CHAPTER 3. *IN VIVO* EFFECTS OF XENOBIOTIC EXPOSURES ON STEROID LEVELS AND STEROID METABOLISM IN MOLLUSCS.

Paper 5

EFFECTS OF 17η-ESTRADIOL EXPOSURE IN THE MUSSEL *MYTILUS GALLOPROVINCIALIS*: THE REGULATING ROLE OF STEROID ACYLTRANSFERASES.

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Effects of 17β-estradiol exposure in the mussel *Mytilus galloprovincialis*: A possible regulating role for steroid acyltransferases

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Abstract

Mussels (*Mytilus galloaprovincialis*) were exposed to different concentrations of estradiol (20, 200, and 2000 ng/L) in a semi-static regime (1-day dosing intervals) for up to 7 days in an attempt to see how mussels deal with exogenous estrogenic compounds. Whole tissue free-estradiol levels were only significantly elevated at the high exposure dose, whereas total-estradiol (free + esterified) sharply increased in a dose-dependent manner, from 2 ng/g in controls to 258 ng/g at the high exposure group. Neither free nor esterified testosterone levels showed significant differences between control and exposed organisms. The results suggest the existence of mechanisms that allow mussels to maintain their hormonal levels stable, with the exception of the high exposure dose, and the important role that fatty acid esterification, e.g. palmitoyl-CoA:estradiol acyltransferases, may play within those mechanisms. Additionally, the activity of 17β-hydroxysteroid dehydrogenase (17β-HSD), 5α-reductase, P450-aromatase, and estradiol-sulfotransferases were investigated in digestive gland microsomal and cytosolic fractions. All these activities were differently affected by estradiol exposure. Overall, the study contributes to the better knowledge of molluscan endocrinology, and defines new mechanisms of regulation of free steroid-levels in mussels.

Keywords: Estradiol; *Mytilus galloprovincialis*; Estradiol acyltransferases; Androstenedione; P450-aromatase; Estradiol sulfation

1. Introduction

There is evidence in the environment that some pollutants, known as endocrine disrupters, interfere with the hormonal function in wildlife. Natural and synthetic estrogens, e.g. phytoestrogens, natural steroids, pharmaceuticals, as well as a variety of industrial chemicals, e.g. 4-nonylphenol, bisphenol-A, plasticizers, herbicides, and pesticides, have been recognized as environmental estrogens (Colborn et al., 1993). The occurrence of estrogenic and xenoestrogenic compounds in inland waters, estuaries and the open sea (Lai et al., 2002; Atkinson et al., 2003), and the existence of related endocrine alterations in several fish species has been demonstrated (Vos et al., 2000).

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Similar to vertebrates, molluscs and other invertebrates may be adversely affected by endocrine disrupters (EDs). In fact, some mollusc species are known to be very sensitive to the androgenic effects of organotin compounds: exposure to tributyltin (TBT) causes imposex (imposition of male sexual characteristics in females) in marine gastropods at a concentration as low as 1 ng/L (Matthiessen and Gibbs, 1998). Exposure to estrogenic effluents increased vitellin-like proteins (a biomarker of estrogenic exposure widely used in fish) in the hemolymph and gonads of the clam *Mya arenaria*, and the mussel *Elliptio complanata* (Gagné et al., 2001, 2002). Environmental estrogens, such as bisphenol-A, 4-*tert*-octylphenol, and ethinylestradiol, have been shown to affect egg or embryo production in prosobranch snails (Oehlmann et al., 2000; Jobling et al., 2003).

However, the mechanisms of action of EDs in invertebrates are not well understood. The interaction of EDs with the estrogen receptor and other sex steroid binding proteins, or the interference with the biosynthesis/metabolism of steroids are potential mechanisms of endocrine alteration. Key steroidogenic pathways have been identified in different mollusc species, i.e. 3β-hydroxysteroid reductase (3β-HSD), 17β-HSD, 5α-reductase, and P450-aromatase (De Longcamp et al., 1974; Hines et al., 1996; Ronis and Mason, 1996; Morcillo et al., 1998; Le Curieux-Belfond et al., 2001). Although the physiological relevance of those vertebrate-type steroids/pathways in invertebrates is still under debate, the existing evidence suggest that the invertebrate endocrine system functions in some aspects similarly to the vertebrate system, e.g. it transduces environmental and endogenous signals to appropriate target sites in order to elicit the required response (LeBlanc et al., 1999). Also, very recently, the complete sequence of the estrogen receptor has been reported in the mollusc *Aplysia californica* (Thornton et al., 2003), supporting the idea that estradiol functions in molluscs through a receptor-mediated mechanism.

Together with biosynthetic pathways, conjugation rates of steroids can play a key role in endocrine homeostasis (Hochberg, 1998; Strott, 1996), and may regulate levels of active steroids within target tissues. However, data regarding conjugation of steroids in molluscs is rather limited and based primarily on in vivo observations. Sulfate conjugates of steroid hormones have been identified in the gastropod *Clione antartica* (Hines et al., 1996), and a decrease of testosterone sulfation was reported for *Littorina littorea* exposed to TBT (Ronis and Mason, 1996). Additionally, apolar conjugation has recently been shown to be a major pathway of testosterone metabolism in the snail *Ilyanassa obsoleta* (Gooding and LeBlanc, 2001), and the conjugation of vertebrate-type steroids, i.e. estradiol and dehydroepiandrosterone (DHEA) with different fatty acids has been reported for the oyster *Crassostrea virginica* (Janer et al., 2004).

Within this context, the study was designed to better characterize the response of the mussel *Mytilus galloprovincialis* to estradiol (as a model estrogenic compound) exposure by looking at key enzymatic pathways involved in both steroid hormone synthesis and clearance in mussels (Fig. 1). Activities of 17β-hydroxysteroid dehydrogenase (17β-HSD), 5α-reductase and P450-aromatase were determined in digestive gland microsomal fractions, since previous studies indicated high specific activity of those enzymes in the digestive gland (R. Lavado, unpublished data). Activities of estradiol-sulfotransferases

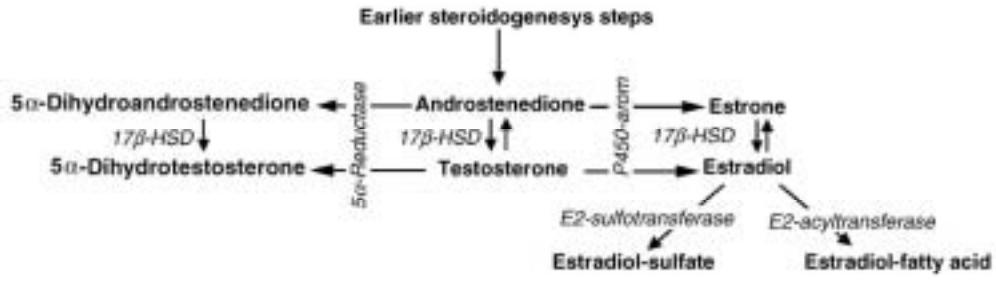

Fig. 1. Key enzymatic pathways involved in steroid hormone synthesis and clearance in mussels investigated in this study. HSD: hydroxysteroid dehydrogenase.

and palmitoyl-CoA:estradiol acyltransferases were also determined. An alteration of those pathways would affect levels of androgens and estrogens within the tissue, and might affect key physiological processes. Tissue levels of testosterone and estradiol were measured in the whole tissue of mussels to assess potential alterations on hormone levels. A mild saponification step was included in the extraction protocol in order to distinguish between free and esterified steroids (released after saponification) (Gooding et al., 2003). The histological analysis of gonads allowed the determination of the reproductive development status of the mussels. Overall, the work aimed at (a) identifying alterations on the endocrine system of mussels as a consequence of exposure to different concentrations of estradiol, and (b) facilitating future assessments of the effects of xenoestrogens in molluscs.

2. Material and methods

2.1. Chemicals

17β-Estradiol, androstenedione, and 5α-dihydrotestosterone were obtained from Sigma (Steinheim, Germany), 5α-dihydroandrostenedione from Steraloids (Wilton, NH). [6,7-^{3}H]17β-estradiol (40–60 Ci/mmol) and [1β-^{3}H]androstenedione (15–30 Ci/mmol) were obtained from PerkinElmer Life Science Inc (Boston, MA).

Radioimmunoassay (RIA) kits for testosterone and 17β-estradiol were obtained from ICN Pharmaceuticals, Inc (Costa Mesa, CA). All solvents and reagents were of analytical grade.

2.2. Experimental design

Mussels (*Mytilus galloprovinciallis*) were collected in June 2001 from the bivalve farms located in the Ebro Delta (NE Spain), carried to the laboratory, and randomly placed into 50 L glass aquaria (40 mussels/tank) filled with filtered sea-water, and fitted with constant air bubbling. The mussels were acclimated in the laboratory for 2 days. Environmental conditions, i.e. temperature (18 °C), salinity (35‰), and photo-period simulated the original conditions of mussels.

After acclimation, mussels were exposed to different concentrations of estradiol: 20 ng/L (L; low dose), 200 ng/L (M; medium dose), and 2000 ng/L (H; high dose), or to the solvent, i.e. 0.004% (v/v) triethyleneglycol (C; control tank). The nominal concentration of the solvent in the exposed tanks was 0.004% (v/v) triethyleneglycol. Water was changed every day, and fresh estradiol added. Mussels were fed every 48 h with a commercially available plankton preparation (Advanced Invertebrate 1, Marine Enterprises, INC, Baltimore, MD, USA). After a 7-day exposure, mussels were dissected; the digestive glands and rest of the tissue stored at −80 °C for determination of enzyme activities and steroid levels, respectively. Gonads of 10 mussels per tank were dissected and placed in buffered formalin (0.1 M sodium phosphate, pH 7.4) for histological examination.

Water samples (500 mL) were collected immediately after dosing, and 24 h later (on days 3rd and 7th after dosing had started); 1% formaldehyde was added, and bottles kept at 4 °C until analysis.

2.3. Sex hormone analysis

Tissue levels of free testosterone and estradiol were analyzed as described in Morcillo et al. (1999). Briefly, tissue samples (1.0–1.5 g wet weight) were homogenized in ethanol, and frozen overnight at −80 °C. Homogenates were then extracted with diethyl ether, followed by two further extractions with diethyl ether:ethanol (4:1). The organic extract was evaporated under nitrogen, and redissolved in 80% methanol. This solution was then washed with petroleum ether to remove the lipid fraction and evaporated to dryness. The dry residue was redissolved in 4 mL milli-Q water and passed through a C18 cartridge (Isolute, International Sorbent Technology, Mid Glamorgan, UK; 1 g, 6 mL), that had been sequentially pre-conditioned with methanol (4 mL) and milli-Q water (8 mL). After finishing the concentration step, cartridges were washed with milli-Q water (8 mL), dried, and connected to a NH2 cartridge (Sep-Pack® Plus; Waters, Milford, MA, USA). The C18–NH2 system was then washed with 8 mL hexane, and the steroids eluted with 9 mL dichloromethane:methanol (7:3). This fraction was collected and evaporated to dryness.

Total testosterone and total estradiol (free + esterified) were measured as described by Gooding et al. (2003), with some modifications. Tissue, homogenized as for free steroid determination (see above), was

extracted with ethyl acetate (3× 2 mL). The organic extract was evaporated under a nitrogen stream, resuspended in 1.0 mL methanol containing 0.1% KOH, and kept at 45 °C for 3 h. After the saponification step, milli-Q water (4.0 mL) was added, and the sample extracted with dichloromethane (3× 3 mL).

The efficiency of the extraction and delipidation procedure was 74 ± 3% for testosterone, and 80 ± 3% for estradiol (Morcillo et al., 1999). The recovery for the purification step (SPE cartridges) was evaluated in this study with radiolabelled steroids, and it was in the range 95–97% for both, testosterone and estradiol.

Water samples were filtered (0.45 μm), and an aliquot (100 mL) extracted with dichloromethane (3× 20 mL), and evaporated to dryness prior to RIA analyses.

Dry extracts (tissue and water samples) were resuspended in 50 mM potassium phosphate buffer pH 7.6 containing 0.1% gelatin, and assayed for estradiol and testosterone concentration using commercial RIA kits. Standard curves with the steroids dissolved in the same phosphate buffer were performed in every run. The limits of detection were of 3 pg/g for estradiol and 30 pg/g for testosterone in tissues, and 0.1 ng/L estradiol in water. Intra-assay coefficients of variation were of 9.5% (testosterone) and 5.5% (estradiol). Inter-assay coefficients of variation were 11.6% (testosterone) and 7.6% (estradiol).

2.4. Subcellular fractionation

Digestive glands (2–3) were homogenized in 5 mL of ice-cold 100 mM KH_2PO4/K_2HPO_4 buffer pH 7.4, containing 0.15 M KCl, 1 mM dithiothreitol (DTT), 1 mM EDTA and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Homogenates were centrifuged at 500 × *g* for 15 min, the fatty layer removed and the supernatant centrifuged at 12,000 × *g* for 45 min. After centrifugation at 100,000 × *g* for 60 min, the supernatant, termed cytosol, was collected and stored at −80 °C until assays were performed. The pellet was resuspended with the same buffer and centrifuged again at 100,000 × *g* for 30 min. Microsomal pellets were resuspended in a small volume of 100 mM Tris–HCl pH 7.4 containing 0.15 M KCl, 20% (w/v) glycerol, 1 mM DTT, 1 mM EDTA and 0.1 mM PMSF. Protein concentrations were determined by the method described by Lowry et al. (1951), using bovine serum albumin as a standard.

2.5. Androstenedione metabolism

Assays were carried out by incubating 0.4 mg of digestive gland microsomal protein in 50 mM Tris–HCl buffer pH 7.4, 10 mM $MgCl_2$, and 0.1 μM ^{3}H-androstenedione (0.1 μCi). Assays were started by addition of NADPH (320 μM), and samples incubated for 60 min in a shaking water bath maintained at 25 °C (final volume 250 μL). Incubations were stopped by adding 250 μL of acetonitrile, and after centrifugation (1500 × *g*, 10 min) 200 μL of supernatant were injected onto the RP-HPLC column.

P-450 aromatase activity was determined by the tritiated-water release method as in Morcillo et al. (1999). Microsomes (0.5 mg protein) were incubated at 25 °C for 3 h in a final volume of 1 mL in the presence of 100 mM Tris–HCl, pH 7.6, 10 μM androstenedione (1 μCi), and 0.2 mM NADPH. Assay blanks containing 100 μL of buffer instead of microsomes were used for every run. The reaction was stopped by placing the tube on ice, and organic metabolites and the excess of substrate were immediately eliminated from the aqueous phase by extraction with methylene chloride (3× 3 mL). The possible remaining tritiated steroids were further eliminated by the addition of a suspension of 2.5% (w/v) activated charcoal and 0.25% dextran in milli-Q water (4 mL). The solution was centrifuged (60 min, 1500 × *g*), and two aliquots of the supernatant (1 mL) were counted for ^{3}H radioactivity (Tri-Carb 2100TR, Packard). The limit of detection of the method was 0.2 pmol/h/mg protein.

2.6. Phase II metabolism of estradiol

Palmitoyl-CoA:estradiol acyltransferase activity was determined by a modification of the method described by Janer et al. (2004). Microsomal proteins (500 μg) were incubated in 0.1 M sodium acetate buffer pH 6.0 with 2 μM [^{3}H]estradiol, 100 μM palmitoyl-CoA (Sigma, Steinheim, Germany), and 5 mM $MgCl_2$ in a final volume of 500 μL. The reaction was initiated by the addition of palmitoyl-CoA, and samples were incubated for 90 min at 35 °C. Reaction was stopped by adding 2 mL of ethyl acetate, and extracted twice. The ethyl acetate fraction was evaporated to dryness,

the dry residue redissolved in methanol, and injected into the HPLC system. The limit of detection of the method was 10 pmol/h/mg protein.

To determine estradiol sulfation, cytosolic proteins isolated from the digestive gland (100 μg) were incubated in 50 mM Tris–HCl buffer pH 9.0, containing 4 mM $MgCl_2$, and 2 mM Na_2SO_3, with 100 nM [^{3}H]estradiol, in a final volume of 150 μL. The reaction was initiated by the addition of 10 μM adenosine 3′-phosphate 5′-phosphosulfate (PAPS; Cal-Biochem, Darmstadt, Germany), and incubated for 60 min at 30 °C. The reaction was stopped with 2 mL methylene chloride, after addition of 200 μL of ice-cold 50 mM Tris–HCl buffer, pH 8.7. The extraction of unconjugated estradiol was completed with 2 mL of methylene chloride, and an aliquot of the aqueous phase was quantified by liquid ^{3}H scintillation counting (Tri-Carb 2100TR, Packard). The limit of detection of the method was 0.8 pmol/h/mg protein.

2.7. HPLC system

HPLC analyses were performed on a PerkinElmer Binary 250 LC pump system equipped with a 250 mm × 4 mm LiChrospher 100 RP-18 (5 μm) reversed-phase column (Merck, Darmstadt, Germany) protected by a guard column LiChrospher 100 RP-18 (5 μm). Separation of androstenedione metabolites was performed at 1 mL/min with a mobile phase composed of (A) 75% water and 25% acetonitrile and (B) 25% water and 75% acetonitrile. The run consisted on a linear gradient from 100% A to 100% B (0–30 min), isocratic mode at 100% B (5 min), linear gradient from 100% B to 100% A (5 min), and isocratic mode at 100% A (5 min). Separation of estradiol and its palmitoyl-ester was performed at 1.2 mL/min with a mobile phase composed of (A) 56% water containing 0.1% acetic acid (pH 3), 13% acetonitrile, and 31% methanol, and (B) 60% acetonitrile and 40% methanol. The run consisted of 9 min isocratic 100% A, 6 min of a linear gradient from 100% A to 100% B, and 25 min isocratic 100% B. Chromatographic peaks were monitored by on-line radioactivity detection with a Radioflow detector LB 509 (Berthold Technologies, Bad Wildbad, Germany) using Flo Scint 3 (Packard BioScience, Groningen, The Netherlands) as scintillation cocktail. Metabolites were quantified by integrating the area under the radioactive peaks. Metabolites were analyzed by gas chromatography–mass spectrometry (EI+) as trimethylsilyl derivatives, and the chemical structures were identified by comparison of the retention times and the mass spectra with authentic standards (Sigma, Steinheim, Germany, and Steraloids, Wilton, NH, USA).

2.8. Histological analysis

Gonad tissue fixed in 10% formaldehyde buffered with 100 mM sodium phosphate buffer pH 7.4, was dehydrated with ethanol, cleared in xylene, and embedded in paraffin. Sections (7 μm) were stained with Haematoxylin Eosin Y (Shandon Inc, Pittsburgh, PA, USA), mounted, and examined by light microscopy. Individuals were classified in six different reproductive stages as described by Hillman (1993). Stage 0: sexual rest, inactive or undifferentiated; stage 1: gametogenesis has begun, no ripe gametes visible; stage 2: follicles and spermatogonia/oogonia visible; stage 3: espermatogonia/oogonia and gametes visible; stage 4: follicles contain mainly ripe gametes, few reserve cells; stage 5: fully mature gonads, almost no reserve cells.

2.9. Statistical procedures

Results are mean values ± S.E.M. of n samples (each sample a pool of 2–3 digestive glands or tissues). Statistical significance was assessed by using one-way ANOVA (followed by Dunnett's test for differences from control).

3. Results

3.1. Estradiol concentration in water

Water estradiol levels determined in the experimental tanks right after dosing were close to nominal concentrations, viz. 20 ± 2, 217 ± 144, and 2988 ± 730 ng/L (values are mean ± range of two different sampling days). However, 24 h after dosing, estradiol levels in water sharply decreased to 17 ± 15, 13 ± 1 and 129 ± 8 ng/L. Detectable levels of estradiol, in the range of 1–12 ng/L, were recorded in the control tank.

3.2. Tissue steroid levels

Steroid levels were determined in the whole tissue of mussels, with or without a saponification step, in order to differentiate between free and esterified forms. No differences in free-estradiol levels were observed between control (C), and low (L) or medium (M) exposure groups (1.1–2.6 pg/g, w/w), whereas the high exposure (H) group exhibited a significant 10-fold increase in free-estradiol levels (13 pg/g, w/w) (Fig. 2A). Nonetheless, total estradiol (free + esterified) increased in a dose-dependent manner, from 2 ng/g in C to 258 ng/g in H (Fig. 2A). Values of free and total estradiol were similar in mussels from tanks C and L, indicating that most of the estradiol within the tissue was in the free form. Only individuals from tanks M & H had significant amounts of esterified estradiol released after a mild saponification step. In contrast, testosterone levels (free and total) did not differ between control and exposed groups (0.9–1.8 pg/g, w/w) (Fig. 2B).

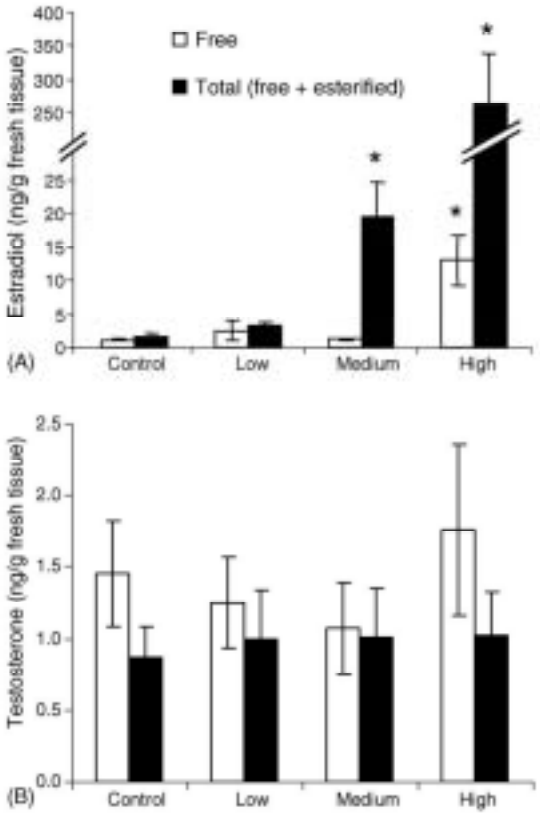

Fig. 2. Whole tissue levels of free and total (A) estradiol and (B) testosterone in control and estradiol-exposed specimens. Data expressed as mean ± S.E.M. ($n = 5$–6). Significant differences with respect to controls indicated by $^{*}p < 0.05$ (one-way ANOVA followed by Dunnet's test).

3.3. Androstenedione metabolism

Mussel digestive gland microsomes metabolized androstenedione mainly to 5α-dihydroandrostenedione (DHA), a step catalyzed by 5α-reductases, and to a minor degree to 5α-dihydrotestosterone (DHT) and testosterone (T) (Fig. 3). The two 5α-reduced metabolites (DHT and DHA) increased in a dose-dependent manner with estradiol exposure (C: 28 pmol/h/mg protein; exposed organisms: 29–78 pmol/h/mg protein). This increase was statistically significant at the medium (DHA formation), and high exposure groups (DHA and DHT formation) (Fig. 4A and B). The synthesis of testosterone from androstenedione was not affected by estradiol exposure (C: 0.9 pmol/h/mg protein; exposed organisms: 0.8–1.3 pmol/h/mg protein) (Fig. 4C).

The endogenous synthesis of estradiol was investigated by measuring the activity of P450-aromatase in control and estradiol-exposed specimens. Depressed P450-aromatase activity was observed in organisms exposed to low estradiol dose (C: 1530 fmol/

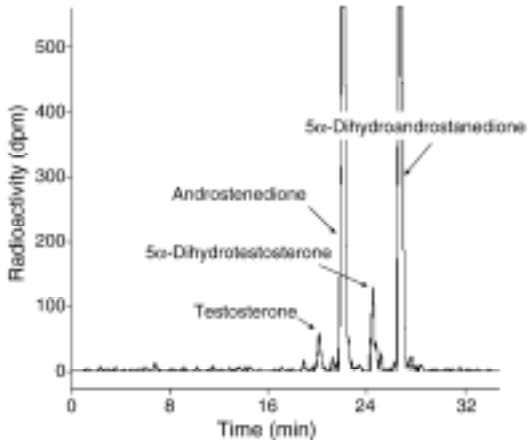

Fig. 3. Radio-HPLC chromatogram obtained for the metabolism of androstenedione by digestive gland microsomal fraction.

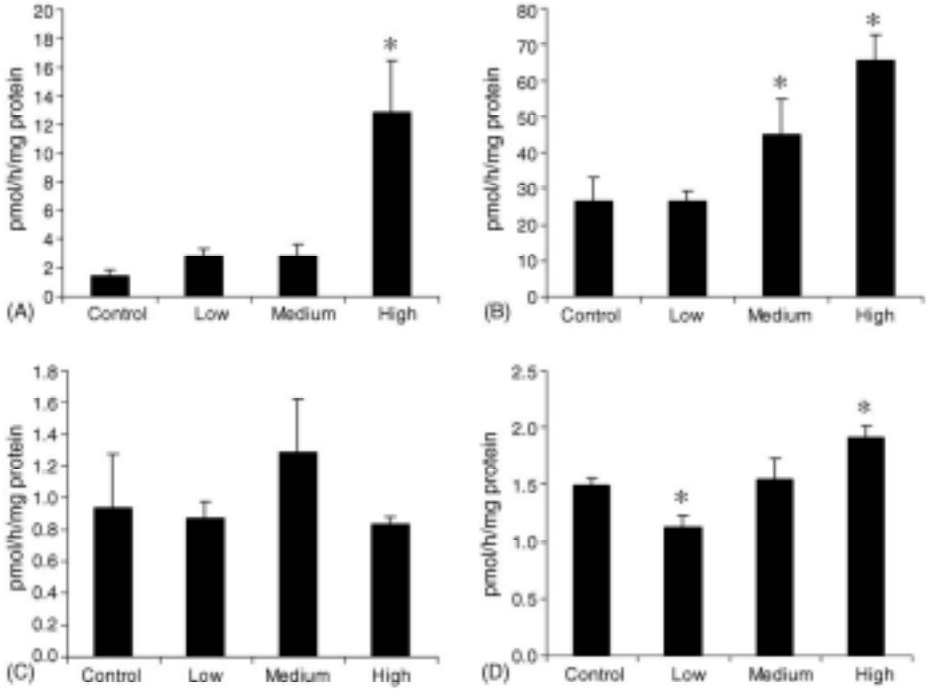

Fig. 4. Androstenedione metabolism by digestive gland microsomal fractions of control and estradiol-exposed specimens. Formation of (A) 5α-dihydrotestosterone, (B) 5α-dihydroandrostenedione, (C) testosterone, and (D) P450-aromatase activity. Data expressed as mean ± S.E.M. ($n = 5$). Significant differences with respect to controls indicated by $^{*}p < 0.05$ (one-way ANOVA followed by Dunnet's test).

h/mg protein; L: 1115 fmol/h/mg protein), but the trend was reversed at the high exposure dose (C: 1530 fmol/h/mg protein; H: 1952 fmol/h/mg protein), where organisms had significantly higher P-450 aromatase activity than controls (Fig. 4D).

3.4. Phase II metabolism of estradiol

Palmitoyl-CoA:estradiol acyltransferase activity increased in a dose-dependent manner (Fig. 5A). The increase was statistically significant at the medium (61 ± 9 pmol/h/mg protein) and high (94 ± 10 pmol/h/mg protein) exposure groups, and this is fully in agreement with the high levels of esterified estradiol detected in mussels from tanks M & H (Fig. 2A). The sulfation of estradiol by digestive gland cytosolic fractions was in the range of 6–12 pmol/h/mg protein, and no differences between control and exposed groups were observed, despite a non-significant increase in estradiol sulfation in mussels from tank H (Fig. 5B).

3.5. Gonad histology

Gonads were examined to assess whether changes in gamete maturation had occurred as a consequence of estradiol exposure. The gonads in most of the individuals (8 out of 10) in tank C were classified as stage 0, thus not developed. However, in the low exposure group, there were fewer individuals with stage 0 gonads (3 out of 10), and correspondingly a higher number of individuals exhibiting mature (stages 3–5) gonads (Fig. 6). This tendency towards higher proportion of mature individuals in tank L was not observed in tanks M & H. Despite the number of individuals analyzed in this study being low (10 animals per tank), the same trend was observed in a replicated experiment.

4. Discussion

The analysis of water revealed daily oscillations in estradiol levels, which right after dosing were close to nominal concentrations (with the exception of thank

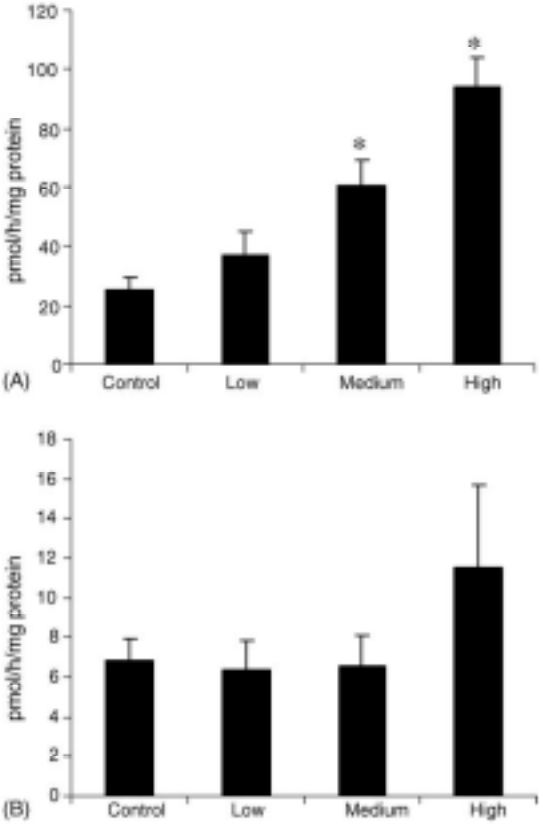

Fig. 5. Phase II metabolism of estradiol. Palmitoyl-CoA:estradiol acyltransferase activity (A), estradiol-sulfotransferase activity (B) in control and estradiol-exposed specimens. Data expressed as mean ± S.E.M. ($n = 5$). Significant differences with respect to controls indicated by $^{*}p < 0.05$ (one-way ANOVA followed by Dunnet's test).

H), but decreased sharply during 24 h of exposure in tanks M & H. The high estradiol levels measured in tank H right after dosing are an indication of a certain build-up of the compound in the experiment. The control tank also had measurable levels of estradiol (1–12 ng/L); the source of estradiol is uncertain as the experiment was carefully designed to avoid cross-contamination between tanks. Similar estradiol levels have been reported in control tanks from other studies, e.g. Halm et al. (2002) reported 1.9–2.3 ng/L; Kramer et al. (1998) had values of 3.5–14.8 ng/L despite of using relatively low stocking densities offish and a high throughput of water. There are no similar data for mussels, but the excretion of estradiol into the water due to

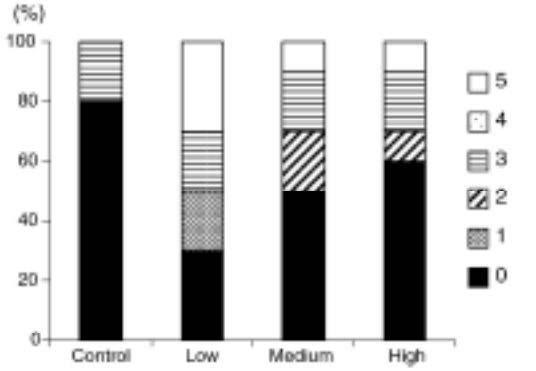

Fig. 6. Distribution of gonad developmental stages in the experimental groups. Gonads were classified in six groups as indicated in Section 2; 10 individuals examined per tank.

handling stress cannot be discarded. Certainly, the presence of endogenous steroids in the tanks is an important issue that should be addressed when designing experiments of this nature.

Free-estradiol levels in mussel tissue were rather stable; they did not increase with estradiol exposure, except for the highest exposure group, that exhibited a significant 10-fold increase. Estradiol levels observed in mussels from tanks C, L & M, but not in those from H, were in the range of the levels naturally occurring in mussels along their reproductive cycle (0.6–1.1 ng/g) (C. Porte, unpublished data).

However, when tissue extracts were saponified in order to release esterified estradiol, mussels from tanks M & H showed a 12- and 160-fold increase in total estradiol (Fig. 2). These results suggest that esterification of the excess of estradiol with fatty acids might act as a homeostatic mechanism to maintain endogenous levels of free-estradiol stable, and this mechanism was only overloaded in mussels from tank H. The increased esterification of estradiol in organisms from tanks M & H is supported by the increased activity of fatty palmitoyl-CoA:estradiol acyltransferase detected in those organisms (Fig. 5).

There is no information on the physiological function of steroid esters in molluscs. Fatty acid conjugation (or esterification) renders steroids to an apolar form, which is retained in the lipoidal matrices of the body, while reducing their activity, bioavailability, and susceptibility to elimination (Borg et al., 1995). It is known that esterified steroids do not bind to steroid receptors, but they have been considered as long-acting

steroids, since they can be hydrolyzed by esterases, and the active hormones can be released whenever needed (Hochberg, 1998). Gooding et al. (2003) indicated that the esterification of testosterone with fatty acids might be a mechanism by which free steroid levels are regulated, and it could represent a target of pollutants toxicity. In fact, they observed that the biocide tributyltin elevates free testosterone levels in the mud snail (*I. obsoleta*) by either decreasing the synthesis or increasing the hydrolysis of testosterone-fatty acid esters.

It is worth mentioning that testosterone levels (free and total) were similar in control and exposed organisms, suggesting that the synthesis and esterification of testosterone were not altered by estradiol exposure.

Additionally, when looking at the metabolism of androstenedione by digestive gland microsomal fractions of control and exposed organisms, we observed that estradiol exposure significantly increased the formation of 5α-reduced metabolites (5α-DHT and 5α-DHA), while testosterone synthesis (a 17β-HSD catalyzed pathway) remained unchanged. This is in accordance with tissue levels of testosterone remaining stable after E2 exposure, and with data on vertebrates that indicated that exposure of cultured adult rat Leydig cells to increasing concentrations of E2 had no effect on testosterone levels (Murono et al., 2001). In mussels, and in agreement with previous data for the gastropod *Marisa cornuarietis* (Janer et al., 2005), 5α-DHT is mainly formed from 5α-DHA, and not from testosterone (Fig. 2). Thus, estradiol exposure affects mainly 5α-reductase activity, with no significant effect on 17β-HSD. The reason why mussels from the M & H groups responded to high estradiol exposure by increasing synthesis of 5α-reduced androgens is unknown. Also the potential physiological role of those 5α-reduced androgens in molluscs has to be thoroughly examined.

The aromatization of androgens into estrogens, which occurs at a very low rate in molluscs, was also affected by exposure. When mussels were exposed to low levels of exogenous estradiol, the activity was significantly reduced, possibly in an attempt to decrease endogenous levels of estradiol. However, the effect was reversed at high estradiol doses; organisms from the H-group had significantly higher P-450 aromatase activity than controls. This is well in agreement with vertebrate studies, which indicate that E2 can both up- and down-regulate aromatase activity depending on dose/time of exposure (Tsai et al., 2001; Halm et al., 2002).

Additionally, the histological study suggested that exposure of mussels to low estradiol doses induced gametogenesis. This is in accordance with an earlier study that reported a similar induction of gametogenesis in oysters due to E2 exposure (Mori et al., 1969). Although mussels from tank L did not show changes in tissue estradiol levels at the end of the exposure experiment, slight estradiol increases might have occurred earlier in the exposure and led to the described effects in gametogenesis.

Finally, estradiol sulfation by digestive gland cytosolic fractions was determined as another potential mechanism that could contribute to regulate steroid levels within the organism. To our knowledge, the in vitro sulfation of steroid hormones has not been studied in molluscs so far, and the obtained data indicate that digestive gland cytosolic fractions can form estradiol sulfates at a rate of 6 pmol/h/mg protein. However, estradiol sulfation was not significantly altered by estradiol exposure.

Overall, these results suggest that at low concentrations, estradiol might behave as an endogenous steroid in mussels and regulate physiological functions. Thus, it inhibits P450-aromatase and stimulates gamete maturation. At high concentrations, estradiol significantly increases the activity of palmitoyl-CoA:estradiol acyltransferases as a mechanism to 'inactivate' the excess of estradiol that is taken up by mussels. It also increases the synthesis of DHA and DHT from androstenedione, and P450-aromatase activity. Moreover, the obtained data suggest the existence of mechanisms that allow mussels to maintain their hormonal levels stable, and the important role that fatty acid esterification may play within those mechanisms. Further research is required to study the esterification of steroids, as well as the esterases responsible for releasing steroids from the fatty acid moiety, and the processes that affect/regulate the equilibrium between synthesis and hydrolysis of this family of steroids. The esterification of estradiol to a fatty acid ester as a strategy for steroid regulation reveals novel potential targets for endocrine disrupters.

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Paper 6

STEROID LEVELS AND STEROID METABOLISM IN THE MUSSEL *MYTILUS EDULIS*: THE MODULATING EFFECT OF DISPERSED CRUDE OIL AND ALKYLPHENOLS.

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Steroid levels and steroid metabolism in the mussel *Mytilus edulis*: the modulating effect of dispersed crude oil and alkylphenols

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ABSTRACT

Significant amounts of oil and alkylphenols are released into the sea by petroleum installations as a result of discharges of produced water. Some of these pollutants elicit estrogenic responses in fish, but their effects on the endocrine system of molluscs are largely unknown. In this study, mussels *Mytilus edulis* were exposed to North Sea oil (O) and the mixture of North Sea oil + alkylphenols (OAP), and the effects on tissue steroid levels and steroid metabolism (P450-aromatase and estradiol-sulfotransferase) were monitored. Levels of free testosterone and free estradiol were much higher in gonad tissue than in peripheral tissue, whereas esterified steroids (released after saponification) were of the same order of magnitude in both tissues. Levels of free steroids determined in gonads were not affected by exposure, but esterified steroids significantly increased in OAP exposed mussels (up to 2.4-fold). The sulfation of estradiol was investigated as a conjugation pathway, and increased activities were observed in digestive gland cytosol of both O and OAP exposure groups (up to 2.8-fold). Additionally, increased P450-aromatase activity was determined in OAP exposed mussels (up to 3-fold, both in gonad and digestive gland), but not in the O group. Altogether, the results indicate that North Sea oil leads to increased sulfation of estradiol, and that in combination with alkylphenols, additional alterations are observed: increased P450-aromatase, and increased levels of esterified-steroids in gonads. Nonetheless, mussels are able to maintain gonad concentrations of free steroids unaltered, possibly via homeostatic mechanisms such as the conjugation with fatty acid or the formation of sulphate conjugates.

Key words: Mytilus edulis, Produced water, Endocrine disruption, P450-aromatase, Estradiol sulfation.

1. INTRODUCTION

In recent years, there has been a growing awareness of the need to detect and assess the adverse effects of the offshore oil and gas industry discharges to the sea. Significant quantities of alkylphenols and aromatic hydrocarbons are released into the sea by petroleum installations as a result of discharges of produced water, i.e. water that occurs naturally in the geological structure, and water that has been injected into the reservoir in order to maintain pressure within the formation. The produced water is cleansed of oil to a maximum content of 40 mg/L, and most of it is then discharged to the sea. In addition to the remaining oil, the water contains other chemicals, i.e. additives used in drilling and pumping operations, and in the oil/water separation process, such as metals, alkylphenols (some of which have the potential to disrupt endocrine processes), and polycyclic aromatic hydrocarbons -PAHs- (Meier et al., 2002; Røe, 1999).

Produced water chemicals can affect and induce the detoxification metabolism in fish (Stephens et al., 2000). Laboratory and field studies have demonstrated the estrogenic effects of alkylphenols in exposed fish, namely, suppression of sex steroids or inhibition of testicular growth among others (Sumpter, 1995; Hecker et al., 2002). Also they have been identified as estrogen receptor agonists by using the yeast estrogen screen assay (Thomas et al., 2004). Thus, there is a great deal of interest in assessing the potentially negative effects of the produced water on the marine environment.

Because of their ability to accumulate organic compounds, filter-feeding bivalves have been used extensively for biomonitoring purposes (Wedderburn et al., 2000; Camus et al., 2003; Andral et al., 2004). Most of the studies have focused on the determination of histo-cytopathological and molecular responses as a consequence of exposure to PAHs and other contaminant mixtures (Au, 2004; Long et al., 2003; Taban et al., 2004; Aarab et al., 2004). However, to our knowledge, no studies have thoroughly investigated the effects of these compounds on the endocrine system of bivalves.

In fact, the issue of endocrine disruption in invertebrates has generated remarkably little interest in the past, compared to research with aquatic vertebrate species. However, with more than 95% of all known species in the animal kingdom, invertebrates constitute a very important part of the global biodiversity, with key species for the structure and function of aquatic ecosystems (Lafont, 2000). However the hormonal system of invertebrates is not as well documented as the vertebrates one. The existing evidences suggest that the invertebrate endocrine system functions in some aspects similarly to the vertebrate system. Sex steroids have been widely detected in molluscs. In particular, estradiol (E2), testosterone (T), and progesterone have been reported in gastropods (Lupo di Prisco et al., 1973; Le Guellec et al., 1987), cephalopods (D'Aniello et al., 1996), and various bivalves, such as the mussel *Mytilus edulis* (Reis-Henriques et al., 1990), and

the oyster *Crassostrea gigas* (Matsumoto et al., 1997). Key steroidogenic pathways have been identified in different mollusc species, i.e. 17η-hydroxysteroid dehydrogenase (17η-HSD), P450-aromatase, 5ζ-reductase, 3η-hydroxysteroid reductase (3η-HSD) (De Longcamp et al., 1974; Hines et al., 1996; Ronis and Mason, 1996; Morcillo et al., 1999; Le Curieux-Belfond et al., 2001). Estradiol binding proteins have been detected in the cytosol of *Octopus vulgaris* (D'Aniello et al., 1996) and the complete sequences of the estrogen receptor has been reported in *Aplysia californica* (Thornton et al., 2003), supporting the fact that estradiol can function in molluscs through a receptor-mediated mechanism.

Some studies have shown that endocrine disruptors can interfere with steroidogenic/metabolic pathways. TBT has been shown to inhibit aromatase activity *in vitro* in the oyster *Crassostrea gigas* (Le Curieux-Belfond et al., 2001). It has been demonstrated that natural estrogens, such as E2, can affect steroid metabolism and gamete maturation in the mussel *Mytilus galloprovincialis* (Janer et al., 2005a). Other environmental xenobiotics, such as bisphenol A, 4-tert-octylphenol, and 17Ŭ-ethynilestradiol, have been shown to affect embryo production in prosobranch snails (Oehlmann et al., 2000; Jobling et al., 2003).

Together with biosynthetic pathways, conjugation rates of steroids can play a key role in endocrine homeostasis, and might regulate levels of active steroids within target tissues. But, at the moment, data regarding conjugation of steroids in molluscs is based primarily on *in vivo* observations. Steroid sulfates have been identified in the gastropod *Clione antartica* (Hines et al., 1996), and a decrease of testosterone sulfation was reported for *Littorina littorea* exposed to TBT (Ronis and Mason, 1996). However, the *in vitro* sulfation of steroid hormones in molluscs has not yet been studied in depth. Janer et al., (2005a) observed that digestive gland cytosolic fractions of *Mytilus galloprovincialis* can form estradiol sulfates at a rate of 6 pmol/h/mg protein, and that this activity was not affected by exposure to 17ɓ-estradiol. Also, it has been observed that sulfatase enzymes in the oyster *C. virginica* are highly expressed in digestive gland, and they may interfere with the in-vitro determination of cytosolic sulfotransferase activity in this mollusc species (Janer et al., 2005b).

Within this context, the study was designed to better characterize the response of the mussel *Mytilus edulis* to North Sea oil, and the mixture of North Sea oil and alkylphenols, and whether those compounds, which are found in produced water from the offshore industry, are able to disrupt the endocrine system of mussels. To this end, testosterone and estradiol levels (free and esterified levels) were measured in both gonad and peripheral tissue (mantle+gills), to assess potential alterations on hormone levels. The activity of the steroidogenic enzyme P450-aromatase, a key enzymatic activity responsible for the conversion of C_{19} androgens into the corresponding C_{18} estrogens, was determined in digestive gland and gonad microsomal fractions. An effort was made

to characterize and determine sulfotransferase activity in digestive gland cytosolic fraction using 17ß-estradiol as a substrate, as a potential pathway involved in steroid clearance in molluscs.

2. MATERIAL AND METHODS

2.1. Chemicals and biochemicals

17ß-Estradiol, androstenedione, and testosterone were obtained from Sigma (Steinheim, Germany); [4-^{14}C]testosterone was purchased from Amersham Pharmacia Biotech, UK; [6,7-^{3}H]17ß-estradiol (40-60 Ci/mmol) and [1ß-^{3}H]androstenedione (15-30 Ci/mmol) were obtained from PerkinElmer Life Science (Boston, MA, USA), Inc. Radioimmunoassay (RIA) kits for testosterone and 17ß-estradiol were obtained from Radim, Inc (Pomezia, Italy). All solvents and reagents were of analytical grade from Merck (Darmstadt, Germany).

2.2. Experimental design

In an experiment performed in the Alkamiljø Center (Stavanger, Norway), mature blue mussels (*Mytilus edulis*), collected in a pristine site (Førlandfjorden) were exposed in November-December 2002 for 3 weeks in a continuous flow-through system to sub-lethal concentrations of North Sea crude oil (0.5 ppm) (O group), North Sea crude oil (0.5 ppm) spiked with a mixture of alkylated phenols (0.1 ppm) and PAHs (0.1 ppm) (OAP group), and to the carrier (2 ppb acetone) (C group). Environmental conditions, i.e. temperature (10-12ºC), salinity (34‰), and photoperiod, simulated their origin conditions. After 3 weeks, mussels were sacrificed, the gonads, digestive gland, and peripheral tissue (gills and mantle) dissected and stored at -80ºC for enzymatic activities (digestive gland and gonads) or steroid determinations (gonads and peripheral tissue).

2.3. Sex hormone analysis

Levels of free testosterone and free estradiol were analyzed as described in Janer et al. (2005a). Briefly, tissue samples (0.5-1.0 g wet weight of gonad tissue and 1.0-2.0 g wet weight of peripheral tissue) were homogenized in ethanol, and frozen overnight at -80ºC. Homogenates were then extracted with ethyl acetate twice. The organic extract was evaporated under nitrogen, and redissolved in 80% methanol. This solution was then washed with petroleum ether to remove the lipid fraction and evaporated to dryness. The dry residue was redissolved in 4 mL milli-Q water and passed through a C18 cartridge (Isolute, 1g, 6 mL), that had been sequentially pre-conditioned with methanol (4 mL) and milli-Q water (8 mL), dried, and connected to a NH2 cartridge (Waters,

Sep-pack™ Plus). The C18-NH2 system was then washed with 8 mL hexane, and the steroids eluted with 9 mL dichloromethane:methanol (7:3). This fraction was collected and evaporated to dryness.

Total testosterone and total estradiol (free + esterified) were determined in the same tissues (gonads and peripheral tissue) after a saponification step as described in Janer et al. (2005a). Tissues, homogenized as for free steroid determination (see above), were extracted with ethyl acetate (3 x 2 mL). The organic extract was evaporated under a nitrogen stream, resuspended in 1 mL methanol containing 0.1% KOH, and kept at 45ºC for 3 hours. After the saponification step, milli-Q water (4 mL) was added, and the sample extracted with dichloromethane (3 x 3 mL).

The efficiency of the extraction and delipidation procedure was 74 ± 3% for T, and 80 ± 3% for E2 (Morcillo et al., 1999), and 95% (E2) to 97% (T) for the purification step (SPE cartridges) (Janer et al., 2005a).

2.4. RIA analyses

Dry extracts were resuspended in 50 mM potassium phosphate buffer pH 7.6 containing 0.1% gelatine, and assayed for 17ϐ-estradiol and testosterone concentration using commercial RIA kits. Standard curves with the steroids dissolved in the same phosphate buffer were performed in every run. The limits of detection were of 9 pg/g for E2 and 20 pg/g for T. Intra-assay coefficients of variation were of 6.1% (T) and 3.3% (E2). Inter-assay coefficients of variation were 9.3% (T) and 3.5% (E2).

2.5. Subcellular fractionation

Gonad tissue and digestive glands were homogenized in ice-cold 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 containing 0.15 M KCl. Homogenates were centrifuged at 500-*g* for 15 min, the fatty layer removed and the supernatant centrifuged at 12,000-*g* for 45 min. After centrifugation at 100,000-*g* for 60 min, the supernatant, termed cytosol, was collected and stored at -80ºC until assays were performed. The pellet was resuspended with the same buffer and centrifuged again at 100,000-*g* for 60 min. Microsomal pellets were resuspended in a small volume of 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 containing 0.15 M KCl and 20% w/v glycerol. Protein concentrations were determined by the method described by Lowry et al. (1951), using bovine serum albumin as a standard.

2.6. Determination of P450-aromatase activity

Aromatase activity was determined in the microsomal fraction of both digestive gland and gonad tissue by the tritiated-water release method as described in Lavado et al. (2004) with some modifications. Microsomes (0.4 mg protein) were incubated at 25ºC for 3 h in a final volume of 1 mL in the presence of 100 mM Tris-HCl buffer pH 7.6, 10 µM androstenedione (1 µCi) and 200 µM NADPH. Assay blanks containing 100 µL of buffer instead of microsomes were used for every run. The reaction was stopped by placing the tub on ice, and organic metabolites and the excess of substrate were immediately eliminated from the aqueous phase by extraction with methylene chloride (3 x 3 mL). The possible remaining tritiated steroids were further eliminated by the addition of a suspension of 2.5% (w/v) activated charcoal and 0.25% dextran in milli-Q water (4 mL). The solution was centrifuged (60 min x 3600 rpm), and two aliquots of the supernatant (1 mL) were counted for ^{3}H radioactivity. The lowest aromatase activity detected by the method was 0.04 pmol/h/mg protein.

2.7. Estradiol sulfotransferase activity

Cytosolic proteins (100 µg) isolated from both digestive glands and gonads were incubated in 50 mM Tris-HCl buffer pH 9.0 containing 4 mM $MgCl_2$ and 2 mM Na_2SO_3, with 110 nM [^{3}H]estradiol (180,000 dpm), in a final volume of 155 µL. The reaction was initiated by the addition of 10 µM PAPS (adenosine 3'-phosphate 5'-phosphosulfate), and incubated for 1 hour at 30ºC. The reaction was stopped with 2 mL methylene chloride, after addition of 200 µL of ice-cold 50 mM Tris-HCl buffer pH 8.7. The extraction of unmetabolized estradiol was completed with 3 mL of methylene chloride, and an aliquot of the aqueous phase, where sulphated estradiol remained, was quantified by liquid ^{3}H scintillation counting. Assay blanks containing water instead of PAPS were used for every run.

For the characterisation of E2 sulfotransferase activity, a wide range of pH buffers (50 mM potassium phosphate at pH 6.5; 50 mM Tris-HCl at pH 7.5, 8.0 and 9.0; and 50 mM sodium acetate at pH 9.5 and 10.0) were used. Concentration of E2 ranged from 10 nM to 30 µM. Reaction times checked were from 15 to 60 min; and protein content in the assay from 0.05 to 0.4 mg. Both gonad and digestive gland tissues were assessed.

2.8. Statistical procedures

Results are presented as mean values ± SEM. Hormone levels statistics were applied on log-transformed data, because dependent variables did not satisfy parametric test's assumptions. Comparison of significant difference (P<0.05) was made with one-way ANOVA followed with Tukey's test multiple range test. All statistics were analysed using the software SPSS v11.0.

3. RESULTS

3.1. Steroid levels

Steroid levels were determined in gonads and peripheral tissues of mussels, with and without a saponification step, in order to differentiate between unesterified (free) and total steroid forms (free + esterified). Free E2 levels in peripheral tissues were significantly higher in oil-

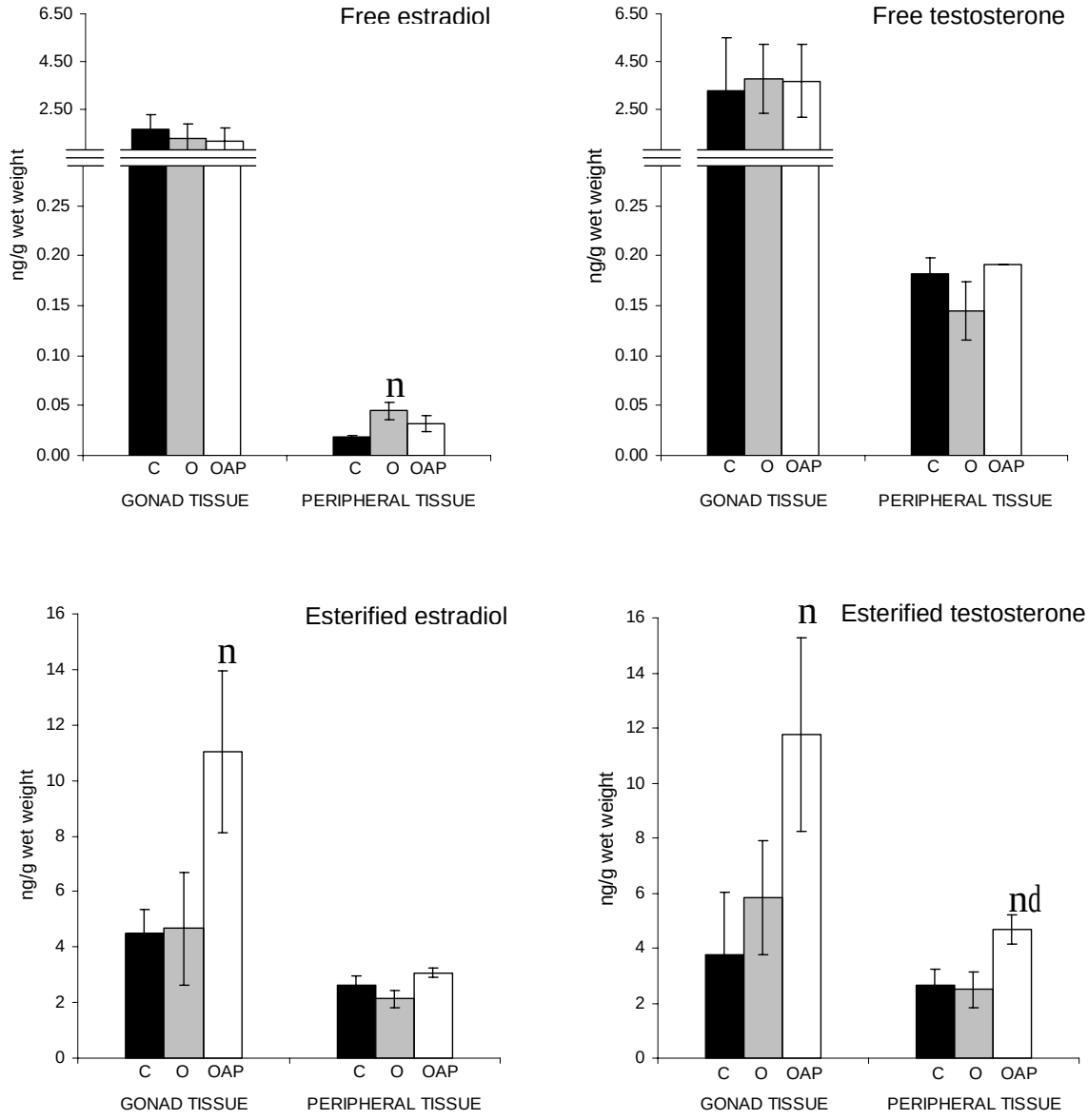

Figure 1. Levels of free and esterified testosterone and 17ß-estradiol in control (C) mussels; mussels exposed to North Sea oil (O); and the mixture of North Sea oil, alkylphenols and PAHs (OAP). Data presented as mean ± SEM (n=6). Significant differences respect to control indicated by 1 $P<0.05$ (one way ANOVA, Tukey's test).

exposed mussels than in controls (Fig. 1). However, in gonads no differences between experimental groups were observed for free E2 levels. Esterified E2 was significantly higher (P<0.05) in gonads of OAP-exposed mussels than in controls (11.0 ± 2.9 vs 4.5 ± 0.9 ng/g w.w., respectively), whereas no differences were observed in peripheral tissues. In all cases, free E2 levels in gonads were much higher than those in peripheral tissues (29 to 92-fold). In contrast, the concentration of esterified E2 was similar in both tissues.

Free levels of T were no significantly altered by exposure to oil or OAP in neither gonad nor peripheral tissues, and similarly to E2, T levels in gonads were higher than in peripheral tissues (18 to 26-fold). Levels of esterified T were similar in both tissues (except in organisms of OAP group). As shown in Figure 1, esterified testosterone was significantly higher in both gonads and peripheral tissues of mussels exposed to OAP when compared to C and O groups (1.7- to 3.1-fold).

3.2. P450-aromatase activity

The synthesis of E2 was investigated by measuring the activity of microsomal P450-aromatase in both gonads and digestive gland. The specific activity of P450-aromatase was up to 3-fold higher in digestive gland than in gonads, in all the studied groups (Fig. 2). A significant 3-fold increase in P-450 aromatase activity was observed in organisms from the OAP group when compared to C and O groups, and this increase was evident both, in digestive gland and gonads.

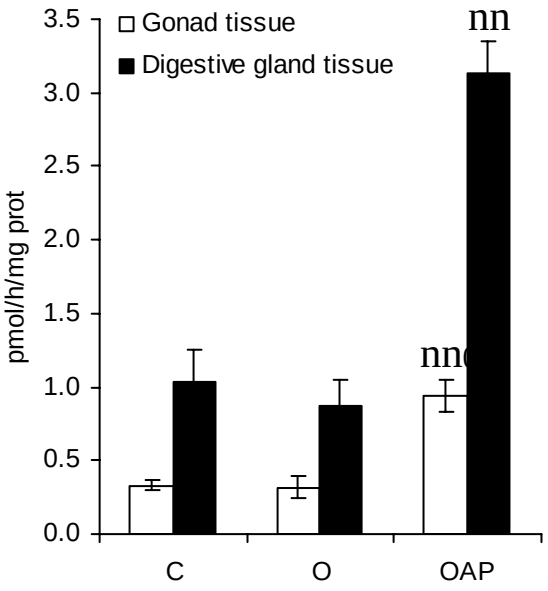

Figure 2. P450-aromatase activity in gonad and digestive gland tissue of control (C) mussels; mussels exposed to North Sea oil (O); and the mixture of North Sea oil, alkylphenols and PAHs (OAP). Data expressed as mean ± SEM (n=6). Significant differences respect to control indicated by ** P<0.01 (one way ANOVA, Tukey's test).

3.3. Sulfotransferase activity towards 17b-estradiol

This activity had been previously determined in other invertebrate species (Hines et al., 1996; Ronis and Mason, 1996; de Knecht et al., 2001), and was found to be rather low in molluscs (Janer et al., 2005b). The first part of the study consisted on the optimization of the assay. The sulfotransferase activity was determined at different pH, ranging from 6.5 to 10.0, using E2 as a substrate at a fixed concentration (110 nM), and both gonad and digestive gland cytosolical

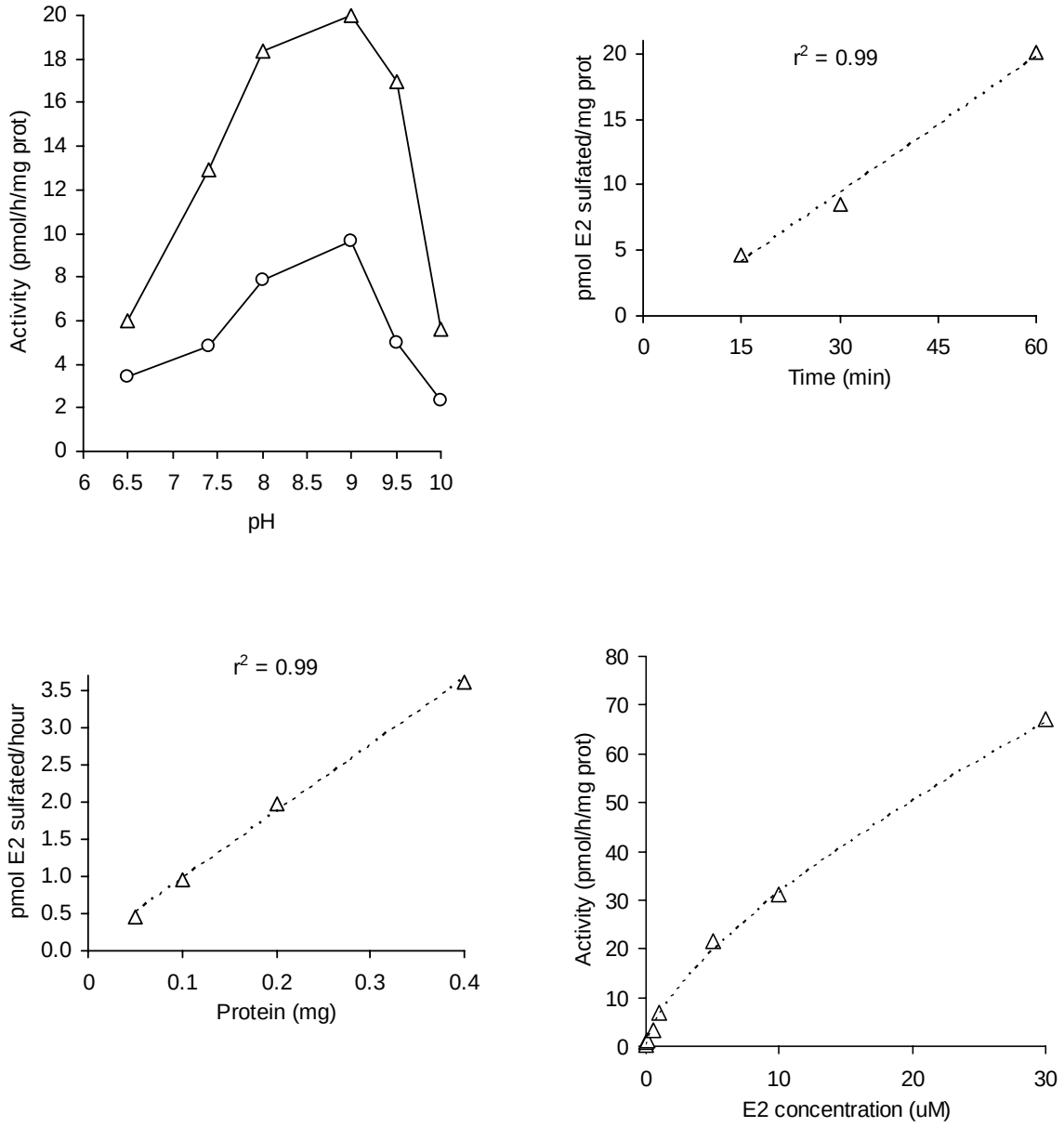

Figure 3. Characterization of E2 sulfotransferase in cytosolic fractions of *M. edulis*. (A) pH dependence in both gonad (ʒ) and digestive gland (r); (B) time course of E2 sulfotransferase in digestive gland; (C) dependence of E2 sulfotransferase on the amount of protein in assay, and (D) dependence of E2 sulfotransferase on the amount of E2 in the assay.

fractions were used as enzyme sources. The enzymatic activity showed a maximum at pH 9.0 in both tissues, and the specific activity was always higher in digestive gland than in gonads (up to 2-fold) (Fig. 3A). At pH 9.0, sulfotransferase activity was lineal for at least 1 hour (r^2=0.99) (Fig. 3B), and at concentration of proteins in the assay ranging from 0.05 to 0.4 mg (Fig. 3C). Finally, different E2 concentrations, ranging from 10 nM to 30 µM, were tested using digestive gland cytosol (0.1 mg proteins in the assay) as enzyme source, and the enzymatic activity was lineal over the range of E2 concentrations tested (Fig. 3D).

Under the selected conditions (0.1 mg of proteins, pH 9.0, 100 nM E2, 1 hour incubation), the sulfation of E2 by digestive gland cytosolic fractions was in the range of 0.5 to 20 pmol/h/mg protein, and significant differences between control and exposed groups were observed (Fig. 4). Exposed mussels (O and OAP group) had increased sulfotransferase activity, that ranged from 3.0 ± 0.7 pmol/h/mg protein in individuals from the C group, to 7.1 ± 2.0 and 8.5 ± 2.6 pmol/h/mg protein in exposed groups (O and OAP, respectively). These differences were statistically significant (P<0.05), and indicated a strong effect of the North Sea oil on this conjugation activity.

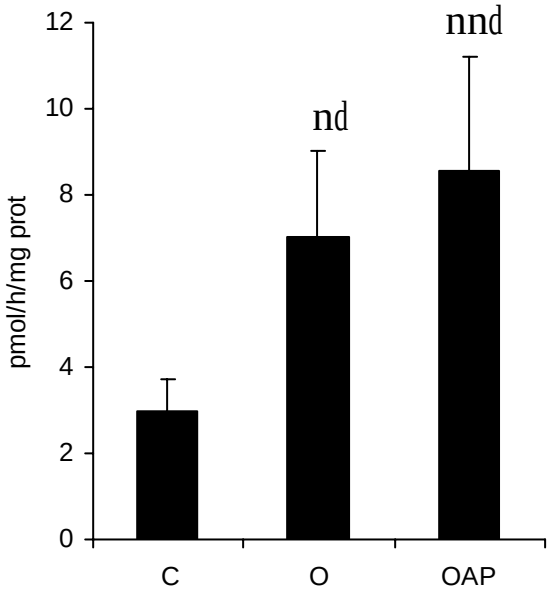

Figure 4. Estradiol-sulfotransferase activity in cytosolic fractions isolated from digestive gland tissue of control (C) mussels; mussels exposed to North Sea oil (O); and the mixture of North Sea oil, alkylphenols and PAHs (OAP). Data expressed as mean ± SEM (n=6). Significant differences respect to control indicated by 1 P<0.05 and 11 P<0.01 (one way ANOVA, Tukey's test).

4. DISCUSSION

The analysis of steroids in this species showed higher levels of free steroids in gonads than in peripheral tissues (18- and 91-fold, for T and E2, respectively), which supports the role of gonads as a target tissue in steroid synthesis. In contrast, levels of esterified steroid were similar in both tissues (1.6-9.6 ng/g w.w.), although they represented a higher proportion of the total levels of steroids in the peripheral tissues (93-98%) than in the gonads (53-73%).

Free steroid levels found in mussel gonads are in the same range of those reported for other invertebrate species. Thus, levels of free testosterone of 1-3 ng/g wet weight have been reported in the mud snail *Ilyanassa obsoleta* (Gooding et al., 2003); 0.7-1.2 ng/g wet weight have been detected in the visceral coil of *Bolinus brandaris* (Morcillo and Porte, 1999), and 0.1-5.5 ng/g in gonads of the sea star *Asterias vulgaris* (Hines et al., 1992). Similarly, free E2 levels are also in the range of those reported in some gastropod (20-600 pg/g w.w. in *Bolinus brandaris*, Morcillo and Porte, 1999) and echinoderm species (40-800 pg/g w.w. in *Asterias vulgaris*, Hines et al., 1992).

Exposure to North Sea oil caused a significant increase in free E2 levels in mussel peripheral tissues, but had no effect in gonads or in free T levels. No effects on free steroid levels were observed in OAP-exposed mussels. Aarab et al. (2004) reported that mussels exposed to North Sea oil had increased expression of some phosphoproteins that corresponded to mussel vitellogenin-like proteins. This increase, that was interpreted as an estrogenic effect caused by North Sea Oil, might be linked to the increase in E2 levels observed in this study. In fact, E2 has been shown to stimulate vitellogenin accumulation in oyster (*Crassostrea gigas*) oocytes (Li et al., 1998).

Although levels of free steroids remained almost unaffected by exposure (apart from E2 in mussels from the O-group), esterified steroids were significantly increased. Mussels from the OAP group showed a 3-fold increase in esterified T and E2 in gonads, and a 2-fold increase in esterified T in peripheral tissue. Fatty acid esterification is recognized as a major biotransformation pathway for sex steroids in molluscs, since a massive biotransformation of testosterone and estradiol to fatty acid conjugates has been reported for the gastropod *I. obsoleta*, and the bivalves *C. virginica* and *M. galloprovincialis* (Gooding and LeBlanc, 2001; Janer et al., 2004; 2005a). The hypothesis exists that fatty acid steroid conjugates may play a key role regulating physiological levels of free steroids in tissues (Gooding and LeBlanc, 2004; Janer et al., 2005a). In fact, when the mussel *M. galloprovincialis* was exposed to different concentrations of estradiol, most of the estradiol in the tissues was detected as fatty acid esters (>78%), which sharply increased in a dose-dependent manner (Janer et al., 2005a). Therefore, the increase in esterified steroid levels in gonads from organisms exposed to the mixture of oil and alkylphenols (OAP-group) suggests that fatty acid conjugation could act as a mechanism to maintain endogenous levels of steroids unchanged, and that enhanced steroidogenesis could be occurring in OAP-exposed organisms. In fact, increases in T production due to exposure to alkylphenols, as 4-tert-octylphenol, had been described in vertebrates (Murono et al., 1999, 2001). Also in zebra mussel (*Dreissena polymorpha*), exposure to municipal effluents where the majority of the compounds had estrogenic activity (E2, 17Ŭ-ethynylestradiol, bisphenol A and alkylphenol polyethoxylates) resulted in an increase in cholesterol in tissues (Quinn et al., 2004), a precursor for the production on sexual steroids in *M.*

edulis (De Longcamp et al., 1974). Thus, although the mechanism is still unknown, increased cholesterol levels have the capacity of being converted into sexual steroids (Quinn et al., 2004).

P450-aromatase activity has been reported in several mollusc species (Morcillo et al., 1999; Le Curieux-Belfond et al., 2001; Janer et al., 2005a) but the aromatization of androgens into estrogens occurs at a very low rate (0.3 to 3.5 pmol/h/mg protein). P450-aromatase specific activity was 3-fold higher in digestive gland than in gonads, indicating a more active role of this tissue in the aromatization of androgens in the mussel *M. edulis*. It was significantly increased (3-fold) by alkylphenols (OAP group) in both gonads and digestive gland. Similarly, vertebrate studies have shown that some xenoestrogens, including PAHs and alkylphenols, can up-regulate and induce P450-aromatase activity (Halm et al., 2002; Kishida et al., 2001; You et al., 2001; Kazeto et al., 2004). The expression of CYP19A2, the aromatase isoform mainly expressed in neural tissues, was enhanced in zebrafish (*D. rerio*) exposed to nonylphenol and benzo[a]pyrene (BaP) (Kazeto et al., 2004). In *M. galloprovincialis*, Janer et al. (2005a) observed that P450-aromatase was significantly reduced when organisms were exposed to low E2 concentration (20 ± 2 ng/L) for 7 days, but the trend was reversed at high E2 concentration (2988 ± 730 ng/L), and organisms had significantly higher P450-aromatase than controls. A decrease of androgen levels as a consequence of increased estrogen production through up-regulation of aromatase enzyme activity might be hypothesized for OAP exposed mussels. However, no such evidence was observed in terms of free or esterifed steroids. In fact, testosterone was elevated in peripheral tissues of OAP-treated mussels, and both esterified testosterone and estradiol were elevated in gonads. Thus, the question rises on whether changes on aromatase activity –that is rather low in molluscs- would have a significant effect on steroid levels and/or whether other mechanisms/pathways may contribute to a greater extent to regulate steroid levels within the organism.

Actually, estradiol sulfation was significantly increased (up to 3-fold) in both O- and OAP-exposed groups when compared to the control group. Sulfation of steroids may inhibit their biological activity by decreasing their affinity for steroid receptors and increasing their rate of elimination (Strott, 1996). Therefore, an alteration of sulfotransferase activity might also have consequences in terms of endogenous levels of free steroids.

The susceptibility of sulfotransferases to modulation by xenobiotics had already been shown in several species. Sulfotransferase and UDP-glucuronosyltransferase, another phase II enzyme, were up-regulated in channel catfish (*Ictalurus punctatus*) exposed to the model PAH (3-methylcholanthrene) (Gaworecki et al., 2004). On the other hand, *in vitro* studies have generally reported an inhibition of estradiol sulfation by alkylphenolic compounds. In fish studies, alkylphenols acted as potent inhibitors of estrone sulfation in vitro, an apparent effect of chain length was observed, the longer chain compounds being the most effective inhibitors in the chub (*Leuciscus leuciscus*) (Kirk et al., 2003). 4-n-octylphenol and 4-n-nonylphenol exerted

concentration-dependent inhibition on the sulfation of 17ϐ-estradiol by zebrafish cytosolic sulfotransferases (Ohkimoto et al., 2003). Nonylphenol inhibited estradiol sulfation by the liver cytosolic fraction of *Cyprinus carpio* (Thibaut and Porte, 2004). Also, in mammals, the sulfation of estradiol has been shown to be inhibited by several xenobiotics as hydroxylated PAHs inhibited the human isoform SULT1E1 (Kester et al., 2000). However, apart from the present study, no data on in-vivo effects of alkylphenol compounds is available to date.

Overall, these results indicate that both, North Sea oil and the mixture North Sea oil + alkylphenols, can affect endogenous levels of steroids in mussels. North Sea oil leads to increased levels of free E2 in peripheral tissues (gills and mantle) and increased activity of E2-sulfotransferase in digestive gland, possibly as a regulatory mechanism to inactivate the excess of estradiol. In mussels exposed to North Sea oil in combination with alkylphenols (OAP-group) additional alterations were observed; namely, increased P450-aromatase activity, increased levels of esterified steroids (T and E2) mainly in gonads but also in peripheral tissue (esterified T), and increased sulfation of E2. Both, sulfation and esterification of steroids appear as potential mechanisms to inactivate the excess of hormones induced by the combined exposure to alkylphenols and oil. Thus, exposure of mussels to chemicals present in produced water can lead to alterations on key biochemical pathways that could have physiological consequences for the organisms but, nonetheless, mussels are able to maintain gonad concentrations of free (active) steroids almost unaltered, possibly via homeostatic mechanisms.

Aknowledgements

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Paper 7

SEXUAL DIMORPHISM IN ESTERIFIED STEROID LEVELS IN THE GASTROPOD *MARISA CORNUARIETIS*: THE EFFECT OF XENOANDROGENIC COMPOUNDS.

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Submitted to Steroids

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Sexual dimorphism in esterified steroid levels in the gastropod *Marisa cornuarietis*: the effect of xenoandrogenic compounds

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Abstract

Molluscs can conjugate a variety of steroids to form fatty acid esters. In this work, the freshwater ramshorn snail *Marisa cornuarietis* was used to investigate sex differences in the endogenous levels of esterified testosterone and estradiol and in the enzyme acyl-CoA:testosterone acyltransferase (ATAT), which catalyzes the esterification of steroids. Testosterone and estradiol in the digestive gland/gonad complex of *M. cornuarietis* were mainly found in the esterified form, and males had higher levels of esterified steroids than females (4 to 10-fold). The ability of several xenobiotics, namely, tributyltin (TBT), methyltestosterone (MT), and fenarimol (FEN) to interfere with the esterification of testosterone and estradiol was investigated. The three compounds induced imposex -appearance of male sexual characteristics in females-. Exposure to TBT led to a decrease in both esterified testosterone (60-85%) and estradiol (16-53%) in females after 100 days exposure, but had no effect on males. FEN and MT did not alter levels of esterified steroids in females or in males, although exposed females developed imposex after 150 days exposure. The decrease in esterified steroids by TBT was not directly related to a decrease in ATAT activity, which was marginally induced in organisms exposed to TBT for 50 days (1.3-fold) and significantly induced in males and females exposed to MT for 50 days (1.8- and 1.5-fold, respectively), whereas no effect on ATAT activity was observed after 150 days exposure.

Keywords: *esterification, testosterone, estradiol, gastropod, imposex, acyl-CoA acyltransferase*

1. Introduction

Sex steroids, such as progesterone, testosterone, and estradiol, are present in molluscs [1], and several studies have demonstrated the ability of these organisms to synthesize sex steroids from precursors, such as cholesterol or pregnenolone [2-5]. Proteins with a high binding affinity for sex steroids have been found in *Octopus vulgaris* [6], and an estrogen receptor ortholog gene has been sequenced in the mollusc *Aplysia californica* [7], suggesting that steroids might function via interaction with steroid receptors in molluscs. In addition, rapid non-genomic effects of estradiol have been demonstrated in mussel neural tissue (increase in nitric oxide release [8]) and in mussel hemocytes (increase in calcium concentrations and changes in the phosphorylation of signal transducers and transcription activators [9]). Although there are still important gaps of knowledge concerning both genomic and non-genomic actions of steroids in molluscs, the above-mentioned observations are suggestive of a physiological role of sex steroids in these organisms. Indeed, various studies have indicated that steroids are involved in the control of mollusc reproduction and sex differentiation. For example, estradiol induces the accumulation of vitellin in the gonads of the scallop *Patinopecten yessoensis* [10]; testosterone administration to castrated male slugs (*Euhadra prelionphala*) stimulates the production of male secondary sex characteristics [11]; and the administration of testosterone to female gastropods results in the development of imposex with extensive penis and vas deferens formation in these organisms [12-13]. In addition, steroid levels vary in some species of molluscs in relation to the reproductive status [3,14-15].

Steroid levels within the organism are at least partially regulated by steroidogenesis and/or biotransformation enzymes. Hydroxylation and polar conjugation (sulfation and glucuronidation), which are major metabolic pathways for testosterone or estradiol in vertebrates, seem to play a minor role in the metabolism of testosterone in molluscs [16-17]. Instead, snails metabolize most of the testosterone into fatty acid conjugates in vivo [16]. Esterification is mediated by a microsomal acyl-coenzyme A acyltransferase enzyme, which, in addition to testosterone [16], can conjugate other steroids, such as estradiol and dehydroepiandrostenedione, with fatty acids [18].

Esterified steroids are not easily excreted from the organism, but stored in fatty tissues. In mammalian species, they are considered potent long acting steroids because, in comparison to unconjugated steroids, they are metabolized and excreted at a very low rate [19]. Esterified steroids do not bind to receptors [20], but can be hydrolyzed by esterases, again liberating the active steroid [19]. Recent studies suggest that esterification might be an important factor in regulating the levels of free hormones in molluscs. Thus, Gooding and LeBlanc [15] showed that exogenous testosterone increased the retention of testosterone as fatty acid esters in *Ilyanassa obsoleta*, while unconjugated testosterone levels did not appreciably change. Similarly, exogenously administered estradiol was extensively esterified by the mussel *Mytilus galloprovincialis*, whereas unconjugated

estradiol levels remained almost unaltered [21]. The balance between conjugated and unconjugated steroids also appeared to control the variations in unconjugated steroid levels during the reproductive cycle. Thus, snails at the onset or end of the reproductive period had high levels of free testosterone coincident with low levels of testosterone fatty acid-esters [15].

Xenobiotic compounds, such as tributyltin (TBT), have been shown to interfere with the esterification of testosterone [22]. TBT is an organotin compound that causes imposex (imposition of male genital characters in females) in several gastropod species at very low concentrations (few ng/L) [23]. Recently, Gooding et al. [22] showed that females experimentally exposed to 10 ng/L TBT for 3 months showed a lower ability to conjugate testosterone with fatty acid moieties; also, females collected in an organotin-polluted site had lower levels of esterified testosterone than those collected in a clean site.

Apart from these studies, there is no data available on endogenous levels of esterified steroids in other mollusc species and/or what kind of pollutants can interfere with the fatty acid esterification of sex steroids. Thus, this study was designed to further investigate the presence and levels of sexual steroids (testosterone and estradiol) in the freshwater gastropod *Marisa cornuarietis*. Free and esterified steroid levels, together with fatty acid conjugating activities, were analyzed and potential sexual dimorphism in these parameters was assessed. In addition, we investigated whether steroid levels were modulated by exposure to xenobiotic compounds and the association with the phenomenon of imposex and other biological effects. Apart from TBT, two other compounds, fenarimol (FEN) and methyltestosterone (MT), were selected for the study.

FEN is a widely used fungicide that has the potential to interfere with the endocrine system through several mechanisms. It acts both as an estrogen agonist and an androgen antagonist, and it is a potent aromatase inhibitor [24]. The complexity of FEN action hampers the prediction of its in-vivo effects. Recently, FEN was reported to have an antiandrogenic effect in rats in vivo [25]. In contrast, the effects observed in exposed fathead minnow did not reflect either aromatase inhibition or androgen receptor antagonism [26]. In the snail *Nassarius reticulatus*, FEN has been shown to induce imposex [27]. The other compound tested, MT, is a synthetic androgen with high affinity for the mammalian androgen receptor [28] that has been shown to cause imposex in *Marisa cornuarietis* [29].

2. Experimental

2.1 Chemicals

Tributyltin chloride, fenarimol, methyltestosterone, testosterone, and estradiol were obtained from Sigma (Steinheim, Germany). [4-^{14}C]testosterone (specific activity 50–60 mCi/mmol) was purchased from NEN Life Science Products, Inc. (Boston, MA).

Radioimmunoassay (RIA) kits for testosterone and 17η-estradiol were obtained from Radim (Rome, Italy). All solvents and reagents were of analytical grade, except tributyltin chloride (96%) and methyltestosterone (above 98%).

2.2 Animals

Ramshorn snail, *Marisa cornuarietis* (Mollusca: Prosobranchia: Ampullariidae), is a subtropical species, originally inhabiting stagnant and slow running freshwater bodies in the northern part of South America. Because ramshorn snails were used as a biological control agent in many tropical countries for molluscs (*Biomphalaria glabrata*) hosting trematodes known to induce intestinal schistosomiasis, *Marisa* can also be found today in the Caribbean, Central and North America, Africa, and Asia. The species is gonochoristic and oviparous, attaining a maximum shell diameter of up to 5 cm. Juvenile snails hatch from the eggs after 8 to 13 days and reach sexual maturity after 6 months. First spawning of females can be observed at an age of 8 months [30].

M. cornuarietis used in this study came from our laboratory breeding stock, which was derived from a stock at Aquazoo Düsseldorf (Germany) in 1991 with regular crossbreeding of wild-caught animals from Florida to avoid inbreeding. Although kept under constant conditions (cf. 2.3 below) *M. cornuarietis* exhibits a clear reproductive seasonality in our laboratory. During the main spawning phase, from late October to early February, snails produce 61.8 ± 21.6 eggs per female and week (mean ± standard deviation). This reproductive output is significantly higher than egg production of 9.7 ± 8.5 eggs per female and week during the rest of the year (Mann Whitney test, $p<0.0001$).

2.3 Experimental design

Twenty-five males and 25 females (VDS Index: 0.5) from the laboratory cultures were collected from March to June to assess sexual dimorphism in steroid levels and steroid metabolism.

For the exposure experiments, two replicate groups of 75 sexually mature snails each were exposed to different nominal concentrations of TBT (0, 30, 60, 125, 250, and 500 ng as Sn/L), MT (0, 30, and 300 ng /L), and FEN (0, 300, and 3000 ng/L) for 5 months (August 2003 to January 2004) in fully reconstituted water at 24±1°C in parallel. Chemicals were added in absolute ethanol; the concentration of ethanol in water was 0.001% in all experimental groups, including the controls.

Test concentrations for TBT, MT and FEN were selected based on reported values in the aquatic environment and on results from earlier effect studies with *M. cornuarietis* and other prosobranch species. TBT and MT had proven to induce imposex in ramshorn snails at nominal concentrations above 50 ng as Sn/L [31] and 100 ng/L [29], respectively. In a sediment test with

the netted whelk *Nassarius reticulatus* FEN induced imposex already at the lowest test concentration of 300 ng/kg dry wt. [27].

Exposure experiments were performed as 24 h (weekends 48 h) semi-static renewal systems in 60 L glass aquaria, provided with an Eheim power filter and additional aeration. Tests were performed under constant conditions regarding temperature and light dark cycles (12 : 12 h). Water parameters (pH, conductivity, temperature, nitrite, O_2 concentration, and saturation) were measured twice a week per tank.

Mortality, numbers of eggs, clutches, and eggs per clutch in the tanks were recorded daily. Fecundity parameters were corrected for number of females per tank by taking into consideration the number of analyzed females as well as mortality data with an assumed 1 to 1 sex ratio. Forty specimens were analyzed at the beginning of the experiment, and 40 animals from every exposure group at day 50, 100, and 150 after the start of the experiment. Among them, 10 males and 10 females were cooled in ice and the digestive gland/gonad complex was dissected, deep-frozen in liquid nitrogen, and stored at -80ºC for determination of steroid levels and ATAT activity. The remaining animals were narcotized (2.5% $MgCl_2$ in distilled water) and the individual shell and aperture height of all animals were measured to the nearest 0.1 mm before the shell was cracked and snails were removed. Malformations such as excrescences on genital and other organs were recorded if present. The size of all sex organs (penis, penis sheath and penis pouch; albumen and capsule gland) were measured to the nearest 0.1 mm under a dissection microscope, and the imposex stage of individual females recorded as described by Schulte-Oehlmann et al. [31]. The vas deferens sequence (VDS) index, calculated as the mean value of all imposex stages in a sample, was used to measure imposex intensities during the experiments (see [31] for details).

2.4 Sex hormone analysis

Tissue levels of testosterone (T) and estradiol (E2) were analyzed in the digestive gland/gonad complexes of *M. cornuarietis* as described in Janer et al. [21], with some modifications. Briefly, individual tissue samples (0.3-0.5 g wet weight) were homogenized in ethanol and frozen overnight at -80ºC. Homogenates were then extracted three times with ethyl acetate. The organic extract was separated into two aliquots for determination of free and total steroid levels.

For the determination of free steroids, the ethyl acetate aliquot was evaporated under nitrogen and redissolved in 80% methanol. This solution was then washed with petroleum ether to remove the lipid fraction and evaporated to dryness. The dry residue was redissolved in Milli-Q water and passed through a C18 cartridge, which was washed with milli-Q water (8 mL), dried, and connected to a NH2 cartridge (Waters, Sep-Pack® Plus). The C18-NH2 system was then washed

with hexane, and the steroids were eluted with dichloromethane:methanol (7 : 3). This fraction was collected and evaporated to dryness (see [21] for details).

For the determination of total steroids (free + esterified), the ethyl acetate aliquot was evaporated under nitrogen and processed as described by Gooding et al. [22]. The dry residue was resuspended in methanol containing 0.1% KOH and kept at 45ºC for 3 hours. After the saponification step, milli-Q water was added, and the sample was extracted three times with dichloromethane.

Dry extracts (free and total steroids) were resuspended in 50 mM potassium phosphate buffer pH 7.6 containing 0.1% gelatin and assayed for estradiol and testosterone concentrations using commercial ^{125}I RIA kits (Radim, Rome, Italy). Standard curves with the steroids dissolved in the same phosphate buffer were performed in every run. The limits of detection of the method were 30 pg/g for E2 and 75 pg/g for T.

2.5 Subcellular fractionation

Digestive gland/gonad complexes were homogenized in ice-cold 100 mM phosphate buffer, pH 7.4, containing 100 mM KCl, 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM phenanthroline, and 0.1 mg/mL trypsin inhibitor. Homogenates were centrifuged at 12,000 *g* for 30 min. After centrifugation at 100,000 *g* for 60 min, the pellet was resuspended in the same buffer and centrifuged again at 100,000 *g* for 60 min. Microsomal pellets were resuspended in a small volume of 100 mM phosphate buffer, pH 7.4, containing 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM phenanthroline, and 0.1 mg/mL trypsin inhibitor, and 20% w/v glycerol.

Protein concentrations were determined by the method described by Lowry et al. [32] using bovine serum albumin as a standard.

2.6 Acyl-CoA:estradiol acyltransferase activity (ATAT)

Assays were carried out on the basis of methods described by Janer et al. [18] with some modifications. Microsomal proteins (150 σg) were incubated in 100 mM sodium acetate buffer (pH 6.0) with 10 σM [^{14}C]testosterone (150,000 dpm) or 10 µM [^{3}H]estradiol (150,000 dpm), 100 σM palmitoyl-CoA, and 5 mM MgCl2 in a final volume of 250 σL. The reaction was initiated by the addition of palmitoyl-CoA, and samples were incubated for 30 minutes at 30ºC. The reaction was stopped by adding 2 mL of ethyl acetate and extracted twice. The ethyl acetate fraction was evaporated to dryness, and the dry residue was redissolved in methanol and injected into the HPLC system that consisted of a PerkinElmer Binary 250 LC pump system equipped with a 250 x 4 mm LiChrospher 100 RP-18 (5 σm) reversed-phase column (Merck, Darmstadt, Germany) protected by a guard column LiChrospher 100 RP-18 (5 σm). Separation of testosterone (or estradiol) and its palmitoyl-ester was performed at 1.2 mL/min with a mobile phase composed of (A) 56% water

containing 0.1% acetic acid (pH 3.0), 13% acetonitrile, and 31% methanol, and (B) 60% acetonitrile and 40% methanol. The run consisted of 9 minutes of isocratic 100% A, 6 minutes of a linear gradient from 100% A to 100% B, and 25 minutes of isocratic 100% B. Chromatographic peaks were monitored by on-line radioactivity detection with a Radioflow detector LB 509 (Berthold Technologies, Bad Wildbad, Germany) using Flo Scint 3 (Packard BioScience, Groningen, The Netherlands) as scintillation cocktail. Metabolites were quantified by integrating the area under the radioactive peaks and identified by comparison of the retention times with authentic standards.

2.7 Statistical procedures

Results are mean values ± SEM. Statistical significance was assessed by using one way ANOVA (Dunnett's test for differences from control) for steroid levels and ATAT activities, the Weir test for incidence of imposex, and the Mann Whitney test for seasonal differences in egg production. Data was log transformed when distribution was not normal (Kolmogorov-Smirnov test). EC10 were calculated with a Weibull distribution model (GraphPad Prism 4.02, San Diego, CA).

3. Results

3.1 Total, free, and esterified steroids in digestive gland/gonad complexes

Steroid levels were determined with and without a mild saponification step in the digestive gland/gonad complexes of adult male and female *M. cornuarietis* sacrificed from March to June (low spawning period). In the absence of the saponification step, unconjugated steroids (free) were determined; free steroids were in the range of 30 to 198 pg estradiol/g tissue and 127 to 2886 pg testosterone/g. Levels of free estradiol were 1.5-fold higher in males than in females ($p<0.05$), whereas the levels of free testosterone were similar in both sexes (Table 1). As a result of this, the ratio between free T and E2 was lower in males than in females (7.0 ± 0.9 vs. 12.7 ± 2.1).

When a mild saponification step was applied to the extraction procedure, the ester bounds of steroid metabolites were cleaved, and total steroids (including both esterified and free steroids) were measured. Total steroid levels were significantly higher in males than in females (4- and 7-fold for estradiol and testosterone, respectively, $p<0.05$). Total estradiol was in the range of 2.7 to 67.8 ng/g in males and 1.0 to 13.1 ng/g in females, and total testosterone ranged from 5.1 to 42.2 ng/g in males and 1.8 to 5.7 ng/g in females. Most of the sex steroids extracted from *M. cornuarietis* digestive gland/gonad complexes were in the esterified form, and free steroids only represented a minor proportion of the steroids extracted from the tissue. In addition, the proportion of testosterone found as fatty acid esters was higher in males (96 ± 1%) than in females (69 ± 4%,

$p<0.05$). The proportion of estradiol in an esterified form was even higher, representing >98% of total estradiol levels in both males and females (Table 1). The differences observed in total steroid levels between males and females were due to sex differences in esterified steroids levels (4- to 10-fold higher in males than in females).

Table 1. Steroid levels (total, free and esterified) in the digestive gland/gonad complex of *M. cornuarietis* (male and female). Values are mean ± SEM (n=20-21) expressed in ng/g w.w. Significant differences between males and females are indicated by * (Student's t-test, $p<0.05$).

	Males	Females
Testosterone		
Free	0.8 ± 0.1	1.0 ± 0.1
Total	22.8 ± 1.9	3.3 ± 0.2*
Esterified	22.0 ± 1.9	2.3 ± 0.2*
% esterified	96.0 ± 0.7	69.6 ± 4.4*
Estradiol		
Free	0.12 ± 0.01	0.08 ± 0.01*
Total	25.2 ± 4.1	5.7 ± 0.8*
Esterified	25.1 ± 4.1	5.6 ± 0.7*
% esterified	99.2 ± 0.2	98.2 ± 0.2*

3.2 Acyl-CoA:steroid acyltransferase

The activity of acyl-CoA acyltransferase, the enzyme that converts steroids to steroid-fatty acid esters, was determined in microsomal fractions isolated from digestive gland/gonad complexes of *M. cornuarietis* collected from March to June (low spawning season). Acyl-CoA acyltransferase activity was first measured using both testosterone and estradiol as substrates to determine whether both compounds were similarly conjugated with palmitic acid. The formation rates of testosterone- and estradiol-palmitoate were similar (19 to 69 pmol/min/mg protein for testosterone and 24 to 105 pmol/min/mg protein for estradiol), and a significant correlation ($r^2= 0.87$, $p<0.05$) was observed between the activities determined using testosterone and estradiol as substrates (Figure 1). Thereafter, only acyl-CoA:testosterone acyltransferase (ATAT) was determined along the different experimental treatments.

ATAT activity was measured in male and female *M. cornuarietis* in order to investigate whether the higher levels of esterified steroids detected in males were related to higher conjugating rates. However, ATAT activity determined in males and females was similar (36 ± 5 and 39 ± 5 pmol/min/mg protein, respectively, n=16).

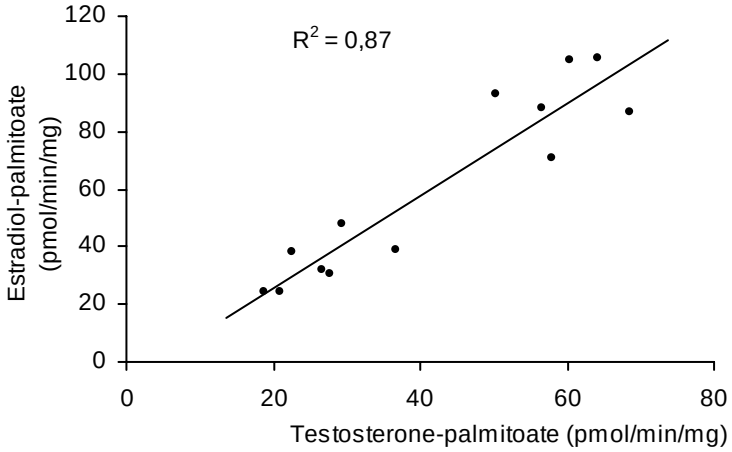

Figure 1. Relationship between the acyl-CoA:estradiol acyltransferase activity and acyl-CoA:testosterone acyltransferase activity evaluated in microsomal fractions isolated from digestive gland/gonad complex of *Marisa cornuarietis*.

ATAT activity was measured in male and female *M. cornuarietis* in order to investigate whether the higher levels of esterified steroids detected in males were related to higher conjugating rates. However, ATAT activity determined in males and females was similar (36 ± 5 and 39 ± 5 pmol/min/mg protein, respectively, n=16).

3.3 Imposex and other biological effects induced by TBT, fenarimol and methyltestosterone

Exposure to different concentrations of TBT, FEN, and MT significantly induced imposex in females of *M. cornuarietis* (Table 2). TBT was the most potent of the three compounds tested, and females exposed to the highest concentration (500 ng as Sn/L) developed imposex after 50 days exposure. At the end of the exposure experiment (150 days), females exposed to all TBT concentrations (30 to 500 ng as Sn/L) and to the highest concentration of FEN (3000 ng/L) and MT (300 ng/L) had significantly higher imposex intensities than controls. The VDS index recorded in females after 150 days of exposure to MT (300 ng/L; VDS: 1.5) and FEN (3000 ng/L; VDS: 1.9) was in the range of those VDS index recorded for medium and low TBT concentrations (30 to 125 ng as Sn/L; VDS: 1.5 to 1.9). The highest potency of TBT in inducing imposex is supported by the EC_{10} for the 3 test compounds with values of 4.16 ng as Sn/L, 26.6 ng/L and 30.0 ng/L for TBT, FEN and MT, respectively.

The concentration ranges of the compounds tested in the experiment did not affect mortality, the size of sex organs in males or females (except for the development of imposex), but caused a significant reduction of fecundity. The clutch size (number of eggs per clutch), number of clutches per female and number of eggs per female were significantly lower in all exposure groups when compared with controls (one way ANOVA with Dunnett's test, $p<0.05$), so that the LOEC

(lowest observed effect concentration) was 30 ng/L for each of the three compounds. EC_{10} values for egg production were 18.1 ng as Sn/L, 11.4. ng/L and 1.73 ng/L for TBT, FEN and MT, respectively, indicating that MT was the most potent compound inhibiting reproduction.

Table 2. VDS index in the different experimental treatments. Number of females (n) is shown in brackets. Significant differences versus control indicated by * (Weir-test, $p<0.05$).

	Concentration	Exposure length		
	(ng/L)	50 days	100 days	150 days
	Control	0.7 (9)	0.7 (17)	0.7 (25)
TBT	30	0.6 (9)	1.5 (18)*	1.5 (12)*
	60	0.7 (10)	1.9 (16)*	1.9 (14)*
	125	0.8 (15)	1.4 (25)	1.5 (21)*
	250	1.2 (17)	1.6 (22)*	2.2 (21)*
	500	2.2 (19)*	2.4 (22)*	2.4 (31)*
Fenarimol	300	0.5 (11)	0.7 (15)	1.1 (20)*
	3000	0.9 (8)	1.3 (20)	1.9 (14)*
MT	30	0.8 (15)	1.0 (23)	0.9 (17)
	300	0.8 (18)	1.2 (16)	1.5 (14)*

3.4 Effect of TBT, fenarimol, and methyltestosterone on steroid levels

Esterified testosterone levels were measured in snails exposed for 100 days to a range of concentrations of TBT, MT, and FEN. Exposure of *M. cornuarietis* to TBT led to a significant decrease of esterified testosterone in females, whereas no significant effect was observed in males (Figure 2). The observed decrease was specific for TBT exposure, since neither MT nor FEN led to a significant change in esterified testosterone levels (Figure 2).

In addition, esterified estradiol was determined in the different treatment groups in order to investigate whether TBT specifically reduced the levels of esterified testosterone or affected those of other sex steroids. Esterified estradiol was also significantly decreased in females exposed to 125 and 500 ng/L TBT, although this decrease (≈50%) was not as pronounced as that observed for esterified testosterone (75-85%). Females exposed to MT (30 and 300 ng/L) showed a marginal, although not significant, decrease in esterified estradiol levels, and no effect was observed in males or in FEN exposed organisms, irrespective of sex (Figure 2).

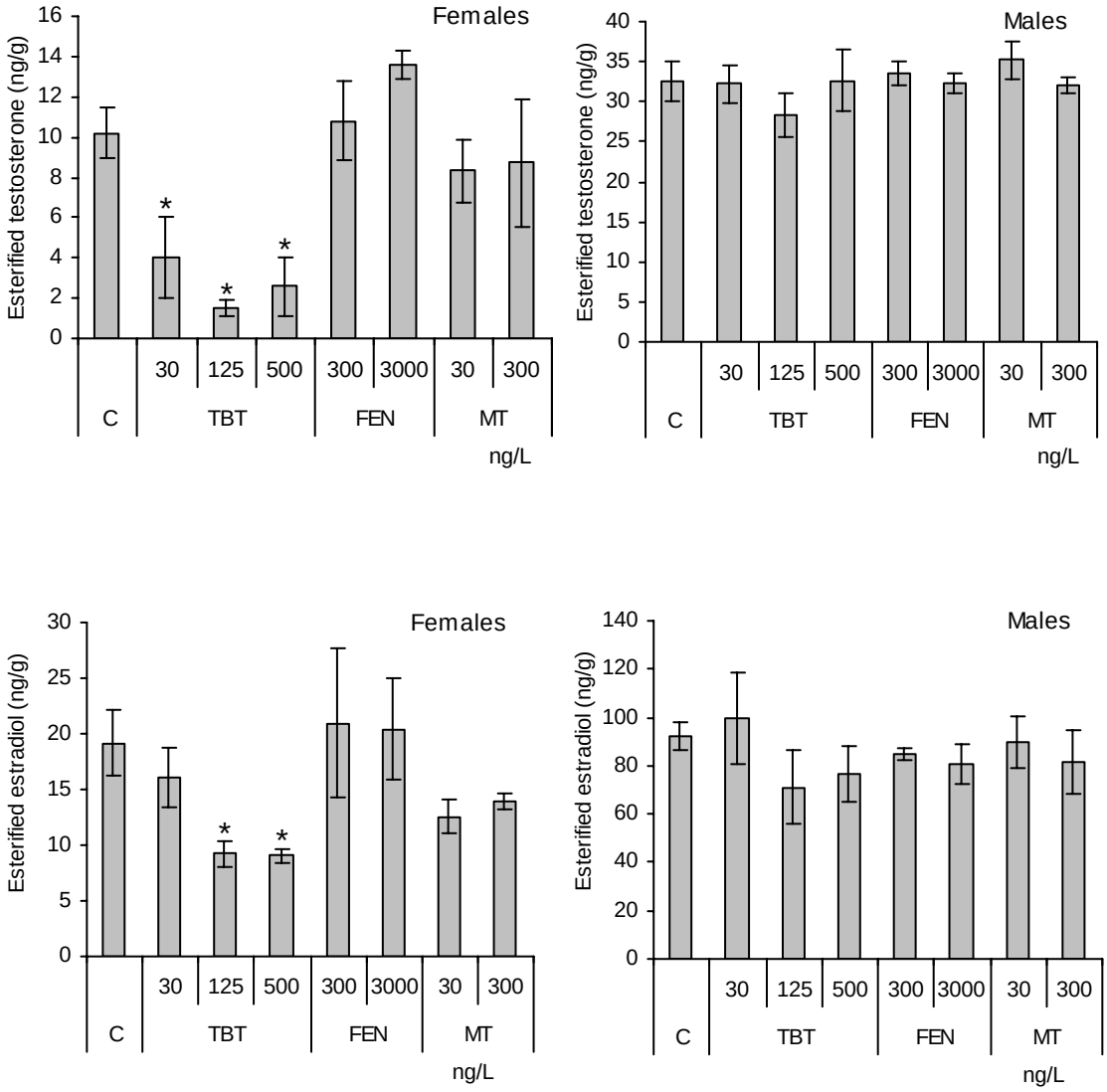

Figure 2. Esterified testosterone (top) and estradiol (bottom) levels determined in the digestive gland/gonad complex of *Marisa cornuarietis* exposed to different concentrations of TBT, fenarimol, and MT for 100 days. Values are mean ± SEM (n= 4). C: control; FEN: fenarimol. Significant differences versus control are indicated by * (ANOVA, Dunnett's test, $p<0.05$).

Thereafter, levels of free (unesterified) testosterone and estradiol were measured in control and exposed animals to investigate whether the changes in esterified steroids affected endogenous levels of free steroids. Levels of free testosterone (0.8 - 1.1 ng/g) and estradiol (0.016 - 0.012 ng/g) were in the range of the values previously determined in this study in specimens collected during the low spawning season (see Table 1) and were not significantly affected by exposure. Nevertheless, the decrease in esterified testosterone levels induced by TBT resulted in an increase in the proportion of free out of total testosterone that was present in female *M. cornuarietis* (Figure 3). This effect was not observed in males exposed to TBT, which showed a non-significant tendency to a lower proportion of free testosterone. Exposure to MT or FEN did not significantly

alter the proportion of free out of total testosterone in males or in females (Figure 3). The proportion of estradiol found in free form was not significantly affected in those experimental groups (0.2 to 0.5% in males and 0.5 to 1.5% in females). Finally, the ratios of free T versus free E2 were evaluated in exposed animals, and no significant differences between controls and exposed organisms were observed, despite a trend towards higher T/E2 ratios in both males and females exposed to TBT and MT (Figure 4).

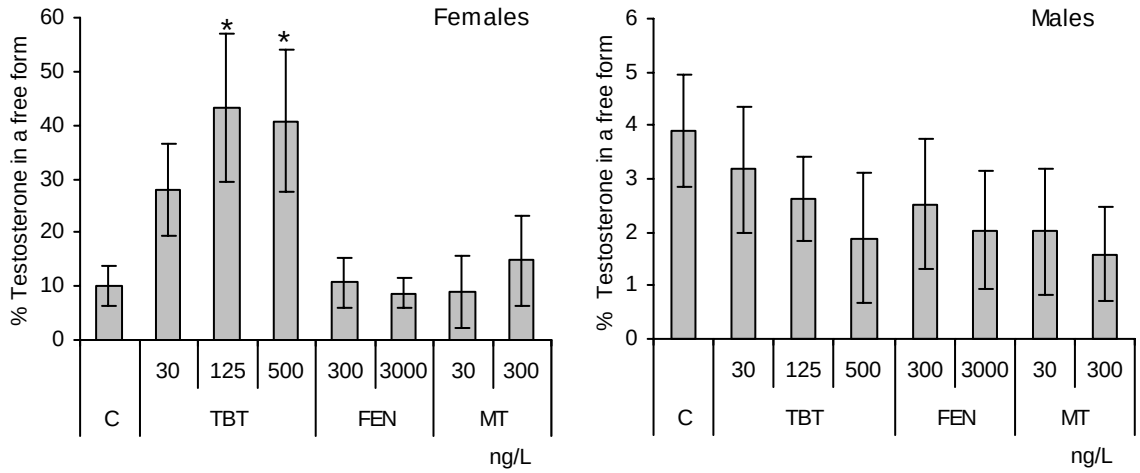

Figure 3. Proportion of testosterone unesterified out of the total testosterone (esterified + unesterified) in the digestive gland/gonad complex of *Marisa cornuarietis* exposed to different concentrations of TBT, fenarimol, and MT for 100 days. Values are mean ± SEM (n= 4). C: control; FEN: fenarimol. Significant differences versus control are indicated by * (ANOVA, Dunnett's test, $p<0.05$).

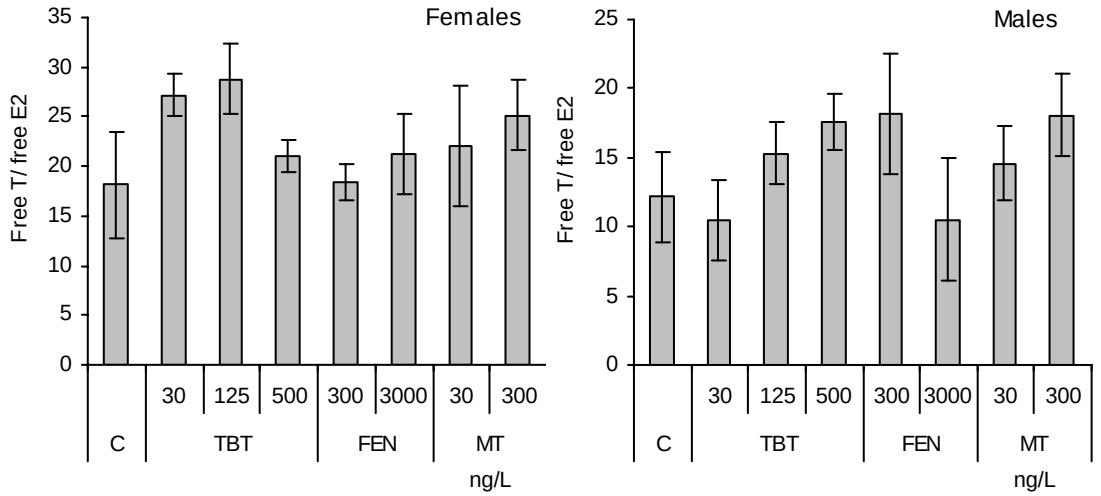

Figure 4. Ratios between free testosterone and free estradiol levels determined in the digestive gland/gonad complex of *Marisa cornuarietis* exposed to different concentrations of TBT, fenarimol, and MT for 100 days. Values are mean ± SEM (n= 4). C: control; FEN: fenarimol. No significant differences versus control were observed (ANOVA, Dunnett's test, $p>0.05$).

3.5 Effects of xenoandrogens on ATAT activity

ATAT activity was measured in microsomal fractions isolated from digestive gland/gonad complexes of *M. cornuarietis* exposed to different concentrations of TBT, MT, and FEN for 50 days. These animals were collected in late October, during the high spawning season, and ATAT activity in all the organisms, including the control group, was up to 5-fold higher than in those collected in the period March to June –low spawning season- (see Table 1). In addition, significant sex differences were observed, males showing higher activities (168 ± 18 pmol/min/mg protein, n=12) than females (90 ± 7 pmol/min/mg protein, n=10).

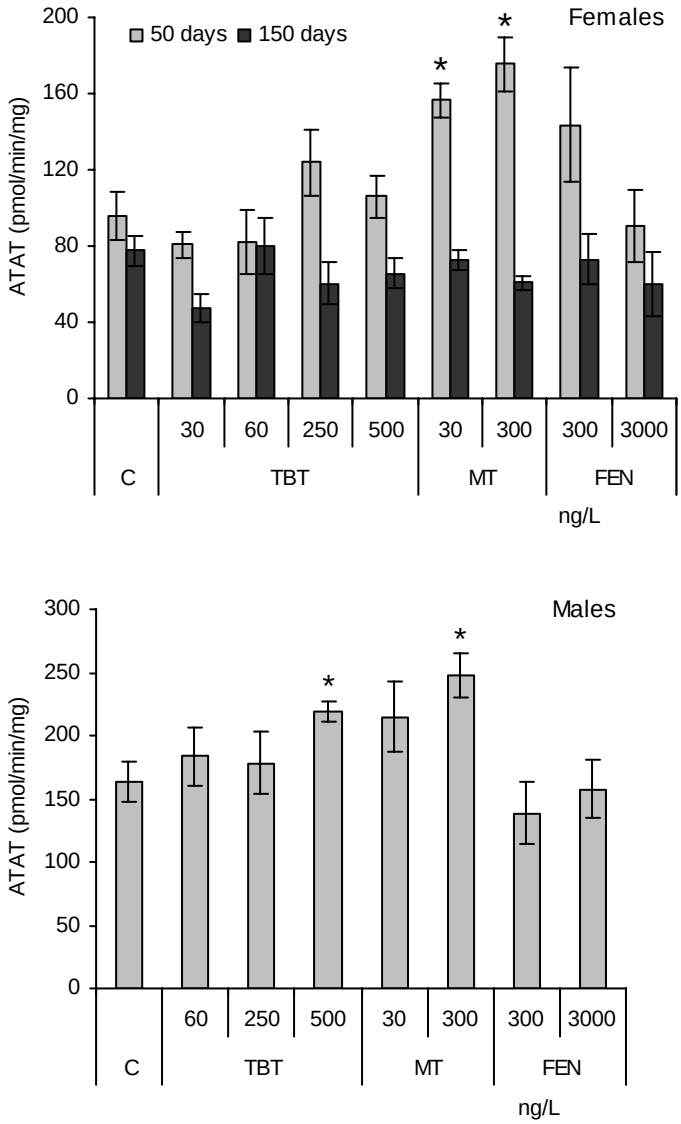

Figure 5. ATAT activity determined in microsomal fractions isolated from the digestive gland/gonad complex of *Marisa cornuarietis* exposed to different concentrations of TBT, FEN, and MT for 50 days (males and females) and 150 days (females). Values are the mean ± SEM (n = 5-6 for females; n = 6 for males). C: control. Significant differences versus control are indicated by * (ANOVA, Dunnett's test, $p<0.05$).

ATAT activity was significantly induced in males and females exposed to MT for 50 days (Figure 5). This induction was concentration-dependent and was higher in females than in males (1.8- and 1.5-fold increase vs. controls in females and males, respectively). ATAT activity measured in MT exposed females (175 ± 14 pmol/min/mg protein) increased up to the values recorded in control males (163 ± 15 pmol/min/mg protein). Exposure to high TBT concentrations (250-500 ng as Sn/L) also induced ATAT activity in males and females, although the increase was only statistically significant in males exposed to 500 ng/L TBT (1.3-fold increase vs. controls). No statistically significant effect was observed for FEN.

ATAT activity was further determined in females after 150 days exposure to the different chemicals. However, the increase in ATAT activity in MT- and TBT-exposed females was no longer observed (Figure 5).

4. Discussion

This study shows that most of the testosterone in *M. cornuarietis* digestive gland/gonad complex extracts is found in an esterified form, confirming previous findings in another gastropod species, *Ilyanassa obsoleta* [15,22]. In addition, it also shows that estradiol is found mainly in an esterified form. Thus, free (unesterified) testosterone represented only 4-30% of the total testosterone measured in the snail tissue, and free estradiol represented 1-2% of total estradiol.

Additionally, the levels of esterified steroids were considerably higher in males than in females, independently of the sampling season (see Table 1 and Figure 2). However, the observed differences in esterified steroid levels were not directly associated to corresponding differences in free steroid levels, i.e., the 4- to 10-fold lower levels of esterified steroids in females when compared to males did not co-occur with markedly higher levels of free steroids in females. Indeed, free steroid levels did not show a clear sexual dimorphism (Table 1). The levels of free steroids measured in this study were similar to the values reported by Schulte-Oehlmann et al. [31] in females of *M. cornuarietis*: 500 to 1900 pg free T/g and 20 to 120 pg free E2/g. Similarly to vertebrates, sex differences in the levels of free steroids during the reproductive season have been found in the echinoderm *Asterias rubens* for progesterone and estrone [33] and in the metazoan *Remilla koellikeri* for estradiol [34], but there is no clear evidence of such a dimorphism in molluscs for progesterone [14,35], testosterone, or estradiol ([10], Porte C, personal communication). Nevertheless, more detailed studies (several samplings along the reproductive cycle) are needed to conclude whether sex differences in free steroids do or do not occur in *M. cornuarietis*.

The equilibrium between esterified and free steroids is probably determined by the action of acyl-CoA steroid acyltransferases and steroid-fatty acid esterases. Existing evidence suggests that acyltransferases specific for sex steroids do exist in mammals: the acyl-CoA acyltransferase enzyme that esterifies cholesterol is not responsible for the esterification of testosterone, dehydroepidandrostenedione, or estradiol [36], and the enzyme that forms corticosterone esters is different from the one that produces estradiol esters [37]. Nevertheless, sex steroid acyltransferases have not yet been fully characterized, and it is uncertain whether they are specific for a single sex steroid or if they can conjugate different sex steroids. In this study, a significant correlation ($p<0.05$) was observed between testosterone and estradiol esterification, possibly indicating that a single enzyme conjugates both steroids. In a previous work, a similar profile of fatty acid esters was found when *C. virginica* digestive gland microsomes were incubated with estradiol and dehydroepiandrosterone in the presence of ATP and coenzyme A (endogenous esterification); and similar conjugation rates and affinity parameters were calculated with different fatty acyl-coenzyme A [18], which supports the hypothesis that a single enzyme conjugates different sex steroids and that it can do so with different fatty acid moieties.

Sexual differences in ATAT activity were only detected during the main spawning season (samples collected from August to January, exposure experiment), and not during the low spawning period (samples collected form March to June, characterization study); the observed differences (2-fold higher specific activity in males) were not as pronounced as those in esterified steroid levels (4- to 10-fold higher levels in males).

Both free and esterified steroid levels have been shown to follow seasonal variations in snails and the highest proportion of steroids conjugated to fatty acids was found during the reproductive period (Gooding and LeBlanc, 2004). Similarly, in this study, esterified steroid levels were higher in males and females collected during the high spawning season –exposure experiment- (Figure 2), than in those collected from March to June –low spawning season- (Table 1). Nevertheless, no significant differences in levels of free steroids between animals collected in both seasons were observed.

Apart from sex and seasonal variations along the biological cycle, certain contaminants can alter levels of free and esterified steroids. Thus, females exposed to TBT for 100 days showed a significant decrease in esterified steroids; the decrease in esterified testosterone was greater than that of esterified estradiol. These changes in esterified steroid levels were not accompanied by statistically significant changes in free steroids, although a higher proportion of testosterone in the free form and a trend towards a higher free testosterone/estradiol ratio was observed in TBT-exposed females (Figures 3 & 4). The relatively low sample size per sex and exposure group (n=4) in combination with the high inter-individual variability observed in the levels of free steroids might have prevented the detection of statistically significant differences in those parameters. In

accordance with the trend observed for the testosterone/estradiol ratio, earlier studies had reported an increase in free testosterone levels in TBT exposed female snails [12-13,22] and in free testosterone/ free estradiol ratios [31]. On the other hand, the finding that TBT decreased esterified testosterone levels confirms previous indications that exposure to TBT might impair the ability of females to esterify testosterone. Thus, *I. obsoleta* exposed to TBT (10 ng/L) for 3 months conjugated less testosterone to fatty acid esters [22].

The decrease in esterified steroids was specific for TBT-exposed females, as no changes in esterified steroids were observed in TBT-exposed males. Neither MT nor FEN exposure led to a significant alteration of esterified steroids. However, exposure to all three compounds caused the development of imposex and a reduction of fecundity in *M. cornuarietis*. TBT was the most potent compound in the induction of imposex, with an EC_{10} (4.16 ng/L), 6- to 7-fold lower than those for FEN or MT, whereas MT was the most potent compound affecting fecundity parameters with an EC_{10} (1.73 ng/L), 7- to 10-fold lower than those for FEN or TBT.

Thus, although the association between the decrease of esterified steroids in females and TBT exposure is clearly demonstrated, the relationship between the observed decrease in esterified steroids and the development of imposex -measured as an increase in the VDS index- is questionable. After 100 days exposure (when steroids were determined) a significant increase in the VDS index was observed in females exposed to 30 and 500 ng/L TBT (VDS = 1.5 and 2.4, respectively), but not in those exposed to 125 ng/L (VDS = 1.4). At that time, the VDS index in females exposed to the highest concentrations of MT (300 ng/L) and FEN (3000 ng/L), which showed no decrease in esterified steroids, were of 1.2 and 1.3 vs 0.7 in controls (Table 2).

Additionally, the precise mechanism by which TBT decreases the esterification of testosterone and estradiol in *M. cornuarietis* is not well established. A previous work did demonstrate that TBT had a rather low inhibitory effect on ATAT activity in-vitro, the enzyme responsible for the fatty acid esterification of testosterone [17], and we have observed here that TBT does not suppress levels of the microsomal enzyme, as determined through enzymatic assays following *in vivo* exposure to TBT. In fact, exposure to TBT (and MT) had an opposite effect, since both compounds significantly induced ATAT activity after 50 days exposure. The possibility remains that prolonged exposure to TBT is required for either inhibition or suppression of the enzyme. Nevertheless, after 150 days exposure the induction was no longer evident and no significant effect was observed, even though all three chemicals have caused the development of imposex. Thus, other factors, such as esterases or the availability of cofactors (i.e., fatty acids and acyl-CoA) that might regulate the equilibrium between free and esterified steroids, might have been affected by TBT-exposure and have led to reduced levels of esterified steroids in females. Apart from alterations in the esterification of sex steroids, other hypotheses exist to explain the mechanism of imposex induction. For instance, TBT might inhibit aromatase activity leading to a

decrease in the ratio estradiol/testosterone in female snails as a result of TBT exposure [31], as suggested in this study (Figure 4). In addition to alterations in steroid hormones, TBT might induce imposex by increasing the secretion of a neuropeptide, a penis morphogenic factor, in female snails [38]. Recently, TBT was shown to bind the RXR receptor in the snail *Thais clavigera*, and the exposure to the RXR ligand 9-cis retinoid acid led to the appearance of imposex in this snail species [39]. The function of RXR in molluscs is not known, however, in vertebrates RXR dimerizes with several other nuclear receptors, including those involved in lipid metabolism [40-42]. Therefore, it might be expected that an interaction of TBT with the RXR receptor led to alterations in lipid profiles and indirectly to alterations in the esterification of steroids.

The physiological role of esterified steroids can only be speculated. Recent studies showed that esterification might act as a homeostatic mechanism in molluscs to help them to maintain levels of free steroids stable [15,21]. In addition to the regulation of free steroids, the high levels of esterified steroids present in snails and the sexual dimorphism observed in the present study support the hypothesis that esterified steroids may have a physiological function. In insects, ecdysteroid esters are transferred to the eggs where they may represent a storage form of ecdysteroid hormone, which supplies free steroid during embryogenesis [43,44]. Thus, it might be that *M. cornuarietis* females transferred esterified steroids to their eggs, leading to lower levels of esterified steroids in maternal tissues. Indeed, levels of testosterone fatty acid esters in *I. obsoleta* were lowest at the end of the egg laying period, and then, most testosterone was found in the free form [15], which might be indicative of the transfer of esterified steroid to the developing embryo.

In conclusion, this study shows that esterified testosterone and estradiol are major steroids in digestive gland/gonad extracts of *M. cornuarietis*. These steroids are present at much higher levels in males than in females, and, in females, they are decreased by exposure to the organotin TBT, but not to other xenobiotics, such as FEN or MT. Additional studies are needed to fully understand the role of esterified steroids in *M. cornuarietis*, the physiological consequences of the strong reduction induced by exposure to TBT, and the possible connection of this reduction to the endocrine disrupting properties of TBT.

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Paper 8

THE EFFECT OF ORGANOTIN COMPOUNDS ON GENDER SPECIFIC ANDROSTENEDIONE METABOLISM IN *M. CORNUARIETIS*.

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The effect of organotin compounds on gender specific androstenedione metabolism in *Marisa cornuarietis*

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ABSTRACT

In a recent study, we demonstrated that androstenedione was mainly converted to testosterone (T) and 5ζ-dihydrotestosterone (DHT) by microsomal fractions isolated from male *Marisa cornuarietis*, whereas it was primarily metabolized to 5ζ-dihydroandrostenedione (DHA) by females. In the present work, the sexual dimorphic metabolism of androstenedione was further investigated, and attributed to a higher 17η-hydroxysteroid dehydrogenase activity in males than in females. Thereafter, the hypothesis was tested that the metabolism of androstenedione might be affected by exposure to tributyltin (TBT) and triphenyltin (TPT), which are known to induce the development of imposex in several gastropod species. The *in vitro* metabolism of androstenedione, particularly the formation of DHA and DHT, was inhibited by both compounds. *In vivo* experiments showed no significant alteration in the metabolism of androstenedione in males, but a marginal (TBT) and a significant (TPT) inhibition of the formation of DHA in females exposed for 150 days to concentrations that had significantly induced imposex. The ratio DHT+T/DHA, a possible indicator of metabolic androgenization, tended to increase (0.43 vs. 0.35, p=0.06) in females exposed to TPT for 50 days. However, these ratios never reached values comparable to those found in males (11 ± 1), regardless of their imposex condition.

Keywords: *androstenedione, steroidogenesis, organotin, imposex, gastropod*

1. INTRODUCTION

A recent study demonstrated that in contrast to vertebrates and other invertebrates, which readily metabolize androstenedione to T through a 17η-hydroxysteroid dehydrogenase (17η-HSD) catalyzed pathway [1], microsomal fractions isolated from digestive gland/gonad complex of the freshwater ramshorn snail *Marisa cornuarietis*, metabolized androstenedione mainly to 5ζ-dihydroandrostenedione (DHA) in females, and to 5ζ-dihydrotestosterone (DHT) and testosterone (T) in males [2].

Androstenedione has been detected in different invertebrate species and tissues [3-6]. In the mussel *Mytilus edulis*, androstenedione was one of the sex steroids found at highest concentrations [6]. Although it is not considered an active androgen in vertebrates due to its low affinity for the androgen receptor, it can be converted to T by the action of 17η-HSD [1]. The function of sex steroids in molluscs is not well known, however, there are indications that androgens are involved in mollusc reproduction and sex differentiation. For example, T administration to castrated male slugs (*Euhadra prelionphala*) stimulated the production of male secondary sex characteristics [7] and caused female gastropods to develop imposex with extensive penis and vas deferens formation [8-10]. Other vertebrate androgens, such as 11-ketotestosterone (11-kT) and DHT, stimulated spermatogenesis in male slugs [11]. In addition, T levels vary in some molluscs species in relation to the reproductive status of the organisms [12-13].

Apart from T, very low concentrations of organotin compounds (few ng/L) have been reported to induce imposex in female gastropods [14]. Besides, some studies have shown that T levels are elevated in female gastropods exposed to organotin compounds [8-9,15]. Based on these observations, several studies have investigated the possible interferences of organotin compounds with the metabolism of T, as a potential mechanism leading to alterations in T levels, and consequently to the induction of imposex. *In vitro* studies showed that TBT inhibited the oxidative metabolism of T and the aromatization of androgens in bivalve molluscs [16-17]. Similarly, the aromatization of T to estradiol [18-19], and its conjugation with sulphate [20] or fatty acid groups [15] were shown to be inhibited in female gastropods exposed to organotin compounds or inhabiting organotin polluted sites. Nevertheless, there is no available information on the effect of organotin compounds on the formation of T from precursors, such as androstenedione, which might as well regulate the levels of T and other androgenic metabolites within the organism. In addition, the finding that androstenedione is metabolized in a sexually dimorphic manner in *M. cornuarietis*, raises the hypothesis that alterations in this pathway might have consequences in sex differentiation and/or that imposex females might have a masculinized metabolism of androstenedione. Indeed, the metabolism of androstenedione is the only sexual dimorphic pathway detected so far in gastropods, since neither aromatization nor sulfation of T showed any clear sexual dimorphism in

M. cornuarietis, and only transient differences were found in the conjugation rate of T with fatty acid moieties (Janer et al., unpublished data).

Thus, in this study, we further characterized the metabolism of androstenedione in male and female *M. cornuarietis* by looking at the kinetics of the enzymatic reactions. Thereafter, we investigated whether the metabolism of androstenedione could be modulated *in vitro* and *in vivo* by organotin compounds. To this end, male and female *M. cornuarietis* were exposed to different concentrations of TBT and TPT (30 to 500 ng as Sn/L) for up to 150 days and the metabolism of androstenedione together with the development of imposex were evaluated.

2. MATHERIAL AND METHODS

2.1. Chemicals

Tributyltin chloride (TBT) was obtained from Sigma (Steinheim, Germany) and triphenyltin chloride (TPT) was purchased from Merck (Darmstadt, Germany). [1ß-^{3}H]-androstenedione (15-30 Ci/mmol) was purchased from Perkin Elmer Life Sciences (Boston, MA, USA). Unlabeled steroids were obtained from Sigma (Steinheim, Germany), and Steraloids Inc (Wilton, NH, USA). NADPH was obtained from Sigma (Steinheim, Germany). All solvents and reagents were of analytical grade, except TBT (96%) and TPT (for synthesis).

2.2. Animals

Ramshorn snails, *Marisa cornuarietis* (Mollusca: Prosobranchia: Ampullariidae), came from our laboratory breeding stock which was derived from a stock at Aquazoo Düsseldorf (Germany) in 1991 with regular cross-breeding of wild-caught animals from Florida (USA) to avoid inbreeding.

2.3. Design of exposure experiments

Exposure experiments were performed as 24 h (weekend 48 h) semi-static renewal systems in 60 L glass tanks, provided with an Eheim filter system and additional aeration. Tests were carried out under constant conditions regarding temperature and light dark cycle (12 : 12 h). Water parameters (pH, conductivity, temperature, nitrite, O_2 concentration and saturation) were measured twice a week.

For the exposure experiments, two replicate groups of 75 sexually mature snails each were exposed to different nominal concentrations of both TBT and TPT (30, 60, 125, 250 and 500 ng as Sn/L for five months in fully reconstituted water at 24 ± 1°C. Chemicals were added in absolute ethanol; the ethanol concentration was of 0.001% in all exposure groups, including control.

At least 40 specimens were analyzed both at the beginning of the experiment and from every exposure group at day 50 and 150 after the start of the experiment for determination of imposex. From these, 10 males and 10 females were cooled in ice, the digestive gland/gonad complex was dissected, deep-frozen in liquid nitrogen and stored at –80°C for determination of androstenedione metabolism. The remaining animals were narcotized (2.5% $MgCl_2$ in deionised water). Individual shell and aperture height of all animals were measured to the nearest 0.1 mm before the shell was cracked and snails were removed. The extension of all sex organs were measured to the nearest 0.1 mm under a dissection microscope and the imposex stage of individual females recorded as described by Schulte-Oehlmann et al. [21]. The vas deferens sequence index (VDSI), calculated as the mean of imposex stages in a sample or population, was used to measure imposex development during the experiment (cf. Schulte-Oehlmann et al. [21] for details).

2.4. Sample preparation

Digestive gland/gonad complexes were homogenized in ice-cold 100 mM phosphate buffer pH 7.4 containing 100 mM KCl, 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM phenanthroline, and 0.1 mg/mL trypsin inhibitor. Homogenates were centrifuged at 12,000-*g* for 30 min. After centrifugation at 100,000-*g* for 60 min, the pellet was resuspended in the same buffer and centrifuged again at 100,000-*g* for 60 min. Microsomal pellets were resuspended in a small volume of 100 mM phosphate buffer pH 7.4, containing 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM phenanthroline, and 0.1 mg/mL trypsin inhibitor, and 20% w/v glycerol.

Protein concentrations were determined by the method described by Lowry et al. [22], using bovine serum albumin as a standard.

2.5. Androstenedione metabolism

Androstenedione metabolism was assessed by incubating microsomal proteins (150-200 σg) in 10 mM potassium phosphate buffer pH 7.4, containing 1.0 mM EDTA, 0.2 σM [^{3}H]-androstenedione (150,000 dpm), and 1.0 mM NADPH, in a final volume of 250 σL. 0.01 to 10 σM [^{3}H]-androstenedione (150,000-300,000 dpm) was used for kinetic analysis. The reaction was initiated by the addition of NADPH and incubated in constant shaking for 60 min at 30ºC. Incubations were stopped by adding 250 µL of acetonitrile and after centrifugation (1,500-g, 10 min), 200 µL of supernatant were injected onto the RP-HPLC column.

In order to investigate the metabolic fate of DHT, T, and DHA, the three metabolites were enzymatically generated by using digestive gland/gonad complex microsomes of *Marisa cornuarietis*, purified by HPLC, and further incubated with digestive gland/gonad complex microsomes of *M. cornuarietis* as described for androstenedione (0.02-0.04 σM; 15,000-30,000 dpm per incubation).

2.6. In vitro effect of organotin compounds on the metabolism of androstenedione

To evaluate the effect of TBT and TPT on androstenedione metabolism, microsomes were pre-incubated for 5 min in the presence of different concentrations of the chemicals (0.01, 0.1, 1.0, and 10 σM). The organotins, dissolved in ethanol, were added into the test tubes, evaporated, and redissolved in assay buffer; the microsomal proteins were added and incubated for 5 min. Thereafter, androstenedione metabolism was assessed as described in 2.4.

2.7. HPLC-radiometric detection

Separation of androstenedione metabolites was performed as described in Janer et al. [2] on a Perkin Elmer Binary LC pump 250 system equipped with a 250 x 4 mm LiChrospher 100 RP-18 (5 µm) reversed-phase column (Merck, Darmstadt Germany) protected by a guard column LiChrospher 100 RP-18 (5 µm). The mobile phase (1 mL/min) was composed of (A) 75% water and 25% acetonitrile and (B) 25% water and 75% acetonitrile. The run consisted on a linear gradient from 100% A to 100% B (0-30 min), followed by isocratic mode 100% B (5 min), linear gradient from 100% B to 100% A (5 min), and isocratic mode 100% A (5 min). Radioactive metabolite peaks were monitored by online radioactivity detection with a Radioflow detector LB 509 (Berthold Technologies, Bad Wildbad, Germany) using Flo-Scint 3 (Packard BioScience, Groningen, The Netherlands) as scintillation cocktail. Metabolites were quantified by integrating the area under the radioactive peaks.

2.8. Data analysis

The Michaelis-Menten parameters (Km and Vmax) were estimated as Y=Vmax*X/(Km+X) using the kinetics module of Prism 4 (GraphPad Software, San Diego, California, USA). Statistical significance was assessed by ANOVA with Dunnett's test for differences from controls (in vitro androstenedione metabolism), Student's *t*-test (in vivo androstenedione metabolism) and by Weir test (imposex index) at a level of significance $p<0.05$.

3. RESULTS

3.1. The metabolism of androstenedione in Marisa cornuarietis

The metabolism of androstenedione by microsomal fractions isolated from the digestive gland/gonad complex of *M. cornuarietis* led mainly to the formation of DHA in females and to T and DHT in males (Table 1). Interestingly, the ratio 17η-reduced metabolites (DHT and T) versus DHA was, in all cases, much higher in males (3 to 42) than in females (0.13 to 0.78), regardless of

their sampling time. Individuals for this study were sampled in the months of March to June and September to December.

The detected metabolites (DHA, DHT and T) were collected from the HPLC system and further incubated with male and female microsomal fractions for 60 min at concentrations of 0.02 to 0.04 σM. Under these experimental conditions, DHA (the major metabolite detected in females) was metabolized to DHT, both in males and females, but this conversion was faster in males (92 ± 3% of the DHA metabolized to DHT, n=4) than in females (48 ± 1%, n=4) (Figure 1). In contrast, when the metabolism of DHT and T was assayed, no metabolites were observed after 60 min incubation.

Table 1. Metabolism of androstenedione in male and female *Marisa cornuarietis*. 200 σg microsomal protein isolated from digestive gland/gonad complex was incubated for 1 h at 30ºC. Values are mean ± SEM (n = 25 males and 35 females). Ranges are given in brackets.

	DHA	DHT	T	(DHT+T)/DHA
Male	4.6 ± 0.6	24.9 ± 1.8	6.0 ± 2.5	11.6 ± 2.1
	(0.6 - 14.4)	(13.6 - 46.4)	(1.6 - 10.1)	(2.9 - 42.1)
Female	28.4 ± 1.5	11.0 ± 1.1	0.8 ± 0.1	0.40 ± 0.02
	(14.3 - 48.6)	(3.0 - 27.1)	(n.d. - 2.1)	(0.13 - 0.78)

n.d.: below detection limit.

3.2. Kinetics and time course of androstenedione metabolism

The kinetics for 5ζ-reductase were evaluated in digestive gland/gonad complex microsomes of *M. cornuarietis* considering the sum of the amount of DHA and DHT formed. Km and Vmax were 1.2 ± 0.5 σM and 250 ± 96 pmol/h/mg, respectively (mean ± SEM, n= 4 animals), and did not differ between males and females. The kinetics for 17η-HSD could not be evaluated because the rate of formation of DHT was greatly determined by the rate of formation of DHA (Figure 1), and the formation of T, which was only formed in a significant amount in males, was linear throughout the range of concentrations of androstenedione tested (0.01 to 10 σM). The metabolism of androstenedione was assessed after different incubation times (ranging from 10 to 120 minutes) and results indicated that the metabolic rate was linear up to 60 minutes incubation.

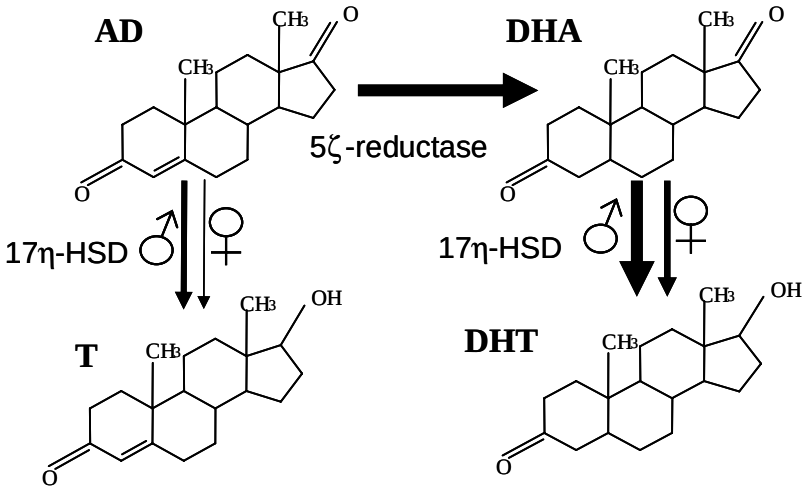


Figure 1. The metabolism of androstenedione by digestive gland/gonad complex of *M. cornuarietis*. Width of the arrows is indicative of the rate of conversion for each metabolite.

3.3. In vitro effects of organotin compounds

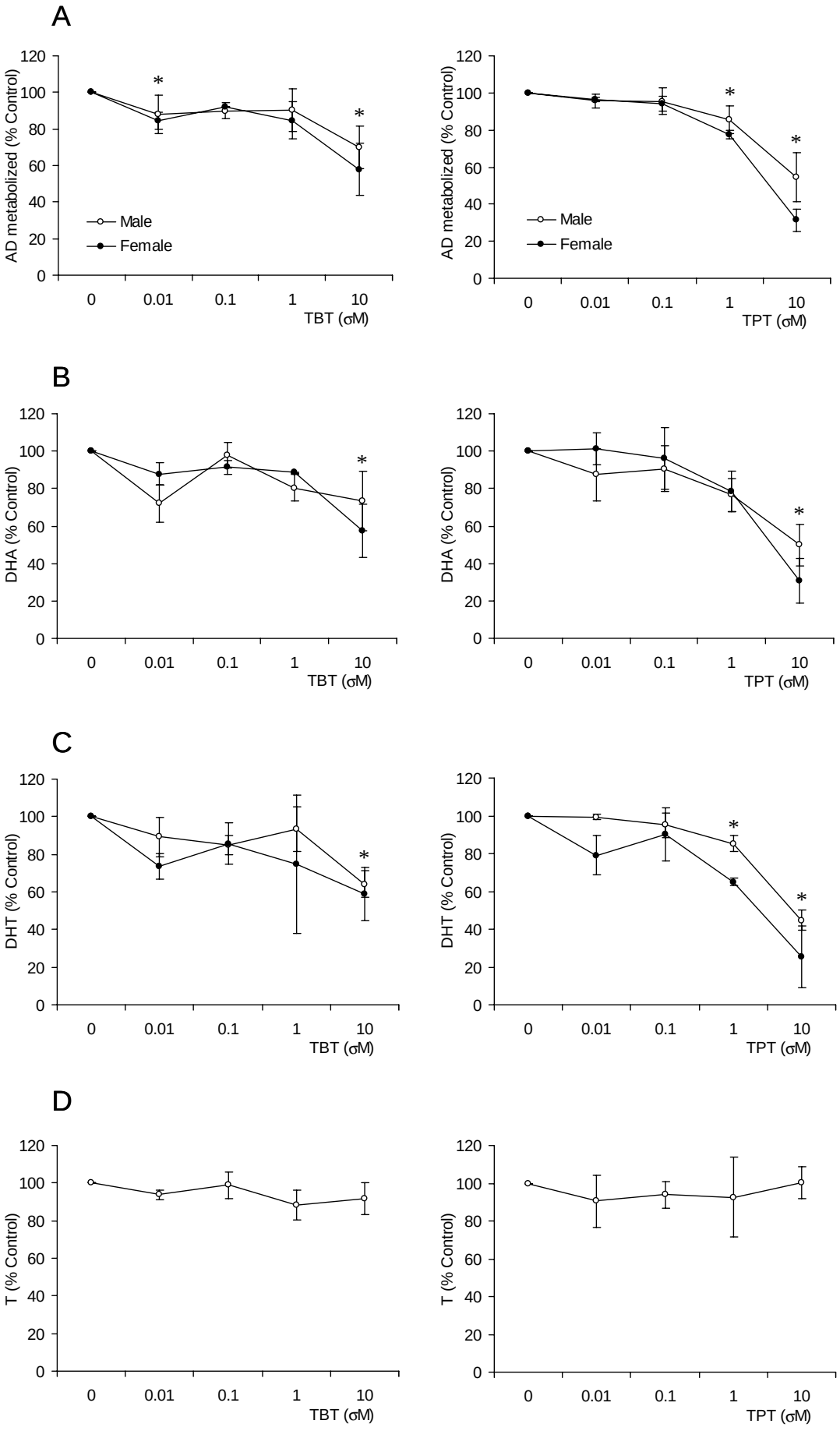
The interaction of TBT and TPT with the metabolism of androstenedione was investigated by incubating *M. cornuarietis* digestive gland/gonad complex microsomes with androstenedione and different concentrations of the organotin compounds (0.01 to 10 σM). The metabolism of androstenedione was significantly inhibited by TBT (0.01 and 10 σM) and TPT (1 and 10 σM) (Figure 2A). TPT caused a stronger inhibition than TBT in both males (50 ± 11% vs. 27 ± 16% for TPT and TBT, respectively) and females (69 ± 12% vs. 43 ± 14%, respectively). Generally, females were more sensitive to inhibition than males. The inhibitory effect on AD metabolism was further evidenced when looking at the formation of DHA and DHT (Figure 2B & C), which was decreased. Nonetheless, the formation of T (only determined in males) was not affected (Figure 2D). These results indicate that the observed decrease in androstenedione metabolism is mainly due to the inhibition of 5ζ-reductase, and that the lower rate of formation of DHT possibly occurred as a consequence of the lower rate of formation of DHA.

3.4. Induction of imposex by TPT and TBT

Females exposed to the highest concentration of TPT and TBT (500 ng as Sn/L) for 50 days developed imposex (VDS index significantly higher than in controls, $p<0.05$). After 150 days exposure, females of the 250 ng TPT as Sn/L group and of all the TBT groups (30 to 250 ng as Sn/L) had also developed imposex (Figure 3). In order to assess the effects of organotin exposure on androstenedione metabolism, and its potential link with imposex development, females were grouped into controls (C), females exposed to concentrations that did not significantly induce

imposex (low concentration group -L-: 30 to 250 ng TPT and TBT as Sn/L after 50 days exposure; 30 to 125 ng TPT as Sn/L after 150 days exposure), and females exposed to concentrations that significantly induced imposex (high concentration group -H-: 500 ng TPT as Sn/L and 500 ng TBT as Sn/L after 50 days exposure; 250 and 500 ng TPT as Sn/L and 30 to 500 ng TBT as Sn/L after 150 days exposure) (see figure 3). Exposed males were classified exactly as described above for females.

Figure 2. (Next page). *In vitro* effect of TBT and TPT on androstenedione metabolism by digestive gland/gonad complex of *Marisa cornuarietis*. Total androstenedione metabolized (A) and formation rate of DHA (B), DHT (C) and T (D) is shown. Males are shown in open circles and females in black circles. Data are expressed as mean ± SEM (n=4 for males) and mean ± standard error (n=2 for females). Testosterone was not quantified in females due to the low formation rate. *Indicates statistically significant differences respect to control (ANOVA, Dunnett's test, $p<0.05$); data for males and females were pooled for statistical analyses.


A
AD metabolized (% Control)
Male
Female
TBT (σM)
TPT (σM)
B
DHA (% Control)
C
DHT (% Control)
D
T (% Control)
0
0.01
0.1
1
10
20
40
60
80
100
120
*

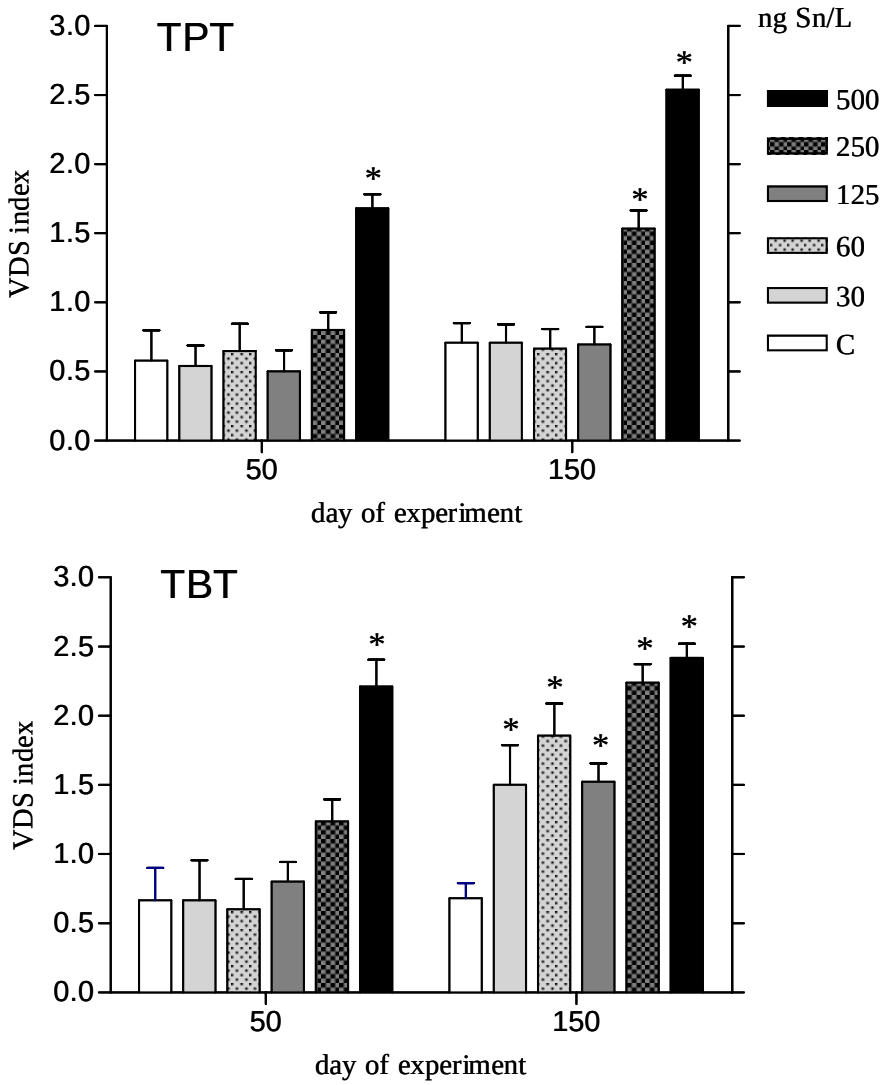


Figure 3. Imposex intensities, measured as the vas deferens sequence (VDS) index, in TPT and TBT exposed female *Marisa cornuarietis*. Exposure concentrations are provided as nominal values. Data are expressed as mean ± SEM (n=9-39). *Indicates statistically significant differences respect to control (Weir test, $p<0.05$).

3.5. In vivo effect of TPT on androstenedione metabolism

Total metabolism of androstenedione in male and female *M. cornuarietis* exposed to TPT for 50 days was not significantly different from that in control organisms, nor were differences found between females having developed imposex (exposed to 500 ng TPT as Sn/L; H group) and exposed females without imposex (30 to 250 ng TPT as Sn/L; L group). The metabolism of androstenedione was also assessed after 150 days exposure, and at this point of time, the rate of metabolism of androstenedione was generally lower in exposed females than in controls, and reached statistically significance ($p<0.05$) in those females that had developed imposex (Figure 4). No significant differences were observed for males.

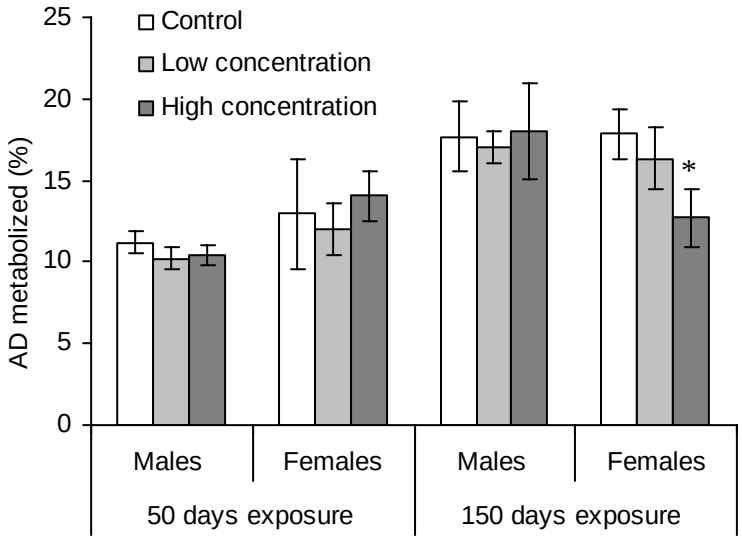


Figure 4. Percentage of androstenedione metabolized by microsomal fractions isolated from digestive gland/gonad complex of male and female *Marisa cornuarietis* exposed to different concentrations of TPT for 50 and 150 days. Data is expressed as mean ± SEM (n = 4 to 17). Low and high concentration groups are defined as those where females had not or had imposex, respectively (see text for further details). *Indicates statistically significant differences respect to control (Student's t-test, $p<0.05$).

When looking at the formation of DHA, no significant differences between control and exposed males were observed after 50 and 150 days exposure. Similarly, the formation of DHA was not affected in females exposed for 50 days, however, it was significantly inhibited ($p<0.05$) in females showing imposex after exposure to TPT for 150 days. The rates of formation of DHT and T were not significantly affected in any of the exposed groups (Figure 5).

Finally, the ratio between 17η-reduced metabolites (T and DHT) and 17η-oxidated metabolites (DHA) formed from androstenedione, which is about 30-fold higher in males than in females, was considered to evaluate a possible 'androgenization' of the androstenedione metabolic pattern. No significant effect on this ratio was observed in any of the experimental groups, although a tendency towards lower ratios was observed in exposed males (i.e. 5 in males exposed for 50 days to high concentration vs. 14 in control males, $p=0.17$) and the opposite tendency was observed in exposed females (0.43 in females exposed for 50 days vs. 0.35 in control females, $p=0.06$) (Figure 6).

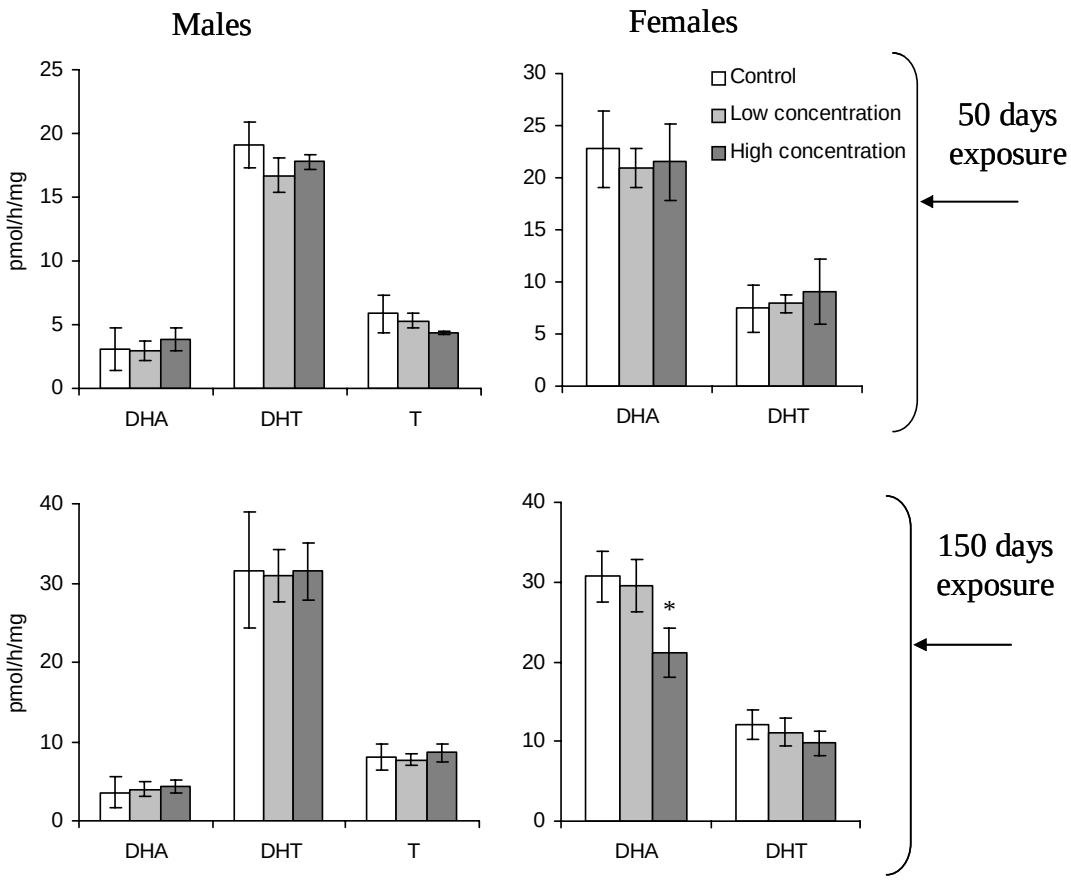


Figure 5. Rate of formation of DHA, DHT and T by microsomal fractions isolated from digestive gland/gonad complex of male and female *Marisa cornuarietis* exposed to different concentrations of TPT for 50 and 150 days. Data are expressed as mean ± SEM (n = 4 to 17). *Statistically significant difference respect to control (Student's t-test, $p<0.05$).

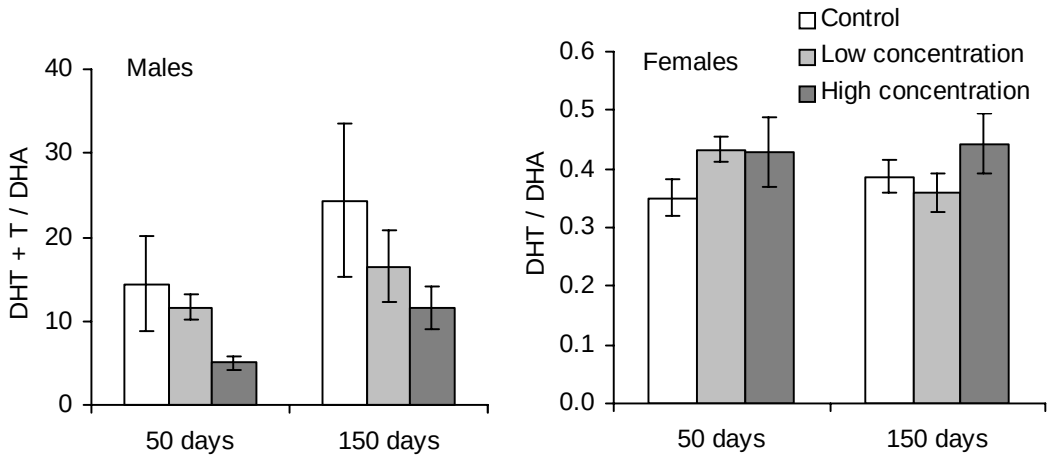


Figure 6. Ratio of 8>η-hydroxylated metabolites (DHT+T) vs. 17-keto metabolites (DHA) in male and female *Marisa cornuarietis* exposed to different concentrations of TPT for 50 and 150 days. Data are expressed as mean ± SEM (n = 4 to 17). No significant differences to controls were observed.

3.6. In vivo effect of TBT on androstenedione metabolism

The *in vivo* effect of TBT on androstenedione metabolism was only evaluated in females, since males had not shown any significant alteration on androstenedione metabolism when exposed to TPT. Exposure to TBT did not significantly alter the metabolism of androstenedione (Figure 7A), however, similarly to the effects observed in TPT-exposed females, the rate of metabolism of androstenedione and the formation of DHA and DHT were generally lower in females exposed to TBT for 150 days (with imposex) than in controls (Figure 7B).

When the ratio between DHT and DHA was assessed, no significant effect was observed (Figure 7C), despite a trend towards lower values in exposed females, which is in contrast with the data obtained for TPT (Figure 6).

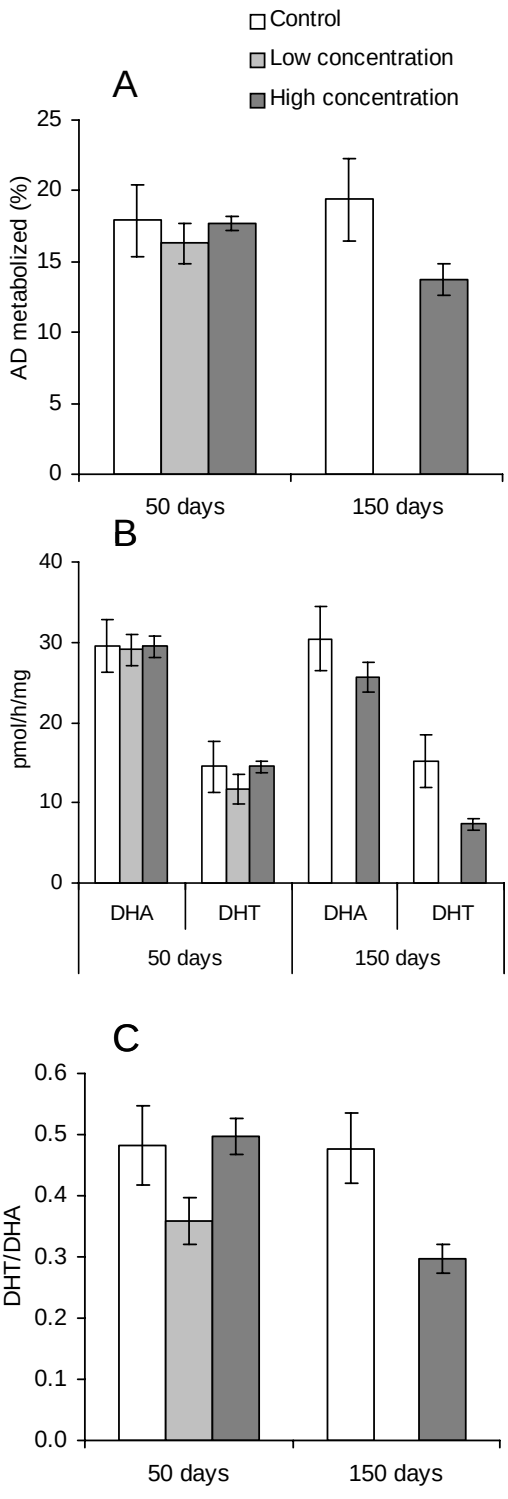


Figure 7. Metabolism of androstenedione by digestive gland/gonad complex of female *Marisa cornuarietis* exposed to different concentrations of TBT for 50 and 150 days. A) Percentage of androstenedione metabolized; B) Rate of formation of DHA and DHT; C) Ratio DHT vs. DHA. Data are expressed as mean ± SEM (n = 4 to 16). After 150 days exposure, all concentrations had induced imposex in females. No significant differences to controls were observed.

4. DISCUSSION

In an earlier study we reported that the profile of metabolites obtained when incubating digestive gland/gonad complex of *M. cornuarietis* with androstenedione was different between males

and females [2]. Thus, the formation of DHT and T occurred at a much higher rate in males than in females. The present study confirmed these findings in all individuals analyzed, regardless of the time of the year when they were sampled. Similarly to other mollusc species [11,24], T was not the major androstenedione metabolite in *M. cornuarietis*. Instead, androstenedione was mainly converted to DHA, through a 5ζ-reductase-catalyzed pathway. This is a major difference with most invertebrate and vertebrate species that readily convert androstenedione to T (e.g. [2,23]). In molluscs, DHA is later metabolized by 17η-HSD to DHT (Figure 1), or by 3ζ/η-HSD to epiandrosterone [24], or by both HSDs to form 5ζ4androstane-3ζ6η,17η-diols [11].

Affinity constants (Km) and specific activities for 5ζ-reductase did not differ between male and female *M. cornuarietis*. The Km obtained (1.2 σM), determined as the sum of DHA and DHT, was similar to that reported for mammalian 5ζ-reductase1 (2.8 σM for androstenedione [25]). Nevertheless, mammalian 5ζ-reductase1 presents similar specific activities for androstenedione and T [25], which contrasts with the low specific activity of *M. cornuarietis* 5ζ-reductase towards T (<0.5 pmol/h/mg protein when incubated at 0.04 σM) versus androstenedione (8 ± 1 pmol/h/mg protein when incubated at 0.04 σM). A higher 5ζ-reductase activity towards androstenedione than towards T has also been found in the bivalve *M. galloprovincialis* (Janer et al., unpublished data), suggesting that this feature might be common to molluscs. Kinetic parameters for 17η-HSD could not be evaluated because the synthesis of DHT was greatly determined by the amount of DHA formed, and the synthesis of T from androstenedione was linear in the range of concentrations tested. However, the data gathered in this study indicate that 17η-HSD activities are higher in males than in females, and that this difference leads to the different metabolic pattern observed between sexes (Figure 1). The substrate specificity of 17η-HSD (i.e., <0.5 to 1.1 vs. 35 ± 4 pmol/h/mg protein for androstenedione and DHA, respectively when incubated at 0.04 σM), contrasts with the high affinity of other invertebrate and vertebrate 17η-HSDs for androstenedione. The unusual substrate affinities of 5ζ-reductase and 17η-HSD suggest that significant differences in androgen metabolism between this and other invertebrate groups exist, and points out the need to investigate the role that 5ζ-reduced androgens, DHT and DHA, might have in molluscs.

The metabolism of androstenedione might be a potential target for organotin compounds, and alterations on its sexually dimorphic pattern might be linked to the development of imposex. Both TPT and TBT (1-10 σM) decreased the rate of formation of DHA and DHT *in vitro*, possibly by inhibiting 5ζ-reductase. In fact, the decreased synthesis of DHT is likely to be a consequence of the lower formation of DHA, rather than an inhibition of 17η-HSD, since the formation of T was not altered (Figure 2). The susceptibility of 5ζ-reductase to inhibition by organotin compounds has already been reported for vertebrate species [23,26]). In contrast, 17η-HSDs were only affected at high concentrations of TPT (4.2 and 10.5 σM for 17η-HSDs vs. 0.95 σM for 5ζ-reductase) in human

microsomes [26]; and they were not affected in fish microsomes incubated with up to 100 σM TBT or TPT [23], in agreement with the observations in *M. cornuarietis*.

However, when the metabolism of androstenedione was investigated in exposed organisms, no significant effect was detected in snails after 50 days, despite of the fact that females had developed imposex (Figure 3). After 150 days, an inhibition of the total metabolism of androstenedione and a decrease in the formation of the major androstenedione metabolite, DHA, was observed in imposex females exposed to TPT (Figure 4 & 5). A marginal inhibition was also observed in imposex females exposed to TBT for 150 days (Figure 7). The fact that TPT had a stronger effect than TBT *in vivo* (31% vs. 16% inhibition of DHA synthesis, respectively) is consistent with the higher ability of TPT to inhibit 5ζ-reductase activity *in vitro*.

The sexually dimorphic metabolism of androstenedione in *M. cornuarietis* is clearly depicted by the ratio between 17η-reduced androstenedione metabolites and DHA (DHT+T/DHA), that is much higher in males (12 ± 2) than in females (0.40 ± 0.02). Thus, an increase of this ratio might be considered as an indication of 'metabolic androgenization'. The use of alterations in steroid metabolic profiles to assess the effect of endocrine disruptors is not a new concept. Thus, other studies [27-29] assessing the effects of endocrine disruptors used an androgenization ratio, defined by Baldwin et al. [27] as the rate of production of reduced and dehydrogenated metabolites of T (preferentially retained in *Daphnia magna* tissues) versus the rate of production of hydroxylated, sulphated and glycosidated metabolites (preferentially excreted). In the present study, the ratio (DHT+T)/DHA was not significantly altered in exposed females, although there was a trend towards a higher ratio in females that have been exposed to TPT for 50 days and in those exposed for 150 days showing imposex (Figure 6). Nevertheless, these nearly significant increases of the ratio (e.g. 0.43 in females exposed to TPT for 50 days vs. 0.35 in control females, p=0.06) never reached values comparable to those found in males (Table 1). Therefore, neither the exposure to organotin compounds nor the development of imposex resulted in a major androgenization of androstenedione metabolism in females.

Overall, this study describing the sexual-dimorphic metabolism of androstenedione in *M. cornuarietis*, shows the susceptibility of 5ζ-reductase to inhibition by organotin compounds, both *in vitro* and *in vivo* (in females), and reports on the inability of organotin compounds to lead to a significant in vivo masculinization of androstenedione metabolism in females (increased synthesis of DHT and T), regardless of imposex induction.

Aknowledgements

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CHAPTER 4. SUMMARY OF RESULTS AND GENERAL DISCUSSION

4. SUMMARY AND GENERAL DISCUSSION

Recapitulating the scope of this dissertation, this thesis evaluated the potential effects of some xenobiotics on steroid metabolic pathways in invertebrate species. Metabolic pathways that may regulate steroid levels in invertebrates were investigated. Thus, phase I and phase II metabolism of sex steroids was identified in an echinoderm (*Paracentrotus lividus*), a crustacean (*Hyalella azteca*) and in five molluscan species (*Mytilus galloprovincialis*, *Mytilus edulis*, *Crassostrea virginica*, *Ilyanassa obsoleta* and *M. cornuarietis*); and the enzyme systems responsible were partly characterized (results in papers 1 to 4). Thereafter, the effect of organotin compounds (TBT and TPT) and fenarimol on those enzymatic pathways was investigated *in vitro* (results in papers 1, 4 and 8). And finally, the effects of several xenobiotics, classified as xenoandrogens (TBT, TPT, and fenarimol), xeno(anti)estrogens (alkylphenols and PAHs), and model steroids (estradiol and methyl-testosterone) were investigated *in vivo* (papers 5 to 8). The main results and conclusions obtained in this dissertation are summarized below.

4.1 Characterization of steroid metabolism in invertebrates

4.1.1 Phase I metabolism of androgens

The existence of different androgen metabolic pathways was demonstrated in the invertebrate species investigated: 5ζ-reductase, 17η-HSD, 3η-HSD, and several P450 isoforms catalyzing different hydroxylations. One of the major differences between the androgen metabolic profiles detected in this study and those reported for vertebrates is the much lower contribution of hydroxylation to the metabolism of testosterone in invertebrates (e.g. Wilson and LeBlanc, 1998; Parks and LeBlanc, 1998). Apart from this, similarities were found between the species used in this study and vertebrate species. Most of the steroid metabolites identified in this thesis are common vertebrate metabolites, with the exception of 4-androstene-3η,17η-diol (formed by *P. lividus* and *H. azteca*).

In addition differences between invertebrate species belonging to different phyla were observed. Thus, the assessment of androstenedione metabolism revealed that important differences exist between molluscs (*M. cornuarietis* and *M. galloprovincialis*) and the other invertebrates tested (*H. azteca* and *P. lividus*). The molluscs formed mainly 5ζ-reduced metabolites, namely, 5ζ-androstanedione and 5ζ-dihydrotestosterone. In contrast, the echinoderm and the crustacean studied readily converted androstenedione to testosterone (Figure 4.1), in a similar manner that

fish and other vertebrates metabolize androstenedione (Thibaut and Porte, 2004). Data available in the literature for other mollusc species, i.e. *Clione antarctica* and *Ariolimax californicus*, also indicated that testosterone is not a major metabolite of androstenedione (Hines et al., 1996; Gottfried and Dorfman, 1970).

Regarding testosterone metabolism, *P. lividus* had a higher and more complex metabolism - involving a variety of pathways- while *M. cornuarietis* had a limited ability to metabolize testosterone. An intermediate situation was found for *H. azteca* (Figure 4.1). Testosterone metabolism was not evaluated in mollusc species other than *M. cornuarietis*, however published work on *M. galloprovincialis* is available (Morcillo et al., 1998) and shows that, similar to *M. cornuarietis*, the ability of this bivalve to metabolize testosterone is limited. The low metabolic activities evidenced in *M. cornuarietis* in comparison with the other species tested, are supported by the fact that no hydroxylated, oxidated or reduced metabolites were observed in the incubation media after *in vivo* exposure of *M. cornuarietis* to ^{14}C-testosterone (Janer et al., unpublished data).

Despite *P. lividus*, *M. cornuarietis* and *M. galloprovincialis* displayed significant levels of 5ζ-reductase and 17η-HSD activities, the isoforms differed in substrate affinity and kinetic parameters (Table 4.1). The low affinity of 17η-HSD for androstenedione in the mollusc species raises the hypothesis that either testosterone is not a major androgen in this gastropod species, or that alternative biosynthetic pathways for T exist (e.g. dehydroepiandrosterone to 3η,17η-androstenediol to T) in these organisms.

Table 4.1. Characteristics of 5ζ-reductase and 17η-HSD in *P. lividus* and *M. cornuarietis.*

	Metabolic pathway catalyzed		Km (σM)	
	P. lividus	*M. cornuarietis*	*P. lividus*	*M. cornuarietis*
5ζ-reductase	T Ą DHT	AD Ą DHA	69 ± 3	1.2 ± 0.5
17η-HSD	T Ą AD Ą T	DHA Ą DHT	-[1]	-[1]

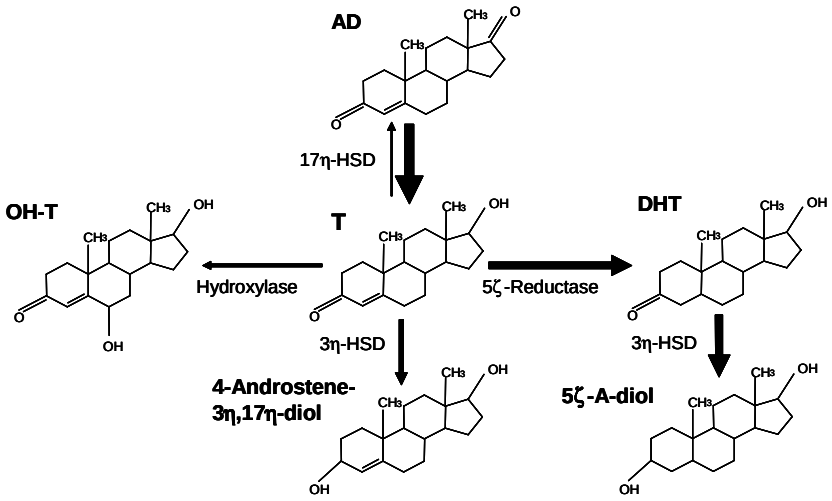
[1]Activity increased linearly in the range of substrate concentrations used, and Km could not be estimated.

The conversion of androgens to estrogens occurs at very low rates in invertebrates, therefore, the tritiated-water-release method, which has a very high sensitivity, was used to determine aromatase activity. The activities recorded for the molluscs investigated were in the range of those reported in earlier studies (0.2 to 0.6 pmol/h/mg protein; Le Curieux-Belfond, 2001; Morcillo et al., 1999).

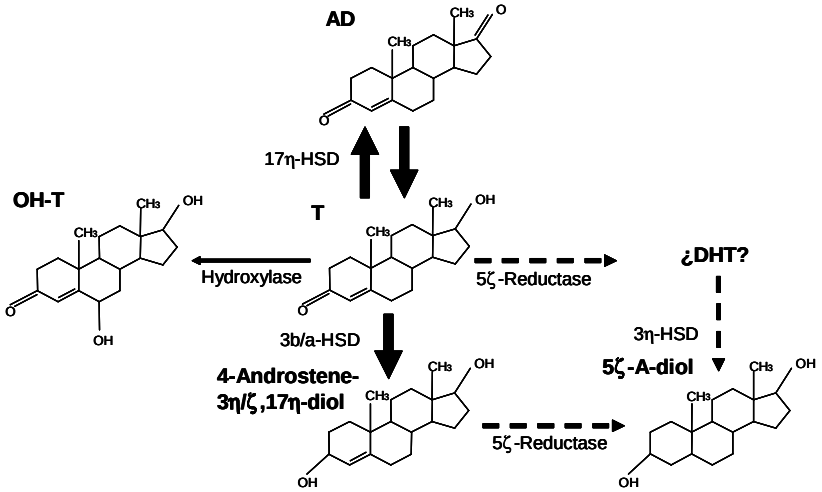
Similar to vertebrates, steroid metabolizing enzymes were differently distributed among tissues in *P. lividus*. Thus, enzymes leading to the biosynthesis of active steroids were mainly found in the gonads, whereas those that inactivate steroid hormones were mainly found in the liver (Norman and Litwack, 1997). In contrast, in the bivalve molluscs, aromatase activity was higher in the digestive gland than in the gonads.

Figure 4.1. *In vitro* metabolism of androstendione and testosterone in *Paracentrotus lividus* (gonad and digestive tube), *Hyalella azteca* (whole body), *Marisa cornuarietis* (digestive gland/gonad complex), and *Mytilus galloprovincialis* (digestive gland). Data for testosterone metabolism in *M. galloprovincialis* is from Morcillo et al., (1998).

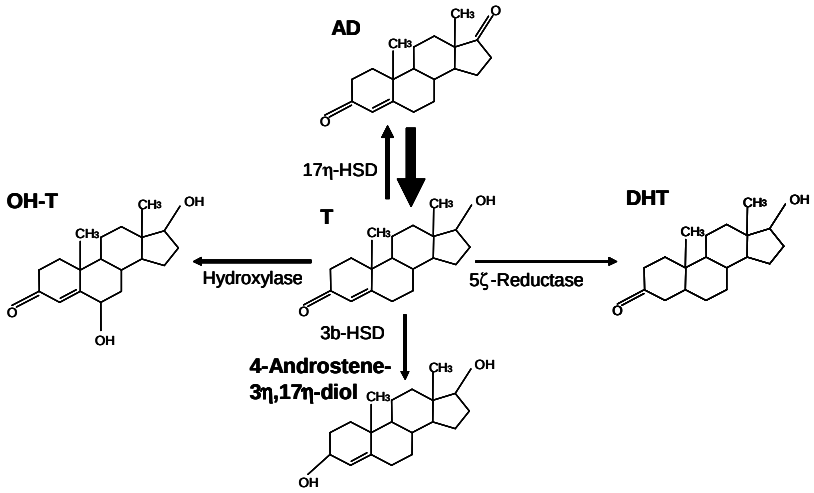
Paracentrotus lividus gonad



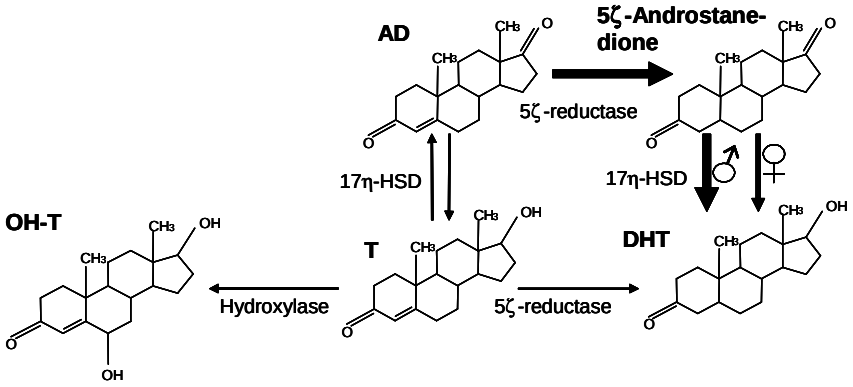
Paracentrotus lividus digestive tube



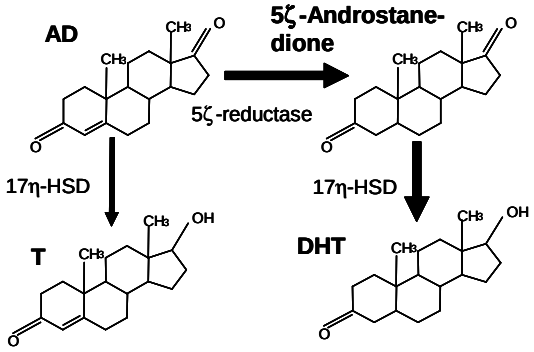
Hyalella azteca



Marisa cornuarietis



Mytilus galloprovincialis



Sex differences in androgen metabolism were observed in *M. cornuarietis* (Figure 4.1) and reflected the pattern observed in vertebrate species, leading to a higher formation of active androgens in males than in females. A trend towards a higher formation of 5ζ-A-diols in female *P. lividus* than in males was also observed, although it was not statistically significant.

Finally, indications were gathered that some of these steroid metabolizing pathways presented seasonal variability. For instance, 5ζ-reductase activity in *M. cornuarietis* varied up to 1.6-fold depending on the sampling season. Although it was not in the scope of this thesis, a detailed study on the seasonal variability of these parameters and their relationship with other reproductive endpoints would be of great interest to progress on the understanding of their physiological function and should be the subject of further research.

4.1.2 Phase II metabolism of steroids

Microsomal fractions isolated from gonads and/or digestive gland of all the invertebrate species studied in this thesis (*M. cornuarietis, Mytilus sp., H. azteca,* and *P. lividus*) had the ability to form fatty acid conjugates of steroids when incubated with fatty acid acyl-CoA. The affinity of acyl-CoA acyltransferase towards testosterone differed between species. Thus, the enzyme saturated at lower concentrations of testosterone (i.e. they had a higher affinity for steroids) in *M. cornuarietis* (4 σM) than in the *H. azteca* or *P. lividus* (19 to 41 σM). The affinity for estradiol did not differ among all mollusc species evaluated (7-10 σM; in *C. virginica*, *M. cornuarietis* and *I. obsoleta*) and it was similar to that for testosterone in *M. cornuarietis*.

The high affinity of molluscan acyltransferases for steroids together with the higher levels of hydroxylases and oxido/reductases described in crustaceans in comparison to molluscs, might explain why fatty acid conjugation has a greater contribution to the fate of sex steroids in molluscs than in crustaceans. Thus, when molluscs were exposed to steroids *in vivo*, they converted these steroids mainly to fatty acid conjugates (this thesis; Gooding and LeBlanc, 2001), whereas fatty acid conjugates were minor metabolites in crustaceans (this thesis; Baldwin and LeBlanc, 1994b; Verslycke et al., 2002).

Several sex steroids were shown to be substrates for acyltransferases. Thus, *C. virginica* microsomes isolated from digestive gland and gonads esterified E2 and DHEA; *I. obsoleta* and *M. cornuarietis* visceral coil microsomes esterified testosterone and estradiol; and a series of additional steroids showed the ability to compete for the esterification of testosterone in *I. obsoleta*, suggesting that they might also be substrates of this enzyme. Similarly, *C. virginica* acyl-CoA acyltransferases could use a variety of fatty acid acyl-CoA substrates, including totally saturated fatty acids (C18:0 and C16:0), monounsaturated fatty acids (C18:1 and C16:1), and polyunsaturated fatty acids (C18:2 and C20:4). Therefore, steroid acyl-CoA acyltransferases seem

to have a broad substrate affinity regarding both the sex steroid and the fatty acid moiety, although a certain degree of substrate specificity existed (e.g. pregnenolone did not interfere with testosterone esterification in *I. obsoleta*).

Similar to phase I metabolic pathways, ATAT activity varied up to 5-fold depending on the sampling season. In addition, ATAT activity was higher in males than in females (1.8-fold) in Spring, when high specific activities were recorded.

In contrast to the widely conserved apolar conjugation, the formation of polar conjugates was only detected in some of the investigated species. Thus, cytosolic sulfotransferase activity towards testosterone was high in the digestive tube of *P. lividus* (137 ± 22 pmol/min/mg at 10 σM testosterone), whereas it was low in *Mytilus* (0.6 pmol/min/mg at 3 σM estradiol) and nearly undetectable in *M. cornuarietis* and *H. azteca* (0.05 to 0.18 pmol/min/mg at 10 σM testosterone) (Table 4.2). SULT exhibited high affinity for testosterone in *P. lividus* (140 pmol/min/mg protein at 5 σM testosterone) although, at high concentrations of the substrate, the conjugation rates decreased, suggesting that testosterone or testosterone-sulfate might act as SULT inhibitors.

These *in vitro* observations are supported by the fact that low rates of *in vivo* testosterone-sulfate conjugation have been reported in both crustacean and mollusc species (Hines et al., 1996; Ronis and Mason, 1996; Baldwin et al., 1998). In addition, the high sulfatase activity that was measured in cytosolic preparations from the oyster *Crassostrea virginica* is likely to interfere with the *in vitro* determination of sulfotransferase activity. Similarly, the presence of sulfatase activity in lobster cytosol (*Homarus americanus*) (Li and James, 2000), and the fact that SULT enzymes are high affinity/low capacity enzymes in crustaceans (de Knecht et al., 2001), might explain the low levels of SULT activity measured in crustaceans in general, and in *H. azteca* in this study.

Additionally, the formation of glucuronyl or glucosyl conjugates was low or undetectable in all tested species (Table 4.2). In contrast, indications that alternative conjugating pathways might occur in molluscs and other invertebrates were gathered. A recent report by Stroomberg et al. (2004) showed that isopods metabolized pyrene to form the conjugate pyrene-1-O-(6”-O-malonyl)glucoside, and opened the possibility that conjugates other than the classical ones (sulfates, glucuronides, glucosides, glutathione derived conjugates, acetylates, and amino acid conjugates; James, 1987) might also be formed in invertebrate species. The observation within this thesis that a conjugate of estradiol was formed when sucrose was present in the microsomal buffer, suggested that new phase II metabolites can be formed in invertebrates. Similarly, the testosterone metabolite that was observed in incubations with *M. cornuarietis* cytosol might be an additional phase II metabolite, possibly unique for this species (Table 4.2).

Table 4.2. Summary of the different conjugates detected in the invertebrate species of interest. The metabolic pathways were classified according to the percentage of the substrate that was metabolized as shown in the legend. M1 is the microsomal metabolite formed in the presence of sucrose and M2 is the cytosolic conjugate formed in the presence of NADPH (see section 2.5). n.a. not assessed.

Species	Sulfate	Glucuronide	Glucose	M1	M2	Fatty acid conjugate
Mollusc						
M. cornuarietis		n.a.				
M. galloprovincialis						
Echinoderm						
P. lividus		n.a.				
Crustacea						
H. azteca		n.a.	n.a.			

% of substrate metabolized	Code
<0.1%	
0.1 to 2%	
2 to 10%	
>10%	

The comparative evaluation in Table 4.2 is based on *in vitro* data, therefore it shows the potential contribution of the different conjugating pathways to steroid metabolism. However, it does not necessarily reflect the situation *in vivo*. In this last case, the subcellular distribution of the enzymes and the substrates, and the availability of cofactors will condition the relative contribution of each pathway to total metabolism. Indeed, the data obtained when *H. azteca* and *M. cornuarietis* were exposed to testosterone *in vivo*, indicated that whereas an unidentified polar conjugate was the major metabolite formed by *H. azteca,* fatty-acid conjugates were the major metabolites formed by *M. cornuarietis*.

4.1.3 Implications for the sensibility to xenobiotics

Altogether, the results on phase I and phase II metabolism of androgens suggest that the pathways involved in the synthesis/metabolism of steroids are notably different among invertebrate species. Some of the metabolic pathways investigated are also involved in the metabolism of xenobiotics. The biotransformation of xenobiotics usually leads to the conversion of the parent compound into a more water soluble form. As a result these more hydrophilic compounds may be more easily excreted from the body than the parent compound. However, in some cases (e.g. conjugation with

fatty acids) the biotransformation product might be less water soluble than the parent compound, and therefore have a higher bioaccumulation potential. In addition, when the chemical structure of a compound is altered, many properties of the compound are likely to be altered as well. Hence the biotransformation product can have different toxic actions and will behave differently within the organism with respect to tissue distribution, bioaccumulation, persistence, route and rate of excretion. Therefore, the differences observed in the metabolic pathways present in the invertebrate species investigated, might also lead to differences in their susceptibility to toxic effects by xenobiotics.

In addition to the above mentioned generalities for all xenobiotics, in the case of endocrine disruptors, it should be added that if they alter one of the metabolic pathways described, they could lead to a significant change in active steroid levels in one species, while not in another species. For example, if a chemical affects the esterification of steroids, the molluscs are likely to be more sensitive to that chemical than the other invertebrate species investigated.

From a risk assessment point of view, the considerations described above suggest that species representing different phyla and thus showing different metabolic activities towards xenobiotics and steroids, should be included in toxicity testing, specially in the case of potential endocrine disrupting substances.

4.2 Steroid levels in molluscs

Levels of free testosterone and estradiol determined in this thesis were in the range of those reported in previous studies with other mollusc species (Table 4.3). This table shows that levels of testosterone are similar to those of 5ζ-DHT and androstenedione in molluscs and this contrasts with the low metabolic rate observed for the conversion of androstenedione to testosterone. Testosterone was identified by GC-MS in *M. edulis*, therefore there is no doubt of its presence in some mollusc species, however, the quantifications reported in Table 4.3 were performed by RIA, therefore, cross-reactivity with other steroids cannot be excluded (e.g. Zhu et al., 2003). If we assume that the testosterone-like compounds identified by RIA are indeed testosterone, then it suggests that alternative pathways that lead to the formation of testosterone, probably from a precursor other than androstenedione exist in molluscs. The second observation in Table 4.3, is that, in agreement with the results we obtained for *M. edulis*, the levels of free estradiol and testosterone are higher in gonadal tissues than in peripheral tissues in molluscs. This suggests that gonads might be either a steroidogenic tissue or a target tissue in *M. edulis*. Finally it should be noticed that similar to previous data for other mollusc species and tissues, levels of testosterone were much higher (about one order of magnitude) than those of estradiol in *M. cornuarietis* (digestive gland-gonad complex) and *M. edulis* (whole tissue excluding digestive gland and gonad). Nevertheless, similar levels of estradiol and testosterone are found in gonads of *M. edulis* (this thesis), and in some reproductive tissues (e.g. prostate) in *Octopus vulgaris* (D'Aniello et al., 1996).

Table 4.3. Sex steroid levels in mollusc species.

Steroid	Method	Specie	Matrix	Levels (ng/g)					Ref
				<0.01	0.01 to 0.1	0.1 to 1	1 to 10	10 to 100	
Testosterone	RIA/IEA	*Octopus vulgaris*	Reproductive tissues[1]				■		D'Aniello et al., 1996
	RIA	*Helix aspersa*	Gonad				■	■	Le Guellec et al., 1987
			Haemolymph			■			Le Guellec et al., 1987
		Marisa cornuarietis	Visceral coil			■	■		This thesis
		Bolinus brandaris	Visceral coil			■	■		Morcillo and Porte, 1999
		Ilyanassa obsoleta	Whole animal				■	■	Gooding and LeBlanc, 2004
		Nucella lapillus	Whole animal			■	■		Santos et al., 2005
		Ruditapes decussata	Whole animal			■			Morcillo et al., 1998
		Mytilus galloprovincialis	Whole animal			■			Morcillo et al., 1999
			Whole animal, except digestive gland				■		This thesis
		Mytilus edulis	Whole animal			■			Reis-Henriques et al., 1990
			Whole animal, except digestive gland & gonad			■			This thesis
			Gonad				■		This thesis
5ζ-DHT	RIA	*Helix aspersa*	Gonad				■		Le Guellec et al., 1987
Androstenedione	RIA	*Mytilus edulis*	Whole animal			■	■		Reis-Henriques et al., 1990
		Helix aspersa	Gonad				■	■	Le Guellec et al., 1987
			Haemolymph		■				Le Guellec et al.,1987

Progesterone	ELISA	*Mya arenaria*	Gonad				■		Siah et al., 2002
	RIA/IEA	*Octopus vulgaris*	Reproductive tissues[1]				■		D'Aniello et al., 1996
	RIA	*Mytilus edulis*	Whole animal			■	■		Reis-Henriques et al.,1990
		Helix aspersa	Gonad				■		Le Guellec et al.,1987
			Haemolymph				■		Le Guellec et al.,1987
Estradiol	RIA/IEA	*Octopus vulgaris*	Reproductive tissues[1]		■	■			D'Aniello et al., 1996
	RIA	*Marisa cornuarietis*	Visceral coil		■	■			This thesis
		Bolinus brandaris	Visceral coil	■	■	■			Morcillo and Porte 1999
		Nucella lapillus	Whole animal			■			Santos et al., 2005
		Ruditapes decussata	Whole animal	■					Morcillo et al., 1998
		Mytilus galloprovincialis	Whole animal		■	■			Morcillo et al., 1999
			Whole animal, except digestive gland			■	■		This thesis
		Mytilus edulis	Whole animal		■				Reis-Henriques et al., 1990
			Whole animal, except digestive gland & gonad		■				This thesis
			Gonad				■		This thesis
Estrone	RIA	*Mytilus edulis*	Whole animal		■				Reis-Henriques et al., 1990

[1]The reproductive tissues analysed were: testis, vas deferens, seminal vesicle, prostate and Needham's sac.

This might be an indication that estradiol is primarily produced in the reproductive tissues, but we cannot explain the apparent contradiction with the observation that aromatase activity was higher in digestive gland than in gonads of *M. edulis*.

In addition, this thesis shows that most testosterone and estradiol are found in an esterified form in the gastropod *M. cornuarietis* digestive gland/gonad complex extracts. Thus, levels of free (unesterified) testosterone and estradiol were only 4-30% and 1-2% of total testosterone and estradiol, respectively. A comparable distribution of free and esterified steroids was observed in the mussel *M. edulis*, although with noticeable tissue differences. Thus, 0.7-2.1% estradiol and 4-7% testosterone were found in the free form in peripheral tissues, whereas this percentage increased to 6-46% and 15-87% (for estradiol and testosterone, respectively) in the gonadal tissues. In contrast, much lower levels of esterified steroids (0 to 27%) were observed in the mussel *M. galloprovincialis*. It is also important to indicate that seasonal variations can occur in the levels of esterified steroids. A recent report (Gooding and LeBlanc, 2004) showed that marked seasonal variations in free and esterified testosterone levels, in parallel with the reproductive cycle, exist in a gastropod snail, and similarly esterified steroid levels in *M. cornuarietis* were higher in individuals collected in Autumn (season with the highest egg production) than in those collected in Spring. The insufficient data on tissue and seasonal variations of esterified steroids in the animals of study prevented a conclusion on whether the differences that were observed between species (e.g. much higher levels of esterified steroids in *M. cornuarietis* than in *M. galloprovincialis*) were real interspecies differences or reflected tissue or seasonal variations.

Independently of the sampling time, the levels of esterified testosterone and estradiol were considerably higher in *M. cornuarietis* males than in females. In *Mytilus sp,* sexual differences were not assessed. The coefficients of variation in the gonad of *Mytilus sp.* (80 to 88%) were similar to those found in *M. cornuarietis* (59 to 72%, variability mainly due to differences between sexes), and much higher than in *Mytilus sp* peripheral tissues (24 to 48%) showing that inter-individual variability in esterified steroid levels was high in *Mytilus sp*. gonads. Whether this variability reflects gender differences, reproductive stages, or other factors requires further investigation. The differences observed in *M. cornuarietis* esterified steroid levels were not associated to correspondent differences in free steroid levels, i.e. higher esterified steroids in males were not associated to lower levels of free steroids. Indeed, free steroid levels did not show a clear sexual dimorphism, and only estradiol was significantly higher in males than in females. As in vertebrates, sex differences in the levels of free steroids during the reproductive season have been found in the echinoderm *Asterias rubens* for progesterone and estrone (Voogt and Dieleman, 1984), and in the anthozoan *Remilla koellikeri*, a primitive invertebrate, for estradiol (Pernet and Anctil, 2002), but there are no clear evidences of such a dimorphism in molluscs for progesterone (Reis-Henriques and Coimbra, 1990; Siah et al., 2002), testosterone or estradiol (Osada et al., 2004; Porte, personal communication). A more detailed study (several samplings along the reproductive cycle) is needed to conclude whether sex differences in free steroids occur in *M. cornuarietis*.

4.3 *In vitro* effects of endocrine disruptors

Organotin compounds showed the ability to interfere with several steroid metabolic pathways. They strongly inhibited 5ζ-reductase activity towards androstenedione in *M. cornuarietis* digestive gland/gonad complex. Nevertheless, TBT and TPT at concentrations as high as 100 σM, did not cause major changes in phase I metabolism of testosterone by microsomal fractions of *P. lividus* and *H. azteca* (Table 4.4.).

The susceptibility of *M. cornuarietis* 5ζ-reductase to inhibition by organotin compounds had already been reported in vertebrate species (Lo et al., 2003; Thibaut and Porte, 2004), and contrasts with the lower sensibility to organotin compounds of other phase I metabolic enzymes, as observed in this thesis and in the literature. For instance, cytosolic 17η-HSD from human placenta was only affected at high concentrations of TPT (Lo et al., 2003); and microsomal 17η-HSD from fish testes was not affected when incubated with up to 100 σM TBT or TPT (Thibaut and Porte, 2004). The fact that 5ζ-reductase was not inhibited in *P. lividus* and *H.azteca* further supports that important differences exist in the characteristics of the 5ζ-reductase enzymes in these species when compared to *M. cornuarietis*.

Table 4.4. *In vitro* effects of organotin compounds and fenarimol on enzymes involved in steroid metabolism in *Marisa cornuarietis*, *Hyalella azteca* and *Paracentrotus lividus*. 3η-HSD and SULT could not be assessed in all species due to very low or undetectable enzymatic activity.

Enzymes	Species	TBT	TPT	Fenarimol
17η-HSD	*M. cornuarietis*	=	=	
	H. azteca	=	=	=
	P. lividus	=	=	=
3η-HSD	*H. azteca*	=	=	=
	P. lividus	Ć	=	=
5ζ-reductase	*M. cornuarietis*	Ć	Ć	
	H. azteca	=	=	=
	P. lividus	=	=	ą
ATAT	*M. cornuarietis*	Ć	=	=
	H. azteca	Ć	=	=
	P. lividus	Ć	Ć	=
SULT	*P. lividus*	Ć	Ć	Ć

Except for *M. cornuarietis*, phase II metabolism of testosterone was much more sensitive to inhibition by TBT and TPT than phase I metabolism (Table 4.4). TBT and TPT strongly inhibited

testosterone esterification in *P. lividus* gonad microsomes, although they had lower or no effect in *M. cornuarietis* and *H. azteca*. SULT activity was inhibited by the organotins in *P. lividus* digestive gland, suggesting that similarly to estradiol sulfation (see Chapter 1, Table 1.4), testosterone sulfation might be a target for endocrine disruptors.

The inhibitory effects observed in *P. lividus* conjugating activities and in *M. cornuarietis* 5ζ-reductase occurred at concentrations of organotin compounds in the range of those reported in vertebrate species for other steroid metabolic pathways. Namely, in mammals, the following IC50 were obtained: 4 σM TPT for 3η-hydroxysteroid dehydrogenase (3η-HSD), 10 σM TPT for 17η-HSD (Lo et al., 2003), 6.2 σM TBT for P450 aromatase (Heidrich et al., 2001), 20 σM TBT for 5ζ-reductase1, and 11 σM TBT for 5ζ-reductase2 (Doering et al., 2002). Concentrations of TBT and TPT higher than 1 mg/kg (–3σM) have been reported in molluscs collected from contaminated sites (Fent, 1996), and although *in vitro* and *in vivo* effective concentrations are not directly comparable, environmental concentrations of TPT and TBT might be sufficiently high (due to the high bioconcentration factors reported for these substances; Fent, 1996) to affect sex steroid conjugating pathways in invertebrates.

Fenarimol did not alter metabolism of testosterone in *M. cornuarietis* or *H. azteca*, but had a strong effect on *P. lividus* enhancing the synthesis of DHT and 5ζ-A-diol and inhibiting testosterone sulfotransferase (Table 4.4). This activation was evident at concentrations higher than 10 µM. *In vitro* activation of steroid-metabolizing enzymes has been reported previously (Korzekwa et al., 1998). The mechanism by which fenarimol enhances 5ζ-reductase activity is not known, and requires further investigation. The *in vitro* effects of fenarimol occurred at comparable concentrations as those reported to inhibit P450-aromatase activity in microsomes of human placenta or rat ovaries (Vinggaard et al., 2000; Hirsch et al., 1987), and ecdysteroid 26-hydroxylase in *Manduca sexta* midgut mitochondria (Williams et al., 2000). The inhibition of P450-aromatase might lead to the reproductive abnormalities observed in male rats exposed to fenarimol (Hirsch et al., 1987), and ecdysteroid 26-hydroxylase might be the cause of the reduced ecdysteroid levels and altered embryo development observed in exposed crustaceans (Mu and LeBlanc, 2002).

4.4 *In vivo* effects of endocrine disruptors

Finally, the effects of endocrine disruptors on both, steroid metabolic pathways and steroid levels, were evaluated after *in vivo* exposures to a series of model steroids and xenobiotics. *Mytilus sp.* were exposed to estradiol (a model estrogen), crude oil (containing a mixture of PAHs), and the mixture of crude oil and alkylphenolic compounds. On the other hand, *M. cornuarietis* were exposed to methyltestosterone (a model androgen), tributyltin, triphenyltin, and fenarimol. An overview of the effects observed is shown in Table 4.5, and is discussed below.

Table 4.5. Summary of the *in vivo* effects on steroid levels and steroid metabolism in mollusc species exposed to model steroids and endocrine disruptors[1].

Endpoint	Species	Estradiol	Oil	Oil & APs	TBT	TPT	FEN	MT
Estradiol (free)	*M. galloprovincialis*	ą						
	M. edulis		ą	=				
	M. cornuarietis				ą、	=	=	=
Testosterone (free)	*M. galloprovincialis*	=						
	M. edulis		=	=				
	M. cornuarietis				=	ą、	=	=
Estradiol (esterified)	*M. galloprovincialis*	ą						
	M. edulis		ą	ą				
	M. cornuarietis				Ć、	Ć、	=	=
Testosterone (esterified)	*M. galloprovincialis*	=						
	M. edulis		ą	ą				
	M. cornuarietis				Ć、	Ć、	=	=
17η-HSD	*M. galloprovincialis*	=						
	M. cornuarietis				=	=	=	ą、
5ζ-reductase	*M. galloprovincialis*	ą						
	M. cornuarietis				=	Ć、	ą、	=
Aromatase	*M. galloprovincialis*	Ć ą						
	M. edulis		ą	ą				
	M. cornuarietis				ą、	ą、	=	=
Sulfotransferase	*M. galloprovincialis*	ą						
	M. edulis		ą	ą				
ATAT	*M. galloprovincialis*	ą						
	M. edulis		=	ą				
	M. cornuarietis				ą、	=	=	ą

[1]Some of the data presented in this table and discussed in the text was not included in the papers from chapters 2 & 3, but has been included in this chapter in order to help providing a general overview of the changes on steroid levels and metabolism induced by the tested xenobiotics.

4.4.1 In vivo effects of exposure to estradiol, crude oil and the mixture of crude oil and alkylphenolic compounds

Mussels exposed to estradiol were able to maintain almost stable tissue levels of free E2, except for the highest exposure group that exhibited a significant 10-fold increase. However, esterified E2 markedly increased in mussels from the M and H exposure tanks, suggesting that esterification of E2 with fatty acids might act as a homeostatic mechanism to maintain endogenous levels of free E2 stable. Similarly, esterification of testosterone is the major factor in the regulation of testosterone levels in snails (Gooding and LeBlanc, 2004). The increased esterification of E2 in organisms from the M and H exposure tanks co-occurred with an increase of fatty acyl-CoA:estradiol acyltransferase activity, suggesting that this was at least one of the factors responsible for the increase of esterified estradiol levels. It is likely that these mechanisms contribute to the regulation of steroid levels in other invertebrates, and it would be of great interest to know which role the esterification of steroids can play in vertebrate species.

E2 exposure significantly increased the formation of androstenedione 5ζ-reduced metabolites (5ζ-DHT and 5ζ-DHA), by digestive gland microsomal fractions of exposed organisms, while testosterone synthesis -a 17η-HSD catalyzed pathway-, remained unchanged.

The aromatization of androgens into estrogens was also affected by E2 exposure. When mussels were exposed to low levels of exogenous E2, the activity was significantly reduced, thus, estrogen synthesis decreased, in an attempt to lower endogenous levels of E2. However, the effect was reversed at high E2 doses; organisms from the H-group had significantly higher P-450 aromatase activity than controls. Similarly, the exposure to the mixture of oil and alkylphenolic compounds led to a significant increase of P450 activity.

Finally, estradiol sulfation marginally increased in animals exposed to the highest concentration of E2, and significantly in animals exposed to crude oil and to the mixture of crude oil and alkylphenolic compounds. Phase II metabolic pathways are known to be induced after exposure to xenobiotics in an attempt to favor the elimination of such compounds (Van der Oost et al., 2005). Thus, in vertebrates, the Ah gene battery (which induces phase I metabolic enzymes that act upon xenobiotics) also comprises phase II genes like GST and UDPGT (Lindros et al., 1998; Okey et al., 2005). Invertebrates do have AhR-homologue genes, but these do not bind dioxins or related chemicals (Hahn, 2002). Nevertheless, the induction of sulfotransferase activity observed suggests that mechanisms exist for the induction of phase II metabolic pathways in order to accelerate the elimination of xenobiotics.

In addition to the described alterations on steroid levels and steroid metabolism, exposure of mussels to low E2 concentrations induced gametogenesis. Gonadal histopathological responses of mussels exposed to the crude oil and the mixture of crude oil and alkylphenols were not analyzed within this thesis, but have been published elsewhere (Aarab et al., 2004). Similarly to the effects observed in mussels exposed to low concentrations of estradiol, larger and more numerous ovarian follicles suggesting a more precocious development were observed in animals exposed to the crude oil. These results were also supported by elevated vitellogenin-like levels in animals of this exposure group (5- to 8-fold) (Aarab et al., 2004). In contrast, gonads of mussels exposed to the mixture of crude oil and alkylphenols displayed numerous degenerating ovarian follicles indicative of a toxic effect of this mixture on gonads (Aarab et al., 2004).

The responses observed in mussels exposed to crude oil and the mixture of crude oil and alkylphenols resembled that of mussels exposed to high doses of estradiol: i.e. aromatase and sulfotransferase activity and levels of esterified estradiol increased. This pattern of response is likely to be related to the estrogenic action of these xenobiotics either directly (alkylphenolic compounds bind the estrogen receptor in vertebrate species; Soto et al., 1991; White et al., 1994) or indirectly (crude oil led to an elevation of free estradiol levels in exposed mussels; see Table 4.5).

Despite similar patterns of response to estrogenic compounds could be observed, none of the parameters evaluated could readily be used as a biomarker of exposure to estrogenic compounds in mussels, for several reasons: the magnitude of the response was limited, there is no knowledge on the natural variability of these parameters, on the physiological relevance of some of them, and on the mechanism by which these parameters are modulated by estrogenic compounds. Further research on basic endocrinology is needed in order to define biomarkers of estrogenic exposure in invertebrates. Until this is accomplished, vitellogenin levels in male fish will probably continue to be the most suitable biomarker of estrogenic exposure in the aquatic environment.

4.4.2. In vivo effects of exposure to triphenyltin, tributyltin, fenarimol and methyltestosterone

Exposure to all four chemicals, i.e. TBT, TPT, MT and FEN, caused imposex in female *M. cornuarietis* (Table 4.6). High doses of TBT and TPT induced imposex after only 50 days exposure and led to the highest VDS index among all experimental groups after 150 days. Throughout the exposure experiment, females exposed to the highest doses of MT (300 ng/L) and fenarimol (3000 ng/L) had VDS index similar to those found in females exposed to low doses of TBT, and they developed imposex after 150 days exposure (Table 4.6).

Table 4.6. VDS Index in female *M. cornuarietis* exposed for different lengths of time to TPT, TBT, fenarimol and methyl-testosterone. Values are mean of n= 9 to 39 females.

Chemical exposure (ng/L)		Exposure length 50 days	100 days	150 days
TPT	30			
	60			
	125			
	250			
	500			
TBT	30			
	60			
	125			
	250			
	500			
FEN	300			
	3000			
MT	30			
	300			

VDS Index	Code
0.5 to 1	
1 to 1.5	
1.5 to 2	
2 to 2.5	

Among the chemical and biochemical parameters investigated, the strongest effect observed in snails exposed to organotin compounds was a decrease in the levels of esterified steroids. Females exposed to TBT and high doses of TPT for 50 to 100 days showed a significant decrease in esterified testosterone and estradiol levels. In contrast, no significant decrease in esterified steroids was observed in males. Thus, neither esterified testosterone nor esterified estradiol were significantly different between control and TBT-exposed males, and esterified testosterone even increased in some groups of TPT-exposed males. Conversely, esterified steroid levels remained stable in males or females exposed to MT and fenarimol. Thus, the effect on esterified testosterone and estradiol levels appears to be specific for organotin compounds.

Previous studies had investigated the effects of tributyltin on the levels of esterified testosterone in female snails (Gooding et al., 2003; Santos et al., 2005). These studies reported a slight decrease of esterified testosterone levels (ℑ5-12%) in exposed females, although it was not statistically significant. Despite the fact that these trends are in agreement with the effects we observed in females, the magnitude of the response is notably different. The differences might be attributed to the time at which the effects were analysed. Indeed, the decrease in esterified testosterone levels in females exposed to TPT was not a permanent effect, but was no longer observed after 150 days exposure. Another relevant difference among the experiments performed in this thesis and those by Gooding et al. (2003) and Santos et al. (2005) is the fact that the digestive gland/gonad complex was used in this thesis, but the whole body tissue was used in the previous studies.

There was not a clear parallelism between the decrease in esterified steroid levels in an exposure group and the future occurrence of imposex. This lack of correlation between alterations in esterified steroid levels and imposex index does not exclude the involvement of such alterations in the development of imposex, but proves that they are not a sufficient factor for the induction of this phenomena. On the other hand, MT- and FEN-exposed females developed imposex without showing alterations in the levels of esterified steroids. Therefore, alterations on esterified steroids appear to be specific for exposure to organotin compounds, and if they are linked to the development of imposex, MT and FEN act upon alternative targets for the induction of imposex.

The changes in esterified steroid levels were not accompanied by statistically significant changes in free steroids, although a trend towards a higher free testosterone/estradiol ratio in TBT-exposed females and in TPT-exposed males was observed. We have to indicate that the relatively low sample size per sex and exposure group (n=4-7) in combination with the high inter-individual variability observed in the levels of free steroids, might have prevented the detection of statistically significant differences in these parameters. In accordance with the trends observed, earlier studies had reported an increase in free testosterone levels in TBT-exposed female snails (Spooner et al., 1991; Bettin et al., 1996; Gooding et al., 2003; Santos et al., 2005), and/or in the free testosterone/ free estradiol ratio (Schulte-Oehlmann et al., 1995).

In vitro studies showed that ATAT activity was inhibited by organotin compounds in *P. lividus* and up to certain extent also in *M. cornuarietis*, which might explain the decreases observed in esterified steroids. Nevertheless, ATAT activity was significantly induced in organisms exposed to MT and TBT. Two hypotheses could explain the apparent incongruity between the effect observed in esterified steroid levels and in ATAT activity. First, it might be that the decrease in esterified steroid levels was not directly related to ATAT, but to other factors, such as esterases or availability of cofactors (i.e. fatty acids or acyl-CoA) that are also involved in the equilibrium between free and esterified steroids, or that earlier steps on the biosynthesis of steroids or the elimination of steroid-esters (e.g. through the eggs) had been affected by TBT or TPT. Alternatively, it might be that ATAT activity had been initially inhibited by organotin compounds leading to a decrease in esterified steroids, and that later, ATAT activity was induced in an attempt to ‘compensate’ this decrease.

The finding that the metabolism of androstendione is sexually dimorphic in *M. cornuarietis*, and that 5ζ-reductase was inhibited by organotin compounds (0.1 to 10 σM) *in vitro*, led us to formulate and test the hypothesis that the metabolism of androstenedione might be altered by organotin exposure, and be possibly related to the phenomena of imposex, or that alterations in androstenedione metabolism might be observed as a consequence of imposex. In fact, the metabolism of androstenedione was not altered in males nor in females exposed to the organotin compounds for 50 days, regardless of the development of imposex. After 150 days exposure, an inhibition of the total androstenedione metabolized and a decrease in the formation of the major

metabolite DHA was observed in females exposed to TPT that had developed imposex (high dose group). A marginal inhibition was also observed in females exposed to TBT for 150 days.

The specific inhibition of 5ζ-reductase observed in the *in vitro* studies might lead to an increased ratio between 17η-reduced androstenedione metabolites and DHA (DHT+T/DHA). This ratio is higher in males (12 ± 2) than in females (0.40 ± 0.02), therefore an increase in this ratio might be considered as a 'metabolic androgenization' of females. Nevertheless, when females were exposed to TBT or TPT for up to 150 days, neither exposure to organotin compounds nor the development of imposex resulted in a major androgenization of androstenedione metabolism in females. It should be mentioned that if changes in metabolism had occurred in a specific reproductive tissue, these might have been diluted by assessing metabolism in the whole digestive gland/gonad complex.

Aromatase activity was induced in *M. cornuarietis* exposed to high doses of TPT and TBT. Evidence supports that organotin compounds inhibit aromatase *in vitro* in vertebrate (Cooke, 2002; Heidrich et al., 2001; Lo et al., 2003) and invertebrate species (LeCurieux-Belfond et al., 2001; Morcillo et al., 1998). However, Nakanishi et al. (2002) showed that TBT and TPT upregulate the expression of aromatase in a dose-dependent manner in three different human choriocarcinoma cell lines, JAR, JEG-3, and BeWo, suggesting that an induction of aromatase might occur *in vivo* as a response of the animals to the inhibition of aromatase activity at the enzyme level, in an attempt to re-establish normal levels of estrogens.

The length of the exposure was shown to be a major factor in the assessment of the effects of endocrine disruptors. As discussed above, aromatase activity and acyl-CoA acyltransferase activity were induced in *M. cornuarietis* after 50 days exposure to organotin compounds or methyltestosterone. Nevertheless, in both cases, no effects were observed after 150 days exposure. In contrast, the effects of organotins on the metabolism of androstenedione only appeared after 150 days exposure.

Figure 4.2 summarizes the effects of TBT, FEN, and MT in *Marisa cornuarietis* and hypothesizes possible connections to the phenomena of imposex. While the biochemical alterations observed differed between the three compounds, all of them induced imposex. *In vitro*, TBT inhibits aromatase (LeCurieux-Belfond, 2001), ATAT, and 5ζ-reductase (this thesis). These alterations might lead to an increase in the levels of free testosterone and a decrease in the levels of free estradiol, as suggested by the trends observed in TBT-exposed females and by the changes in steroid levels shown in earlier studies (Bettin et al., 1996; Schulte-Oehlmann et al., 1995). On the other hand, ATAT and aromatase activity were induced after *in vivo* exposure to TBT/TPT for 50 days. These responses might be explained as homeostatic responses of the organism in an attempt to regulate the altered levels of free estradiol, and the altered balance between free and

conjugated steroids. The major effect on steroid metabolism detected in snails exposed to MT was the induction of ATAT activity. The ability of MT to inhibit ATAT activity *in vitro* has not been tested, but it is likely that MT is a potent inhibitor of ATAT because other androgens (e.g. DHT and 5ζ-androstane-3η,17η-diol) were ATAT inhibitors in *I. obsoleta* (see Section 2.3). Despite the fact that esterified steroid levels were not significantly decreased in MT-exposed snails, there was certainly a trend towards lower values than in controls (levels in MT-exposed females were 69 to 84% of those in control females). Therefore, this data would be consistent with a certain inhibition of ATAT activity by MT, and the later upregulation of this enzyme observed after 50 days exposure. All these hypotheses are based on the involvement of elevated testosterone and/or decrease estradiol levels in the development of imposex. The action of these steroids might be mediated through steroid receptors. The androgen receptor has not been found in invertebrates yet, despite intensive efforts by several research groups. However, an estrogen receptor-like has been cloned in *Aplysia californica* (Thornton et al., 2003) and a partial sequence of this receptor has also been obtained in *M. cornuarietis* (Jobling et al., personal communiation).

In addition to the targets evaluated, it is likely that these xenobiotics also acted through other targets, which might also be involved in the development of imposex. Thus, TBT was shown to bind the RXR receptor in the snail *Thais clavigera*, and the exposure to the RXR ligand 9-cis retinoid acid led to the appearance of imposex in this snail species (Nishikawa et a., 2004). The function of RXR in molluscs is not known, however it might be linked to some of the alterations observed in this study. For instance, in vertebrates RXR modulates aromatase activity (Yanase et al., 2001) and dimerizes with several other nuclear receptors, including those involved in lipid, xenobiotic and hormone metabolism (Krey et al., 1995; Chawla et al., 2001; Ahuja et al., 2003; Kliewer, 2003). Therefore, it might be expected that an interaction of TBT with the RXR receptor led to alterations on steroid metabolism and in lipid profiles, which indirectly could alter the esterification of steroids. Currently, the lipid profiles are being analyzed in tissues of snails exposed to TBT, FEN and MT in order to further understand the possible connections between the induction of RXR receptor and the alterations in the esterification of steroids and/or the phenomena of imposex.

In order to evaluate whether the targets *in vitro* were indeed primary targets *in vivo*, a much shorter exposure should have been performed so that regulatory mechanisms were not masking the direct effects of the chemicals evaluated. In order to do so, the effects of a 7-days exposure to TPT on *M. cornuarietis* are currently being evaluated.

Overall, the *in vivo* studies showed that the xenobiotics included in the study induced imposex and altered a series of biochemical targets, that some of these effects differed between males and females and that most of them were transient effects. Therefore, it is evidenced that the endocrine system of these molluscs was altered by the exposure to the xenobiotics, and that indirect regulatory mechanisms were activated to set physiological parameters at their normal levels.

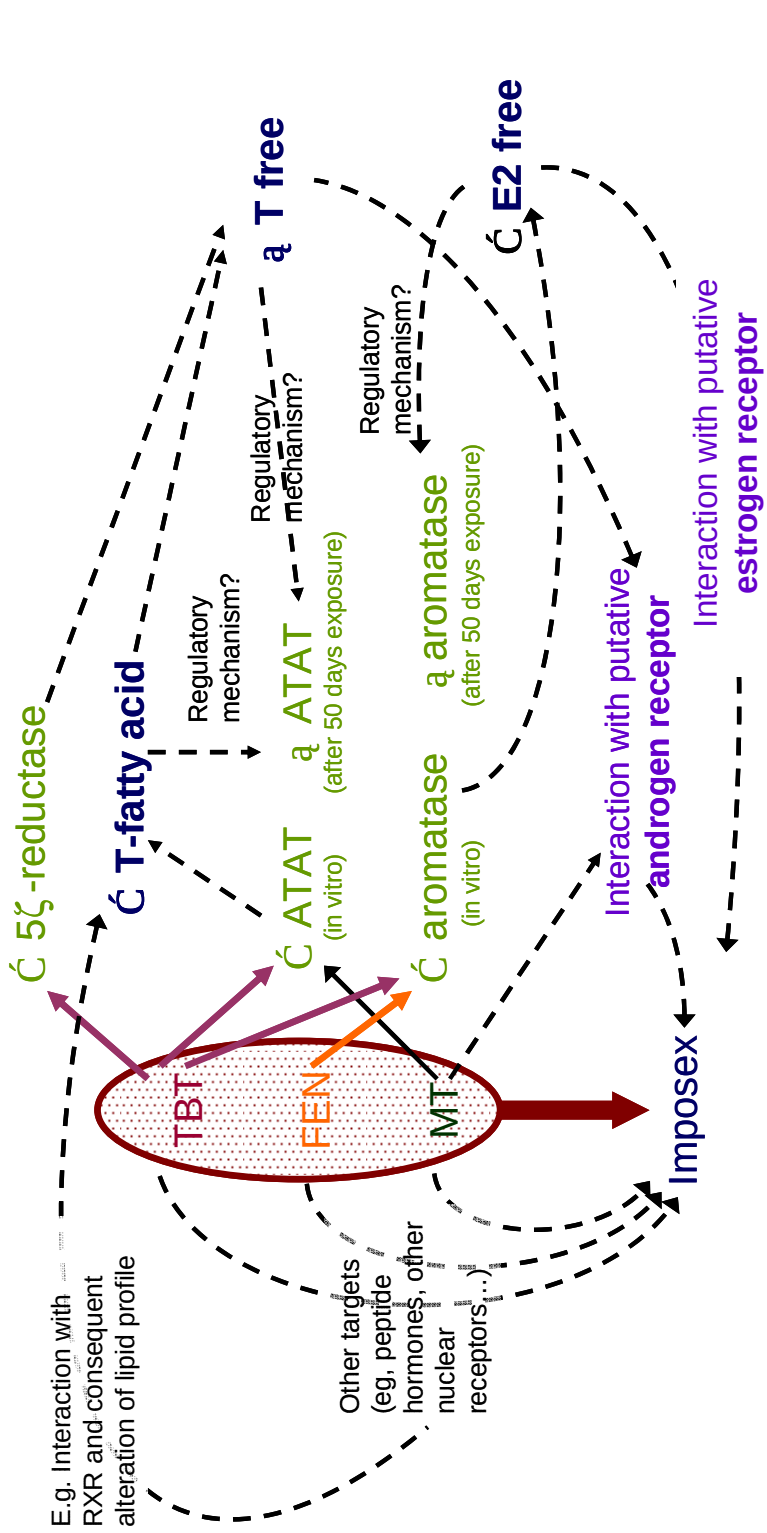


Figure 4.2. Possible relationship between the biochemical effects caused by TBT, FEN and MT and the phenomena of imposex in *M. cornuarietis*.

CHAPTER 5. CONCLUSIONS

5. CONCLUSIONS

1. Invertebrates are able to form a series of phase I metabolites of testosterone that are also found in vertebrates, with the exception of 4-androstene-3η,17η-diol, a metabolite formed by *P. lividus* and *H. azteca*. One of the major differences with vertebrate species was the lower contribution of hydroxylases in the metabolism of testosterone in all the invertebrates investigated.

2. The metabolism of androstenedione in molluscs, which leads mainly to 5ζ-dihydroandrostenedione and 5ζ-dihydrotestosterone, differed from that in crustaceans, echinoderms and vertebrates, which leads mainly to testosterone.

3. Steroid levels and some metabolic pathways presented tissue and sex differences as well as seasonal variations in some invertebrate species. These data represent additional evidence for a physiological role of sex steroids in invertebrates.

4. Low or undetectable levels of sulfo-, glucosyl- and glucuronyl-transferases towards steroid substrates are present in the molluscs species investigated. In contrast, indications were obtained that these invertebrates are able to form polar metabolites through alternative pathways, such as conjugation with C_6 sugar chains in the presence of sucrose, which had not been previously reported in the literature.

5. The conjugation of steroids with fatty acid moieties is a well-conserved pathway through evolution. Acyl-CoA:steroid acyltransferases presented broad substrate affinity both towards steroids and fatty acid moieties. Nevertheless, they appeared to have a special relevance in the regulation of steroid levels in molluscs.

6. Some xenobiotics modulate steroid metabolic pathways in invertebrates *in vitro*. Among the pathways investigated, 5ζ-reductase and sulfotransferase showed the highest sensitivity to inhibition/activation by organotin compounds and fenarimol. The *in vitro* effects on these parameters occurred at concentrations similar to other *in vitro* effects that have been mechanistically associated to physiological abnormalities in *in vivo* exposures.

7. Estradiol, crude oil, and the mixture of crude oil and alkylphenolic compounds induced a series of alterations in steroid levels and steroid metabolism in the bivalve molluscs *Mytilus sp*. The effects of estradiol depended greatly on the concentration. Some of the responses observed in mussels exposed to crude oil and the mixture of crude oil and alkylphenols were similar to those observed in mussels exposed to the highest concentrations of estradiol.

8. Organotin compounds decreased esterified steroid levels in females of *M. cornuarietis*, but not in males. This effect was not induced by other chemicals that had also induced imposex in this species. In addition, the strong decrease in esterified steroid levels was not observed after a longer exposure time and the changes observed in these levels were not coincident with parallel changes in free steroid levels. Overall, the connection between a decrease of esterified steroids and the phenomena of imposex could not be proved.

9. A long-term exposure to TPT inhibited 5ζ-reductase in female *M. cornuarietis*. However, neither TPT nor TBT altered the sexual dimorphism in androstenedione metabolism that exists in the digestive gland/gonad complex of this snail species.

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7. REFERENCES

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ANNEX

ANNEX. A MOLECULAR APPROACH TO STUDY ENDOCRINE DISRUPTION IN INVERTEBRATES.

A.1. Introduction

It can be expected that almost all pollutants that cause adverse effects will do so by interfering with gene expression. Thus, chemicals that cause endocrine and/or reproductive alterations are likely to interfere with some of the genes involved in gonadogenesis, gametogenesis, and/or steroidogenesis. An increasing number of studies on mammalian models and fish focus on the effect of environmental chemicals at the transcription level, with the aim to analyze cellular response mechanisms to a particular toxicant (e.g. Roberts et al., 2003; Yokota et al., 2005; Vetillard et al., 2005), or to develop new biomarkers (e.g. George et al., 2004; Lampe et al., 2004). However there is virtually nothing known about transcriptomic effects of environmental chemicals in echinoderm species.

One of the major difficulties when working with echinoderms is the limited number of genes for which partial or complete sequences are available. The genome project for the echinoid *Strongylocentrotus purpuratus* is now ongoing (*http://sugp.caltech.edu*), and will become a major contribution to echinoderm molecular biology.

Paracentrotus lividus, an echinoid, is an edible species of great commercial interest, and is widely used in embryotoxicity tests. For these reasons it was selected as model echinoderm species in the COMPRENDO project. *P. lividus* and *S. purpuratus* belong to the same order, but not to the same family. Therefore, similarities in their genome are expected, but only to a certain extent when it comes to sequence identities.

The last period of the doctoral training was directed to learn the techniques and approaches used in molecular biology to study the effect of toxicants on gene transcription. The study was performed as part of a bigger project, which is developing a microarray for analysing gene expression in several aquatic species, including *P. lividus*, after exposure to endocrine disruptor compounds. The specific objectives of this work were to generate sequence information for some of the genes to be included in the microarray.

The genes chosen for study included genes known to play a role in gonadogenesis and/or steroidogenesis in vertebrates and which potentially have similar roles in invertebrates. In addition, beta actin, a housekeeping gene, was chosen to serve as a positive control.

A.2. Materials and Methods

Animals

Paracentrotus lividus were collected from the Ligurian coast (Italy) and taken to the laboratory. Gonads were dissected and immediately deep-frozen and stored at -80ºC until processed for RNA extraction.

RNA-extraction

Total RNA of female (n= 4) and male (n= 4) adult *P. lividus* was isolated from the gonads using TRI® reagent method according to the manufacturer's instructions (Sigma, Germany). Similarly, total RNA was isolated from whole juvenile *P. lividus* (n= 3). The RNA content was determined using a GeneQuant photometer (Pharmacia Biotech, UK) and the purity was examined on a 1 to 2 % agarose gel using gel-electrophoresis (Fig. A.1). RNA (2µg) was treated with DNase (Promega, UK), followed by a reverse transcription using M-MLV reverse transcriptase (Promega, UK).

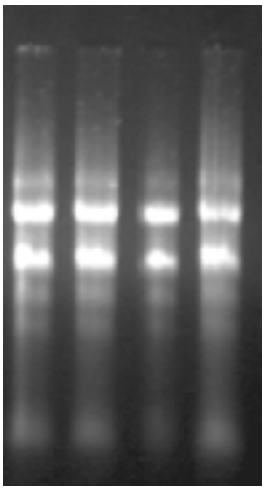


Figure A.1. Example of an agarose gel with RNA extracted from the sea urchin.

Selection of Genes

The genes chosen for study included genes known to play a role in gonadogenesis and/or steroidogenesis in vertebrates and which potentially have similar roles in invertebrates. In addition, beta actin, a housekeeping gene, was chosen to serve as a positive control.

Literature and sequence databases were screened for information on genes that potentially could play a role in reproductive development of *P. lividus* and the genes selected for this study are summarised in Table A.1.

Table A.1. Genes selected for the study

	Nomenclature used in this study	Other names in the NCBI database
Related to gonadogenesis and/or steroidogenesis		
Steroidogenic Acute Regulatory protein	StAR	
Cytochrome P450 aromatase	CYP19	CYP19A1
Cyt. P450 steroid 17Ŭ-hydroxylase/17,20 lyase	CYP17	CYPc17
Steroidogenic factor 1	SF-1	NR5A1; FTZF1; FTZ1
Vasa	Vasa	DEAD/H BOX 4; DDX4
Double-sex- and MAB3-related transcription factor 1	DMRT1	DMT1
Control gene		
η-actin	η-actin	ACTB

The key function(s) of each of the chosen genes and the current status on their presence in vertebrates and invertebrates is summarised below:

- Steroidogenic Acute Regulatory protein (StAR). All steroid hormones are synthesised from a common precursor: cholesterol. Cholesterol is a lipophilic substrate that cannot move freely inside the aqueous intracellular environment. StAR is the major protein involved in cholesterol transport from the outer membrane to the inner membrane of mitochondria (Stocco and Clark, 1996). The gene encoding for StAR has been cloned and sequenced in several vertebrates, and a mln64 N-terminal homolog (which shares the StAR box) has been cloned in *C. elegans* (gi:25148448).

- Cytochrome P450 steroid 17Ŭ-hydroxylase/17,20 lyase (CYP17). CYP17 is a key regulator of steroidogenesis. Depending on the presence/absence of each of the CYP17 activities, it can direct pregnenolone towards C-21 17-deoxysteroids (e.g. aldosterone), C-21 17-hydroxysteroids (e.g. cortisol), or C-19 steroids (e.g. androstenedione) (Miller et al., 2002). The gene encoding for CYP17 has been cloned and sequenced in several vertebrates, but not in invertebrates.

- Cytochrome P450 aromatase (CYP19). The CYP19 gene encodes an enzyme controlling the synthesis of estrogens from androgens, by catalysing the conversions of androstenedione to estrone, and of testosterone to 17ƀ-estradiol. Sequence data for CYP19 is available for several vertebrates, but not in invertebrates.

≠ Steroidogenic factor 1 (SF-1). SF-1 is a nuclear orphan receptor and a member of the steroid receptor super-family (Parker and Schimmer, 1997). Proteins in this family share a common structural organisation which includes a DNA binding domain, a consensus binding recognition site, a ligand binding domain, and an activation domain. These domains in SF-1 are highly conserved across many taxa, except for the ligand-binding domain (Parker and Schimmer, 1997). The extraordinary conservation of the SF-1 protein sequence suggests that it arose early in evolution, and there are highly conserved functions for this receptor. Indeed, SF-1 was first described in invertebrates (e.g. gi:8980870; 31213472), but has now been sequenced in several vertebrates species.

≠ Vasa. The *Drosophila* gene *vasa* has a central role in several aspects of germ cell development. It encodes a member of the DEAD (Asp-Glu-Ala-Asp) box family of ATP-dependent RNA helicases. The vasa protein appears to regulate the translation of multiple downstream mRNAs. Vasa protein is an essential component of germ plasm, a poorly understood complex of RNA and proteins that is required for germ cell determination. The *vasa* gene has been sequenced in several vertebrate and invertebrate species (e.g. 40891624; 10039328).

≠ Double-sex- and MAB3-related transcription factor 1 (DMRT1). The DMRT1 gene is implicated in testis determination and has a gonad-specific and sexually dimorphic expression profile during embryogenesis in vertebrates and invertebrates (insects and nematodes), therefore DMRT1 must represent an ancient, conserved component of the sex-determining pathway (Smith et al., 1999). DMRT1 was first described in invertebrates (e.g. 24644933), but it has also been cloned in numerous vertebrate species.

≠ η-actin. η-actin is a component of the cytoskeleton and functions as mediator of internal cell motility. Because of this, it is widely employed as internal control gene, with the assumption that it is expressed constitutively to similar degrees in different cells and tissues and under different experimental conditions. Several actin genes have been cloned in echinoderms (Kissinger et al., 1997), but not in *P. lividus*.

Primer Design

Aminoacid and nucleotide sequences of phylogenetically distant organisms were obtained from NCBI databases and aligned (ClustalW, EBI), in order to identify conserved regions, and degenerated primers or specific primers towards a related species were designed. More detailed information on the choice of primers can be found in the results and discussion section.

Amplification and isolation of the genes of interest

The PCR reactions were performed using PCR Master Mix X2 (Promega, UK), 1 µl primer (100 pmol/µl) and 2 to 10.5 µl *P. lividus* cDNA to a final volume of 25µl per reaction. The general conditions for the PCR were 94°C for 5 minutes, 30 to 45 cycles of 94°C for 30 seconds, 42°C for 30 seconds, and 72°C for 1 minute, followed by 72°C for 10 minutes. PCR products were analysed on 1.5 to 2 % agarose gels and bands of interest were cut out of the gel and purified using the Wizard ® DNA Clean-Up System (Promega, UK), according to the supplier's instructions. An aliquot of the purified product was sequenced (Lark Sequencing, UK).

Cloning

The purified PCR products were cloned with TOPO TA Cloning (Invitrogen, UK) using One Shot® TOP10 chemically competent *E. coli* according to the manufacturer's instructions.

Briefly, the (purified) PCR products (2 σL) were incubated for 30 min at room temperature with the vector (0.5 σL) and the salt solution (0.5 σL). 2 σL of the mixtures were added to the cells (50 σL) to allow for transformation, and subsequently cells were incubated for 30 min on ice and heat shocked for 15 to 20 seconds at 42ºC. Finally, 250 σL of SOC medium (Invitrogen, UK) were carefully added to the cells, and the cell mixtures were incubated in a horizontal orbital shaker-incubator for 2 hours at 37ºC.

The cultures were spread on Agar plates (containing 100µg/ml ampicilin and 1.6 mg X-Gal) and incubated overnight at 37°C. PCRs using the following conditions: 94°C for 10 minutes, 20 cycles of 92°C for 1 min, 50 °C for 1 min, 72°C for 1 minutes, and a final step of 72 °C for 10 minutes, were performed on 10 to 15 white colonies using specific primers for the vector (M13Forward and M13Reverse). The PCR products were separated on an agarose gel and three colonies containing the insert of interest were selected and incubated in L-Broth in a horizontal orbital shaker-incubator overnight at 37°C. Lysates were made from these cultures and the plasmids were isolated using the Wizard ® Plus SV Miniprep DNA Purification System (Promega, UK).

The sequencing of the plasmids was performed by Lark technologies (UK). The obtained sequences were blasted in nucleotide and aminoacid databases (http://www.ncbi.nlm.nih.gov/BLAST/) in order to compare these sequences with previously characterised genes, based on sequence similarities.

A.3. Results/discussion

Selection of genes and primer design

Highly conserved regions were found for StAR in vertebrates, but when the StAR-like sequence for *C. elegans* was added to the alignment, the conserved regions were too short for designing highly conserved primers. In addition, no match for this gene could be identified on the *Strongylocentrotus purpuratus* genome database. Therefore, no further work on StAR was performed.

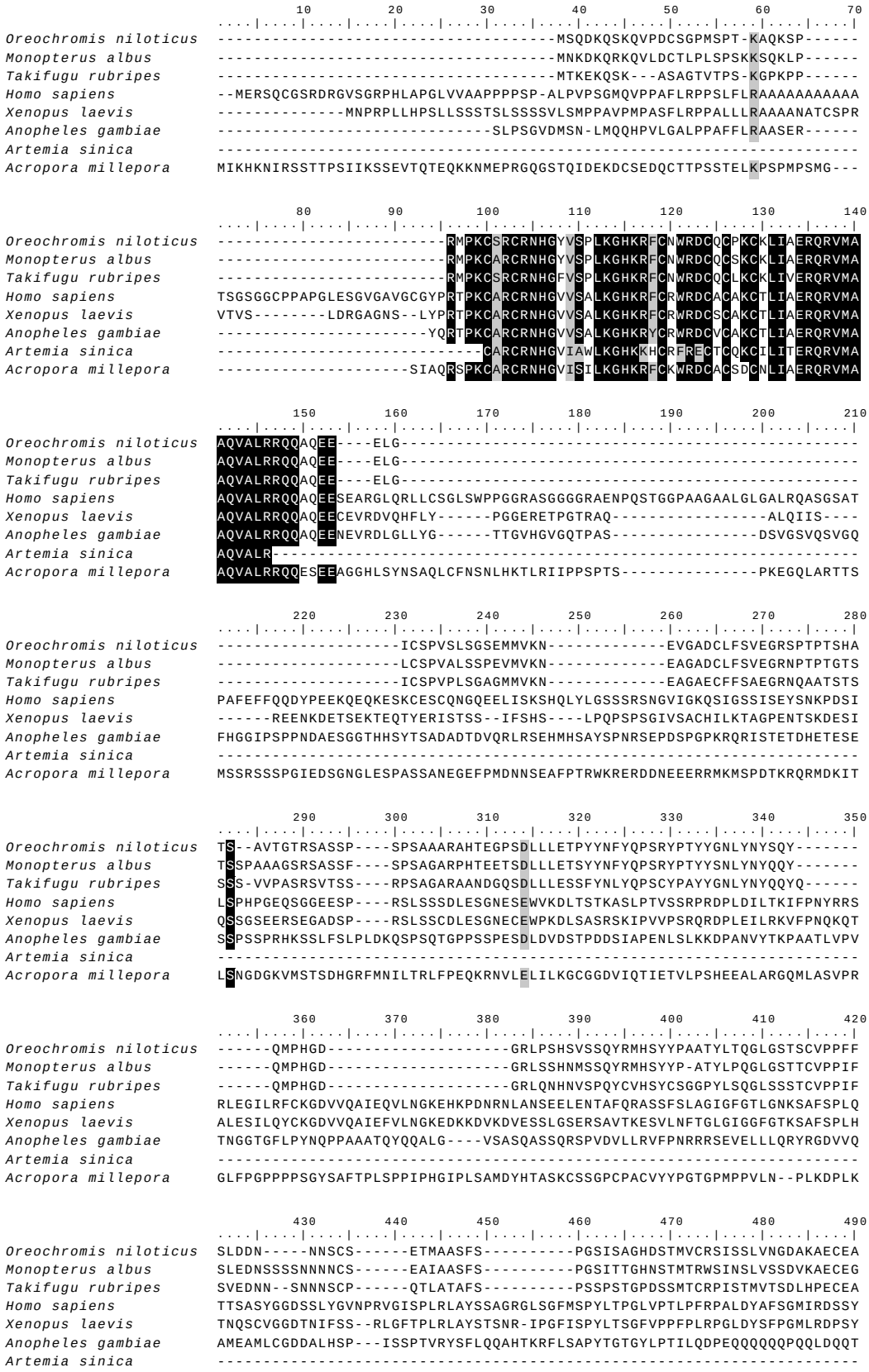
Gene sequence for CYP19-aromatase is only available for vertebrates. Indeed, searches in the genome of *Caenorhabditis elegans*, *Drosophila melanogaster* or *Ciona intestinalis* could not identify any CYP19 homolog (Baker et al., 2004). Therefore, it is quite unlikely that CYP19 is present in echinoderms and no further work on it was performed.

DMRT has been cloned in several vertebrate and invertebrate species, and it is likely that it is also present in echinoderms. One of the major difficulties in working with DMRT is the limited length of the conserved region (about 50 aminoacids). This is probably the reason why this gene has not yet been identified in the *S. purpuratus* genome database. Moreover, we could not identify a single work that had used a primer approach to first identify this gene in a species. Instead, published work used cDNA libraries (e.g. Kondo et al., 2002; Veith et al., 2003). Since a cDNA library approach was not feasible in this study, degenerated primers were designed on both ends of the conserved region by aligning (ClustalW, EBI) eight aminoacid sequences of phylogenetically distant organisms (obtained from NCBI databases) (Figure A.2.A).

SF1 has been cloned in several vertebrate and invertebrate species, it contains highly conserved regions, and it is likely that it is present in echinoderms. As an initial attempt, primers were designed on the sites used for SF1 isolation in other species (Li et al., 2000), by aligning aminoacid sequences of six phylogenetically distant organisms (Figure A.2.B). In addition, in order to reduce the number of degeneracies of the primers, seven nucleotide sequences were aligned and two further sets of primers were designed (Figure A.3.A). There is no rule on the number of degeneracies that a primer should have. Deciding on the number of degeneracies is always a compromise between minimizing non-specific primer sequences, primer dimerisation, and dilution of the specific primer in a pool of non-specific primers, and maximizing the probability that the sequence of interest will be present in one of the pool of primer sequences. When exploring the sea urchin genome database, no matched for SF1 were found.

Figure A.2. Aminoacid sequence alignments for (A) DMRT and (B) SF1.

A) DMRT



B) SF-1

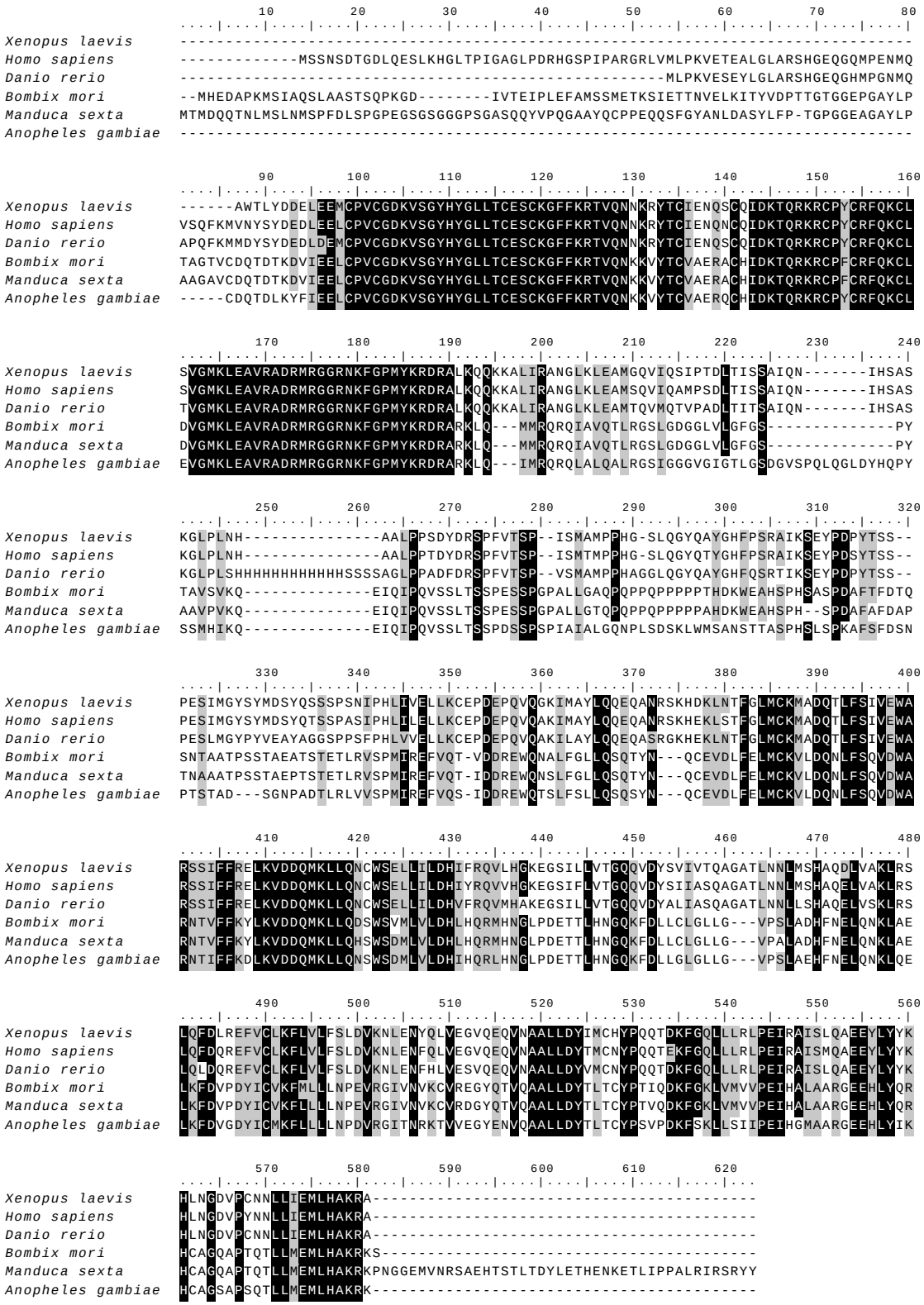
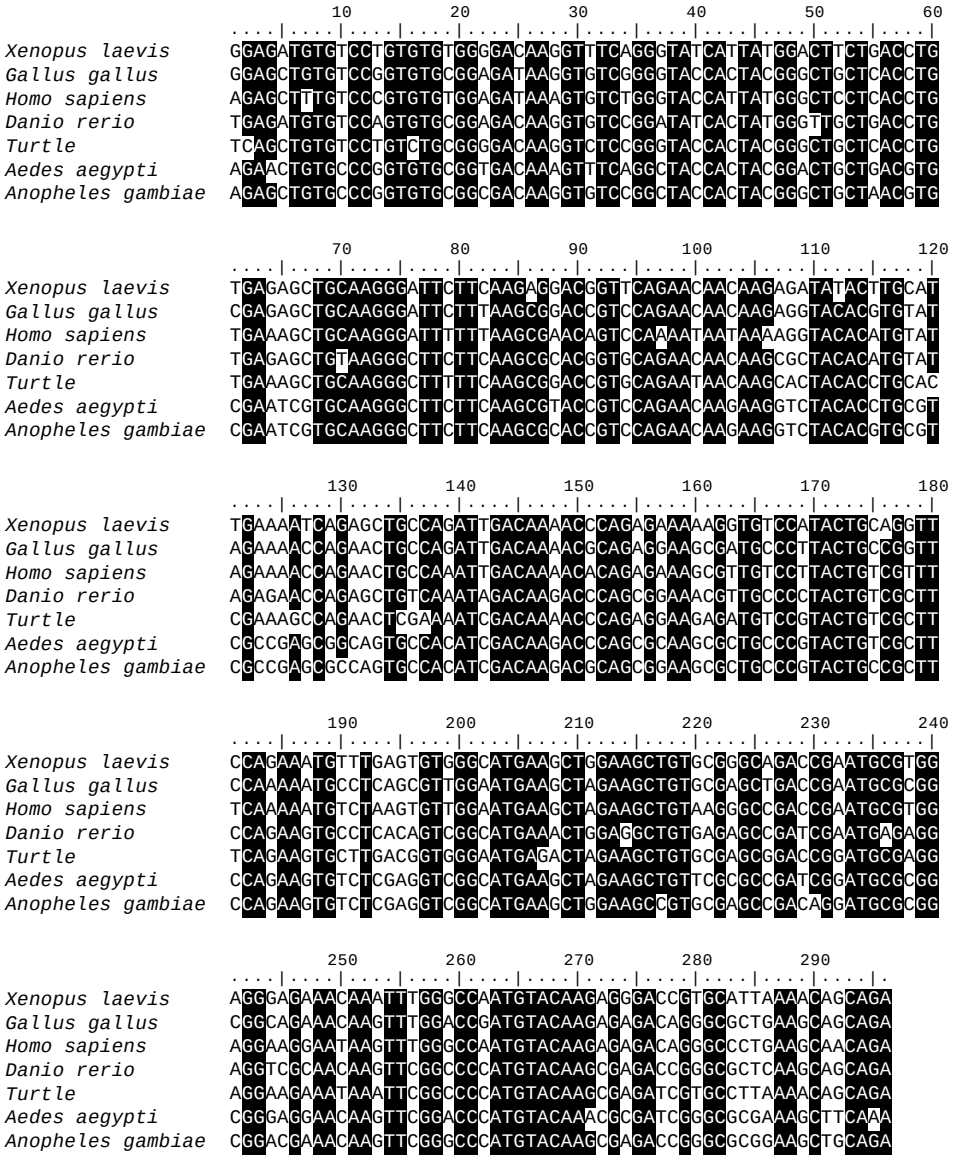
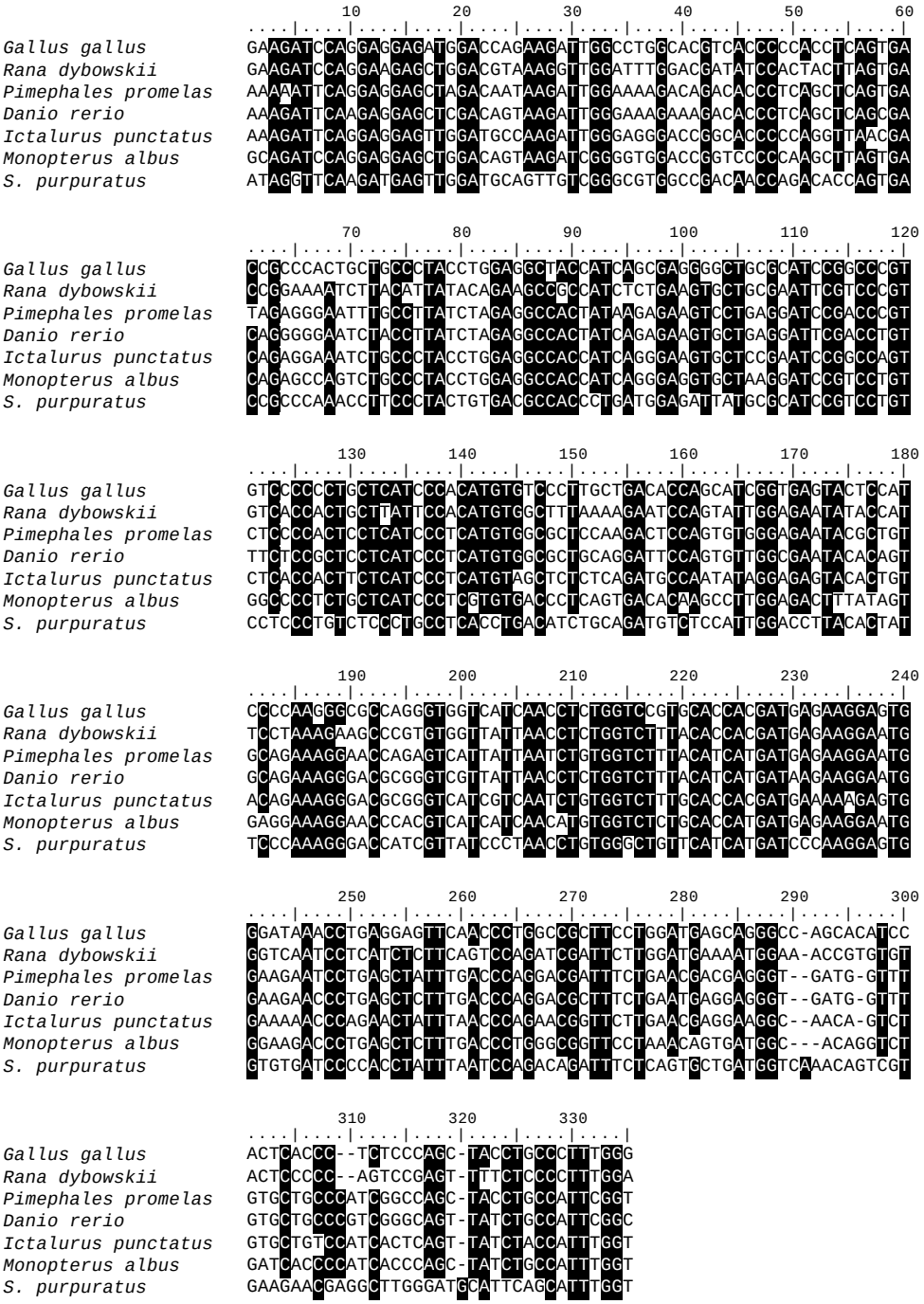

Xenopus laevis
Homo sapiens
Danio rerio
Bombix mori
Manduca sexta
Anopheles gambiae

Figure A.3. Nucleotide sequence alignments for (A) SF1, (B) CYP17 and (C) vasa.

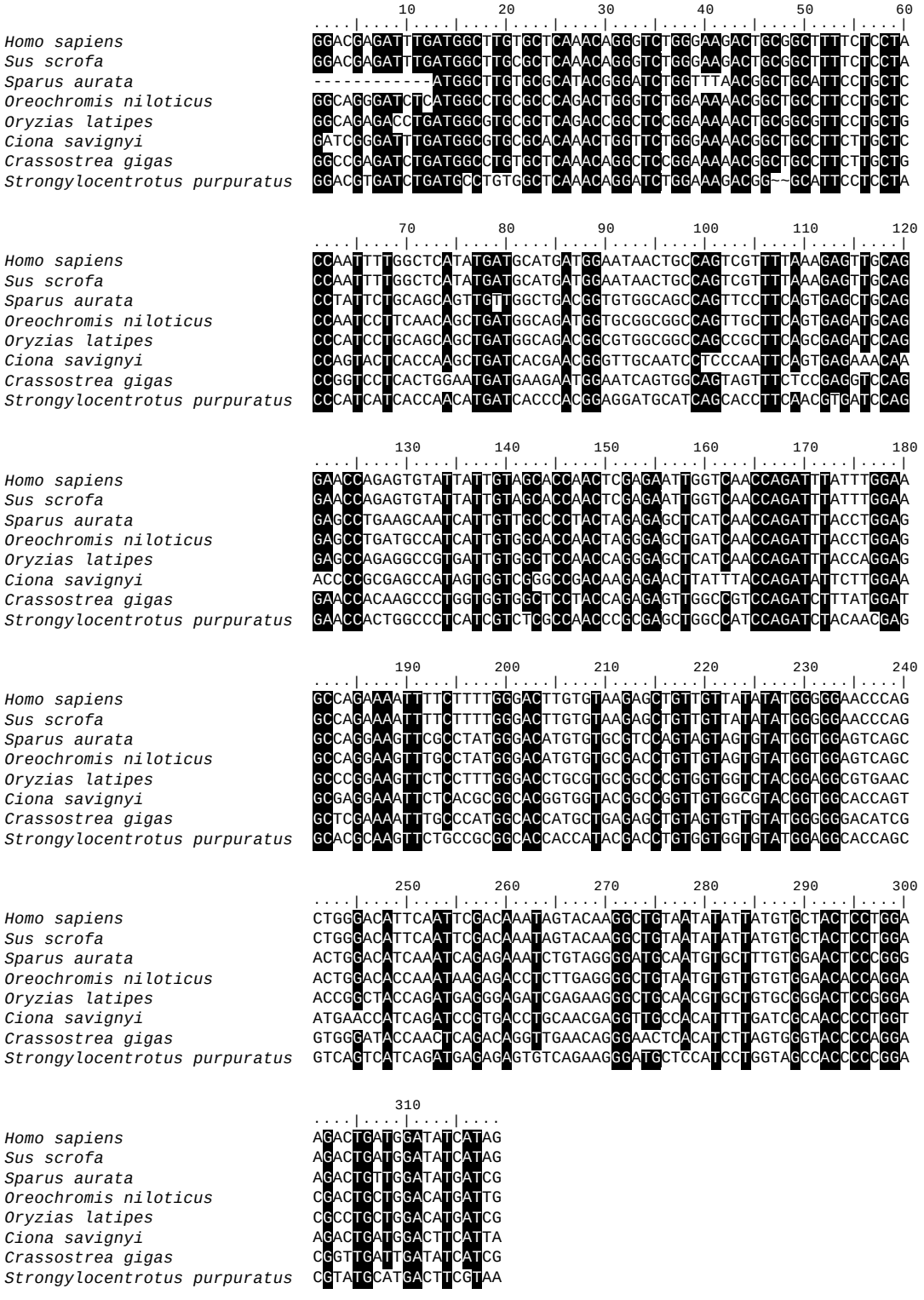
A) SF1



B) CYP17


Gallus gallus
Rana dybowskii
Pimephales promelas
Danio rerio
Ictalurus punctatus
Monopterus albus
S. purpuratus

C) Vasa


Homo sapiens
Sus scrofa
Sparus aurata
Oreochromis niloticus
Oryzias latipes
Ciona savignyi
Crassostrea gigas
Strongylocentrotus purpuratus

CYP17 has up to now only been cloned in vertebrate species. Indeed, searches in the genome of *Caenorhabditis elegans*, *Drosophila melanogaster* or *Ciona intestinalis* could not identify any CYP17 homolog (Baker et al., 2004). However, there are genomic sequences in the *S. purpuratus* database with high homology to CYP17. Therefore, we selected the genomic sequence with lower E score (gnl\ti\201957509) and aligned it towards seven vertebrate nucleotide sequences for CYP17 to identify highly conserved regions. Then, we designed specific primers towards *S. purpuratus* on these regions (Figure A.3.B).

Vasa has been cloned in several vertebrate and invertebrate species, suggesting that this gene is present in echinoderms. In addition, there are a few genomic sequences in the *S. purpuratus* database with high similarity to vasa. Therefore, we selected the genomic sequence with lower E score (gnl\ti\250606936) and aligned it together with eight nucleotide sequences for vasa of phylogenetically distant organisms in order to identify highly conserved regions (Figure A.3.C). Then, we designed specific primers for *S. purpuratus* for these regions. One of the problems faced when working with genomic databases is the presence of introns. In fact, despite that the vasa fragment available for *S. purpuratus* consisted on 1199 nucleotides, there were only two conserved regions and they were separated by an intron.

η-actin is a highly conserved gene, therefore specific primers for *Pimephales promelas* η-actin, already available in the laboratory, were used.

All primer sequences designed are presented in Table A.2. For all genes, the expected length of the fragments of interest ranged from 111 to 960 Bp .

Amplification, isolation and cloning of the genes of interest.

For most PCR reactions, a band corresponding to a gene fragment of expected size was obtained and purified (Figure A.4). Sometimes a regular (single) PCR did not result in bands of expected size and a second PCR was performed using 1σl of the first PCR product as template. The PCR conditions and combinations of primers used for each of the obtained gene fragments are summarised in Table A.3.

Table A.2. Primer sequences.

Gene of interest	Primer ID	Primer sequence (5'-3')	Based on[a]	Degeneracies (length)
b-actin	b-act-f	ATG AGA CCG CTT ACA ACA GC	1	0 (20)
	b-act-r	ATC CAC ATC TGC TGG AAG GT	1	0 (20)
SF1	SF1-f1	GY CCN GTN TGY GGN GAY AAR	2	1024 (20)
	SF1-f2	AT NGG NCC RAA YTT RTT NCK	2	1024 (20)
	SF1-f3	ACS TGY GAR WSS TGY AAG GG	3	128 (20)
	SF1-f4	SST GYA AGG GMT TYT TYA AG	3	64 (20)
	SF1-r1	GN AAY AAR TTY GGN CCN ATG	2	1024 (20)
	SF1-r2	TG NAR NAR YTT CAT YTG RTC	2	1024 (20)
	SF1-r3	GCM CKR TCB CKY TTG TAC AT	3	96 (20)
	SF1-r4	TAC ATB GGB CCR AAY TTR TT	3	72 (20)
DMRT	DMRT-f	CCN AAR TGY KCN MGN TGY MG	2	4098 (20)
	DMRT-r	AR NGC NAC YTG NGC NGC CAT	2	1024 (20)
CYP17	CYP17-f	GTTCAAGATGAGTTGGATGC	4	0 (20)
	CYP17-f	TCATGATGAACAGCCCACAG	4	0 (20)
vasa	vasa-f	AACAGGATCTGGAAAGACGG	4	0 (20)
	vasa-r	ATGAGGGCCAGTGGTTCCTG	4	0 (20)

[a]1: Fathead minnow η-actin sequence; 2: Multispecies aminoacid sequences alignment; 3: Multispecies nucleotide sequences alignment; 4: *Strongylocentrotus purpuratus* BACs.

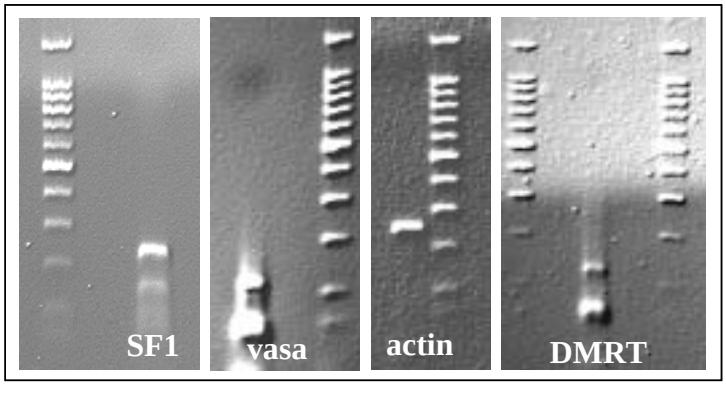


Figure A.4. Agarose gels of the PCR products obtained using SF1, vasa, η-actin and DMRT primers. All gels included a 100 bp ladder.

After two consecutive PCR reactions, a band of expected size was obtained for CYP17, but this band was very faint (possibly due to primer depletion by other non-specific products) and it was not possible to purify it (Figure A.5).

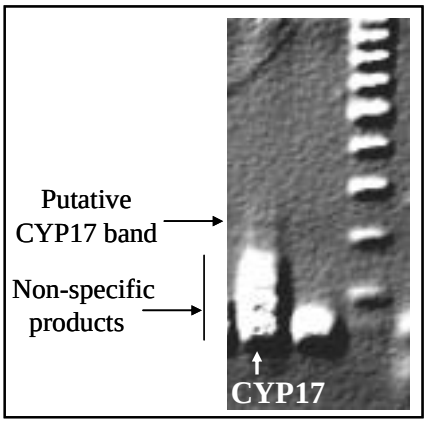
Table A.3. PCR products obtained.

Gene of interest	Primer ID	Expected size	PCR conditions[1]	Approximate size of the bands obtained
b-actin	b-act-f b-act-r	247	35 c: 94ºC (30''), 42ºC (30''), 72ºC (1')	250
SF1	SF1-f4 SF1-r3	197	35 c: 94ºC (30''), 42ºC (30''), 72ºC (1')	140
DMRT	DMRT-f DMRT-r	134	2 PCR reactions, 35 c.: 94ºC (30''), 37ºC for 7 c. and 42ºC for 28 cycles (30''), 72ºC (1'). 35 c.: 94ºC (30''), 42ºC (30''), 72ºC (1')	130
CYP17	CYP17-f CYP17-f	224	2 PCR reactions, both 35 c.: 94ºC (30''), 37ºC (7 c.) and 42ºC (28 c.) (30''), 72ºC (1').	220
vasa	vasa-f vasa-r	111[2]	40 c.: 94ºC (30''), 52ºC (30''), 72ºC (1').	110

[1]c: cycles; ':minutes; '':seconds

[2]Once introns have been excluded from the *S. purpuratus* genomic sequence.

Figure A.5. Agarose gel of the PCR product obtained with CYP17 primers.



Direct sequencing was performed for those genes where enough quantity of product was obtained after purification. Alternatively, the bands were cloned. In addition, when the direct sequencing confirmed that the PCR product belonged to the gene of interest, it was cloned in order to obtain a longer sequence. For all cloning reactions, white and blue colonies were obtained. While colonies were collected, the plasmids were amplified by PCR using M13 primers, and the bands were separated in agarose gels (1%) (as an example, see Figure A.6). Up to three colonies containing the right size insert were selected, grown overnight, purified, and sequenced.

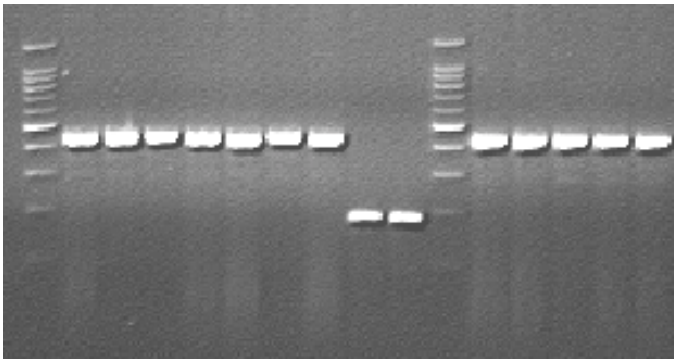


Figure A.6. Cloning of η-actin. Colonies 1 to 7 and 10 to 15 contain inserts of the right size, whereas colonies 8 and 9 do not contain inserts.

Sequence analysis

The nucleotide sequence obtained for vasa showed high similarity to the vasa-like gene from *S. purpuratus.* Two different nucleotide sequences were obtained for actin and they both showed high similarity to actin genes of the echinoderm and/or fish species. The nucleotide sequences of the putative DMRT and SF1 gene fragments did not result in high similarities with the sequence information available for these genes (see Table A.4).

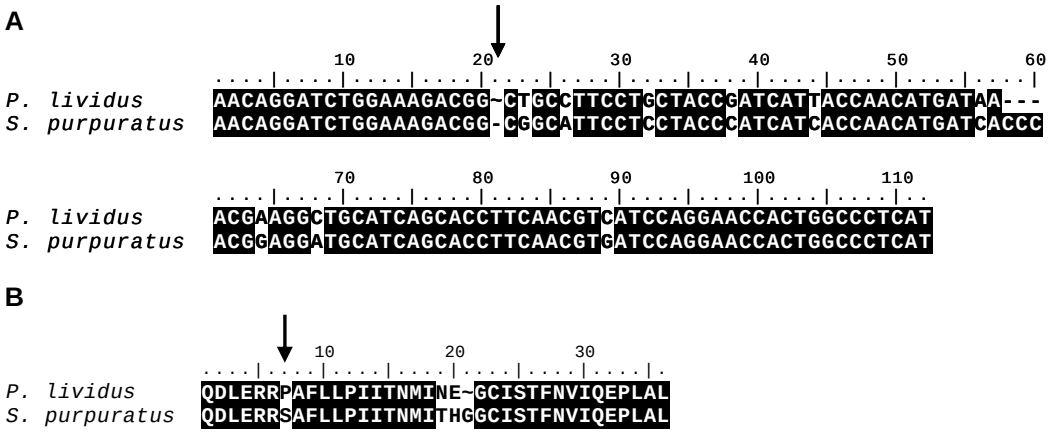
Two different sequences were obtained using the η-actin primers. One of the sequences was similar to *S. purpuratus* CyIIb (gi: 47551036; 95% and 100% nucleotide and aminoacid identities, respectively) and to vertebrate η-actin genes (e.g. in *Fundulus heteroclitus,* gi:52430329, 90% and 96% nucleotide and aminoacid identities, respectively). The second sequence was similar to fish ζ-actin (e.g. in *Sphyraena idiastes,* gi: 27733680, 88% and 96% nucleotide and aminoacid identities, respectively). A definitive identification of the first actin gene as CyIIb homolog is not possible due to the high similarity among the different cytoplasmic actin genes in *S. purpuratus* (97 to 99% identity; Kissinger et al., 1997), and therefore we identified this gene as '*P. lividus* η-actin-like'. Similarly, the identification of the second gene is problematic since it shares 81 to 85% identities with other *S. purpuratus* actins (CyIIIa, CyIIIb, and CyIIb), therefore, we identified it as '*P. lividus* ζ-actin-like'. Unfortunately, the nomenclature for actin genes is not standardised in echinoderms. The sequences obtained can be used to design specific primers against *P. lividus* actin genes and obtain their complete sequences by a Race approach. Actin genes are widely used as control genes in transcriptomic studies. However, a basic characterization of their expression in different tissues and developmental/reproductive stages in *P. lividus* would be desirable prior to their use as control genes.

Table A.4. Sequences of the cloned fragments. Primers are bolded.

Gene	Nucleotide sequence	Significant Blast results ($E<10^{-5}$)
DMRT	10 20 30 40 50 60 70 **AGTGCAACTTGTGCTGCCATAGGGGTATAGGTAGAGGCGAAATGTCTGGCAGCCCCTTCGATGAAGAATC** 80 90 100 110 120 130 140 **ATCTCGAAGATTGGGTAAGGAATCCGATCATTGGATAGGCCATTGATGTGTCCAAATATTCCTTCCCAGG** **GCGAATTCA**	None
SF-1	10 20 30 40 50 60 70 **GGTGCAAGGGCTTTTTCAAGAGTTACCTCTCACGGGAAATGGACCAGCAAGCGAACAGTCTGTCCCAAAA** 80 90 100 110 120 130 140 **TGATCAACCAAAATTGGATAAGCACATAATGAATGGGGATGGTCATTCACCATTGAAGGACTCTTCTCGA** 150 160 170 180 190 200 210 **AGTGAACATTTGGTTACGTCTCCAGTTATAGAATCGGCCAGGCAACCGACGACTGGGCACACGAGCCTTA** 220 **AAAAATCCCTTACACC**	None
η-actin	10 20 30 40 50 60 70 **ATGAGACCGCTTACAACAGCATCATGAAGTGCGATGTTGACATCCGTAAGGATCTGTACGCCAACACCGT** 80 90 100 110 120 130 140 **CCTCTCTGGAGGTTCCACCATGTTCCCAGGAATCGCCGACAGGATGCAGAAGGAGATCACCGCCCTTGCT** 150 160 170 180 190 200 210 **CCACCAACCATGAAGATCAAGATCATCGCCCCACCAGAAAGGAAATACTCTGTATGGATCGGAGGCTCCA** 220 230 240 **TCCTTGCTTCTCTCTCCACCTTCCAGCAGATGTGGAT**	Actin Similar to echinoderm CyIIb
η-actin	10 20 30 40 50 60 70 **TAGAGACCGCTTACAACAGCATCATGAAGTGTGACGTAGACATCCGTCGTGACCTCTACGCCAACACAGT** 80 90 100 110 120 130 140 **CCTATCAGGAGGCACCACCATGTACCCTGGCATTGCTGATCGTATGCAGAAGGAGATCACTGCACTGGCT** 150 160 170 180 190 200 210 **CCAAGCACCATGAAGATCAAGATCATTGCTCCACCCGAGAGGAAATACTCCGTCTGGATCGGTGGCTCCA** 220 230 240 **TCCTTGCTTCCCTCTCTACCTTCCAGCAGATGTGGAT**	Actin Similar to fish ζ-actin
vasa	10 20 30 40 50 60 70 **AACAGGATCTGGAAAGACGGCTGCCTTCCTGCTACCGATCATTACCAACATGATAAACGAAGGCTGCATC** 80 90 100 **AGCACCTTCAACGTCATCCAGGAACCACTGGCCCTCAT**	Vasa

The sequence obtained with the vasa primers was aligned with that of *S. purpuratus* (Figure A.7). The high similarity between the two sequences (83% identities, both in nucleotide and aminoacid sequences) indicates that this is a well-conserved gene. The obtained fragment is part of the centre of the gene (according to sequences for other organisms), with 1000 to 1400 bp towards it 3' end, and 1100 to 1800 bp towards its 5' end. Despite the fact that the length of this sequence is relatively short, it allows for the design of specific nested primers to undertake a 3'Race and 5'Race approach in order toobtain the complete sequence for this gene.

Figure A.7. Nucleotide (A) and aminoacid (B) alignment of *P. lividus* and *S. purpuratus* vasa sequences. The arrow indicates where the intron in *S. purpuratus* was located.



A.4. Future work

Further work should focus on obtaining the whole lengths of vasa and actin genes, and thereafter characterise their expression, in order to include them in the microarray.

For DMRT, a band of the expected size was obtained, however, the clone sequenced did not correspond to DMRT. The aminoacid sequence from which the primers were designed was highly specific for DMRT, however, the low temperatures used during the PCR reaction combined with the high level of degeneracies (thousands) included in the primers, can explain why unspecific products were obtained. By increasing the annealing temperature in the PCR reaction, the specificity of the PCR towards DMRT would increase, and there is the possibility that the DMRT fragment could be cloned.

For CYP17, a band of the expected size was obtained, however at a very low yield. The low amplification of the band of interest could be due to the low presence of CYP17 template in the reaction, or to the competition for the primers by unspecific short bands. In a PCR reaction, shorter

bands are amplified faster, and can decrease the amount of primer available for longer bands—in this case, the gene of interest. Future work would need to design other primers, with a higher specificity towards CYP17.

For SF1, we could never obtain a band of approximately the expected size. Since, multiple PCR conditions and templates were already used without success, new primers should be designed to continue the work. Alternatively, the sea urchin EST database could be further explored for possible matches with SF1 that were not identified in the searches performed within this study.

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RESUM / ABSTRACT

RESUM

El invertebrats constitueixen el 95% de les espècies animals i, com en el cas dels vertebrats, són susceptibles a l'efecte dels disruptors endocrins (DEs). Tot i això, la comprensió d'aquest fenomen està limitada pel desconeixement del sistema endocrí dels invertebrats. L'alteració de la síntesis i metabolisme d'hormones esteroides és un dels possibles mecanismes d'acció dels DEs. En aquesta tesi s'ha caracteritzat el metabolisme d'hormones esteroides en diferents espècies d'invertebrats per tal d'avaluar l'efecte *in vitro* de DEs en aquestes espècies, i finalment investigar l'efecte *in vivo* de DEs en els nivells i el metabolisme d'esteroides en espècies d'un filum seleccionat (Mol·lusca). Les espècies investigades (Mollusca: *Marisa cornuarietis*, *Mytilus sp.*, i/o *Crassostrea virginica*; Crustacea: *Hyalella azteca*; i Echinoderma: *Paracentrotus lividus*) van metabolitzar la testosterona a una sèrie de metabolits de fase I que també són formats per vertebrats, amb l'excepció de 4-androstene-3η,17η-diol, un metabolit format per *P. lividus* i *H. azteca*. Una de les diferències més importants amb els vertebrats va ser la baixa contribució de les hidroxilacions en el metabolisme de testosterona, i en el cas dels mol·luscs, el metabolisme d'androstenediona (reducció en 5ζ# en comptes de reducció en 17η). Pel que fa al metabolisme de testosterona de fase II, les sulfotransferases es trobaven en nivells alts en *P. lividus*, però baixos o indetectables en *H. azteca* i totes les espècies de mol·luscs investigades. En canvi, tots els invertebrats estudiats presentaven una gran capacitat de conjugació d'hormones esteroides amb àcid grassos, una via que sembla que ha estat ben conservada al llarg de l'evolució. Els nivells d'hormones esteroides i algunes de les vies enzimàtiques que les metabolitzen van mostrar diferències estacionals, entre diferents teixits i entre sexes, fet que recolza la possible funció fisiològica de les hormones esteroides en invertebrats. Alguns xenobiòtics van interferir *in vitro* amb els enzims que metabolitzen les hormones esteroides. Entre les vies investigades, la 5ζ-reductasa i la sulfotransferasa, van mostrar la sensibilitat més alta a inhibició/activació per tributilestany (TBT), trifenilestany (TPT) i fenarimol. Per últim es va investigar l'efecte de l'exposició a estradiol, petroli, i la barreja de petroli i compostos alquilfenòlics en bivalves *Mytilus sp.*, i l'exposició a metiltestosterona, TBT, TPT i fenarimol en el gasteròpode *M. cornuarietis*. El tipus d'efectes induïts per l'estradiol van dependre en gran mesura de la seva concentració. Algunes de les respostes observades en els musclos exposats a petroli o la barreja de petroli i alquilfenols, van ser similars a les que s'havien observat per concentracions altes d'estradiol. En aquests experiments es va observar també el possible rol que juga la conjugació d'esteroides amb àcid grassos en la regulació dels nivells d'esteroides lliures. L'exposició als compostos organoestànnics (TBT i TPT) va causar l'aparició d'imposex i va resultar en una reducció del nivell d'esteroides en forma esterificada en femelles de *M. cornuarietis*, però no en mascles. Els altres compostos

avaluats (metiltestosterona i fenarimol) van induir imposex en femelles, però no van mostrar alteracions en els nivells d'esteroides esterificats, de manera que no es va poder demostrar la connexió entre la davallada dels nivells d'esteroides conjugats amb àcids grassos i el fenomen de l'imposex. L'exposició a TPT va inhibir 5ζ-reductasa en femelles de *M. cornuarietis*. Tot i així, cap dels dos organoestànnics va alterar el dimorfisme sexual en el metabolisme d'androstenedione que existeix en el complex glàndula digestiva/gònada d'aquest gasteròpode. En resum, aquesta tesi contribueix al coneixement del metabolisme d'andròg ens en invertebrats i identifica dianes on poden actuar els disruptors endocrins.

ABSTRACT

Ninety-five percent of all animal species are invertebrates, and like vertebrates, they are susceptible to endocrine disruption. Nevertheless, there are important gaps on the knowledge of the endocrine system of invertebrates that hinder the understanding of the endocrine disruption phenomena in those species. Steroid synthesis and metabolism is one of several possible targets of endocrine disruptors. This thesis aimed to characterize sex steroid metabolism in different invertebrate species, to test the *in vitro* effect of model endocrine disruptors in those species, and to test the *in vivo* effect of model endocrine disruptors on steroid levels and metabolism in species from a selected phyla (Mollusca). The species investigated (Mollusca: *Marisa cornuarietis*, *Mytilus sp.*, and/or *Crassostrea virginica*; Crustacea: *Hyalella azteca*; and Echinoderma: *Paracentrotus lividus*) were able to form a series of phase I metabolites of testosterone that are also formed by vertebrates, with the exception of 4-androstene-3η,17η-diol, a metabolite formed by *P. lividus* and *H. azteca*. One of the major differences with vertebrate species was the lower contribution of hydroxylases in the metabolism of testosterone, and the metabolic fate of androstenedione in molluscs (5ζ-reduction instead of 17η-reduction). Regarding phase II metabolism of testosterone, sulfotransferases were found at high levels in *P. lividus*, but low or undetectable levels were present in *H. azteca* and the molluscan species investigated. In contrast, the conjugation of steroids with fatty acid moieties was present in all invertebrates investigated, showing that acyl-CoA:steroid acyltransferases are well-conserved through evolution. Steroid levels and the enzymatic activities for some metabolic pathways showed differences between males and females, among tissues, and between seasons in some invertebrate species, which adds further evidence for a physiological role of sex steroids in invertebrates. Some xenobiotics modulated steroid metabolic pathways in invertebrates *in vitro*. Among the pathways investigated, 5ζ-reductase and sulfotransferase showed the highest sensitivity to inhibition/activation by tributyltin (TBT), triphenyltin (TPT) and fenarimol. Finally, the effects of the exposure to estradiol, crude oil, and the mixture of crude oil and alkylphenolic compounds in *Mytilus sp.* and the exposure to methyltestosterone, TBT, TPT and fenarimol in the gastropod *M. cornuarietis* were investigated. The type of effects induced by estradiol depended greatly on the concentration. Some of the responses observed in mussels exposed to crude oil and the mixture of crude oil and alkylphenols were similar to those observed in mussels exposed to the highest concentrations of estradiol. Those experiments also showed that esterification seems to play a key role in the regulation of free steroid levels in bivalve molluscs. The exposure to the organotin compounds (TBT and TPT) caused imposex and decreased esterified steroid levels in females of *M. cornuarietis*, but not in males. Methyltestosterone and fenarimol induced imposex in females, but did not lead to

alterations in esterified steroid levels. Therefore, the connection between a decrease of esterified steroids and the phenomena of imposex could not be proved. The exposure to TPT inhibited#ζ-reductase in female *M. cornuarietis*. However, neither TPT nor TBT altered the sexual dimorphism in androstenedione metabolism that exists in the digestive gland/gonad complex of this snail species. Overall, this thesis contributes to the knowledge of androgen metabolism in invertebrate species and identifies possible targets of endocrine disruption.