Universitat Autònoma de Barcelona

Programa de Doctorat de Farmacologia, Terapèutica i Toxicologia

STEROID LEVELS, STEROID METABOLIC PATHWAYS AND THEIR MODULATION BY ENDOCRINE DISRUPTORS IN INVERTEBRATES

Tesi doctoral

Gemma Janer Gual

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ACRONIM LIST

ACRONIM LIST

11-kT: 11-keto-testosterone
2,4-D: 2,4-dichlorophenoxyacetic acid
4-A-diol: 4-androsten-3η,17η-diol
NP: nonylphenol
5ζ-A-diol: 5ζ-androstan-3η,17η-diol
5ζ-R: 5ζ-reductase
AACs: androgenic and antiandrogenic compounds
AD: androstenedione
AhR: aryl hydrocarbon receptor
APCI: atmospheric pressure chemical ionization
APE: alkylphenol ethoxylate
ATAT: acyl-CoA acyl transferase
BBP: butylbenzyl phthalate
BPA: bisphenol A
BSA: bovine serum albumine
BSTFA: bis(Trimethylsilyl)trifluoroacetamide
cAMP: adenosine 3',5'-cyclic monophosphate
CNS: central nervous system
CoA: coenzyme A
CYP: cytochrome P450
DBP: di-n-butyl-phthalate
DDE: dichlorodiphenyldichloroethylene
DDT: dichlorodiphenyltrichloroethane
DEHP: di-(2-ethylhexyl)phthalate
DES: diethylstilbestrol
DHA: dihydroandrostenedione
DHEA: dehydroepiandrosterone
DHT: dihydrotestosterone
E1: estrone
E2: estradiol
ED: endocrine disruptor compounds
EDSTAC: endocrine disruptor screening and testing advisory committee
EDTA: ethylenediaminetetraacetic acid
EE2: ethynylestradiol
EI: electron impact
ELISA: enzyme-linked immunosorbent assay
EPA: environmental protection agency
ER: estrogen receptor
ERE: estrogen response element
ESI: electrospray ionization
FEN: fenarimol
GC: gas chromatography
HCB: hexachlorobenzene
HCH: hexachlorocyclohexane (lindane)
HPLC: high performance liquid chromatography
HPTE: 2,2-bis[p-hydroxyphenyl]-1,1,1-trichloroethane
HRE: hormone response element
HSD: hydroxysteroid dehydrogenase
IC50: inhibitory concentration 50%
JH: juvenile hormone
LC-MS: liquid chromatography-mass spectrometry
LLE: liquid liquid extraction
LOEC: lowest observed effect concentration
MAPK: mitogen-activated protein kinase
METI: ministry of energy, trade and industry
MS: mass spectrometry
MT: methyl testosterone
NADPH: η-nicotinamide adenine dinucleotide phosphate
NCI: negative chemical ionization
NGO: non-governamental organization
OECD: organization for economic cooperation and development
OH-T: hydroxylated testosterone metabolites
P450arom: cytochrome P450 aromatase
P450c17: cytochrome P450 17ζ-hydroxylase and C_{17-20}-lyase

P450scc: cytochrome P450 side chain cleavage
PAH: polyaromatic hydrocarbon
PAPS: adenosine 3Ñphosphate 5Ñphosphosulfate
PBB: polybrominated biphenyl
PCB: polychlorinated biphenyl
PCP: pentachlorophenol
RIA: radioimmunoassay
RXR: retinoid X receptor
SHBG: sex steroid binding protein
SPE: solid phase extraction
StAR: steroidogenic acute regulatory protein
STAT: signal transducers and activators of transcription
SULT: sulfotransferase
T: testosterone
TBT: tributyltin
TCDD: tetrachlorodibenzodioxin
TLC: thin layer chromatography
TMS: trimethylsilyl
TPT: triphenyltin
UDP: uridine diphosphate
UGT: uridine diphosphate (glucuronosyl or glucosyl) transferase
VDS Index: vas deference sequence index
WHO: world health organization
XRE: xenobiotic response element

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CHAPTER 1. GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1 Global framework in endocrine disruption research and origin of the COMPRENDO project

Global concerns have been raised in recent years over the adverse effects that may result from exposure to chemicals that have the potential to interfere with the endocrine system (endocrine disruptors, ED). Wildlife and human health effects of EDs were first proclaimed by Rachel Carson in 1962, and based on growing body of knowledge, those concerns have increased. As a consequence of it, in the late 90s, national and international organizations established a series of programs to investigate and monitor the effects of ED, and to develop screening tests to evaluate new chemicals (WHO, 2002).

In 1996, a European workshop organized in the UK brought together scientists and policy-makers from the EU, USA and Japan, international organizations such as the OECD and the WHO, and several NGOs. They concluded that (i) there was evidence that testicular cancer rates were increasing; (ii) the apparent decline in sperm counts in some countries was likely to be genuine; (iii) endocrine-disrupting effects observed in birds and mammals could be due to the use of chemical substances with endocrine-disrupting properties, (iv) exposure to endocrine disrupters should be dealt with measures in line with the “precautionary principle” (http://europa.eu.int/comm/research/endocrine/ index_en.html).

As a result of the workshop findings and due to increasing public concern, in 1998**,** the European Parliament adopted a report and a resolution calling upon the European Commission to take specific actions to improve the legislative framework, reinforce research efforts, and make information available to the public (http://europa.eu.int/comm/research/endocrine/index_en.html).

In 1999, the European Commission established short-, medium- and long-term strategies to manage the problem of ED in a rapid and effective manner. Within the actions included in the short-term strategy, there was the establishment of a priority list of substances, the communication to the public and international cooperation. The medium term strategies include the identification and evaluation of EDs, and research and development. Finally, as a long-term strategy, the information obtained from these two strategies, would be used for legislation action.

In 2001, as part of the medium-term strategies, the European Commission launched a call focused on endocrine disrupters that culminated in the formation of the CREDO cluster, which consists of four projects: EDEN, FIRE, EURISKED and COMPRENDO, and seven additional associated projects (http://europa.eu.int/comm/research/endocrine/index_en.html).

Similar to the European Union, the U.S. Environmental Protection Agency (EPA) and the Ministry of Energy, Trade and Industry (METI) of Japan have established specific programs for the investigation of EDs. EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) that is establishing a series of toxicological and ecotoxicological tests for the identification and characterization of the effects of EDs (http://www.epa.gov/scipoly/oscpendo/). The american system is based on two levels, a first screening level which uses *in vivo* and *in vitro* tests, and a second level that includes *in vivo* tests and second generation studies. METI has been developing the 3-D QSAR system, which will be used to prescreen thousands of industrial chemicals. Additionally, *in vitro* and *in vivo* screening methods are also being developed (Oikawa and Matsumoto, 2003).

Finally, it is worth mentioning the creation by WHO, in collaboration with other international organisms, of a global inventory of endocrine disruption (http://endocrine.ei.jrc.it); and, in 2002, the publication by OECD of a report that established the basis for the development of OECD guidelines (currently used as the quality standard in most toxicological experiments) for the evaluation of ED effects (OECD, 2001).

1.2 Evidences of endocrine disruption

1.2.1 Evidences in vertebrates

A number of adverse human health effects in which EDs may be implicated have been observed, e.g. decreased sperm counts, increasing incidence of certain congenital malformations in children, and increasing incidence of hormone-related cancers (such as breast and testicular cancer) (WHO, 2002). However, epidemiological studies have not established a causal relationship between these observations and exposure to EDs.

In contrast to the uncertainty of cause-effect relationships in humans, studies on wildlife and laboratory animals have provided stronger evidence that EDs have the potential to interfere with the development of the endocrine system, including the reproductive tract.

Male fish living downstream of sewage treatment plants were reported to have high concentrations of the female egg-yolk protein, vitellogenin, not normally present in males (Figure 1.1A). Some male fish also had female-like oocytes within their testis resulting in gonads that are intermediate between male and female (Figure 1.1B). These effects probably resulted from natural and

synthetic estrogenic chemicals (e.g. estradiol excreted in urine, alkylphenolic compounds and birth control pills synthetic hormones), which are released into the aquatic environment via sewage treatment plant effluents (Vos et al., 2000).

Pulp and paper mill effluents are another identified cause of reproductive alterations in fish. In this case, the exposure is related to reduced gonad weight, dysfunction of steroid synthesis and metabolism, decreased plasma steroid levels, masculinization (including secondary sex characteristics and behavior; Figure 1.1C), altered sex ratio, reduced egg production and hatchability, delayed sexual maturity, reduced capability to spawn, and increased vitellogenin expression (Munkittrick et al., 1998).

Amphibian declines have been documented in many parts of the world. Environmental contamination probably accounted for the historical and geographical trends in the gonadal alterations (intersexuality) observed in frogs and likely contributed to the decline of the species (Reeder et al., 2005).

Despite the fact that most of the evidences come from aquatic environments, endocrine disruptors are also suspected to be related to the observed decreased fertility in birds and mammals (Colborn et al., 1993); reproductive impairment of panthers (Facemire et al., 1995); reduced hatching success in birds and turtles; and the feminization and masculinization of birds (Colborn et al., 1993).

1.2.2 Evidences in invertebrates

Invertebrates represent approximately 95% of all animal species. The current knowledge about endocrine regulation of development and reproduction in aquatic invertebrates is limited, and progress on understanding endocrine disruption in invertebrates has been hampered by the lack of detailed insights in their endocrinology. In spite of this, there are several evidences that environmental pollutants are interfering with the endocrine system of invertebrates.

A widespread development of male sexual organs in female marine gastropods, such as the dog whelk, has been attributed to the exposure to tributyltin (TBT) and other organotin compounds such as triphenyltin (TPT), constituents of marine anti-fouling paints (Fent, 1996). The imposition of male sexual organs on females is known as ‘imposex’ (Figure 1.1D), and often results in sterilization of females, leading to population declines in many harbors and coastal waters. TBT initiates the induction of imposex at a seawater concentration of 1 ng/L. Besides the effects on gastropods, organotin compounds are known to induce morphological abnormalities in oyster shells at concentrations as low as 2 ng/L, and to be responsible for dramatic declines of oyster populations during the late 1970s in Arcachon Bay (Fent, 1996).

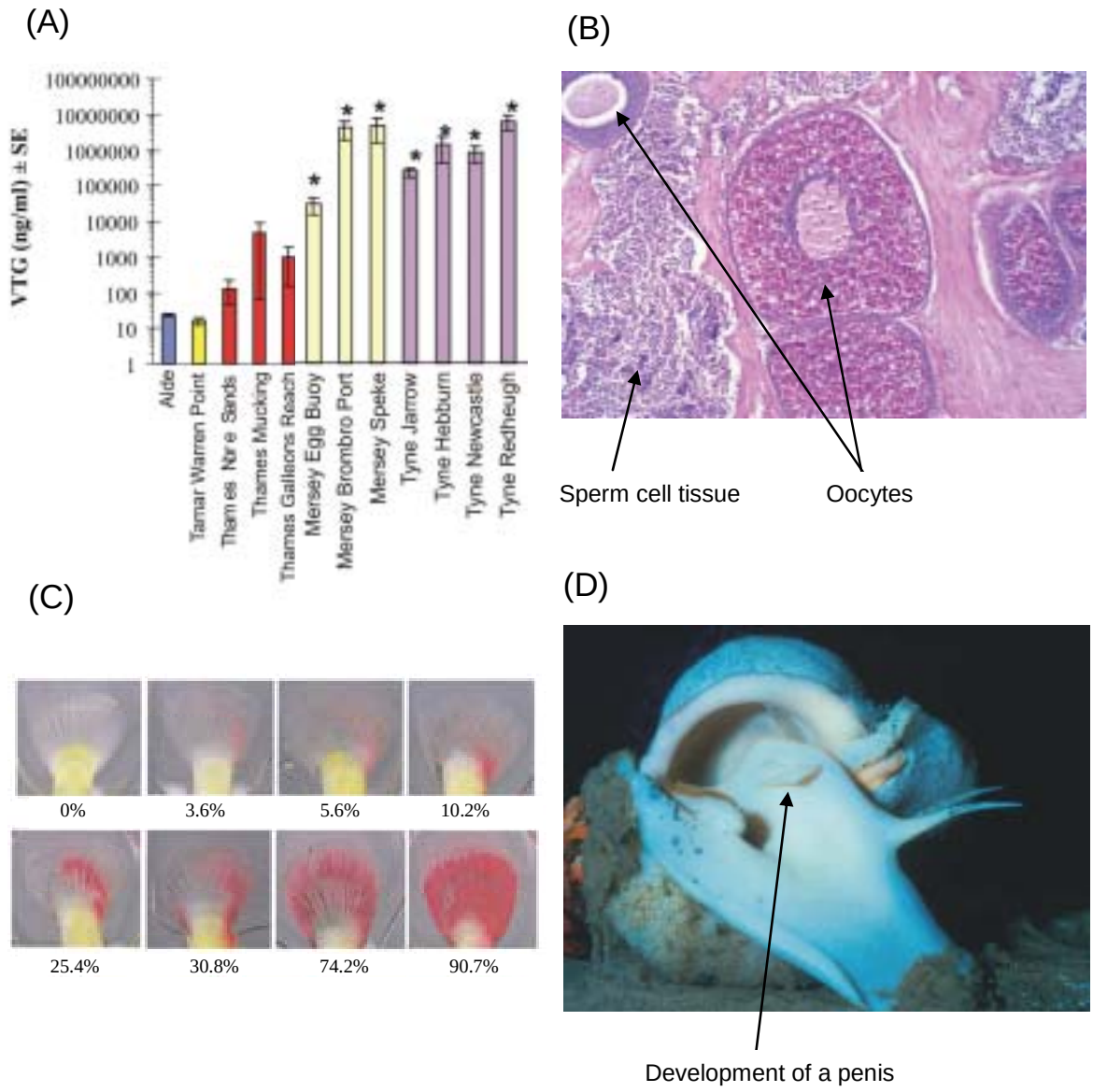


Figure 1.1. Examples of endocrine disruption in wildlife. (A) Mean concentrations of plasma VTG in male flounder (*Platichthys flesus*) from various U.K. estuaries sampled in 1997. Asterisks indicate significant difference from the clean reference estuary, Alde (extreme left) (WHO, 2002). (B) Testis section from a male flounder (*Platichthys flesus)* sampled in the U.K. Mersey estuary in 1996, showing several large secondary oocytes alongside abnormal sperm cell tissue (ovotestis) (WHO, 2002). (C) Tail fins of female guppies exposed to increasing concentrations of pulp mill effluent and showing increasing levels of redness (% red area); the last two are exposed to methyltestosterone (Larsson et al., 2002). (D) Imposex in the common whelk (*Buccinum undatum* L) (WHO, 2002).

In contrast to molluscs, there is minimal evidence of the impact of TBT on crustaceans. Effects reported are enhanced testosterone metabolism, retardation of regenerative limb growth, retardation of molting, and inhibition of calcium resorption from the exoskeleton (Mathiessen et al., 1999). Crustaceans appear to have greater ability than molluscs to metabolize TBT (Fent, 1996), and probably, the target for the effect of TBT has a greater physiological relevance in molluscs than in crustaceans.

TBT and TPT showed sperm and embryo toxicity in echinoderm species at concentrations of few σg/L, affecting embryo growth at concentrations as low as 10 ng/L (Marin et al., 2000; Novelli et al., 2002). In addition, the regeneration of ophiods has been reported to be altered by low concentrations of these organotins (Walsh et al., 1986). Apart from the effects on regeneration and embryo growth, the information on the endocrine effects of organotin compounds in echinoderms is limited by the lack of studies looking at developmental or reproductive parameters.

In addition to organotin compounds, other groups of compounds have been related to physiological disturbances in invertebrate species that might be endocrine-mediated. A brief overview of the disturbances linked to some chemical groups: namely heavy metals, herbicides, and xenoestrogenic/phytoestrogenic compounds is offered below.

Heavy metals have been shown to have significant impacts on crustaceans (molting, growth, and reproduction) and echinoderms (oogenesis and gonadal development) (Mathiessen et al., 1999).

Various pesticides disturb endocrine processes in invertebrates at concentrations considerably below those that are overtly metabolically toxic. For example, triazine herbicides disturbed the fecundity and growth in *Daphnia pulex*, impaired reproduction and development in the malacostracan *Gammarus fasciatus*, and reduced frequency of molting in *D. pulex* (Mathiessen et al., 1999). The organochlorine 1,1,1-trichloro-2,2-bis[4-chlorophenyl]ethane (DDT), an insecticide, caused a substantial reduction in fecundity in the snail *Lymnaea stagnalis* (Mathiessen et al., 1999). The pesticide heptachlor affected ecdysis by an alteration of ecdysteroid levels (Snyder and Mulder, 2001). Vinclozolin, a fungicide, caused similar effects as the antiandrogen cyproterone acetate in three snail species. Both vinclozolin and cyproterone acetate reduced the length of the penis and of accessory male sex organs, and an advancement of the sexual repose phase (Tillmann et al., 2001). Deformities of the mouthparts and other head capsule features of chironomid larvae have been suggested to arise as a consequence of exposure of the organisms to pesticides and heavy metals. The underlying mechanism for these deformities remains elusive, but they appear to originate in relation to developmental processes, and this has led to their consideration as indicators of EDs exposure (Mathiessen et al., 1999). Additionally, it should be noted that some insecticides have been purposely synthesized to disrupt the endocrine system of

a number of insects to aid their control, and apart from the target species, it is likely that they will alter the endocrine system of closely related species.

A range of estrogenic compounds also affect the reproductive system of invertebrates. Thus, nonylphenol decreased the fecundity in *Daphnia magna* (Baldwin et al., 1997), induced cyprid major protein, which resembles vitellin, in the juvenile barnacle *Balanus amphitrite* (Billinghurst et al., 2000), delayed the development of the oyster *Crassostrea virginica* (Nice et al., 2000), and increased the production of eggs in the polychaete *Dinophilus gyrociliatus* (Price and Depledge, 1998). Diethylstilbestrol (DES) and endosulfan, inhibited molting in juvenile *Daphnia magna* (Mathiessen et al., 1999). The exposure to the xeno-estrogen bisphenol A (BPA) stimulated ovarian maturation in the copepod *Acartia tonsa* (Andersen et al., 1999), induced asexual reproduction while suppressing sexual reproduction in *Hydra oligactis* (Fukuhori et al., 2005), and caused alterations in molting and embryonic development in *Daphnia magna* (Mu et al., 2005). BPA and octylphenol at the lowest concentrations tested (1 σg/L) induced a series of alterations in female snails, namely the formation of additional female organs, an enlargement of the accessory pallial sex glands, gross malformations of the pallial oviduct section resulting in increased female mortality, and a massive stimulation of oocyte and spawning mass production (Oehlmann et al., 2000). Similarly, BPA, nonylphenol and octylphenol, affected development, including sexual maturity, in the copepod *Tigriopus japonicus* (Marcial et al., 2003). Phytoecdysteroids also demonstrated hormonal activity in insects, thus, effluents from pulp and paper mills, which contain phytoecdysteroids, and effluents from a tannery were shown to influence molting in aquatic insects (Mathiessen et al., 1999; WHO, 2002). Chlordane-treated shrimps (*Neocaridina denticulate*) showed an induction of vitellogenin-like proteins (Huang and Chen, 2004). In *D. magna*, disturbances in testosterone metabolism and the development of secondary sex characteristics, occurred after exposure to different endocrine active substances (Baldwin and LeBlanc, 1994a; Baldwin et al., 1995, 1997; Olmstead and LeBlanc, 2000). Similarly, *Neomysis integer* exposed to nonylphenol and methoprene (a synthetic insect juvenile hormone analog) displayed alterations in testosterone metabolism (Verslycke et al., 2004). Some of these effects might not be associated to an estrogenic activity of the xenobiotics, but to interferences with ecdysteroid/juvenoid regulated processes. For instance, the effects of BPA on daphnids were mediated through an enhancement of the activity of the crustacean juvenoid hormone methylfarnesoate (Mu et al., 2005).

Additionally, reproductive and morphological abnormalities have been described in invertebrates inhabiting polluted sites. For example, intersexuality and demasculinization was observed in *Echinogammarus marinus* collected from a site characterized by high levels of polychlorinated biphenyls (PCBs), heavy metals and receiving the effluent from a paper mill (Ford et al., 2004); disturbed gametogenesis and exoskeleton integrity was observed in *Gammarus sp*. collected at polluted sites (harbours and areas with ferry traffic) (Gagné et al., 2005); abnormal sex ratios were noted in harpacticoid copepods collected in the vicinity of sewage discharges (Moore and

Stevenson, 1991); and an increased incidence of embryonic malformations was found in the amphipod *Monoporeia affinis* from sites receiving industrial effluents (Sundelin and Eriksson, 1998). In these cases, it is difficult to discriminate the causal factor for the abnormalities observed, and most likely the effects are mediated through multiple actions of the mixture of xenobiotics to which the animals are exposed.

These evidences suggest that several groups of substances might cause endocrine-related disturbances in invertebrates. However, the mechanisms underlying most of the effects described above are not known.

1.3 Endocrine disruptor compounds: definition and examples.

An environmental endocrine disruptor may be defined as an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body, that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior (EPA, 1997).

Numerous substances are suspected of acting as endocrine disruptors. Several national and international institutions are currently trying to establish a list of priority substances. The EU draft of substances with endocrine disruptor properties includes 564 chemicals, to which several other chemicals (e.g. flame retardant and cosmetics such as UV-filters; Schlecht et al., 2004; Kuriyama et al., 2005) should be added in the view of new evidences. A simplified list of the compounds included in priority groups 1 and 2 in the EU draft, together with production, persistence and availability of endocrine disruption data, is shown in Table 1.1. It should be noted that some of the chemicals included in the table (e.g. PCBs, phthalates, or organotin compounds) actually represent groups of substances with similar chemical structure, but that can have different potency as endocrine disruptors. Some additional details are summarized below for the chemicals included in Table 1.1 that have attracted more attention during the last years, as indicated by the high number (>100) of studies published in scientific journals that were retrieved from the SciFinder® database containing the concept of endocrine disruption (search performed on March 14th 2005). These substances or groups of substances were, ordered from highest to lowest number of journal papers retrieved: BPA, PCBs, phthalates, dioxins, DDT, alkylphenols, atrazine and lindane. In addition, a special emphasis will be focused on the chemicals investigated in this thesis: the organotin compounds and fenarimol.

Table 1.1. Substances classified by the European Commission as prioritary for further evaluation of their role in endocrine disruption (Adapted from BKH, 2000 and Olea et al., 2002). See acronim list for abreviations.

	Production	Persistence	Cathegory[1]	Group[2]		Production	Persistence	Cathegory	Group		Production	Persistence	Cathegory	Group
Pesticides										**Industrial compounds**				
Chlordane		*	1	I	Nitrofen	*		1	II	Styrene	*		1	I
Mirex		*	1	I	Endosulfan	*	*	2	II	HCB	*		1	I
Toxaphene		*	1	I	Aldrin		*	2	II	Bisphenol A	*		1	I
Maneb	*		1	I	Endrin		*	2	II	Organotins	*		1	I
Metam Natrium	*		1	I	Heptachlor		*	2	II	3,4-Diclhoroaniline	*		1	I
Thiram	*		1	I	Carbendazim	*		2	II	Resorcinol	*		1	I
Zineb	*		1	I	Prochloraz	*		2	II	Dioxins		*	1	I
Lindane	*		1	I	Iprodione	*		2	II	Alkylphenols	*		1	II
Linuron	*		1	I	Ziram	*		2	II	PCBs		*	1,2	I,II
Atrazine	*		1	I	Diuron	*		2	II	PBBs		*	1,2	I,II
Alachlor	*		1	I	Diazinon	*		2	II	Furans		*	1,2	I,II
Kepona		*	2	I	Dimethoate	*		2	II	Phthalates	*		1,2	I,II
DDT		*	2	I	Malathion	*		2	II	Chlorophenols		*	2	II
Vinclozolin	*		2	I	Parathion	*		2	II	o-phenylphenol	*		2	II
Dieldrin		*	1	II	Simazine	*		2	II	Carbon dishulphide	*		2	II
2,4-D	*		1	II	Triadimefon	*		2	II					
Dicofol	*		1	II	Bromomethane	*		2	II	Perchloroethylene	*		2	II
Aminotriazol	*		1	II	Propanil	*		2	II					

[1]Cathegory 1: evidence of endocrine disruption in an intact organism. Cathegory 2: potential for endocrine disruption (in vitro evidences, in vivo effects that may, or may not, be ED-mediated, structural analyses).

[2]Group 1: Cathegory 1 + high concern in terms of human and wildlife exposure. Group 2: Cathegory 1 + medium concern in terms of human and wildlife exposure; or Cathegory 2. Both, groups 1 and 2 contained only substances highly persistent and/or high production volume.

Bisphenol A. Bisphenol A (BPA, Figure 1.2) is an industrial chemical, used to manufacture polycarbonate and numerous plastic articles. However, recent studies have shown that it can leach from certain products, including the plastic lining of cans used for food, polycarbonate bottles and tableware, and white dental fillings and sealants.

The estrogenic activity of BPA has been demonstrated *in vitro* and *in vivo*. BPA binds to the estrogen receptor, and can act as an antiandrogen by blocking the action of dihydrotestosterone in a yeast screen containing a human androgen receptor (Sohoni and Sumpter, 1998).

Low levels of BPA (2 σg/Kg/day) induced increases in prostate weight in male mice (Nagel et al., 1997). In addition, it was suggested that BPA could cause greater effects at low dose levels than at higher doses. This launched the debate on the “low dose theory” or the inverted U-shaped dose response curve. Afterwards, several studies examining the low level effects of BPA have been published, some have replicated the findings and some have not (usually financed by the industry). Two explanations of the difficulties to replicate the effects of BPA were suggested by Ashby et al. (2003): first, it might be that BPA possesses subtle low-dose ED toxicities that only become evident under certain undefined experimental conditions, which are not yet understood; second, natural variability among control parameters monitored in ED studies allows artefactual positive results (Ashby et al., 2004). In addition to the effects observed in rats, other studies have reported effects at low levels of BPA in wildlife, supporting the first of the two explanations mentioned. Indeed, sex ratios were altered (skewed to females) in frog tadpoles (*Xenopus laevia*) exposed to 23 µg/L (Levy et al., 2004); and egg production was stimulated (up to sterilization) in freshwater ramshorn snails exposed to 1 σg/L (*Marisa cornuarietis*) (Oehlmann et al., 2000).

PCBs. Polychlorinated biphenyls (PCBs, Figure 1.2) are a family comprising of 209 chemically related compounds that were widely used more than 25 years ago in a variety of industrial applications due to their insulating and fire retardant properties. Thereafter, the concern over possible adverse effects on the environment and on human health resulted in the cessation of PCB production and an ultimate ban on manufacture in most countries.

The effects of PCBs are complex because the different congeners differ in their activity. Indeed, there are both estrogenic and antiestrogenic congeners, and their hydroxylated metabolites have the ability to interfere with sex steroid and thyroid hormone metabolism (Morse et al., 1993; Kester et al., 2000).

Rats exposed to PCB mixtures early in life developed reproductive effects similar to those caused by DES exposure (Birnbaum, 1994; Li and Hansen, 1996). Epidemiologic studies have also associated PCB exposure to deficits in neurodevelopment (Schantz et al., 2003), and delayed pubertal development (genital development and public hair growth) (Den Hond et al., 2002).

Phthalates. Phthalates (Figure 1.2) are synthetic chemicals that are widely used as plasticizers in the production of plastics, and as solvents in inks used in food packaging and in certain cosmetics (Fisher, 2004).

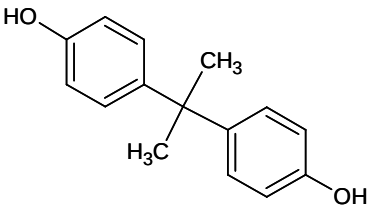
Some phthalate esters (e.g. butylbenzyl phthalate –BBP-, diethylhexyl phthalate –DEHP-, and di-isononyl phthalate) are able to induce reproductive tract abnormalities after in utero exposure in rats (Gray et al., 2000). The abnormalities observed (i.e. reduced anogenital distance, nipple and areola retention, cleft phallus, hypospadias, and undescended testes) are suggestive of under-virilisation of the Wolffian duct and urogenital sinus (Fisher, 2004). Recently, a study reported that some of these reproductive abnormalities observed in rats, are also observed in babies of mothers with high levels of phthalates in their urine (Swan et al., 2005).

BBP, DEHP and dibutyl phthalate (DBP) stimulate the proliferation of MCF7 and ZR-75 cells and bind to the estrogen receptor (Jobling et al., 1995; Zacharewski et al., 1998; Blom et al., 1998), however, they do not seem to be estrogenic *in vivo* (Zacharewski et al., 1998). Phthalates do not interact with the androgen receptor, but interfere with the transcription of several key genes involved in both, cholesterol transport and the biosynthesis of testosterone (Thompson et al., 2004).

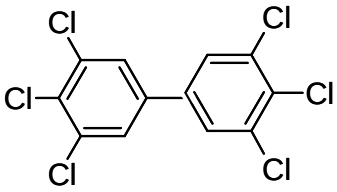
Dioxins. Dioxins (Figure 1.2) are unwanted by-products generated during the synthesis of other compounds and in incomplete combustion processes.

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and its related compounds induce pleiotropic biochemical and toxicological effects, such as induction of drug-metabolizing enzymes, teratogenesis, immunosuppression due to thymic involution, tumor promotion, and liver damage (Fujii-Kuriyama and Mimura, 2003). The major AhR (arylhydrocarbon receptor) is a receptor type transcription factor and has been believed to mediate these effects as a cellular factor. Extensive studies of AhR have revealed that most, if not all, of the toxic effects of TCDD are mediated by AhR (Fujii-Kuriyama and Mimura, 2003). Several cross-talk interactions between the AhR and steroid receptors, mostly estradiol receptor, have been described (reviewed in Pocar et al., 2005) and might explain the antiestrogenic effects of dioxins.

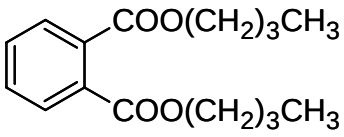
DDT. Dichlorodiphenyltrichloroethane (DDT, Figure 1.2) is no longer used in developed countries, though it is used in various parts of the developing world, particularly for mosquito control, but also in general agriculture. DDT is metabolized in the body to dichlorodiphenyldichloroethylene (DDE), and both these compounds persist in the body fat. Numerous epidemiological studies have investigated the association between DDT levels and risk of breast cancer; however, there is no clear evidence yet for such an association (Mendez and Arab, 2003).



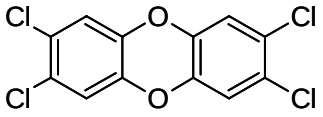
Bisphenol A



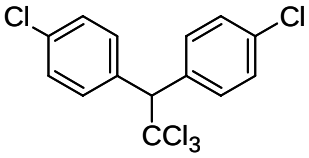
3,3',4,4',5,5'-hexachlorobiphenyl (example of **PCB**)



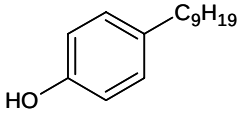
Di-n-butyl-phthalate (DBP) (example of **phthalate**)



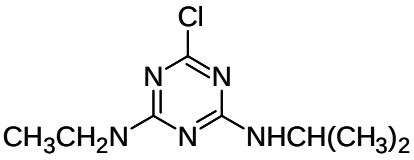
2,3,7,8-tetrachlorodibenzo-p-dioxin (example of **dioxin**)



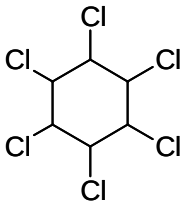
DDT



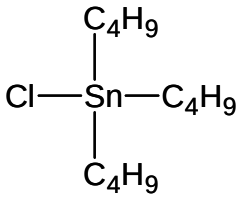
Nonylphenol (example of **alkylphenol**)



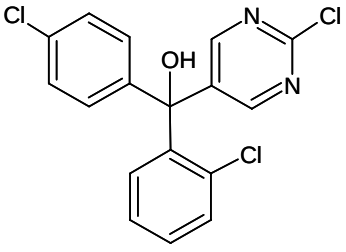
Atrazine



Lindane



TBT



Fenarimol

Figure 1.2. Chemical structure of some endocrine disruptor compounds.

Several isomers of DDT, including *o,p'*-DDT and *p,p'*-DDT, are estrogenic in MCF-7 breast cancer cells (the E-SCREEN assay) (Soto et al., 1995). In addition, the major and persistent DDT metabolite, *p,p*'-DDE, inhibited androgen binding to the androgen receptor, androgen-induced transcriptional activity, and androgen action in developing, pubertal and adult male rats (Kelce et al., 1995), and accordingly it is also anti-androgenic in the Hershberger assay (Kang et al., 2004).

Alkylphenolic compounds. Alkylphenols such as nonylphenol (Figure 1.2) and octylphenol are mainly used to make alkylphenol ethoxylate (APE) surfactants (detergents), though alkylphenols themselves can be used as plasticizers, and its derivatives (alkylphenol phosphites) can be used as UV stabilizers in plastics. It is estimated that 60% of the APEs find their way into the aquatic environment as nonylphenol and octylphenol, the major degradation products. APEs are not persistent and do not accumulate in food chain, nevertheless, the production levels are high and they are detected at relatively high levels in the aquatic environment (up to ~50 σg/L; Petrovic et al., 2002; Zoller et al., 2004). This explains why most studies assess the effects of these compounds on aquatic organisms. Rainbow trout exposed to environmentally-relevant concentrations of 4-nonylphenol (0.1 to 1 µg/L) showed a reduction of semen production, alterations in embryo development (Lahnsteiner et al., 2005), and the induction of vitellogenin in juveniles (Ackermann et al., 2002). Few studies have investigated the effects of alkylphenolic compounds in rodents, and they showed that they stimulated estrogen-dependent uterine growth in prepubertal rats, but had no effect on sex differentiation of rat brain (Bicknell et al., 1995). In adult male rats, chronic administration of 80 mg/kg octylphenol caused shrinkage of the testes and male accessory sex organs, spermatogenesis was disrupted, sperm deformities were seen and luteinizing hormone, follicle stimulating hormone, prolactin, and testosterone secretion were altered in these animals (Boockfor and Blake, 1997). In another study (Nagao et al., 2001), octylphenol caused significant delays in acquisition of puberty in male and female rats (50 mg/Kg octylphenol) and decreased relative prostate weight in males (12.5 mg/kg).

Alkylphenolic compounds bind to the estrogen receptor and elicit estrogenic responses (Soto et al., 1991; Jobling and Sumpter, 1993; White et al., 1994). Estrogenic effects are observed at tissue concentrations of 0.1 µM for octylphenol and 1 µM for nonylphenol (Soto et al., 1995). Octylphenol, stimulates vitellogenin gene expression in trout hepatocytes, estrogen receptor transcription in mammalian and chicken cells, and growth of breast cancer cells in cultured cell lines (White et al., 1994). In addition, they are potent inhibitors of sulfotransferase activity, probably leading to a reduction in the sulfation of endogenous estrogens (Harris et al., 2000).

Atrazine. The 2-chloro-*s*-triazine family of herbicides (Figure 1.2) is used in large amounts to control weeds, particularly on maize crops, in North America and Europe. It is relatively persistent to abiotic and biotic breakdown. Epidemiological and laboratory (female rats) studies have

associated long-term exposures to triazine herbicides with increased risk of ovarian and breast cancer (Sanderson and van den Berg, 2003). Numerous studies have investigated the effects of atrazine in gonadal abnormalities in frogs and in secondary sex characters (e.g. size of laryngeal dilator muscle), however, results are not consistent between studies and there is no consensus in the scientific community on whether exposure to atrazine is related to the abnormalities observed in the environment in frogs (e.g. Hayes et al., 2002; Carr et al., 2003; Coady et al., 2004).

Initially, investigations into the mechanism of this apparently estrogenic effect were directed towards the estrogen receptor. However, consistent interactions of triazine herbicides with the estrogen receptor or effects on receptor-mediated responses were never demonstrated. In contrast, several 2-chloro-*s*-triazine herbicides (atrazine, simazine, and propazine) and a number of their common metabolites (atrazine-desethyl and atrazine-desisopropyl) induced human aromatase activity and gene expression in vitro in H295R adrenocortical carcinoma cells (Sanderson and van den Berg, 2003).

Lindane. Lindane (Figure 1.2) is an organochlorine insecticide and fumigant mainly comprised of the gama-isomer of hexachlorocyclohexane (HCH) that has been used on a wide range of soil-dwelling and plant-eating insects. It is commonly used on a wide variety of crops, in warehouses, in public health to control insect-bone diseases, and as seed treatment. Lindane is also presently used in lotions, creams, shampoos for the control of lice and mites in humans (extoxnet, 1996; http://extoxnet.orst.edu/pips/lindane.htm). Lindane is absorbed through respiratory, digestive or cutaneous routes and accumulates in fat tissues.

Several studies have demonstrated that lindane disrupts the reproductive function in male and female animals. In male rats, chronic exposure to lindane markedly reduces serum testosterone levels, epididymal sperm counts, and sperm motility, whereas in guinea pigs, it damages seminiferous tubules and completely arrests spermatogenesis. Similarly, in female mice and rabbits, lindane reduces serum estrogen and progesterone levels, whereas in pregnant mice and minks, it decreases whelping rate and litter size (Walsh and Stocco, 2000). The decline in serum steroid levels likely contributes to the pathogenesis of lindane-induced infertility. Lindane blocks the conversion of cholesterol to pregnenolone (Sircar and Lahiri, 1990) without affecting P450scc, probably by reducing the expression of the StAR protein (Walsh and Stocco, 2000).

TBT. TBT (Figure 1.2) is an organotin compound used primarily as a biocide and as a biocidal preservative for wood, cotton, textiles, paper, and paints and stains for residential homes. TBT gained widespread application as an effective antifouling paint biocide on pleasure boats, large ships, and docks in the 1970s and 1980s. In the late 1970s, antifouling paints were found to cause detrimental environmental impacts. As TBT leaches directly from paints into water, high contamination of harbors and coastal areas resulted. The studies by Alzieu and co-workers (e.g. Alzieu, 1991) and Bryan and Gibbs (1991) showed the detrimental impact of this toxicant to oyster

and gastropods, and contributed to the adoption of regulatory standards for TBT in antifouling paints. The International Maritime Organization decided in autumn 2001 to ban the application of TBT-based paints on all boats by January 2003 and the presence on ship hulls by January 2008. Antifouling alternatives are needed in order to prevent an increase in fuel consumption and an associated increase in atmospheric emissions, as well as the transportation of invasive species (Abbott et al., 2000). Chemical substitutes of organotin compounds include copper and organic biocides (e.g. irgarol and diuron) but mechanical and biological (extracts of the natural repellents used for marine organisms as antifouling) are also being considered.

TPT has also been employed as a cotoxicant with TBT in some antifouling paints. However, the major employment of TPT compounds lies in agriculture, where they are used as fungicides in crop protection. TPT compounds enter the aquatic environment via leaching and runoff from agricultural fields, but it was also suggested that a proportion of TPT applied in agriculture is volatilized.
Organotin compounds are bioaccumulated in biota. They are not metabolized or slowly metabolized by organisms, although this varies among species. Of aquatic organisms, bivalves showed the highest accumulation of TBT and TPT, probably due to their low metabolic capacity.

Lethal concentrations of organotin compounds are around 1 µg TBT/L (Table 1.2). Histological studies have shown that the toxicity of organotin compounds is based on their eye-and skin-irritating activity, on their activity against the immune system, renal and neuronal tissues (Fent, 1996). Growth rate is reduced at lower concentrations (~0.1 µg/L), and imposex is the most sensitive response being already observed at concentrations as low as 1 ng TBT/g w.w (Bryan et al., 1987; Gibbs et al., 1988).

Organotins exert a number of important cellular, biochemical, and molecular effects (Table 1.2) that may lead to the physiological consequences described. Nevertheless, there is not a clear understanding yet, of the mechanisms of toxic and endocrine disruption action of organotins.

Fenarimol. Fenarimol [(ζ-(2-chlorophenyl)- ζ-(4-chlorophenyl)-5-pyrimidenemethanol)] (Figure 1.2) is a systemic fungicide widely used in the industrialized world within horticulture and agriculture, on a wide range of fruits, vegetables, hops, and wheat (WHO, 1995). It is rapidly adsorbed onto soil and sediments and is highly persistent, but not mobile in the environmental matrices (WHO, 1995). However, fenarimol bioaccumulates to a very limited degree.

Table 1.2. Toxicological effects of tributyltin. Data was extracted from Fent (1996) and ESI (1999), except otherwise indicated[1].

Organism	Concentration / Dose[2]	Effect
Algae, zooplankton, amphipods, mollusks, crustacean, fish	0.4 – 5.5 σg/L	Acute and chronic toxicity (LC50)
Bivalve mollusks, crustacean	0.12 -1.2 µg/g	Growth rate inhibition
Fish	0.09 to 0.5 µg/L	Growth rate inhibition
Fish	1 µg/g diet	Masculinization of females [1]
Fish	1 µg/g	Altered male sexual behaviour [2]
Fish	1.8 µg/g	Reduced gonadal development in male fish
Fish	0.1 ng/L	Male-biased population with low quality sperm [3]
Fish	11.2 ng/L	Decrease in sperm counts [4]
Several mollusk species	0.5 – 21 µg/g	Sterilization due to imposex
Several mollusk species	5 to 300 ng/g	Imposex
Several mollusk species	244 ng/g	Intersex [5]
Polychaete worm	1.2 µg/g	Significant reductions in fecundity and juvenile production
Pacific oyster	2.1 µg/g ovaries	Reduced rate of fertilization and development
Pacific oyster	2 ng/L	Shell thickening
Fish hepatoma cell line	10^{-7} M	Cytotoxicity
Thymocytes	10^{-7} M	Disturbance of Ca2+ homeostasis and induction of apoptosis
Rats, bacteria, chloroplasts	10^{-7} M	Inhibition of mithocondrial oxidative phosphorylation and ATP-synthesis
Fish gills; thymocytes; erythrocytes	10^{-7} M	Inhibition of ion pumps and cell membrane damage
Fish; rats (in vitro and in vivo)	10^{-5} M / 16 mg/kg	Inhibition of Cyt P450 system
Mammals, fish, molluscs	10^{-6} M / 24 ng/L	Alterations in steroid metabolism[3]
Mollusc RXR ligand binding domain	10^{-5} M	Binding to RXR [6]
Mollusc	10^{-9} M	Abnormal release of penis morphogenic factor [7]

[1] References: [1] Shimasaki et al., 2003; [2] Nakayama et al., 2004; [3] McAllister and Kime, 2003; [4] Haubruge et al., 2000; [5] Bauer et al., 1997; [6] Nishikawa et al., 2004; [7] Féral and Le Gall, 1983.

[2] Dose/concentrations of TBT are expressed in levels of TBT as cation versus fresh body weight. The following conversion factors have been used in order to homogenize the units at which concentration/dose data were expressed in the different studies: wet weight = 5 x dry weight (Stephan et al., 1985); TBT(g): 290 / 325.5 TBTCl(g); TBT(g): (290 x 2) / 596.1TBTO(g); TBT(g): 290 / 119 Sn(g).

[3]Further details will be included in section 1.5.4.

The effects of fenarimol are summarized in Table 1.3. Fenarimol has shown estrogenic, androgenic and antiandrogenic properties *in vitro*; and inhibits P450 aromatase activity as well as other enzymes of the CYP gene family involved in the metabolism of steroids. The *in vivo* effects of fenarimol include reproductive, teratogenic, and oncogenic effects in experimental animals. The alterations observed in the reproductive parameters in rats are characteristic of antiandrogenic compounds.

Table 1.3. Toxicological effects of fenarimol

Species (experimental design)	Dose/ Concentration	Effect	Ref
Intact and castrated testosterone-treated male rats*	200 mg/kg	Reduced weight of seminal vesicles, musc. levator ani/bulbocavernous, and bulbourethral glands	Vinggaard et al., 2005
Castrated testosterone-treated male rats*	200 mg/kg	Serum T4 level decreased	Vinggaard et al., 2005
Microsomes of Sprague-Dawley rats exposed in vivo	200mg/Kg	Induction and decreases of a range of CYP-dependent reactions	Paolini et al., 1996
MCF7 cell proliferation assay	3 σM 5 σM	Estrogenic activity (IC50) Estrogenic activity (LOEC)	Vinggaard et al., 1999 Andersen et al., 2002
MCF-7 cells	84 and 85 σM 151 and 212 σM	ERζ and ERη mRNA (LOEC) Decrease of estrogen-like effects on the ERζ#mRNA level	Grünfeld and Bonefeld-Jorgersen, 2004
ER-transactivation assay	5 σM	Estrogenic activity (LOEC)	Andersen et al., 2002
Yeast estrogen screen	13 σM	Estrogenic activity (IC50)	Vinggaard et al., 1999; Andersen et al., 2002
AR-transactivation assay	10 σM	Androgenic activity (LOEC)	Andersen et al., 2002
AR reporter gene assay in CHO cells	19 σM	Androgen receptor inhibition (IC50)	Vinggaard et al., 2005
Rats (in vivo)	350 mg/kg (diet)	Inhibition of aromatase	Hirsch et al., 1987
Rats (microsomes)	4.1 σM	Inhibition of aromatase (IC50)	Hirsch et al., 1987
Human placental microsomes	10 σM 50 σM	Inhibition of aromatase Inhibition of aromatase (83% inhibition)	Vinggaard et al., 2000 Andersen et al., 2002
JEG-3 human choriocarcinoma cells	2 σM	Inhibition of aromatase (IC50)	Vinggaard et al., 2000
Fungi	3 σM	Inhibition of ergosterol synthesis by blocking sterol C-14 demethylation	Henry and Sisler, 1984

*Hershberger assay

1.4 Endocrine disruption escapes from classical toxicology

After a decade of intensive research efforts, endocrine disruption has proved to be a very complex matter to evaluate and to manage, due to several intrinsic characteristics, which differ from classical toxicology. Some of these aspects will be pointed out in this section.

Atypical dose-response relationships. Toxicological research and policies are based on the principle that dose makes the poison, introduced by Paracelsius (1491-1541). Thus, regulatory toxicology is based on the establishment of monotonic dose-effect curves to determine thresholds (or acceptable risks for non-threshold chemicals such as mutagenic compounds) followed by the application of safety factors and usually has different considerations for short and long term exposures. Nevertheless, when it concerns endocrine disruptors, low-dose effects and transient exposure can be as dangerous as higher doses and prolonged exposure. One of the reasons is that EDs often act by mimicking or antagonizing the actions of naturally occurring hormones, which are already at physiologically functional concentrations. In addition, the increasing exposure to some EDs swamps the endocrine system and prevents or reduces dysfunction (i.e. an inverted U dose-response), while other EDs exhibit effects at both high- and low- doses, but not in between (i.e. a U- or J-shaped dose-response) (e.g. Alsmtrup et al., 2002). In classical toxicology low doses are rarely included in the experimental design, and the expected effects at those low doses is extrapolated from those observed at the high doses. The finding that endocrine disruptors can be effective at very low doses (e.g. Melnick et al., 2002) and that those effects can not be inferred from the effects of higher doses by using monotonic dose-effect curves, challenges both the toxicological designs (which are forced to increase the experimental doses) and the exposure assessment.

Critical windows of exposure and complexity of feedback mechanisms. Timing of exposure is also critical to understanding the effects of EDs. Exposure to EDs during the period when “programming” of the endocrine system is in progress may result in a permanent change of function or sensitivity to stimulatory/inhibitory signals. For example, endocrine disruption of the developing brain can permanently alter behavior, whereas similar exposures to a fully differentiated brain could have no effect. Exposure to the same level of an endocrine signal during different life history stages or during different seasons may produce different effects (e.g., effects in wildlife are critical during the breeding season). The complexity of the endocrine system, with numerous feedback mechanisms and environmental signals that might interfere with it, hampers the development of predictive biomarkers, slows down the scientific understanding of the cause-effect relationships, and might be responsible for the non-linear dose-response relationships and for the conflicts in the scientific community raised because of the inability to replicate the findings of some studies. In addition, because of cross talk between different components of the endocrine system, effects may occur unpredictably in endocrine target tissues other than those predicted to be affected (WHO, 2002). Thus, for most associations reported between exposure to EDs and a

variety of biologic outcomes, the mechanism(s) of action are poorly understood. This makes it difficult to distinguish between direct and indirect effects and primary versus secondary effects of exposure to EDs.

Causality is difficult to be established by classical epidemiological studies. Epidemiological studies have frequently failed to find causal associations in the field of endocrine disruption. Several factors explain why elucidating the presence/absence of such causality relations is not a straightforward process. First of all, environmental endocrine disruptors are ubiquitous, and therefore epidemiologists face difficulties in defining control and exposed groups. Second of all, they have usually attempted to relate exposure to endocrine disruptors to diseases of multifactorial causes, transgenerational, and usually of delayed appearance. However, causative agents might not be present any longer in the tissues when the disorders become manifest. Finally, there is almost no information on the combined effect of chemical mixtures (e.g., synergy, additivity, or antagonism) to which the human population is widely exposed. Therefore, the regulations need to be set under the umbrella of the precautionary principle and not the epistemological evidence.

Multiple mechanisms of action require multiple screening systems. The screening of the potential endocrine disruptors is extremely complex and expensive due to the multiple mechanisms by which endocrine disruptors can exert their effects. *In vitro* screening systems have been developed although usually they only provide information for a specific mode of action, e.g. the estrogen-receptor binding assays provides information on the binding affinity of xenobiotics for the estrogen receptor, but not on other mechanisms of action (further details in section 1.5). *In vivo* systems, are more integrative, the uterotrophic assay for (anti)estrogenicity, and the Hershberger assay for (anti)androgenicity, and the so-called “enhanced subacute test” (TG 407) for (anti)estrogenicity, (anti)androgenicity, and (anti)thyroid effects, have been developed by the OECD and are currently undergoing validation studies (Gelbke et al., 2004). However, these *in vivo* studies might not include the critical windows of exposure to ED. It is believed that ED can act during the development and affect the hormonal homeostasis in critical periods, and that the alterations in the endocrine function can be manifested in any organ at any time of the life of the individual. Although the definition of such critical windows of exposure and the establishment of the critical lenght of the tests is still under debate, two generation studies are undertaken in order to encompass all critical life stages and processes in the testing of endocrine disruptors. Finally, the selection of the endpoints that will be assessed in these tests is also complex, because ED might induce several possible dysfunctions: e.g. cancer susceptibility, birth defects, and many subtle effects such as immunological dysfunction, suppression of secondary sex characteristics, decreased fertility, increased aggression, decreased mental capacity and focus, disrupted brain development, etc... The need for multiple *in vitro* and *in vivo* studies leads to important cost and time limitations to assess the extensive list of compounds considered as potential endocrine disruptors.

1.5 Potential pathways of endocrine disruption

Steroid hormones are synthesized in the gonadal cells. Hormones are then secreted into the blood and become available to the cell through diffusion or may be transported bound to sex-hormone binding globulin (SHBG). The free steroid hormone diffuses into the perinuclear region of the cell, where unoccupied receptors are located. In some cases, the chemical secreted into the blood is a prohormone that is metabolized in the cell to the active hormone. For example, in some tissues, testosterone is metabolized by aromatase to E2, whereas in others the enzyme 5Ŭ-reductase converts it to DHT. The hormone binds the receptor, which undergoes a conformational change, exposing key protein binding sites, and forms homodimers. The homodimers accumulate transcriptional factors (tf), forming a transcriptional complex, which binds to specific sequences on the DNA of hormone-dependent genes, known as hormone response elements (HRE). The transcriptional complex then initiates mRNA synthesis (mRNA), which is transported out of the cell into the cytoplasm. In association with amino acids bound to specific tRNAs and ribosomes, proteins are synthesized from the mRNA template. In addition, steroids can bind the membrane receptors and initiate intracellular signalling cascades, known as steroid non-genomic actions.

The complexity or this hormone action cascade enables several possible targets and mechanisms of action of environmental contaminants (Figure 1.3). EDs can bind to the steroid hormone receptors and either mimic (i.e. estrogenic or androgenic compounds) or antagonize (i.e. antiestrogenic or antiandrogenic compounds) the effect of endogenous hormones; interfere with sex steroid binding proteins; modify hormone receptor levels; and alter the pattern of synthesis and metabolism of endogenous hormones (Danzo et al., 1997; Sonnenschein and Soto, 1998). In addition, xenobiotics can interfere with the non-genomic responses of steroid hormones (Bulayeva and Watson, 2004).

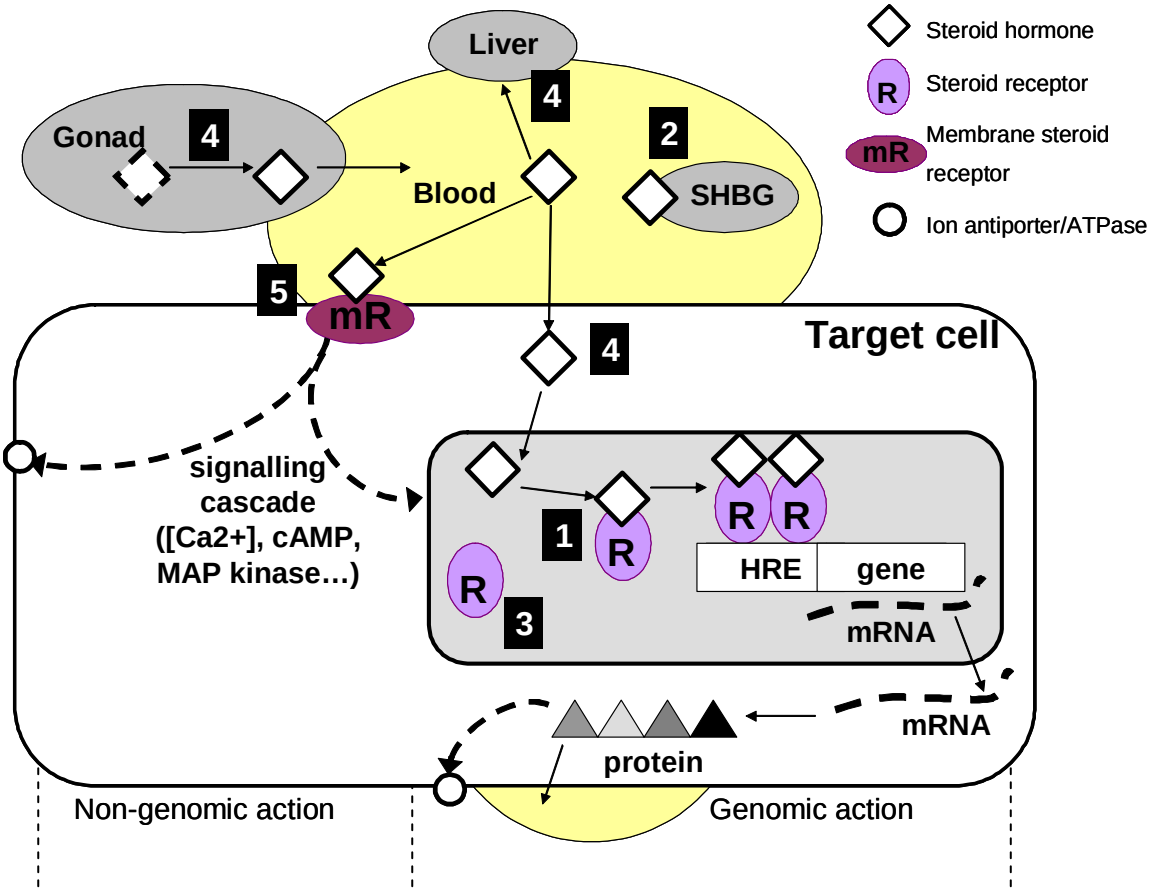


Figure 1.3. Action of steroid hormones and possible mechanisms of action of endocrine disruptors: 1) Binding to steroid receptors; 2) Binding to steroid human binding globulin; 3) Alteration of steroid receptor levels; 4) Alterations in the synthesis or metabolism (by the target cell or the liver); 5) Interaction with the non-genomic action of steroid hormones. Adapted from WHO (2002) and Falkenstein et al. (2000).

1.5.1 Binding to steroid receptors

Hormones elicit genomic responses through direct interactions with nuclear receptors. Nuclear receptors regulate gene transcription in a ligand-dependent manner through their interaction with specific DNA sequences (response elements).

A large number of environmental agents and phytochemicals interact with steroid hormone nuclear receptors either as agonists or antagonists. Hydroxylated PCBs, DDT, alkylphenols, bisphenol A, methoxychlor, and chlordecone, as well as phytoestrogens such as coumestrol or genistein, compete with 17η-estradiol (E2) binding to the estrogen receptor (ER). Other chemicals, vinclozolin and the DDT metabolite p,p'-DDE, act as antiandrogens by binding the androgen receptor and blocking testosterone-induced cellular responses (Kelce et al., 1994, 1995). Complexity increases

when EDs bind to more than one type of receptor. E.g. nonylphenol and the metabolite of methoxychlor, 2,2-bis[p-hydroxyphenyl]-1,1,1-trichloroethane (HPTE), have the ability to inhibit binding of estrogen, progesterone, and androgen to their receptors with similar affinities (Laws et al., 1995).

1.5.2 Interference with sex steroid binding proteins

In most vertebrate species, sex steroid hormones circulate bound to a specific high affinity sex steroid-binding protein (SHBG) and low affinity proteins such as corticosteroid-binding proteins and albumin. In the blood, typically 97–99% of the estrogens and androgens are bound to these proteins (Rosner, 1990).

Xenobiotics can bind to the sex steroid binding proteins and modulate the bioavailability of endogenous steroids. Thus, synthetic estrogens (e.g. EE2, DES) and industrial chemicals (e.g. octylphenol, nonylphenol, bisphenol A) are able to displace endogenous sex steroids from the sex steroid-binding site of the SHBG (Danzo et al., 1997; Tollefsen, 2002). The binding affinity of SHBG for industrial compounds is about 10^3 to 10^6 lower than for endogenous sex steroids (Déchaud et al., 1999; Tollefsen, 2002), nevertheless if xenobiotics are present at high enough concentrations they can displace endogenous sex steroid hormones from SHBG binding sites as shown by Déchaud et al. (1999).

1.5.3 Alteration of receptor levels

Steroid hormone receptor activation can be modified by indirect mechanisms such as a down-regulation of the receptor as seen after TCDD exposure (Safe et al., 1991, Safe and Krishnan, 1995). TCDD does not inhibit the ER synthesis at the transcriptional level, but enhances the expression (via xenobiotic response element, XRE) of enzymes that degrade the ER.

The inhibition of ER binding to the estrogen response element (ERE) is another mechanism for the anti-estrogenic effect of TCDD, PCBs, and PAHs. These compounds bind to the arylhydrocarbon receptor (AhR), which thereafter binds to sequences adjacent or overlapping to the ERE that are similar to the XRE (Klinge et al., 1999). Thus, binding of xenobiotics to the AhR may inhibit ER transactivation by a competition of the binding between AhR-agonist/AhR and E2/ER to DNA, and alter the levels of functional steroid receptors. This type of interaction may provide an explanation for the antiestrogenic activity of AhR agonists (Ramamoorthy et al, 1999; Navas and Segner, 2000).

1.5.4 Changes in hormone biosynthesis and metabolism

Most metabolic pathways can act upon both exogenous and endogenous compounds, including steroid hormones. Xenobiotics can regulate P450 isozyme expression (e.g., induction or inhibition

of gene expression) and/or can cause reversible (e.g. acting as substrates) or irreversible inhibition of reductases and phase II enzyme systems, leading to changes in the metabolism of endogenous steroids (You, 2004). In addition, other less direct effects on steroidogenic enzyme activities, such as modulation by the hypothalamic-pituitary-gonadal axis, may also occur (Sanderson and van der Berg, 2003).

Various methods are available to investigate effects on steroidogenic enzymes, starting with the choice of a biological system. This can be the simple isolated enzyme, if available, or a microsomal fraction of tissues that express the enzyme of interest. On an increasingly more complex level, cell lines, primary cells in culture, tissues slices, or whole animals may be used. To answer the straightforward question on whether a compound can inhibit a specific enzymatic reaction, simple systems, such as purified enzyme, microsomes, or cell lines, may suffice. For questions regarding the effects of chemicals on the expression of steroidogenic enzymes, more complex systems are required. Cell lines and primary cultures may provide information on intracellular regulation, co-cultures may shed light on intra and intercellular regulation, and *in vivo* studies will be necessary to investigate possible effects of chemicals on steroidogenesis by affecting the hypothalamic-pituitary-gonadal/adrenocortical axes (Sanderson and van der Berg, 2003).

Several studies have assessed the ability of EDs to alter steroid hormone metabolism. Table 1.4 presents a brief overview of such studies. Organotin compounds inhibited steroid biosynthesis and metabolism in a variety of organisms *in vitro*, except when cell lines were used, where an induction of different enzymes was observed. *In vivo* studies were only found for molluscs and showed decreases of several steroid metabolizing enzymes. Plasticizers and surfactants, such as bisphenol A, phthalates and alkylphenolic compounds inhibit some steroidogenic pathways, evidenced in studies with purified enzymes or cell fractions, but some of them, possibly through interactions with the hypothalamic-pituitary-gonadal axis, also were able to induce key enzymes, such as aromatase or 5ζ-reductase. Other industrial chemicals, such as PAHs, PCBs, and pharmaceuticals, such as DES, lead to inhibition of certain steroidogenic pathways. A whole range of responses are observed in animals exposed to pesticides. Thus, triazine pesticides (e.g. atrazine) induce aromatase activity, whereas imidazole fungicides decrease aromatase activity in vertebrate cell lines.

In addition to the data presented in Table 1.4, numerous studies have reported that exposure to some xenobiotics led to alterations in sex steroid levels. It is likely that most of these xenobiotics acted upon steroidogenic or steroid metabolic pathways, and consequently altered steroid levels.

Table 1.4. Steroid metabolic pathways affected by environmental agents.[1]

In vitro

Compound/ Exposure	Organism used[2]	Metabolic pathway and effect		Ref
Organotins (TBT, TPT and/or DBT)	Human arom + P450 reductase supersomes	P450-arom	Ć	Cooke, 2002
	Human choriocarcinoma cell lines	P450-arom	ą	Nakanishi et al., 2002
	Human: adrenal / testis / placenta /liver / prostate /brain	3η-HSD2, 17η-HSD3, 17η-HSD1 DHEA-SULT, P450-arom, 5ζ-R	Ć	Heidrich et al., 2001; Doering et al., 2002; Lo et al., 2003; Alléra et al., 2004
	Rat: testis	3η-HSD, 17-hydroxylase	Ć	McVey and Cooke, 2003
	Fish: testis / liver	5ζ-R, T-UGT, E2-UGT, E2-SULT	Ć	Thibaut and Porte, 2004
	Mollusc: gonad+digestive gland	P450-arom, 17η-HSD	Ć	Morcillo et al., 1998; LeCurieuxBelfond et al., 2001
PAHs	Fish: ovary	P450-17,20-lyase	Ć	Rocha Monteiro et al., 2000
OH-PAHs, OH-PCBs	Human SULT1E1	E2-SULT	Ć	Kester et al., 2000; Kester et al., 2002
Aroclor 1254	Mammal/Ave/Reptile: adrenal glands	Progesterone 21-hydroxylation	Ć	Goldman and Yawetz, 1991
p,p'-DDE	Human: placenta / prostate	P450-arom, 5ζ-R	Ć	Alléra et al., 2004
chlordecone, 4-NP, OH-PCB	Fish: testis	11-kT synthesis	Ć	Loomis and Thomas, 2000
Dicofol	Fish: testis / liver	17η-HSD, 5ζ-R	Ć	Thibaut and Porte, 2004
	Fish: ovary	20η-HSD	ą	
Alkylphenols	Fish: liver	E1-SULT	Ć	Kirk et al., 2003
Nonylphenol	Fish: testis	E2-UGT, T-UGT, E2-SULT	Ć	Thibaut and Porte, 2004
DEHP, DBP	Fish: testis	5ζ-R	Ć	Thibaut and Porte, 2004
DES	Fish: testis	11-kT synthesis	Ć	Loomis and Thomas, 2000
Imidazole fungicides	H295R Human adrenocortical carcinoma cells	P450-arom	Ć	Sanderson et al., 2002
Vinclozolin / atrazine /Zimazine/ propazine		P450-arom	ą	Sanderson et al., 2000; 2002
Atrazine	Fish: gonads	5ζ-R, 20ζ-HSD	Ć	Thibaut and Porte, 2004
	GST-TS Turtle cell lines	P450-arom	ą	Keller and McClellan-Green, 2004
Letrozole	Human: placenta	P450-arom	Ć	Alléra et al., 2004
Fenarimol	Human: placenta / prostate / liver	P450-arom, 5ζ-R; DHEA-SULT	Ć	Alléra et al., 2004
	Fish: testis	E2-UGT, T-UGT	Ć	Thibaut and Porte, 2004

Prochloraz	Human: placenta / prostate / liver	P450-arom, 5ζ-R, DHEA-SULT	Ć	Alléra et al., 2004
Roundup / Lindane/ Dimethoate	Mouse MA-10 Leydig tumor cell line	StAR	Ć	Walsh and Stocco, 2000; Walsh et al., 2000a; 2000b
Lindane / Malathion	Rat: prostate	5ζ-R	Ć	Simic et al., 1992.
Cadmium	Porcine granulosa cell line	P450scc	ą	Henson and Chedrese, 2004
Manganese	Primary Leydig cells	StAR, P450scc, 3η-HSD	Ć	Cheng et al., 2003

In vivo

Compound/ Exposure	Species used[2]	Metabolic pathway and effect		Ref
TBT/organotin polluted sites	Crustacea: whole body	17η-HSD, metabolic androgenization[4]	ą	Verslycke et al., 2003
		T-SULT	Ć	
	Mollusc: digestive gland	P450-arom	Ć	Morcillo et al., 1998; 1999; Santos et al., 2002
	Mollusc: visceral coil	ATAT, T-SULT, Metabolic androgenization[4]	Ć	Gooding et al., 2003; Ronis and Mason, 1996; Oberdörster et al., 1998
Bisphenol A	Fish: brain	P450-arom mRNA	ą	Kishida et al., 2001
Nonylphenol polyethoxylate	Crustacea: whole body	T UDP, T-SULT	Ć	Baldwin et al., 1998
Octylphenol	Rats: testis	P450c17 mRNA / protein	Ć	Majdic et al., 1996
DBP	Rats: testis	StAR, P450scc, 3b-HSD, P450c17 mRNA and protein levels	Ć	Lehmann et al., 2004; Thompson et al., 2004
DEHP	Rat: testis	5ζ-R; T 16ζ and 6η-hydroxylases	ą	Kim et al., 2003 Akingbemi et al., 2001
		P450-arom mRNA; 17η-HSD	Ć	
TCDD	Mouse: tesis	P450scc mRNA / protein	Ć	Fukuzawa et al., 2004
PCP	Rat: liver	DHEA-SULT	Ć	Boles and Klaassen, 1998
DES	Fish: brain	P450-arom mRNA	ą	Kishida et al., 2001
	Mouse: testis	StAR protein; P450c17	Ć	Guyot et al., 2004
Aldrin	Rats	3η-HSD, 17η-HSD	Ć	Chatterjee et al., 1988
Endosulfan	Mice: liver	T hydroxylases	ą	Wilson and LeBlanc, 1998
Malathion	Crustacea: whole body	T hydroxylases	Ć	Baldwin and LeBlanc, 1994a
Contaminated lakes	Reptile: liver	T hydroxylases, oxidoreductases, UDP-glucuronyl transferase	[5]	Gunderson et al., 2001
Cadmium	Rats: testis	StAR protein	Ć	Gunnarsson et al., 2004

[1]Studies that did not use steroids as the metabolic substrate when assessing the effects of xenobiotics have not been included, despite steroids being possible substrates for the studied enzymes. See acronim list for full names.

[3]Tissue used is specified, otherwise metabolism was assessed *in vivo*.

[4]Metabolic androgenization is defined as the ratio between reduced and dehydrogenated metabolites (which are preferentially retained in the organisms), and hydroxylated and conjugated metabolites (which are preferentially eliminated) (Baldwin et al., 1998).

[5]Altered sexual dimorphism

1.5.5 Interactions with the non-genomic action of steroid hormones.

Increasing literature is underlying the importance of the rapid, non-genomic action of steroids (reviewed in Falkenstein et al., 2000). The exact pathways for this form of steroid signaling are not fully elucidated, and few studies have reported on the ability of xenobiotics to mediate nongenomic steroid actions. Buleyava and Watson (2004) demonstrated that several xenoestrogens (i.e. dieldrin, endosulfan, DDE, and *p*-nonylphenol) can rapidly activate extracellular-regulated kinases (ERKs) in the pituitary tumor cell line GH3/B6/F10, which expresses high levels of the membrane receptor for ER-Ŭ (mER). Similarly, Canesi et al. (2004) showed that DES, bisphenol A, and nonylphenol affected the phosphorylation state of signal transducers and activators of transcription suggesting that these chemicals may lead to changes in gene expression as a consequence of the modulation of kinases/phosphatases.

1.6 The endocrine system in invertebrates

Hormonal regulation of biological functions is common to both vertebrates and invertebrates. While a basic endocrine strategy to regulate biological processes has been widely conserved, individual components intrinsic to the endocrine system have undergone significant evolutionary divergence resulting in distinct strategies of the endocrine systems of various taxa. Evolutionary biologists generally agree that animals diverged into two discrete lineages, protostomes and deuterostomes, during early evolution of the animal kingdom. Accordingly, significant divergences in endocrine strategies would be expected between deuterostomes (vertebrates and echinoderms) and protostomes (most invertebrate groups). Furthermore, through evolution, invertebrate species have developed a huge diversity of life histories and hence a multitude of unique approaches to growth, development and reproduction. These approaches include processes of metamorphosis, diapause, and pupation, which are life history traits not evident in vertebrates (LeBlanc et al., 1999).

In general, the available information on invertebrate endocrine systems is more limited than that on vertebrates, and has resulted from two opposite approaches: a classical approach, which starts from a biological function and a putative endocrine gland, and results in the isolation and identification of a hormone; and a reverse approach, which takes profit of the classical work performed in vertebrates to investigate the presence of similar molecules in invertebrates; then what has to be determined is the source of such substances and their biological functions (Lafont, 2000). The classic approach has advanced in the characterization of neuropeptide signaling, and of ecdysone and juvenile hormone, which are important hormones in a number of invertebrate phyla. On the other hand, the reverse approach has focused mainly on sex steroid hormones. This is reflected in Table 1.5, which presents a list of hormones identified in molluscs, crustaceans, and echinoderms, together with their source and their possible function. As it can be observed in this table, the information that we have on the synthesis and role of steroid hormones is much more limited than that for peptide hormones, which seem to be the most common hormones in invertebrates (Lafont, 2000).

Table 1.5 Hormones in crustaceans, molluscs and echinoderms. Data was extracted from LeBlanc et al. (1999) and Barrington et al. (1996).

Hormone	***Neurosecretory body***	***Controlled functions***
	Molluscs	
Peptide hormones		
Egg-laying hormone	Abdominal ganglia	Gonad maturation, egg mass production and egg-laying behaviour
Dorsal body hormone(s)	Light-green cells (caudo-dorsal cells) of the cerebral ganglion	Development of female accessory sex organs, gonad maturation and ovulation
Molluscan insulin-like peptides	CNS	Growth, development and metabolism
FMRFamide	Nervous system [1]	Regulation of heartbeat
APGWamide	Cerebral ganglia	Male sexual behaviour [2]
Somatostatin-like[1]	Unknown	Growth
Vasotocin- and vasopressin-like[1]	Unknown	Fluid balance
Gonadotropin hormone	Dorsal bodies [3]	Control of reproduction and brain functions [4]
Terpenoids	Unknown	Questionable role
Steroids		
Sex steroids	Unknown	Reproduction in prosobranchs
Ecdysteroids	Unknown	Questionable role
	Echinoderms	

Peptide hormones		
Gonad stimulating substance	Radial nerve cords	Stimulation of spawning
1-Methyl adenine	Gonads	Maturating promoting factor
Maturating promoting factor	Unknown	Oocyte germinal vesicle breakdown
Steroids		
Progesterone	Pyloric caeca and gonads	Reproduction
Testosterone	Pyloric caeca and gonads	Reproduction
Estradiol	Pyloric caeca and gonads	Stimulation of vitellogenesis and ovarian growth
Crustaceans		
Peptide hormones		
Molt inhibiting hormone	Eyestalk	Regulation of the synthesis of ecdysteroids
Vitellogenesis inhibiting hormona	Eyestalk	Inhibition of vitellogenesis
Mandibular organ inhibiting hormona	Sinus gland	Inhibition of the secretion of methyl farnesoate
Androgenic hormone	Androgenic gland (terminal region of male gamete ducts)	Sexual differentiation Inhibition of vitellogenesis
Retinal pigment hormones	Eyestalk	Control of coloration
Retinoic acid	Unknown	Limb regeneration
Limb autotomy factor	Eyestalk	Regeneration and molting
Methyl farnesoate	Mandibular organ	Stimulation of reproduction Molt cycle
Terpenoids		
Juvenile hormone	Unknown	Questionable role
Steroids		
Ecdysteroids	Y-organ	Molting; Vitellogenesis; Embryo development
Testosterone	Unknown	Sex differentiation
Progesterone	Unknown	Maturation and spawning
Estradiol	Unknown	Questionable role

References: [1] Croll et al., 2003; [2] de Lange and Van Minnen, 1998; [3] Lafont, 2000; [4] Iwakoshi-Ukena et al., 2004

1.6.1 Neuropeptides

Neuropeptides act on membrane receptors and therefore stimulate various types of second messengers within the cell. Invertebrates are likely to have much in common with vertebrates in what relates to peptide hormones (Barrington, 1986). Thus, positive results have been obtained for a wide range of vertebrate-like peptide material in the alimentary tract and cerebral ganglia of the ascidian *Ciona*. Cells in the alimentary epithelium of Branchiostoma (amphioxus) react to antisera against a number of mammalian peptides, including insulin, glucagon, pancreatic polypeptide, somatostatin, secretin, vasoactive intestinal polypeptide, pentagastrin and neurotensin, and show specific distribution patterns. Monoaminergic and peptidergic neurons have been identified, while a central neurohaemal area is thought to be comparable to the median eminence and neurohypophysis of vertebrates. There is ample evidence that many vertebrate neurotransmitters are also active in insects (the invertebrates that have been most closely studied). E.g. acetylcholine, glutamic acid, somatostatin, insulin, glucagon and gastrin. Even in molluscs, immunoreactive somatostatin was found in two species of pond snail and has been thought to be a growth factor, but it does not seem to be chemically identical to synthetic somatostatin. Again, the ganglia of the mollusc *Aplysia* contain a neurohypophysial peptide-like material which resembles vasotocin and vasopressin, but is actually neither. It is suggested, although quite hypothetically, that this material might act as a neurotransmitter or neurohormone in the regulation of fluid balance (Barrington, 1986).

Strong evidence for insulin-like material in a number of invertebrates does exist. The function of insulin-like materials in invertebrates remains to be discovered. They have been thought to be involved in the regulation of carbohydrate metabolism in bivalve molluscs, but probably they do not have a glucostatic role in the lobster *Homarus americanus* (Barrington, 1986).

In addition, to the vertebrate-like peptide hormones, a series of peptide hormones exist, regulating functions specific for some invertebrates (e.g. the molt inhibiting hormone in crustaceans). Some of the peptidic hormones reported in invertebrates are summarized in Table 1.5.

Finally, it is worth mentioning, that one of the characteristics of peptide hormones, in contrast to other classes of hormones, is their interspecific variation. Thus extraction and characterization of potent hyperglycaemic hormones from several crustaceans have shown them to be peptides with 50-58 residues. Cross-reaction studies have shown in this case that the receptors have evolved side by side with the hormones (Barrington, 1986).

1.6.2 Ecdysteroids and juvenoids

Ecdysteroids are the molting hormones of insects and crustaceans. Ecdysone is secreted by the Y-organ and rapidly hydroxylated to 20-hydroxyecdysone by several tissues. In addition to the regulation of molting, ecdysteroids have been implicated in vitellogenesis (LeBlanc et al., 1999).

The juvenile hormone (JH) regulates metamorphosis and reproduction in insects. Current data indicates that JH is probably not present in crustaceans. It appears that the precursor to the insect JH, methyl farnesoate may be acting as the crustacean JH. Methyl farnesoate seems to have a permissive or stimulatory effect upon reproduction in both female and male crustaceans.
Ecdysteroids and juvenoid hormones have been reported in a few mollusc species but remain unconfirmed in the majority of molluscs and in echinoderms (LeBlanc et al., 1999).

1.6.3 Sex-steroid hormones

There is no doubt that vertebrate-like sex steroids, e.g. testosterone, androstenedione, and estradiol, occur in several groups of invertebrates (Table 1.6). However, occurrence is not enough to conclude that sex steroids function as hormones in invertebrates. First, those molecules are widespread. Many plant species contain vertebrate-like sex steroids (Milanesi et al., 2001) and it was initially thought that sex steroids in invertebrates were taken up via the diet. However, several studies have demonstrated that some groups of invertebrates are able to synthesize sex steroids from precursors such as cholesterol or pregnenolone (further details are given in section 1.7.3).

In vertebrates, sex steroids act via genomic (binding to steroid hormone receptors) and non-genomic processes. Nuclear receptors are found in vertebrates and invertebrates. Genetic and phylogenetic analyses suggest that vertebrate steroid receptors arose in a deuterostome (Escriva et al 1997, 2000; Baker 1997, 2001a) and evolved from an ancestor steroid receptor gene, which would be similar to an estrogen receptor (Baker, 2003). This is supported by the fact that no androgen- or progestin-like receptors have been found in invertebrates (Baker, 2003), and by the recent identification of an estrogen-like receptor in the mollusc *Aplysia californica* reported by Thornton et al. (2003). The isolation of this estrogen receptor ortholog indicates that steroid receptors are extremely ancient and widespread, and that, except for ecdysozoans, many other invertebrates also have steroid receptors resembling that of estradiol. *Aplysia californica* estrogen receptor did not specifically bind estradiol, and its ligand binding domain activated transcription constitutively (while human ERζ-ligand binding domain requires the presence of estrogens to activate expression). However, the authors predicted that the loss of estrogen-dependent activation is recent and unique to the estrogen receptors of the Opisthobranchs (Thornthon et al., 2003).

Table 1.6. Sex steroid unequivocally identified in invertebrate species.

Specie	Matrix	Method[1]	Steroid	Ref
Mytilus edulis	Whole animal	GC-MS (EI)	Androstenedione, testosterone, progesterone, estradiol, estrone	Reis-Henriques et al., 1990
Astacus leptodactylus	Hemolymph	GC-MS (NCI)	Pregnenolone, 17ζ-hydroxypregnenolone, testosterone, cholesterol and 6η-hydroxyprogesterone	Ollevier et al., 1986
Astacus leptodactylus	Hemolymph	GC-MS (NCI; SIM)	Androstenedione, 5ζ-dihydrotestosterone, 11-ketotestosterone, 11η-hydroxytestosterone	Ollevier et al., 1986
Sarcophaga bullata	Hemolymph	GC-MS (NCI)	Testosterone, progesterone	De Clerck et al., 1983
Mya arenaria	Gonad	LC-MS/MS (ESI; PI)	Progesterone	Siah et al., 2002
Neomysis integer	Whole animal	LC-MS (APCI; PI)	Testosterone, androstenedione	Verslycke et al., 2002
Arion ater rufus	Eggs	GC-UV TLC-UV[2]	11-Ketotestosterone, testosterone, 17ζ-hydroxyprogesterone, cortisone	Gottfried and Lusis, 1966
Asterias rubens	Pyloric caeca	GC-MS (EI; SIM)	Estradiol	Voogt et al., 1992
Helix aspersa	Gonad	GC-MS (EI)	Dehydroepiandrosterone, estradiol, estriol	Le Guellec et al., 1987
Helix aspersa	Gonad	GC-MS (EI; SIM)	Androsterone, androstenedione, 3ζ-androstanediol, estrone	Le Guellec et al., 1987
Nephrops norvegicus	Ovary	GC-MS (EI; SIM)	5ζ-Dihydrotestosterone, testosterone, pregnenolone, 20ζ-hydroxypregn-4-en-3-one	Fairs et al., 1989
Nephrops norvegicus	Eggs and haemolymph	GC-MS (EI; SIM)	Estradiol	Fairs et al., 1989
Arion ater rufus	Spermatheca gland	GC-UV[2]	Estrone and estradiol	Gottfried et al., 1967

[1] EI: electron impact mode; NCI: negative ion chemical ionization; ESI: electrospray ionization; APCI: atmospheric pressure chemical ionization; PI: positive ion mode; NI: negative ion mode ; SIM : selected ion monitoring. MS analyses were performed in full scan except otherwise indicated.
[2]Several systems and derivatizations were used for identification

Despite the limited genetic evidence for steroid receptors in invertebrates, binding proteins for vertebrate steroids have been described in several invertebrates, including nematodes, echinoderms, crustaceans, and molluscs (Kiser et al 1986; de Waal et al 1982; Paesen and De Loof 1989; Di Cosmo et al., 1998, 2002; Keshan and Ray, 2001; Stenberg and LeBlanc, personal communication). However, it has not yet been demonstrated that this binding is coupled to a biological response.

Recent studies have shown that estradiol can act through nongenomic mechanisms in molluscs. Nongenomic action of steroid hormones gene transcription is regulated through cell surface receptors in association with steroid hormone-binding proteins located in the blood, using signal transduction pathways analogous to those used by peptide hormones. In contrast to genomic action, nongenomic steroid effects are principally characterized by their insensitivity to inhibitors of transcription and protein synthesis, and by their rapid onset of action (within seconds to minutes). These rapid effects may be initiated at either membrane or cytosolic locations and can result in both direct local effects (such as modification of ion fluxes) and regulation of gene transcription secondary to activation of kinase cascades (involvind cAMP, MAPKs, PKC and PKA, PI-3K, etc.) (Falkenstein et al., 2000). This nongenomic steroid action is likely to be mediated through receptors with pharmacological properties distinct from those of the intracellular steroid receptors. Elicited responses depend upon the cell type studied and the conditions used; however, rapid changes in phosphorylation state of mitogen activated protein kinases (MAPKs) and in cytosolic Ca^{2+} are among the most common events observed in nongenomic effects of E2 (Falkenstein et al., 2000). The nongenomic effects of estradiol have been investigated in the mollusc *Mytilus galloprovincialis*. Canesi et al. (2004) reported that E2 caused a rapid and significant increase in hemocyte cytosolic Ca^{2+}, and affected the phosphorylation state of the components of tyrosine kinase-mediated signal transduction MAPK- and STAT-like proteins (signal transducers and activators of transcription). Stefano et al. (2003) reported that E2 (and also BSA-E2) induced an immediate concentration-dependent release of NO by the pedal ganglia.

Whether the action of sex steroids is receptor-mediated or not, a number of studies suggest that, at least in some invertebrate phyla, e.g. molluscs and echinoderms, sex-steroids might have a role in reproduction and sex differentiation. Thus, temporal variations in steroid titers and some steroid biosynthetic pathways that coincide with reproductive stages have been reported (De Longcamp et al., 1974; LeGuellec et al., 1987; Reis-Henriques and Coimbra, 1990; Xu and Barker, 1990; Voogt et al., 1991; den Besten et al., 1991; Hines et al., 1992; Bose et al., 1997; Siah et al., 2002, Pernet and Anctil, 2002), and alterations in sexual characteristics or reproduction have been observed due to exposure to exogenous testosterone in molluscs (Takeda, 1979; Spooner et al., 1991; Sakr et al., 1992; Bettin et al., 1996; Oberdoerster et al., 1998), and crustaceans (Nagabhushanam and Kulkarni, 1981). Exposure to estradiol has been associated to an induction of female gonad maturation in echinoderms (Takahashi and Kanatani, 1981) and in molluscs (Mori et al., 1969).

However, much more information is needed to fully understand the physiological function of these steroid hormones. The most common approaches used in vertebrates to study the role of sex steroids have been manipulating the receptors (e.g. observing the physiological differences associated to genetic alterations or polymorphisms, or using knock-out animals) or blocking specific steroid biotransformation pathways (e.g. finasteride and letrozole are potent inhibitors of 5ζ-reductase type II and aromatase, respectively). In order to use similar approaches in invertebrates, further research on their steroidogenic pathways and the presence and characteristics of the putative steroid receptors is still needed.

In summary, while there is no doubt about the presence of sex steroids in invertebrates, the evidence gets weaker when we consider more relevant levels necessary to consider sex steroids as functional hormones in invertebrates (Figure 1.4).

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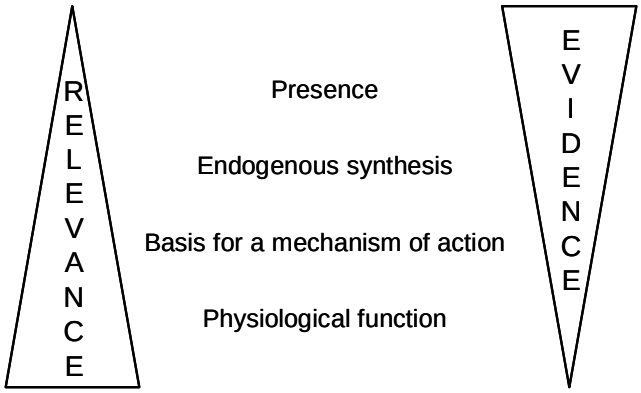


Figure 1.4. Evidence for existence and function of sex-steroids in invertebrates

1.7 Sex steroids

1.7.1 Steroid structure and classes

Steroid hormones are derivatives of cholesterol that are synthesized by a variety of tissues, most prominently the adrenal gland and gonads. Steroids have a common structure consisting of four fused rings, named A, B, C and D. The first three rings (A, B and C) resemble phenanthrene to which a cyclopentane (ring D) is attached. This basic perhydrocyclopentanophenanthrene ring structure and carbon numbering system of all steroid hormones is shown in Figure 1.5. Steroid hormones and their precursors and metabolites differ in number and type of substituted groups,

number and location of double bonds, and stereochemical configuration. Atoms or groups attached to the ring are termed ζ, if they lie below the horizontal plane, or η if they lie above the horizontal plane. At each ring junction of the steroid molecule, there is a single hydrogen atom attached to each of the carbon atoms forming a junction. These hydrogens may be orientated on opposite sides of the plane of the ring (ζ3η), in which case the junction is referred to as trans, or they may both be on the same side of the ring plane (ζ3ζ or η3η), which is referred to as cis. A trans junction results in a planar molecule, whereas a cis junction gives a bent molecule. In all naturally occurring vertebrate hormones, the ring junction B/C and C/D are trans so that rings B, C, and D always form a planar structure. Junction A/B, however, can be either cis or trans, directing the final shape of the hormone (Butt et al., 1975).

There are three sex steroid classes: the progestins, the androgens and the estrogens.

The progestins are C_{21} steroids (Figure 1.5). Progesterone is the active progestin in mammals and is responsible for the maintenance of pregnancy and milk secretion, whereas 17ζ,20η-dihydroxy-4-pregnen-3-one is the active progestin in fish and is responsible for the maturation of occyte and germinal vesicle breakdown. The presence of progesterone has been reported in invertebrates (see section 1.6.3).

All androgens have the basic C_{19} ring configuration, and the differences occur in the type and position of the moieties which are attached to the basic C_{19} unit (Figure 1.5). Androgens are responsible for male sexual differentiation, spermatogenesis, and the development of secondary sexual characteristics. The main active androgens in vertebrates are testosterone, dihydrotestosterone and 11-keto-testosterone (11-kT). Testosterone was initially considered to be the most biologically active androgen, but it is now generally accepted that the androgenic potency of T occurs as a result of its very active conversion to DHT in target tissues. In teleost fish, 11-kT is considered the major androgen. These three androgens: testosterone, 11-kT and DHT have also been found in invertebrate tissues (see 1.6.3).

All estrogens have a basic C_{18} structure with an oxygen substituent at C_{17} and a characteristic aromatic A ring (Figure 1.5). Estrogens influence the growth, development differentiation and function of peripheral tissues of the female and male reproductive system such as the mammary gland, uterus, vagina, ovary, testis, epididymis and prostate. It has also been known that estrogens play an important role in bone maintenance, in the cardiovascular system and in the central nervous system. Estrogens are synthesized from androgenic precursor (androstenedione and testosterone) by demethylation and aromatization. Estrogen metabolism has been implicated in the risk of hormone-dependent diseases and has demonstrated physiological significance. Estradiol is the most potent naturally occurring estrogen in all vertebrate species. Its presence has been described in invertebrates (see 1.6.3).

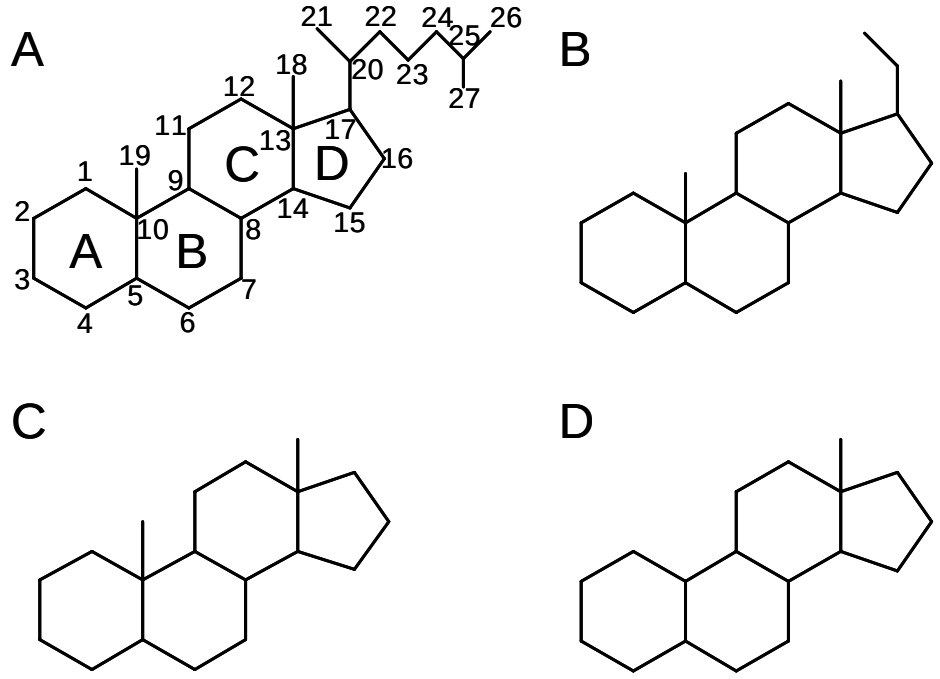


Figure 1.5. Chemical structure of steroids. A: perhydrocyclopentanophenanthrene ring structure; B: progestins; C: androgens; D: estrogens.

1.7.2 Steroidogenesis in vertebrates

The synthesis of steroid hormones is a complex process involving a parent compound, cholesterol, from which biologically active steroids are derived (Figure 1.6). All steroid transformations are catalyzed by enzymes, which are members of either the cytochrome P450 superfamily or members of the steroid dehydrogenase family (reviewed in Miller, 1988; Norman and Litwack, 1998, Payne and Hales, 2004).

The P450 enzymes are membrane-bound proteins associated with either the mitochondrial membranes or the endoplasmic reticulum (microsomal). These P450 enzymes are members of a superfamily of heme-containing proteins found in bacteria, fungi, plants, and animals. They derive their name from the characteristic that, when complexed *in vitro* with exogenous CO, they have a maximum absorbtion of light at 450 nm. In the biosynthesis of steroid hormones from cholesterol, cytochrome P450 enzymes catalyze the hydroxylation and cleavage of the steroid substrate. They function as monooxygenases utilizing reduced NADPH as the electron donor for the reduction of molecular oxygen.

The hydroxysteroid dehydrogenases (HSDs) belong to the same phylogenetic protein family, namely the short-chain alcohol dehydrogenase reductase superfamily. They are involved in the reduction and oxidation of steroid hormones requiring NAD^+/$NADP^+$ as acceptors and their reduced forms NADH/NADPH as donors of reducing equivalents. One of the major differences between the P450 enzymes and the HSDs is that each of the P450 enzymes is a product of a single gene, whereas there are several isoforms for the 3ηHSDs and for the 17ηHSDs, each a product of a distinct gene. The number of isoforms or isozymes varies in tissue distribution, catalytic activity (whether they function predominantly as dehydrogenases or reductases), substrate and cofactor specificity, and subcellular localization.

The rate-limiting step in the biosynthesis of steroid hormones is the transport of free cholesterol from the cytoplasm into the mitochondria. The transport of cholesterol is facilitated by the binding of gonadotropins (follicle stimulating hormone and luteinising hormone) to their receptors on the membrane of the steroidogenic cells, which results in the activation of adenylate cyclase, followed by an increased production of cAMP. Within mitochondria, cholesterol is converted into pregnenolone by CYP11A1 (also called P450-linked cholesterol side chain cleaving enzyme (P450scc), or desmolase). Pregnenolone itself is not a hormone, but the immediate precursor for the synthesis of all of the steroid hormones. Pregnenolone diffuses across the mitochondrial membranes and may be then further metabolized by enzymes that are associated with the smooth endoplasmic reticulum to yield either mineralcorticoids, glucocorticoids or sex steroids. The conversion of pregnenolone into one of those groups of steroids is dependent on which of the two biosynthesis pathways, e.g. the ÷^{5}-3-hydroxyl pathway or the ÷^{4}-3-oxo pathways, is activated. One of the two enzymes responsible for determining the followed pathway is 3η-hydroxysteroid dehydrogenase/÷^{5}4÷^{4} isomerase (3η-HSD). 3η-HSD catalyses the 3η-hydroxysteroid dehydrogenation, and 5-ene-4-ene isomeration of the double bond from the B ring to the A ring of steroids. Thus, in the presence of 3η-HSD -activity, pregnenolone is converted into progesterone, a steroid of the ÷^{4}-3-oxo pathway. While the production of mineral- and glucocorticoids is strictly dependent on the activation of the ÷^{4}-3-oxo pathway, there are alternative routes for the synthesis of sex steroids (Figure 1.6).

Pregnenolone can be hydroxylated at its C_{17} position by the action of cytochrome P450 17ζ-hydroxylase,17,20-lyase (P450c17). The product of this catalysis is 8>ζ-hydroxypregnenolone, a steroid of the ÷^{5}-3-hydroxyl pathway. This can then undergo cleavage of the C_{17}-C_{20} carbon bond to yield dehydroepiandrosterone. This reaction is also catalyzed by P450c17 and it leads to the formation of C19 steroids (e.g. androgens). P450c17 can also act on the ÷^{4}-3-oxo pathway, converting progesterone into 17ζ-hydroxyprogesterone (17ζ-hydroxylation) and subsequently into androstenedione ($C_{17\text{-}20}$ lyase activity). Having both 17ζ-hydroxylase and $C_{17\text{-}20}$ lyase activity, P450c17 is positioned at a key branch in steroid hormone synthesis. Pregnenolone is converted into mineralcorticoids when P450c17 remains inactive and pregenolone is used as a substrate by

3η-HSD instead. Pregnenolone is converted into glucocorticoids when P450c17 only displays 17ζ-hydroxylase activity, or into sex steroids when P450c17 displays both activities, 17ζ-hydroxylation and C_{17-20} cleavage.

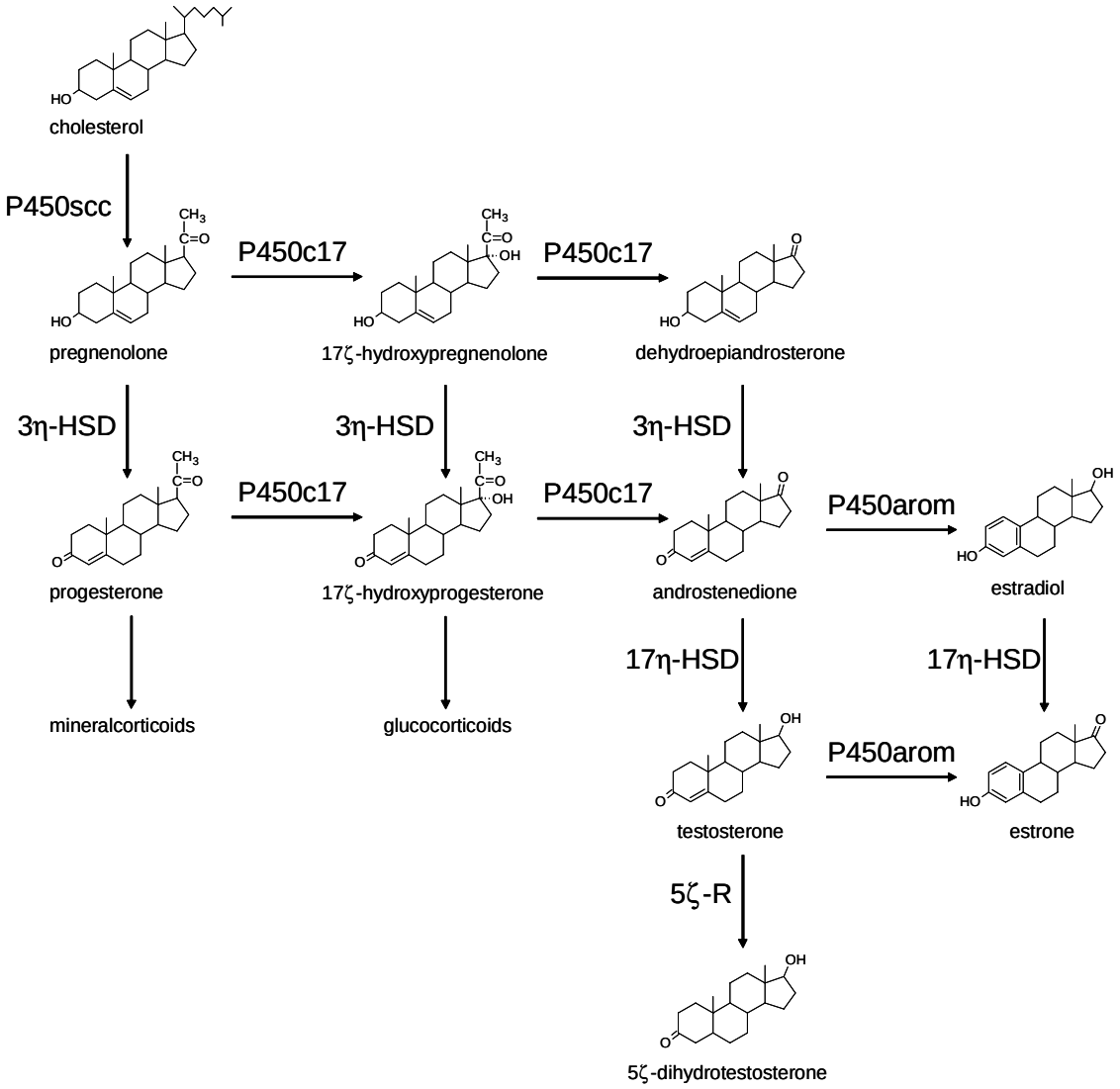


Figure 1.6. Sex steroid biosynthesis pathway in vertebrate gonads/adrenal glands. P450scc: P450 side-chain cleavage; P450c17: P450 17ζ-hydroxylase and C_{17-20}-lyase; 17η-HSD: 17η-hydroxysteroid-dehydrogenase; P450arom: P450 aromatase; 3η-HSD: 3η-hydroxysteroid-dehydrogenase.

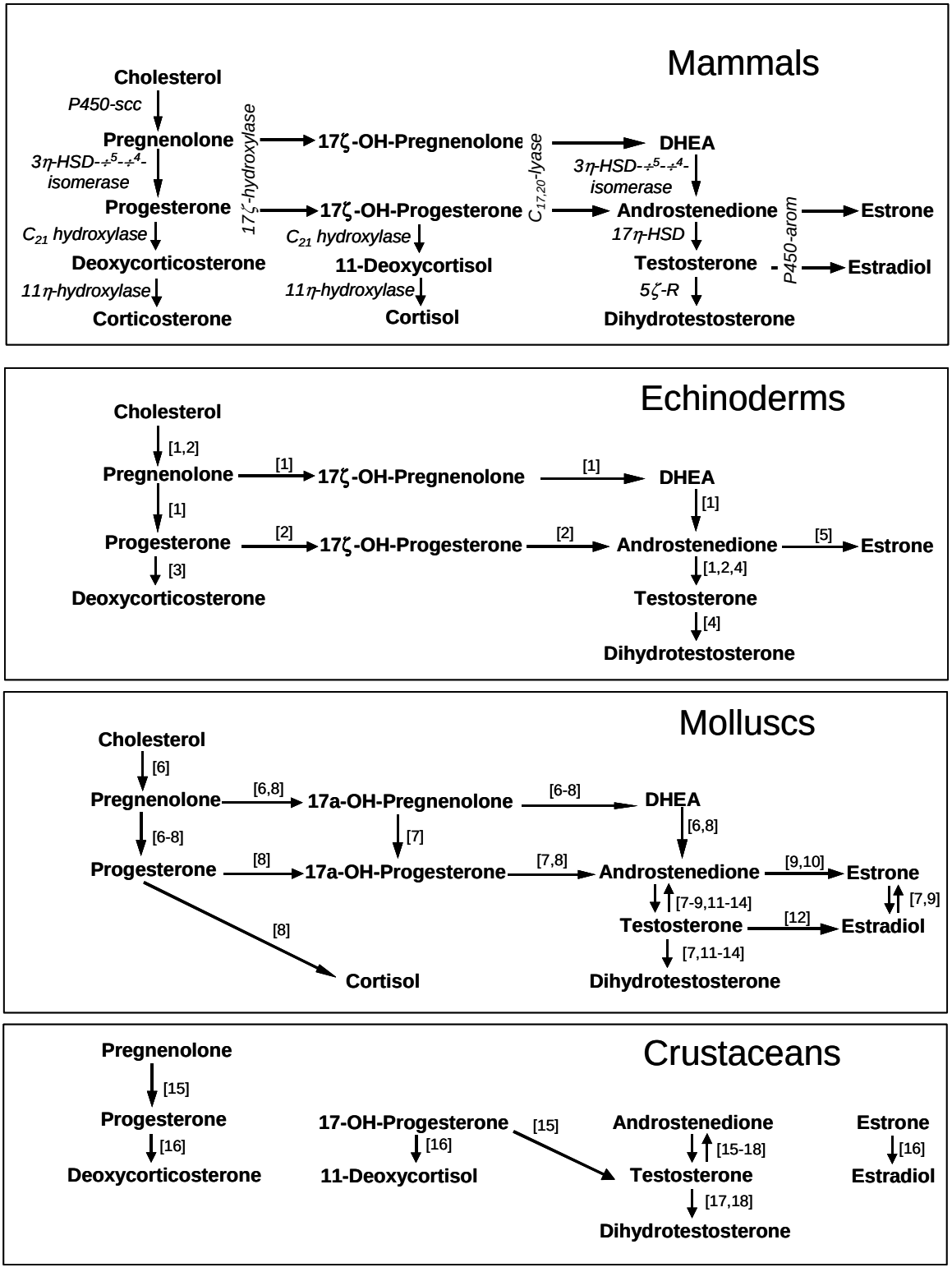
Dehydroepiandrosterone and androstenedione can be converted into androstenediol and testosterone, respectively by 17η-HSD.

The conversion of C_{19} androgens into C_{18} estrogens is mediated by aromatase, a cytochrome P450 enzyme (P450arom). Aromatase converts aromatisable androgens into estrogens by three subsequent hydroxylations at the C_{19}-methylgroup, which leads to the aromatization of the A-ring of these steroids.

1.7.3 Steroidogenesis in invertebrates

Most steps of the steroidogenic pathways described above for vertebrates have been demonstrated to occur in invertebrates by exposing/incubating animals/homogenates to steroid precursors (Figure 1.7). Thus, the key steps of steroidogenesis leading to androgens and estrogens have been found in echinoderm (Schoenmakers, 1979; Voogt and Rheenen, 1986; Voogt et al., 1990; Hines et al 1992; Wasson et al., 1998; Wasson and Watts, 2000) and mollusc species (de Longcamp 1974; Krusch et al., 1979; Lupo di Prisco and Dessi'Fulgheri, 1975; Le Guellec et al., 1987; Hines et al., 1996; Ronis and Mason., 1996; Morcillo et al., 1999; le Curieux-Belfond et al., 2001; Morcillo et al., 1998; Oberdörster et al., 1998). Crustacea have also been subject of numerous studies, however, it can be observed in Figure 1.7 that several steroidogenic pathways remain to be demonstrated (Swevers et al., 1991; Blanchet et al., 1972; Baldwin et al., 1998; Verslycke et al., 2002). An interpretation of this could be that crustaceans are not actively synthesizing vertebrate-type steroids.

Figure 1.7. (next page) Basic mammalian steroidogenic pathways present in different invertebrate phylogenetic groups: echinoderms, mollusks, and crustacea. *Although no reports of aromatase activity in echinoderms were found, studies in our lab suggest that aromatase is present in these organisms. **The formation of dihydrotestosterone has not been reported in echinoderms, but 5ζ-androstan-diols (DHT metabolites). P450scc: P450 side-chain cleavage; 17η-HSD: 17η-hydroxysteroid-dehydrogenase; P450arom: P450 aromatase; 3η-HSD: 3η-hydroxysteroid-dehydrogenase. References: top figure adapted from Norman and Litwack, 1998; [1] Voogt et al., 1990; [2] Schoenmakers et al., 1979; [3] Wasson and Watts, 2000; [4] Wasson et al., 1998; [5] our group, unpublished results; [6] Gottfried and Dorfman, 1970; [7] De Longcamp et al., 1974; [8] Lupo di Prisco and Lessi'Fulgheri, 1975; [9] Le Curieux-Belfond et al., 2001; [10] Morcillo et al., 1999; [11] Morcillo et al., 1998; [12] Le Guellec et al., 1987; [13] Ronis and Mason, 1996; [14] Oberdörster et al., 1998; [15] Swevers et al., 1991; [16] Blanchet et al., 1972; [17] Verslycke et al., 2002; [18] Baldwin and LeBlanc, 1994b.


Mammals
Cholesterol
P450-scc
Pregnenolone
3β-HSD-Δ5-Δ4-isomerase
Progesterone
C21 hydroxylase
Deoxycorticosterone
11β-hydroxylase
Corticosterone
17α-hydroxylase
17α-OH-Pregnenolone
17α-OH-Progesterone
C21 hydroxylase
11-Deoxycortisol
11β-hydroxylase
Cortisol
C17,20-lyase
DHEA
3β-HSD-Δ5-Δ4-isomerase
Androstenedione
17β-HSD
Testosterone
5α-R
Dihydrotestosterone
P450-arom
Estrone
Estradiol
Echinoderms
Cholesterol
[1,2]
Pregnenolone
[1]
17α-OH-Pregnenolone
[1]
DHEA
[1]
Progesterone
[2]
17α-OH-Progesterone
[2]
Androstenedione
[5]
Estrone
[3]
Deoxycorticosterone
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Testosterone
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Dihydrotestosterone
Molluscs
Cholesterol
[6]
Pregnenolone
[6,8]
17a-OH-Pregnenolone
[6-8]
DHEA
[6-8]
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Progesterone
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17a-OH-Progesterone
[7,8]
Androstenedione
[9,10]
Estrone
[7-9,11-14]
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[8]
Testosterone
[12]
Estradiol
[7,11-14]
Cortisol
Dihydrotestosterone
Crustaceans
Pregnenolone
[15]
Progesterone
[16]
Deoxycorticosterone
17-OH-Progesterone
[15]
[16]
11-Deoxycortisol
Androstenedione
[15-18]
Testosterone
[17,18]
Dihydrotestosterone
Estrone
[16]
Estradiol

1.7.4 Androstenedione and testosterone metabolic pathways.

Steroid metabolism plays an important role in the regulation of active steroids. Most of the enzymes involved in steroid metabolism can metabolize a variety of steroids and, some of them (e.g. hydroxylases, phase II enzymes) can also metabolize a wide range of structurally unrelated molecules. Because of its chemical structure, testosterone can be a substrate for most of these metabolic pathways and has been extensively used as model substrate. An overview of the major pathways involved in metabolism of sex steroids (depicted in figures 1.8 and 1.9) is described below. For each of them, the existing information on the invertebrate groups considered in this thesis is presented.

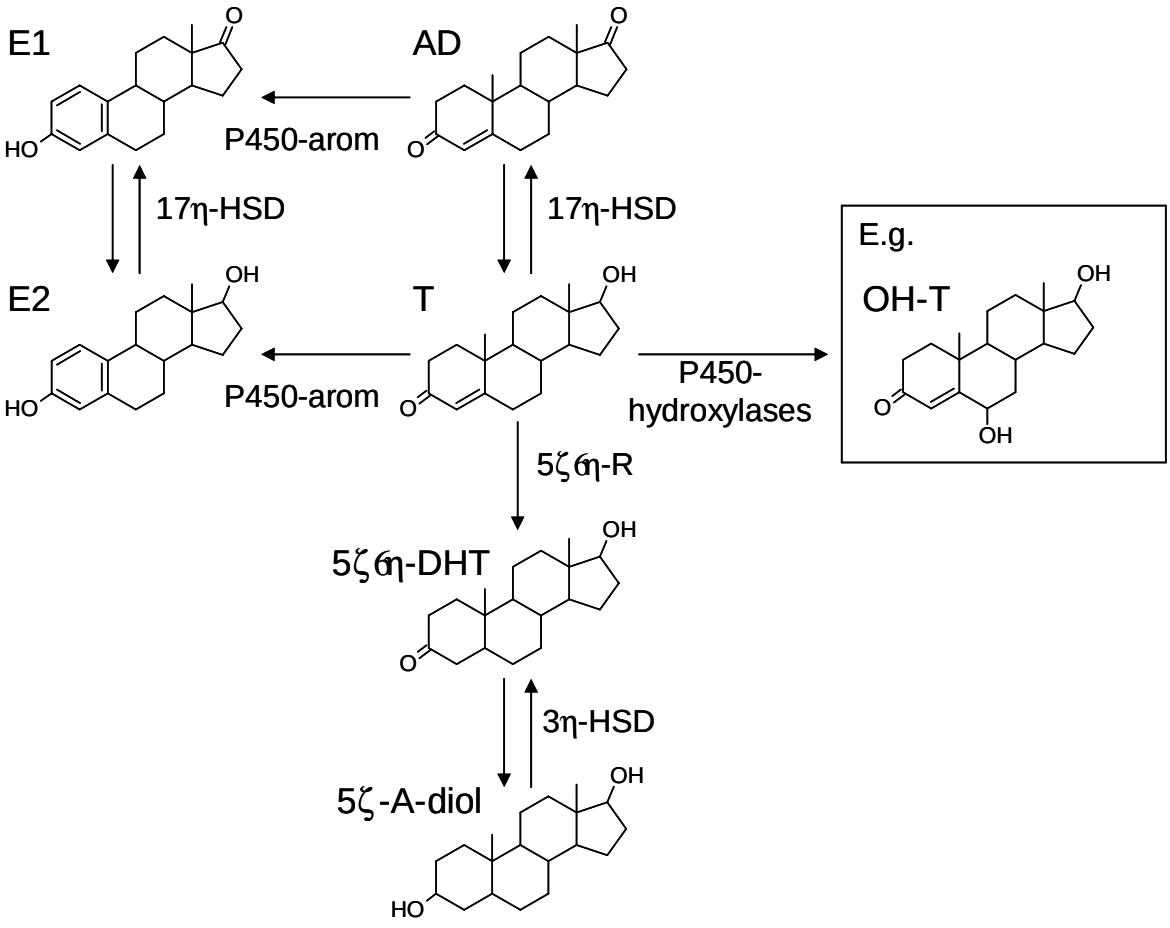

Figure 1.8. Main pathways of Testosterone phase I metabolism. E1: estrone; AD: androstenedione; E2: estradiol; T: testosterone; DHT: dihydrotestosterone; 5ζ-A-diol: 5ζ-androstan-3ζ/η,17η-diol; 6η-OH-T: 6η-hydroxytestosterone.

5ζ-Reductase. The enzyme acts upon steroids containing a 4-en,3-keto configuration by reducing the double bond in the A ring. Thus testosterone can be converted to 5ζ-dihydrotestosterone (DHT), which is the most potent of the human male steroid hormones, with an androgenic activity that is 10 times that of testosterone (Wilson, 2001). The reaction exhibits an absolute cofactor requirement for NADPH, which provides the hydride ion for carbon-5, and a proton from water attaches to carbon-4 (Wilson, 2001). Reversal of the reaction does take place in some bacteria (Florin et al., 1996), but not in mammals. Two 5ζ-reductase genes have been described. The enzymes codified by these genes differ in pH optimum, substrate affinity and in their response to finasteride, which inhibits type 2 but not type 1. The enzyme activity has been located in a wide variety of tissues including liver, prostate, sweat and sebaceous glands, and genital skin fibroblasts (Wilson, 2001).

The 5ζ-reduction of 4-en,3-keto steroids plays a role in the action of testosterone, brassinolides, androstaneol/androstenol and, in some species, progesterone (Wilson, 2001). Evidence is clear that testosterone and DHT act via a single androgen receptor, and that DHT binds more tightly than testosterone to the hormone-binding domain of the androgen receptor. However, it is not clear whether conversion of testosterone to DHT results in the formation of a different hormone or simply amplifies a hormonal signal; that is, whether the DHT–receptor complex is sufficiently different to perform unique function(s). In addition, 5ζ-reduction precludes the aromatization of androgens to estrogen and promotes intracellular accumulation of androgen (Wilson, 2001).

5ζ-reductase has been reported in echinoderm, molluscs, and crustacea. In echinoderms, 5ζ-reductase has been found in incubations with gonads, pyloric caeca and body wall of asteroids or echinoids species (Voogt and Rheenen, 1986; Hines et al., 1992; Wasson et al., 1998). In studies using androstenedione or progesterone, 5ζ-reductase was one of the major metabolic pathways (Voogt et al., 1986; Hines et al., 1992; Wasson et al., 1998). However, it should be mentioned that DHT has never been reported within the 5ζ-reduced metabolites produced. In molluscs, 5ζ-reductase has been found in *in vivo* metabolism experiments with the gastropods *Clione antarctica* or *Ilyanassa obsoleta* (Hines et al., 1996; Oberdörster et al., 1998), and in *in vitro* incubations of digestive gland microsomes from the bivalve *R. decussata* (Morcillo et al., 1998), the gastropod *L. littorea* (Ronis and Mason, 1996), and in gonad whole homogenates of the gastropod *H. aspersa* (Le Guellec et al., 1987). In contrast to echinoderms, DHT was observed in most studies. In crustacea, DHT has been found in *in vivo* metabolism experiments (Baldwin and LeBlanc, 1994b; Verslycke et al., 2001), but not in *in vitro* incubations (Swevers et al., 1991).

5η-reductase. Similar to 5ζ-reductase, this enzyme acts upon 4-en-3-oxosteroids reducing the double bond in the A ring. 5η-reductase is a NADPH-dependent cytosolic enzyme and has been purified in mammals and avian species (Okuda and Okuda, 1984; Sugimoto et al., 1990). It has been suggested that 5η-reductase is more important in bile acid biosynthesis than steroid

metabolism (Kondo et al., 1994), however, the physiological role of 5η-reductase has not been thoroughly studied. In invertebrates, 5η-reductase has been reported in pyloric caeca of *Asterias vulgaris* (Hines, 1992).

Hydroxysteroid dehydrogenases (HSD). HSDs convert keto-steroids into hydroxy-steroids or viceversa.

17ƃ-HSD. The last steps of steroid synthesis and its primary metabolism are catalyzed by 17ƃ-hydroxysteroid dehydrogenases (17ƃ-HSD). These enzymes convert inactive 17-keto-steroids into their active 17ƃ-hydroxy-forms or vice versa. In vertebrates, androgens and estrogens with C17 alcohols are active steroids, while those with C17-ketones have substantially less activity (Labrie et al., 1997). Thus, testosterone is formed by dehydrogenation of androstenedione by 17ƃ-HSDs. At least eight distinct 17ƃ-HSD isoforms have been described in mammalian species (Baker, 2001b). Although in principle each 17η-HSDs can catalyze oxidation or reduction of substrates, *in vivo* each enzyme is either an oxidase or a reductase. Some 17η-HSDs have a preference for reduction of androgens (type 3); others for reduction of estrogens (type 1 and type 7); and yet others for oxidation of androgens and estrogens (type 2) (Blomquist, 1995; Baker, 2001b). In addition, they differ in tissue distribution, subcellular localization, and mechanisms of regulation. Thus, in gonads, the enzymes catalyze the reduction of steroids to active metabolites, while an oxidative process to inactivate steroids metabolites is favored in peripheral tissues (Luu-The, 2001).

The presence of 17η-HSD has been reported in the three invertebrate phyla discussed here (see Figure 1.7). This is not surprising, since most 17η-HSD belong to the short chain dehydrogenase/reductase family of enzymes, which are known to be present in bacteria, fungus, plants and animals (Baker, 2001b; Lanisnik-Kizner and Zaeklj-Mavric, 2000).

3ƃ-HSD. In contrast to the other HSD enzyme families, 3η-HSD are enzyme complexes that catalyze both the reduction/oxidation of the 3-keto/hydroxyl and the $\div^5$-$\ddot{3}^4$-isomerization. Thus, they catalyze the transformations of all 5-ene-3η-hydroxy-steroids (that is with a double bond between carbon 5 and 6, the $\div^5$ steroids) into 4-ene-3-oxosteroids (double bond between carbons 4 and 5, the $\div^4$ steroids) and are involved in the synthesis of all classes of active steroids (Peng et al., 2002). Several 3η-HSD isoforms have been described in mammals (Simard et al., 1991; Peng et al., 2002) and have different tissue distribution. E.g., 3η-HSD (type 1) is found in placenta, and skin, and 3η-HSD (type 2) is found in adrenals and gonads. The 4-ene-3-oxosteroids are involved in gonad development and differentiation processes (Labrie et al., 1994).

The presence of 3η-HSD has been reported in the three phyla of invertebrates discussed here. Most reports found 3η-dehydrogenation in ‘androstane’ or ‘pregnane’ steroids, which do not have a

4-ene structure (Wasson et al., 1998; Wasson and Watts, 2000; Ronis and Mason, 1996; Oberdörster et al., 1998). Other reports, described the 3η-dehydrogenation in 5-en steroids, however, the product obtained was also a 5-en steroid (Hines et al., 1996; Morcillo et al., 1998). The ÷^{5}-3^{4}-isomerase activity associated to 3η-HSD has only been demonstrated using pregnenolone as the substrate in a few studies including species of the three phyla we are interested in (De Longcamp et al., 1974; Lupo di Prisco and Dessi'Fulgheri, 1975; Schoenmakers, 1979; Swevers et al., 1991).

3ζ-HSD. This family of enzymes catalyzes the reduction/oxidation of the keto/hydroxyl group at the 3- position. They generally act upon 5-reduced steroids, thus, they have an important regulatory function by inactivating DHT to the 3ζ-A-diol. There are 4 soluble 3ζ-HSDs described in humans (Penning, 2003).

3ζ-HSD are possibly present in the invertebrate species discussed here. Thus, 3ζ-OH metabolites have been described in molluscs and echinoderms (Morcillo et al., 1998; Wasson et al., 1998; Wasson and Watts, 2000). Other studies have identified 3ζ/η-OH metabolites in invertebrates, however, have not distinguished between 3ζ and 3η isoforms (Ronis and Mason, 1996; Oberdörster et al., 1998).

Cytochrome P450 dependent biotransformations: aromatization and hydroxylations. Cytochrome P450 monooxygense enzymes (CYP) comprise an ancient and widely distributed protein superfamily. P450-type enzymatic activities have been reported in crustaceans, molluscs and echinoderms (Livingstone et al., 1989; Livingstone, 1991; James and Boyle, 1998). The highest P450 activity is found in digestive gland, pyloric caeca, and hepatopancreas of mollusc, echinoderm and crustaceans, respectively (Snyder, 2000). Typically, total P450 protein and associated enzymatic activities in invertebrates are found to be 10-fold lower than in mammals (Livingstone, 1991). In addition, several studies have failed to measure P450-catalyzed activities *in vitro* in crustacea (Singer et al., 1980; James and Little, 1984; Baldwin and LeBlanc, 1998) possibly because of the presence of endogenous inhibitors in microsomal preparations (James, 1989). Therefore, the *in vitro* detection of both aromatase and hydroxylation activities in crustaceans is also expected to be affected by similar problems.

Aromatase. The conversion of the C19 steroids to estrogens is catalysed by a CYP450 enzyme. It is expressed in a variety of tissues including ovarian granulosa cells, placenta, testicular Sertoli and Leydig cells, brain and adipose tissue. This enzyme requires NADPH as cofactor, and its action involves hydroxylations and dehydrations that culminate in aromatization of the A ring of the androgens (Simpson and Davis, 2001).

Aromatase activity has been reported in molluscs (Morcillo et al., 1999; Le Curieux-Belfond, 2001; Horiguchi et al., 2004), and studies in our laboratory have detected aromatase activity in echinoderms (unpublished results). However, the aromatization rates reported are rather close to the detection limit of the methods used to assess the activity. The tritiated water release assay, which is based on the quantification of the tritiated water released during [^{3}H]-androstenedione aromatization (Morcillo et al., 1999; Le Curieux-Belfond, 2001), and analysis by GC-MS of the differential estradiol levels before and after incubating tissue slices with testosterone have been used to measure aromatase activity in molluscs (Horiguchi et al., 2004).

Hydroxylases. Hydroxylation is one of the possible pathways to render testosterone inactive. Testosterone is a substrate for many P450s, and it is hydroxylated in a regiospecific and stereospecific manner by different P450 isozymes, allowing simultaneous analyses of multiple P450 enzymes (Waxman et al., 1983). For example, in rat CYP3A1 predominantly hydroxylates testosterone at the 6ƀ position, whereas CYP2A1 hydroxylates testosterone at the 6ζ and 7ζ positions. Mammalian and fish hepatic microsomes generally produce six or more hydroxytestosterone metabolites (Wilson and LeBlanc, 1998; Parks and LeBlanc, 1998).

Testosterone hydroxylation has been studied *in vivo* in the crustaceans *Daphnia magna*, and *Neomysis integer*. At least 10 polar metabolites of testosterone were excreted by daphnids exposed to testosterone (Baldwin and LeBlanc, 1994b). Similarly, at least 11 polar testosterone metabolites were produced by *N. integer* (Verslycke, 2002). In molluscs, testosterone hydroxylation has been demonstrated in incubations of digestive gland microsomes. At least 5 OH-metabolites were found in *R. decussata* incubations (Morcillo et al., 1999), and at least 3 OH-metabolites in *L. littorea* (Ronis and Mason, 1996). We are not aware of any report on hydroxylations in echinoderms, in fact, as far as we know, testosterone has never been used as a substrate in studies with species of this phylogenetic group.

UDP-(glucuronosyl or glucosyl) transferases (UGTs). The UDP-glucuronosyl transferases are a family of membrane-bound isozymes that catalyze the transfer of UDP-glucuronic acid to a variety of endogenous (including steroid hormones) or exogenous aglycone compounds, and constitute a major pathway for their metabolic degradation and excretion. In humans, steroid UGT activities are found in the liver and several extrahepatic tissues including the prostate, mammary gland and ovary (Hum et al, 1999). Different isozymes have been described including a 17η-hydroxysteroid UGT and a 3ζ-hydroxysteroid UGT.

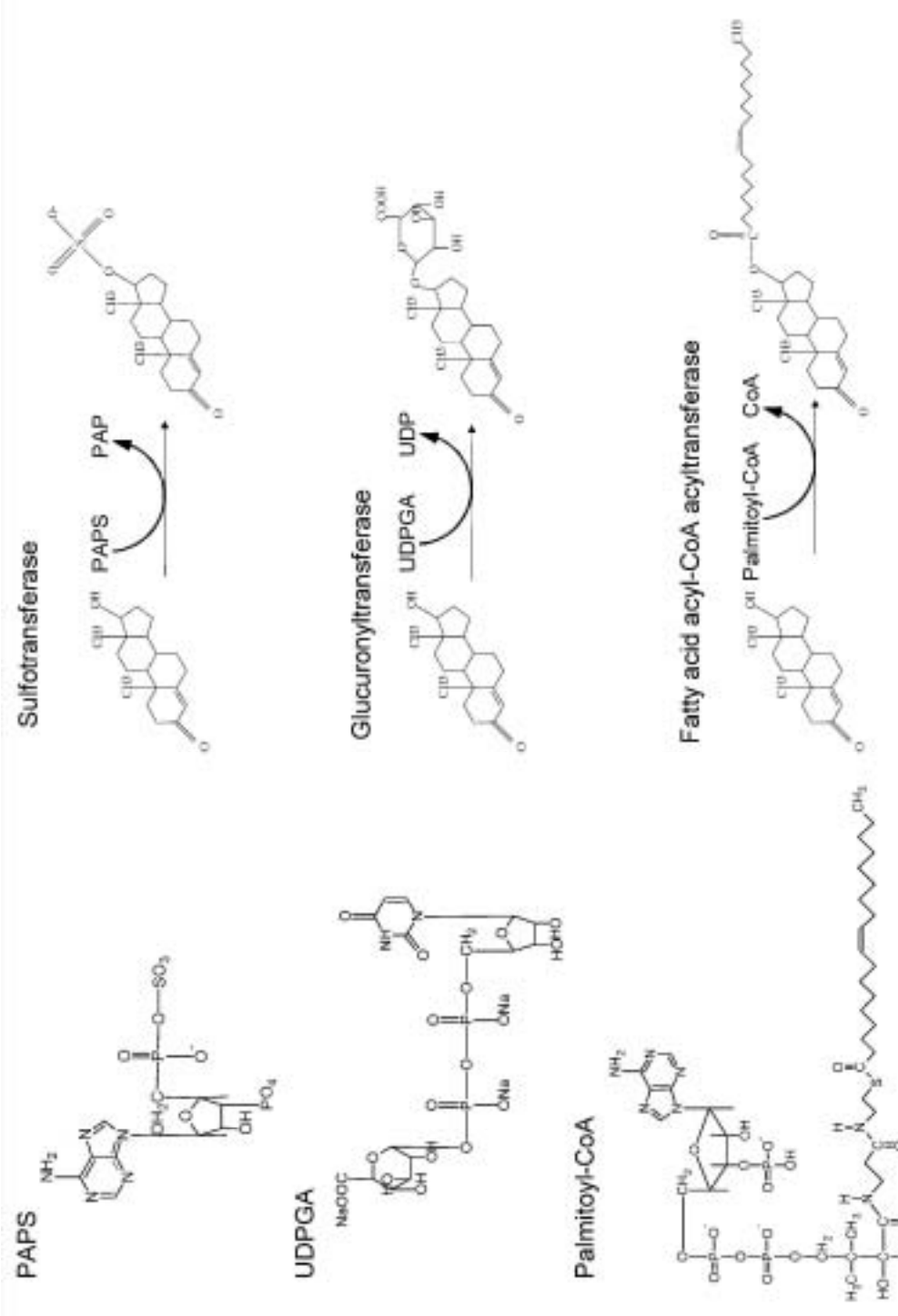

Figure 1.9. Phase II metabolism of testosterone: cofactors and metabolic reactions.

Despite the recent report showing that the polychaete *Nereis diversicolor* conjugated 1-hydroxypyrene to its glucuronide (Giessing and Lund, 2002), several studies suggested that glucosidation is a more important pathway than glucuronidation in invertebrates (James, 1987). It has been shown that phenolic compounds are conjugated to polar metabolites with sulfate and glucosyl moieties in invertebrates (Dutton, 1966; Elmamlouk and Gessner, 1978; Foster and Crosby, 1986; Schell and James, 1989; de Knecht et al., 2001), suggesting that polar conjugates of steroid hormones are also formed by sulfation and glucosidation. However, we have not found any report on the formation of steroid glucosides in invertebrates, except for those where glucosides were identified based on their hydrolysis by glucosidases (e.g. Baldwin and LeBlanc, 1994b).

Sulfotransferases. Sulfate conjugation is a major pathway of detoxification or bioactivation of foreign compounds and it is important in modulating the metabolism and biological activity of endogenous substances, including steroids (Strott, 1996). Sulfonation of low molecular weight compounds such as hydroxysteroids, estrogens and catecholamines is catalysed by cytosolic sulfotransferases belonging to a gene superfamily designated as SULT (Weinshilboum et al., 1997). These cytosolic enzymes utilize 3' phosphoadenosine 5'-phosphosulfate (PAPS) as the sulfuryl donor in sulfonation reactions (Strott, 1996). Mammalian SULTs are classified into two major families, the phenol (SULT1) and the hydroxysteroid sulfotransferase (SULT2) (Coughtrie and Johnston, 2001). Estrogens are sulfated by a SULT1E1, while androgens and other hormones are sulfated by isozymes from the SULT2 family, DHEA being the most rapidly conjugated steroid (Strott, 1996).

The sulfation of steroids is considered to have an important role in inhibiting their biological activity and increasing their excretion. The sulfated form of the steroids may also serve as a soluble, inactive transport form, from which the active steroid may be regenerated by sulfatase activity (Strott, 1996).

Sulfate conjugates have been frequently identified as the major metabolites of hydroxylated xenobiotics in invertebrates (James, 1987). Sulfate conjugates of steroid hormones have been observed in crustaceans (Swevers et al., 1991; Baldwin and LeBlanc,1998), and in molluscs (Hines et al., 1996; Ronis and Mason, 1996). However, their identification was only based on their susceptibility to hydrolysis by sulfatases. Few studies have measured *in vitro* sulfation in crustaceans (Li and James, 2000; de Knecht et al., 2001). Sulfonation of steroid hormones has not been studied in molluscs and echinoderm species.

Fatty acid acyl-CoA acyltransferase. Fatty acid conjugation (or esterification) renders steroids to an apolar form, which is retained in the lipoidal matrices of the body, while reducing their bioactivity, bioavailability, and susceptibility to elimination (Borg et al., 1995). Thus, esterification might have a regulatory function by inactivating steroids or preventing the formation of biologically active steroids.

Steroid esters do not bind receptors, but they can be hydrolyzed by esterases liberating again the active steroid. Thus, they are considered to be long-acting steroids (Hochberg, 1998). The fatty acid esterification of testosterone by mammals typically represents a minor process in the biotransformation of testosterone (Borg et al., 1995).

Esterification is known to occur in both, vertebrates and invertebrates. Several studies have focused on ecdysteroid esterification (Connat et al., 1984; Slinger et al., 1986; Slinger and Isaac, 1988; Whiting and Dinan, 1989; Zhang and Kubo, 1992). Sex-steroid esters have also been reported in molluscs (Gooding and LeBlanc, 2001) and echinoderms (Voogt and Van Rheenen, 1986; Voogt et al., 1990). It has been suggested that esterification is the major biotransformation pathway for testosterone in snails, based on the report that exogenously-provided testosterone is converted to fatty acid esters by the mud snail *Ilyanassa obsoleta* and retained in the tissues of the organism (Gooding and LeBlanc, 2001). In addition, steroid esterification might be implicated in the regulation of free steroid levels. Gooding and LeBlanc (2004) observed that, irrespective of the amount of testosterone administered to the snails, the amount of free testosterone measured in the tissues of the organism remains relatively constant and all excess of testosterone is converted to the fatty acid ester.

1.8 Investigating steroids and steroid metabolism. A methodological remark.

An important part of this thesis will be based on the determination of steroid levels and steroid metabolism in invertebrate tissues. The methodologies that can be used for both purposes will be briefly described below.

1.8.1 The identification and quantitative determination of sex steroids

The first step in analyzing sex steroids is to extract them from their matrix. Several extraction and purification steps are usually necessary prior to the identification and/or quantification. The complexity of the extraction and purification procedures will depend on the matrix studied and the identification technique to be applied (table 1.7). Haemolymph, because of its resemblance to vertebrate plasma, is an attractive matrix to measure steroid levels in invertebrates. However, obtaining haemolymph is not possible in some animals, particularly in very small species, and sometimes tissues extracts are the only possibility.

Tissues are usually homogenized in an aqueous or organic phase. Thereafter, the haemolymph or the tissue homogenates are further extracted with an organic solvent (table 1.7). To quantitatively extract the analyte(s) in a liquid-liquid extraction system (LLE), the chosen solvent should completely dissolve the compounds of interest, and also be capable of breaking associations to proteins. These requirements are seldom satisfactorily met in practice and low extraction efficiencies are often seen (Appelblad and Irgum, 2002). Moreover, other non-steroidal lipids are

co-extracted and may well cause interference in the ensuing operations (Appelblad and Irgum, 2002). Despite these drawbacks, LLE extraction procedures are still widely used (Table 1.7). New techniques are emerging, such as solid phase extraction, molecular imprinted polymers or restricted access materials (Appelblad and Irgum, 2002). To the best of our knowledge, these techniques have not been applied to extract steroids from animal tissues, although solid phase extraction is commonly used in later steps as described below.

After LLE, purification procedures can be applied to reduce the lipid content, salts and other possible substances in the extract that might interfere with the detection method. Thus, additional LLE steps are sometimes introduced, however, these methods are time consuming, use large quantities of organic solvents, furthermore their recoveries for steroids may be low (Appelblad and Irgum, 2002). Alternatively, several chromatographic techniques can be applied for sample purification. Thin layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) allow the isolation of the steroids of interest from other possible interfering compounds. Over the last two decades, the use of SPE has gained in popularity, and is today considered as the technique of choice for sample work-up. Despite the wide variety of sorbents available, the SPE material most commonly used for clean-up of steroid samples is bonded silica with either C_8 or C_{18} functionality (Table 1.7). Gels, such as Sephadex LH-20, Lipidex-5000 or Lipidex-1000, Dowex AGI X_2, and Bio-beads SX-3 have also been used in gel permeation chromatography. Briefly, the dry extract from the previous step is redisolved and applied to a glass column filled with the equilibrated gel, eluted with further solvent, and the small fraction, known to contain the steroid investigated, is isolated (Wolthers and Kraan, 1999). Saponification and acid treatment have also been applied to remove fatty components, and recently a freezing-lipid filtration method has been described for the elimination of lipids (Seo et al., 2005).

Once steroids have been extracted and partially purified, several methods are available for measuring steroid hormone levels. The technique most commonly used is radioimmunoassay (RIA). The advantages of this method include availability of commercial reagents and kits, high sensitivity, and the capacity to analyze large numbers of samples relatively quickly and inexpensively (McMaster et al., 2001). Nonradioactive methods, including fluorescent immunoassay or enzyme-linked immunosorbant assay (ELISA), are increasing in popularity. The lack of specificity is the major problem associated to immunoassays. Thus, the cross-reactivity of the antibodies with other steroids, and the interference of other substances with the antigen-antibody binding should be carefully revised. This is of especial concern when working with invertebrates, due to the limited information available on the profile of endogenous steroids in the organisms under study, which might cross react with the antibodies used in the assay. Indeed, Zhu et al. (2003) reported that in addition to estradiol, extracts of *M. edulis* gonads contained a second molecule that had a high binding affinity for estradiol antibodies. Chromatographic techniques, on the other hand, although not as sensitive as biological techniques, enable simultaneous screening of free steroids, conjugates and other compounds. In addition, in contrast to immunoassays, some

Table 1.7. Extraction, purification and analytical methods used for the identification and quantification of sex steroids

	Extraction	Purification	Analyt. Method	Detection limit[1]	Ref
Tissue	Homogenization (CH_3OH+acetic acid), sonication	C_{18} SPE -- HPLC	GC-MS (EI) D[2]: HFBA	1 ng/g	Liere et al., 2000
	Homogenization (CH_3OH : H2O), freeze, extraction	C_{18}SPE, Column chromatography (Dowex AGI-X2), extraction of ketosteroids (trimethylacethyldrazide ammonium chloride), HPLC	GC-MS (EI) D: BSTFA/TMCS	Qualitative	Le Guellec et al., 1987
	Lyophilization, lipid extraction, homogenization (CH_3OH), frozen, reextraction (CH_3OH 80%)	Column chromatography (Dowex AGI X_2), reextraction ($CHCl_3$ vs. H_2O), TLC / HPLC	GC-MS (EI)	4 ng/g*	Reis-Henriques et al. 1990a; 1990b
	Homogenization (Acetone : CH_3OH), sonication, reextraction (CH_2Cl_2 : H_2O)	Delipidation (CH_3OH : H_2O vs. hexane), C18, TLC	GC-MS (EI; SIM) D: TSIM	Qualitative	Voogt et al., 1992
	Homogenization (hexane : propan-2-ol), filtration (glass wool), rehomogenization of filtrate	Delipidation (MeOH : H_2O vs. hexane), Lipidex 1000 gel, Sep-Pak C_{18}, Lipidex 5000, LC	GC-MS (EI; SIM)	60 pg/g*	Fairs 1989
	Homogenization (CH_3OH), extraction (hexane)	C_{18} SPE	LC-MS (NI; SIM)	0.5 ng	Shimada et al., 2001
	Homogenization (H_2O), extraction (ethyl acetate)		LC-MS (PI; SIM)	Qualitative	Verslycke et al., 2002
	Homogenization (H_2O), acid hydrolisis (pH 2), neutralization (pH 7.4), extraction (CH_2Cl_2)		LC-MS (PI; SIM) ELISA	Qualitative 2 ng/g*	Siah et al., 2002
	Lyophilization, lipid extraction, homogenization (CH_3OH), frozen, reextraction (CH_3OH 80%)	Column chromatography (Dowex AGI X_2)	RIA	0.02 ng/g*	Reis-Henriques et al. 1990a;
	Homogenization (buffer), extraction (CH_2Cl_2)		RIA	1.5 ng/g*	Reis-Henriques et al. 1990b
	Homogenization (H_2O), digestion (HCl), neutralization (Na_2HPO_4)	C_{18} SPE	RIA	2 pg/mL 0.1 ng/g	Wasson et al., 2000

Tissue	Homogenization (H_2O), extraction (diethyl ether)	With /without HPLC-UV	Several immunoassays	0.1 ng/g*	D'Aniello et al., 1996
	Homogenization (buffer), extraction (diethyl ether)		RIA	0.02 ng/g*	Xu and Barker, 1990
	Homogenization (buffer), sonication, extraction (hexane)		RIA	0.7 ng/g*	Dieleman and Schoenmakers, 1979
	Homogenization (C_2H_5OH), extraction (diethyl ether-C_2H_5OH)	Delipidation (CH_3OH : H_2O vs. petroleum ether), GPC (Bio-beads SX-3)	RIA	3 pg/g*	Morcillo and Porte, 1999
	Homogenization (H_2O), sonication, digestion (HCl), neutralization (Na_2HPO_4), extraction (CH_2Cl_2)	Sep-Pak C18	RIA	4 pg/g*	Hines et al., 1992
	Homogenization (buffer), sonication, extraction (CH_2Cl_2)	TLC	RIA	0.2 ng/g*	Voogt et al., 1987
Haemolymph	Extraction (C_2H_5OH, CH_3OH)	Delipidation (CH_3OH : H_2O vs. hexane), Lipidex 1000 gel, Sep-Pak C_{18}, Lipidex 5000, LC	GC-MS (EI; SIM) D: MO, TMS, MO-TMS	0.8 ng/mL*	Fairs et al., 1989
	Extraction (CH_2Cl_2)	Delipidation (H_2O : C_2H_5OH vs. CH_2Cl_2), Sephadex LH 20, paper chromatography, silica gel column	GC-MS (NCI) D: OPFB, HFB	0.9 ng/mL*	De Clerck et al., 1983
	Extraction (ether)	Saponification (NaOH and H_2O), Sephadex LH 20 column	RIA	2.5 pg/mL*	
	Homogenization (buffer), sonication, extraction (hexane)		RIA	2 pg/mL	Dieleman and Schoenmakers, 1979
	Extraction (CH_2Cl_2)		RIA	33 ng/mL*	Bose et al., 1997

[1] In those studies where detection limit was not mentioned, the lowest level reported in that work is included and indicated by *.

[2] D: derivatization; describes the derivatizating agent: HFBA: heptafluorobutyric acid anhydride; OPFB: O-pentafluorobenzyloxime; HFB: heptafluorobutyrylester; MO: methyloxime; TMS: trimethylsilyl; BSTFA: bis(trimethylsilyl)trifluoroacetamide

detection systems coupled to chromatographic techniques (e.g. mass spectrometry) provide a highly reliable identification of the steroids. Thus, the use of HPLC, gas chromatography-mass spectrometry (GC-MS), or liquid chromatography-mass spectrometry (LC-MS) has increased in recent years. Due to its high resolving power, excellent performance, and its relative ease of use, gas chromatography is the most commonly used separation of steroids. However, LC-MS, unlike GC-MS, is not limited by the non-volatility and high molecular weight of analytes, and enables the determination of both conjugated and unconjugated steroids. Derivatization is not necessary for analysis by LC-MS, however, it can be used to enhance the ionization efficiencies of some steroids, leading to high sensitivity and specific detection (Higashi and Shimada, 2004). Nonetheless, the limitations of using LC- or GC-MS for the determination of steroids in tissues are the interferences of the matrix and the high detection limits of these methods. Thus, mass spectrometry has seldom been used to quantify vertebrate-type sex steroids in invertebrates (Table 1.7).

In addition to unconjugated steroids, some studies require the quantification of conjugated steroids. In these cases, steroid-glucuronids and –sulfates can be hydrolysed using preparations of η-glucuronidase and sulfatase. Hydrolysis may also occur in solvents such as ether or ethyl acetate (solvolysis).

1.8.2 The investigation of sex steroid metabolism

The metabolism of vertebrate-type steroids can be studied *in vivo*, or most frequently *in vitro* either using cell lines or incubating tissue homogenates, tissue minces, tissue slices or whole isolated organs.

In vivo methods are based on the administration of a labeled precursor and the follow-up of the metabolites in urine (non-invasive methods), blood or tissues (invasive methods).

In vitro methods are based on the incubation of tissue minces, tissue slices or whole isolated organs. The organs where steroidogenesis occurs can be removed from the animal and kept viable, therefore providing an isolated organ method for assessing steroidogenic pathways. Once isolated, these organs can be used as a whole or further processed into sections or minced organ preparations. Although the organ has been removed from the animal, the integrity and interrelationship of the cells and tissues within the organ remain intact. Nevertheless, *in vitro* incubations, particularly when tissue homogenates are used, are carried out in artificial media which must inevitably contain very different relative concentrations of proteins, hormones, co-factors, etc. from those which are present *in vivo*. Finally, *in vitro* metabolism can also be assessed in cell lines. Most of the steroidogenic pathways are found in specific cells in the ovary (ovarian follicle cells) and testis (Leydig cells). Therefore, isolated and cultured cells can be used to assess

steroidogenesis. Numerous cell lines are available for mammalian models, however, for most species primary cell cultures is the only alternative.

Different results can be obtained depending on the matrix incubated (e.g. whole animal, tissue slices, whole homogenates, or sub-cellular fractions). The aims of the study and the characteristics of the animals will determine the most adequate approach. Higher metabolic rates have been obtained in tissue homogenates compared to tissue pieces (Voogt et al., 1986), and limited diffusion rate of the precursor into the intact tissue has been suggested to be responsible for this difference. However, while homogenates indicate the metabolic potency of the tissue, it might not be representative of the situation *in vivo*. Incubation parameters, such as time, substrate concentration or pH, may affect the profile of metabolites obtained. This has to be taken into consideration when different studies are compared.

After the incubation, steroids are extracted, identified and quantified, by similar procedures as the ones described above. The complexity of the extraction and purification procedures depends on the matrix used in the metabolic assay, the nature of the precursor used, and the identification technique to be applied. For instance, the extraction and purification will be more complex if metabolism is assessed in whole organ incubations than if microsomal incubations are used, and if non-radiolabeled precursors instead of radiolabeled precursors are used.

Identification of steroids usually relies on chromatographic techniques (thin layer chromatography –TLC- or high pressure liquid chromatography –HPLC-) coupled with radiometric, UV or mass spectrometry detectors.

1.8.3 Thin layer chromatography

Thin-layer chromatography consists of a stationary phase (usually silica or alumina) immobilized on a glass, metal, or plastic plate and a solvent. The sample, either liquid or dissolved in a volatile solvent, is deposited as a spot on the stationary phase. One edge of the plate is then placed in a solvent reservoir and the solvent moves up the plate by capillary action. When the solvent front reaches the other edge of the stationary phase, the plate is removed from the solvent reservoir. The different components in the mixture move up the plate at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase. When radiolabeled precursors are used, the separated spots can be visualized by autoradiography (either developed in X-ray film or using a Radioimager).

For a definite identification of a metabolite, comigration with standards should be tested in several solvent systems. Some standards and non-radioactive metabolites (e.g. those that have an $\div^4$-3-oxo group) can be visualized by ultraviolet light, otherwise they can be visualized by placing the

plate in iodine vapor or by acid spraying followed by charring. Alternatively, the metabolites can be extracted out of the plate and processed for mass spectrometry analysis. When metabolites are radiolabeled, recrystallization to constant specific radioactivity can also be used as a reliable identification method.

1.8.4 High pressure liquid chromatography

High Performance Liquid Chromatography (HPLC) is an analytical technique where a liquid permeates through a porous solid stationary phase and elutes the solutes usually into a flow-through detector. In Reversed Phase separations organic molecules are separated based on their degree of hydrophobicity. A radiometric, a UV, or a MS detector can be used to visualize the metabolites. A radiometric detector has usually higher sensitivity, and fewer problems with interferences. In addition, some metabolites can not be visualized in a UV detector. On the other hand, MS provides definite identification of the metabolites, whereas the radiometric and UV detection methods do not. In the later cases, in order to reach definite identification of the metabolites similar procedures as described for the identification of TLC metabolites are required (i.e. comigration with standards in several solvent systems, recrystallization to constant specific radioactivity, mass spectrometry analysis).

1.9 The test species

1.9.1 Mollusca

Molluscs form a highly diverse invertebrate phylum that includes bivalves, gastropods, cephalopods, limpets, slipper shells, and tusk shells. In this study, we have selected the marine bivalves *Crassostrea virginica*, *Mytilus galloprovincialis* and *Mytilus edulis* (bivalvia), and the fresh-water snail *Marisa cornuarietis* (gastropoda; Figure 1.10).

- Bivalves such as ***Crassostrea virginica*** and ***Mytilus sp.*** are widely used in monitoring programs to assess environmental health of coastal environments. Indeed, several characteristics make them ideal organisms for biomonitoring programs. They are widespread (found in littoral communities of most seas), sessile, have very low metabolic rates for xenobiotics, which together with their feeding behavior (filtrators), leads to high bioaccumulation factors.

- ***Marisa cornuarietis*** naturally occurs in lakes, rivers and other freshwater bodies in Central and North America. The snail occupies the upper water levels and tends to follow leafy vegetation. In Puerto Rico, *Marisa sp.* has been found to thrive in both non-polluted and heavily polluted waters. Eggs are laid in gelatinous clusters in the water typically

among aquatic vegetation. *M. cornuarietis* is a dioic species. *M. cornuarietis* females develop imposex when exposed to organotin compounds, and has been shown to be sensitive to other mechanistically diverse endocrine disruptor compounds, such as cyproterone acetate and vinclozolin, bisphenol A and octylphenol (Oehlmann et al., 2000; Tillmann et al., 2001). In addition, they are large enough to allow the measurement of steroids and enzyme activities.

1.9.2 Echinodermata

Echinoderms are a major phylum of deuterostome invertebrates that include a number of species playing a key-role in numerous marine ecosystems. By their occurrence in coastal and estuarine waters, they are directly exposed to anthropogenic contaminants. A number of these contaminants have been shown to affect several aspects of their physiology, such as reproduction (den Besten et al., 1989), or early development (Pagano et al., 2000). In this study, we selected as a model echinoderm the sea urchin ***Paracentrotus lividus*** (echinoidea, Figure 1.10), a well-known key-species of commercial importance in the Atlantic ocean and the Mediterranean. This organism is a very sensitive model to a variety of pollutants during the first stages of development (Graillet et al., 1993; Pagano et al., 2000), and has been widely used in embryotoxicity tests.

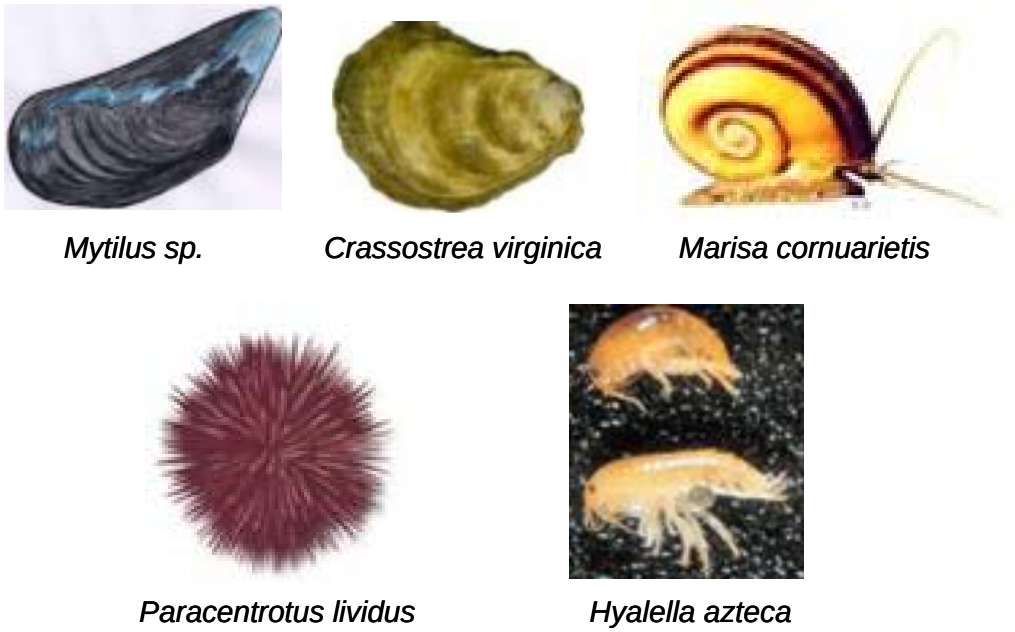

Figure 1.10. The test species: *Mytilus sp*., *Crassostrea virginica*, *Marisa cornuarietis*, *Paracentrotus lividus*, and *Hyalella azteca*.

1.9.3 Arthropoda

Within arthropoda, crustaceans are potential indicator species of endocrine disruption due to their economic importance, ecological significance, and extensive use as model invertebrates in laboratory toxicity tests. Several examples of possible endocrine disruption as a consequence of exposure to anthropogenic substances in crustacean species have been discussed in section 1.2.1. This study selected the amphipod ***Hyalella azteca*** (crustacea, Figure 1.10), because it represents a primary food source for fish, birds, amphibians, and other invertebrates, and is commonly used as a test species in standardized (sediment) toxicity tests. Thus, it can also be considered ecologically relevant for use as a sentinel species for the evaluation of environmental endocrine disruption.

1.10. Objectives of this thesis

The aim of this thesis is to establish whether environmental pollutants can cause endocrine disruption in invertebrate species by altering sex steroid metabolism, and consequently steroid levels. For this purpose, the steroid metabolism was characterized and possible targets of endocrine disruption in different invertebrate species were identified. Next, the effects of model xenobiotics on steroid metabolism were evaluated *in vitro*. And finally, in a selected phylum, the molluscs, exposures to xenobiotics were performed in order to assess alterations in steroid metabolism and steroid levels *in vivo*.

The results of this research are presented in two chapters (chapters 2 and 3). The first one focuses on the characterization of steroid metabolism and the effects of environmental pollutants *in vitro*. The second chapter investigates the effects caused by the *in vivo* exposure to a variety of xenobiotics in different mollusc species. The publications included in these two chapters are briefly presented below.

Chapter 2. *Characterization of steroid metabolism in invertebrate species and the effects of environmental pollutants in vitro.*

Paper 1 characterizes phase I metabolism of androstenedione and testosterone in three invertebrate species: *Hyalella azteca* (crustacean), *Marisa cornuarietis* (mollusc) and *Paracentrotus lividus* (echinoderm), and investigates the *in vitro* effect of organotin compounds and fenarimol on those pathways.

> Janer, G., LeBlanc, G.A., Porte, C., 2005. A comparative study on androgen metabolism in three invertebrate species. General and Comparative Endocrinology, in press.

Paper 2 reports on the presence of high levels of estradiol and dehydroepiandrosterone (DHEA) acyl-CoA acyltransferase in digestive gland and gonad of the oyster *Crassostrea. virginica*, and presents a basic enzymatic characterization, including kinetic parameters for different fatty acyl-CoA.

Janer G, Mesia-Vela, S, Porte C, Kauffman, FC. Esterification of vertebrate-like steroids in the oyster *Crassostrea virginica*. Steroids 69, 129-136.

Paper 3 describes the detection of low levels of sulfotransferase activity found in *C. virginica*, and investigates the hypothesis that the presence of high sulfatase activity in the digestive gland of molluscs may hamper the determination of sulfotransferase activity.

Janer G, Mesia-Vela, S, Kauffman, FC, Porte C. Sulfatase activity in the oyster *Crassostrea virginica*: its potential interference with sulfotransferase determination. Aquatic Toxicology, in press.

Paper 4 characterizes testosterone sulfotransferase and acyl-CoA acyltransferase activities in three invertebrate species: *Hyalella azteca* (crustacean), *Marisa cornuarietis* (mollusc) and *Paracentrotus lividus* (echinoderm), and investigates the *in vitro* effect of organotin compounds and fenarimol on those pathways.

Janer, G., Stenberg, R.M., LeBlanc, G.A., Porte, C., 2005. Steroid conjugating activities in invertebrates: are they target for endocrine disruption? Aquatic Toxicology, 71: 273-282.

Additional experiments on chapter 2. This section compiles additional experiments that complement the data presented in papers 1 to 4. Particularly, it reports on the minor contribution of common polar phase II metabolic pathways, i.e. glucuronidation, glucosidation and sulfation, and on the existence of new phase II metabolites in molluscs, and investigates the specificity of acy-CoA acyltransferase for different steroid substrates.

Chapter 3. *In-vivo effects of xenobiotic exposures on steroid levels and steroid metabolism in molluscs.*

Paper 5 presents the effects of estradiol exposure on the levels of free (unesterified) and esterified steroids and on different steroid metabolic pathways in *Mytilus galloprovincialis*.

Janer, G., Lavado, R., Thibaut, R., Porte, C. 2005. Effects of 17η-estradiol exposure in the mussel *Mytilus galloprovincialis*: the regulating role of steroid acyltransferases. Aquatic toxicology. in press.

Paper 6 reports on the effects in *Mytilus edulis* of exposure to crude oil and the mixture of crude oil and alkylphenols on the levels of free (unesterified) and esterified steroids and on different steroid metabolic pathways.

Lavado, R., Janer, G., Porte, C. 2005. Steroid levels and steroid metabolism in the mussel *Mytilus edulis*: the modulating effect of dispersed crude oil and alkylphenols. Aquatic toxicology. submitted.

Paper 7 characterizes levels of free (unesterified) and esterified steroids, as well as acyl-CoA steroid acyltransferases in *Marisa cornuarietis*, investigates the presence of sexual dimorphisms in these parameters, and describes the effects of a long-term *in vivo* exposure to TBT on the levels of free and esterified steroids and on acyl-CoA:testosterone acyltransferase, and compares them with the responses induced by exposure to methyl-testosterone and fenarimol (a model androgen and a fungicide, respectively).

Janer, G., Lyssimachou, A., Beachman, J., Oehlmann, J., Schutle-Oehlmann, U., Porte C., 2005. Sexual dimorphism in esterified steroid levels in the gastropod *Marisa cornuarietis*: the effect of xenoandrogenic compounds. Steroids, submitted.

Paper 8, based on the results obtained in paper 1, further characterizes the sexual dimorphism in androstenedione metabolism in *Marisa cornuarietis*, and investigates the effects of TBT and TPT *in vitro* and *in vivo* on these enzymatic pathways.

Janer, G., Bachmann, J., Oehlmann, J., Schutle-Oehlmann, U., Porte C., 2005. The effect of organotin compounds on gender specific androstenedione metabolism in *Marisa cornuarietis*. Journal of Steroid Biochemistry and Molecular Biology, submitted.

CHAPTER 2. CHARACTERIZATION OF STEROID METABOLISM IN INVERTEBRATE SPECIES AND THE EFFECTS OF ENVIRONMENTAL POLLUTANTS *IN VITRO*.

Paper 1

A COMPARATIVE STUDY ON ANDROGEN METABOLISM IN THREE INVERTEBRATE SPECIES.

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A comparative study on androgen metabolism in three invertebrate species

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Abstract

A comparative approach was taken in this study to evaluate androgen (androstenedione and testosterone) metabolism in three invertebrate species: the gastropod *Marisa cornuarietis*, the amphipod *Hyalella azteca*, and the echinoderm *Paracentrotus lividus*. The existence of 17β/3β-hydroxysteroid dehydrogenase (HSD) and 5α-reductase catalyzed reactions was demonstrated in all three species. Androstenedione was primarily converted to 5α-androstanedione in *M. cornuarietis*, while it was primarily metabolized to testosterone in *P. lividus* and *H. azteca*. In addition, and consistent with vertebrate findings, tissue specific pathways and sexual dimorphism in androgen metabolism were observed. Namely, testosterone was metabolized to dihydrotestosterone in *P. lividus* gonads (via 5α-reductase), and metabolized to 4-androstene-3β,17β-diol in the digestive tube (via 3β-hydroxysteroid dehydrogenase). Furthermore, the synthesis of 17β-reduced metabolites of androstenedione (testosterone and dihydrotestosterone) was 3- to 4-fold higher in males of *M. cornuarietis* than in females. Organotin compounds, which have been shown to interfere with some aspects of androgen metabolism, had no major effect on testosterone metabolism in any of the three species. Fenarimol enhanced 5α-reductase-mediated catalysis in gonads of *P. lividus*. Overall, results demonstrate the ubiquity of some androgen biotransformation processes in invertebrates and reveals interphyla differences in androgen metabolic pathways, and different sensitivity of these pathways to some xenobiotics.

Keywords: Testosterone; Androstenedione; Metabolism; Mollusc; Crustacean; Echinoderm

1. Introduction

Current knowledge on endocrine regulation of development and reproduction in aquatic invertebrates is rather limited. The endocrine systems of vertebrates and invertebrates function similarly: it transduces signals, either environmental or endogenous, to appropriate target sites to regulate gene transcription (LeBlanc et al., 1999). However, most lineages of invertebrate phyla diverged from vertebrates early in evolution and, accordingly, significant divergences in endocrine strategies with vertebrates, and among invertebrates, are likely. Vertebrate-like sex steroids, e.g., testosterone, androstenedione, and estradiol (Reis-Henriques et al., 1990; Verslycke et al., 2002), and proteins with specific binding to those steroids (Di Cosmo et al., 2002; Keshan and Ray, 2001) occur in several invertebrate groups.

Key steps of steroidogenesis leading to the synthesis of androgens or estrogens have been measured in invertebrate species. For example, pregnenolone is converted to progesterone through the action of 3β-HSD/Δ^4–Δ^5 isomerase in *Asterias rubens* (Schoenmakers, 1979), *Cancer pagurus* (Swevers et al., 1991), and *Mytilus edulis* (De Longcamp et al., 1974). 17β-HSD catalyzes the reversible conversion of androstenedione to testosterone in *Crassostrea gigas* (Le Curieux-Belfond et al., 2001), and *Lytechinus variegatus* (Wasson et al., 1998). The hydroxy-

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lation of testosterone and progesterone at various positions of the steroid ring has been described in *Daphnia magna* (Baldwin and LeBlanc, 1994a), *Rudipates decussata* (Morcillo et al., 1998), and *A. rubens* (Voogt et al., 1987), suggesting the involvement of multiple P450 isoenzymes in the metabolism. 5α-Reductases acting upon testosterone, but also upon progesterone or androstenedione, are found in *L. variegatus* (Wasson et al., 1998) and *R. decussata* (Morcillo et al., 1998). And 5α-reduced steroids are substrates of 3β-hydroxysteroid dehydrogenases (3β-HSD) in *L. variegatus* (Wasson and Watts, 2000), *D. magna* (Oberdörster et al., 1998), and *Littorina littorea* (Ronis and Mason, 1996).

As evidenced above, our understanding of steroid metabolic pathways among invertebrate phyla is fragmentary. Methodological differences (e.g., in vivo vs. in vitro studies) and different assay conditions (e.g., substrate concentrations), prevent an adequate comparison of the reported activities among invertebrate phyla. This fragmented knowledge on invertebrate endocrine systems and the variety of endocrine strategies within different invertebrate groups, are some of the factors that hamper the progress on understanding endocrine disruption by environmental chemicals in these species.

The present study was designed to assess androgen metabolism in three invertebrate species representing different phyla using both testosterone and androstenedione as model substrates. In addition, potential differences in susceptibility of the metabolic pathways to perturbation by xenobiotics—tributyltin (TBT), triphenyltin (TPT), and fenarimol—were evaluated. These comparative studies were performed using the echinoid *Paracentrotus lividus* (Echinodermata, Deuterostome), the gastropod *M. cornuarietis* (Mollusca, Protostome), and the amphipod *Hyalella azteca* (Arthropoda, Protostome). The echinoid and gastropod were sufficiently large to allow evaluations of metabolic activities associated with specific tissues. In these cases, gonads, considered the main tissue for steroidogenesis and steroid action, and digestive tube, involved in the metabolism of endogenous and exogenous compounds, were evaluated. Total tissue preparations of the amphipod were used in the metabolic assays due to their diminutive size.

2. Methods

2.1. Chemicals

Organotin compounds and fenarimol were obtained from Sigma (Steinheim, Germany). [1β-^{3}H]androstenedione (15–30 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA, USA), and [4-^{14}C]testosterone (50–60 mCi/mmol) was purchased from Amersham (Buckinghamshire, England). Unlabeled steroids were obtained from Sigma (Steinheim, Germany) and Steraloids (Wilton, NH, USA). NADPH was obtained from Sigma (Steinheim, Germany). All solvents and reagents were analytical grade from Merck (Darmstadt, Germany).

2.2. Animals

Adult *P. lividus* (35–50 mm ambital diameter) were collected from the Ligurian coast (Italy), taken to the laboratory, and gonads and digestive tube were immediately dissected, deep-frozen in liquid nitrogen, and stored at −80 °C. Sex was determined by microscopic evaluation of gonadal smears. Adult *M. cornuarietis* were obtained from Live-Aquaria (Rhinelander, WI, USA) and acclimated to laboratory conditions for at least 1 week prior to dissection. Gonad-digestive gland complex was dissected, deep-frozen in liquid nitrogen, and stored at −80 °C. Sex was determined macroscopically by the presence of the albumen gland (females) or the penis and testis (males). Adult *H. azteca* were obtained from Environmental Consulting and Testing (Superior, WI, USA) and acclimated to laboratory conditions for at least 1 week prior to use in experiments.

2.3. Sample preparation

Selected tissues of *P. lividus* individuals, *M. cornuarietis* (2–3 pooled individuals), and whole tissue of *H. azteca* (15–20 pooled individuals) were homogenized in ice-cold 100 mM potassium phosphate buffer, pH 7.4, containing 100 mM KCl (150 mM KCl for the sea urchin), 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM phenanthroline, and 0.1 mg/mL trypsin inhibitor. Amphipod homogenates were centrifuged at 9000*g* for 30 min, and the supernatant (S9-fraction) freshly used for the biochemical assays. Snails and sea urchin homogenates were centrifuged at 10,000*g* for 20 min. After a further centrifugation at 100,000*g* for 60 min, the supernatant, termed cytosol, was collected and stored at −80 °C. The pellet was resuspended in homogenization buffer and centrifuged again at 100,000*g* for 60 min. After centrifugation, the pellet, termed microsomal fraction, was resuspended in microsomal buffer, consisting of 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM phenanthroline, and 0.1 mg/mL trypsin inhibitor, and 20%w/v glycerol. Microsomal buffer for sea urchin also contained 150 mM KCl. Microsomes were stored at −80 °C until assays were performed. Protein concentrations were determined by the method of Bradford, 1976, by using commercially available reagents (Bio-Rad, Hercules, CA) and bovine serum albumin as standard.

2.4. Androgen metabolism

Androstenedione metabolism was assessed by incubating microsomal, cytosolic, and S9-fraction proteins (200–

400 μg) in 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, 10 μM [^{3}H]androstenedione (150,000 dpm), and 1.0 mM NADPH, in a final volume of 250 μL. A concentration of 0.2 μM [^{3}H]androstenedione (150,000 dpm) was used for the assays with *M. cormuarietis* due to the low K_m of the enzymatic reaction. The reaction was initiated by the addition of NADPH and incubated in constant shaking for 15 min (*H. azteca*, *P. lividus*) and for 60 min (*M. cormuarietis*) at 35 °C. Incubations were stopped by adding 250 μL of acetonitrile and after centrifugation (1500g, 10 min), 200 μL of supernatant was injected onto the RP-HPLC column.

Testosterone metabolism was assessed by the method described by Baldwin and LeBlanc (1994a) with some modifications. Microsomal, cytosolic and S9-fractions (200–400 μg protein) were incubated in 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, 10 μM [^{14}C]testosterone (150,000 dpm), and 1.0 mM NADPH, in a final volume of 400 μL. The reaction was initiated by the addition of NADPH, and incubated in constant shaking for 60 min (20 min for *P. lividus*) at 35 °C. To assess linearity, samples were incubated for different periods of time (5–60 min). Incubations were stopped by adding ethyl acetate (2 mL × 2), the ethyl acetate fraction was collected and evaporated under a nitrogen stream, and the extract spotted onto thin layer chromatography (TLC) plates. In addition, for a further identification of the metabolites, some of the samples were analyzed by HPLC. In that case, the incubation was stopped by adding 250 μL acetonitrile, and after centrifugation (1500g, 10 min), 200 μL of supernatant was injected onto the RP-HPLC column.

To evaluate the effect of model pollutants on testosterone metabolism, microsomes (*P. lividus* and *M. cormuarietis*) and S9-fraction (*H. azteca*) were pre-incubated for 5 min in the presence of TPT, TBT, and fenarimol. Chemicals were first investigated at a concentration of 100 μM and when a striking effect was detected, assays were repeated at 100, 30, 10, 3, and 1 μM. The xenobiotics were delivered to the assay solutions in absolute ethanol. The concentration of ethanol in the assay was below 1%, and was kept constant in all the assays. Control microsomes were incubated with the carrier alone.

2.5. HPLC-radiometric detection

HPLC separations were performed on a PerkinElmer Binary LC pump 250 system equipped with a 250 × 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), 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). Separation of testosterone metabolites was performed at 1 mL/min with a mobile phase composed of (A) 75% water and 25% acetonitrile and (B) 45% water and 55% acetonitrile. The run was set as follows: 0–40 min, linear gradient from 100% A to 100 % B, followed by 10 min, 100% B. Radioactive metabolite 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. Identification of the metabolites was based on the comparison of their retention times to those obtained for standards commercially available and monitored at 254 nm using a UV-detector (Knauer LC-photometer), and their analyses by gas chromatography–mass spectrometry (GC–MS).

2.6. Thin layer chromatography-autoradiography

The dry residues obtained after testosterone metabolism assays were redissolved twice in 35 μL of ethyl acetate and spotted with a micropipette onto 20 × 20 cm aluminum-backed silica gel thin layer chromatography (TLC) plates (Whatman, Maidstone, Kent, England). Metabolites were resolved using methylene chloride:acetone (4:1 v/v) followed by chloroform:ethyl acetate:ethanol (4:1:0.7 v/v/v) (Baldwin and LeBlanc, 1994a) (TLC system A), and visualized and quantified by electronic autoradiography (Packard, Instant Imager, Downers Grove, IL). Metabolites were quantified individually, except for hydroxylated testosterone products, which, due to their low formation rates, were quantified together as hydroxylated metabolites (Parks and LeBlanc, 1998). Metabolite identification was based on co-migration with commercial standards in different solvent systems. The solvent systems used were as follows—B, two consecutive migrations in methylene chloride:acetone (8:2); C, chloroform:ethyl acetate:ethanol (4:1:0.7, v/v/v); D, methylene chloride:acetone (1.8:1, v/v); E, methylene chloride:ethyl ether (9:1, v/v); and F, *t*-butyl-methyl-ether:hexane (6:4). Unlabeled steroids were visualized under UV light, and by spraying the TLC plate with sulphuric acid:methanol (1:1) and charring (2–4 min at 70 °C). Unknown metabolites were extracted from the TLC plates, and analysed by gas chromatography–mass spectrometry (GC–MS).

2.7. Gas chromatography–mass spectrometry

The metabolites were analyzed by gas chromatography–mass spectrometry (GC–MS) (EI+) as trimethylsilyl (TMS) derivatives and the chemical structures were identified by comparison of the retention times and the mass spectra with authentic standards. Silylation was

achieved using 100 μL bis(trimethylsilyl)trifluoroacetamide (BSTFA) and allowed to stand 1 h at 70 °C. After the reaction, the samples were evaporated to dryness under a nitrogen stream, and redissolved in isooctane before injection. Mass spectra were obtained on a Fisons GC 8000 Series chromatograph coupled to a Fisons MD800 mass spectrometer fitted with a HP-5MS (30 m × 0.25 mm i.d., crosslinked 5% PH ME siloxane) column (Hewlett–Packard). The carrier gas was helium at 1 mL/min. The oven temperature was programmed as follows: 90–140 °C at 12 °C/min and from 140 to 320 °C at 6 °C/min. The injector temperature was 280 °C and the ion source and the analyzer were maintained at 200 and 250 °C, respectively.

2.8. Data analyses

The Michaelis–Menten parameters (K_m and V_{max}) were estimated by analyzing Lineweaver–Burk plots. Statistical significance for inhibition studies was assessed by using one way ANOVA (Dunnett's test for differences from control). Sex differences were assessed by Student's t test. Level of significance was $P < 0.05$.

3. Results

3.1. Androgen metabolism in P. lividus

Subcellular fractions (cytosol and microsomes) isolated from the gonad and digestive tube of *P. lividus* readily converted androstenedione to testosterone in the presence of NADPH. Androstenedione was more actively metabolized by digestive tube than by gonads. After 15 min incubation, 92 ± 4% (microsomes) and 95 ± 1% (cytosol) of the androstenedione was metabolized to testosterone in the digestive tube, and 32 ± 10% (microsomes) and 54 ± 11% (cytosol) in gonads ($n = 3$). Due to the rapid conversion of androstenedione to testosterone, further studies were performed using testosterone as a substrate.

Gonad microsomal fractions metabolized testosterone to several oxido-reduced and hydroxylated metabolites in the presence of NADPH. The major metabolite detected was 5α-androstan-3β,17β-diol (5α-A-diol), together with minor amounts of dihydrotestosterone (DHT), androstenedione (AD), and several hydroxylated metabolites (T-OHs) (Table 1). The formation of all metabolites was NADPH-dependent, and under the assay conditions, product formation occurred at a linear rate for up to 60 min.

Cytosolic fractions metabolized testosterone to an unknown metabolite (UNK1) and AD in the presence of NADPH (Table 1). No metabolites were formed in the absence of NADPH. The rate of formation of AD was linear for up to 60 min, while the formation rate of UNK1 decreased after 20 min incubation. When UNK1 was extracted from the TLC plate, and incubated with cytosolic proteins, either in the presence or in the absence of NADPH, testosterone was formed, indicating that the pathway was reversible. Retention factors (R_f)

Table 1
Metabolism of testosterone by the three invertebrate species tested

	P. lividus		*M. cornuarietis*	*H. azteca*[a]
	Gonad	Digestive tube	Gonad-digestive gland	Whole animal
Microsomes				
5α-Dihydrotestosterone	6.4 ± 1.6	n.d.[c]	n.d.	n.d. to 1.4
5α-Androstane-3β,17β-diol	22.8 ± 3.9[b]	—[b]	n.d.	n.d.
4-Androstene-3β,17β-diol	—[b]	111 ± 12[b]	n.d.	3.7 ± 0.4
Androstenedione	1.5 ± 0.4	25 ± 3	0.8 ± 0.1	7.0 ± 1.1
Σ Hydroxytestosterone	2.4 ± 1.2	4.7 ± 1.6	1.4 ± 0.5	4.3 ± 1.2
Total metabolism	31 ± 5	141 ± 15	2.2 ± 0.5	15 ± 2
Cytosol				
Androstenedione	6.6 ± 1.2	6.7 ± 1.7	0.8 ± 0.1	
Androstenedione[d]	n.d.	92 ± 21	n.d.	
4-Androstene-3β,17β-diol[e]	15 ± 2	13 ± 2	n.d.	
4-Androstene-3α,17β-diol	n.d.	5.2 ± 0.9	n.d.	
Polar metabolite (UNK2)	n.d.	n.d.	4.0 ± 0.1	
Total metabolism	21.8 ± 1.3	25 ± 2	4.8 ± 0.1	

Values are mean ± SEM ($n = 4$–6) expressed as pmol/min/mg protein.

[a] S9-fraction instead of microsomal fraction was used. Data were obtained from two pools of 10–15 organisms.

[b] 5α-Androstane-3β,17β-diol partially comigrated with 4-androstene-3β,17β-diol in the TLC system A and values given in the table include the sum of both metabolites. HPLC analyses showed that 4-androstene-3β,17β-diol was the major metabolite in digestive tube microsomes (>90%), and than 5α-androstane-3β,17β-diol was the major metabolite in gonad microsomes (>80%).

[c] n.d.: not detected.

[d] Determined in the absence of NADPH.

[e] First mentioned in the text as UNK1.

for UNK1 in the different TLC solvent systems (see Section 2 for details) were as follows: 0.66 in solvent system A, 0.56 in B, 0.50 in C, 0.47 in D, 0.14 in E, and 0.19 in F.

The identification of UNK1 was accomplished by GC-MS, and the spectrum obtained is shown in Fig. 1. The *m/z* 434 ion indicates that the metabolite had a molecular weight of 290 (434 − (2 × 73) + 2). The *m/z* 344 and 354 ions originated from loss of the TMS-OH groups (−90); the *m/z* 239 and 329 ions indicate the loss of the CH_3 group. The most abundant ions, *m/z* 73 correspond to the TMS group [$(CH_3)_3Si$], and *m/z* 143 originated from the fragmentation at the A ring (Fig. 1). The formation of this ion requires both, the presence of a 4-ene structure, and a hydroxyl group at the 3-position. Thus, 4-androstene-3α,17β-diol and 4-androstene-3β,17β-diol were analyzed as possible candidates. The mass spectra of both isomers were almost identical, but the retention time allowed to discriminate between the 3α and 3β isoform, and the identification of the metabolite formed in gonad cytosol as 4-androstene-3β,17β-diol.

Testosterone was also actively metabolized by digestive tube microsomal fractions of *P. lividus*, the specific activity was up to 4.5-fold higher than that measured in gonads (Table 1). The major metabolite formed was 4-A-diol, together with minor amounts of 5α-A-diol, AD, and T-OHs. The metabolism was NADPH dependent, and linear up to 60 min for AD, although the synthesis of 4-A-diol and T-OHs decreased after 20 and 40 min incubation, respectively. Similarly, cytosolic fractions isolated from digestive tube metabolized testosterone to 4-A-diol, AD, and an additional metabolite which was identified as 4-androstene-3α,17β-diol. These metabolites were formed in the presence of NADPH, but in the absence only AD was formed (Table 1). This pathway (testosterone → AD), that was reversed when NADPH was added to the incubation mixture, was not detected in gonad cytosol, where no metabolites where formed in the absence of NADPH.

The metabolism of testosterone was assessed separately in males and females, and no statistically significant differences were observed in the rate of metabolites formed.

3.2. Androgen metabolism in H. azteca

S9-fractions isolated from *H. azteca* and incubated in the presence of NADPH readily metabolized AD to testosterone, which was further converted to several metabolites. In fact, 75 ± 6% of the androstenedione was metabolized to testosterone after 15 min incubation. Therefore, further assays were performed using testosterone as substrate, which in the presence of NADPH, was metabolized to AD and 4-A-diol—that represented 47 and 25% of the metabolites formed, respectively—and minor amounts of T-OHs and dihydrotestosterone (Table 1). The total testosterone metabolism was of 15 pmol/min/mg protein. No metabolites were formed in the absence of NADPH.

3.3. Androgen metabolism in M. cornuarietis

Microsomal fractions isolated from the gonad-digestive gland complex of *M. cornuarietis* metabolized androstenedione to 5α-androstanedione, 5α-dihydrotestosterone, testosterone, and hydroxylated metabolites

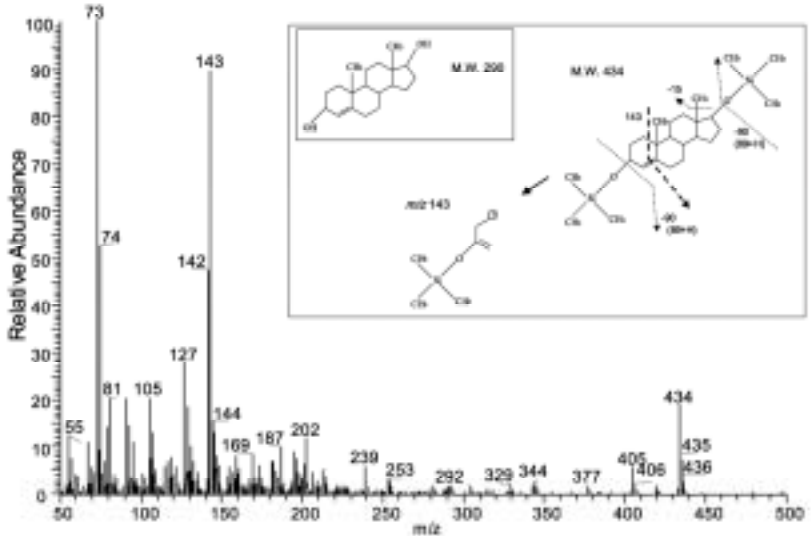

Fig. 1. Mass spectra of the gonad cytosol metabolite after derivatization, identified as 4-androstene-3β,17β-diol and proposed fragmentation.

(Table 2). The metabolism of androstenedione was NADPH-dependent and linear up to 60 min. Significant differences between sexes were observed: males produced DHT and testosterone at a significantly greater rate than females (35 pmol/h/mg protein vs. 9 pmol/h/mg protein), whereas females produced 5α-androstanedione at a significantly greater rate than males (25 pmol/h/mg protein vs. 3 pmol/h/mg protein) (Table 2). When 5α-androstanedione was isolated and further incubated with digestive gland-gonad microsomal fractions, we observed the formation of DHT, suggesting that sexual dimorphism in the metabolism of androstenedione was mainly due to higher 17β-HSD activity in males (5α-androstanedione → DHT; AD → testosterone). No metabolites were observed when androstenedione was incubated with cytosolic fractions.

Testosterone was metabolized to AD, and hydroxylated metabolites by digestive gland-gonad microsomal fractions at an average rate of 2 pmol/min/mg protein (Table 1), while cytosolic fractions formed AD, and an additional metabolite, whose formation was NADPH-dependent, and proved to be highly polar, but could not be identified (UNK2).

3.4. Effects of organotins and fenarimol on testosterone metabolism

TPT and TBT, at concentrations of 100 μM, did not significantly affect the metabolism of testosterone in any of the organisms tested, except for a 12% inhibition of *P. lividus* gonad 5α-reductase caused by TPT (Fig. 2). In contrast, 100 μM fenarimol significantly increased the formation of 5α-reduced metabolites in microsomal fractions isolated from gonads of *P. lividus*. The formation of DHT and 5α-A-diol was significantly increased (122 ± 11 and $20 \pm 4\%$, respectively; $n = 3$) when *P. lividus* gonad microsomes were incubated in the presence of 100 μM fenarimol. The activation of 5α-reductase, the enzyme catalyzing the conversion of testosterone to dihydrotestosterone, was evident at concentrations as low as 10 μM fenarimol, and increased in a concentration-dependent manner (Fig. 3). Kinetic studies indicated that fenarimol increased the V_{max} of 5α-reductase from 230 ± 4 to 347 ± 4 pmol/min/mg ($P < 0.05$), without a major change in the K_m (69 ± 3 μM vs. 58 ± 2 μM). Fenarimol did not alter the metabolism of testosterone in *M. cornuarietis* nor *H. azteca* (Fig. 2).

Table 2
Metabolism of androstenedione by male and female *M. cornuarietis*

Metabolite	Male	Female
5α-Androstanedione	3.3 ± 0.7	$24.6 \pm 2.2^*$
5α-Dihydrotestosterone	27.9 ± 3.4	$8.2 \pm 1.1^*$
Testosterone[a]	7.4 ± 0.8	$0.7 \pm 0.2^*$
Hydroxylated metabolites[a]	0.7 ± 0.3	0.4 ± 0.3

Values are mean ± SEM ($n = 12$) expressed as pmol/h/mg protein.
[a] Those samples that were below detection limit were assigned the value of 0.1 pmol/h/mg protein, which is the detection limit divided by 2.
* Indicates significant differences with males ($P < 0.05$).

4. Discussion

Different androgen metabolic pathways were detected in microsomal fractions isolated from gonads and digestive tube of the echinoderm *P. lividus*. Thus, 5α-reductase, 17β-HSD, 3β-HSD, and several P450 isoforms catalyzing different hydroxylations were observed in microsomal fractions, whereas 17β-HSD, and 3α/β-HSD catalyzed activities were detected in the cytosol (Figs. 4A and B). Some of those pathways, 5α-reductase, 17β-HSD, and 3α/β-HSD-catalyzed reactions, have been previously reported for other echinoderm species (Hines et al., 1992; Voogt et al., 1986; Wasson et al., 1998; Wasson and Watts, 2000). However, the identification of 4-androstene-3β,17β-diol, as the major testosterone metabolite detected in digestive tube and gonads (cytosol), had not been previously reported in echinoderms. Its 3α-isoform, 4-androstene-3α,17β-diol, was formed by catfish intestinal and hepatic microsomes incubated with testosterone (Lou et al., 2002), but it is still unknown whether any of the two isomers play a physiological role in fish and echinoderms.

Androgen metabolizing enzymes were found to have a different tissue distribution in *P. lividus*. Thus, enzymes leading to the biosynthesis of active steroids were mainly found in the gonads, where testosterone was metabolized to 5α-dihydrotestosterone (5α-reductase catalyzed pathway), and further metabolized to 5α-androstane-3β,17β-diol (3β-HSD) (Fig. 4A), while in the digestive tube, no DHT was detected, and testosterone was readily metabolized to 4-androstene-3β,17β-diol or to androstenedione in the absence of NADPH (Fig. 4B). DHT is a potent androgen in humans (Wilson, 2001) and in most vertebrates, including fish (Sperry and Thomas, 1999a,b). However, up to now, there is not information available on whether DHT might have a similar role in echinoderms. This study shows that DHT, synthesized by gonad microsomal fractions, was further metabolized to 5α-androstane-3β,17β-diol, a strong β-estrogen receptor agonist in vertebrates (Pak et al., 2005; Weihua et al., 2002). Wasson et al. (2000) found that echinoid ovaries synthesized more 5α-A-diols than testes. Indeed, the same trend was observed in this study (28 ± 5 and 17 ± 4 pmol/min/mg protein in females and males, respectively), although differences were not statistically significant.

For *H. azteca*, the metabolism of androstenedione and testosterone by S9 fractions indicated the presence of 17β-HSD, 3α/β-HSD, and 5α-reductase catalyzed

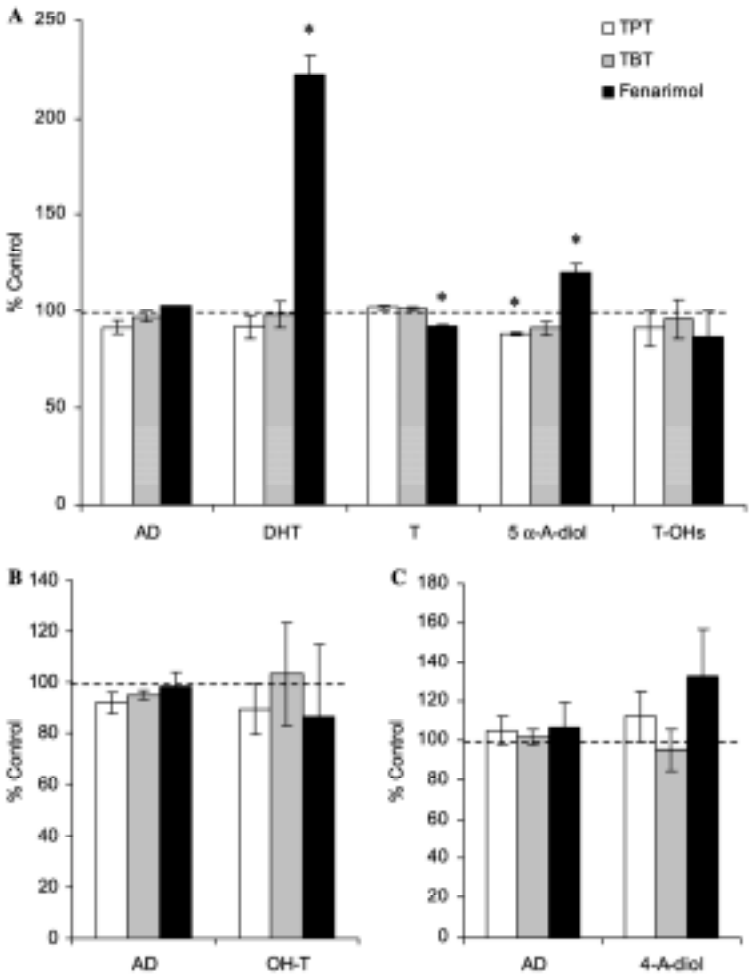

Fig. 2. Effect of TPT, TBT and fenarimol (100 μM) on testosterone metabolism in *P. lividus* (A), *H. azteca* (B) and *M. cornuarietis* (C). Values are mean and SEM ($n = 3$). *Denotes significant differences with respect to controls ($P < 0.05$).

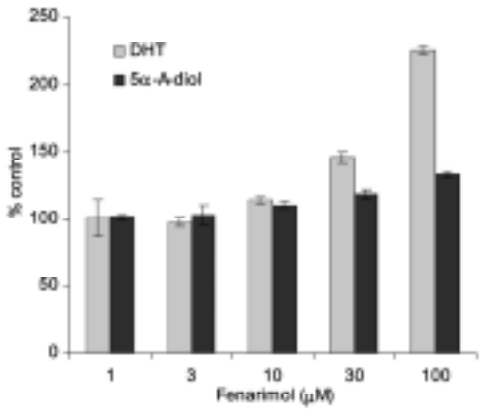

Fig. 3. Effect of different fenarimol concentrations (1–100 μM) on the formation of 5α-reduced metabolites (DHT and 5α-A-diol) by *P. lividus* gonads. Values are mean range ($n = 2$).

pathways, together with several P450-isoforms catalyzing different hydroxylations (Fig. 5). This is in agreement, with previous work that described HSDs as very active enzymes in steroid metabolism in several crustacean species (Baldwin and LeBlanc, 1994b; Blanchet et al., 1972; Swevers et al., 1991).

A different androgen metabolic profile was detected for *M. cornuarietis* (Fig. 6). The major metabolic pathway was the 5α-reduction of androstenedione to form 5α-androstanedione, which was further reduced in position 17 to form DHT. This pathway and the formation of low levels of testosterone from androstenedione, suggested the presence in the microsomal fractions of 17β-HSDs with higher affinity for 5α-androstanedione than for androstenedione. In addition, the formation of dihydrotestosterone and testosterone was 3- to 4-fold higher in males than in females, indicating sexual dimorphism in androgen metabolism. The low met-

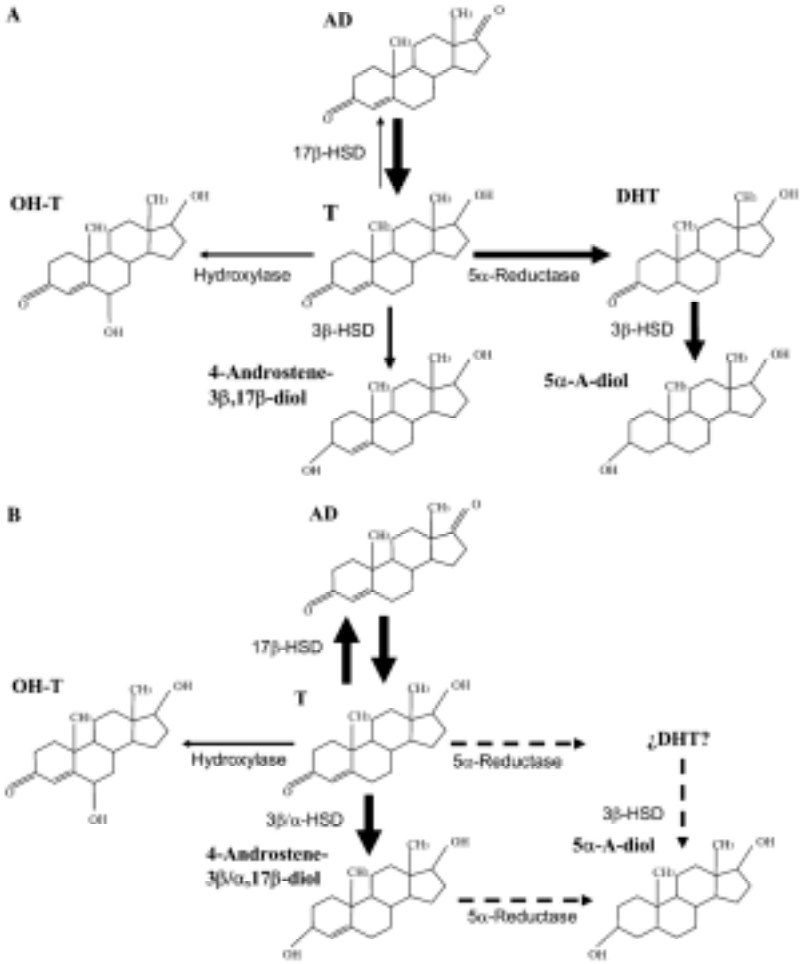

Fig. 4. Androstenedione and testosterone metabolic pathways in *P. lividus* gonad (A) and digestive tube (B). Width of the arrows indicates relative metabolic rates. Discontinuous lines indicate possible formation pathway for an identified metabolite. HSD: hydroxysteroid dehydrogenases: T, testosterone; AD, androstenedione; DHT, dihydrotestosterone; 5α-A-diol, 5α-androstane-3β,17β-diol; OH-T, hydroxylated metabolites.

abolic activities evidenced in *M. cornuarietis* (for both androstenedione and testosterone) in comparison with the other species tested, are supported by the fact that no hydroxylated nor reduced metabolites, and only minor amounts of oxidated metabolites (<10%) were observed in the incubation media after in vivo exposure of *M. cornuarietis* to [^{14}C]testosterone (data not shown). Indeed, *M. cornuarietis* bioaccumulated most of the radioactivity as fatty acid conjugates (84–95%), similarly to what was earlier observed in the gastropod *Ilyanassa obsoleta* (Gooding and LeBlanc, 2001).

Finally, the potential effects of organotin compounds and fenarinol on the in vitro metabolism of testosterone were tested. TBT and TPT, at concentrations as high as 100 μM, did not cause major changes in phase I metabolism of testosterone on microsomal fractions of *P. lividus* and *M. cornuarietis*, or S9-fraction of *H. azteca*. Organotin compounds, and particularly TBT and TPT, have been shown to inhibit 5α-reductases, 3β-HSD, and 17β-HSD in vitro in subcellular fractions isolated from human or rat tissues (Doering et al., 2002; Lo et al., 2003; McVey and Cooke, 2003). Nonetheless, steroid substrate concentrations used in those mammalian studies were 1–3 orders of magnitude lower than the ones reported in this study (8 nM–1 μM vs. 10 μM), that were selected after kinetic studies were performed, and a higher ratio xenobiotic/endogenous substrate might have resulted in higher sensitivity to inhibition in the mammalian studies. But also, earlier studies on molluscs reported no effects, or contradictory results for the effect of TBT on 5α-reductases or hydroxysteroid dehydrogenases. Both an increase and a decrease of testosterone oxidation to androstenedione were reported in *Littorina littorea* (Ronis and Mason, 1996) and *R. decussata* (Morcillo et al., 1998). 5α-reductase

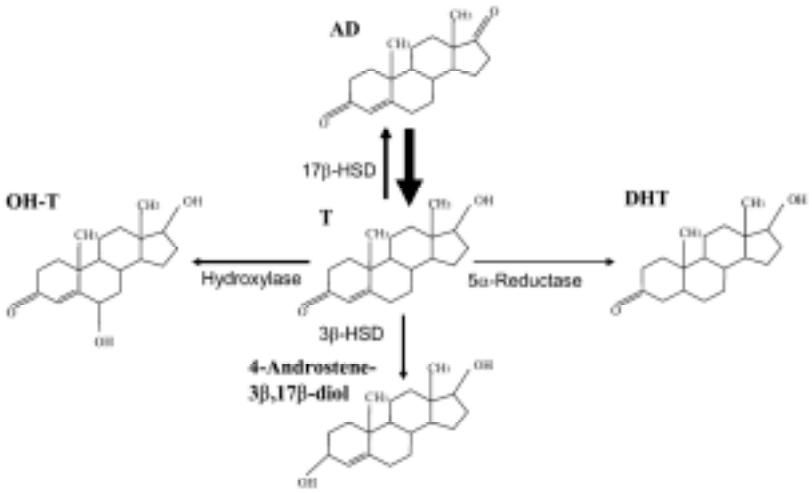

Fig. 5. Androstenedione and testosterone metabolic pathways in *H. azteca*. Width of the arrows indicates relative metabolic rates. Abbreviations: same as in Fig. 4.

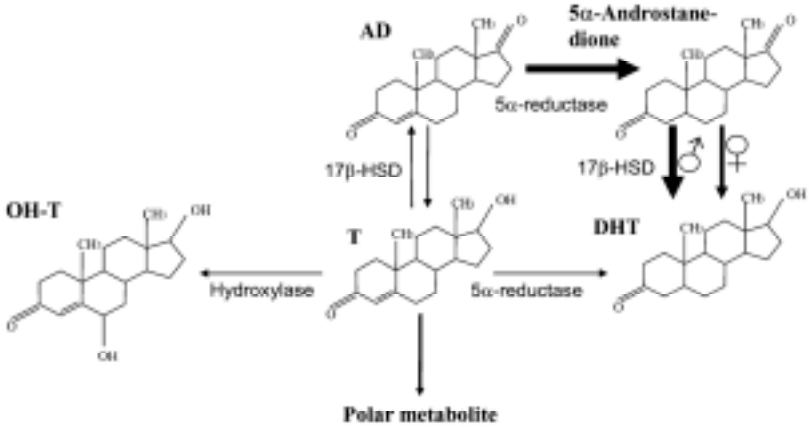

Fig. 6. Androstenedione and testosterone metabolic pathways in *M. cornuarietis* gonad-digestive gland complex. Width of the arrows indicates relative metabolic rates. Abbreviations: same as in Fig. 4.

was inhibited in *L. littorea* exposed to TBT (Ronis and Mason, 1996) but not in *R. decussata* (Morcillo et al., 1998). Only P-450 aromatase was consistently inhibited by TBT in all the studies reported so far (Le Curieux-Belfond et al., 2001; Morcillo and Porte, 1999; Ronis and Mason, 1996). Thus, 5α-reductase and hydroxysteroid dehydrogenases, are probably not specific targets for organotin compounds 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 5α-Adiol. 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 5α-reductase activity is not known, and requires further investigation. Ongoing in vivo exposure experiments of *P. lividus* to fenarimol will reveal whether the increase in 5α-reductase activity, and consequently in the levels of DHT, is also observed in vivo, and the potential physiological consequences if any. For comparative purposes, the effect of fenarimol on 5α-reduction of androstenedione by *M. cornuarietis* was investigated. In contrast to the results obtained in *P. lividus*, 5α-reductase was not activated by fenarimol, but inhibited in the snail (data not shown).

To summarize, the metabolism of androstenedione revealed the existence of significant differences between *M. cornuarietis* (Fig. 6) and the other two invertebrate species, *P. lividus* and *H. azteca* (Figs. 4 and 5), which similarly to vertebrates readily converted androstenedione to testosterone. Regarding testosterone metabolism,

P. lividus had a rather active and complex metabolism, involving a variety of pathways, while *M. cornuarietis* had a limited ability to metabolize testosterone, and an intermediate situation (metabolic rate and number of metabolites formed) was found for *H. azteca*. One of the major differences between the androgen metabolic profiles detected in this study and those reported for vertebrates is the rather low levels of hydroxylated testosterone metabolites detected in invertebrates, whereas they are usually major metabolites of testosterone in mammals and fish (e.g., Parks and LeBlanc, 1998; Wilson and LeBlanc, 1998). Apart from this, strong similarities were found between the species used in this study and vertebrate species. Most of the metabolites identified in this study are common vertebrate metabolites, with the exception of 4-androstene-3β,17β-diol.

Altogether, these results indicate the existence of (a) both similarities and differences in the pathways involved in the metabolism of androgens in the three invertebrate species tested, (b) tissue specific pathways in androgen metabolism in *P. lividus*, and (c) sex differences in androstenedione metabolism in *M. cornuarietis*, leading to a higher formation of testosterone and dihydrotestosterone in males than in females. Moreover, despite the limited number of chemicals tested in vitro, the obtained data suggest the existence of further differences in sensitivity among different invertebrate groups. Thus, from a risk assessment point of view, species representing different phyla and thus showing different pathways of steroid metabolism should be included when testing endocrine disrupting substances.

Acknowledgments

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Paper 2

ESTERIFICATION OF VERTEBRATE-LIKE STEROIDS IN THE OYSTER *CRASSOSTREA VIRGINICA*

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Esterification of vertebrate-type steroids in the Eastern oyster (*Crassostrea virginica*)

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Abstract

Characteristics of acyl-coenzyme A (acyl-CoA):steroid acyltransferase from the digestive gland of the oyster *Crassostrea virginica* were determined by using estradiol (E2) and dehydroepiandrosterone (DHEA) as substrates. The apparent K_m and V_{max} values for esterification of E2 with the six fatty acid acyl-CoAs tested (C20:4, C18:2, C18:1, C16:1, C18:0, and C16:0) were in the range of 9–17 μM E2 and 35–74 pmol/min/mg protein, respectively. Kinetic parameters for esterification of DHEA (K_m: 45–120 μM; V_{max}: 30–182 pmol/min/mg protein) showed a lower affinity of the enzyme for this steroid. Formation of endogenous fatty acid esters of steroids by microsomes of digestive gland and gonads incubated in the presence of ATP and CoA was assessed, and at least seven E2 fatty acid esters and five DHEA fatty acid esters were observed. Some peaks eluted at the same retention times as palmitoleoyl-, linoleoyl-, oleoyl/palmitoyl-, and stearoyl-E2; and palmitoleoyl-, oleoyl/palmitoyl-, and stearoyl-DHEA. The same endogenous esters, although in different proportions, were produced by gonadal microsomes. The kinetic parameters for both E2 (K_m: 10 μM; V_{max}: 38 pmol/min/mg protein) and DHEA (K_m: 61 μM; V_{max}: 60 pmol/min/mg protein) were similar to those obtained in the digestive gland. Kinetic parameters obtained are similar to those observed in mammals; thus, fatty acid esterification of sex steroids appears to be a well-conserved conjugation pathway during evolution.

Keywords: Esterification; Steroid; *Crassostrea virginica*; Digestive gland; Gonad

1. Introduction

Vertebrate-like sex steroids, e.g. testosterone, androstenedione, and estradiol, have been found in several groups of invertebrates [1–5], including molluscs [6,7]. Although their origin is still a subject of controversy, a number of studies point to an endogenous source and a physiological role of these steroids in molluscs. Evidence in support of this possibility include the following: (i) several steroid biosynthetic pathways present in vertebrates have been identified [8–10]; (ii) a temporal variation in steroid titers and some biosynthetic pathways coinciding with reproductive stages have been found [8,11]; and (iii) alterations in sexual characteristics or reproduction have been observed when molluscs are exposed to estrogenic or androgenic compounds: e.g. 1-methyl-1,4-androstadiene-3,17-dione caused imposex (the imposition of male sexual characteristics in females) in the whelk *Nassarius reticulatus* [12], and estradiol-induced vitellogenesis in the pacific oyster *Crassostera gigas* [13].

Phase I metabolism of sex steroids, i.e. hydroxylation and reduction, has been detected in several protostome invertebrates. The presence of 20α-, 17β-, and 3β-hydroxysteroid dehydrogenases have been demonstrated in crustacea, and C_{17}–C_{20} lyase was found in the gonads of the crab *Cancer pagurus* [14,15]. Several monohydroxy-testosterone metabolites have also been detected in *Neomysis integer* and *Daphnia magna* [5,16], and 17β-hydroxysteroids were demonstrated in *Homarus americanus* [17]. In mollusca, the presence of similar steroidogenic enzyme systems has been described. Steroid reductases and dehydrogenases were found in the gastropod *Clione antartica* [18], while 17β-hydroxysteroid dehydrogenase, 5α-reductase, 3α-hydroxysteroid dehydrogenase, and an aromatization system were found in the snail *Helix aspersa* [6]. In bivalve molluscs, the presence of 3β- and 17β-hydroxysteroid dehydrogenases, C_{17}–C_{20} lyase, and 5α-reductase were also demonstrated in gonad homogenates of *Mytilus edulis* incubated with labeled precursors [8]. Aromatase activity

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was found in microsomal fractions isolated from the digestive gland of the mussel *Mytilus galloprovincialis* [10] and in gonad–digestive gland homogenates of the oyster *Crassostrea gigas* [9].

Despite the available information on Phase I metabolism of sex steroids in invertebrates, data regarding their conjugation are rather limited and based primarily on in vivo observations. For example, conjugated 5α-dihydrotestosterone was detected in the ovary and the hemolymph of the decapod *Nephrops norvegicus*, although the nature of the conjugate was not described [19]. Glucuronyl and sulfate conjugates of progesterone metabolites (based on hydrolysis procedures) were observed in the gastropod *C. antartica* after exposure to [^{3}H]progesterone [18]. Several studies have shown that other phenolic compounds are conjugated to polar metabolites with sulfate and glucosyl moieties in invertebrates [20–24], suggesting that polar conjugates of steroid hormones are also formed by sulfation and glucosidation.

Apart from this, apolar conjugation may also play a key role in invertebrates. Recently, apolar conjugation was shown to be the major pathway of testosterone metabolism (more than 70%) in the snail *Ilyanassa obsoleta* exposed to 1 μM [^{14}C]testosterone in water [25]. In addition, several invertebrates have been shown to readily convert sterols to fatty acid conjugates, although most of the knowledge is restricted to ecdysteroids [26–28]. Fatty acid esters of ecdysteroids are formed enzymatically in various insects species and are common metabolites in larvae and adults [29–31]. Also, gastropods have been shown to esterify sterols, such as cholesterol, brassicasterol, campesterol, sigmasterol, and β-sitosterol [32,33].

The present work was designed to assess the esterification (acyl-CoA:steroid acyltransferase activity) of two model steroids, estradiol and dehydroepiandrosterone, by microsomal fractions isolated from both the digestive gland and gonads of the Eastern oyster *Crassostrea virginica*. The digestive gland was selected because it is the tissue with a major metabolic role in bivalves, and gonads were chosen because they are considered to be the site for steroid synthesis. Estradiol and dehydroepiandrosterone were selected as model steroids because they are conjugated at different sites: DHEA is conjugated at the 3-OH position, and E2 is conjugated at the 17-OH position [34]. In addition, these steroids are normally used when assessing acyl-CoA:steroid acyltransferase in vertebrate species [35] and, therefore, facilitate inter-phyla comparisons.

2. Experimental

2.1. Chemicals

[2,4,6,7,16,17-^{3}H]Estradiol (110 Ci/mmol) and [1,2,6,7-^{3}H]dehydroepiandrosterone (60 Ci/mmol) were purchased from NEN Life Science Products Inc. Estradiol, dehydroepidandrosterone, and the lithium salt of acyl-CoA fatty acids (oleoyl-CoA (C18:1), stearoyl-CoA (C18:0), arachinoyl-CoA (C20:4), palmitoyl-CoA (C16:0), linoleoyl-CoA (C18:2), and palmitoleoyl-CoA (C16:1)) were purchased from Sigma. All solvents were of HPLC grade and were purchased from Fisher Scientific.

2.2. Tissue preparation

Eastern oysters (*C. virginica*), 4 years old, were obtained from Prince Edward Island (Canada). They were maintained up to a month in a recirculating saltwater system and fed *Isochrysis galbana* and fish food pellets daily until dissection.

Digestive glands and gonads were dissected, frozen in liquid nitrogen, and kept at −80 °C until used. Cellular fractions were prepared as described previously [36]. Samples were homogenized in ice-cold 10 mM Tris–HCl, pH 7.6, containing 150 mM KCl and 0.5 M sucrose, and centrifuged at 12,000 × *g* for 45 min at 4 °C. After centrifugation at 100,000 × *g* for 90 min, pellets were washed and centrifuged for further 30 min. Finally, the pellet, termed microsomal fraction, was resuspended in 20 mM Tris–HCl, pH 7.6, containing 20% (w/v) glycerol.

Protein content was determined with the BCA™ Protein Assay kit (Pierce Chemical Co.) according to the supplier's instructions using bovine serum albumin as a standard.

2.3. Enzyme assays

Assays for the esterification of estradiol and dehydroepiandrosterone were based on the method described by Xu et al. [37] with slight modifications. Briefly, microsomes (25–100 μg protein) were incubated at 30 °C for 30 min in a final volume of 0.25 ml 100 mM sodium acetate buffer, pH 6.0, in the presence of E2 (0.1–50 μM; 0.05–2 μCi) or DHEA (0.1–150 μM; 0.5–2 μCi) and 100 μM of a fatty acid acyl-CoA. Endogenous conjugation was assayed in the presence of 1 mM CoA and 10 mM ATP. The reaction was stopped by the addition of 0.3 ml of ice-cold sodium acetate buffer, pH 6.0, and 4 ml ethyl acetate. The samples were vortexed immediately and centrifuged for 10 min at 3000 × *g*. The ethyl acetate extract was removed and evaporated to dryness under a stream of nitrogen. Each resulting residue was dissolved in 130 μl of methanol and analyzed by HPLC.

Separation and measurement of the esterified metabolites were achieved by HPLC with a Spherisorb ODS column (5-μm particle size, 250 mm × 4.6 mm i.d.). The HPLC system consisted of a Waters 600E solvent gradient programmer, a Waters Lambda-Max model 481 UV detector, and a radioactive flow detector (β-ram from IN/US) as described by Xu et al. [37]. The solvent system consisted of acetonitrile/0.1% acetic acid in H_2O/methanol. The solvent gradient used for elution of the E2 esters from the column was: 12 min isocratic at 30/6/64; 6 min with a number 10 convex gradient to 60/0/40; 15 min isocratic at 60/0/40; 2 min with a number 2 convex gradient to 20/0/80; 5 min isocratic at

20/0/80; and the column was then returned to initial conditions over 15 min. The flow rate was 1.2 ml/min. DHEA esters were eluted in the isocratic mode with 100% methanol at the same flow rate.

3. Results

3.1. Optimization of the esterification assay

The activity of acyl-CoA:steroid acyltransferase in microsomal fractions isolated from the digestive glands of *C. virginica* was characterized using estradiol and oleoyl-CoA as substrates. The assay for E2 esterification was linear up to 0.1 mg microsomal protein/ml in the presence of 200 nM E2 (Fig. 1A), and at least up to 0.4 mg/ml in the presence of 25 μM E2 (data not shown). Thereafter, all assays were performed using 0.1 mg microsomal protein/ml, except for endogenous esterification, which was studied using high protein concentrations to achieve high sensitivity (up to 1 mg/ml). The formation of oleoyl-E2 was measured as a function of the incubation time, and the reaction rate was linear for at least 40 min (Fig. 1B). All subsequent incubations were for 30 min. The reaction rate increased linearly with temperature over the range of 25–37 °C (Fig. 1C). An incubation temperature of 30 °C was selected for all further assays. Finally, the pH dependence of the enzymatic activity was studied, and its profile is shown in Fig. 1D. Acyl-CoA:steroid acyltransferase was active over a pH range of 4.0–9.0 with an optimum around 6.0. Although optimization assays were performed at pH 5.0, which was the optimum for mammals, all subsequent assays for the molluscan enzyme were performed at pH 6.0.

In addition, microsomal protein was incubated in the absence of cofactors (CoA + ATP or fatty acid acyl-CoA), and the formation of E2 esters was also observed, although the activity measured was 20-fold lower than when a fatty acid acyl-CoA was added. When assays were performed using heat-inactivated microsomes (100 °C for 5 min), no esters were formed.

3.2. Esterification of estradiol in the digestive gland of *Crassostrea virginica*

E2 was esterified to the corresponding fatty acid ester when incubated with any of the six fatty acid acyl-CoAs used: arachidonoyl-CoA, palmitoleoyl-CoA, linoleoyl-CoA, oleoyl-CoA, palmitoyl-CoA, and stearoyl-CoA (Fig. 2). The retention time, and the apparent K_m and V_{max} for each of the different esters are shown in Table 1. K_m for all the esters ranged from 9 to 17 μM; V_{max} differed over two-fold between esters and ranged from 35 to 74 pmol/min/mg protein.

Rates of conjugation were determined using saturating or near saturating amounts of E2 (25 μM) and results are shown in Fig. 3. The rate of formation of linoleoyl-E2 conjugates was significantly lower (about two-fold difference, $P < 0.05$) than noted with the other esters, which occurred at similar rates.

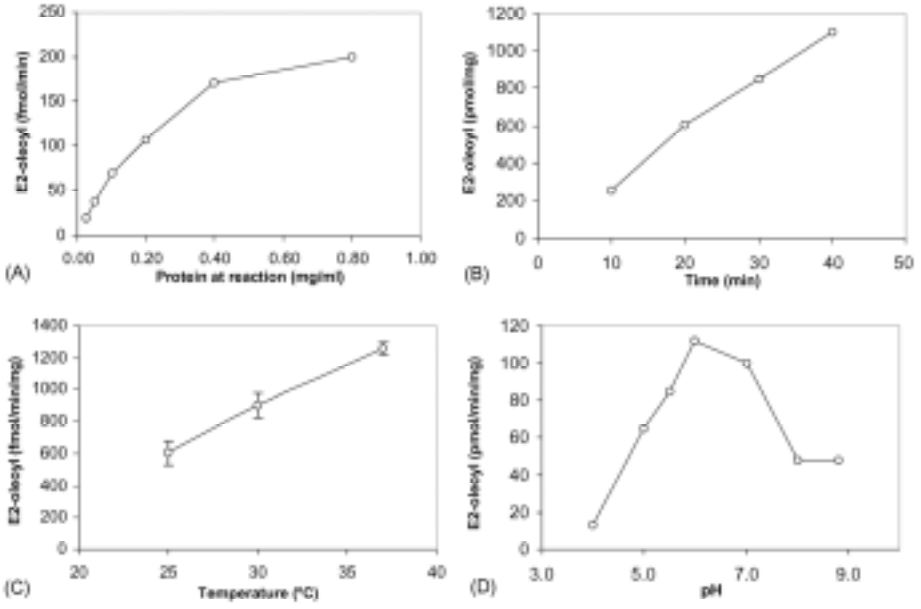

Fig. 1. Effect of the amount of microsomal protein in the reaction system (A), the incubation temperature (B), the incubation time (C), and the pH (B) on acyl-CoA:steroid acyltransferase activity using E2 and oleoyl-CoA as substrates. The concentration of E2 in the assay was 200 nM in experiments A and C and 25 μM in experiments B and D.

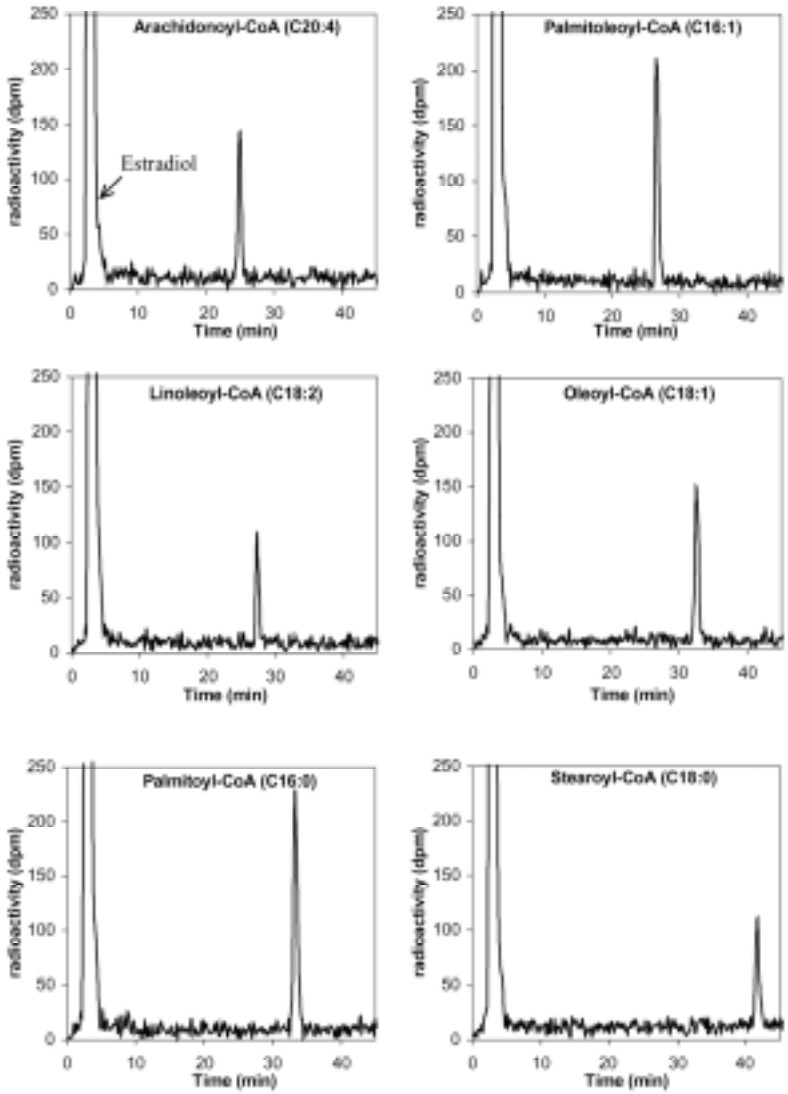

Fig. 2. Esterification of estradiol induced by the addition of specific fatty acid acyl-CoAs. In each experiment, 75 μg of microsomal protein was incubated for 30 min with 25 μM E2 at 30 °C.

Table 1
Retention times of E2 esters and kinetic parameters for esterification of E2 using different fatty acid acyl-CoAs as cofactors

Ester	Retention time	K_m	V_{max}
Arachidonoyl-E2	24:40	9.4 ± 1.8	55 ± 4
Palmitoleoyl-E2	26:33	11 ± 3	66 ± 8
Linoleoyl-E2	27:15	17 ± 5	35 ± 4
Oleoyl-E2	32:22	15.8 ± 1.6	53 ± 2
Palmitoyl-E2	33:12	10.1 ± 1.9	74 ± 6
Stearoyl-E2	41:34	12.3 ± 1.2	44.6 ± 1.7

Values (mean ± S.D.) were obtained from reactions using five concentrations of E2 ranging from 3 to 50 μM.

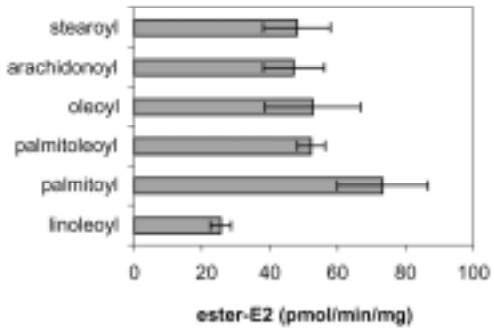

Fig. 3. Esterification of estradiol (25 μM) with six different fatty acid acyl-CoAs. Values are the mean ± S.E.M. ($n = 3$).

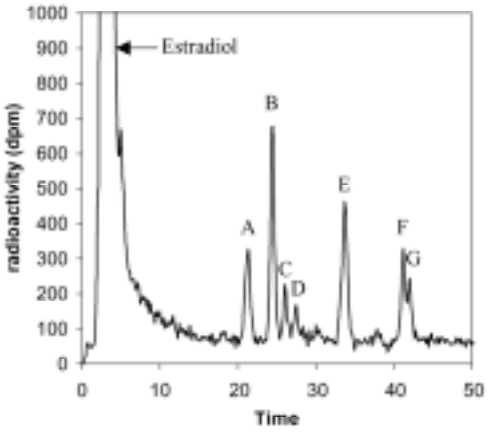

Fig. 4. Endogenous esterification of estradiol. 250 μg of digestive gland microsomal protein was incubated in the presence of 200 nM E2, 10 mM ATP, and 1 mM CoA for 30 min at 30 °C. A and B: unknown; C: palmitoleoyl-E2; D: linoleoyl-E2; E: oleoyl/palmitoyl-E2; F + G: stearoyl-E2.

Endogenous esterification of E2 assessed by incubation of microsomal fractions in the presence of ATP and CoA resulted in the formation of up to seven peaks (Fig. 4). Peaks C, D, E, and F + G were tentatively identified as palmitoleoyl-E2, linoeloyl-E2, oleoyl/palmitoy-E2, and stearoyl-E2, respectively, by comparison of their retention times with those obtained in the presence of specific fatty acid acyl-CoA standards (Table 1). Peaks A and B are unknown. No qualitative differences were observed in the chromatogram profiles when microsomes were incubated in the presence of low (200 nM) and high (25 μM) E2 concentrations. Esterification rates increased linearly with E2 concentration in the range of 200 nM–25 μM.

3.3. Esterification of dehydroepiandrosterone in the digestive gland of Crassostrea virginica

Similarly to E2, DHEA was esterified to the corresponding fatty acid ester when incubated with any of the six fatty acid acyl-CoA substrates studied. The apparent K_m and V_{max} for each of the different esters were calculated, and results are shown in Table 2. K_m values ranged from 45 to 120 μM, and V_{max} values ranged from 30 to 182 pmol/min/mg protein. At a DHEA concentration of 25 μM, the esterification rate was lower than that of E2 for all the esters tested (2.7 ± 0.5-fold).

Table 2
Retention times of DHEA esters and kinetic parameters for esterification of DHEA using different fatty acid acyl-CoAs as cofactors

Ester	Retention time	K_m	V_{max}
Arachidonoyl-DHEA	11:51	109 ± 27	52 ± 8
Palmitoleoyl-DHEA	13:14	120 ± 16	182 ± 14
Linoleoyl-DHEA	14:15	79 ± 17	51 ± 5
Oleoyl-DHEA	18:02	45 ± 10	30 ± 4
Palmitoyl-DHEA	18:29	54 ± 6	81 ± 4
Stearoyl-DHEA	26:38	60 ± 8	87 ± 5

Values (mean ± S.D.) were obtained from reactions using five concentrations of DHEA ranging from 3 to 150 μM.

Up to five peaks were observed when microsomal protein was incubated with DHEA in the presence of ATP and CoA (Fig. 5). Peaks C, D, and E were tentatively identified as palmitoleoyl-DHEA, oleoyl/palmitoyl-DHEA, and stearoyl-DHEA, respectively, whereas peaks A and B are unknown.

3.4. Esterification of estradiol and dehidroepiandrosterone in gonads of Crassostrea virginica

Gonadal microsomes incubated in the presence of ATP and CoA formed the same number of peaks at similar retention times as those detected previously in the digestive gland microsomes. However, the profile of the esters was different (Fig. 5).

When the esterification of E2 was assayed in the presence of palmitoyl-CoA, we found a K_m of 9.7 ± 0.9 μM and a V_{max} of 38 ± 2 pmol/min/mg protein. This K_m was similar to that found in the digestive gland (Table 1). In contrast, when esterification of DHEA was assayed in the presence of palmitoleoyl-CoA, the K_m was 61 ± 22 μM and the V_{max}

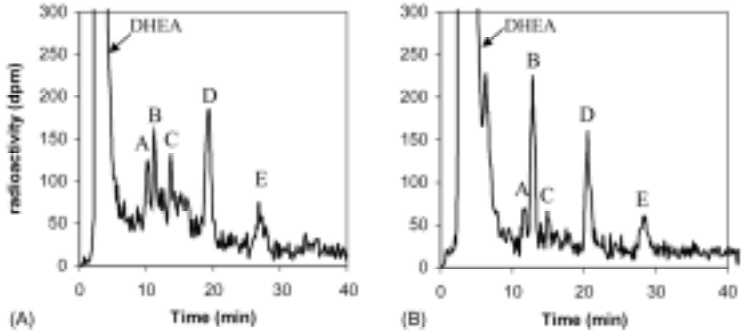

Fig. 5. Endogenous esterification of dehydroepiandrosterone by digestive gland (A) and gonad (B) microsomes. 80 μg of microsomal protein was incubated in the presence of 1 μM DHEA, 10 mM ATP, and 1 mM CoA for 30 min at 30 °C. A and B: unknown; C: palmitoleoyl-DHEA; D: oleoyl/palmitoyl-DHEA; E: stearoyl-DHEA.

was 60 ± 10 pmol/min/mg protein. This K_m was lower than that found in the digestive gland for the same conjugate, although it is in the range of other DHEA esters (Table 2). The V_{max} values for the two conjugative reactions assayed in the gonads were lower than those found in the digestive gland.

4. Discussion

In the present study, we characterized the acyl-CoA:steroid acyltransferase activity in the microsomal fraction of digestive glands and gonads from the oyster *C. virginica*. Activity increased linearly with time (10–40 min), amount of protein (25–100 μg/ml), and temperature (25–37 °C), and was active over a broad range of pH values. The profile of the pH dependence of the activity was very similar to that found for the fatty acyl-CoA:ecdysteroid-22-*O*-acyltransferase in the tobacco budworm *Heliothis virescens* [26], with very low activities at pH values less than 4 and considerable activities at basic pH values. The optimum pH, 6.0, was also in the same range of those reported earlier for the fatty acyl-CoA:ecydsteroid acyltransferase in *H. virescens* and the fatty acyl-CoA:estradiol acyltransferase in rats [26,37].

Oyster tissue microsomes isolated from digestive glands and gonads esterified E2 and DHEA with all the fatty acid acyl-CoA substrates tested, which included 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). Several steroid fatty acid esters (at least seven E2 esters and five DHEA esters) were formed when digestive gland or gonad microsomal fractions were incubated with ATP and CoA. According to their retention times, the esters formed were putatively palmitoleoyl-, stearoyl-, and oleoyl/palmitoyl-DHEA as well as palmitoleoyl-, linoeloyl-, oleoyl/palmitoyl-, and stearoyl-E2. This indicates the presence of similar enzymatic systems in both tissues. The fatty acid esters of steroids found in this study have been reported to be major fatty acid esters in several molluscan species [38–40]. Namely, palmitoyl, stearoyl, palmitoleoyl, and oleoyl represented 17.9, 6.2, 7.2, and 9.1%, respectively, of the total fatty acids detected in the bivalve *M. galloprovincialis* [40]. Some other esters (peaks A and B in Figs. 4 and 5) were formed in the presence of ATP and CoA (endogenous esterification), but they did not coelute with any of the six esters analyzed in this study. Their retention times suggest that they might be polyunsaturated esters.

We demonstrated the ability of preparations from the digestive gland and gonads of the Eastern oyster to esterify both E2 and DHEA. Since DHEA can only be esterified at the 3β-OH position, and assuming that, similar to mammals, E2 is esterified at the 17β-OH position [34], our results would indicate that the oyster can esterify sex steroids at both the 17β-OH and the 3β-OH positions. The K_m values for DHEA were higher than those for E2. In mammals, there is still controversy with respect to whether there are specific enzymes for the esterification of 3-OH and 17-OH positions or not. DHEA has been shown to act as a competitive inhibitor of E2 esterification, suggesting that the same enzyme esterifies both steroids [41]. A similar fatty acid composition for both steroid esters has also been reported supporting the hypothesis of a single enzyme isoform [35]. In general, the HPLC chromatograms we obtained for endogenous conjugation (Figs. 4 and 5) also showed a similar fatty acid composition for both steroids esters and comparable rates of conjugation between different CoA esters for both steroids.

Apolar metabolism of steroids is known to occur in molluscs. De Souza and De Oliveira [32] reported a 15.4% esterification of [^{14}C]cholesterol when incubated in the hemolymph of the mollusc *Biomphalaria glabrata* in the absence of cofactors for 24 h at 37 °C. Several studies suggest that the esterification of cholesterol and other steroids occurs via separate forms of acyl-CoA acyltransferase. In mammals, the profiles of the cholesterol esters formed differ from those of the estradiol esters. Moreover, a potent acyl-CoA:cholesterol acyltransferase inhibitor had no effect on the esterification of E2 or DHEA [34,42].

In contrast to the limited information on the esterification of sex steroid metabolism in molluscs and other invertebrates, there exists an extensive literature on esterification of ecdysteroids in several arthropod species [26,27,43–45], which might provide some insight on a possible role for other steroid esters. In the cricket *Acheta domesticus* [44] and the cockroach *Periplaneta americana* [45], the esters are synthesized in the ovary or transferred to it and then to the eggs, where they represent a storage form of the molting hormone [43] that supplies free steroid during embryogenesis [46]. In fact, the storage role of steroid esters is not restricted to insects. Steroid esters have been used pharmacologically for decades as potent long-acting pharmaceuticals [47] that require enzymatic hydrolysis by esterases to exert their endocrine actions [48].

From a comparative approach, apolar conjugation of steroids in the oyster *C. virginica* saturates at similar concentrations (viz. K_m was 13 μM versus 8 μM E2 for E2 oleoate formation in the oyster digestive gland and rat liver, respectively) and occurs at a similar rate to that found in mammals. At 25 μM E2, 53 and 40 pmol oleoyl-E2/min/mg protein were formed by oyster digestive gland and rat liver, respectively (Mesia-Vela et al., unpublished data). These kinetic parameters were also very similar to those reported for acyl-CoA:ecdysteroid-22-*O*-acyltransferase (K_m: 10 μM; V_{max}: 85 pmol/min/mg) in midgut tissues of the tobacco budworm *H. virescens* [26].

To our knowledge, no physiological differences related to the nature of the fatty acid moiety of a steroid ester are known. If the affinity of steroid esterase(s) differed for different esters, the fatty acid composition of the steroid esters could have physiological relevance. The available information on steroid esterases is rather restricted to mammals. A study using the MCF-7 breast cancer cell line showed that long chain esters, such as the ones studied here, are

hydrolyzed by specific esterases distinct from those that act upon shorter chain esters of E2 (e.g. acetate, propionate, etc.), which are nonspecific esterases [48]. Certainly, further work is needed to elucidate whether invertebrate esterases act upon long or short chain esters and to determine the affinities of these enzymes for the various esters.

Both acyl-CoA acyltransferases and esterases can be modulated by drugs [37]. Compounds that alter esterification or ester cleavage will subsequently modulate hormone availability and activity and may well act as endocrine disrupting substances. In this sense, the effect of different concentrations of TBT on the activity of fatty acid acyl-CoA:steroid acyltransferase was explored in preliminary studies in our laboratory. Preliminary data showed that TBT in the low micromolar range inhibited esterification of E2 and DHEA in vitro, with the esterification of E2 being more sensitive. TBT is an antifouling agent that causes imposex in some gastropod species. Its ability to interact with different steroid metabolic pathways, aromatase, and 5α-reductase, has already been shown [49–51]. However, interaction with conjugation enzymes has been hypothesized as a more plausible mode of action to explain the increased testosterone levels detected in some TBT exposed molluscs [25,52]. The interference of TBT in the esterification of free hormones can affect levels of active steroids within tissues and may be one of the responsible mechanisms of the reported androgenization/feminization phenomena. In fact, esterification of testosterone has already been considered as a possible site of action for the endocrine disrupter TBT [25,53], although further experiments to test this hypothesis are warranted.

In summary, data obtained on *C. virginica* shared many similarities with data available for vertebrate and ecdysteroid esterification in insects, suggesting that esterification is a highly conserved conjugation pathway in evolution. Together with other biosynthesis and conjugation pathways, esterification could modulate endogenous steroids levels in molluscs. Clearly, further research to gain better understanding of the physiological function of the esterification of steroids in marine organisms is needed.

Acknowledgements

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Paper 3

SULFATASE ACTIVITY IN THE OYSTER *CRASSOSTREA VIRGINICA*: ITS POTENTIAL INTERFERENCE WITH SULFOTRANSFERASE DETERMINATION.

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Short communication

Sulfatase activity in the oyster *Crassostrea virginica*: Its potential interference with sulfotransferase determination

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Abstract

Two sulfatase isoforms, a soluble one with an optimum pH of 5.0, and a microsomal one with an optimum pH of 7.6, were observed in digestive gland, gonads, mantle and gills of the oyster *C. virginica*. The highest sulfatase activity was recorded in the digestive gland cytosol and is likely to interfere with the in vitro determination of sulfotransferase activity. Indeed, the sulfatase inhibitor Na_2SO_3 led to an increase of measured sulfotransferase activity ($31 \pm 9\%$), suggesting that those sulfatases might be partially responsible for the low sulfotransferase activities found in *C. virginica*.

Keywords: Sulfatase; Sulfotransferase; Mollusk; Metabolism

Sulfation modulates the biological activity of many xenobiotics and endogenous compounds including steroid hormones. The sulfation pathway is reversible and comprises two enzyme systems: the sulfotransferases, which catalyse the sulfation reaction and are located in the cytoplasm (Kauffman, 2004), and the sulfatases, which catalyse the hydrolysis of sulfate esters and are located in the lysosomes, the nucleus, the mitochondria or the endoplasmic reticulum (Coughtrie et al., 1998; Zhu et al., 1998). The determination of sulfotransferase activity in hepatopancreas/digestive gland cytosol of marine mollusk and crustacean species has indicated low or undetectable activities (Schell and James, 1989; Li and James, 1993; Janer et al., 2005) and has lead to the hypothesis that inhibitors of sulfotransferase or high levels of sulfatases present in hepatopancreas/digestive gland interfere with the determination of sulfotransferase activity in vitro. Indeed, hepatopancreas cytosol of the lobster *Panulirus argus* inhibited sulfotransferase activity present in the sheepshead minnow (*Cyprinodon variegates*) (Schell and James, 1989), and digestive gland cytosol of *Mytilus galloprovincialis* inhibited sulfotransferase activity in red mullet (*Mullus barbatus*) liver cytosol (G. Janer and C. Porte, unpublished data). Several sulfatase enzymes and genes have been reported in mollusks (Spaulding

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and Morse, 1991; Wittstock et al., 2000), and are possibly involved in the catabolism of sulfated polysaccharides in their herbivorous diet. However, there is still little information about the enzyme kinetics and subcellular distribution of sulfatases. Thus, this work was designed to determine sulfatase in the cytosolic and microsomal fractions of the oyster *Crassostrea virginica*, and to explore its possible interference with the measurement of sulfotransferase activities in vitro.

Digestive gland, gonads, gills and mantle of Eastern oysters (*C. virginica*, 4 years old) were dissected, frozen in liquid nitrogen, and kept at −80 °C until used. Subcellular fractions (cytosol, microsomes) were prepared as described previously (Morcillo et al., 1998), with the addition of a second ultracentrifugation step to wash the microsomes. The protein content was determined with the BCA™ Protein Assay kit (Pierce Chemical Co.).

Sulfatase activity was measured as in Zhu et al. (1998) using estrone-sulfate (E_1SO_4) as substrate. The assay tubes contained 20 mM Tris–HCl buffer pH 7.6 (microsomes) or 20 mM sodium acetate buffer pH 5.0 (cytosol), 4 mM $MgCl_2$, 30 μM E_1SO_4 (0.2 μCi; 50 Ci/mmol NEN Life Science Products Inc., Boston, MA), and 30–45 μg of microsomal/cytosolic protein, in a final volume of 150 μL. After 20 min incubation at 30 °C the reaction was stopped by adding 200 μL of ice-cold water, and 3 mL of toluene. The tubes were vortexed, centrifuged (2500 × *g* for 15 min), and 400 μL of the supernatant was counted for [^{3}H] in a β-counter. Sulfatase activity was calculated by the difference of radioactivity in the aqueous phase in the system incubated at 30 °C compared to the same system kept in ice. Blanks without proteins were included in each assay to validate the extraction efficiency.

Sulfatase activity towards E_1SO_4 was linear with up to at least 0.3 mg protein/mL and with time for at least 30 min. Activities also increased linearly with temperatures ranging between 22 and 37 °C. Dependence on pH was investigated and different curves were obtained for the cytosolic and microsomal forms (Fig. 1). The cytosolic activity was optimum at pH 5.0, decreased sharply at pH 6.0 and a very low activity was found at pH 7.0 (6% of that at pH 5.0). In contrast, two optimal pH peaks were observed at pH 5.0 and 7.6 using microsomes as a source of sulfatase. The peak at pH 5.0 might be due to the presence of some residual cytosol in the microsomal fraction, despite of the fact that microsomes were carefully washed. The apparent Km and Vmax were 62 ± 14 μM and 2,263 ± 204 pmol/min/mg protein ($n = 4$) for sulfatase activity in the cytosolic fraction (pH 5.0), and 130 ± 21 μM and 958 ± 53 pmol/min/mg protein ($n = 4$) in the microsomal fraction (pH 7.6). These activities are in the range of those reported in rats towards the same substrate (Zhu et al., 1998), and the pH optimum found in the cytosol and microsomal fractions are similar to those reported for soluble and membrane bound sulfatases, respectively, in numerous species (Hanson et al., 2004). The identification of sulfatase activity in the microsomal fraction indicates the presence of a sulfatase isoform located in the endoplasmatic reticulum, as previously reported for vertebrate species. These results contrast with existing data for invertebrate species that indicate the existence of only soluble isoforms (Hanson et al., 2004). Certainly, the highest sulfatase activity was observed in the cytosolic

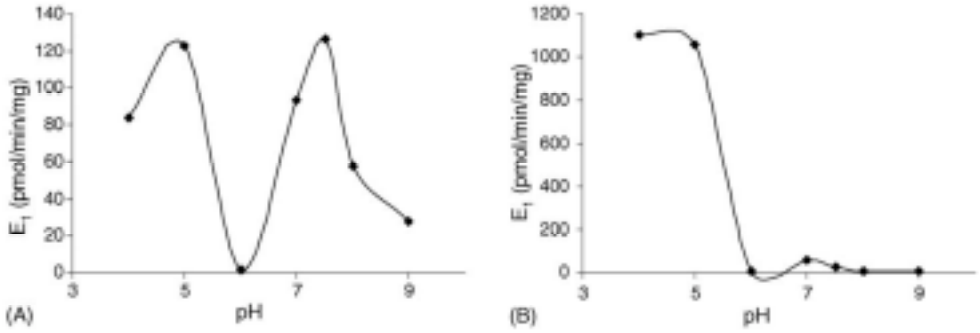

Fig. 1. pH dependence of (A) microsomal and (B) cytosolic E1-SO_4 sulfatase activity. Values are mean of duplicates.

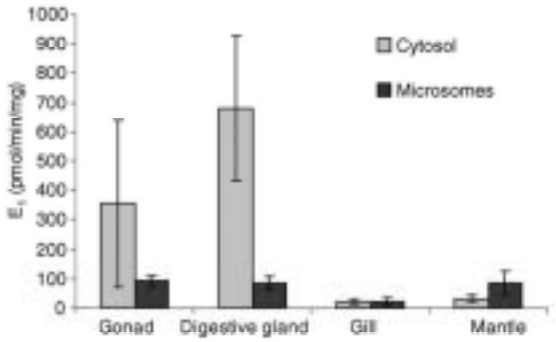

Fig. 2. Tissue distribution of cytosolic and microsomal sulfatase activity (mean ± S.E.M., $n = 3$).

fraction; this corresponds to a soluble isoform, possibly associated to lysosomes, which are very abundant in digestive cells of mollusks (Lobo-da-Cunha, 2000).

Tissue distribution of microsomal and soluble (cytosol) sulfatase activity was assessed by looking at different tissues, namely mantle, gills, gonads and digestive gland. The highest sulfatase activity was detected in digestive gland cytosol, followed by that of the gonad cytosol (Fig. 2). Mantle and gill cytosolic fractions had low sulfatase activity (<5% that of digestive gland). No significant differences in tissue distribution were observed for the microsomal isoform, except for a lower sulfatase activity in gills compared to the other tissues (three- to four-fold, $p < 0.05$) (Fig. 2).

Altogether the data indicate that at least two sulfatase enzymes are present in *C. virginica*. One of them, the soluble isoform, is highly expressed in digestive gland, and is likely to interfere with the determination of sulfotransferases. In order to investigate this interaction, sulfotransferase activity was determined in digestive gland, with *p*-nitrophenol (*p*-NP) as substrate (a model substrate for phenol sulfotransferase SULT1A in vertebrates; Nagata and Yamazoe, 2000). The PAP^{35}S barium precipitation assay (Foldes and Meek, 1973) was used to measure sulfotransferase activity. The assay tubes contained 20 mM Tris–HCl buffer (pH 7.5), 4 mM $MgCl_2$, 5 μM ^{35}S-PAPS (0.05 μCi), cytosolic protein (30 μg) and *p*-NP (5 μM to 1.3 mM), in a final reaction volume of 150 μL. After a 30 min incubation at 30 °C, the reaction was terminated by the addition of 200 μL of 0.1 M barium acetate, 200 μL of 0.1 M barium hydroxide, and 200 μL of 0.1 M zinc sulfate. Tubes were vortex-mixed and centrifuged at 10,000 × *g* for 2 min. An aliquot (500 μL) of the supernatant was directly assayed for [^{35}S] content by liquid scintillation counting.

The apparent Km and Vmax for *p*-NP sulfotransferase in the digestive gland cytosol were 1.1 ± 0.5 mM and 73 ± 18 pmol/min/mg protein, respectively. This Km, although similar to that found in the lobster *Panulirus argus* (0.73 ± 0.03 mM) (Schell and James, 1989), is 500-fold higher than those reported for vertebrates using the same substrate (Honma et al., 2001). The high Km, and the comparatively low Vmax obtained for *C. virginica* (0.073 ± 0.018 versus 1.5 ± 0.5 nmol/min/mg protein in *P. argus*), suggest that sulfotransferase is a low affinity enzyme in mollusks.

When sodium sulphite (Na_2SO_3), an inhibitor of sulfatase, was added to the assay systems at a concentration of 2 mM, it inhibited 84 ± 14% ($n = 3$) of the digestive gland cytosol sulfatase activity, whereas sulfotransferase activity increased (31 ± 9%; $n = 3$). These results suggest that soluble sulfatases interfere with the assessment of phenolic sulfotransferases in vitro. However, even in the presence of the sulfatase inhibitor, the sulfotransferase activity measured was very low. Additional assays were performed to evaluate the activity of other sulfotransferase isoforms, using 50 μM 4-methylumbelliferone, dehydroepiandrostenedione and estradiol as substrates. Similarly to the results obtained for *p*-NP, the sulfation rates towards these substrates were rather low (undetectable to 14 pmol/min/mg protein), indicating that sulfotransferases might play a minor role in xenobiotic and steroid metabolism in *C. virginica*.

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Paper 4

STEROID CONJUGATING ACTIVITIES IN INVERTEBRATES: ARE THEY TARGET FOR ENDOCRINE DISRUPTION?

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Testosterone conjugating activities in invertebrates: are they targets for endocrine disruptors?

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Abstract

Testosterone conjugation activities, microsomal acyltransferases and cytosolic sulfotransferases, were investigated in three invertebrate species, the gastropod *Marisa cornuarietis*, the amphipod *Hyalella azteca*, and the echinoderm *Paracentrotus lividus*. The goals of the study were to characterize steroid conjugation pathways in different invertebrate phyla and to assess the susceptibility of those processes to disruption by environmental chemicals. All three species exhibited palmitoyl-CoA: testosterone acyltransferase activity (ATAT) in the range of 100–510 pmol/min/mg protein. Despite similarities in specific activities, kinetic studies indicated that ATAT had a higher affinity for testosterone but a lower V_{max} in *M. cornuarietis* than in *P. lividus*, and intermediate values were found for *H. azteca*. In contrast, the activity of testosterone sulfotransferase (SULT) was rather low (0.05–0.18 pmol/min/mg protein) in *M. cornuarietis* and *H. azteca*. The low activity precluded kinetic analyses and inhibition studies with these species. *P. lividus* digestive tube displayed high SULT activity (50–170 pmol/min/mg protein) at moderate testosterone concentrations, but was inhibited at high testosterone concentrations. The interference of model pollutants (triphenyltin (TPT), tributyltin (TBT), and fenarimol) with these conjugation pathways was investigated in vitro. Both TPT and TBT (100 μM) inhibited ATAT in *P. lividus* (68 and 42% inhibition, respectively), and appeared to act as non-competitive inhibitors. ATAT activity in *M. cornuarietis* was less affected by organotins, and a significant inhibition (20% inhibition) was detected only with TBT. Fenarimol (100 μM) did not affect ATAT in any of the species tested. Sulfation of testosterone was suppressed by the organotins as well as fenarimol when using cytosolic preparations from *P. lividus*. These results demonstrated the existence of interphyla differences in testosterone conjugation, and revealed that these processes can serve as targets for endocrine disrupting chemicals.

Keywords: Sulfotransferase; Acyl-CoA: testosterone acyltransferase; Echinoderm; Crustacea; Mollusc

1. Introduction

It is now generally accepted that endocrine-disrupting chemicals (EDCs) are at least partially

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responsible for reproductive and developmental alterations in some wildlife populations (Vos et al., 2000). Invertebrates represent 95% of all animal species, and similar to vertebrates, are susceptible to the action of EDCs (LeBlanc et al., 1999). In fact, the development of imposex in gastropods exposed to tributyltin is considered one of the clearest examples of chemically-induced endocrine disruption (WHO, 2002). However, progress in understanding the mechanisms of action of these compounds and the relevance of the observed effects has been hampered by the lack of knowledge on invertebrate endocrinology.

Existing evidence suggest that steroid hormones have a role in endocrine signaling in invertebrates (LeBlanc et al., 1999). Conjugation rates of steroids, together with biosynthetic and deconjugation rates, regulate steroid levels, and play a key role in endocrine homeostasis. However, up to now, limited attention has been paid to phase II metabolism of steroids in invertebrates, and their ability to form polar (sulfated and glucosylated conjugates) or apolar (fatty acid conjugates) metabolites.

The sulfation of steroids may inhibit their biological activity by decreasing their affinity for steroid receptors and increasing their rate of elimination (Strott, 1996). Active steroids may be regenerated from sulfate conjugates by the action of sulfatase enzymes (Strott, 1996). Sulfate conjugates have been frequently identified as the major metabolites of hydroxylated xenobiotics in invertebrates (James, 1987). Sulfate conjugates of steroid hormones have been observed in crustaceans (Swevers et al., 1991; Baldwin et al., 1998) and molluscs (Hines et al., 1996; Ronis and Mason, 1996). The identification of these sulfate conjugates was based on their susceptibility to hydrolysis by sulfatases. Some in vitro studies have been performed in crustaceans using phenolic compounds as substrates for sulfotransferases (Li and James, 1993, 2000; De Knecht et al., 2001). However, to our knowledge, the in vitro sulfation of steroid hormones has not yet been studied in invertebrates.

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). Steroid esters do not bind receptors, but they can be hydrolyzed by esterases to liberate the active steroid (Hochberg, 1998). Several studies have focused on ecdysteroid esterification in insects (Slinger and Isaac, 1988; Whiting and Dinan, 1989; Zhang and Kubo, 1992), and sex-steroid esters have also been reported in molluscs (Gooding and LeBlanc, 2001; Janer et al., 2004a) and echinoderms (Voogt and Van Rheenen, 1986; Voogt et al., 1990).

The interference of xenobiotics with the synthesis and clearance of key sex hormones may alter bioavailable amounts of active hormones within the organism, and be a potential mechanism of endocrine disruption. Studies in vertebrates have shown that several xenobiotics (PAHs, fungicides, alkylphenols) interact with steroid synthesis and clearance (Rocha Monteiro et al., 2000; Kester et al., 2000; Noaksson et al., 2003). Similarly, studies in invertebrates have shown that these pathways are modulated in vivo by exposure to xenobiotics, mainly organotin compounds. Thus, P450 aromatase activity was decreased in *Mytillus galloprovinciallis*, *Baccinum undatum*, and *Rudipates decussata* exposed to tributyltin or inhabiting organotin polluted sites (Morcillo et al., 1998, 1999; Santos et al., 2002). In addition, other studies suggest that the conjugation of steroids is a potential target for xenobiotic compounds. Testosterone sulfation was reduced in *Neomysis integer* and *Littorina littorea* exposed to TBT (Verslycke et al., 2003; Ronis and Mason, 1996) and in *Daphnia magna* exposed to pentachlorophenol (Parks and LeBlanc, 1996). Testosterone-glucose and sulfate conjugation were reduced in *D. magna* exposed to 4-nonylphenol and nonylphenol polyethoxylate (Baldwin et al., 1997; Baldwin et al., 1998). The ability of snails to accumulate testosterone-fatty acid esters in vivo was reduced by exposure to TBT (Gooding et al., 2003).

This work was designed to study steroid conjugating activities in three invertebrate species representing different phyla, and to characterize the effect of (anti)androgenic compounds on these enzymatic pathways, in an attempt to identify potential mechanisms of endocrine disruption. The selected species were the echinoid *Paracentrotus lividus* (echinodermata, deuterostome), the gastropod *Marisa cornuarietis* (mollusca, protostome), and the amphipod *Hyalella azteca* (arthropoda, protostome). The selected xenobiotic compounds for the study were the organotins tributyltin (TBT) and triphenyltin (TPT), and the imidazole-like fungicide fenarimol. Both TPT and TBT induce imposex in gastropods (Spooner et al.,

1991), and spermatogenesis in the ovary of the female abalone *Haliotis gigantea* (Horiguchi et al., 2002), among other effects. Several hypothesis, without consensus, have been postulated concerning the mechanism of action of organotin compounds in invertebrates, including an increase of testosterone levels associated with the inhibition of phase II metabolism (sulfation and esterification) (Ronis and Mason, 1996; Gooding and LeBlanc, 2001). Organotin compounds are embryotoxic in *P. lividus* (Novelli et al., 2002) and have been shown to inhibit arm regeneration in the brittle star *Ophioderma brevispina* (Walsh et al., 1986). Fenarimol is used in agriculture as a systemic fungicide (HIARC, 2001) and has shown reproductive, teratogenic, and oncogenic effects in experimental animals (Hirsch et al., 1987; Gray and Ostby, 1998). It inhibits P450 aromatase activity (Hirsch et al., 1987; Vinggaard et al., 2000; Andersen et al., 2002), affects other enzymes of the CYP gene family involved in the metabolism of steroids (Paolini et al., 1996), and it has both estrogenic and antiandrogenic properties (Vinggaard et al., 1999; Andersen et al., 2002).

2. Methods

2.1. Chemicals

Tributyltin (chloride), triphenyltin (chloride), and fenarimol were obtained from Aldrich Chemical Co. (Steinheim, Germany). [4-^{14}C]Testosterone (specific activity 50–60 mCi/mmol) was purchased from NEN Life Science Products, Inc. (Boston, MA). Unlabeled steroids were obtained from Sigma (Steinheim, Germany). PAPS (3′-phosphoadenosine 5′-phosphosulfate) was obtained from Calbiochem (Darmstadt, Germany) and palmitoyl-CoA from Sigma (Steinheim, Germany). All solvents and reagents were analytical grade from Merck (Darmstadt, Germany).

2.2. Organisms

Adult *P. lividus* (35–50 mm ambital diameter) were collected from the Ligurian coast (Italy). Gonads and digestive tube were dissected, deep-frozen in liquid nitrogen, and stored at −80 °C. Sex was determined by microscopic evaluation of gonadal smears. Adult *M. cornuarietis* were obtained from Live-Aquaria (Rhinelander, WI) and acclimatized to laboratory conditions for at least 1 week prior to dissection. Gonadal-digestive gland complex was dissected, deep-frozen in liquid nitrogen and stored at −80 °C. Sex was determined macroscopically by the presence of the albumen gland (females) or the penis and testis (males). Adult *H. azteca* were obtained from Environmental Consulting and Testing (Superior, WI) and acclimatized to laboratory conditions for at least 1 week.

2.3. Subcellular fractioning

Selected tissues of sea urchin (individual samples), and snail (2–3 pooled individuals), and whole body of amphipods (15–20 pooled individuals) were homogenized in ice-cold 100 mM potassium phosphate buffer pH 7.4 containing 100 mM KC1 (150 mM KCl for sea urchin), 1.0 mM EDTA, 1.0 mM dithiotreitol, 0.1 mM phenanthroline, and 0.1 mg/mL trypsin inhibitor. Homogenates were centrifuged at 12,000 g for 20 min, and the supernatant was collected and further centrifuged at 100,000 × *g* for 60 min. The supernatant, termed cytosol, was collected and stored at −80 °C. The pellet was resuspended in homogenization buffer and centrifuged again at 100,000 × *g* for 60 min. After centrifugation, the pellet, termed microsomal fraction, was resuspended in microsomal buffer, consisting on 100 mM potassium phosphate buffer (150 mM KCl for sea urchin) pH 7.4 containing 1 mM EDTA, 1.0 mM dithiotreitol, 0.1 mM phenanthroline, and 0.1 mg/mL trypsin inhibitor, and 20% w:v glycerol. Microsomal protein concentration was measured (Bradford, 1976) using commercially available reagents (Bio-Rad, Hercules, CA) and bovine serum albumin as a standard. Microsomes were stored at −80 °C until assays were performed.

2.4. Palmitoyl-CoA: testosterone acyltransferase activity (ATAT)

Enzyme assays for the fatty acid esterification of testosterone were based on the methods described by Janer et al. (2004b) with some modifications. Microsomal proteins (100–200 μg) were incubated in 0.1 M sodium acetate buffer pH 5.5 with 10 μM [^{14}C]testosterone (150,000 dpm), 100 μM palmitoyl-CoA, and 5 mM magnesium chloride in a final

volume of 400 μL. The reaction was initiated by the addition of palmitoyl-CoA, and incubated for a selected time (30–60 min) at 35 °C. Ethyl acetate (2 mL) was added, and tubes were vortexed to stop the reaction. After centrifugation at $1000 \times g$ for 10 min, the ethyl acetate fraction was collected. The extraction with ethyl acetate was repeated, and the two extracts were pooled and evaporated to dryness under nitrogen.

The dried residues were redissolved twice in 35 μL of ethyl acetate and spotted with a micropipette onto 20 cm × 20 cm aluminum-backed silica gel thin layer chromatography (TLC) plates (Whatman Ltd., Maidstone, Kent, England). Testosterone palmitoate and testosterone were resolved using hexane:methyl-*tert*-butyl-ether:palmitic acid (4:1:0.1) (Gooding et al., 2003), and were visualized and quantified by electronic autoradiography (Packard, Instant Imager, Downers Grove, IL). The detection limit was 2 pmol/min/mg protein.

2.5. Sulfotransferase (SULT) activity

Cytosolic proteins (50 μg for *P. lividus* and 150 μg for *H. azteca* and *M. cornuarietis*) were incubated in 50 mM Tris–HCl buffer pH 7.4, containing 4 mM $MgCl_2$ and 2 mM Na_2SO_3, with 2.5 μM [^{14}C]testosterone (40,000 dpm), in a final volume of 150 μL. The reaction was initiated by the addition of PAPS (20 μM), incubated for 30 min at 35 °C, and stopped by the addition of 200 μL of ice-cold Tris–HCl buffer (50 mM, pH 8.7). Free steroids were immediately extracted twice with 2 mL of methylene chloride. An aliquot of the aqueous phase, where sulfated testosterone remained, was counted for ^{14}C radioactivity in a scintillation counter. The detection limit was 0.04 pmol/min/mg protein.

2.6. Effect of xenobiotics on testosterone conjugating activities

To evaluate the effect of (anti)androgenic compounds on testosterone metabolism, microsomal or cytosolic fractions were pre-incubated for 5 min with triphenyltin (TPT), tributyltin (TBT), or fenarimol. The xenobiotics were delivered to the assay solutions in absolute ethanol. Control microsomes were incubated with the carrier alone at the same concentration used in the treatments (<1%).

2.7. Data analyses

The Michaelis–Menten parameters (K_m and V_{max}, 95% confidence intervals) were estimated as $Y = V_{max} \times X/(K_m + X)$ using the kinetics module of Prism 4 (GraphPad Software, San Diego, California, USA). Statistical significance was assessed by using one way ANOVA (Dunnett's test for differences from control). Level of significance was $p < 0.05$.

3. Results

3.1. Palmitoyl-CoA: acyltransferase activity

ATAT was assessed in microsomal fractions isolated from *H. azteca* (whole body), *M. cornuarietis* (gonadal-digestive gland complex), and *P. lividus* (gonads and digestive tube). The activity was linear up to 0.5 mg protein/ml, and at least up to 60 min incubation in all three species. The specific activity ranged from 100 to 510 pmol/min/mg microsomal protein, and was rather similar among the three species (Table 1). The highest specific activity was observed in the digestive

Table 1
Microsomal palmitoyl-CoA: testosterone acyltransferase and cytosolic testosterone sulfotransferase specific activities (pmol/min/mg protein)

Species	Tissue	Palmitoyl-CoA: testosterone acyltransferase	Testosterone sulfotransferase
Hyalella azteca	Whole organism	174 ± 4	0.05 ± 0.01
Marisa cornuarietis	Gonad-digestive complex	102 ± 13	0.18 ± 0.01
Paracentrotus lividus	Gonad	100 ± 14	0.80 ± 0.40
	Digestive tube	510 ± 195	137 ± 22

Values are mean ± S.E.M. ($n = 4$–6), except for *Hyalella azteca*, where 2 pools of 15–20 organisms were analyzed.

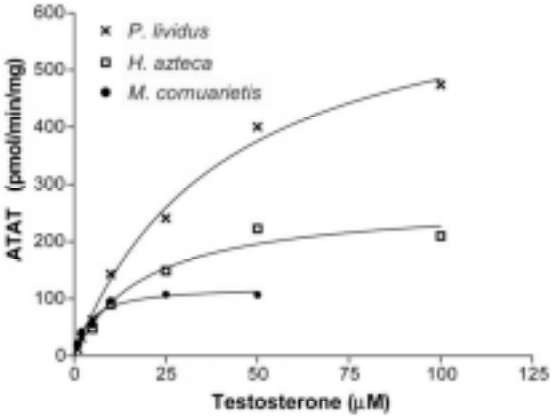

Fig. 1. Palmitoyl-CoA: testosterone acyltransferase activity vs. testosterone concentration measured in microsomes isolated from whole body of *H. azteca*, gonadal-digestive gland complex of *M. cornuarietis*, and gonads of *P. lividus*. Values are mean of duplicates.

tube of the sea urchin. K_m and V_{max} values were 19 ± 6 μM and 270 ± 31 pmol/min/mg protein for *H. azteca*, 4.2 ± 1.1 μM and 122 ± 9 pmol/min/mg protein for *M. cornuarietis*, and 41 ± 6 μM and 682 ± 46 pmol/min/mg protein for *P. lividus* (gonads) (Fig. 1).

3.2. SULT activity

SULT was assessed in cytosolic fractions isolated from *H. azteca* (whole body), *M. cornuarietis* (gonadal-digestive gland complex), and *P. lividus* (gonads and digestive tube). SULT specific activity ranged from 0.05 to 137 pmol/min/mg cytosolic protein, and the digestive tube of *P. lividus* showed highest specific activity (Table 1).

Due to the rather low specific activities recorded in *M. cornuarietis*, *H. azteca*, and gonads of *P. lividus*, kinetic studies were only performed in *P. lividus* digestive tube. The activity did not follow a typical Michaelis–Menten curve, decreasing sharply at concentrations of testosterone higher than 5 μM (Fig. 2). The decrease was not due to limiting levels of the cofactor (PAPS), since similar activity curve was obtained even when PAPS concentration was increased up to 150 μM in the assay.

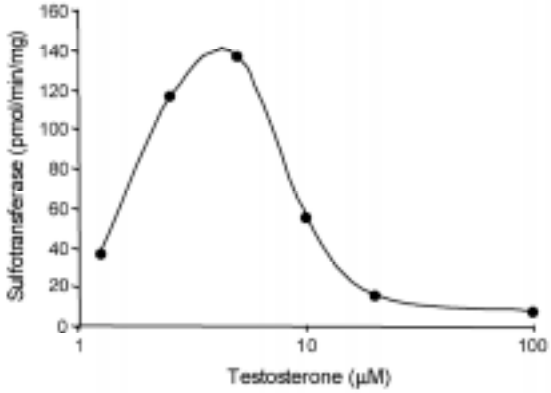

Fig. 2. Effect of testosterone concentration on sulfotransferase specific activity determined in digestive tube cytosolic fractions of *P. lividus*. Values are mean of duplicates.

3.3. Effect of xenobiotics on testosterone conjugating activities

ATAT activity was inhibited by organotins, however, differences between organisms were observed. Thus, both TPT and TBT significantly inhibited ATAT in *P. lividus* (68 ± 3% and 42 ± 5%, respectively), while only TBT inhibited ATAT in *M. cornuarietis* (22 ± 4%) and *H. azteca* (19 ± 3%). Fenarimol had no inhibitory effect on ATAT activity in any of the organisms tested (Fig. 3).

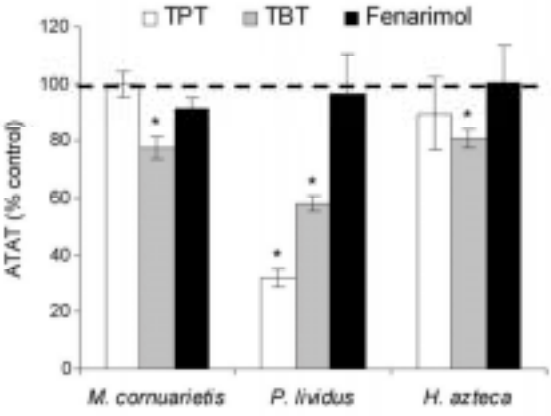

Fig. 3. Inhibitory effect of 100 μM triphenyltin, tributyltin, and fenarimol on ATAT activity in microsomal fractions of *P. lividus* (gonads), *M. cornuarietis* (gonadal-digestive gland complex), and *H. azteca* (whole animal). Asterisks denote significant differences from controls ($p < 0.05$). Values are mean ± S.E.M ($n = 3$–4).

The effect of TPT, the strongest inhibitor of ATAT, was further investigated using *P. lividus* gonad microsomes. Inhibition was observed at concentrations as low as 3 μM TPT (11 ± 2%) and increased in a concentration-dependent manner (31 ± 8% at 10 μM; 55 ± 16% at 30 μM and 70 ± 11% at 100 μM). Experiments using a range of testosterone (Fig. 4A) or palmitoyl-CoA concentrations (Fig. 4B) showed that the inhibitory effect was not ameliorated by higher testosterone or palmitoyl-CoA concentrations, which suggests that TPT does not exert its inhibition by competing with either substrate. ATAT reached its maximum activity in a relatively narrow range of palmitoyl-CoA concentrations, as previously described for other species (Xu et al., 2001). Additionally, the degree of inhibition increased with increasing incubation time (Fig. 4C). Similar results were obtained for TBT when microsomal fractions were co-incubated in the presence of different concentrations of testosterone, palmitoyl-CoA, and different incubation times. The obtained results suggest that both organotins have similar mechanisms of action in *P. lividus*.

All three xenobiotics significantly inhibited SULT activity in the digestive tube of *P. lividus*, when tested at concentrations of 100 μM. The percentage inhibition was of 29 ± 11% for TPT, 43 ± 9% for TBT, and 46 ± 5% for fenarimol. The effects of TBT and fenarimol were further evaluated at lower concentrations; and both compounds inhibited the sulfation of testosterone in a concentration-dependent manner in the range of 1–100 μM (Fig. 5).

4. Discussion

ATAT specific activity was similar among the three species studied despite the different tissues used. Nonetheless, the enzyme saturated at lower testosterone concentrations in *M. cornuarietis* than in *H. azteca* or *P. lividus* (K_m: 4 μM versus 19 μM and 41 μM, respectively), demonstrating that ATAT has a higher affinity for testosterone in the snail than in the sea urchin or the amphipod. The V_{max} and K_m values obtained for *M. cornuarietis* were similar to the values reported (V_{max}: 74 ± 6 pmol/min/mg protein; K_m: 10 ± 2 μM) for palmitoyl-CoA: estradiol acyltransferase in the oyster *Crassostrea virginica* (Janer et al., 2004b).

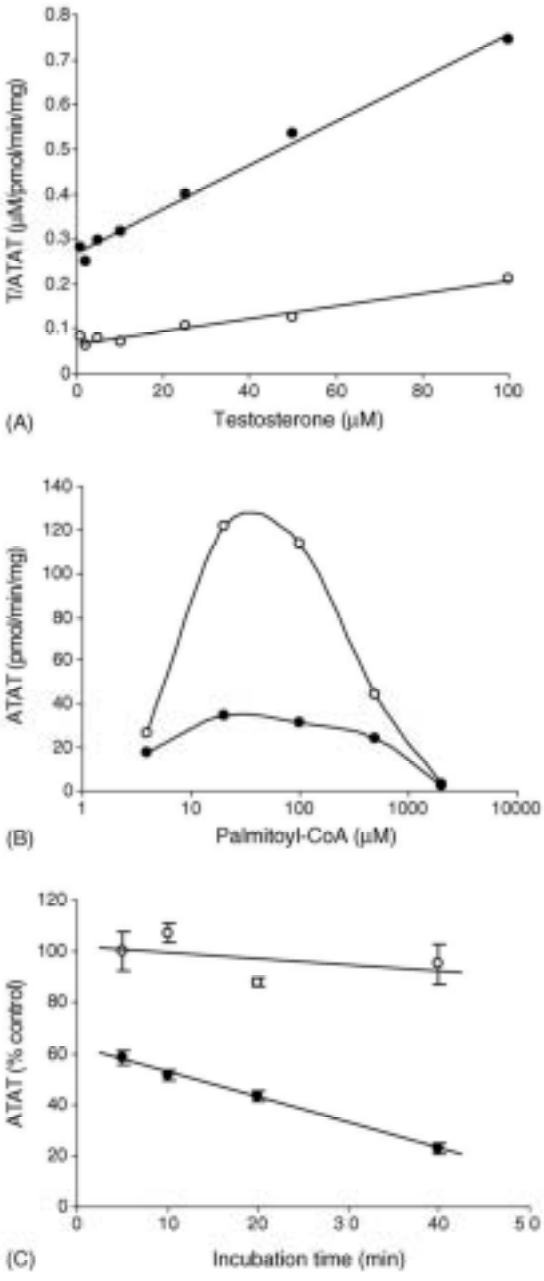

Fig. 4. Inhibition of ATAT by triphenyltin in microsomal fractions of *P. lividus* (gonad). Effect of (A) testosterone concentration (Hanes Plot); (B) palmitoyl-CoA concentration; (C) incubation time. Open circles: control; black circles: 100 μM TPT. In graph C, all values are expressed as % of control at 5 min incubation. Error bars in graph C account for range ($n = 2$).

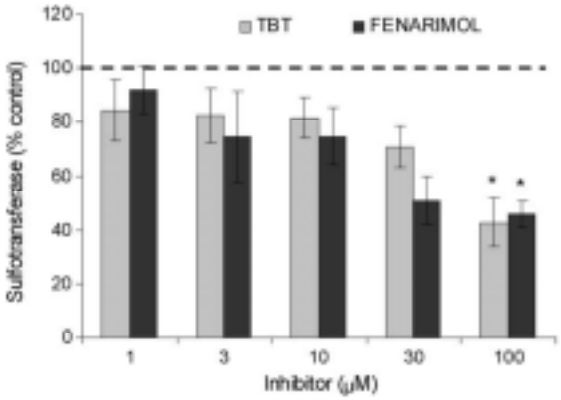

Fig. 5. Concentration-dependent inhibition of testosterone sulfotransferase (digestive tube) by TBT and fenarimol in cytosolic fractions of *P. lividus*. Values are mean ± S.E.M. ($n = 3–5$). Asterisks denote significant differences from controls ($p < 0.05$).

Fatty acid esterification is recognized as a major biotransformation pathway for sex steroids in some molluscs (Gooding and LeBlanc, 2001; Janer et al., 2004a), and has also been reported in echinoderms. Indeed, fatty acid conjugates of pregnenolone and testosterone were the most abundant metabolites formed when *Asterias rubens* pyloric caeca was incubated in the presence of pregnenolone and androstenedione, respectively (Voogt et al., 1990; Voogt and Van Rheenen, 1986). Fatty acid conjugation of ecdysteroids is a relevant metabolic pathway in arthropods (Slinger and Isaac, 1988), and this study suggests that, similarly to ecdysteroids, sex steroids are conjugated to fatty acids in crustacean species. In fact, the affinity of acyl-CoA acyltransferase for testosterone in *H. azteca* was similar to that previously reported for 22-O-ecdysteroids (K_m: 10 μM) in the tobacco budworm *Heliothis virescens* (Zhang and Kubo, 1992).

Despite the similarities observed in ATAT activity measured in vitro, in vivo exposure to radiolabeled steroids suggest that the formation of fatty acid conjugates might be more predominant in molluscs than in crustaceans. A massive biotransformation of testosterone and estradiol to fatty acid conjugates was found for the gastropod *Ilyanassa obsoleta* and the bivalve *Mytilus galloprovincialis*, respectively (Gooding and LeBlanc, 2001; Janer et al., 2004a). In contrast, when *N. integer* and *D. magna* were exposed to radiolabelled testosterone, most of the testosterone absorbed was excreted in the form of hydroxylated metabolites and polar conjugates (Baldwin and LeBlanc, 1994; Verslycke et al., 2002). Nonpolar metabolites were observed in the organic extract of *N. integer* (Verslycke et al., 2002), and although the authors did not identify those metabolites, their chromatographic properties suggest that they were fatty acid conjugates of testosterone. The higher levels of hydroxylases and oxidoreductases described in crustaceans in comparison to molluscs (Janer et al., unpublished data), might explain why fatty acid conjugation has a greater contribution to the fate of testosterone in molluscs than in crustaceans.

Cytosolic sulfotransferase specific activity towards testosterone was highest in the digestive tube of *P. lividus*, whereas it was nearly undetectable in *M. cornuarietis* and *H. azteca*. SULT exhibited high-affinity for testosterone in *P. lividus* although, at high concentrations of the substrate, the conjugation rates decreased, suggesting that testosterone or testosterone-sulfate might act as SULT inhibitors.

Low rates of in vivo testosterone-sulfate conjugation have been reported in both crustaceans and molluscs (Hines et al., 1996; Ronis and Mason, 1996; Baldwin et al., 1998). In addition, high sulfatase activity was measured in cytosolic preparations from the oyster *C. virginica* (Janer et al., unpublished data) and lobster (*Homarus americanus*) (Li and James, 2000). The high level of sulfatase activity associated with some tissues of molluscs and crustaceans coupled with the fact that SULT enzymes are typically of high-affinity/low capacity (de Knecht et al., 2001) might explain the low levels of SULT activity measured in *M. cornuarietis* and *H. azteca*.

Organotin compounds altered both the esterification of testosterone and its sulfation, but strong differences among species were observed. 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*. Further experiments performed in *P. lividus* indicated that TPT and TBT act as non-competitive and probably irreversible inhibitors.

SULT activity was inhibited by the three tested xenobiotics in *P. lividus* digestive gland, suggesting that similarly to estradiol sulfation, testosterone sulfation might be a common target for endocrine disruptors. Sulfation of estradiol is inhibited by a variety of xenobiotics, e.g. hydroxylated PAHs and hydroxylated PCBs inhibited the human isoform SULT1E1 (Kester

et al., 2000, 2002), and nonylphenol, TBT and TPT inhibited estradiol sulfation by the liver cytosolic fraction of *Cyprinus carpio* (Thibaut and Porte, 2004).

The inhibitory effects observed in *P. lividus* conjugating activities occurred at TPT concentrations in the range of those reported in vertebrate species for other steroid metabolic pathways. In mammals, the following IC50 were obtained: 4 μM TPT for 3β-hydroxysteroid dehydrogenase (3β-HSD), 10 μM TPT for 17β-HSD (Lo et al., 2003), 6.2 μM TBT for P450 aromatase (Heidrich et al., 2001), 20 μM TBT for 5α-reductase1, and 11 μM TBT for 5α-reductase2 (Doering et al., 2002). Concentrations of TBT and TPT higher than 1 mg/kg (≈3 μM) 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 to affect sex steroid conjugating pathways in invertebrates. Indeed, the obtained in vitro results are in agreement with the fact that exposure of the snail *I. obsoleta* to TBT reduced the accumulation of testosterone esters and increased levels of free testosterone (Gooding et al., 2003); and that exposure of the mollusc *L. littorea*, and the crustacea *N. integer* to TBT reduced the excretion of sulfated metabolites (Ronis and Mason, 1996; Verslycke et al., 2003). Altered testosterone metabolism might have important physiological consequences, and is considered to be a possible cause of the phenomena of imposex in snails.

The inhibition of SULT by fenarimol occurred at comparable concentrations to 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 mithocondria (Williams et al., 2000). These in vitro effects were reflected in experimental exposures to fenarimol in reproductive abnormalities in male rats (Hirsch et al., 1987), and reduced ecdysteroid levels and altered embryo development in crustaceans (Mu and LeBlanc, 2002).

Overall, the study shows that fatty acid and sulfate conjugation are significant contributors to steroid metabolism in echinoderms, molluscs and crustaceans. These conjugation pathways were directly inhibited by some environmental chemicals, and might be important targets of EDCs.

Acknowledgements

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Complementari work on chapter 2

PHASE II METABOLISM OF SEX STEROID IN MOLLUSCS

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2.5 Additional experiments within Chapter 2.

In addition to the results presented in papers 1 to 4, other data was generated during this thesis regarding phase II metabolism of steroids in molluscs. Despite of the fact that this data has not yet been assembled for publication, we considered that it was worth including it in this thesis. These additional experiments are presented in three sections: section 2.5.1 shows the results obtained in the attempts to measure enzymatic activity for classical steroid phase II metabolic pathways in molluscs; section 2.5.2 reports on the existence of novel phase II metabolites of steroids in molluscs; and finally, section 2.5.3 investigates the specificity of acyl-CoA acyltransferase for different steroid substrates.

2.5.1 Minor contribution of glucosyl-, glucuronyl- and sulfo-transferases to steroid metabolism in molluscs.

The conjugation of sex steroids with polar moieties in molluscs has not yet been reported *in vitro*, but metabolic studies performed *in vitro* in species from other phyla have shown the formation of sulfate-, glucuronide- and glucosyl- metabolites of sex steroids (see introduction, section 1.7.4). Moreover, *in vivo* metabolic studies in molluscs showed that some conjugates formed were susceptible to hydrolysis by sulfatases, glucuronidases and glucosidases (Hines et al., 1996; Ronis and Mason, 1996). Therefore, the objective of this work was to assess the presence of glucosyltransferase, glucuronyltransferase and sulfotransferase activity in two mollusc species, *Mytilus galloprovinciallis* and *Crassostrea virginica*.

Digestive glands and gonads of *M. galloprovinciallis* (collected from the Ebro Delta, Spain) and *C. virginica* (collected from Prince Edward Island, Canada) were dissected, frozen in liquid nitrogen, and kept at -80ºC until used. Cellular fractions were prepared as described previously (Livingstone, 1988), and protein content was determined by the method of Lowry et al (1951) using bovine serum albumin as a standard.

Two different methods were used to assess sulfotransferase activity, one that used a radiolabelled steroid substrate and another one that used a radiolabelled cofactor. The first method was based on the method described by Wilson and LeBlanc (2000) with some modifications. Cytosolic proteins (25 to 200 σg) were incubated with 100 σM [^{14}C]testosterone/[^{3}H]estradiol (100,000 dpm, NEN Life Science Products, Inc) in Tris-HCl buffer (50 mM, pH 7.4), containing 10 mM $MgCl_2$. The reaction was started by the addition of 10-50 σM PAPS (CalBiochem). After 20 to 120 min incubation the reaction was stopped by adding ice-cold water and dichloromethane. The second

method for assessing sulfotransferase was based on the method of Foldes and Meek (1973), and estradiol and dehydroepiandrosterone (DHEA) were used as substrates. In this case, cytosolic proteins (25 to 200 σg) were incubated with 50-100 σM estradiol/DHEA in Tris-HCl buffer (20 mM, pH 7.5), containing 4 mM $MgCl_2$. The reaction was started by the addition of 5 σM [^{35}S]PAPS (30,000 dpm). After 20 to 120 min incubation the reaction was terminated by the of 200 σL of 0.1M barium acetate, 200 σL of 0.1M barium hydroxide, and 200 σL of 0.1 zinc sulfate. Tubes were vortex-mixed and centrifuged at 10,000 g for 2 min. An aliquot (500 σL) of the supernatant was directly assayed for [^{35}S] content by liquid scintillation counting.

Glucosyltransferase and glucuronyltransferase were carried out on the basis of methods described by Wilson and LeBlanc (2000). Microsomal proteins (25 to 200 σg) were incubated with 100 σM [^{14}C]testosterone (100,000 dpm) in Tris-HCl buffer (50 mM, pH 7.4), containing 10 mM $MgCl_2$. The reaction was started by the addition of 2.5 mM uridine diphosphate glucuronic acid (UDPGA, Sigma) or uridine diphosphate glucose (UDPG, Sigma). After 20 to 120 min incubation the reaction was stopped by adding ice-cold water and ethyl acetate and vortexing. The aqueous and ethyl acetate phases were separated by centrifugation. They ethyl acetate fraction was removed and extraction of the aqueous phase was repeated a second time with an additional 2 mL ethyl acetate. An aliquot of the aqueous phase, containing the steroid conjugates, was counted for [^{14}C] liquid scintillation.

The experiments performed to determine glucuronyltransferase and glucosyltransferase using testosterone as substrate did not result in any measurable conjugating activity, indicating that these conjugating pathways are not present in the molluscs investigated, or that they are present at very low levels. Sulfotransferase activity towards the sex steroids tested was found in both molluscs, although the activities recorded were always close to the detection limits of the methods and hampered a proper characterization of this enzymatic pathway. For comparative purposes the assays were also performed with subcellular fractions obtained from the liver of vertebrate species (*Cyprinus carpio* obtained from the Ebro River, Spain, for UGT, and *Rhesus rhesus* liver cytosol obtained from CellzDirect, for sulfotransferase). The activities detected in these vertebrates, using the same methods as in molluscs, were orders of magnitude higher than those found in molluscs (Table 2.1). Similarly the activities found for these conjugating pathways are much lower than those found for acyl-CoA acyltransferase, another phase II enzyme, in the same bivalve species (Table 2.1). Therefore, this data might suggest either that glucosyl-, glucuronyl- and sulfo-transferases play a limited role in the metabolism of steroids in these mollusc species, or that the formation of such metabolites cannot be adequately traced *in vitro* (e.g. low stability of the enzyme, presence of hydrolytic enzymes cleaving the conjugate formed, presence of proteases…).

Table 2.1. Sulfotransferase and glucuronyl/glucosyl-transferase activity in bivalve molluscs. Comparison with acyltransferase, and with sulfotransferase and glucuronyl-transferase in vertebrate species. Values are expressed in pmol/min/mg protein as mean ± SEM (n=3 to 6). E2: estradiol; T: testosterone.

	Molluscs	Vertebrates
Sulfotransferase	*Crassostrea virginica*; digestive gland	*Rhesus rhesus;* liver
(10 σM E2, cytosol)	0.9 ± 0.3	103 ± 6
UGT	*Mytilus galloprovincialis*; digestive gland[1]	*Cyprinus carpio;* liver[2]
(100 σM T, microsomes)	<d.l.[4]	(1.2 ± 0.4)*10[3]
Acyl-CoA acyltransferase	*Crassostrea virginica*; digestive gland[3]	
(10 σM E2, microsomes)	29 ± 4	

[1]Using either UDP-glucose or UDP-glucuronid acid as cofactor.

[2]UDP-glucuronic acid was used as cofactor.

[3]Data from paper 2.

[4]Limit of detection of the method was 5 pmol/min/mg protein.

2.5.2 Formation of two new polar sex steroid conjugates by mollusc species.

During the experiments performed within this thesis looking at sex steroid metabolism by subcellular fractions of digestive gland/gonads isolated from different mollusc species, two polar metabolites were observed. These metabolites could not be unequivocally identified, however, the experiments performed and the information that was generated on their characteristics will be described below.

Metabolite 1

The first of these two metabolites was a major testosterone metabolite formed by cytosolic proteins isolated from the digestive gland/gonad complex of *M. cornuarietis* (homogenization performed as in paper 1).

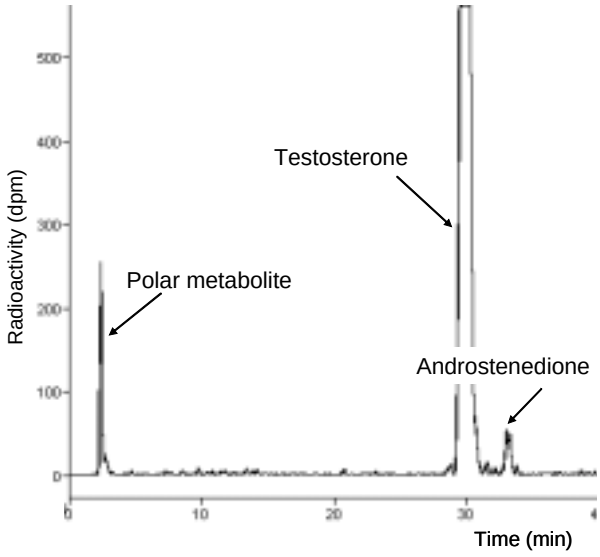


Figure 2.1. HPLC chromatogram obtained when ^{14}C-testosterone (1 σM, 150,000 dpm) was incubated with *M. cornuarietis* visceral coil cytosol in the presence of NADPH. HPLC chromatographic conditions were as described in paper 1.

The retention time of this metabolite (R_t: 2.4 min, figure 2.1) and the fact that it remained in the water phase when steroids were extracted with ethyl acetate or dichloromethane, suggests that it is a highly polar metabolite. This high polarity is characteristic of steroids conjugated with polar moieties. The formation of the metabolite depended on the presence of NADPH (1 mM), suggesting that testosterone undergoes a first conversion that is NADPH-dependent, and the product of this conversion is rapidly conjugated.

Among the phase II enzymes that conjugate steroids in other vertebrate or invertebrate species, only sulfotransferases and glutathione-transferases occur in the cytosol. Glutathione conjugates have been described for estradiol, but they only occur after the formation of cathecol-estrogens and quinones (Hachey et al., 2003). In order to test whether the metabolite could be a sulfate or a glutathione conjugate, its formation was assessed in the presence of NADPH and the cofactors involved in sulfation and glutathione conjugation: PAPS (50 σM) and glutathione (1 mM). However, none of these cofactors increased the formation of this metabolite. This might suggest that the metabolite is neither a sulfate- nor a glutathione-conjugate, or that the concentration of cofactor already present in the cytosolic fraction of the organism was enough for an optimal yield of the reaction.

The solvent system in the HPLC elution was modified by addition of 0.01% trifluoroacetic acid (resulting in a pH ℑ 2.5) in order to test whether the retention time of the metabolite could be changed. If the metabolite had been a weak acid, such as a glucuronide, a change in retention time would have been expected. In contrast, if the metabolite had been, for instance, a strong acid, such as a sulfate, its retention time would be unaltered. No change in retention time was observed in this experiment.

In an attempt to further characterize the NADPH-dependent metabolite, its susceptibility to cleavage when submitted to different treatments, particularly, incubation with a sulfatase-glucuronidase extract (from *Helix pomatia*, Merk) and saponification, was assessed. The metabolite of interest (10,000 dpm) was isolated by removing the unconjugated testosterone with dichloromethane extraction, evaporated, redissolved in 1 mL potassium phosphate buffer (pH 6.2) and divided into three aliquots. One aliquote was incubated with sulfatase-glucuronidase (20 µL of sulfatase-glucuronidase extract), the second aliquot was saponified by adding 330 µL of 2 M potassium hydroxide, and the third aliquot was used as a control, in order to account for possible unspecific cleavage during the incubation step. After incubation of the three tubes at 37ºC for two hours, dichloromethane was added and used to extract unconjugated steroids. An aliquot of the organic phase was injected onto the HPLC, and two aliquots of the water phase were counted for ^{14}C content in a η-counter. Results showed that the metabolite was not cleaved by the saponification treatment (radioactivity in the aqueous phase was 105 ± 4% that of controls),

whereas it was partially cleaved by the sulfatase-glucuronidase treatment (32 ± 5% hydrolisis). Therefore, the metabolite might be a sulfate or a glucuronide, and the low yield of the reaction obtained might be due to suboptimal conditions for the enzyme in the incubation. Indeed, in a later experiment with increased amount of sulfatase-glucuronidase extract and incubation time (40 σL and 3 hours), the proportion of the metabolite that remained unaltered decreased to 32%. A parallel incubation at pH 5.0 with bovine liver glucuronidase (EC 3.2.1.31; Sigma) did not result in any detectable cleavage of the conjugate, suggesting that the sulfatase/glucuronidase extract acted upon a sulfate moiety. The steroid liberated after cleavage by this extract was not testosterone, but a slightly more polar steroid that could not be identified, supporting the hypothesis that two steps take place in the formation of this conjugate from testosterone (i.e. testosterone suffered an initial NADPH-dependent metabolic alteration to an intermediate metabolite that is then subject to conjugation).

Attempts were performed to identify this polar conjugate by mass spectrometry. In order to do so, approximately 100 ng of the metabolite were generated enzymatically and purified by liquid-liquid extractions (buffer vs. dichloromethane), solid phase extraction (NH_2), and HPLC. An incubation without NADPH was performed and processed in parallel in order to be used as a blank for the compounds present in the cytosolic extract. The purified extract was then injected into the LC-MS system. HPLC was performed with an HP 1100 autosampler fitted with a 100 σL loop and an HP 1090 A binary pump, both from Hewlett-Packard (Palo Alto, CA, USA) equipped with a 250 x 4 mm reversed-phase column (LiChrospher 100 RP-18, 5 σm) preceded by a guard column (4 x 4 mm, 5 σm) (Merck, Darmstadt, Germany). The chromatographic conditions consisted on a 0.8 mL/min linear gradient from 60% MeOH containing 0.2% formic acid and 40% H_2O containing 0.2% formic acid to 100% MeOH containing 0.2% formid acid. The extraction voltage used in both negative ionization (NI) and positive ionization (PI) modes was 90 V. Detection was carried out using an HP 1040M diode array UV-visible detector coupled in series with an LC-MSD HP 1100 mass-selective detector, equipped with an electrospray (ESI) interface.

We could not identify any ion correspondent to sulfated testosterone ($[M+H]^+$: 370, $[M+2H]^{2+}$: 185, $[M+Na]^+$: 392, or $[M+2Na-H]^+$: 414 in PI mode; $[M-H]^-$: 368 or $[M-2H]^{2-}$: 183 in NI mode), or sulfated hydroxylated metabolites of testosterone (+16), except for *m/z* 193 ($[M+2H]^{2+}$), which was present in the extract from the incubation with NADPH, but not in the incubation blank. This information is suggestive of, but does not prove, the presence of a hydroxylated-testosterone sulfate. Analysis by MS/MS should be perfomed in order to obtain additional structural information on the *m/z* 193 ion.

In summary, the data obtained in all the above described experiments supports, but does not definitely prove, that the metabolite observed is a sulfate conjugate of a metabolite of testosterone (probably hydroxylated testosterone). Interestingly, this metabolite seems to be specific for this species or maybe for gastropod molluscs, since it was not formed by fish (*Mullus barbatus*),

crustaceans (*Hyalella azteca*), echinoderms (*Paracentrotus lividus*), or other non-gastropod molluscs (*Mytilus galloprovincialis*).

Metabolite 2

The second polar metabolite observed, was formed by the digestive gland microsomes of the mussel *Mytilus galloprovincialis* (Ebro River, Spain) when incubated in sodium acetate/acetic acid buffer (pH 6) (Figure 2.2). This metabolite was dependent on the presence of sucrose during the homogenization and subcellular preparation process, which had been widely used in the subcellular fractioning of molluscs (Livingstone, 1988). Indeed, when an alternative homogenization procedure with no sucrose was used (Gooding et al., 2003), the formation of this metabolite was no longer observed. The conjugation of estradiol with sucrose was mediated by a microsomal enzyme, since the metabolite was not observed in blanks without microsomes.

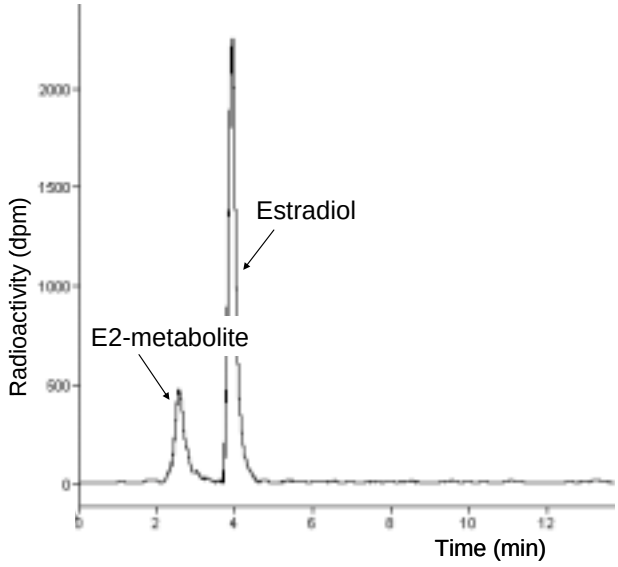


Figure 2.2. HPLC chromatogram obtained when ^{3}H-estradiol (2 σM, 150,000 dpm) was incubated with *M. galloprovincialis* digestive gland microsomes in the presence of NADPH. HPLC chromatographic conditions were as follows: 1ml/min linear gradient from A (water:acetonitrile; 30:70) to B (acetonitrile) in 30 minutes.

In order to further investigate the role of sucrose in this reaction, we incubated microsomes (prepared with sucrose-free buffers) with increasing concentrations of sucrose (0.01 to 250 mM) and 2 σM estradiol. The Km obtained was of 2.1 ± 1.1 mM sucrose, and maximal yield of the reaction (3.4 ± 0.3 pmol/min/mg) was achieved at 50 mM sucrose. The affinity of the enzyme for estradiol was also assayed using 50 mM sucrose and increasing concentrations of estradiol (0.01 to 400 σM). The kinetic parameters were calculated to be 62 ± 15 σM (Km) and of 113 ± 36 pmol/min/mg (Vmax).

Further assays demonstrated that this metabolite was also formed when *P. lividus* and *M. cornuarietis* were homogenized in sucrose buffer and incubated as described for *M. galloprovincialis*. In contrast, *Mullus barbatus* microsomes did not form the metabolite when

microsomes were incubated in the presence of 50 mM sucrose, suggesting that the formation of this metabolite might be characteristic of invertebrate species.

In order to identify the metabolite, incubations with microsomes isolated from the digestive gland of *M. galloprovincialis* and homogenized in the presence of sucrose were performed to generate approximately 1 σg of unlabelled metabolite. The metabolite was partially purified by collecting its corresponding HPLC fraction and it was analysed in full scan by ESI-MS/MS by Dr. Debrauwer in 'Laboratoire des Xénobiotiques' INRA, Toulouse. Sucrose is a disacharide formed by glucose and fructose. There was no response in MS/MS for the molecular ion $[M-H]^-$: 595 (which would correspond to estradiol-sucrose), therefore rejecting the possibility that the conjugate was with sucrose. The MS/MS spectrum obtained for the molecular ion $[M-H]^-$: 433 (which would correspond to estradiol-glucose/fructose) is shown in Figure 2.3. The *m/z* 271 ion corresponds to the estradiol moiety $[E2-H]^-$. The difference between both ions (433-271= 162) indicates the molecular weight of the polar moiety attached to estradiol in the metabolite. The two sugar moieties in sucrose have a molecular weight of 180, which with a loss of a water molecule (180-18= 162) correspond to the difference between the *m/z* of the conjugate and that of estradiol.. Therefore, this mass spectrum indicates that the metabolite observed was a fructosyl- or glucosyl-estradiol. In order to know which of the two sugar moieties (fructose or glucose) were present in the conjugate, its mass spectrum and/or its retention time should be compared to those of standards. Nevertheless, neither of the two conjugates are commercially available and the identification of this metabolite could not be completed.

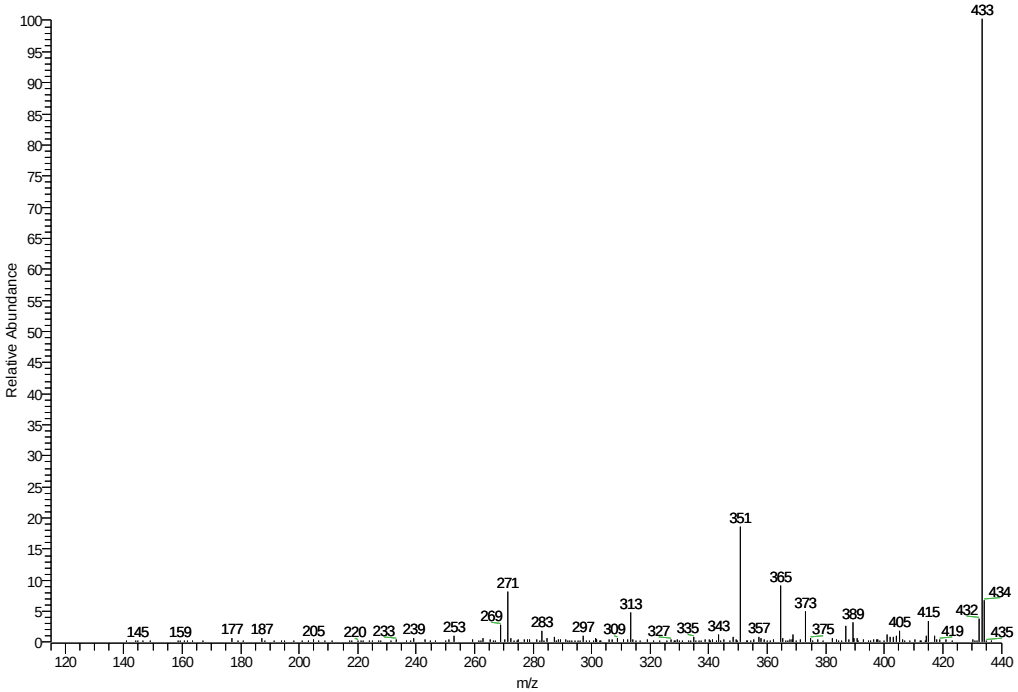


Figure 2.3. MS/MS spectrum (negative ionization) of the ion *m/z* 433 obtained from the metabolite formed by *M. galloprovincialis* microsomes in the presence of sucrose.

Recently, Stroomberg et al. (2004) reported the formation of a glucose-malonate metabolite of 1-hydroxypyrene by terrestrial isopods. Glucose-malonate is a known conjugating molecule in plants (Lin et al., 2000), but had not been previously reported in animals. The authors hypothesized that isopods could activate glucose-malonate for use as a co-factor, in the same way as glucose is activated by UDP, which is then used to perform a conjugating reaction. Sucrose might be acting similarly as glucose-malonate in the incubations with *M. galloprovincialis* microsomes, and thus be used as a cofactor and transfer the glucose or fructose unit to estradiol. Another hypotheses is that sucrose is used as such to form UDP-sucrose, and that one of the sugar rings is then taken up in the conjugation reaction.

The *in vivo* relevance of this conjugation pathway will depend on the presence of sucrose in the diet of the invertebrates, and on whether sucrose is incorporated and available for the enzymes catalyzing this conjugation.

2.5.3 Substrate specificity of acyl-CoA:testosterone acyltransferase

There are some evidences that the esterification of steroids is involved in the regulation of free steroid levels in mollusc species (Gooding et al., 2004; this thesis). In order to better understand the role that steroid acyltransferases may play in the regulation of sex steroid levels, it is important to know whether acyltransferases have a broad substrate affinity (conjugating a wide range of steroids) or whether they specifically conjugate certain steroids. Mammalian acyl-CoA acyltransferases act upon several sex steroids (Hochberg, 1998; Xu et al., 2002), and the ability of mollusc species to esterify estradiol, dehydroepiandrosterone and testosterone has also been demonstrated (Gooding et al., 2001; this thesis, paper 4). However, it is still under discussion whether specific acyltransferases for some steroids do exist.

Differences in the fatty acid composition of the esters of cholesterol, corticosteroids and that of sex steroids have been shown, suggesting that the enzymes that catalyze the esterification of cholesterol or corticosteroids are probably different from those that esterify sex steroids (Pahuja and Hochberg, 1989; 1995). Nevertheless, these putative sex steroid acyltransferases seem to have a relatively broad substrate affinity, a single enzyme being able to conjugate different sex steroids. Thus, DHEA, testosterone and 5-androstene-3η,17η-diol were shown to act as competitive inhibitors of estradiol esterification in bovine placenta microsomes (Martyn et al., 1988). In addition, similar profiles of fatty acid conjugates were obtained when the esterification of estradiol and dehydroepiandrosterone was investigated in *Crassostrea virginica* in the presence of Acyl-CoA and ATP (i.e., using endogenous fatty acid moieties) (this thesis, paper 4).

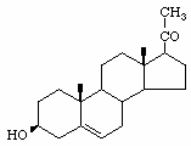
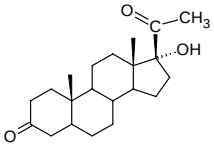
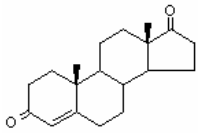
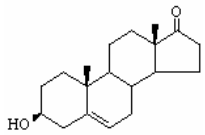
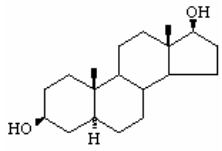
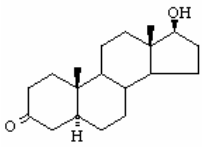
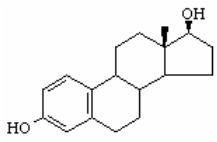
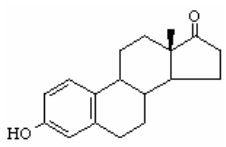
In this work we investigated the substrate specificity of acyl-CoA:testosterone acyltransferase (ATAT) present in microsomal fractions isolated from the digestive gland/gonad complex of the gastropod snail *Ilyanassa obsoleta*. To this end, we assessed the ability of several sex steroids to compete for acyl-CoA acyltransferase towards testosterone; and we evaluated whether the acyl-CoA acyltransferase activities towards testosterone and estradiol were correlated.

The steroids used for the competition study were selected so that they would include different structural features (Table 2.2). Thus, the steroids included in the study were two progestins (pregnenolone and progesterone), two estrogens (estradiol and estrone), and four androgens (dihydrotestosterone -DHT-, dehydroepiandrosterone -DHEA-, androstenedione -AD-, and 5Ŭ-androstane-3ƀ,17ƀ-diol -5ζ-A-diol-). Pregnenolone, the two estrogens and DHEA have a hydroxyl group at the C_3, although the two estrogens do not seem to be esterified at this position (Mellon-Nussbaum et al., 1982). 5-A-diol, which has two hydroxyl groups and can be esterified in both positions by human breast tumor microsomes (Hochberg, 1998). Dihydrotestosterone only differs from testosterone by the absence of the double bond at C_5 and has a hydroxyl group at C_{17}, that can only be esterified by certain tissues (Hochberg, 1998). Progesterone has a hydroxyl group at C_{17} but in mammals is not subject to esterification (Hochberg, 1998). Similarly androstenedione and estrone are not esterified in mammals (Xu et al., 2002). The synthesis of all these steroids from precursors has been demonstrated in mollusc species (see Introduction, Figure 1.5), therefore it is likely that these steroids are present in mollusc tissues.

Digestive gland/gonad complex of *Ilyanassa obsoleta* (collected from Bald Head Island Creek, Bald Head Island, and Bird Shoals in the Rachel Carson Reseve, Morehead City, NC, USA) were homogenized as described in Gooding et al. (2003). Protein concentrations were determined by the method described by Bradford et al. (1976), using commercially available reagents (Bio-Rad) and bovine serum albumin (Sigma) as a standard.

In a first experiment the ability of several sex steroids to inhibit acyl-CoA:testosterone acyltransferase activity (ATAT) was assessed. ATAT carried out on the basis of methods described by Gooding et al. (2003), with some modifications. Microsomal proteins (250 σg) were incubated in 100 mM potassium phosphate buffer (pH 7.6) with 50 σM [^{14}C]testosterone (100,000 dpm, NEN Life Science Products, Inc.) and 150 σM palmitoyl-CoA (Sigma) in a final volume of 500 σL, in the presence/absence of 50 σM of an additional sex steroid (Table 1). The reaction was initiated by the addition of palmitoyl-CoA, and samples were incubated for 30 minutes at 37ºC. Reaction was stopped by adding 2 mL of ethyl acetate, and extracted twice. The extract was dried under nitrogen and then spotted onto 20 × 20 cm aluminum-backed silica thin layer chromatography (TLC) plates (Whatman Ltd., Maidstone, Kent, England). [^{14}C]testosterone- and [^{14}C]estradiol- fatty acid ester was resolved from free testosterone using a mobile phase of hexane:methyl-*tert*-butyl ether:formic

Table 2.2. Steroids used in the competitive assay with ATAT. Data was extracted from Hochberg (1998) and Xu et al. (2002).

Steroid	Structure	Relevant information on its esterification
C_{21} steroids		
Pregnenolone		It is esterified by rat brain and liver microsomes. The fatty acid composition of its esters differed from that of testosterone esters
Progesterone		It is not esterified by a breast cancer cell line (ZR-75-1)
C_{19} steroids		
Androstenedione		Does not have any potential group that can be esterified
Dehydroepi-androsterone		It is esterified by microsomal fractions of several tissues and mammalian species
5ζ-Androstane-3η,17η-diol		A similar diol (androstenediol) can be esterified at the two hydroxyl groups by human breast tumor microsomes
5ζ-Dihydro-testosterone		It is esterified by rat brain microsomes but not by a breast cancer cell line (ZR-75-1)
C_{18} steroids		
Estradiol		It is esterified by microsomal fractions of several tissues and mammalian species only at the 17-hydroxyl group
Estrone		It is not esterified by rat liver microsomes

acid (80:20:2 v/v/v), and quantified by electronic autoradiography (Instant Imager; Packard, Downers Grove, IL).

ATAT follows a Michaelis-Menten curve and the concentration of testosterone used in the assay (50 µM) is above the Km of the enzyme (4 µM in the gastropod *M. cornuarietis*; paper 4), therefore, at this concentration most of the active sites of the enzyme are occupied by the substrate, and its activity can only marginally increase in the presence of additional substrate. In order to know the magnitude of this increase, and as a reference value, the assay was performed with additional testosterone (100 µM total testosterone concentration). As expected only a moderate increase in ATAT activity was observed (1.1 vs. 0.9 nmol/min/mg at 100 and 50 σM testosterone, respectively). Figure 2.3 shows ATAT activity in the presence of other sex steroids. The area limited by the slash-dot line (calculated as half of the ATAT activity measured using 100 σM testosterone, with its standard deviation) indicates the expected ATAT activity if the additional substrate had an equal affinity than testosterone for the enzyme. Thus, the presence of a substrate able to compete for the enzyme would be expected to inhibit the measured activity above or below the slash-dot line area depending on whether its affinity was lower or higher than testosterone, respectively.

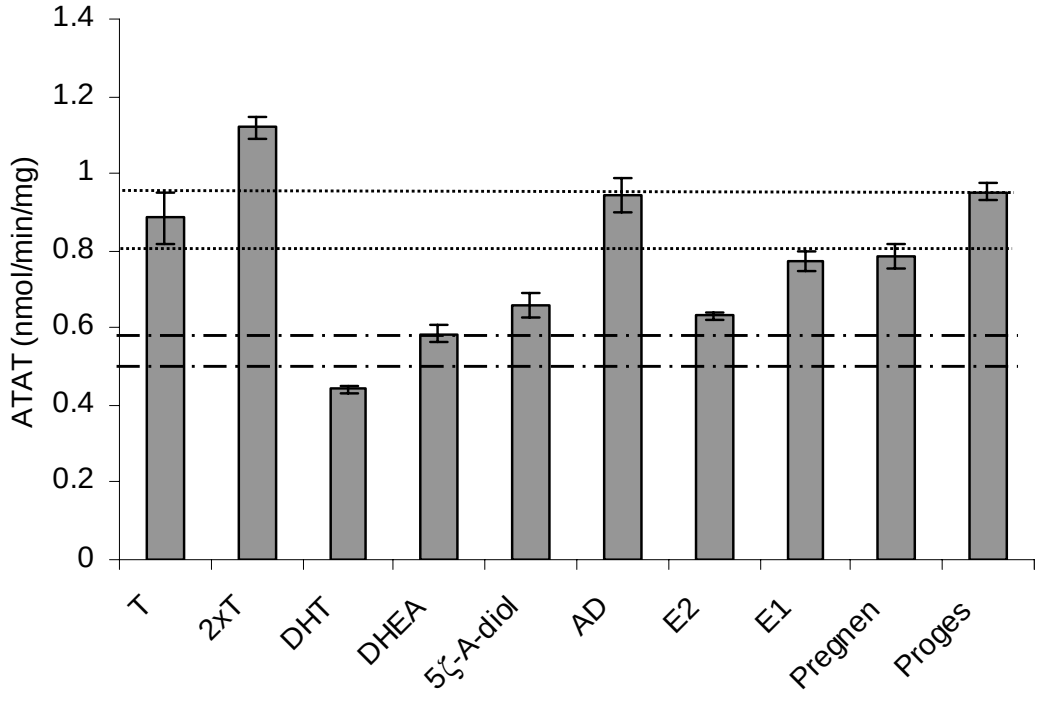


Figure 2.3. Acyl-CoA:testosterone acyltransferase activity in the presence of an equal concentration of a second sex steroid. Values are mean ± SEM (n= 3). Area limited by doted line indicates expected ATAT activity if the substrate does not compete with testosterone. Area limited by slash-doted line indicates expected ATAT activity if the substrate competes with testosterone and both had similar affinities for the enzyme. T: testosterone, DHT: 5ζ-dihydrotestosterone, DHEA: dehydroepiandrosterone, 5ζ-A-diol: 5ζ-androstane-3η,17η-diol, AD: androstenedione, E2: estradiol, E1: estrone, Pregnen: pregnenolone, Proges: progesterone.

The addition of androstenedione, pregnenolone, progesterone, and estrone to the incubation mixture, did not decrease ATAT activity, suggesting that these steroids had no affinity or much lower affinity than testosterone for the acyl-CoA acyltransferase enzyme. Indeed, androstenedione, progesterone and estrone were not expected to be conjugated by acyl-CoA acyltransferases (Table 2.2). Pregnenolone could theoretically be esterified by acyl-CoA acyltransferase. Nevertheless, the observed data suggests that its affinity for ATAT is much lower than that of testosterone, therefore, it might either be esterified by a different enzyme than ATAT or not be esterified in *I. obsoleta*. Estradiol, dihydrotestosterone, dehydroepiandrosterone and 5Ŭ-androstan-3ƀ,17ƀ-diol decreased ATAT activity. Estradiol, 5Ŭ-androstan-3ƀ,17ƀ-diol and dehydroepiandrosterone caused lower or similar decreases than those expected for testosterone, suggesting that, despite they interact with ATAT their affinity is lower than that of testosterone. In contrast, dihydrotestosterone showed higher affinity for ATAT than testosterone.

In a second experiment, visceral coil microsomes isolated from different individuals were assayed for ATAT and acyl-CoA: estradiol acyltransferase (AEAT) in order to investigate whether both activities were correlated. AEAT was assayed as described above for ATAT, but [^{3}H]estradiol (100,000 dpm, NEN Life Science Products, Inc.) was used as substrate. No correlation was observed between the esterification rates for these two steroids (figure 2.4). These results might suggest that two different enzymes are in charge of the esterification of estradiol and testosterone. Nevertheless, it should be noticed that the interindividual variability for both ATAT and AEAT in the subsample used for this assay was always lower than 2-fold and this hampered the assessment of their correlation. Indeed, an experiment performed with *Marisa cornuarietis* with an interindividual variability up to 5-fold showed a positive correlation (R^2: 0.87) between ATAT and AEAT (see paper 7).

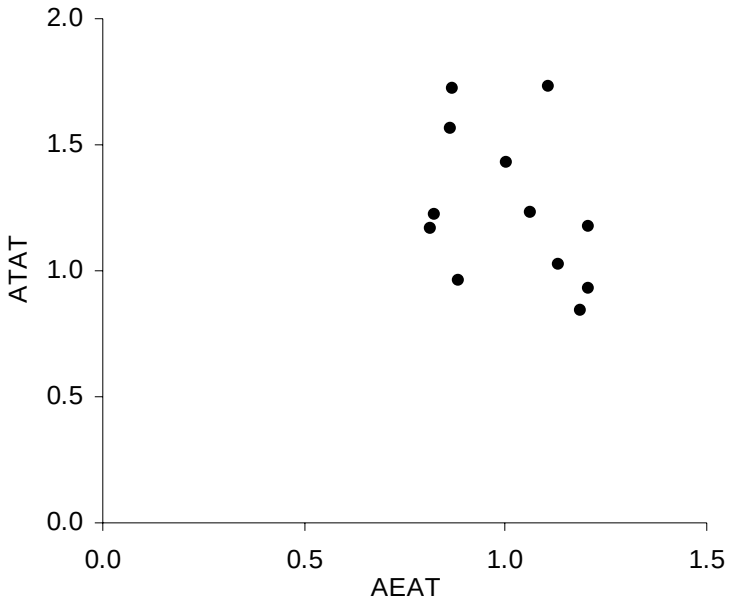


Figure 2.4. Relationship between acyl-CoA:testosterone acyltransferase and acyl-CoA:estradiol acyltransferase in microsomes isolated from *I. obsoleta* visceral coil. Values are mean of duplicates.

Overall, the results suggest that the enzyme(s) that esterifies testosterone in *I. obsoleta* is also able to conjugate/interact with other androgens and estrogens, but not to C_{21} steroids; and that, out of the tested steroids, dihydrotestosterone is the one showing the highest affinity for this enzyme. Therefore, despite that acyl-coA acyltransferases seem to be specific for certain classes of steroids, there is an overlap in its affinity for steroid substrates.