on two freshwater pulmonate gastropods of the *Physidae* family (i.e., *Physa cubensis* and *P. acuta*), which belong to the same monophyletic order of Basommatophora like *L. stagnalis*, displayed similar results. Indeed, *P. cubensis* and *P. acuta* sampled in a pond in Guadeloupe, featured comparable BCF values (between 140 to 480) as CLD concentrations measured were 195.40 ± 15.03 ng/L in the pond water and were 62.2 ± 22.4 ng/g of wet weight in snail tissues. These bioconcentration factors are particularly low compared to BCFs reported in vertebrate and invertebrates species (up to 60000 in fish) (IPCS 1984). In oysters *Crassostreas gigas* exposed to 0.03 and to 0.39 µg/L of chlordecone in a flow though system, tissue concentrations were 10000 times higher than water concentrations within 19 days. However, depuration in *C. gigas* was rapid as within 7 to 20 days after the end of exposure, no chlordecone could be detected in oyster tissues. Moreover, CLD concentrations could be measured in faeces and pseudofaeces of oysters fed with contaminated algae (Bahner et al. 1977). These observations are consistent with our results, which suggest that *L. stagnalis* and

Physidae sp. can either metabolise or excrete chlordecone in order to decrease the body burdens. Excretion of unmetabolised chlordecone in gastropods might occur through faeces as observed in oyster. Moreover, an increase in mucus production could constitute a detoxification process in *L. stagnalis*, thereby reducing body burdens. Indeed, in our laboratory experiment, exposure of *L. stagnalis* to high chlordecone concentrations increased the production of mucus that accumulated on the walls of the beakers, as previously reported in TBT experiments (Giusti et al. 2013a; Part 2: chapter 2). Two water samples, with or without mucus, were taken from each exposure beaker of the 19.6 and 49.1 µg/L treatments. Samples that contained a high proportion of mucus showed that chlordecone concentrations were 3.3 ± 0.7 times higher than in water samples that were mucus free. As bioconcentration factors were similar for every chlordecone concentration tested, along with the increase in mucus productions, which was concentration dependent, it remains to be investigated whether *L. stagnalis* excretes chlordecone in order to reduce its toxicity.

Part 4:

Investigation of the mechanisms of action of EDCs in Lymnaea stagnalis

The analyses of the impacts of different endocrine disrupting chemicals on the reproduction of the hermaphroditic freshwater gastropod *Lymnaea stagnalis* reported in the previous chapters have highlighted that some of the tested molecules could lead to adverse impacts on the mollusc reproduction.

The organotin TBT significantly reduced oviposition and fecundity in *L. stagnalis* exposed to concentrations of 94, 197 and 473 ng Sn/L. The frequency of abnormal eggs and the frequency of polyembryonic eggs among the total abnormal eggs produced per individual were increased in the range of concentration tested (from 19 to 473 ng Sn/L). Furthermore, modifications of the activity of enzymes associated to detoxification mechanisms (i.e., PROD and GSTs) as well as adverse impacts on shell calcification were reported in snails exposed to 197 and 473 ng Sn/L of TBT for 21 days.

Oviposition and fecundity were significantly reduced in individuals exposed to chlordecone from a concentration of 19.6 µg/L. At lower concentrations, this chemical also significantly increased the frequency of abnormal eggs produced per snail. Polyembryony was the major egg abnormality observed among the total egg abnormalities*.* A mortality rate of 30 % was observed at the highest concentration tested (49.1 µg/L), which allowed to calculate a LC50 at 21 days of 104.5 µg/L (Part 2: chapter 4).

In *L. stagnalis* exposed to testosterone and cyproterone acetate, oviposition and fecundity were not altered. However, the proportion of abnormal eggs produced per snails was significantly increased following exposure to 6.8 and 14.8 ng/L of testosterone and to 1.1 and 2.5 µg/L of cyproterone acetate. Furthermore, exposure to these steroids elicited a U-shaped dose-response curve for this endpoint. U-shaped and inverted U-shaped dose-response relationships are observed in many studies that aim to investigate the impacts of EDCs on reproduction, mainly following exposure to oestrogens. Therefore, these chemicals might induce adverse impacts on the egg formation, which is under the control of the neuro-endocrine system, through interactions with the endocrine system of this species. Furthermore, polyembryony was again the major egg abnormality reported following exposure to these chemicals. The proportion of polyembryonic eggs over the total abnormal eggs produced per snail over 21 days was significantly increased from 0.3 ng/L and 2.5 µg/L of testosterone and cyproterone acetate, respectively.

Our results showed that the two non-steroid anti-androgenic molecules, fenitrothion and vinclozolin, did not alter oviposition and fecundity of *L. stagnalis* in the concentration range tested (µg/L and ng/L, respectively). Both molecules tended to increase the frequency of abnormal eggs produced and an increase of polyembryony was also reported. However, the impacts of these compounds on reproductive endpoints assessed in *L. stagnalis* were less clear than the impacts of the other anti-androgenic molecule tested, i.e., the synthetic steroid cyproterone acetate.

Based on these results, further analyses of the mechanisms of action of the putative endocrine disruptors were performed on snails that were exposed to TBT, CLD, T and CPA at concentrations summarised in the table 7.1.

Chemicals	Nominal concentrations	Average exposure concentrations (AEC)
Testosterone (T)	2 ng/L	0.3 ng/L
	10 ng/L	1.4 ng/L
	50 ng/L	6.8 ng/L
Tributyltin (TBT)	45 ng Sn/L	19.2 ng Sn/L
	220 ng Sn/L	94.2 ng Sn/L
Cyproterone acetate (CPA)	2 µg/L	1.1 µg/L
	50 µg/L	28.7 µg/L
Chlordecone (CLD)	4,5 µg/L	2.1 µg/L
	50 µg/L	19.6 µg/L

Table 7.1: Nominal and average exposure concentrations of chemicals selected for the investigations of their mechanisms of action on *Lymnaea stagnalis*.

In Chapter 5, we will analyse endogenous concentrations of testosterone, free and esterified forms, in the reproductive organs of *Lymnaea stagnalis* exposed for 21 days to testosterone, tributyltin, cyproterone acetate, and chlordecone. Testosterone is the natural ligand of the androgen receptor in vertebrates. This steroid is found in molluscs' tissues and its endogenous synthesis is suggested in molluscs.

In chapter 6, proteomic experiments will be used to identify proteins differently expressed in reproductive tract of *L. stagnalis* exposed to testosterone, tributyltin, cyproterone acetate, and chlordecone for 21 days.

Modifications of endogenous concentrations of testosterone and of the expression of proteins involved in *L. stagnalis* will be compared to the impacts induced by these compounds on snail reproduction observed in previous chapters (2,3 and 4).

Chapter 5:

Impacts of EDCs on endogenous concentrations of testosterone in Lymnaea stagnalis

Publication 4:

Testosterone levels and fecundity in the hermaphroditic aquatic snail *Lymnaea stagnalis* exposed to testosterone and endocrine disruptors

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Abstract

Endocrine disruptors are known to alter endogenous free and/or esterified levels of androgenic and oestrogenic steroid hormones in aquatic molluscs. However, there is still a controversy on the origin of steroids in these animals. In this study, free and esterified testosterone concentrations were measured in the hermaphroditic aquatic gastropod *Lymnaea stagnalis* exposed to molecules known for their androgenic (testosterone and tributyltin), anti-androgenic (cyproterone acetate) and oestrogenic (chlordecone) properties by reference to their mode of action in vertebrates. In parallel, snail oviposition and fecundity were followed over the 21-day exposure period. Testosterone exposure resulted in increased esterified testosterone levels whereas free testosterone concentrations remained stable. In contrast, cyproterone acetate increased significantly the free form of testosterone with no changes in the esterified form whereas chlordecone showed a tendency to reduce esterified testosterone concentrations without changes in free testosterone levels. Finally, tributyltin did not alter testosterone homeostasis. The production of egg-clutches and eggs was significantly reduced only in the snails exposed to the highest concentrations of chlordecone (19.6 µg/L) and tributyltin (94.2 ng Sn/L). Overall, this study demonstrates that uptake of testosterone from the exposure medium occurs in *L. stagnalis*. Moreover, it shows that cyproterone acetate and chlordecone can alter endogenous testosterone levels in this freshwater snail. However, relationship between hormonal changes and snail reproduction has not been established.

Keywords: Gastropod; Testosterone esterification; Cyproterone acetate; Chlordecone; Tributyltin

1. Introduction

Endocrine disruptors (EDs) are known to interfere with endocrine systems in various ways, extensively described in vertebrates (Kortenkamp et al. 2011; RTI 2011). Knowledge of mollusc endocrinology is far too limited to unequivocally infer mechanisms of endocrine disruption in this group from the altered molecular and metabolic endocrine pathways described in vertebrates. To date, research on invertebrate endocrine systems has focussed on arthropod development and mollusc reproduction. Experimentally, it has been shown that vertebrate sex steroids can adversely affect the fertility of adult molluscs (Kortenkamp et al. 2011). Alteration of sex steroid tissue concentrations was also observed following laboratory and field exposure to EDs, the most studied example being the exposure to tributyltin (TBT) in prosobranch gastropods (Sternberg et al. 2010). Evidence of vertebrate-like steroid hormone synthesis and biological activity have been reported in bivalves, gastropods and cephalopods (Köhler et al. 2007; Lafont and Mathieu 2007; Porte et al. 2006; Sternberg et al. 2010). Fatty acid esterification of such steroids (i.e., testosterone, estradiol) is recognised as the major process of androgens and oestrogens homeostasis in molluscs (Gooding and LeBlanc 2001; Janer et al. 2006; Janer et al. 2004; Labadie et al. 2007; Sternberg and LeBlanc 2006). However, synthesis and activity of steroids in molluscs are still controversial. Very recently, Scott (2012) has questioned the ability of molluscs to biosynthesise vertebrate-type steroids, arguing that they might only take up these steroids from the environment. This however does not preclude the ability of molluscs to readily conjugate steroids to fatty acids, providing that the molecules have a reactive hydroxyl group (e.g., the C-17β-hydroxyl group of both testosterone and estradiol) (Gooding and LeBlanc 2001; Scott 2012; Sternberg et al. 2010).

Endogenous, free and/or esterified, concentrations of androgens and oestrogens in molluscs can be altered by EDs, which can lead to hormonal homeostasis disruption (Abidli et al. 2012; Fernandes et al. 2010; Gooding et al. 2003; Janer et al. 2006; Sternberg and LeBlanc 2006). To date, studies mainly focussed on gonochoric gastropods (mostly marine species), in which steroid concentrations and metabolism exhibit clear sex specific responses following exposure to EDs (Sternberg et al. 2010). In contrast, impacts of EDs on the reproduction and the steroid levels and biosynthesis in hermaphrodite gastropods are still poorly documented. *Lymnaea stagnalis*, a

simultaneous hermaphrodite freshwater gastropod, is probably the best-suited species for filling this gap. Indeed, it has been shown to be sensitive to EDs. Exposure to different EDs (e.g., TBT and vinclozolin) can alter *L. stagnalis* reproduction by reducing oviposition and fecundity, by impairing embryonic development, or by reducing egg hatching success (Czech et al. 2001; Ducrot et al. 2010; Giusti et al. 2013a; Part 2: chapter 2; Leung et al. 2007; Segner et al. 2003). Furthermore, the neurohormonal control of reproduction is reasonably well understood in this species (Jarne et al. 2010; Koene 2010), and Scott considers it as one of the species that should be "revisited" in order to definitively conclude on the ability of molluscs to synthesise vertebrate steroids, considering that it has already been reported to carry out critical transformations of radioactive vertebrate steroid precursors in good yields (Scott 2012).

In the present study, endogenous free and esterified testosterone concentrations were measured in adult *L. stagnalis* exposed to waterborne testosterone and to EDs (tributyltin, cyproterone acetate and chlordecone) for 21 days. Reproductive endpoints (oviposition and fecundity) were also followed during the exposure period. The ability of *L. stagnalis* to absorb vertebrate testosterone from water but also to esterify its endogenous testosterone following exposure to EDs with possible consequences on snail reproduction is discussed.

2. Material and methods

2.1. Animal rearing and exposure

The RENILYS strain of *Lymnaea stagnalis* used for the present investigation has been cultured under laboratory conditions for approximately ten years as described elsewhere (Jumel et al. 2002). Young adults of homogenous size (22.5 ± 2.5mm) and age (4 ± 0.5 months) were sampled from the culture and acclimatised to the test conditions during 48 h prior to exposure. Snails were exposed to testosterone and to each ED (Tributyltin hydride, CAS Nr 688-73-3; Cyproterone acetate, CAS Nr 427-51-0; Chlordecone, CAS Nr 143-50-0) for 21 days at the concentrations shown in Table 1. Spiking solutions were prepared in analytical grade acetone (99.9% purity) poured into the exposure water in order to obtain a solvent concentration of 0.01% (OECD 2000).

For each concentration of the tested compounds and the water and solvent controls, six replicates were used. Each replicate consisted in five snails in a glass beaker filled with 1 L of dechlorinated, charcoal-filtered tap water (pH = 7.7 ± 0.2, conductivity = 623 ± 60 µS/cm, dissolved oxygen = 7.3 ± 2 mg/L and water hardness = 254 ± 7 mg CaCo3/L). Water was renewed every 2 days to maintain exposure concentrations and physico-chemical properties of the test water. Tests were conducted at 20.5 ± 0.6°C and a 14:10 L:D photoperiod as previously described (Ducrot et al. 2010). Snails were fed every other day with *ca.* 2.5 g of rinsed organic lettuce (National AB–Agriculture Biologique and International Ecocert certifications) per replicate. After 21 days of exposure, four out of the six replicates were randomly chosen per experimental condition and one snail was randomly sampled in each of these four replicates. They were frozen and kept at -80°C until testosterone measurements.

2.2. Chemical analysis

Exposure water was sampled in triplicates. Each replicate consists in a pool of 100 mL of water sampled from two beakers, 15 min and 48 h after water renewal, and was frozen until chemical analysis. Testosterone and cyproterone acetate (CPA) were extracted using C-18 solid phase extraction (SPE) and quantified by high-resolution liquid chromatography-mass spectrometry (HPLC-MS- MS). Chlordecone (CLD) was extracted by handshaking 5 mL of exposure water with a volume of 5 mL of dichloromethane. Tributyltin (TBT) was derivatised and extraction was performed as described in the ISO method for organotin analysis in water (ISO 2004). CLD analysis and quantifications were performed by capillary gas chromatography-electron capture detection (GC-MS-ECD), and TBT was analysed and quantified using capillary gas chromatography-mass spectrometry (GC-MS-MS). Actual exposure concentrations (AEC) were calculated as the time-weighted average of measured concentrations over the test period (Belgers et al. 2011) (Table 6.2).

2.3. Testosterone quantification

Free and total (free + esterified) testosterone extractions were performed on whole snail soft tissues as previously described (Gooding et al. 2003), and analysed by

RadioImmunoAssay (RIA) using a RIA testosterone commercial Kit (Immunotech, Beckman Coulter, France). Testosterone was measured according to the RIA Kit protocol. Supplier's information indicates that the assay is highly (100%) specific for testosterone. Cross-reactivity with other steroids has been estimated at 10% for 5α-dihydrotestosterone, 5% for 19-nortestosterone and 2% for 11β-hydroxytestosterone, 5α-androstane-3β, 17β-diol, and methyltestosterone. It is below 0.1% for all other steroids. Extraction efficiencies of testosterone from *L. stagnalis* tissues were ≥ 90%, which is consistent with other studies (Gooding and LeBlanc 2004; Gust et al. 2009). Therefore, no corrections of testosterone concentrations were performed. In our conditions, detection and quantification limits of the RIA method were 25.3 and 61.4 pg/g w.w., respectively. All the testosterone concentrations measured in *L. stagnalis* tissues were above the limit of quantification. Esterified testosterone levels were obtained by subtracting free from total testosterone measured in each replicate. Steroid levels were normalised to sample wet weight and expressed as pg/g tissue wet weight.

2.4. Reproductive endpoints

Effects of testosterone and EDs on snail reproduction were estimated through the monitoring of oviposition (cumulative number of egg-clutches per snail) and fecundity (cumulative number of eggs per snail) over the whole exposure period (21 days). Every other day, egg-clutches were gently removed from the wall of the vessels using a sharp-edge spoon, and counted. The number of eggs in each egg-clutch was then counted under a stereomicroscope.

2.5. Statistical Analysis

Results are presented as means ± standard-error to the mean (SEM). Testosterone concentrations were expressed as the mean concentrations measured in the tissues of the four snails sampled in four independent replicates. Reproductive endpoints were expressed as the mean numbers of egg-clutches and of eggs laid per snail for the six replicates per treatment. Data were checked for normality using the d'Agostino and Pearson test (D'Agostino and Pearson 1973) and for homoscedasticity using the Bartlett and Kendall test (Bartlett and Kendall 1946). Differences between treatments were

tested using One-Way ANOVA, as previously used for comparing steroid levels in muricid gastropods (Abidli et al. 2012), and followed by a Dunnett's *post-hoc* test. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

3. Results

Actual exposure concentrations (AEC) to testosterone and EDs are given in table 7.2. For the control, testosterone was detected in only one out of the six replicates, so that the resulting AEC was estimated at 0.02 ng/L. On average, AECs were 2.2-fold lower than the nominal concentrations. Thereafter, AECs instead of nominal concentrations are used when referring to the exposure concentrations to testosterone and EDs.

Mortality and growth were not affected by the exposure as compared to the controls (data not shown). The highest mortality (10%) was observed in the solvent-treated group. However, no significant differences were found between water controls and solvent controls for survival and growth ($p > 0.05$; Mann-Whitney test). Similarly, for the reproductive endpoints, the cumulative numbers of egg-clutches and eggs laid by individual over the exposure period were not different between the two control conditions ($p > 0.05$; Mann-Whitney test).

Chemicals	Nominal concentrations	Average exposure concentrations (AEC)
Testosterone	2 ng/L	0.3 ng/L
	10 ng/L	1.4 ng/L
	50 ng/L	6.8 ng/L
Tributyltin (TBT)	45 ng Sn/L	19.2 ng Sn/L
	220 ng Sn/L	94.2 ng Sn/L
Cyproterone acetate (CPA)	2 µg/L	1.1 µg/L
	50 µg/L	28.7 µg/L
Chlordecone (CLD)	4,5 µg/L	2.1 µg/L
	50 µg/L	19.6 µg/L

Table 7.2: Nominal and time-weighted average exposure concentrations (AEC) of testosterone and endocrine disruptors in water.

Mean (± SEM) endogenous testosterone levels in control snails were 425 ± 45 pg/g w.w. for total testosterone, 200 ± 25 pg/g w.w. for the free form, and 238 ± 57 pg/g w.w. for esterified testosterone. Exposure of *L. stagnalis* to 1.4 and 6.8 ng/L of testosterone led to a significant dose- dependent increase of total and esterified testosterone concentrations ($p < 0.001$) whereas the levels of free testosterone were not different from the controls (Fig. 7.1).

Figure 7.2 shows the levels of total, free and esterified testosterone concentrations in *L. stagnalis* exposed to EDs. Snails exposed to 28.7 µg/L CPA showed significant increases of total and free testosterone concentrations ($p < 0.01$). The level of testosterone fatty esters in those snails was also increased, but not significantly as compared to the controls. In contrast, exposure to 1.1 µg/L CPA had no impact on testosterone levels. None of the tested concentrations of TBT (19.2 and 94.2 ng Sn/L) had an impact on endogenous testosterone levels in *L. stagnalis*. Finally, CLD induced no significant effect on testosterone concentrations in *L. stagnalis*, although the exposure to 19.6 µg/L resulted in fatty acid ester levels five times lower than the concentrations measured in control snails (46 ± 45 pg/g w.w.).

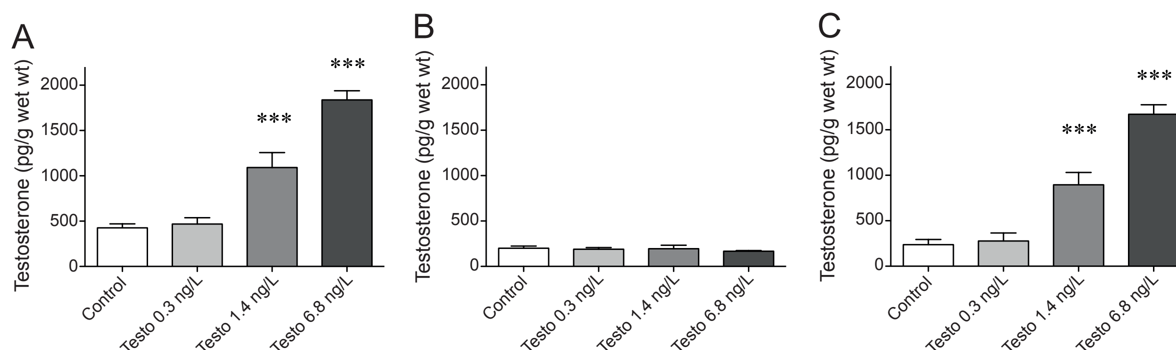

Fig. 7.1: Internal testosterone concentrations (mean ± SEM, pg/g tissue wet weight) in *Lymnaea stagnalis* exposed to testosterone (T) for 21 days: (A) Total testosterone, (B) Free testosterone and (C) Esterified testosterone. One-Way ANOVA Dunnett's post-hoc test statistical significance (n = 4); ∗: p<0.05; ∗∗: p<0.01; ∗∗∗: p<0.001.

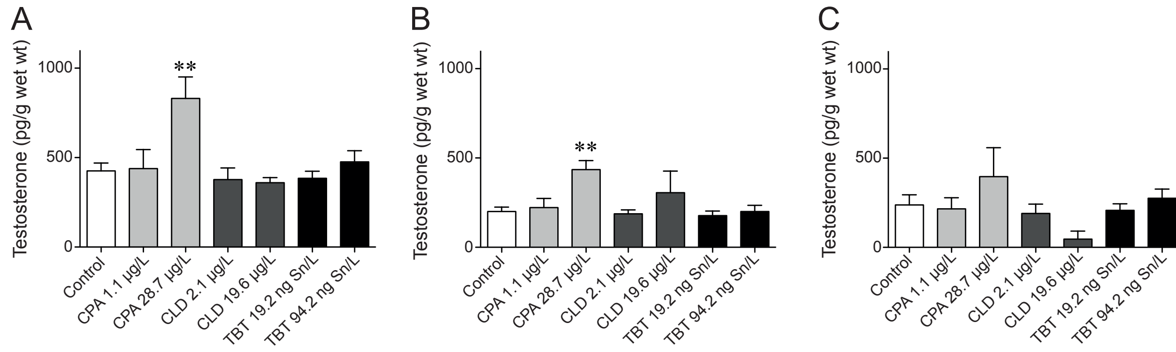

Fig. 7.2: Internal testosterone concentrations (mean ± SEM, pg/g tissue wet weight) in *Lymnaea stagnalis* exposed to cyproterone acetate (CPA), chlordecone (CLD) and tributyltin (TBT) for 21 days: (A) Total testosterone, (B) Free testosterone and (C) Esterified testosterone. One-Way ANOVA Dunnett's post-hoc test statistical significance (n = 4); ∗: p<0.05; ∗∗: p<0.01; ∗∗∗: p<0.001).

Reproduction of *L. stagnalis* was not affected by the exposure to testosterone and CPA (Table 7.3). In contrast, CLD and TBT significantly reduced the production of both egg-clutches and eggs by the exposed snails (Table 7.3). As compared to the controls, snails exposed to 19.6 µg/L CLD and 94.2 ng Sn/L TBT showed significantly reduced oviposition and fecundity.

Exposure condition		Number of egg-clutches per individual	Number of eggs per individual
Control	Water	3,87 ± 0,46	196,9 ± 26,4
	Acetone	3,29 ± 0,55	172,5 ± 31,1
Testosterone	0.3 ng/L	3,24 ± 0,50	153,2 ± 26,7
	1.4 ng/L	3,38 ± 0,31	159,3 ± 16,6
	6.8 ng/L	3,67 ± 0,56	170,0 ± 23,4
Cyproterone acetate (CPA)	1.1 µg/L	3,27 ± 0,40	153,8 ± 21,6
	28.7 µg/L	3,25 ± 0,40	159,3 ± 19,9
Chlordecone (CLD)	2.1 µg/L	2,93 ± 0,39	151,4 ± 23,3
	19.6 µg/L	2,56 ± 0,28 *	55,8 ± 11,6 *
Tributyltin (TBT)	19.2 ng Sn/L	2,93 ± 0,29	133,5 ± 12,8
	94.2 ng Sn/L	2,31 ± 0,18 *	91,5 ± 11,1 *

Table 7.3: Mean (± SEM) cumulated numbers of egg-clutches and eggs per individual over the 21-d exposure period of *Lymnaea stagnalis* to testosterone and endocrine disruptors. (∗): Statistically different from acetone control, ANOVA Dunnett's *post hoc* (p<0,05).

4. Discussion

Endogenous testosterone levels (total, free and esterified) in control *L. stagnalis* are comparable to levels reported in some molluscs species, e.g., the hermaphroditic snail *Valvata piscinalis*, the gonochoric gastropods *Nassarius reticulatus* and *Hinia reticulata*, or the bivalves *Ruditapes decussatus* and *Mytilus Galloprovincialis* (Abidli et al. 2012; Barroso et al. 2005; Bettin et al. 1996; Fernandes et al. 2010; Gauthier-Clerc et al. 2006; Gust et al. 2009; Janer et al. 2006; Ketata et al. 2007; Morcillo and Porte 1999; Morcillo et al. 1998). It has to be noted that, as for other mollusc species investigated for steroid levels, testosterone in *L. stagnalis* was found predominantly as fatty esters (Abidli et al. 2012; Gooding and LeBlanc 2004; Janer et al. 2006; Lavado et al. 2006; Lazzara et al. 2012; Riva et al. 2010).

Exposure of *L. stagnalis* to testosterone through water had no effect on oviposition or fecundity, as determined from the number of clutches and eggs produced over 21 days. This was confirmed in an independent experiment where no significant effects of testosterone could be highlighted after 56 days of exposure in the range 0.1 to 10000 ng/L (unpublished data). In the present study, exposure to testosterone led to a significant dose-dependent increase of total testosterone concentrations in snail tissues. This is in agreement with observations reported by other authors on gastropods and bivalves, and supports Scott's assumption regarding the ability of molluscs to pick up testosterone from water exposure (Scott 2012). The internal level of esterified testosterone increased with the exposure concentration whereas the internal concentration of free testosterone remained at the control level. This shows that *L. stagnalis* is able to convert testosterone into its esterified form, thus regulating the levels of the free form of this sex steroid. Since first demonstrated in *Ilyanassa obsoleta* (Gooding and LeBlanc 2004; Gooding and LeBlanc 2001), this esterification has been shown in a number of gonochoric gastropod species (Fernandes et al. 2010; Gust et al. 2010; Sternberg et al. 2010). The present study provides strong evidence that testosterone esterification also acts in the hermaphroditic *L. stagnalis* as a homeostatic regulation that enables the snails to maintain endogenous levels of the free form of the hormone.

CPA was purposely designed for antagonistic interaction with the human androgen receptor. It has progestagenic and anti-gonadotropic properties in vertebrates (Raudrant and Rabe 2003). The effects of CPA on testosterone metabolism have never been studied in hermaphrodite molluscs. We observed that in the concentration range of 1.1 to 28.7 µg/L, CPA has no effect on the reproduction (expressed as number of egg-clutches and eggs produced per individual; Table 7.3) of *L. stagnalis*. This result was confirmed in an additional experiment where snails were exposed to CPA in the range 10 to 1000 µg/L during 21 days (Giusti et al. 2010; Part 2: chapter 1). Other studies showed that water exposure to a mixture of this anti-androgen and 17α-ethinyloestradiol has been shown to stimulate egg production (Weltje et al. 2003). Exposure of the gonochoric prosobranch species *Nucella lapillus* to 1.25 mg/L CPA increased free estradiol levels in females (Santos et al. 2005). Our data show a significant increase of total internal testosterone concentrations in *L. stagnalis* exposed to 28.7 µg/L CPA. This results from an increase in both free and esterified forms of the steroid, though the concentration in fatty acid esters in the exposed snails was not significantly different from that of the controls (Fig. 7.2). These observations indicate that *L. stagnalis* can synthesise testosterone. Even if accidental presence of testosterone in the exposure medium cannot be ruled out (Scott 2012), its concentration is expected to be the same at all CPA concentrations tested. An increase in internal testosterone levels in the presence of 28.7 µg/L CPA thus indicates that there was a stimulation of testosterone production by *L. stagnalis*.

A different pattern was obtained when *L. stagnalis* was exposed to CLD, a potent agonist of oestrogen receptors in vertebrates (Kuiper et al. 1998). It had no significant impact on testosterone concentrations, although the fatty acid ester concentration was reduced, though not significantly, at 19.6 µg/L CLD (Fig. 6.2). Yet, CLD exposure (concentration range: 2.1 – 49.1 µg/L) resulted in a dose-dependent reduction of egg-clutch and egg production in *L. stagnalis* (Table 7.3). It has to be noted that mortality (30%) only occurred when snails were exposed to 49.1 µg/L CLD whereas solvent controls showed 90% survival. Therefore, at 19.6 µg/L, reproduction impairment is unlikely to be due to the acute toxicity of CLD. CLD also acts as an aromatase inhibitor (Benachour et al. 2007; Hammond et al. 1979). Cytochrome-P450 aromatase enzymes are responsible for the conversion of androgens to oestrogens (Simpson et al. 1994). Inhibition of the aromatase activity could thus reduce the conversion of testosterone to

estradiol, resulting in an increase in testosterone concentrations. However, in our study, concentration of total testosterone was not modified by CLD and the decrease in the internal level of esterified testosterone in CLD-exposed snails was not statistically significant. In such conditions, the link between the impairment of reproduction and steroid metabolism in *L. stagnalis* exposed to CLD is not straightforward.

Interestingly, TBT had no effect on the levels of testosterone, whatever its form, in *L. stagnalis* whereas it significantly reduced snail oviposition and fecundity at concentrations of 94.2 ng Sn/L up to 473 ng Sn/L (Giusti et al. 2013a; Part 2: chapter 2). It is now well established that exposure of gonochoric gastropods to TBT not only induces imposex in females but can also significantly increase testosterone levels in these organisms, although the mechanistic link between the two phenomena has not been established (Sternberg et al. 2010). In the gonochoric species studied so far, TBT elevates testosterone tissue concentrations through several enzymatic pathways, which have been extensively investigated (Sternberg et al. 2010). Generally, TBT increases free testosterone concentrations and/or decreases the esterified testosterone levels in females of most of the studied species but, in some species, the fatty acid ester concentrations can be increased (Abidli et al. 2012; Gooding and LeBlanc 2001; Gooding et al. 2003; Janer et al. 2006; Santos et al. 2005). In males, TBT had no effect on the concentration of any form of testosterone, as shown in *Hexaplex trunculus* and *Bolinus brandaris* (Abidli et al. 2012). The absence of change of the testosterone tissue concentration found in this study for *L. stagnalis* exposed to TBT could therefore be species-specific, or linked to its mode of reproduction. Exposure duration may also have influenced snail responses as it has been shown that the increase of total testosterone concentrations in *N. lapillus* and *Hinia reticulata* was not only species- dependent but also linked to the duration of TBT exposure (Bettin et al. 1996).

This study provides evidence of testosterone uptake from the environment by *L. stagnalis* as a dose-dependent increase of total testosterone concentrations was observed when the snails were exposed to waterborne steroid. Other exogenous sources of testosterone were quite limited since the lettuce provided to the snails was rinsed under flowing charcoal-filtered tap water, and the snails, food and experimental devices were handled with gloves. The exposure of *L. stagnalis* to CPA shows, for the first time, that a synthetic steroid compound known for its anti-androgenic properties in vertebrates is able to increase the internal steroid levels in a hermaphroditic gastropod.

The endogenous origin of testosterone in *L. stagnalis* exposed to 28.7 µg/L CPA is highly probable considering the limited exogenous sources of testosterone. Moreover, the exposure of *L. stagnalis* to 19.6 µg/L CLD, which acts as both an oestrogen agonist and an aromatase inhibitor in vertebrates, tended to decrease the level of esterified testosterone. Overall, this study demonstrates that chemicals, which exhibit agonist and antagonist interactions with steroid receptors in vertebrates, can alter endogenous homeostasis of testosterone in the hermaphoroditic gastropod *L. stagnalis*. Furthermore, androgenic, anti-androgenic and oestrogenic molecules act on testosterone metabolism in different ways (e.g., increase of total/free testosterone by CPA, decrease of esterified testosterone by CLD, and unchanged testosterone titre in TBT-exposed snails) with variable linked alterations of snail reproduction (e.g., no effect of CPA on fecundity, concentration-dependent decrease of egg production in CLD- and TBT-exposed snails).

In conclusion, our study clearly shows that chemicals that act as endocrine disruptors in vertebrates can also alter the testosterone levels in hermaphroditic gastropods. The expression of such alterations in terms of internal testosterone levels apparently depends on the mode of reproduction of these molluscs, as TBT does not alter testosterone homeostasis in the hermaphrodite *L. stagnalis* whereas it disrupts steroid metabolism in many gonochoric species. Nevertheless, the mechanistic link between alteration of steroid tissue concentrations by EDs in *L. stagnalis* and changes in snail reproduction remain to be demonstrated.

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Chapter 6:

Analyses of the alterations of expression of proteins involved in the reproduction of* Lymnaea stagnalis *following exposure to EDCs

Publication 5:

Analysis of differential protein expression in the hermaphroditic gastropod *Lymnaea stagnalis* exposed to different endocrine disrupting chemicals

Prepared for publication submission in Aquatic Toxicology

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Protein analysis

Abstract

Many studies have reported perturbations of mollusc reproduction following exposure to low concentrations (ng/L range) of endocrine disrupting chemicals (EDCs). However, the mechanisms of action of these molecules on molluscs are still poorly understood. Investigation of the modification in protein expression in organisms exposed to chemicals can provide a broader and more comprehensive understanding of adverse impacts of pollution on organisms. In this study we have investigated the impacts of four chemicals that exhibit different endocrine disrupting properties in vertebrates on the proteome of the hermaphroditic freshwater pulmonate gastropod *Lymnaea stagnalis* after 21 days of exposure. Testosterone, tributyltin, chlordecone and cyproterone acetate were chosen as tested compounds as they can induce adverse effects on the reproduction of this snail. The 2D-DIGE method was used to identify proteins, which expression was affected by these compounds. In addition to modifying the expression of proteins involved in the structure and function of the cytoskeleton, chemicals had impacts on the expression of proteins involved in the reproduction of *L. stagnalis*. Exposure to 19.2 μg/L of chlordecone increased the abundance of ovipostatin, a peptide transmitted during mating through seminal fluid, which reduces oviposition. The expression of yolk ferritin, the vitellogenin equivalent in *L. stagnalis*, was reduced after exposure to 94.2 ng Sn/L of tributyltin. The identification of yolk ferritin and the modification of its expression in snails exposed to chemicals were refined using western blot analysis. Our results showed that the tested compounds influenced the abundance of yolk ferritin in the reproductive organs. Alteration in proteins involved in reproductive pathways (e.g., ovipostatin and yolk ferritin) could constitute relevant evidence of interaction of EDCs with reproductive pathways that are under the control of the endocrine system of *L. stagnalis*.

Keywords: 2D-DIGE; Yolk Ferritin; Ovipostatin; Mollusc; Endocrine Disruption; Oestrogens; Androgen; Tributyltin

1. Introduction

An Endocrine Disrupting Chemical (EDC) is defined as an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations (IPCS 2002). A wide variety of chemicals are known to be endocrine disruptors: natural and synthetic hormones, pesticides, industrial by-products and other chemicals produced in the plastic industry (IPCS 2002). Mechanisms of action of EDCs can be mediated through receptor binding, inhibition of hormone synthesis, metabolism and transport (IPCS 2002; US-EPA 1997). Impacts of EDCs on wildlife are numerous and differ highly between taxa, species and even between reproductive statuses of organisms.

Many compounds are able to induce adverse impacts in mollusc species in the environment, e.g., superfeminisation and imposex in gastropod species (Oehlmann et al. 2006; Oehlmann et al. 2000), intersex in bivalves (Gomes et al. 2009; Ortiz-Zarragoitia and Cajaraville 2010). Furthermore, laboratory and field studies have shown that mollusc reproduction is altered by exposure to low concentrations (ng/L range) of these molecules, indicating that mollusc species are particularly sensitive to EDCs. Therefore, some mollusc species are already used as bio-indicators in the field (e.g. *Nucella* lapillus, *Littorina littorea*) (Bauer et al. 1997; Oehlmann et al. 1998) or as test species in laboratory (e.g., *Potamopyrgus antipodarum*, *Marisa cornuarietis*) (Duft et al. 2003b; Tillmann et al. 2001). The hermaphroditic gastropod *Lymnaea stagnalis* has been shown to be responsive to EDCs such as organotin compounds (i.e., TBT and TPT), some pesticides and industrial products (e.g., vinclozolin, nonylphenol) (Czech et al. 2001; Ducrot et al. 2010; Giusti et al. 2013a; Part 2: chapter 2; Leung et al. 2007). Even though the knowledge on mollusc endocrinology is rather limited, the neurohormonal control of reproduction is reasonably well understood in *L. stagnalis* (Geraerts 1976; Koene 2010; Rittschof and McClellan-Green 2005). Moreover, genomic resources have recently been improved by pyrosequencing of cDNAs from *L. stagnalis*, expending the databases for protein identification in genomic and proteomic approaches for this species (Bouétard et al. 2012). Finally, this species has been proposed as a candidate species for the development of guidelines for the testing and the assessment of endocrine disruptors in molluscs (OECD 2010).

Protein analysis

Proteomic analyses in ecotoxicology aim to obtain a comprehensive and quantitative description of protein expression and modification (e.g., abundance, activity, structure, post-translational modification) following exposure to environmental stress conditions (Cao et al. 2009). Temperature fluctuation, parasitism, and exposure to environmental pollutions lead to alterations of the protein expression in stressed animals (Sanchez et al. 2011; Wright et al. 2012). Investigation of the modification of protein expression in organisms exposed to chemicals can provide a broader and more comprehensive understanding of adverse impacts of pollution on the organisms. As proteomic is a large-scale, hypotheses free approach, knowledge on the exact mechanisms of action of the chemicals is not required (Apraiz et al. 2006; Rodríguez-Ortega et al. 2003). Therefore analysis of the impacts of one or more environmental contaminants on the proteome of a species can provide insights on the mechanism of action of molecules and pinpoint to a number of potential biomarkers of exposure to specific pollutions (Ankley et al. 2009; Dowling and Sheehan 2006; Sanchez et al. 2011). Even though few studies have investigated the impacts of chemicals on mollusc using proteomic methods, specific biomarkers and protein expression signatures were observed following exposure to particular toxicants (Apraiz et al. 2006; Campos et al. 2012; Shepard et al. 2000; Thompson et al. 2012a). However, the majority of these studies were conducted on bivalve species and the impacts of endocrine disrupting chemicals on protein expression have rarely been reported in molluscs (Zhou et al. 2010a).

In this study we have investigated the impacts of four chemicals (i.e., testosterone, cyproterone acetate, tributyltin, and chlordecone), which exhibit different endocrine disrupting properties in vertebrates, on the proteome of *Lymnaea stagnalis* after 21 days of exposure. The analysis of the impacts of chemicals on the protein expression in the reproductive organs of exposed subjects was performed using the two-dimensional differential fluorescence in gel electrophoresis method (2D-DIGE). Beside structural proteins such as actin and tubulin, chemicals affected the expression of proteins involved in different pathways of mollusc reproduction such as yolk ferritin, the main egg yolk protein, and ovipostatin, a peptide transmitted between mates through seminal fluid. Western Blot analysis was used to further confirm the modification of expression of yolk ferritin induced by the molecule tested. The impacts of chemicals on proteins involved in the reproduction of *L. stagnalis*, particularly on yolk ferritin, are discussed.

2. Material and Methods

2.1. Animals and exposure experiment

L. stagnalis (Linnaeus, 1758) (Mollusca, Gastropoda, Pulmonata, Heterobranchia), RENILYS® strain, has been reared at the INRA Experimental Unit of Aquatic Ecology and Ecotoxicology (Rennes, France) under laboratory conditions as previously described (Coutellec and Lagadic 2006). Young adults of homogenous size (22.5 ± 2.5 mm) and age (4 ± 0.5 months) were sampled from the culture and acclimatised to test conditions for 48 hours prior to chemical exposure.

Analytical standards of testosterone (≥98% purity, CAS Nr 58-22-0), cyproterone acetate (≥98% purity, CAS Nr 427-51-0), tributyltin hydride (≥97% purity, CAS Number 688-73-3) and chlordecone (Pestanal® grade, CAS Nr 143-50-0) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Exposure media consisted in dechlorinated, charcoal-filtered tap water (i.e., similar to culture medium; pH = 7.7 ± 0.2, conductivity = 623 ± 60 µS/cm, dissolved oxygen = 7.3 ± 2 mg/L, water hardness = 254 ± 7 mg $CaCo_3$/L) contaminated with stock solutions prepared in analytical grade acetone (99.9 % purity). Concentrations of stock solutions were chosen to ensure addition of the same amount of solvent among treatments (i.e., 100 µL/L, except for the water control) as recommended by (OECD 2000). Six replicates (each with five snails in 1 L glass beaker) of each chemical concentration, water control and solvent control were randomly distributed in the exposure room. Tests were conducted at constant temperature (20.5 ± 0.6°C) and photoperiod (14:10 Light:Dark) as previously described (Ducrot et al. 2010). Snails were fed every other day with 2.5g of rinsed organic lettuce per exposure beaker. Exposure media, as well as controls and solvent controls, were renewed every 2 days to ensure maintaining exposure concentration and physico-chemical properties of test water during the experiment duration. After 21 days of exposure, snails were frozen at -80°c until proteomic analysis.

2.2. Chemical analysis

Exposure water was sampled at the beginning, mid-term and end of the experiment. Three replicates (200mL) were taken per concentration. Each replicate consists in a

pool of 100 mL of water sampled from two beakers. Samples were taken 15 minutes and 48 hours after water renewal and stored in glass flasks at -20°C until analysis. Testosterone and cyproterone acetate were extracted using C-18 solid phase extraction (SPE) and determined by high-resolution liquid chromatography – mass spectrometry (HPLC-MS-MS). Chlordecone was extracted by manually handshaking 5 mL of water with a volume of 5 mL of dichloromethane. Extraction of tributyltin was performed as described in the ISO method for organotin analysis in water (ISO 2004). Analysis and quantification of chlordecone and tributyltin concentrations were performed by capillary gas chromatography - mass spectrometry (GC-MS-MS). Actual exposure concentrations were calculated as the time-weighted average of measured concentrations (AEC) over the test period (Belgers et al. 2011) and were further used instead of nominal concentrations (Table 8.1).

Chemicals	Nominal concentration	Average exposure concentration (AEC)
Testosterone (T)	2 ng/L	0.3 ng/L
	10 ng/L	1.4 ng/L
	50 ng/L	6.8 ng/L
Cyproterone acetate (CPA)	2 µg/L	1.1 µg/L
	50 µg/L	28.7 µg/L
Chlordecone (CLD)	4,5 µg/L	2.1 µg/L
	50 µg/L	19.6 µg/L
Tributyltin (TBT)	45 ng Sn/L	19.2 ng Sn/L
	220 ng Sn/L	94.2 ng Sn/L

Table 8.1: Nominal and time-weighted average exposure concentrations (AEC) of chemicals in water.

2.3. 2D-DIGE (Two-Dimensional Differential In-Gel Electrophoresis)

2.3.1. Protein extraction

After 21 days of exposure, four of the six replicates were randomly chosen per experimental condition and one snail was randomly sampled in each of these four replicates and frozen at -80°c until protein extraction. Reproductive tissues were isolated by dissection of frozen individuals and weighted. Protein extraction was

performed as described in previous studies conducted on arthropods and bivalves (Francis et al. 2010; Leroy et al. 2010; Leung et al. 2011). Samples were crushed in a volume of lysis buffer ASB 14 (7 M urea, 2 M thiourea, 30 mM Tris pH 8.5 buffer including 2% ASB14) equivalent to ten times the fresh weight of the sample. A hundred microlitres of homogenate were taken and submitted to subsequent extraction steps. Samples were sonicated for 10 min, incubated 15 min with 1 µL benzonase nuclease (≥250 units/µL, Sigma-Aldrich, Schnelldorf, Germany) and centrifuged at 20000 g at 4°c for 30 min. Supernatants were collected and proteins were precipitated using a 2D Clean-Up Kit according to the manufacturer's instructions (GE Healthcare, Diegem, Belgium). Protein pellets were suspended in 100 µL of lysis buffer and pH was adjusted to 8.5 with NaOH. Protein quantification was carried out using the RC DC Protein assay kit (Bio-Rad, Nazareth, Belgium). Samples were stored at -80°C until subsequent steps.

2.3.2. CyDye labelling

Two-dimensional Differential In-Gel separation method uses fluorescent cyanine dyes, i.e., CyDyes, as protein staining prior to protein separation. Twenty-five micrograms of proteins were labelled with 200 pmol of either Cy3 or Cy5 (GE Healthcare, Diegem, Belgium); per experimental condition, two replicates were labelled with Cy3 and two replicates with Cy5. Internal standard was obtained by pooling equal amounts of proteins (25 µg) of each biological sample and labelled with Cy2 (GE Healthcare, Diegem, Belgium). Following 30 min of incubation in darkness, the labelled samples were quenched with the addition of 0.2 µL of 10 mM Lysine (Sigma-Aldrich, Schnelldorf, Germany) and submitted to another 10 min incubation in the obscurity.

2.3.3. Protein separation

Pairs of randomly chosen Cy3 and Cy5 samples were mixed and pooled with 25 µg of Cy2-labelled internal standard. After the addition of 1% DTT (1M) and 2% of IPG Buffer 3-11 NL (GE Healthcare, Diegem, Belgium), the volume was adjusted to 450 µL with rehydration buffer 3-11 NL ASB14 (7 M urea, 2 M thiourea, 2% w/v ASB 14, 25 mM DTT, and 0.6% v/v pH 3–11 NL IPG buffer). Protein focussing was performed at 20 °C on a 24 cm strip (pH 3-11 NL, GE Healthcare, Diegem, Belgium) for 23 h 45 min using an

Ettan IPGphor II isoelectric focussing System (GE Healthcare, Diegem, Belgium) at 500 V for 1 h, followed by a gradient from 500 to 1000 V in 3 h. During the next 3 hours the voltage increased from 1000 V to 8000 V and the voltage was held at 8000 V to achieve 85000Vh.

Focussed strips were equilibrated for 15 min in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol (v/v), 1.6% SDS (w/v)) with 1% DTT and a second equilibration step was performed for another 15 min in equilibration buffer with 5 % of iodoacetamide. For the second dimension of electrophoresis, strips were loaded on a 12.5% (w/v) polyacrylamide gel and migration was carried out at 30°c at 1W/gel and 80V for 1 hour followed by 2W/gel and 50V overnight until completion of migration using an Ettan Dalt-6 system (GE Healthcare, Diegem, Belgium).

2.3.4. Image acquisition and analysis

Gels were scanned using a Typhoon 9400 Laser scanner (GE Healthcare, Piscataway, NJ, United States) with excitation and emission wavelengths specific to each CyDye (Cy2: 488/520; Cy3: 523/580 and Cy5: 633/670 nm). 2D-Gel images were analysed using Decyder 2D Differential analysis software (v.7.0, GE Healthcare, Piscataway, NJ, United States). Differential In-Gel Analysis (DIA) module was used for the automation of spot detection and abundance measurement for each sample. Internal control was used to match the 22 gels acquired and the differentially expressed proteins were detected using Biological Variance Analysis (BVA) module.

2.3.5. Protein identification

Protein spots that showed a significant fold change of at least 1.5 (Student's *t*-test, $p<0.05$) in reproductive organs of *L. stagnalis* exposed to chemicals were submitted to identification. Two preparative gels were loaded with 250 µg of unlabelled proteins from either controls or solvent controls and 25 µg of the internal standard. Gels were run under the same conditions as analytical gels. Spots of interest were excised using an Ettan Spotpicker robot (GE Healthcare, Piscataway, NJ, United States) and transferred to a 96-Wells plate (Eppendorf, Hamburg, Germany). Proteins were digested with 20 ng/L of trypsin (Roche, porcine, proteomic grade) for 4 h at 37°C using a Janus Robot (Perkin

Elmer, Waltham, MA, USA) (Collodoro et al. 2012). Resulting peptides were extracted and rehydrated in 10 µL of formic acid (1%). Automated spectra acquisition was performed using an Ultraflex II MALDI mass Spectrometer (Bruker Daltonics, Billerica, MA) under control of Flex Control v.3.0. and Flex Analysis v.3.0. software (Bruker Daltonics, Billerica, MA) in MS mode for peptide mass fingerprint (PMF, spectra acquisition mass range: 70-4000 *m/z*) followed by the MS-MS mode for peptide sequencing. Peptides identification was managed using Biotools v.3.1. software (Bruker Daltonics, Billerica, MA) with an in-house hosted Mascot v2.2.2. server. Metazoa taxonomy was used for database search with the following parameters: peptide mass tolerance of 100 ppm precision, charge state of 1+ and a maximum number of missed cleavages of 1. Carbamidomethylation of cysteines was used as a fixed modification and oxidation of methionine as variable modification. Identification was significant for peptide mass fingerprint with a $p<0.05$ and a Mascott protein score ≥75.

Further protein identification was performed using genomic resources obtained by pyrosequencing *L. stagnalis* individuals from our laboratory cultures (Bouétard et al. 2012). Open reading frames (ORFs) were searched among all the snail contigs available (Great Pond Snail Contig Browser: http://genotoul-contigbrowser.toulouse.inra.fr:9095/Lymnaea_stagnalis/index.html) with the EMBOSS getorf free open source software (http://emboss.open-bio.org/wiki/Appdocs). Translation of regions of minimum 30 nucleotides was performed between methionine (start codon) and stop codons. ORFs were also searched in the reverse sequence. The ORFs obtained in Pearson FASTA format were included in our Mascott library and protein identifications were performed using the previously described parameters. Significantly identified contigs were searched in NCBI database for sequence homology with a cut-off e-value of 1^{-5} as previously described (Bouétard et al. 2012).

2.4. Western Blot

2.4.1. Antibody design and production

For the antibody production, a peptide was selected in the *L. stagnalis* yolk ferritin protein sequence (Uniprot accession number: P42578) based on the hydrophobicity, antigen index and on secondary structure using dedicated software (Eurogentec, Liege, Belgium) and searched in the NCBI database to avoid cross detection of other proteins. The synthetic peptide (H2N-LRSFEQGSGNNYKLGC-CONH2) was coupled with ovalbumin carrier protein and was injected in rabbit. Total rabbit serum was recovered after 28 days (Eurogentec, Liege, Belgium).

2.4.2. Protein extraction and separation

Four of the six replicates were randomly chosen per experimental condition and one snail was randomly sampled in each of these four replicates and frozen at -80°c until protein extraction. Reproductive tissues were isolated by dissection of frozen individuals and weighted. Proteins were extracted by homogenisation in a lysis buffer (Tris Base 20 mM, 150 mM NaCl, 2 mM EDTA and 0.1% Triton-X with a cocktail of protease inhibitor (Roche, Meylan, France)). Samples were sonicated for 30 min at room temperature and were centrifuged at 20000 g at 4°c for 30 min. Total proteins concentrations were measured using Pierce 660 nm protein assay (Fisher Scientific Inc., Rockford, IL).

A constant amount of protein (25 µg) and 5 µL of a pre-stained molecular weight ladder (PageRuler Plus Prestained Protein Ladder 10-250kDa, Fisher Scientific Inc., Rockford, IL) were loaded on a 12% polyacrylamide gel. Gels were run with running buffer (25 mM Tris pH 8.8, 192 mM glycine, 2% SDS (w/v)) using a Bio-Rad Mini Protean system (Bio-Rad, Hercules, CA, USA) for 1 h at 150V. Proteins were transferred on nitrocellulose membranes (Hybond ECL, Amersham/GE Healthcare, Diegem, Belgium) at 2 mA/cm^2 during 1 h using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA). Total proteins were stained with Ponceau red (0.2%) in 5% acetic acid and membranes were scanned for total proteins using an Image Scanner (Amersham Pharmacia Biotech, Orsay, France). Non-specific binding sites were blocked

with 3% of powdered skim milk in TBS-T (pH 7.6) (20 mM Tris Base, 150 mM NaCl and 0.05% Tween-20). Membranes were incubated overnight at 4°c with anti-yolk ferritin antibody (Eurogentec, Liege, Belgium) (dilution of 1/10 in 3% powdered skim milk in TBS-T). After 3 washings in TBS-T, nitrocellulose membranes were incubated in darkness for 1 h at room temperature with anti-rabbit antibody conjugated with Alexa Fluor Cy2. After 3 washings in TBS-T followed by 1 washing in TBS (20bmM Tris and 150 mM NaCl), membranes were dried at 37°c for 1 h and were scanned using a Typhoon 9400 Laser scanner (GE Healthcare, Piscataway, NJ, United States) with excitation and emission wavelength specific to the CyDye (488/520 nm). Analyses were performed in triplicates for each biological sample.

2.4.3. Protein quantification and analysis

For yolk ferritin quantification, membranes were scanned as TIFF files (8 Bit, 300 dpi). Ponceau S reversible protein staining, usually applied as quality control of membrane transfer, was used to quantify total amount of protein loaded per condition by scanning membranes prior to antibody incubation. In comparison to the quantification using the “housekeeping” proteins method, which generally uses ß-actin and/or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amongst other proteins whose level is thought to be stable between conditions, Ponceau staining normalisation avoid the possibility that these housekeeping proteins abundance would also be altered by the experimental conditions (Aldridge et al. 2008; Romero-Calvo et al. 2010).

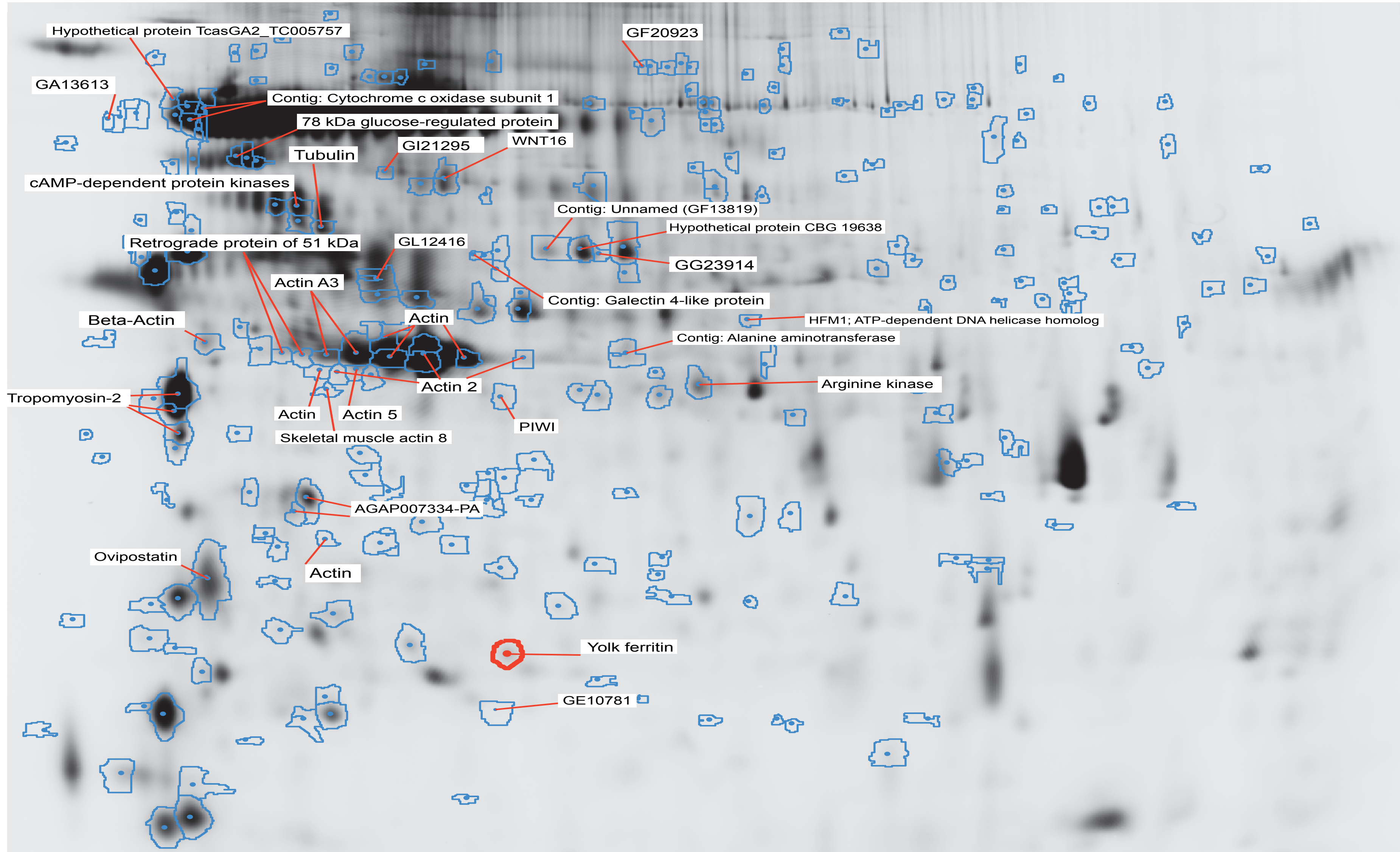
Total proteins and yolk ferritin bands were quantified using the 1D gel analysis of ImageQuantTL software (GE Healthcare, Piscataway, NJ, United States). The inter-gel normalisation of the results was performed using a positive control (i.e., proteins extracted from eggs sampled in our laboratory breeding stock) as 100% reference. Therefore the results of yolk ferritin expression in samples were expressed as percentage of the yolk ferritin expression quantified in the positive control ran in the same gel.

3. Results

3.1. 2D-DIGE

In this study we have investigated the impact of four chemicals, which have different endocrine disrupting properties in vertebrates, on the expression of proteins in the reproductive organs of the hermaphrodite gastropod *Lymnaea stagnalis*. From the master gel image it was possible to detect more than 4000 spots containing protein species expressed in the reproductive organs of *L. stagnalis* over the selected pH range (pH 3-11) (Fig. 8.1). All tested chemicals had a significant impact on the expression of several proteins extracted from the reproductive organs. According to the statistical threshold ($p<0.05$, Student's *t*-test) and the 1.5 fold change criterion, a total of 117 and 141 proteins were detected as differently expressed in treated samples compared to controls and to solvent controls, respectively (Fig. 8.2). Solvent alone had an impact on the expression of 18 proteins compared to controls, amongst which 9 proteins were not altered following exposure to the chemicals tested. Proteins significantly altered in the solvent controls were mainly upregulated (12) compared to the controls whereas after chemical exposure, the majority of proteins were downregulated when compared to controls (Table 8.2 and 8.3).

Fig. 8.1: Gel master and location of the protein spots picked (polygons are protein spots with at least 1.5-fold expression change, $p<0.05$, student *t*-test) and proteins significantly identified with the NCBI Metazoa database and with the *Lymnaea stagnalis* contig data base.



Proteins responsive to at least 2 of the tested chemicals were always altered in a same way (i.e., either up- or downregulation). The abundance of 58 protein spots was significantly altered following exposure to testosterone compared to controls whereas tributyltin, chlordecone and cyproterone acetate had an impact on 37, 35 and 20 protein spots, respectively (Fig. 8.2 A). Compared to the protein expression in solvent controls, chlordecone, tributyltin and cyproterone acetate had an impact on a greater number of protein spot (79, 44 and 40 spots, respectively), while exposure to testosterone altered 49 spots (Fig. 8.2 B). Interestingly, we observed that most of the proteins altered following exposure to EDCs were specifically disrupted by a single compound as 78 and 65% of spots were only disrupted by one chemical compared to controls and to solvent controls, respectively (Fig. 8.2). Only 1 and 5 proteins were simultaneously altered by all four tested chemicals, compared to the controls or the solvent controls, respectively (Supplementary material provided in the CD-Rom).

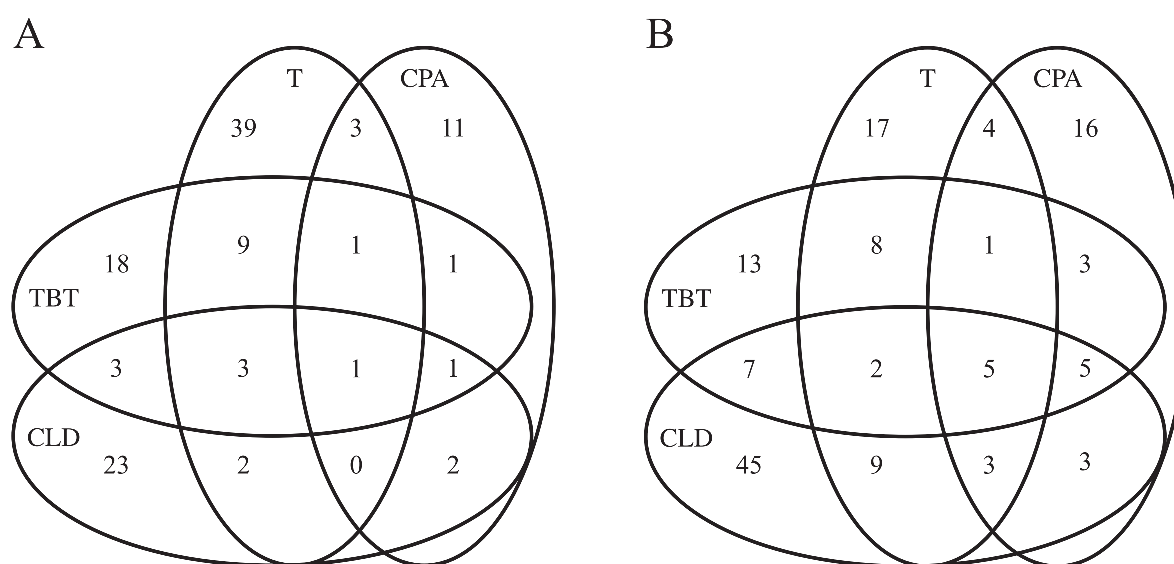


Fig. 8.2: Venn diagrams of proteins with at least 1.5-fold expression change ($p<0.05$, student *t*-test) in reproductive organs of *Lymnaea stagnalis* exposed to testosterone (T), cyproterone acetate (CPA), tributyltin and chlordecone during 21 days compared to (**A**) controls and (**B**) solvent controls.

Treatment		Number of proteins spots		
Chemicals	Concentration	≥ 1,5 Fold Change	≤ 1,5 Fold Change	Total of altered proteins
Solvent Control	-	12	6	18
Testosterone	0,3 ng/L	8	11	19
	1,4 ng/L	6	17	23
	6,8 ng/L	7	17	24
Cyproterone acetate	1,1 µg/L	2	6	8
	28,8 µg/L	4	8	12
Tributyltin	19,2 ng Sn/L	9	10	19
	94,2 ng Sn/L	7	18	25
Chlordecone	2 µg/L	6	12	18
	19,6 µg/L	6	14	20

Table 8.2: Number of protein spots with at least 1.5-fold expression change ($p<0.05$, student *t*-test) in reproductive organs of *Lymnaea stagnalis* after 21 days of exposure to chemicals compared to controls.

Treatment		Number of proteins spots		
Chemicals	Concentration	≥ 1,5 Fold Change	≤ 1,5 Fold Change	Total of altered proteins
Control	-	5	13	18
Testosterone	0,3 ng/L	2	9	11
	1,4 ng/L	7	13	20
	6,8 ng/L	3	21	24
Cyproterone acetate	1,1 µg/L	4	20	24
	28,8 µg/L	5	15	20
Tributyltin	19,2 ng Sn/L	1	16	17
	94,2 ng Sn/L	8	27	35
Chlordecone	2 µg/L	8	36	44
	19,6 µg/L	12	35	47

Table 8.3: Number of protein spots with at least 1.5-fold expression change ($p<0.05$, student *t*-test) in reproductive organs of *Lymnaea stagnalis* after 21 days of exposure to chemicals compared to solvent controls.

3.2. Protein identification

Among the 144 protein spots excised, the Mascott search within the metazoan database provided 57 significant identifications, among which MS-MS significantly identified only 13 proteins. Forty proteins were identified as homologous to invertebrate proteins and 57 % of these proteins were significantly identified in mollusc species (Table 8.4). The DNA contigs obtained after pyrosequencing of the genome of *L. stagnalis* were used to generate translated open reading frames (ORFs), which were

added to the Mascott library, in order to improve the protein identifications. However, only 20 ORFs showed a significant matching score and only 7 proteins were newly identified. Half of these ORFs were identified by MS-MS analyses (Supplementary material provided in the CD-Rom).

Most of the spots identified were structural proteins mostly involved in the structure and function of the cytoskeleton (e.g., actin and tropomyosin) (Table 8.4). Expression of these proteins in the reproductive organs of *L. stagnalis* was downregulated by at least one of the tested chemical. Only one protein spot (i.e., spot n° 1411), corresponding to a retrograde protein of 51 kDa involved in intermediate filament constitution in *L. stagnalis,* was upregulated in individuals exposed to the highest concentration of chlordecone (19.6 μg/L) compared to solvent controls. At a lower chlordecone concentration (2.1 μg/L), 2 spots (i.e., spots n° 2141 and 2154) were downregulated and identified as retrograde protein. Among the structural protein identified as being altered following exposure to chemicals, 2 spot (i.e., spots n° 2129 and 2154) were downregulated following exposure to at least one concentration of each chemical tested (Supplementary material). A second major group of proteins, involved in transcription and in post-transcriptional modifications of proteins, was downregulated after exposure to the chemicals. The molecules also affected proteins involved in signal transduction, cell division, energy storage, detoxification and oxidative phosphorylation (Table 8.4).

Finally, proteins involved in reproduction showed significantly altered expressions in *L. stagnalis* exposed to the endocrine disrupting chemicals tested. In reproductive organs of individuals exposed to 1.1 μg/L of cyproterone acetate, to 94.6 ng Sn/L of TBT and to 19.6 μg/L of chlordecone, the expression of PIWI, a key protein in germline stem cell differentiation (Cox et al. 2000), was significantly increased when compared to solvent controls (Fig. 8.3 A). An increase in the expression of another protein involved in *L. stagnalis* reproduction, ovipostatin (produced in the prostate gland (Koene et al. 2010)), was observed in individuals exposed to 19.6 μg/L of chlordecone compared to controls (Fig. 8.3 B). However, this effect was not significant when compared to solvent controls. Lastly, yolk ferritin was downregulated compared to both controls following exposure to 94.6 ng Sn/L of TBT (Fig. 8.3 C). In *L. stagnalis*, yolk ferritin is the major egg yolk protein (Bottke et al. 1988), suggesting that a reduction of expression of this protein could be responsible for a decrease in egg production or of the egg quality (i.e.,

reduction of energetic reserves for embryonic development). Therefore, to confirm the results obtained by 2D-DIGE, further analysis of the yolk ferritin expression was conducted using western blot, which is a more specific method for protein expression analysis.

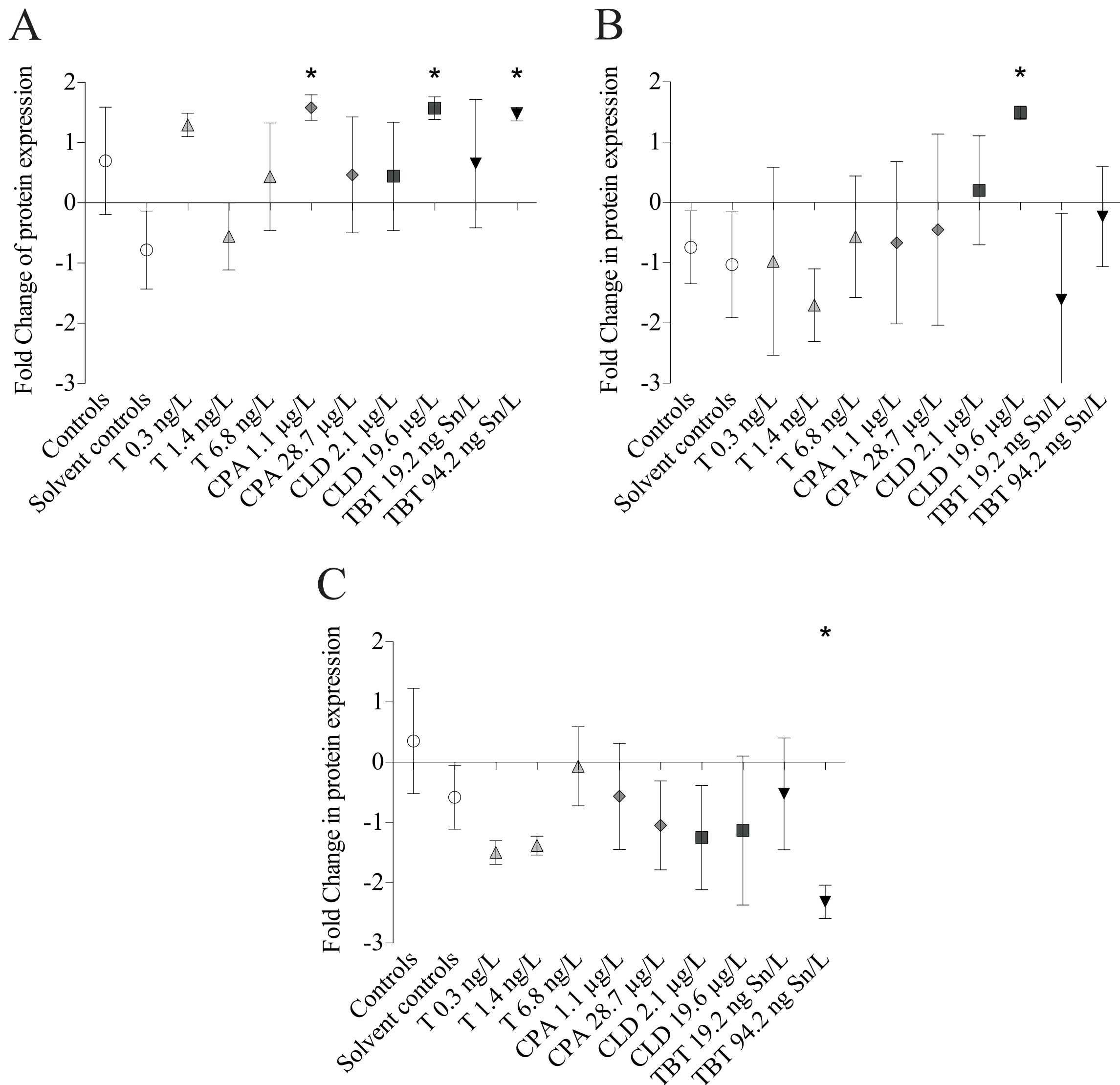


Fig. 8.3: Fold change of protein expression (mean ± SEM) in the reproductive organs of *L. stagnalis* after 21 days of exposure to testosterone (T), cyproterone acetate (CPA), chlordecone (CLD) and tributyltin (TBT) in the 2D-DIGE analysis. (A) PIWI; (B) ovipostatin; (C) yolk ferritin. Student's *t*-test statistical significance (n = 4) (*: $p<0.05$; (B) vs. Controls, (A-C) vs. Solvent controls).

Spot number	MW	p*I*	Score Mascott	MS Coverage	p-value	Protein identification	Accession number	Organism	Taxonomy (Phylum, Class)	Functions	Identification
1411	51678	5.2	95	31	6.1^{-4}	Retrograde protein of 51 kDa	gi|74912853	*Lymnaea stagnalis*	Mollusca, Gastropoda	Cytoskeleton and muscle movements	PMF
1474	49148	4.9	103	43	9.6^{e-5}	Tubulin	gi|156383445	*Nematostella vectensis*	Cnidaria, Anthozoa		PMF
1736	136747	7.1	90	22	0.002	GL12416	gi|195157178	*Drosophila persimilis*	Arthropoda, Insecta		PMF
1991	42081	5.3	91	41	<0.05	Actin	gi|113290	*Aplysia californica*	Mollusca, Gastropoda		MS-MS
	8125	9.9	34	18	<0.05	Contig: Actin	GW7IPVU02F2ZX3	*Crassostrea gigas*	Mollusca, Bivalvia		MS-MS
2067	42036	5.3	141	64	1.5^{e-8}	Beta-actin	gi|110617755	*Mizuhopecten yessoensis*	Mollusca, Bivalvia		PMF
2114	42252	5.3	132	7	<0.05	Actin A3	gi|1881572	*Biomphalaria glabrata*	Mollusca, Gastropoda		MS-MS
	16066	6.6	100	20	<0.05	Contig: Actin	GW7IPVU02F1G9T	*Placopecten magellanicus*	Mollusca, Bivalvia		MS-MS
2123	42164	5.4	245	14	<0.05	Actin A3	gi|483321	*Aplysia californica*	Mollusca, Gastropoda		MS-MS
	16066	6.6	164	28	<0.05	Contig: Actin	GW7IPVU02F1G9T	*Placopecten magellanicus*	Mollusca, Bivalvia		MS-MS
2106	42002	5.3	154	52	7.7^{e-10}	Actin 2	gi|18565104	*Crassostrea gigas*	Mollusca, Bivalvia		PMF
	8125	9.9	21	18	<0.05	Contig: Actin	GW7IPVU02F2ZX3	*Crassostrea gigas*	Mollusca, Bivalvia		MS-MS
2141 mixture	51678	5.2	105	40	6.1^{e-5}	Retrograde protein of 51 kDa	gi|74912853	*Lymnaea stagnalis*	Mollusca, Gastropoda		PMF
	41950	5.1	85	45	6.1^{e-3}	Actin	gi|14010639	*Heliothis virescens*	Arthropoda, Insecta		PMF
2150	42164	5.2	231	11	<0.05	Actin	gi|483321	*Aplysia californica*	Mollusca, Gastropoda		MS-MS
	16066	6.6	140	20	<0.05	Contig: Actin	GW7IPVU02F1G9T	*Placopecten magellanicus*	Mollusca, Bivalvia		MS-MS
2154	51678	5.2	141	37	1.5^{e-8}	Retrograde protein of 51 kDa	gi|74912853	*Lymnaea stagnalis*	Mollusca, Gastropoda		PMF
2158	42081	5.3	200	15	<0.05	Actin	gi|113290	*Aplysia californica*	Mollusca, Gastropoda		MS-MS
	16405	4.6	82	11	<0.05	Contig: Actin	GW7IPVU02F1J4U	*Biomphalaria obstructa*	Mollusca, Gastropoda		MS-MS
2174	42002	5.3	268	20	<0.05	Actin 2	gi|18565104	*Crassostrea gigas*	Mollusca, Bivalvia		MS-MS
	16066	6.6	63	7	<0.05	Contig: Actin	GW7IPVU02F1G9T	*Placopecten magellanicus*	Mollusca, Bivalvia		MS-MS
2218	42185	5.4	82	32	0.011	Actin	gi|47116422	*Biomphalaria pfeifferi*	Mollusca, Gastropoda		PMF
2219	42002	5.3	75	49	0.05	Actin 2	gi|18565104	*Crassostrea gigas*	Mollusca, Bivalvia		PMF

Table 8.4: Proteins with at least 1.5-fold expression change ($p<0.05$, student *t*-test) in reproductive organs of *Lymnaea stagnalis* after 21 days of exposure to chemicals significantly identified with the NCBI Metazoa database and with the *Lymnaea stagnalis* contig data base.

Spot number	MW	p*I*	Score Mascott	MS Coverage	p-value	Protein identification	Accession number	Organism	Taxonomy (Phylum, Class)	Functions	Identification
2220	42147	5,3	92	22	1.2^{e-3}	Actin 5	gi|728796	*Limulus polyphemus*	Arthropoda, Merostomata	Cytoskeleton and muscle movements	PMF
2235	42002	5.3	182	67	1.2^{e-12}	Actin 2	gi|18565104	*Crassostrea gigas*	Mollusca, Bivalvia		PMF
	14602	5.2	74	52	1.6^{e-3}	Contig: Actin	GW7IPVU02HXDAX	*Nasonia vitripennis*	Mollusca, Gastropoda		PMF
2294	42310	5,16	84	25	7.8^{e-3}	Skeletal muscle Actin 8	gi|207298839	*Homarus americanus*	Arthropoda, Malacostraca		PMF
2310	32663	4,58	193	48	<0.05	Tropomyosin-2	gi|1174755	*Biomphalaria glabrata*	Mollusca, Gastropoda		MS-MS
2393	32663	4,58	170	48	<0.05	Tropomyosin-2	gi|1174755	*Biomphalaria glabrata*	Mollusca, Gastropoda		MS-MS
2506	32663	4,58	119	11	<0.05	Tropomyosin-2	gi|1174755	*Biomphalaria glabrata*	Mollusca, Gastropoda		MS-MS
2844	49769	5,8	78	26	0.027	Similar to AGAP007334-PA, partial	gi|91095419	*Tribolium castaneum*	Arthropoda, Insecta		PMF
2927	49769	5.8	85	31	6.5^{e-3}	Similar to AGAP007334-PA, partial	gi|91095419	*Tribolium castaneum*	Arthropoda, Insecta		PMF
3036	42081	5,3	91	40	1.6^{e-3}	Actin	gi|113290	*Aplysia californica*	Mollusca, Gastropoda		PMF
514	79811	9.3	80	17	0.02	GF20923	gi|194763888	*Drosophila ananassae*	Arthropoda, Insecta	Post-transcriptional modifications	PMF
748	19214	5,37	91	54	1.6^{e-3}	GA13613	gi|198472675	*Drosophila pseudoobscura*	Arthropoda, Insecta		PMF
1050	73710	4,8	146	5	<0.05	78 kDa glucose-regulated protein	gi|3023914	*Aplysia californica*	Mollusca, Gastropoda		MS-MS
1375	12501	8,27	78	61	0.034	cAMP-dependent protein kinase	gi|204305500	*Nasonia longicornis*	Mollusca, Gastropoda		PMF
1579	5046	5,5	68	13	6.1^{e-3}	Contig: Galectin 4-like protein transcript variant	GW7IPVU02IO057	*Haliotis discus hannai*	Mollusca, Gastropoda		PMF
3769	42530	6,57	76	24	0.05	GE10781	gi|195504707	*Drosophila yakuba*	Arthropoda, Insecta		PMF
732	133246	8,66	87	14	3.8^{e-3}	hypothetical protein TcasGA2_TC005757	gi|91088617	*Tribolium castaneum*	Arthropoda, Insecta	Chromosome segregation	PMF
1163	200851	6,34	78	17	0.031	GI21295	gi|195124758	*Drosophila mojavensis*	Arthropoda, Insecta		PMF
3529	80576	6,75	81	14	0.015	Similar to ATP-dependent DNA helicase PIF1	gi|221111106	*Hydra magnipapillata*	Cnidaria, Hydrozoa		PMF
	2435	5,4	73	32	2.2^{e-3}	Contig: Unnamed	GW7IPVU02HXBVQ	-	-		PMF
4576	99375	9,47	83	20	9^{e-3}	PIWI	gi|157116679	*Aedes aegypti*	Arthropoda, Insecta		PMF

Table 8.4: Proteins with at least 1.5-fold expression change (p<0.05, student *t*-test) in reproductive organs of *Lymnaea stagnalis* after 21 days of exposure to chemicals significantly identified with the NCBI Metazoa database and with the *Lymnaea stagnalis* contig data base. (Continued).

Spot number	MW	p*I*	Score Mascott	MS Coverage	p-value	Protein identification	Accession number	Organism	Taxonomy (Phylum, Class)	Functions	Identification
861	2060	7	60	18	0.042	Contig: Cytochrome c oxidase subunit 1	GW7IPVU02G8789	*Albinaria caerulea*	Mollusca, Gastropoda	Oxidative phosphorylation	PMF
888	155613	6,34	82	21	0.014	Hypothetical protein CBG19638	gi|268563428	*Caenorhabditis briggsae*	Namatoda, Secernentea		PMF
	2060	7	60	18	0.041	Contig: Cytochrome c oxidase subunit 1	GW7IPVU02G8789	*Pulmonata sp.*	Mollusca, Gastropoda		PMF
4572	28835	9,05	80	44	0.021	WNT16	gi|284157247	*Capitella teleta*	Annelida, Polychaeta	Signalisation	PMF
2298	39305	7,12	125	6	<0.05	Arginine kinase	gi|3183057	*Liolophura japonica*	Mollusca, Polyplacophora	Energy storage	MS-MS
2089	15152	5,3	59	14	0.05	Contig: Alanine aminotransferase 2	GW7IPVU02IYGTU	*Crassostrea gigas*	Mollusca, Bivalvia	Detoxification	PMF
1627	108020	5,32	76	20	0.043	GF13819	gi|194758182	*Drosophila ananassae*	Arthropoda, Insecta	RNA Silencing	PMF
	2576	7,1	21	40	<0.05	Contig: Unnamed	GW7IPVU02FY4MI	-	-	-	MS-MS
3255	18856	4,6	47	5	<0.05	Ovipostatin	gi|283137716	*Lymnaea stagnalis*	Mollusca, Gastropoda	Egg Laying inhibition	MS-MS
3567	27606	5,42	45	10	<0.05	Yolk Ferritin	gi|1169743	*Lymnaea stagnalis*	Mollusca, Gastropoda	Egg Yolk protein	MS-MS
	12457	5,2	24	13	<0.05	Contig: Yolk Ferritin	GW7IPVU02F8ZI0	*Lymnaea stagnalis*	Mollusca, Gastropoda		MS-MS

Table 8.4: Proteins with at least 1.5-fold expression change ($p<0.05$, student *t*-test) in reproductive organs of *Lymnaea stagnalis* after 21 days of exposure to chemicals significantly identified with the NCBI Metazoa database and with the *Lymnaea stagnalis* contig data base. (Continued).

3.3. Western Blot

3.3.1. Antibody efficiency

Rabbit serum was used without purification of the anti-yolk ferritin antibody. The specificity of the yolk ferritin detection was confirmed by the identification of a band at the corresponding molecular weight (i.e., 27KDa) with further confirmation using positive and negative controls. Yolk ferritin is the main constituent protein of egg albumen in *L. stagnalis* (Bottke et al. 1988; Bottke and Sinha 1979). Therefore, egg proteins were extracted and loaded on the gels as positive control whereas the prostate gland was used as negative control. Results showed a strong expression of a band corresponding to a protein with a molecular weight between 25 and 35 KDa, that was observed in eggs but was weak or absent in the prostate samples. The pre-immune rabbit serum, sampled before the immunisation with the synthetic peptide, was tested on egg proteins and compared to the post-immune serum response. A weak detection of a band similar to the yolk ferritin protein was detected in egg samples after incubation with pre-immune serum, however the intensity of the band was much stronger in samples incubated with the post-immune serum (Fig. 8.4). Finally, we have tested the serum on reproductive organs samples with or without the prostate gland. The removal of the prostate gland improved the detection of yolk ferritin (Fig. 8.4). Therefore, in order to reduce unspecific binding, we have removed the prostate gland from the rest of the reproductive organs and negative controls (i.e., prostate gland proteins) were added to each gel.

3.3.2. Yolk ferritin expression in *Lymnaea stagnalis* exposed to EDCs

Results of the yolk ferritin quantification were normalised between gels as percentages of the yolk ferritin expression signal detected in the positive control (i.e., proteins extracted from eggs sampled in our laboratory breeding stock), which was identical in every gel. Our results showed that, as previously observed during the method validation, a low but significant expression of yolk ferritin immunolabelling was found in the prostate gland (36.6 ± 6.3 % of the positive control yolk ferritin expression)

(Fig. 8.5). No statistical differences were observed between controls and solvent controls (p= 0,092, Mann-Whitney test). Therefore controls and solvent controls were pooled for subsequent statistical analysis. The results obtained following exposure of *L. stagnalis* to EDCs were compared to the results obtained in the negative controls (i.e., proteins of a prostate gland from a snail from our laboratory breeding stock).

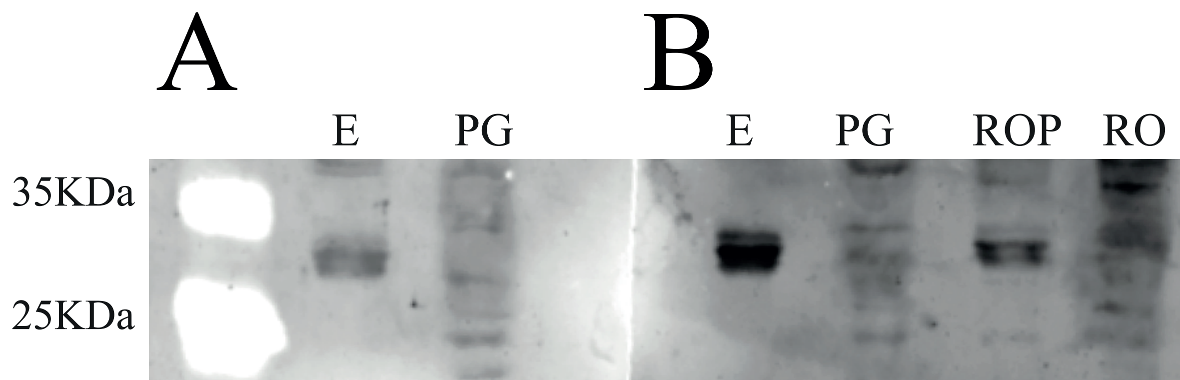


Fig. 8.4: Western blot gel of 25 µg of proteins extracted from eggs (E), prostate gland (PG) and reproductive organs with (RO) and without prostate gland (ROP) incubated with (A) pre-immune rabbit serum and (B) post-immune rabbit serum.

Only individuals exposed to 0.3 and 6.8 ng/L of testosterone and to a concentration of 2.1 µg/L of chlordecone showed a significantly higher percentage of protein expression compared to the negative controls (i.e., prostate). Individuals exposed to the highest tested concentration of testosterone (6.8 ng/L) produced significantly more yolk ferritin than controls ($p<0.05$, ANOVA Dunnett's *post hoc* test). A U-shaped dose response curve was observed in testosterone treatments (Fig. 8.5). The highest tested concentration of cyproterone acetate reduced the yolk ferritin expression compared to controls although this reduction was not significant. Conversely to cyproterone acetate, the lowest concentrations of TBT (19.2 ng Sn/L) and chlordecone (2.1 µg/L) tended to increase the quantity of yolk ferritin expressed in reproductive organs, while higher concentrations had no impacts on the protein expression of this protein. These results highlight that the different molecules tested had concentration dependent impacts on the yolk ferritin expression in the reproductive organs of *L. stagnalis* after 21 days of exposure. Conversely to the downregulation in the yolk ferritin expression observed in 2D-DIGE following exposure to 94.2 ng Sn/L of TBT, results of the western blot analysis showed that this treatment had no impacts on the protein expression, whereas an upregulation was observed at the lower concentration.

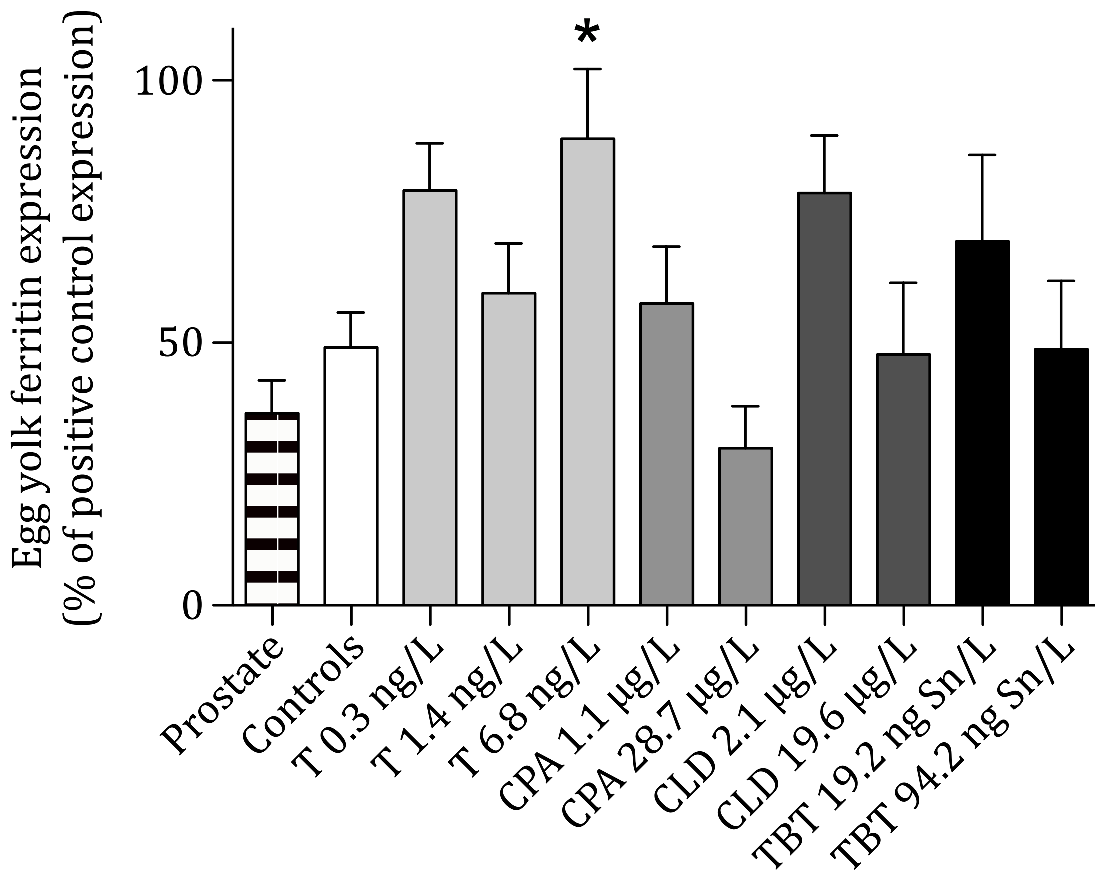


Fig. 8.5: Yolk ferritin expression in reproductive organs of *L. stagnalis* exposed to testosterone (T), cyproterone acetate (CPA), chlordecone (CLD) and tributyltin (TBT) for 21 days. Results are expressed as percentage of expression measured in positive controls (egg samples) run with each gel. Error bars are SEM (*n*=4). Statistical differences from the controls; ★: $p<0.05$.

4. Discussion

4.1. Differential proteomic analysis

Our study is the first to investigate the impacts of putative endocrine disrupting chemicals on the proteome of the hermaphroditic freshwater gastropod *Lymnaea stagnalis* using 2D-DIGE analysis, a method that allows to determine the modification in expression levels of a large number of proteins with a good reproducibility (Berth et al. 2007). Our results suggest that the four molecules tested, which can interact differently with the endocrine system of vertebrates (e.g., androgenic, anti-androgenic and oestrogenic), are able to alter the protein expression profile in reproductive organs

extracts. As compared to the solvent controls, the exposure of *L. stagnalis* to chlordecone had an impact on the expression of a greater number of proteins than the other compounds tested after 21 days of exposure. Moreover, the results of this study showed that proteins responsive to at least 2 chemicals were always altered in a same way (i.e., either up- or downregulation), suggesting that alteration in the expression of these proteins could be the result of a general stress response to chemical exposure (Rodríguez-Ortega et al. 2003). Our results show that chemicals have impacts on different proteins suggesting that particular protein expression signature (PES) could be associated to each molecule as observed in other molluscs species exposed to different chemicals (Apraiz et al. 2006; Bradley et al. 2002; Dondero et al. 2010; Shepard et al. 2000).

4.2. Impacts of EDCs on reproductive pathways

Many studies have reported that identification of specific protein expression signature (PES) is useful to propose new biomarkers of exposure to chemicals (Bradley et al. 2002; Shepard et al. 2000). Moreover, further identification of proteins disrupted by chemicals is essential to understand their mechanisms of action. The recent pyrosequencing of the genome of *L. stagnalis* (Bouétard et al. 2012) has helped to increase the number of proteins identified. Thus, seven more proteins were identified with this new genetic information additionally to proteins previously identified using other Metazoa databases. Furthermore, Bouétard et al. (2012) have reported that only 34.2 % of the contigs obtained in *L. stagnalis* produced significant identification using available databases. Therefore, some protein spots that significantly matched to the contigs could not be identified in this study. The majority of identified proteins were related to structure and function of the cytoskeleton (i.e., actin and tropomyosin). The high proportion of cytoskeleton proteins identified was also reported in other studies on mollusc species (Chora et al. 2009; Thompson et al. 2012b). This is probably due to both their high abundance in molluscs databases and to their identification as major target of oxidative stress following exposure to pollutants in molluscs (Chora et al. 2009; McDonagh et al. 2005; Miura et al. 2005; Rodríguez-Ortega et al. 2003). These results support the need for gene annotation in gastropods to magnify the results obtained from high throughput methods such as “omics” (Pascoal et al. 2012).

As previously observed on the proteome of different tissues of molluscs exposed to pollutants (Chora et al. 2009; Manduzio et al. 2005; McDonagh et al. 2005; Rodríguez-Ortega et al. 2003), actin and tropomyosin proteins were downregulated in the reproductive organs of *L. stagnalis* following a 21-day exposure to testosterone, cyproterone acetate, tributyltin and chlordecone. These proteins are highly abundant in muscle and non-muscle cells and impacts of toxicants on the expression of these proteins was suggested to be a consequence of the production of reactive oxygen species due to the toxicity of the compounds (Apraiz et al. 2009; Chora et al. 2009; McDonagh et al. 2005; Zhou et al. 2010b). Therefore, the impacts of the tested chemicals on these proteins could not be attributed to endocrine disruption.

However, exposure to the different chemicals also significantly altered expression of some proteins related to the reproduction in *L. stagnalis*. An increase in PIWI expression was observed in reproductive organs of individuals exposed to cyproterone acetate (1.1 µg/L), tributyltin (94.6 ng Sn/) and chlordecone (19.6 µg/L) compared to solvent controls. PIWI is an evolutionary conserved protein, which is involved in the regulation in germ stem cell divisions (Cox et al. 1998; Cox et al. 2000; Zhou et al. 2007). It was observed in *Drosophila sp.* mutants that an overexpression of PIWI increased the division of germ stem cells, therefore increasing the number of gametes produced (Cox et al. 2000). It was later reported that the role of PIWI was associated with spermatogenesis in vertebrates (Beyret and Lin 2011) as well as in invertebrates, such as the hermaphroditic *Caenorhabditis elegans* (Wang and Reinke 2008). Upregulation in genes involved in spermatogenesis was observed in the gonochoric gastropod *Nucella lapillus* exposed to 100 and 200 ng Sn/L of TBT, using transcriptomic methods (Pascoal et al. 2012). Therefore, it is reasonable to hypothesise that the increase in PIWI expression we observed in reproductive organs of *L. stagnalis* could indicate an increase in the gamete production, especially through spermatogenesis, hypothesis that would be interesting to investigate in further studies.

The expression of another protein, ovipostatin, was significantly increased in the reproductive organs of individuals exposed to 19.6 µg/L of chlordecone compared to controls. This protein was recently isolated from seminal fluid of *L. stagnalis* and was reported to decrease oviposition when injected to the animals (Koene et al. 2010). It is to be noted that at the end of the 21 days of exposure to chlordecone, a significant

reduction of the number of clutches laid by snails exposed to 19.6 µg/L was observed (Part 2: chapter 4). These observations indicate that exposure to chlordecone could increase ovipostatin production by the prostate gland in *L. stagnalis*, which could induce a reduction of oviposition. Koene et al. (2010) suggested that a reduction in clutch production could be a side effect of the protein, which would generally decrease the female function to provide more fitness to male donor. However, a recent study showed that ovipostatin also induces oligospermia in receptor individuals (Hoffer 2012). In our study, five individuals were exposed per 1L beaker, which allowed mating during the experiment duration. This implies that the decrease in the number of clutches produced per snails (Part 2: chapter 4) could also result from a side effect of chlordecone on male allohormone production. In this case, the increase of ovipostatin production will have no impact on single individual if mating occurs. Further analyses of the impacts of EDCs on ovipostatin production by the prostate gland of *L. stagnalis* are required to confirm our results, as the change in abundance was not observed when compared to solvent controls. Furthermore, the mechanisms of action of ovipostatin on snail reproduction through endocrine pathways have to be further investigated.

In *L. stagnalis* exposed to 94.2 ng Sn/L of tributyltin for 21 days, the yolk ferritin expression was downregulated in the 2D-DIGE experiment. This protein was found to be the main yolk protein in oocytes in this species (Bottke 1986; Bottke et al. 1988), whereas vitellogenin (Vtg), the precursor of egg yolk proteins in many species of vertebrates and invertebrates (Agnese et al. 2013), has never been reported in freshwater gastropods. Alterations of Vtg production were reported in male fish affected by intersex in field and laboratory studies (Björkblom et al. 2011; Donohoe and Curtis 1996; Purdom et al. 1994). Nowadays, this protein is used in fish as biomarker of exposure to oestrogenic molecules in the environment (Harries et al. 1999; Sumpter and Jobling 1995) and is currently used to screen oestrogenicity of chemicals in the OECD test guidelines Test No. 229: Fish Short Term Reproduction Assay (OECD). Laboratory and field studies have reported modifications of Vtg expression in mollusc species exposed to oestrogenic compounds (e.g., 4–nonylphenol, 17ß-oestradiol) (Andrew et al. 2008; Andrew et al. 2010; Andrew-Priestley et al. 2012; Matozzo and Marin 2008). Moreover, in the scallop *Chlamys farreri*, transcriptomic analysis have reported that low concentrations of benzo(a)pyrene (0.4 and 2 µg/L) increased Vtg gene transcription

whereas a higher concentration (10 µg/L) tended to reduce Vtg genes (Zhang et al. 2012). Vtg expression was proposed as biomarker of exposure to oestrogens in bivalves populations (Matozzo et al. 2008).

Therefore, alterations of yolk ferritin expression observed by 2D-DIGE analysis in our study suggest that EDCs could interact with oocyte production and egg quality in *L. stagnalis.* Western blot analysis was used to confirm the protein identification (Damodaran et al. 2007) and to provide quantitative results on yolk ferritin expression after exposure to chemicals. In this study we showed that further isolation of anti-yolk ferritin antibody from rabbit serum is needed in order to avoid the unspecific binding we observed. The western blot analysis has shown that yolk ferritin expression was modified in the reproductive organs of snails exposed to EDCs for 21 days. Therefore, our results suggest that in the hermaphrodite gastropod species *L. stagnalis*, the endocrine disrupting chemicals tested are able to induce alterations of the expression of the main egg yolk protein in the reproductive organs of individuals exposed to for 21 days. The U-shaped dose response relationship that we have observed for the impact of testosterone on yolk ferritin expression, as well as inverted U-shaped dose-response relationship, are regularly observed when assessing the impacts of EDCs, mainly following exposure to oestrogens (e.g., Bisphenol A) (Weltje et al. 2005). Together, these results further support that the tested molecules might act as endocrine disruptors in *L. stagnalis*.

Our results highlight that chemicals known for their endocrine disrupting properties in vertebrates can significantly alter proteins involved in reproduction of *Lymnaea stagnalis*. Some of the proteins identified are involved in different mechanisms such as gamete production (i.e., PIWI), oviposition (i.e., ovipostatin) or vitellogenesis (yolk ferritin). As oviposition was decreased (Part 2: chapter 4) while ovipostatin expression was upregulated, chlordecone might be considered to act as an endocrine disruptor through alterations of the oviposition pathways. In the same treatment condition, an increase in PIWI expression was observed, which suggest an increase in gamete production. These results highlight that chemicals can act on reproduction through different mechanisms of action, especially in hermaphrodite species. Further analysis of the alterations by EDCs of the expression of proteins identified in this study

using more specific methods (e.g., western blot) are needed to better understand the mechanisms of action of EDCs on the reproduction of *L. stagnalis* and will help to define whether those molecules acts as endocrine disruptors in gastropods.

5. Conclusions

Our results showed that the high throughput proteomic method 2D-DIGE allows to investigate the impacts of chemicals, known for their endocrine disrupting properties in vertebrates, on the protein expression in the reproductive organs of the freshwater hermaphrodite gastropod *Lymnaea stagnalis*. This method allowed the identification of proteins involved in the reproduction that were differentially expressed following exposure to the chemicals, therefore suggesting possible endocrine disruption mechanisms. Among these proteins, yolk ferritin appeared to be a potential biomarker of exposure to different EDCs as its homolog, vitellogenin, in fish and other molluscs. *Lymnaea stagnalis* is proposed as candidate species for the development of an OECD guideline on reprotoxicity tests with molluscs. This guideline will cover level 4 and level 5 tests (i.e., *in vivo* assays on entire animals, that provide data on adverse effects on endocrine relevant endpoints), as defined in the revised version of the OECD Conceptual Framework for Endocrine Disruptors Testing and Assessment (OECD 2012). The analysis of yolk ferritin expression could be a complementary tool to screen the EDC potential of toxicants at the sub-individual level (i.e., level 3 tests: *in vivo* assays providing data about selected endocrine mechanisms/pathways). For chemicals suspected to be endocrinally active based on screening test, combining reproductive toxicity test on entire snails and proteomic analysis in animals sampled from this reprotoxicity test would provide the full body of proof needed to show that a given toxicant actually matches the definition of an endocrine disruptor in the snail. Further analysis of the impacts of EDCs on this protein should be conducted in order to better understand the alterations that the different molecules induce in this species.

Acknowledgements

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Part 5:

General Discussion and Perspectives

1. Impact of EDCs on oviposition and fecundity of *Lymnaea stagnalis*

In this thesis, we have investigated the impacts of chemicals, which present different endocrine disrupting properties in vertebrates, on the reproduction of the freshwater gastropod *Lymnaea stagnalis*. This hermaphrodite species is a candidate species for the development of OECD test guidelines for reprotoxicity tests of EDCs, along with other chemicals, on molluscs (Matthiessen 2008; OECD 2010). Therefore, testing methods need to be standardised in order to provide reliable and sensitive tools that will provide comprehensive information on the apical effects of chemicals on the reproduction of this species (OECD 2010). Even though such tests will not necessarily diagnose the mechanisms of action of the molecules, a better understanding of the interactions of the molecules tested with physiological pathways in organisms will contribute to improve the quality of the future guideline (OECD 2010).

In the present work, we used the experimental set up proposed for the partial life cycle test on adult reproduction with *L. stagnalis*, which is currently under validation in different laboratories throughout Europe. Since this test is designed to assess the effect of any toxicant, and not specifically EDCs, we performed minor changes to the protocol in order to improve the sensitivity of the test to EDCs. Instead of using fully-grown adults, we conducted this partial-life cycle tests on snails having just reached sexual maturity. Indeed, young adults are supposed to be more sensitive to putative endocrine disruptors than juveniles, sub-adults and adults (Ducrot et al. 2010; OECD 2010). We have also modified the duration of exposure to chemicals, as snails were exposed for 21 days instead of the 28 days required in the experimental set up submitted for validation of the test guideline (OECD 2010). Results of the range finding test have highlighted that this exposure period was sufficient to assess adverse impacts of different molecules on the reproduction of *L. stagnalis* (Part 2: chapter 1).

Based on the results of the range finding test, we conducted another test using an adjusted concentration range for each chemical under similar experimental conditions (Part 2: chapters 2 and 4). Even though the exposure medium of controls and solvent controls was renewed weekly during the range finding test and every other day during the subsequent test, we observed similar reproductive outputs in the controls of these two tests (Fig. 9.1). A stimulation of oviposition due to the water renewal was previously reported in *L. stagnalis* (Ter Maat et al. 1983), however in our test conditions we did not

observe any clear water stimulus (Fig. 9.1 a). These observations emphasise the repeatability of the results obtained from two independent tests, therefore suggesting that under similar laboratory conditions, reliable comparisons of the results obtained with different repeatable tests can be performed as required for test guidelines.

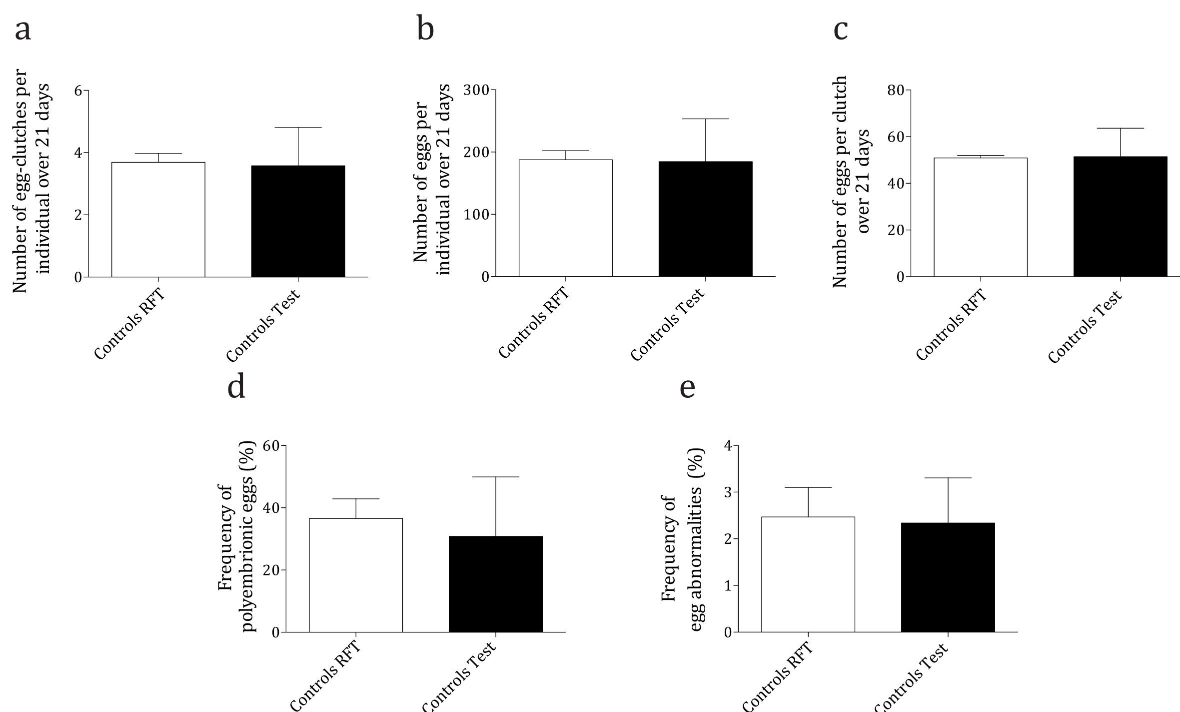

Fig. 9.1: (a) Mean cumulated number of egg-clutches, (b) mean cumulated number of eggs laid per individual, (c) mean number of eggs per clutch, (d) frequency of abnormal eggs over the total number of eggs laid, and (e) frequency of polyembryonic eggs over total number of abnormal eggs in controls from the range finding test (white) and from the test (black) over 21 days. Error bars are standard deviations (SD).

Our results highlighted that the classical reproductive endpoints assessed in this species (i.e., number of clutches and number of eggs) were decreased after 21 days of exposure to TBT, TPT and chlordecone but not after exposure to the other chemicals (i.e., testosterone, cyproterone acetate, fenitrothion and vinclozolin) in the chosen concentration range tested.

Exposure of *L. stagnalis* to TBT significantly decreased oviposition and fecundity from 94 ng Sn/L to 473 ng Sn/L (Part 2: chapters 1 and 2). Impacts of TBT on mollusc reproduction were mainly investigated in gonochoric species. In prosobranch gastropods, the development of imposex was induced at concentrations as low as 1 ng Sn/L. At higher concentrations, this molecule did not only sterilise females (e.g.,

inhibition of mating due to modifications of the oviduct), it also suppressed oogenesis and induced spermatogenesis (Brick et al. 1996; Gibbs and Bryan 1996; Matthiessen and Gibbs 1998). In bivalves, the production and the release of larvae were reduced after exposure to approximately 250 ng Sn/L of TBT (Ruiz et al. 1995; Thain 1986; Thain et al. 1986). Therefore, results of the present work suggest that the hermaphroditic species *L. stagnalis* might be as sensitive as gonochoric mollusc species to TBT.

Even though TBT concentrations that affected mollusc reproduction had no impact on growth and survival, a decrease in feeding was observed. A concentration dependant increase in mucus production was also reported (Part 2: chapter 2). Moreover, the results obtained from experiments measuring the enzymatic activity of biomarkers following exposure to chemicals (i.e., PROD and GSTs; Part 2: chapter 3) have highlighted that TBT interacts with the detoxification process from 197 ng Sn/L. In animals, the energy assimilated from the food is allocated to somatic maintenance (e.g., growth), and to maturation maintenance (e.g., courtship, mating, oviposition) (Fig. 9.2) (Jager and Zimmer 2012; Kooijman 2010). Toxic compounds can influence the acquisition and/or the use of the energy by the organisms, which can therefore induce adverse impacts on both somatic and maturation maintenance (Jager and Zimmer 2012). The results obtained from the enzymatic activity experiments, coupled to the observed increase in mucus production and the reduction in feeding, suggest that the reduction in oviposition and fecundity observed after 21 days of exposure of *L. stagnalis* to TBT from 197 ng Sn/L is likely due to a change in energy allocation. These observations reinforce the hypothesis that TBT exhibits sublethal toxic effects on *L. stagnalis*, which is reflected on both growth and reproduction (Part 2: chapters 1 and 2).

Egg production and oviposition are under the control of two different hormonal pathways in *Lymnaea stagnalis* (Koene 2010). In addition to results showed in chapter 2, we observed a decrease in the number of eggs per clutch laid by snails exposed from 19.2 ng Sn/L of TBT (Part 2: chapter 2; table 9.1). These results suggest that TBT impacts egg production as well as egg-laying. A change in energy allocation, after exposure to high TBT concentrations, might affect one or even both pathways (Zonneveld 1992). In isolated *L. stagnalis*, snails not only laid less clutches but also produced less eggs per clutch than individuals reared in groups. Isolated snails also invested more energy in growth and in male functions, which is reflected by a larger prostate gland (Koene and Ter Maat 2004). These observations suggest that a change in

energy allocation can be responsible for the decrease in both endpoints as observed in the TBT treatments.

The triphenylated organotin TPT significantly reduced both oviposition and fecundity at 265 ng Sn/L while a concentration of 590 ng Sn/L induced 100 % mortality within 3 days (Part 2: chapter 2). Similarly to TBT, food consumption was reduced at high TPT concentrations, suggesting that the total energy assimilated is reduced. These results support the hypothesis that this organotin also induces acute toxicity in *L. stagnalis*. The reduction in oviposition observed following exposure to 265 ng Sn/L of TPT might also be due to modifications of the allocation of the energy between somatic and maturation maintenance. However, TPT induced an adverse effect on fecundity only by reducing oviposition as the mean number of eggs per clutch was only decreased at the highest concentration tested (Table 9.1).

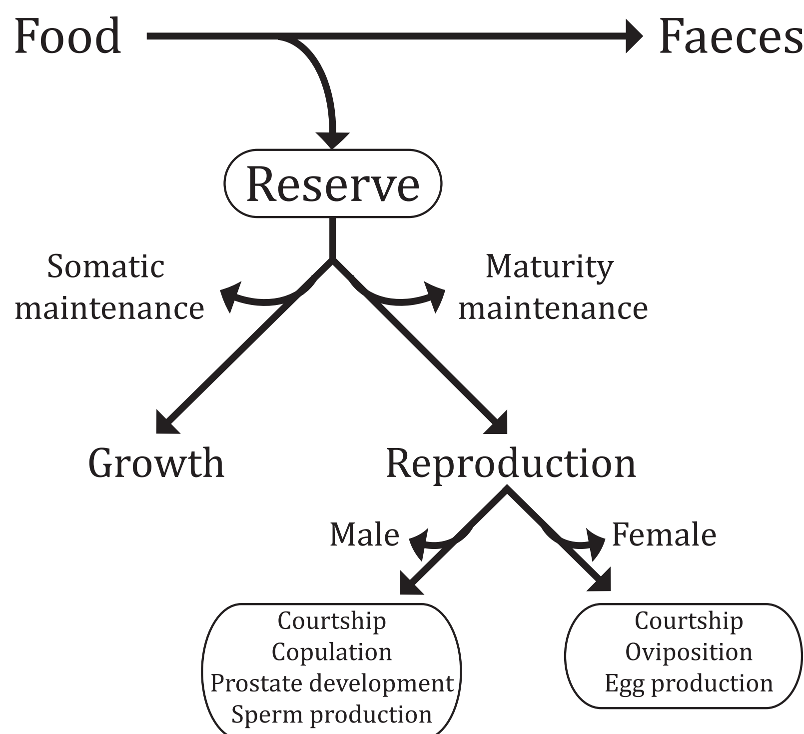

Fig. 9.2: Energy allocation in *L. stagnalis* (Modified from Jager et Zimmer 2012).

Treatments		Mean number (± SD) of egg-clutches per individual	Mean number (± SD) of eggs per individual	Mean number (± SD) of eggs per clutch
Controls	0	3.9 ± 1.1	196.9 ± 64.5	50.9 ± 10.6
Solvent Controls	0	3.3 ±1.3	172.5 ± 76.3	52.1 ± 14.1
TBT (ng Sn/L)	19.2	2.9 ± 0.7	133.5 ± 31.2	45.5 ± 12.2 **
	42.6	3.1 ± 1.0	139.9 ± 43.3	45.6 ± 12.0 **
	94.2	2.3 ± 0.5 *	91.46 ± 27.1 **	39.7 ± 12.7 ***
	196.7	0.2 ± 0.2 ***	5.8 ± 7.6 ***	34.6 ± 16.0
	473.4	0.1 ± 0.2 ***	5.5 ± 9.8 ***	41.0 ± 15.2
Controls	0	3.5 ± 0.7	193.7 ± 47.9	57.7 ± 12.5
Solvent Controls	0	2.7 ± 0.6	141.8 ± 21.2	53.9 ± 12.9
TPT (ng Sn/L)	45	4.1 ± 0.5 ***	243.8 ± 31.9 *	60.0 ± 13.2 **
	74	3.2 ± 0.3	162.2 ± 25.8	52.3 ± 13.4
	187	3.8 ± 0.3 **	211.7 ± 21.5 *	54.7 ± 12.5
	265	1.7 ± 0.3 **	91.2 ± 16.8 ***	55.2 ± 12.6
	590	0.5 ± 0.3 ***	20.6 ± 12.5 ***	41.3 ± 15.9 *

Table 9.1: Mean number of egg-clutches (oviposition) and of eggs (fecundity) produced per individuals (± SD) and mean number of eggs per clutch (± SD) produced by *L. stagnalis* over 21 days of exposure to chemicals. Kruskal-Wallis with Dunn's *post hoc* test statistical significance; ∗: p<0.05; ∗∗: p<0.01; ∗∗∗: p<0.001).

Exposure to chlordecone induced adverse impacts on both oviposition and fecundity from 11.3 µg/L (Part 2: chapters 1 and 4). Similarly to TPT, the reduction in fecundity observed after exposure to chlordecone was a consequence of the decrease in oviposition (Part 2: chapters 1 and 4). At high concentrations (from 49.1 µg/L) this organochlorinated pesticide reduced the survival of *L. stagnalis*. Moreover, and similarly to TBT, a concentration dependent increase in mucus production was observed over 21 days (Part 2: chapter 4). It can be hypothesised that the increase in mucus production might constitute a detoxification process used by the snail to reduce chlordecone body burdens. This hypothesis is supported by the low bioconcentration factor measured in the tissues (as compared to vertebrate species) (Part 3). These results suggest that, as we observed for the two organotins, the reduction in reproductive endpoints observed in the higher concentration range might be due to a greater allocation of energy for detoxification processes. However, conversely to TBT and TPT, we did not observe an impact on food consumption in the chlordecone treatments.

In chapter 6, we highlighted that expression of ovipostatin, a protein synthesised in the prostate gland of *L. stagnalis*, was upregulated in individuals exposed to 19.6 μg/L of chlordecone. This protein is transferred between mates through the seminal fluid during copulation. Laboratory experiments have shown that the injection of this protein reduced oviposition and induced oligospermia (i.e., reduction in the amount of sperm transferred by the recipient individual to the mating partner) (Hoffer 2012; Koene et al. 2010). Therefore, this protein is involved in sexual competition in this hermaphrodite species, as copulation serves not only to transfer sperm to the recipient mate, but also reduce the male function of competitors (Hoffer 2012). Thus, an increase in ovipostatin produced by individuals exposed to chlordecone, and therefore an increase in the quantity transferred to the recipient mate, may be responsible for the decrease in oviposition observed at 19.2 μg/L. Conversely, TBT had no impact on ovipostatin expression in the reproductive organs of individuals exposed to 19.2 and 94.2 ng Sn/L.

Together, results from enzymatic biomarkers (Part 2: chapter 3) and 2D-DIGE (Part 4: chapter 6) support the hypothesis that the mechanisms involved in the reduction in fecundity and oviposition are different following exposure to either organotins (i.e., TBT and TPT) and the organochlorine chlordecone. The organotin compounds might alter the oviposition behaviour through a reduction in the energy available for the reproduction processes, as reflected by the reduction in feeding and the enzymatic activity (Part 2: chapter 2). This energetic hypothesis might also be true for high chlordecone concentrations. The increase in mucus production, possibly due to a detoxification process, could require more energy, therefore reducing the amount of energy available for reproduction (Part 2: chapter 4). At lower concentrations, for which increased mucus production was not observed, the reduction in oviposition might be due to an increase in ovipostatin produced in the prostate of *L. stagnalis*. This observation suggests that chlordecone might act on this gland through interactions with the endocrine system of the snail (Part 4: chapter 6). Further analysis of the activity of enzymatic biomarkers of exposure to toxic compounds (e.g., GSTs and PROD) as well as food consumption analysis and energy allocation under chemical exposure can provide evidence of the concentrations of the molecules that induce toxic impacts on this species. Moreover, an assessment of the modification of ovipostatin expression in *L. stagnalis* exposed to a wider range of concentrations while using more accurate

methods for the identification and quantification of the protein (e.g., western blot, HPLC quantification (Koene et al. 2010)) is required in order to elucidate the impacts of the chemicals on this protein. These experiments will provide new insights on the mechanisms of action of chemicals on the production of eggs and clutches in *Lymnaea stagnalis*.

2. Impacts of EDCs on the egg quality of *Lymnaea stagnalis*

The assessment of fertilisation failures (e.g., number of unfertilised eggs) was suggested as an additional endpoint that might reflect an alteration of endocrine functions induced by the exposure to EDCs (OECD 2010). Therefore, in addition to the classical reproductive endpoints generally followed in the assessment of the impacts of EDCs on *L. stagnalis*, i.e., oviposition and fecundity (Czech et al. 2001; Ducrot et al. 2010; Leung et al. 2007; Segner et al. 2003), we have investigated the impacts of the different molecules on egg quality. The different egg abnormalities were assessed through the quantification of unfertilised eggs, polyembryonic eggs and eggs with depleted albumen quantity (i.e., albumen atrophied and single embryo) produced by individuals exposed to the different chemicals tested (Part 2: chapters 1, 2 and 4). The analysis of the impacts of the molecules on these different egg abnormalities might provide indications on the interactions between the chemicals and different physiological pathways involved in egg production in *L. stagnalis*. Indeed, reproduction, and its neuro-endocrine control, has been particularly well studied in this species, as in other basommatophoran gastropods. Egg production starts in hermaphroditic organs with the production and release of gametes, and, after fertilisation, proceeds along the female reproductive tract until oviposition, each step being under the neurohormonal control of different hormones (Part 1: 3.1).

Our analysis of the impacts of tested EDCs on egg quality has shown that these chemicals increase the frequency of abnormal eggs (Part 2: chapters 1, 2 and 4). In contrast, TPT had no impact on this endpoint (Part 2: chapter 2). Lack of impacts of TPT on egg quality, at every concentration tested, and of TBT at high concentrations (Part 2: chapter 2), reinforces the hypothesis that these chemicals induce adverse impacts on reproduction through modification of the energy available for reproduction. In contrast, effects on egg production observed following exposure to the other chemicals, as well as TBT at lower concentrations, might reflect interactions between chemicals and different physiological pathways involved, e.g. gametogenesis, suggesting possible endocrine disrupting effects. Among the different egg abnormalities described, the depletion of albumen quality (i.e., albumen atrophied with single embryo) was predominant in

controls whereas polyembryony prevailed in abnormal eggs produced by snails exposed to the different chemicals (Part 2: chapters 1, 2 and 4). This indicates that the chemicals tested might interact with egg production through gamete production or vitellogenesis.

Moreover, 2D-DIGE analysis has shown that the expression of yolk ferritin, the vitellogenin equivalent in *L. stagnalis*, in reproductive organs of *L. stagnalis* is affected by 21 days of exposure to the chemicals (Part 4: chapter 6). As the expression of this protein was significantly reduced after exposure to 94.2 ng Sn/L of TBT, it would thus be interesting to test the hypothesis that the decrease in this protein is actually responsible for the decrease in the number of eggs produced per snail. Moreover, TBT increased, in a dose-dependent relationship, the frequency of polyembryonic eggs among the total number of abnormal eggs produced per individual (Part 2: chapter 2). Therefore, alterations in the abundance of egg yolk protein might induce a reduction in the number of eggs produced as well as an increase in egg abnormalities due to alterations of vitellogenesis. Furthermore, alteration in the expression of another protein, identified using 2D-DIGE, might contribute to explain the development of polyembryonic eggs observed following chemical treatments. PIWI, an evolutionary conserved protein found in plants, invertebrates, and vertebrates, is involved in the regulation of germ stem cells division (Cox et al. 1998; Cox et al. 2000). In vertebrates, and invertebrates, this protein was shown to regulate gametogenesis, and more specifically spermatogenesis (Wang and Reinke 2008; Zhou et al. 2007). Therefore, an upregulation of expression of this protein could increase gamete production, both male and females, hence increasing fertilisation, which in turn leads to an increase in embryo production.

The dose response curves observed for abnormal eggs and polyembryony frequency (Part 2: chapters 1, 2 and 4) were compared with the results of PIWI expression, obtained in the 2D-DIGE experiment, and with the yolk ferritin expression, obtained in the western blot experiment, to further discuss the possible mechanisms of action of the different chemicals involved in oogenesis (Part 4: chapter 6;).

Exposure of *L. stagnalis* to testosterone concentrations ranging from 0.3 to 14.8 ng/L led to a U-shape dose response increase in the frequency of abnormal eggs produced (Part 2: chapter 4). In the three treatments analysed in the proteomic experiments (i.e., 0.3, 1.4 and 6.8 ng/L), yolk ferritin induced a similar U-shaped dose

response curves (Part 4: chapter 6). These results suggest that overexpression of this protein might be responsible for the increase in abnormal eggs production. In contrast, the increase in the frequency of polyembryonic eggs in testosterone treatments was similar at every concentration tested (Part 2: chapter 4), suggesting that yolk ferritin not only affected polyembryony but also induced other egg abnormalities. Interestingly, the expression of the PIWI protein also followed a U-shaped dose response curve. Therefore, the monotonic dose response curve observed for the frequency of polyembryonic eggs could result from the upregulation of PIWI, which might be partly counterbalanced by an increase in yolk ferritin expression.

Exposure of *L. stagnalis* to the synthetic steroid cyproterone acetate led to a U-shaped dose-response curve increase in the frequency of abnormal eggs. In contrast, a concentration dependent increase in the frequency of polyembryonic eggs was observed (Part 2: chapter 4). Proteomic analyses have shown that exposure to 1.1 µg/L of cyproterone acetate increased PIWI expression but had no effect on yolk ferritin expression. At a higher concentration (i.e., 28.7 µg/L), the abundance of yolk ferritin in reproductive organs was reduced while the expression of PIWI was not affected. Therefore, at a low concentration the increase in PIWI expression might still be partly counterbalanced by the yolk ferritin quantity whereas at a higher concentration the reduced amount of albumen is overwhelmed by the number of embryo produced, which finally increase the frequency of abnormal eggs in both cases.

Chlordecone increased the frequency of abnormal eggs as well as the expression of yolk ferritin at 2.1 µg/L but not at 19.6 µg/L (Part 2: Chapter 4; Part 4: Chapter 6). Conversely, both polyembryony frequency and PIWI expression were not different from controls at a low concentration but were increased at 19.6 µg/L. This suggests that at 2.1 µg/L of chlordecone, the increase in the frequency of abnormal eggs produced could be linked to an increase in the amount of yolk ferritin produced, which affects all types of abnormal eggs. At a higher concentration, the overexpression of PIWI could increase the number of embryo produced which can not be compensated by an increase in available albumen, therefore increasing the proportion of polyembryonic eggs produced. This process could also be responsible for the slight decrease in the number of eggs per clutch observed at 19.6 µg/L (Part 2: chapter 4).

Finally, western blot analysis has shown that yolk ferritin expression, in reproductive organs of snails exposed to 19.2 ng Sn/L of TBT, was increased. In this treatment, PIWI expression was not modulated. At a higher concentration (i.e., 94.2 ng Sn/L), PIWI expression was significantly increased but yolk ferritin expression remained unchanged compared to controls (Part 4: chapter 6). Results obtained on egg quality showed that TBT significantly increased the frequency of polyembryonic eggs in a dose-dependent relationship (Part 2: chapter 2) whereas the frequency of abnormal eggs was only significantly increased after exposure to 19.2 ng Sn/L. These results suggest that the increase in yolk ferritin quantity might be responsible for the increase in the frequency of abnormal eggs produced at a low concentration. At 94.2 ng Sn/L, the increased number of embryo observed, probably due to an increase in PIWI expression, could be responsible for the increase in the proportion of polyembryonic egg proportion, which was not, however, reflected by an increase in the frequency of abnormal eggs.

These results highlight that abnormal egg production, and more particularly polyembryonic eggs, might be explained by the impacts of the different chemicals on the expression of PIWI and yolk ferritin. Finally, the different chemicals, as well as the different concentrations tested, induced different impacts on these reproductive endpoints, which might be associated with the expression of these 2 proteins. However, further analysis of the impacts of a wider range of concentrations of these putative EDCs on the expression of these proteins are required in order to have a better understanding of the relationships between reproductive endpoints and the dose response curves observed. Results from chlordecone and TBT experiments emphasise the complex interactions of these chemicals with several different physiological pathways (i.e., energy allocation, vitellogenesis, gametogenesis and ovulation).

The neuro-endocrine pathways involved in the synthesis of the different proteins identified using proteomic methods (Part 4: chapter 6) remain to be investigated. In Basommatophora, egg production and oviposition are under the control of different neuropeptides synthesised in different regions of the cerebral ganglia (Part 1: 3.1). In this species, ovulation is under the control CDCH, a hormone produced by the

caudodorsal cells (CDCs) (Ter Maat et al. 1983). This neuropeptide also controls the stimulation of perivitellin fluid production. Therefore, a reduction in the release of CDCH might reduce ovulation of gametes but also decrease the quantity of vitellin fluid available for egg packaging. Dorsal bodies (DB) of the cerebral ganglia secrete the dorsal body hormone (DBH). Analyses of the role of this hormone on the reproduction of *L. stagnalis* led to conflicting observations. Geraerts and Joose (1975) observed that the removal of DB inhibited vitellogenesis but did not affect gamete production. *In vitro* experiments have reported that DBH stimulates polysaccharide synthesis in the albumen gland, as observed for CDCH, suggesting a stimulation of vitellogenesis (Wijdenes et al. 1983). Therefore, the analysis of the modification in the expression of ovipostatin, yolk ferritin and PIWI coupled with the assessment of reproductive endpoints induced by cauterisation of the different regions of the cerebral ganglia might provide new insights on the neuro-endocrine control of the production of these proteins in the different reproductive organs of snails and their role on *L. stagnalis* reproduction. These results will also help identifying probable targets of the different endocrine disrupting compounds.

3. Impacts on endogenous concentrations of testosterone

Endogenous concentrations of testosterone, along with other vertebrate-like sex steroid hormones (i.e. oestradiol and progesterone), were measured in molluscs from the field and laboratory cultures (Croll and Wang 2007; Gauthier-Clerc et al. 2006; Ketata et al. 2007; Ronis and Mason 1996). Variations in the endogenous concentrations of these sex steroid hormones were shown to vary according to gender, species and reproductive status in different gonochoric species of molluscs (Part 1: 2.2). Even though steroids might be taken up from the environment (Scott 2012), *in vivo* and *in vitro* evidence support that these hormones might be synthesised *de novo* in molluscs, as the presence and the activity of different enzymes involved in steroidogenesis in vertebrates were reported in different mollusc species (De Longcamp et al. 1974; Fernandes et al. 2011; Gottfried and Dorfman 1970; Janer et al. 2005b; Lavado et al. 2006). These observations suggest that vertebrate-like sex steroids might play a physiological role in the mollusc reproduction, however no clear interactions between these hormones and reproductive processes have been established. In chapter 5, we have investigated the impacts of four chemicals on the synthesis and esterification of testosterone, after a 21-day exposure period. The chemicals tested were selected based on their binding affinities to steroid receptors in vertebrates (Part 2: chapter 1) and the concentrations assessed were selected based on their impacts on *L. stagnalis* reproduction (Part 2: chapters 2 and 4). In chapter 5, we have shown a dose-dependent increase in the endogenous concentrations of testosterone after 21 days of exposure to water borne testosterone in the ng/L range. These results confirm that *L. stagnalis* might uptake steroids from the aquatic environment, as previously suggested in other mollusc species (Scott 2012). Moreover, testosterone was further metabolised to fatty acid esters. This metabolisation is the major homeostatic regulation of the steroids in molluscs (Part 1: 2.2; Fig. 9.3).

Furthermore, our results highlighted that the synthetic anti-androgen steroid cyproterone acetate was able to stimulate endogenous synthesis of testosterone, as reflected by the increase in testosterone concentrations in snail tissues. Results of the chemical analysis have shown that cyproterone acetate bioaccumulates in the tissues of *L. stagnalis* (Part 3). Metabolisation of cyproterone acetate was never reported in molluscs. However, in humans this pharmaceutical is metabolised into 15ß-

hydroxycyproterone acetate by a cytochrome P450 enzyme, CYP3A4, before its excretion (Part 3; (Kuhl 2001)). Bioconcentration factor measured in the present work highlight that *L. stagnalis* is not able to metabolise and excrete this molecule efficiently. These observations show that the increase in endogenous testosterone concentrations reported in chapter 6 is not due to a metabolisation of cyproterone acetate into testosterone. Our results support the hypothesis that *L. stagnalis* can synthesise testosterone *de novo*. Exposure to chlordecone tended to reduce the concentration of the esterified form of the steroid, whereas the free form remained unchanged. This hydrolysis of testosterone fatty acid esters might reflect a homeostatic response to a decrease in the synthesis of testosterone.

Our results suggest that the endogenous synthesis of testosterone might be affected differently by the anti-androgenic and the oestrogenic molecules tested. The neuro-endocrine control of steroidogenesis is still poorly understood in invertebrates. In vertebrates, the synthesis of sex steroids (i.e., testosterone in males and oestradiol in females) is regulated by the hypothalamic-pituitary-testicular axis (IPCS 2002). Low testosterone or oestradiol concentrations induce the release of the gonadotropin-releasing hormone (GnRH) by the hypothalamus. This hormone stimulates the production of follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary gland. These gonadotropin hormones, in turn, act on their respective target cells in the gonads, thereby stimulating steroidogenesis. The increase in steroid concentrations finally induces a negative feedback control of the release of GnRH, thus stopping steroidogenesis (IPCS 2002). GnRH was identified in different mollusc species (e.g., *Octopus vulgaris*, *Helisoma trivolvis*) (Iwakoshi et al. 2002; Young et al. 1999). In *Lymnaea stagnalis*, this hormone was found in the lateral and ventral lobes of the male part of the cerebral ganglia, whereas it was found only in the lateral lobes in the female part of the cerebral ganglia (Young et al. 1999). Even though the physiological role of GnRH is not known, the innervation pattern suggests that this hormone might play a role in the reproduction of *L. stagnalis*. However, it remains to be investigated if this hormone regulates steroidogenesis in molluscs and whether chlordecone and cyproterone acetate can interact with the synthesis/release of this hormone or on its target organs.

Moreover, it might be hypothesised that chlordecone and cyproterone acetate interact with enzymes involved in steroidogenesis, either by stimulating or repressing their activity (Fig. 9.3). A study of *in vitro* exposure of rat cells to chlordecone has reported that the pesticide was able to inhibit the conversion of pregnenolone to 20α-dihydroprogesterone (Warner 1987), suggesting an inhibition of hydroxysteroids dehydrogenases (HSDs). In molluscs, different HSDs were identified (Part 1: 2.2; Fig. 9.3)(Fernandes et al. 2010), which supports the hypothesis that chlordecone induces a reduction in steroid concentrations, testosterone and oestradiol, through inhibition of HSDs. The impacts of cyproterone acetate on steroidogenesis were investigated *in vitro* on adrenal cells of guinea pigs. Cyproterone acetate increased the production of 17-hydroxyprogesterone and of dehydroepiandrosterone (DHEA) at low concentrations (<2μg/mL), probably through interactions with enzymes such as HSDs (Pham-Huu-Trung et al. 1984). This increase in steroid precursors of testosterone, which were also identified in mollusc species (Part1: 2.2; Fig. 9.3; (Gottfried and Dorfman 1970)), could be responsible for the increase in endogenous testosterone concentrations observed in *L. stagnalis* tissues. Even though our results have highlighted that cyproterone acetate increases the *de novo* synthesis of testosterone, the testosterone fatty acid ester concentrations were not increased. These results suggest that cyproterone acetate can induce testosterone synthesis but can also inhibit the homeostatic regulation of testosterone through esterification. This homeostatic regulation of testosterone is mediated by the activity of fatty acid acyl-CoA:testosterone acyltransferase (ATAT). Therefore, it is possible that cyproterone acetate also inhibits the activity of these enzymes, resulting in the inhibition of testosterone esterification reported in this study (Fig. 9.3).

Finally, the organotin TBT had not impacts on testosterone synthesis. Studies, led on the mechanisms of action involved in the development of imposex in gonochoric gastropod species, have highlighted that this chemical was able to alter endogenous concentrations of steroids (i.e., testosterone, oestradiol). TBT alter the endogenous steroid concentrations through either an inhibition or repression of the activity of enzymes involved in the metabolisation of testosterone (i.e., aromatase, acyl:coA acyltransferase) (Bettin et al. 1996; LeBlanc et al. 2005; Ronis and Mason 1996; Sternberg and LeBlanc 2006). However, gender- and species specific differences in

endogenous concentrations of steroids were observed. Our results suggest that TBT does not alter steroidogenesis in this *L. stagnalis*.

Further analyses of the levels of oestradiol and progesterone in individuals exposed to the different molecules, and *in vitro* or *in vivo* investigations on the modification in the activity of enzymes involved in steroidogenesis (i.e., HSDs, ATAT, aromatase), could help to provide new insights on the interactions of theses chemicals with steroidogenesis in this hermaphrodite species and in other mollusc species.

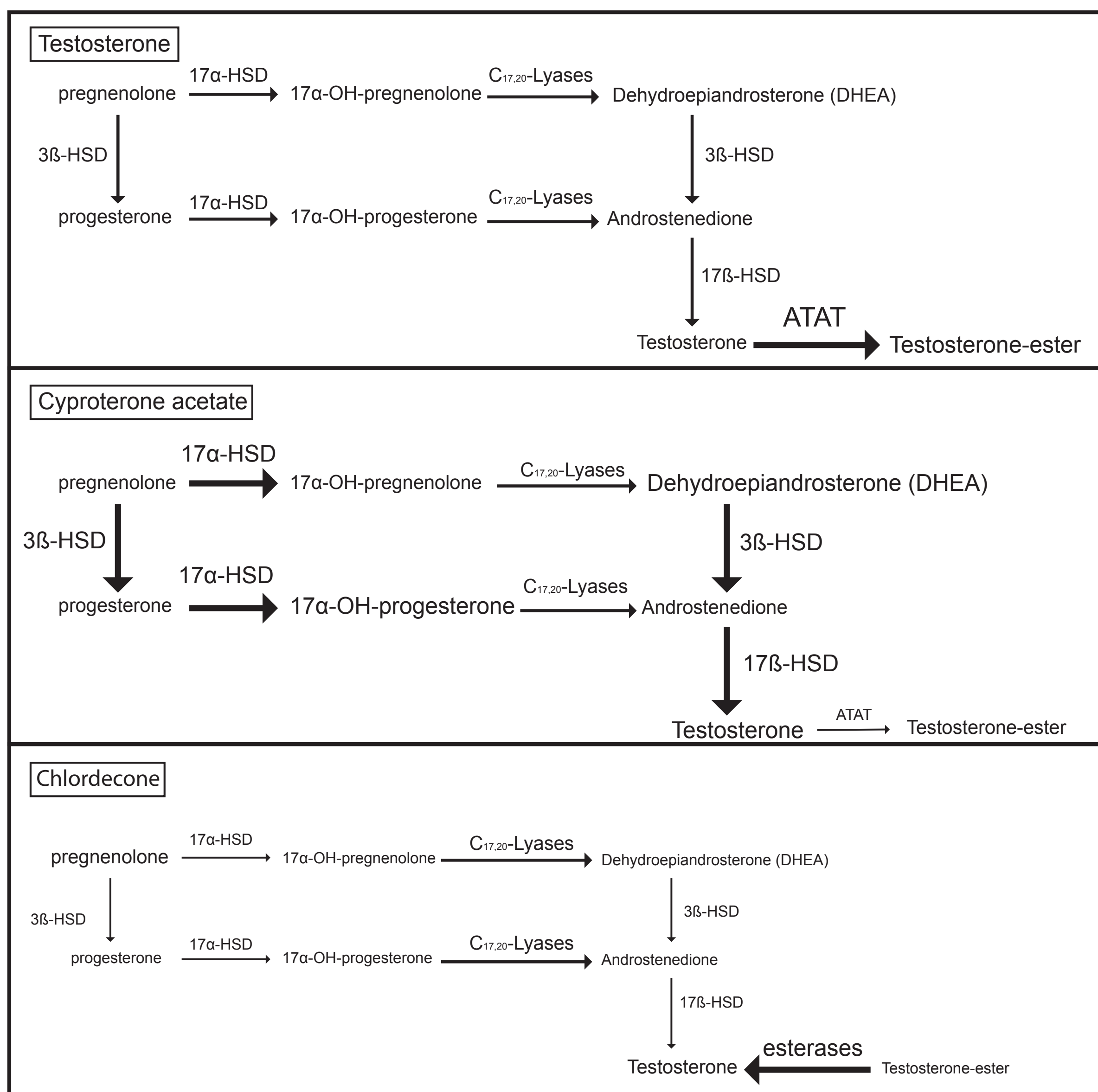

Fig. 9.3: Hypothesis on the impacts of testosterone, cyproterone acetate and chlordecone on steroidogenesis in *Lymnaea stagnalis*. Arrow thickness reflects the enzyme activity. Font size reflects the concentration of metabolites. 3ß-HSD: 3ß-hydroxysteroid dehydrogenases, 17α/ß-HSD: 17α/ß-hydroxysteroid dehydrogenases, ATAT: fatty acid acyl-CoA:testosterone acyltransferase.

Even thought androgen receptors have never been identified in any mollusc species (Reitzel and Tarrant 2010), a few publications have reported impacts of steroids on the reproduction and the development of sex organs. In the sea scallop, *Placopecten magellanicus*, *in vitro* exposure to 10^{-6}M of testosterone facilitated the sperm release from male gonads, however egg release from female gonads was not affected. It was reported as well that oestradiol and progesterone stimulated egg and sperm release in both genders (Wang and Croll 2003). Furthermore, *in vivo* injections of steroids increased the differentiation of gonads in juveniles, and a significant change of the sex ratio, towards males, was observed following exposure to testosterone, progesterone and oestradiol (Wang and Croll 2004). The synthetic androgen, methyltestosterone, increased the male/female ratio in clams, *Mulinia lateralis* (Moss 1989). This androgen also induced the development of imposex in juvenile females *Marisa cornuarietis* from 100 ng/L, whereas it failed to increase male penis size (Schulte-Oehlmann 2004). Finally, exposure to 500 ng/L of testosterone for 5 months induced imposex development at a similar degree to TBT-induced imposex in *N. lapillus* and *H. reticulata* (Bettin et al. 1996). Together, these reports suggest that vertebrate-like sex steroids play a physiological role on the reproduction of some gonochoric species. In the present thesis, concentration of the free, putatively bioactive, form of testosterone was only increased after exposure to cyproterone acetate. This pharmaceutical had no impact on oviposition and fecundity of *L. stagnalis*. Moreover, its impacts on the quality of eggs were similar to those reported after exposure to testosterone, chlordecone and TBT. Therefore, it is possible that testosterone does not play any physiological activity on the reproduction of *Lymnaea stagnalis*.

Further investigations on the impacts of chemicals on other steroid hormones are required to understand the differences of endogenous synthesis and homeostasis of androgens and oestrogens. Moreover, histological analyses of reproductive organs of *L. stagnalis* exposed to vertebrate-like steroid hormones might provide precious information on the role of these hormones in the development of the male and female organs as observed in other mollusc species.

4. Conclusion

The aim of this thesis focused on identifying and describing the impact of molecules, which present different endocrine disrupting properties in vertebrates (i.e., testosterone, tributyltin, cyproterone acetate, fenitrothion, vinclozolin and chlordecone), on the reproduction of the hermaphroditic pulmonate gastropod *Lymnaea stagnalis*. The results obtained through our laboratory experiments have highlighted that some of these chemicals could adversely alter mollusc reproduction by altering the production of eggs. The quality of eggs appears to be an essential parameter in the assessment of the reprotoxicity of chemicals, including EDCs. Indeed, we observed that the different chemicals tested in this work could adversely impact the quality of eggs, and this at lower concentrations than those affecting the classical reproductive endpoints assessed in this species. Furthermore, along with the identification of alterations of proteins involved in gametogenesis, vitellogenesis and oviposition, the types of egg abnormalities might provide new insights on the mechanisms of action of these compounds. Moreover, investigations of the mechanisms of actions of these putative EDCs showed that endogenous synthesis and homeostasis of testosterone, a vertebrate-like steroid, was disrupted in individuals exposed to testosterone, cyproterone acetate and chlordecone but not to tributyltin. The results obtained in the present work provide evidence of interactions of the chemicals tested with the endocrine system of this hermaphrodite species. *Lymnaea stagnalis* is proposed as a candidate species for the development of OECD guidelines for the Testing and the Assessment of EDCs in molluscs (OECD 2010). Therefore, the additional sensitive reproductive endpoints described in this thesis (i.e., egg quality) as well as the identification of particular proteins (i.e., ovipostatin, yolk ferritin and PIWI) and their differential expression after exposure to chemicals should provide reliable tools to screen the potency of toxicants to be endocrine disruptors in a partial life cycle test on the freshwater gastropod *Lymnaea stagnalis*. This thesis highlights that, due to a lack of knowledge on mollusc endocrinology, the mechanisms of action of putative EDCs are still poorly understood. The actions exerted by neuro-endocrine peptides on the different physiological pathways, involved in egg production and oviposition, remain to be investigated. Moreover, understanding the interactions of chemicals with

steroidogenesis, and elucidating the physiological roles exerted by these vertebrate-like steroid hormones in mollusc reproduction is required in order to provide new insights on the mode of actions of these compounds.

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