INTEGRATED TESTING STRATEGY FOR THE STUDY OF THE EFFECTS OF THE HUMAN PHARMACEUTICAL DUTASTERIDE ON FISH

A thesis submitted for the degree of Doctor of Philosophy

by

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DECLARATION

The work submitted in this thesis was conducted between 2007 and 2011 at Brunel University (Uxbridge, West London, UK). This work was carried out independently and has not been submitted for any other degree.

ABSTRACT

In recent years, a growing number of human pharmaceuticals have been detected in the aquatic environment, generally at low concentrations (sub-ng/L to low μg/L). These compounds are characterised by highly specific mechanisms of action, high potency and prolonged activity in order to minimise dosing requirements and potential toxicity in patients. Among the various classes of pharmaceuticals, steroids and anti-steroids are widely used, as shown by the analysis of their clinical use carried out at the beginning of this Ph.D. project. Although the amounts used are much lower than the amounts of some other pharmaceuticals (e.g. analgesics), their ability to affect important physiological processes in fish (e.g. reproduction) at very low concentrations (ng/L) suggest that this class of compounds should represent a high priority for ecotoxicological research. In particular, this Ph.D. project addressed the question of whether or not dutasteride, a human pharmaceutical mainly used to treat benign prostatic hyperplasia, may cause adverse effects in the teleost fathead minnow (*Pimephales promelas*) by inhibiting the activity of both isoforms of 5α-reductase (5αR), the enzyme which convert testosterone into dihydrotestosterone (DHT). The theoretical framework used to guide the design of the experimental studies was based on the combination of several conceptual approaches, including the study of the evolutionary degree of conservation and functionality of the drug target in non-target species, and the cross-species extrapolation of pharmacological and toxicological information generated during pre-clinical and clinical studies in mammals during drug development. The results obtained during the first phase of this Ph.D. project strongly suggested that DHT has a physiological role in the fathead minnow. In fact, 5αRs are evolutionary conserved in this species, 5αRs genes are expressed in tissues such as the testis, and DHT circulates in fathead minnow plasma at concentrations similar to those detected in humans. These findings represented the rationale for testing the effects of dutasteride in the fathead minnow. Dutasteride caused significant adverse effects in all the *in vivo* studies performed in order to evaluate its potential toxicity on fish, including early life stage and short term reproduction studies, and all the tested life stages were sensitive to the inhibition of 5αRs activity; however, none of the observed adverse effects occurred at concentrations of exposure lower than 32 μg/L (measured concentration). The results also showed that female fish are highly sensitive to disruption of the androgenic pathways, highlighting their utility for the evaluation of potential adverse effects caused by anti-androgens on fish. In conclusion, the results presented in this Thesis suggest that, at present, the potential presence of dutasteride in the environment does not represent a risk to wild fish populations, due to the high concentrations required to elict significant adverse effect (LOEC = 32 μg/L) and the low volume of drug prescribed every year (5.07 kg in UK in 2006). However, the high bioaccumulation factor of dutasteride suggest that further studies should be conducted to elucidate the role played by the bioaccumulation process in the toxicity responses observed in fish.

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PUBLICATIONS

The results reported in this thesis which were not protected by the confidentialty status were published in the following peer reviewed papers and conference posters:

- **Margiotta-Casaluci, L.**, Sumpter, J.P. (2011) 5α-Dihydrotestosterone is a potent androgen in the fathead minnow (*Pimephales promelas*). *General and Comparative Endocrinology*, 171:309-318.

- Runnalls, T.J.*, **Margiotta-Casaluci, L.***, Kugathas, S. and Sumpter, J.P. (2010) Pharmaceuticals in the aquatic environment: steroids and anti-steroids as high priorities for research. *International Journal of Human and Ecological Risk Assessment*, **6**: 1318-1338. (*Co-first authorship).

- **Margiotta-Casaluci, L.,** Sumpter, J.P. (2008) Design and first results of a project aimed at determining if human pharmaceuticals inhibiting 5-alpha-reductase might adversely affect fish. *SETAC Europe 18th Annual Meeting, Warsaw, Poland.*

Additional publications taken from research not reported in this thesis include:

- Brian J.V., Beresford, N., **Margiotta-Casaluci, L.**, Sumpter J.P. (Accepted) Preliminary data on the influence of rearing temperature on the growth and reproductive status of fathead minnows (*Pimephales promelas*). *Journal of Fish Biology*.

- **Margiotta-Casaluci, L.,** Carnevali, O. (2009) Can estrogenic compounds enhance the activity of cathepsin D and cathepsin L in the mussel, Mytilus galloprovincialis? *Chemistry and Ecology,* 25(1): 49-60.

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ABBREVIATIONS

5αR:	5 alpha-Reductase
ACTH:	Adrenocorticotropic Hormone
ADI:	Acceptable Daily Intake
AMH:	Anti-Müllerian Hormone
API:	Active Pharmaceurtical Ingrdient
AR:	Androgen Receptor
ASTM:	American Society for Testing and Materials
AUC:	Area Under the Curve
BLAST:	Basic Local Alignment Search Tool
BPH:	Benign Prostatic Hyperplasia
BRENDA:	BRaunschweig ENzyme Database
CAO:	Cortico Alveolar Oocyte
cDNA:	Complementary Deoxyribonucleic Acid
CI:	Condition Index
COX:	Cyclo-oxygenase
DHP:	17α,20β-dihydroxy-4- pregnen-3-one
DHT:	5α-Dihydrotestosterone
DMF:	N,N-Dimethilformamide
DMSO:	Dimethyl Sulfoxide
DOC:	Deoxycorticosterone
DTT:	Dithiothreitol
DWC:	Dilution Water Control
E2:	Estradiol
EBI:	European Bioinformatics Institute
EC:	Environmental Concentration
EDTA:	Ethylenediaminetetraacetic Acid
EE2:	Ethinyl Estradiol
EGF:	Epidermal Growth Factor
ELISA:	Enzyme-Linked Immunosorbent Assay
ELS:	Early Life Stage
EMBL:	European Moecular Biology Laboratory
EPA:	Environmental Protection Agency
ER:	Estrogen Receptor
EST:	Expressed Sequence Tag
EVO:	Early Vitellogenic Oocyte
FDA:	Food and Drug Administration
FSH:	Follicle-Stimulating Hormone

FSHR: Follicle-Stimulating Hormone Receptor
$F_{SS}PC$: Fish Steady State Plasma Concentration
GABA: Gamma-Amino Butyric Acid
GC: Gas Chromatography
GDF: Growth and Differentiation Factor
GH: Growth Hormone
GMP: Good Manufacturing Practice
GnRH: Gonadotropin-Releasing Hormone
GR: Glucocorticosteroid Receptor
GSI: Gonado-Somatic Index
GSK: Glaxo Smith Kline
GVBD: Germinal Vescicle Break Down
HIS: Hepato-Somatic Index
HPG: Hypothalamic-Pituitary-Gonadal
HPLC: High-Performance Liquid Chromatography
HRT: Hormone Replacement Therapy
H_TPC: Human Therapeutic Plasma Concentration
IGF: Insulin Growth Factor
IMS: Industrial Methylated Spirits
KEGG: Kyoto Encyclopedia of Genes and Genomes
KT: 11-Ketotestosterone
LC: Liquid Chromatography
LH: Leuteinizing Hormone
LHR: Leuteinizing Hormone Receptor
LIN: Linearity
LOD: Limit of Detection
LOEC: Lowest Observed Effect Concentration
LOQ: Limit of Quantification
MB: Maximum Binding
MCA: Medicines Control Agency
$MgCl_2$: Magnesium Chloride
MIS: Maturation-Inducing Steroid
M-LVO: Mid-Late Vitellogenic Oocyte
MMLV RT: Moloney Murine Leukemia Virus Reverse Transcriptase
MOT: Motility
MPF: Maturation Promoting Factor
MR: Mineralcorticosteroid Receptor

MRDH:	Maximum Recommended Human Dose
MS/MS:	Tandem Mass Spectrometry
MS:	Mass Spectrometry
MS-222:	Ethyl 3-Aminobenzoate Methanesulfonate Salt
NaCl:	Sodium Chloride
NaOH:	Sodium hydroxide
$NaPO_4$:	Sodium Phosphate
NCBI:	National Center for Biotechnology Information
ND:	Not Detected
NHS:	National Health Service
NOEC:	No Observed Effect Concentration
NSAID:	Nonsteroideal Anti-Inflammatory Drug
NSB:	Non Specific Binding
NTP:	Nucleoside Triphosphate
OECD:	Organisation for Economic Co-operation and development
PANTHER:	Protein Analysis Through Evolutionary Relationship
PBS:	Phosphate Buffered Saline
PCPT:	Prostate Cancer Prevention Trial
PCR:	Polymerase Chain Reaction
PEC:	Predicted Environmental Concentration
PGC:	Primordial Germ Cell
pKa:	Acid Dissociation Constant
PNEC:	Predicted No Effect Concentration
PNO:	Perinucleolar Oocyte
PP:	Peroxisomal Proliferator
PPAR:	Peroxisomal Proliferator-Activated Receptor
PPDBSA:	Prescription Pricing Division of the Business Services Authority
RACE:	Rapid Amplification of cDNA Ends
RF:	Radio Frequency
RIA:	Radioimmunoassay
RNA:	Ribonucleic Acid
RNase:	Ribonuclease
RT:	Reverse Transcriptase
SAB:	Steroid Assay Buffer
SC:	Solvent Control
SERM:	Selective Estrogen Receptor Modulator
SHBG:	Steroid Hormone Binding Globulin
SPE:	Solid Phase Extraction
SSC:	Secondary Sexual Characteristic

SSRI:	Selective Serotonin Reuptake Inhibitor
STR:	Straightness
T:	Testosterone
TBA:	Trenbolone Acetate
TBE:	Tris borate EDTA buffer
TMHMM:	Trans-Membrane Hidden Markov Model
UV:	UltraViolet
VAP:	Average Path Velocity
VCL:	Curvlilinear Velocity
VSL:	Straight Line Velocity
VTG:	Vitellogenin
WWTP:	Waste Water Treatment Plant

CHAPTER 1 : GENERAL INTRODUCTION

1.1 Human pharmaceuticals in the environment

Pharmaceutical active compounds are a large and varied group of molecules showing different physiochemical and biological properties and functionalities (Kümmerer, 2008). These molecules are sometimes called "active pharmaceutical ingredients" (APIs), and constitute the active part of medicines, which in turn, are defined by the US Food and Drug Administration (US FDA) as "substances intended for use in the diagnosis, cure, mitigation, treatment, or prevention of diseases". The importance and the wide usage of these compounds world-wide are easily deducible. According to IMS Health (Market Prognosis, 2011), the value of the global pharmaceutical market is expected to grow in 2011 up to US$ 880 billion. In OECD countries, spending on pharmaceuticals represents a significant proportion of total health spending. Among the European countries in 2008, Greece was at the top of the rank of "*per capita* spending" on pharmaceutical products, with spending of EUR 584, compared with an OECD average of EUR 376 (OECD, 2010) (Fig. 1.1). It has been estimated that over 3000 APIs are licensed for human use in the UK, and only in England, the number of dispensed prescription items was 886 million (NHS, 2010), with an estimated usage exceeding 10 tonnes per year for all the 25 most used APIs and greater than 100 tonnes per year the for the top three compounds (paracetemol, metformin hydrochloride and ibuprofen) (Jones *et al*., 2002).

An inevitable consequence of this large consumption of pharmaceuticals is their increased discharge into the environment, through patient use and disposal. Indeed, over the past years a growing number of studies have detected trace levels of various APIs in waste water treatment plant (WWTP) effluents, surface and ground waters, and, in some countries, even in drinking water supplies (e.g. Daughton and Ternes, 1999; Fent *et al*., 2006; Herberer, 2002; Loos *et al*., 2009; Jones *et al*., 2002; Kümmerer, 2009; Ternes, 2001).

Other than the large consumption volume, APIs are designed and developed in order to have a highly specific biological activity in the body, and hence some of them can exert their effects at concentrations (few ng/L) much lower than the ones of other environmental pollutants (μg/L) (Kümmerer, 2008). Some APIs act through mechanisms of action (MoA) that can be evolutionary conserved across animal phyla, so that molecules designed to induce a specific effect in humans may also be biologically active in wildlife species (Gunnarsson *et al*., 2009). Furthermore, APIs are usually characterized by their ionic nature, and some of them have high lipophilicity (which increases their potential of bioaccumulation in the organism), and low biodegradability rate in the WWTPs (which increase their persistence in the environment) (Fent *et al*., 2006). These intrinsic properties represent the main reason for the concern of

potential adverse effects arising from the presence of human pharmaceuticals in the environment, both for human health and wildlife.

Expenditure per person (in Euro) **Expenditure as a share of GDP**

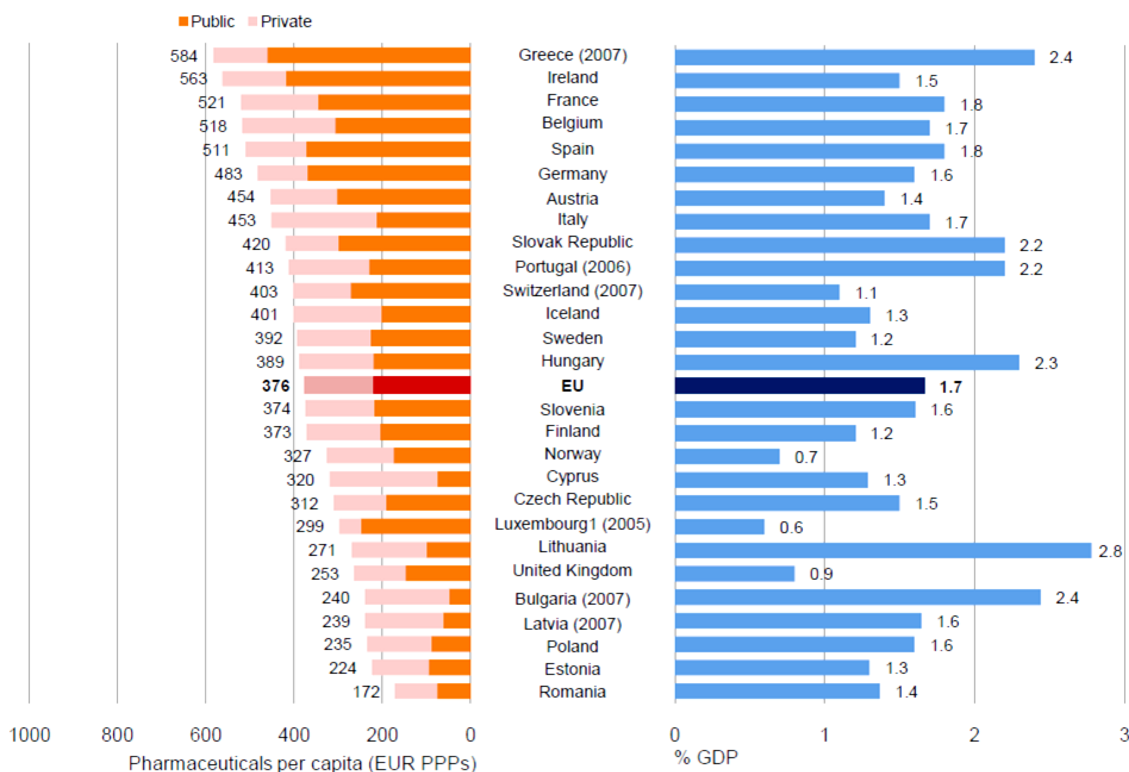


***Figure 1.1**. Expenditure on pharmaceuticals per capita, 2008. 1, prescribed medicines only (from OECD, 2010).*

1.2 Sources of human pharmaceuticals in the environment

Human pharmaceuticals and their metabolites are released into the environment via a number of routes. After administration to patients, pharmaceuticals face several changes in their chemical structure, as a consequence of the interaction with microorganisms in the digestive tract, or with enzymes of human metabolism, producing various metabolites. There is a large variability in the degree of metabolism of each drug, so that some pharmaceuticals are largely metabolized, others only partially metabolized, and others yet are completely unchanged by human metabolism (Bound & Voulvoulis, 2004; Lienert *et al.*, 2007). The resulting metabolites show different pharmacological properties compared to the parent compound, and although it is normally assumed that drug metabolism will lead to decreased toxicity, sometimes metabolites show increased toxicity (eg. sulfatation can lead to mutagenicity), and play important roles in the mediation of toxic effects (Kümmerer, 2009; Ohbuchi *et al.*, 2009).

The metabolic pathways in the human body consist of Phase I and Phase II hepatic metabolism. Phase I reactions involve oxidation, reduction and/or hydrolysis, while Phase II involves conjugation (e.g. the addition of glucuronic acid, sulphate, acetic acid or amino acid), which produce water-soluble polar metabolites that can be more easily excreted from the body. Successively, the parent compound and its metabolites are excreted in urine or feces, reaching the sewage collection system (from hospitals, homes etc.), and shortly after that, the WWTPs, where, after unefficient or incomplete removal, are finally released into the collection water body (rivers, lakes, sea) through the WWTP effluents.

Other than human consumption, other sources of release of APIs in the environment include accidental events (e.g. transport accidents), release from pharmaceutical manufacturer sites, disposal of pharmaceutical from the supply chain, and disposal of unused pharmaceuticals by patients or health care facilities (Bound and Voulvoulis, 2005; Kümmerer, 2009; Slack *et al.,* 2005). However, these sources are generally considered of modest impact when compared to the amount of APIs directly excreted by patients. For example, the activity of drug manufacturing sites in Western countries is regulated by Good Manufacturing Practice (GMP), and these sites are regularly inspected by the Medicines Control Agency (MCA) to ensure the quality and safety of products (Ayscough *et al*, 2000). This strict regulatory and controlling system usually minimizes the chances of release of APIs from these sites into the environment. Exceptions to this generally modest impact are present in India, where concentrations up to several mg/L for single compounds can be detected in effluents of local WWTPs (Larsson *et al*., 2007). In this country, the presence of several areas with high density of manufacturing sites and the lack of strict safety regulations mean that there is potentially a high risk situation for both human health and wildlife.

Finally, the disposal of expired/unused drugs by household waste, or via sink or toilet, may represent a potentially significant source of release of APIs in the environment; for example, in the US, a significant proportion of prescribed drugs are not administered and are disposed directly into waste waters, so that an estimated $1 billion of prescription drugs are discarded each year from hospitals, care facilities, and pharmacies (Cunningham *et al*., 2008; Strom, 2005). The significance of this environmental source could be strongly minimised by improving patient education (Kümmerer, 2009).

1.3 Degradation processes in WWTPs

Generally, the concentrations of pharmaceuticals detected in the aquatic environment are relatively low, usually in the ng/L to µg/L range (Daughton and Ternes, 1999; Fent *et al*.,

2006; Halling-Sorensen *et al*., 1998; Kümmerer, 2009; William and Cook, 2007), and in surface waters their concentrations rarely exceed 100 ng/L (Trudeau *et al*., 2005). In the last few years, the understanding of the occurrence of pharmaceuticals in the environment has been strongly enhanced thanks to the improvement of analytical techniques able to detect APIs at trace quantities in various matrixes, such as liquid chromathography or gas cromathography coupled to tandem mass spectrometry (LC-MS/MS and GC-MS/MS, respectively), but information about their fate in the environment is still limited (Fatta *et al*., 2007; Fent *et al*., 2006).

About 160 different APIs have been detected so far in WWTP's effluent, surface water, groundwater, and even drinking water (Fatta-Kassinos *et al*., 2010; Monteiro and Boxall, 2010; Kümmerer, 2010; Santos *et al*., 2010), but very little is known about the occurrence and fate of APIs metabolites. In particular, a key issue would be to understand if conjugated forms, like glucoronides, methylates, glycinates, and sulphates can be deconjugated by microbial metabolism in WWTP sludge or in the environment, bringing back to full activity the parent APIs (Kumar *et al*., 2011; Kümmerer, 2008).

In the WWTPs, the majority of drugs are removed during conventional secondary sewage treatments by biotransformation/biodegradation and abiotic removal by adsorption to suspended solid sludge particles (Jelic *et al.*, 2011; Petrovic *et al.,* 2009). Some APIs, however, such as the anticonvulsant carbamazepine, the lipid regulator gemfibrozil, and the analgesic diclofenac are not effectively degraded in most WWTPs (Zhang *et al*., 2008). For each API the hydrophobic properties and electrostatic interactions with particulate matter determine the predominance of one of the two main removal process, biodegradation or adsorption (Fent *et al*., 2006). For example, acidic pharmaceuticals such as the non-steroidal anti-inflammatory drug (NSAID) acetylsalicylic acid do not adsorb readily to particulate matter in the sludge, therefore elimination via microbial degradation is the most significant process (Ternes *et al*, 2004).

Many studies have attempted to establish removal rates of APIs from WWTPs, but the values obtained are highly variable, since they depend on the specific operating parameters of individual WWTP, such as hydraulic retention time, sludge retention time, microbial community structure, treatment technology and performance, temperature, and atmospheric weather at the time (Fent *et al*, 2006; Tauxe-Wuersch *et al*., 2005; Vieno *et al*, 2007; Williams, 2005). Consequently, pharmaceutical elimination is often partial; for example, ibuprofen has a high elimination rate, generally >90%, and is rapidly degraded (Buser *et al*., 1999; Metcalfe *et al*., 2003; Suarez *et al*., 2010), whereas, on the other hand, carbamazepine

has an elimination rate of only 4–8% (Herberer *et al*., 2002; Clara *et al*., 2004). Kanda *et al*. (2003) investigated the presence of a number of pharmaceuticals in six UK WWTPs, finding that many pharmaceuticals occurred in influent at ng/L levels and were removed by wastewater treatment processes with different efficiencies. Ibuprofen removal at different WWTPs was generally between 80% and 100%, with the exception of one site where removal was poor (14.4% to 44%). Moreover, Li *et al*. (2003) analyzed samples from eight WWTPs in Canada for 11 acidic drug compounds, including salicylic acid, ibuprofen, and diclofenac, recording removal rates from the influent between 0% and 98%.

Improving each WWTP with additional treatment technologies, such as activated carbon, oxidation by chlorination or ozonation, and membrane filtration can increase APIs removal rates (Boyd *et al*., 2003; Heberer *et al*., 2002; Yoon *et al*., 2006; Ternes *et al*., 2003). However, the most persistent APIa may still not be completely removed, reaching the surface waters when the WWTP effluent is released.

Contamination of soil and groundwater can also happen if WWTPs effluents are used for irrigation, as happens in some regions of the United States characterized by aridity and water depletion (Daughton, 2004; Kinney *et al*., 2006; Loraine and Pettigrove, 2006; Toze, 2006). In the case of hydrophobic APIs, the compound adsorbed on the sludge particles could be released into the environment if that sludge is used as agricultural fertilizer, since the API could transfer to surface waters via particulate matter runoff, to groundwater, or accumulate in soil (Boxall *et al*., 2002; Diaz-Cruz *et al*., 2003; Golet *et al*., 2003; Heberer, 2002).

The load of APIs received by WWTPs will vary among different regions according to several factors, including population density, average health status, average age, and economic status, which determine differences in the amount of pharmaceuticals used. For example, in 2002 in the U.S. the percentage of women of reproductive age using the Pill as contraception method - containing an estrogen, ethinyl estradiol (EE2), and a progestin, e.g. norethindrone - varied from 19% (Puerto Rico) to 42% (South Dakota) (Bensyl *et al*., 2005).

1.4 Degradation processes in the environment

Once the APIs reach surface water through WWTPs effluents, further degradation processes can occur. The abiotic transformation of a pharmaceutical in surface waters may occur mainly by photodegradation, caused by both ultraviolet (UV) and visible light (Kümmerer *et al*, 2009), which can play a major role in the degradation of some drugs, like diclofenac (Buser *et al*., 1998). The direct photolysis of a compound can be modulated by the presence of humic

acids or nitrates, which can act as photosensitizers for some drugs, thanks to the formation of hydroxyl radicals and singlet oxygen (Andreozzi *et al*., 2003; Doll and Frimmel, 2003). The degree of photodegradation depends also on the strength of the solar irradiation, and therefore on latitude and season (Fent *et al*., 2006).

The variations of pH occurring in different environments (e.g. transition freshwater/seawater) will influence the dissociation behaviour of APIs according to the acid dissociation constant (pKa) (Fent *et al*., 2006; Scheytt *et al*., 2005). This parameter will determine the ratio of ionized/un-ionized species of a compound present in solution, and the degree of ionization of the drug at a particular pH will affect its bioavailability, as well as its chemical and physical reactivity, since an ionized compound will be more water soluble than its un-ionized form (Kümmerer, 2009). Little information is available about the bioaccumulation potential of pharmaceuticals in food webs, with the exception of diclofenac, accumulating in the prey of vultures (Oaks *et al*., 2004), and fluoxetine, sertraline and their metabolites detected in fish (Brooks *et al*., 2005). Diclofenac bioconcentration factors were 10–2700 in the liver of fish and 5–1000 in the kidney, depending on exposure concentrations (Schwaiger *et al*., 2004).

Finally, biotic degradation by microbial communities can occur, even if this process is generally considered of moderate importance when compared to abiotic degradation (Fent *et al*., 2006).

1.5 Pharmaceutical concentrations in the aquatic environment

The determination of APIs concentrations in aqueous environmental matrix is mainly based on solid phase extraction (SPE), followed by liquid or gas chromatography, combined with mass spectrometry for identification and sensitive quantification. In the past few years, the improvement of all the steps of this process has allowed a limit of quantification (LOQ) for LC-MS/MS in the low ng/L range to be obtained, while with GC-MS/MS coupled with a derivatization pre-treatment the LOQ further decreases by up to 10-fold (Kümmerer, 2009).

Many studies are available in the literature concerning the screening of APIs residues in WWTP effluents and surface waters. Since the list grows at a very high rate, and new papers are continuously published, in this section only some general considerations about this issue we will provided (among the most recent papers see Fatta-Kassinos *et al*., 2010; Monteiro and Boxall, 2010; Kümmerer, 2010; Santos *et al*., 2010).

According to Monteiro and Boxall (2010), APIs concentrations in WWTP effluent are generally between “not detected” and a few μg/L, with some exceptions, like salicylic acid

(13 mg/L) (Spain). From the information provided into the paper, taking into account the highest concentrations reported, it is possible to outline of the following situation. In surface water, the concentrations are usually below 1 µg/L, often in the low ng/L range, except for acetaminophen (10 µg/L), ibuprofen (5.04 µg/L), naproxen (2 µg/L), diclofenac (4.2 µg/L), salicylic acid (8.8 µg/L), and oestrone (1.6 µg/L). Unsurprisingly, the concentration of analgesics and anti-inflammatory drugs were relatively high compared with other pharmaceutical classes, reflecting the higher volumes used by patients, thanks partly to the availability of a number of non-prescribed over-the-counter formulations. Groundwater can be contaminated by APIs up to a few µg/L, in particular the most frequently reported drugs in groundwater are in the classes of analgesic/anti-inflammatory (e.g. diclofenac, dimethylaminophenazone, propyphenazone), and sulphonamide antibiotics. Finally, in drinking water, the compounds detected at concentrations higher than the LOQ were the analgesic and anti-inflammatory phenazone (0.9 µg/L) and propyphenazone (0.12 µg/L), the antiepileptic carbamazepin (0.358 µg/L), the iodinated X-ray contrast media diatrizoate (0.085 µg/L), iopamidol (0.079 µg/L) and iopromide (0.086 µg/L); the lipid regulator benzafibrate (0.027 µg/L), and lipid regulator metabolites like colfibric acid (0.07 µg/L) and fenofibric acid (0.042 µg/L).

1.6 Effects of pharmaceuticals in the aquatic environment

1.6.1 Modes of action of pharmaceuticals

Pharmaceuticals exert their therapeutic effect by interacting with high specificity with molecular targets in the organism. A drug target is classically defined as a molecular structure (chemically definable by a molecular mass) that specifically interacts with chemicals called "drugs" because they are administered to treat or diagnose a disease (Imming *et al*., 2006). However, this definition is sometimes too "static", since the effects caused by drugs are highly dynamic, and all the drugs interfere somehow with signal transduction, receptor signalling and biochemical equilibrium. Moreover, many drugs interact with more than one target, causing simultaneous changes in several biochemical signals, and their relative feedback reactions. This dynamicity can be observed in many drugs, causing clinical effects that are not readily deducible from single molecular effects, and it is enhanced by drug combinations (Imming *et al*., 2006).

In 2006, Imming *et al*. catalogued 218 molecular targets for approved drugs; in the same year Zheng *et al*. identified 268 targets in the Therapeutic Targets Database, and Overington *et al*. (2006), finally proposed a consensus number of 324 drug targets for all classes of approved

therapeutic drugs, combining all the earlier reports into a single comprehensive survey. The majority of drug targets are proteins, and these are classified into 4 major groups: receptors, ion channels, enzymes and transporters (Fig.1.2) (Rang *et al*, 2003).

Knowledge of the mechanisms of action of different APIs detected in the environment has a critical importance in the risk assessment process, since the evolutionary conservation of drug targets in wildlife species would suggest the possibility that human pharmaceuticals present in aquatic environments, even if at low concentrations (ng/L), may cause toxicological effect acting through the same targets as they do in humans (Ankley *et al.*, 2007; Christen *et al.*, 2010; Gunnarsson *et al*, 2008; Kostich and Lazorchak, 2008; Seiler, 2002). Knowledge of the degree of target conservation could help in elucidating the sensitivity of different taxa, or species, to each API, thus giving precious information in the risk assessment process (Christen *et al.*, 2010; Owen *et al.*, 2007). A more detailed discussion of this topic will be provided in Chapter 2.

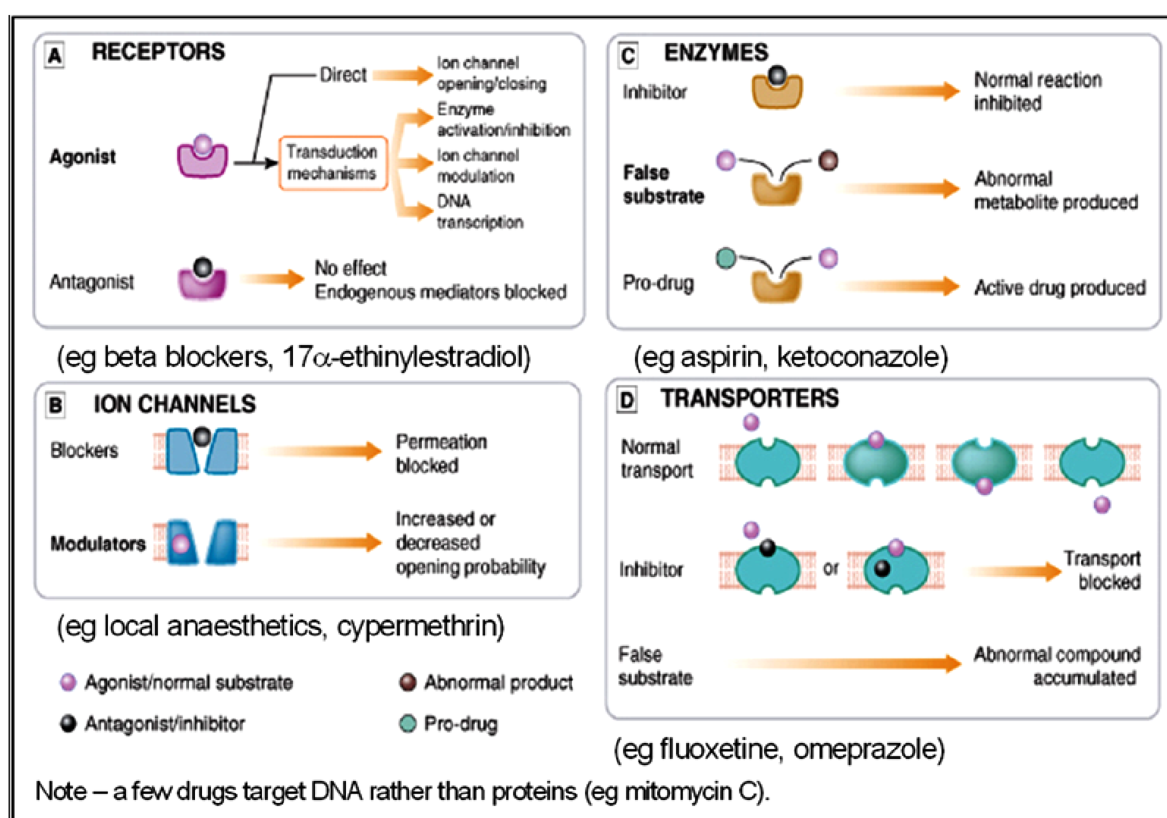


Figure 1.2. *The majority of drug targets are proteins, and are classified into 4 major groups: receptors, ion channels, enzymes and transporters (from Rang et al., 2003).*

1.6.2 Effects of pharmaceuticals on fish

The story of pharmaceuticals in the environment started in 1978, after the accidental discovery of intersex fish in an English river (Sweeting, 1981). After that a link between intersex fish and estrogens (in particular EE2) was established, the issue of estrogenic chemicals in the aquatic environment, and of their possible effects on fish and other wildlife, became a topic of very considerable interest until today, as described in the fascinating story published by Sumpter and Johnson (2008) in their "Reflections on endocrine disruption in the aquatic environment: from known knowns to unknown unknowns". Thousands of research papers have been dedicated to the varied aspects - both chemical and biological - of this issue (Matthiessen, 2003).

The issue of EE2 in the aquatic environment could be considered as a general model of reference in the study of pharmaceutical in the environment. In fact, this synthetic estrogen is one of the active ingredients of the contraceptive "Pill." EE2 is an extremely potent estrogen; concentrations less than 1 ng/L have adverse effects on reproduction of fish, and only slightly higher concentrations prevent fish reproducing (e.g., Lange *et al*., 2001; Nash *et al*., 2004; Parrott and Blunt, 2005), leading to population crashes (Kidd, *et al*. 2007). One of the key lessons to come out of all the environmental research on EE2 is that very biologically active chemicals can be present in the environment, and they have the capability to cause effects at extremely low concentrations (Sumpter and Johnson, 2005). This seems to be due to a combination of factors, specifically (1) that EE2 is readily taken up by fish from the water (Maunder *et al*. 2007; Scott *et al*. 2005), (2) that it bioconcentrates to a reasonable degree in fish (Lange *et al*. 2001), (3) that estrogen receptors (ERs) to which it binds very avidly exist in fish, just as they do in humans taking the drug as a contraceptive, and (4) that these receptors play key roles in regulating reproduction.

These "lessons" can be translated into several general assumptions: 1) fish can take up pharmaceuticals from the surrounding environment in a number of ways, for example through dermal and gill surfaces, orally through the diet, or maternally, via the transfer of contaminants through the lipid reserve of eggs (Corcoran *et al*., 2010), and some of these drugs can be bioaccumulated in the organisms; 2) a medium-high degree of evolutionary conservation of drug molecular targets exists between fish and humans (Gunnarsson *et al*., 2008); 3) the conservation of the targets suggest an high probability of functional interaction between drug and target, which in turn will cause some (potentially) negative physiological

effects. The latter point has been confirmed by several studies, and the amount of evidence is continuously growing (e.g. Giltrow *et al*., 2009; Kugathas and Sumpter, 2011; Paulos *et al*., 2010; Runnalls *et al*., 2007; Winter *et al*., 2008).

Finally, two more factors should be taken into consideration. Firstly, differences in the capacity of fish to metabolize specific xenobiotics compared to mammalian metabolism, in particular during early life stages, could also play a role in the response of fish to human pharmaceuticals (Wolf and Wolfe, 2005; Andersson and Forlin, 1992), and secondly, from the review of the studies concerning the effects of pharmaceuticals in fish, a potentially large inter-species variability seems to exist in fish with regard to the sensitivity of each species to a specific API, at least for some pharmaceuticals, such as flutamide (Jensen *et al*., 2004; OECD, 2006).

Detailed reviews of the effects of human pharmaceuticals in fish have been published by Fent *et al*. (2006), and recently by Corcoran *et al*. (2010), whereas the effects of steroids and anti-steroids in fish will be discussed in Chapter 2. Moreover, the effects of anti-androgenic drugs in fish and other animal models will be the main topic of discussion in Chapters 5 and 6. Hence, in this section only some general considerations will be provided.

Among all the therapeutic classes, the largest set of toxicity data available for fish is the one concerning the effects of steroid agonists. Both natural and synthetic steroids show the lowest LOEC values in fish among all the tested drugs. For example, EE2 has been shown to act on fish even at sub-ng concentrations (Parrot and Blunt, 2005), inducing feminization of male fish and affecting reproduction (e.g. Kidd *et al.*, 2007; Nash *et al.*, 2004; Orn *et al.*, 2003, 2006); progestagens, like levonorogestrel and norethindrone, adversely affected fathead minnow reproduction at 0.8 ng/L and 1.2 ng/L, respectively (Paulos *et al.*, 2010; Zeilinger *et al.*, 2009), while androgens and glucocorticoids exerted their effect at higher concentration, but still in the low ng/L range; for example, trenbolone caused irreversible masculinisation of zebrafish at 9.2-15.5 ng/L (Morthorst *et al.*, 2010) and the glucocorticoid beclomethasone dipropionate caused an increase of plasma glucose at 100 ng/L (Kugathas, personal communication).

Anti-steroids, including steroid receptor antagonists (e.g tamoxifen, flutamide) and steroidogenic enzyme inhibitors (e.g. fadrozole, ketoconazole), generally produce adverse effects in fish only when their concentrations are in the range of µg/L (Ankley *et al.*, 2007; Jensen *et al.*, 2004; Panter *et al.*, 2004; Sharpe *et al.*, 2004; Sun *et al.*, 2007; Williams *et al.*, 2007), except cyproterone actetate, which produced a decrease of plasma testosterone (T) and

11-ketotestosterone (KT) in female mummichog (*Fundulus heteroclitus*) at concentrations as low as 10 ng/L (Sharpe *et al.*, 2004), although it has never been tested on fish reproductive functions or other major endpoints. All the other therapeutic classes caused adverse effects on fish only at high concentrations (µg/L or mg/L), with few exceptions. Generally these concentrations are not environmentally relevant.

The widely used nonsteroidal anti-inflammatory drugs (NSAIDs), such as diclofenac, naproxen and ibuprofen, act by inhibiting cyclooxygenases (COXs), the enzymes that catalyse the synthesis of prostaglandins, via the oxidation of arachidonic acid, inducing a reduction of inflammation and pain. Some NSAIDs have been reported to produce adverse effects on fish, but only at relatively high concentrations. For example, indomethacin disrupted the process of oocyte maturation and ovulation in zebrafish at 100 mg/L (Lister and van der Kraak, 2008) and ibuprofen altered the pattern of reproduction in Japanese medaka at concentrations of 100 µg/L (Flippin *et al.*, 2007). Diclofenac, which brought the collapse of Asian vulture populations by inducing renal failure in individual birds (Oaks *et al.*, 2004; Green *et al.*, 2004), caused some effects also in fish exposed to the drug in the low µg/L range. For example, diclofenac induced glomeruloneophritis, necrosis of endothelial cells, and hyaline droplet degeneration in the kidney of rainbow trout, and also pillar cell necrosis, epithelial lifting, hyperplasia, and hypertrophy of epithelial chloride cells in gills at exposure concentrations between 1 and 5 µg/L (Triebskorn *et al.*, 2004; Schwaiger *et al.*, 2004).

Also the selective serotonin reuptake inhibitor (SSRI) antidepressants, such as fluoxetine, are consumed in large amounts by humans and exert their therapeutic effects mainly through the inhibition of the reuptake of the neurotransmitter serotonin at presynaptic neuronal membranes (Hiemke and Hartter, 2000), causing an increase of serotonin concentration and the time when present in the synaptic gap (Fent *et al.*, 2006). This alteration of serotonin levels *in situ* is generally considered beneficial to cure symptoms of depression and other psychiatric disorders (Brooks *et al.*, 2003). Both serotonin and its receptors have been identified in several fish species (Caamano-Tubio *et al.*, 2007; De Lucchini *et al.*, 2001; Yamaguchi and Brenner, 1997). Furthermore, serotonin seems involved in the mediation of a number of physiological processes in fish, such as behaviour (aggression, appetite), and reproduction (Alanara *et al.*, 1998; Overli *et al.*, 1998; Winberg *et al.*, 1997). With regard to the effects casued by SSRIs in fish, fluoxetine is the SSRI which has received the highest amount of attention. Exposure to this drug typically altered fish behaviour, such as territoriality and aggressiveness (at 6 µg/g/day for 2 weeks) (Perreault *et al.*, 2003; Semsar *et al.*, 2004), and feeding behaviour (between 23.2 and 170 µg/L) (Gaworecki and Klaine, 2008;

Stanley *et al.*, 2007). Exposure of medaka to fluoxetine (0.1-0.5 µg/L) caused an increase in the concentrations of plasma E2, and impaired growth (Brooks *et al.*, 2003), confirming the involvement of serotonin in the system of steroidogenic feedbacks (Khan and Thomas, 1992; Somoza and Peter, 1991). However, other studies did not confirm these effects in fish (Foran *et al.*, 2004).

Beta blockers, such as atenolol and propranolol, are used to treat or manage several conditions, including cardiac conditions (angina pectoris, heart failure, arrhythmia, hypertension), glaucoma, hyperhidrosis. They work as competitive β-adrenergic receptor (β-AR) antagonists (Owen *et al.*, 2007). β-ARs are present in both cardiac and extra-cardiac tissues in fish (e.g. gills, liver, brain, muscles) (Lortie and Moon, 2003; Nickerson *et al.*, 2001; Zikopoulos and Dermon, 2005). From the toxicological point of views, the studies available in the literature suggest that beta blockers produce adverse effect on fish only at high concentrations (µg/L or mg/L). For example, propranolol caused decreased growth in medaka (500 µg/L) and juvenile rainbow trout (10 mg/L) (Huggett *et al.*, 2002; Owen *et al.*, 2007), other than a decrease of egg production and hatching success in medaka (5 µg/L), and egg production in fathead minnow (1 mg/L) (Huggett *et al.*, 2002; Giltrow *et al.*, 2009). Another beta-blocker, atenolol, affected growth in fathead minnow larvae, but only at very high concentrations (10 mg/L) (Winter *et al.*, 2008).

Lipid regulators, in particular fibrate drug and statins, are also commonly detected in surface waters. These compounds act as peroxisomal proliferators (PPs), and thanks to their ability to lower lipid levels in the blood, are used to treat hypercholesterolemia. Fibrates are agonist of the peroxisomal proliferator-activated receptor alpha (αPPAR), a nuclear transcription factor. The activation of this pathway produces regulation of the expression of some genes coding for enzymes involved the lipid metabolism (Gervois *et al.*, 2000), which when activated, coupled with increased peroxisomal volume, leads to the removal of fatty acids and cholesterol from the blood. There are only a few studies on the effects of fibrates on fish, but there is evidence that the PPAR pathway has an important function also in this taxon (Ruyter *et al.*, 1997; Andersen *et al.*, 2000; Ibabe *et al.*, 2002). Runnalls *et al.* (2007) did not observe any effect on the lipid metabolism of fathead minnow exposed to clofibric acid (0.01–1 mg/L), however, the drug adversely affected the reproductive axis, in particular reducing sperm count and motility (at 1 mg/L).

Finally, other therapeutic classes on which only very limited environmental research has been conducted include antibiotics, chemotherapy and antiepileptic drugs. There is no reported evidence of toxicity induced by antibiotics in fish, except for tetracyclines that can have a

suppressive effect on the immune systems in fish at µg/L concentrations (Grondel *et al.*, 1985), but it is unlikely that these compounds would have direct physiological effects on fish, since they mainly target physiological or molecular processes of bacteria. However, the main risk of antibiotics in the environment is their involvement in the development of antibiotic resistance in bacteria (Kim and Aga, 2007). Predicted environmental concentrations of cytotoxic chemotherapy drugs are in the range of 5-50 ng/L, but no toxicity data are available in the literature yet (Johnson *et al.*, 2008). Similarly, no data are available for antiepileptic drugs toxicity.

1.7 5α-Reductase and DHT

5α-Reductase (EC 1.3.99.5, 3-oxo-5alpha-steroid 4-dehydrogenase, 5αR) is a microsomal enzyme that catalyzes the conversion of T into DHT. The discovery, functions, and clinical importance of 5αR and of its inhibitor drugs have been extensively described by Frye (2006) and Marks (2004). In the following sections the key aspects of 5αR biological relevance will be described, with the aim to provide a useful background for the interpretation of the experimental results described later in this thesis.

1.7.1 History and clinical importance

The rise of 5αR as a clinically important target in prostate diseases is due, as for many important scientific discoveries, to a casual observation made by Imperato-McGinley and colleagues at beginning of the 1970s, when they discovered the existence of pseudo-hermaphrodite children in an isolated village of the Dominican Republic, locally called “guevedoces”, literally “penis at 12” (years of age). This term was used by locals to indicate biological males born with female looking external genitalia, which then develop typical male genitalia at puberty (Fig. 1.3). The explanation for this pathological condition was a congenital deficiency of the enzyme 5αR. Interestingly, in these individuals the prostate remained small throughout life (Imperato-McGinley *et al.*, 1974, 1979, 1992; Peterson *et al.*, 1977). This discovery led to the synthesis of a new class of drugs, the 5α-reductase inhibitors (5αRIs). Finasteride was the first 5αRI, approved in 1992 (Gormley *et al.*, 1992, 1994), followed by dutasteride, approved by US FDA in 2002 (Bakshi *et al.*, 1995; Roehborn *et al.*, 2002).

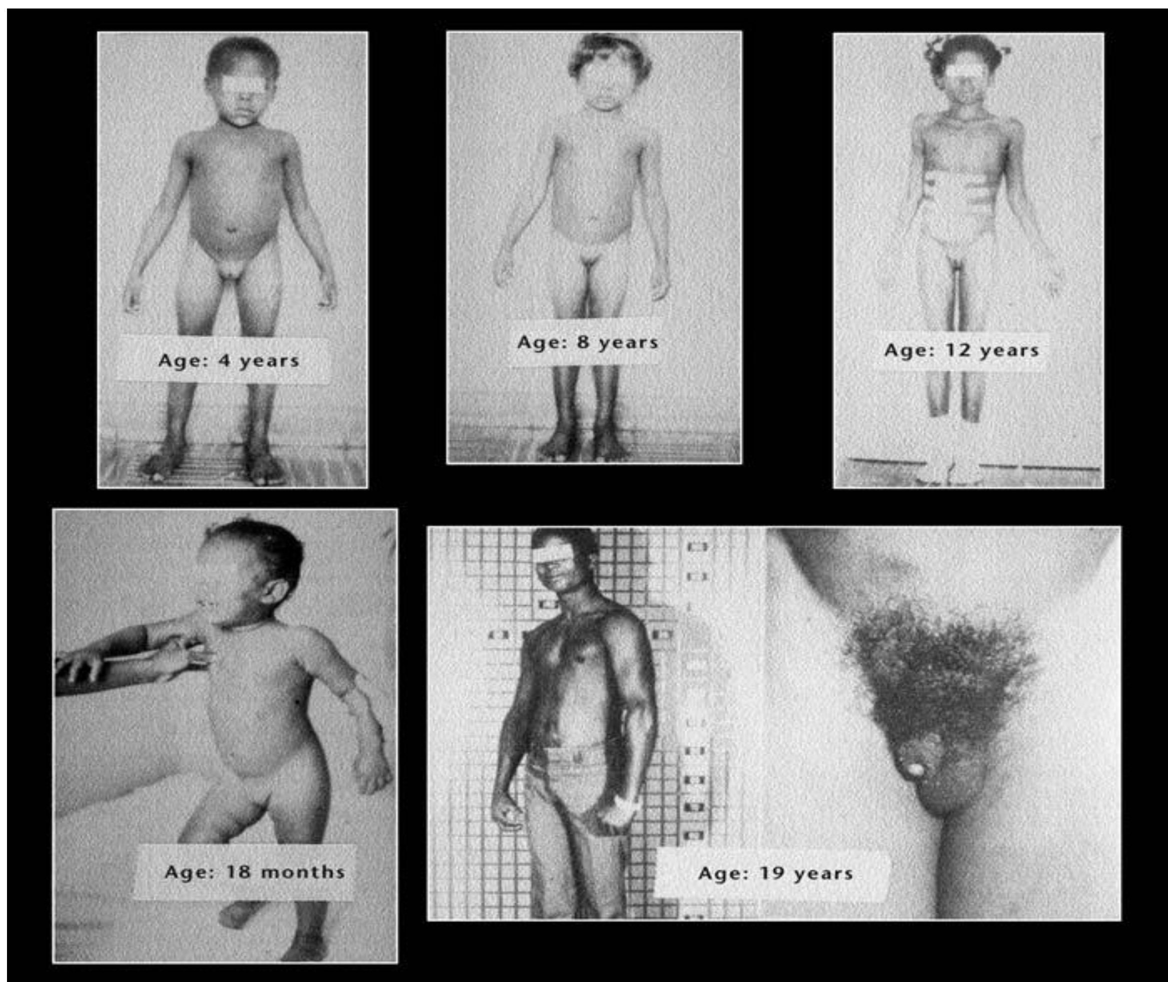


Figure 1.3. *"Guevedoces (literally, penis at 12) are biological males with female lacking external genitalia from birth until puberty; at puberty, they develop a penis, the testes descend, muscles and a male psychosexual orientation develop. Throughout life, the prostate remains small, because the congenital deficiency of 5αR leaves affected individuals with markedly suppressed levels of DHT, which is the major androgenic stimulus for the prostate" (from Peterson et al., 1977; Marks, 2004).*

Benign prostatic hyperplasia (BPH) and prostate cancer are both age-related proliferative diseases which result in significant morbidity and mortality in affected males (Geller, 1991; Isaacs, 1990). These conditions are extremely common in men; in fact, BPH occurs in the majority of individuals over the age of 60 and microscopic hyperplasia is a nearly universal finding on autopsy (Frye, 2006). Prostate cancer is the most common cancer in male population, and in 2008 around 37,000 men were diagnosed with prostate cancer in the UK, and around 338,000 in Europe. In the UK, it constitutes - after lung cancer - the second most common cause of cancer death in men, and in 2008 it causes 10,170 deaths, albeit more than 80% of them occurred in men aged 70 and over (Cancer Research UK, 2011). Both the incidence of BPH and prostate cancer increase steadily from age 50 onward; current knowledge suggest that they originate in different regions of the prostate, although they share a common dependence upon androgens for development and progression (Frye, 2006).

In the prostate it is possible to recognize three anatomically distinct zones – the peripheral, the central and the transition zone. In BPH, the transition zone is the one that exhibits nodular expansion via proliferation and branching of epithelial glandular tissue and, to a lesser extent, prostatic stromal elements (Lee and Pheel, 2004). The growth of the transition zone causes the typical constriction of the urethra by compression, producing difficulty in urination, and sometimes acute urinary retention, which can be treated only by surgery (Fig. 1.4) (Frye *et al.*, 2006). BPH is strictly dependent on testicular function and androgen synthesis (Moore, 1944). T is converted into DHT by the two isoforms of 5αR, and DHT is the major androgen acting on the prostate. 5αR Type 2 deficiency, the same one as was observed in "guevedoces", produced a dramatic decrease in the development and growth of the prostate (Imperato-McGinley *et al.*, 1974), confirming that DHT is involved in the aetiology of BPH. This observation led Petrow and Padilla (1984) to the theory that 5αR could be a target enzyme for treatment of prostate cancer. This intuition was confirmed by some results obtained from the Prostate Cancer Prevention Trial (PCPT), showing that treatment with finasteride caused a decrease of the incidence of prostate cancer by 25% when compared to a placebo (Thompson *et al.*, 2003). Dutasteride was also proposed for prostate cancer prevention, and tested in a similar clinical trial called "Reduction by Dutasteride of Prostate Cancer Events" (REDUCE). However, in March 2011 the US FDA, following recommendations from its Oncologic Drugs Advisory Committee, rejected both proposals, since dutasteride, although reducing the incidence of prostate cancer in men at high risk of developing the disease, also caused a slightly higher risk of developing a more aggressive form of prostate cancer (NCI Cancer Bullettin, 2011).

In addition to BPH and prostate cancer, DHT mediates also the development of some diseases of the skin, such as acne, idiopathic female hirsutism and androgenetic alopecia (male pattern baldness) (Tenover *et al.*, 1991).

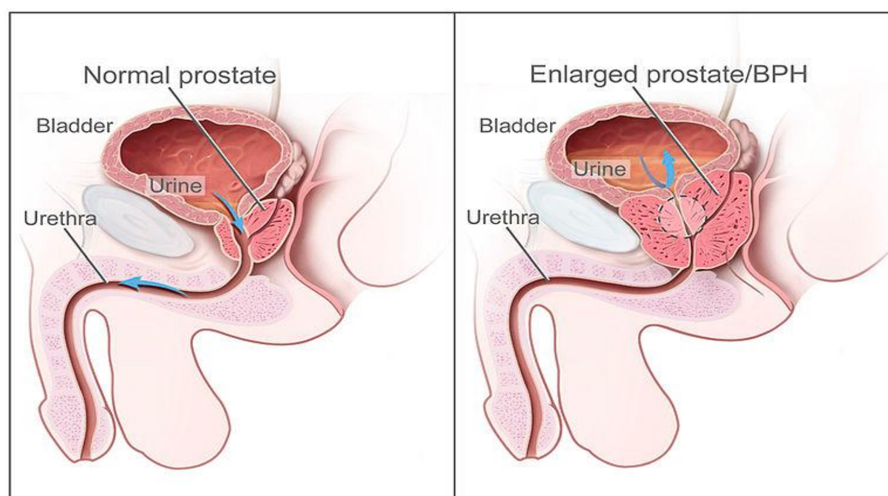


Figure 1.4. *Benign prostatic hyperplasia, characterized by hyperplasia of prostatic stromal and epithelial cells, resulting in the formation of large nodules in the peri-urethral region of the prostate. When sufficiently large, the nodules compress the urethral canal to cause partial, or sometimes virtually complete, obstruction of the urethra, which interferes with the normal flow of urine.*

1.7.2 Role of 5α-reductase in human physiology

The two isoforms of 5αR catalyse the NADPH-dependent reduction of $\Delta^{4,5}$ unsaturated steroids (Russel and Wilson, 1994). The Type 1 and 2 isozymes differ in their tissue distribution, optimum working conditions (e.g. pH), and sensitivity to inhibitors (Table 1.1) (Frye, 2006). Both isozymes are hydrophobic proteins of 259 and 254 amino acids, respectively, and present 50% of identity in their amino acid sequences. In men, 5αR Type 2 is mainly expressed in genital tissues (prostate, epididymis, seminal vesicle, genital skin) and liver, while the Type 1 isozyme is predominantly expressed in sebaceous glands, liver and non-genital skin (Table 1.1) (Levine *et al.*, 1996; Patel *et al.*, 1996; Russel and Wilson, 1994). Inter-species comparisons of the amino acid sequences of 5αR Type 1 and Type 2 showed only moderate homologies, suggesting important differences in forms, functions, and/or regulation. For example, the Type 1 human and rat isozymes have approximately 61% amino acid sequence identity, while the two corresponding 5αR Type 2 are 75% homologous. Tissue distributions of the two isozymes between rat and human also differ (Levy *et al.*, 1995; Normington and Russe, 1992).

Both expression of the 5αR genes and enzymatic activity are regulated by several factors, such as androgens, glucocorticoids and IGF1, in tissues such as prostate, epididymis, liver and brain (Antonipillai *et al.*, 1995; Andersson *et al*, 1989; Henderson, 2006; Horton *et al.*, 1993; Robaire and Henderson, 2006). Some evidence also suggests the occurrence of a significant regulation at the post-transcriptional level (El-Awady *et al.*, 2004).

Table 1.1. *Biochemical properties and localization of human 5a-reductase Type 1 and 2 (from Frye, 2006).*

Properties	**5αR Type 1**	**5αR Type 2**
k_{cat}, s^{-1} (testosterone)	0.52	0.0001
K_m, μM (testosterone)	6.3	0.0063
k_{cat}/K_m $M^{-1}s^{-1}$	8.2×10^4	1.6×10^4
pH optima	6-8.5	5
<u>Localization</u>		
prostate, epididymis, seminal vescicle, genital skin	absent	abundant
testis, ovary, adrenal, brain, kidney	absent	absent
liver	present	present
non-genital skin	abundant	absent

The classic 5αR substrate is the androgen T, subsequently converted to DHT, a metabolite with higher androgenic potency (Fig. 1.5). Both androgens are essential for male phenotypic sexual differentiation and maturation, exerting their effects through the androgen receptor (AR) (Josso, 1994; Wilson; 1989). Initial male reproductive development depends on the synthesis of T and anti-müllerian hormone (AMH) by the fetal testes (Josso, 1994). The masculinization of the external genitalia is controlled by the conversion of T into DHT in the tissues of the urogenital sinus, and deficiency in 5αR (Type 2) causes an incomplete form of male pseudohermaphroditism (Andersson *et al.*, 1991; Imperato-McGinley *et al.*, 1974; Thigpen *et al.*, 1992; Walsh *et al.*, 1974). The 5αR deficient males undergo a partial masculinisation during puberty, including testicular descent, penal enlargement and development of male musculature; however, these individuals continue to present reduced body and facial hair, decreased temporal regression of the hairline (which regresses in non-deficient men), and reduced size of the prostate (Imperato-McGinley *et al.*, 1992).

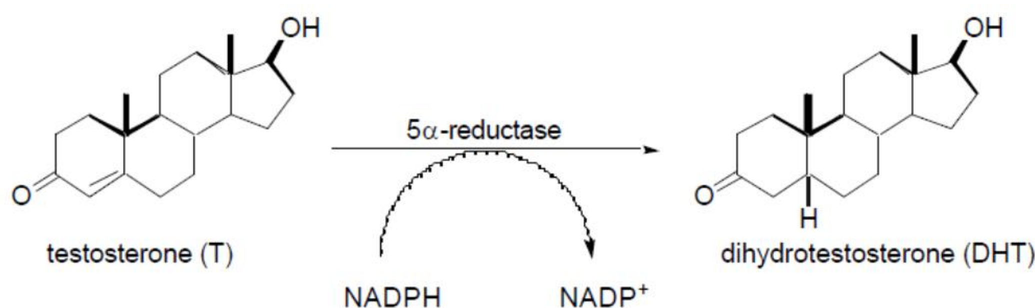


Figure 1.5. *5α-Reductase catalyses the conversion of T into DHT (from Frye, 2006).*

The 5α-reduction of T into DHT in the testis is important for the maintenance or restoration of spermatogenesis in adult rats affected by low levels of testicular T. Since in pubertal male rats T levels are lower than in adult males, it is possible to hypothesize that DHT may also be important for initiating spermatogenesis during puberty (Killian *et al.*, 2003; O'Donnell *et al.*, 1996, 1999). In female mice, 5αR deficiency shifts the steroidogenic pathway toward the conversion of androgen into estrogens, which when in excess can, in turn, cause fetal death by estrogen toxicity (Mahendroo *et al.*, 1997). These findings lead to the conclusion that 5α-reduced androgens are required for normal reproduction in female mice. Indeed, 5αR Type 1 gene silencing causes a parturition defect in mice, and this defect can be reversed by administration of 5α-reduced androgens (Mahendroo *et al.*, 1996). Some studies performed on the mouse also highlighted a potential sex-specific relevance of the two enzymatic isoforms, suggesting that the Type 1 enzyme may have a greater importance role in females, while 5αR Type 2 may be more important in males (Mahendroo and Russel, 1999).

Other than sexual development, 5αRs and DHT are involved in other physiological processes, as suggested by the high activity of these enzyme in several tissues, including liver, skin, and central nervous system. The functions mediate by 5αRs in these organs are not completely clear: in the liver, the enzymatic activity could exert a catabolic function (Josso, 1994), in the skin has been shown to mediate sebum production (Imperato-McGinley *et al.*, 1993), while in the brain it has been suggested that 5α-reduced metabolites of progesterone alter GABAA receptor function, playing a role in sexual differentiation of the fetal brain (Melcangi *et al.*, 1994).

Other than the conversion of T into DHT, 5αR also catalyzes the reduction of other steroids with the 3-oxo-$\Delta^{4,5}$ structure, including progesterone, androstenedione, 11-deoxycorticosterone, and cortisol (Fig. 1.6) (Tian *et al.*, 1994; BRENDA Database). For example, it has been shown that 5αR Type 1 controls androstanediol formation in immature mouse testes (Mahendroo *et al.*, 2004). Androstanediol seems to be involved in the regulation of male behaviour in some species (Mann *et al.*, 1998, Mahendroo *et al.*, 2004), and is present at high concentrations in the immature testes of species like mice (Chase and Payn, 1983) and golden hamsters (Frungieri *et al.*, 1999). The 5α-reduction of T to DHT in target tissues is a key step in androgen action, but in some circumstances DHT is formed by an alternative steroidogenic pathway involving the oxidation of androstanediol synthesized in the testes (Shaw *et al.*, 2000). Whether androstanediol has any specific biological action, other than being a precursor for DHT, is unclear (Frye *et al.*, 1996).

5αRs also convert progesterone and deoxycorticosterone (DOC) to their respective 5α–reduced derivatives providing the substrates for 3α-hydroxysteroid dehydrogenase, the enzyme responsible for the formation of 3α-hydroxy–5α–pregnan-20-one (allopregnanolone), and 3α,21-dihydroxy-5α-pregnan-20-one (tetrahydroDOC). These compounds can act as potent neurosteroids, whose action is mediated by allosteric modulation of the γ-aminobutyric acid–A receptor complex ($GABA_A$-R) (Mellon and Griffin, 2002). 5α-reduced metabolites of progesterone have often been detected in *in vitro* metabolism studies performed using mammary tissue from mouse (Mori and Tamaoki, 1977, 1980), rat (Eechaute *et al.*, 1983; Mori *et al.*, 1978), cat (Shiota *et al*, 1991), dog (Shiota *et al.*, 1991; Tanaka *et al.*, 1994) and human (Lloyd, 1979; Verma *et al.*, 1978; Wiebe *et al.*, 2000). These 5α-reduced metabolites (5α-pregnanes) have been shown to be present at significantly higher concentration in human breast cancer tissue than in non-tumorous tissue, indicating an increased 5αR activity in the carcinoma (Lewis *et al.*, 2004; Wiebe *et al*, 2000; Wiebe and Muzia, 2001,).

Finally, the pathways of cortisol metabolism includes irreversible inactivation by 5αR. Increased peripheral inactivation of cortisol caused by enhanced 5α-reductase activity may interfere with negative feedback suppression of adrenocorticotrophic hormone (ACTH) secretion, and it is involved in the aetiology of some human pathological conditions, like polycystic ovary syndrome (Tsilchorozidou *et al.*, 2003).

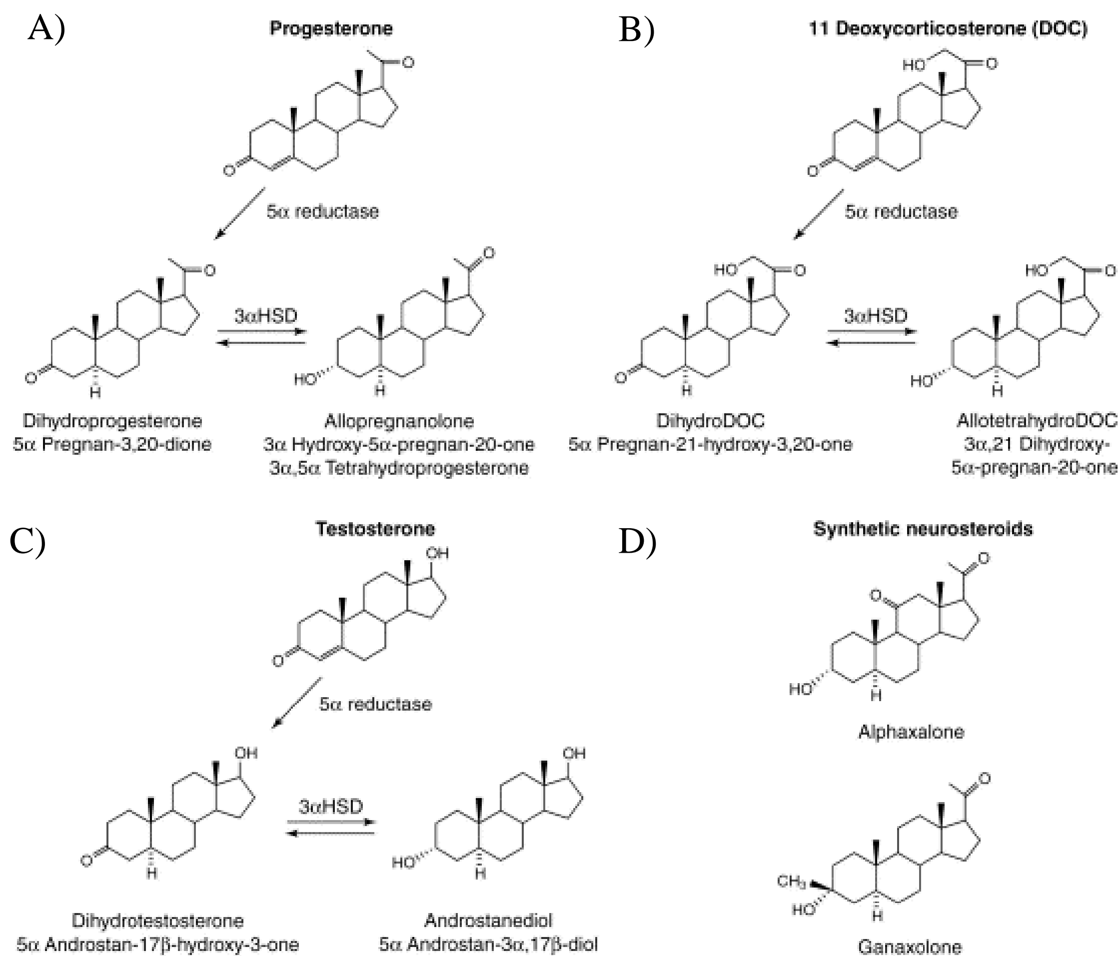


Figure 1.6. *Structure of neuroactive steroids. Metabolism of (A) progesterone, (B) 11 deoxycorticosterone (DOC) or (C) T by the enzymes 5αR and 3α-hydroxysteroid dehydrogenase (3αHSD) leads to the production of the neuroactive steroids allopregnanolone, tetrahydroDOC or androstanediol. The chemical names for these steroids are shown below the trivial names. The dotted triangle indicates that the hydrogen or hydroxyl group at C3 or C5 is referred to as α; the solid triangle indicates that the hydrogen or hydroxyl group is referred to as β. The related structures of two synthetic steroids that are neuroactive (D) are shown for comparison with the endogenously synthesized neurosteroids (from Mellon and Griffin, 2001).*

1.8 Dutasteride, a dual inhibitor of 5α-reductases

Dutasteride (CAS N°: 164656-23-9), a synthetic 4-azasteroid, is a competitive and specific inhibitor of both the Type 1 and Type 2 isoforms of 5αR; it is manufactured by GSK and marketed with the brand name "Avodart", or "Combodart" when combined with tamsulosin, an α_{1a}-selective alpha blocker. Its extended chemical name is (5α,17β)-N {2,5-bis(trifluoromethyl)phenyl}-3-oxo-4-azandrost-1-ene-17-carboxamide, its molecular weight is 528.5 and its chemical structure is characterized by the N substitute at C4, and by the bis(trifluoromethyl)phenyl group, which increase the stability and lipophilicity of the drug, and is involved in its mechanism of action (Fig. 1.7) (Tian *et al.*, 1995).

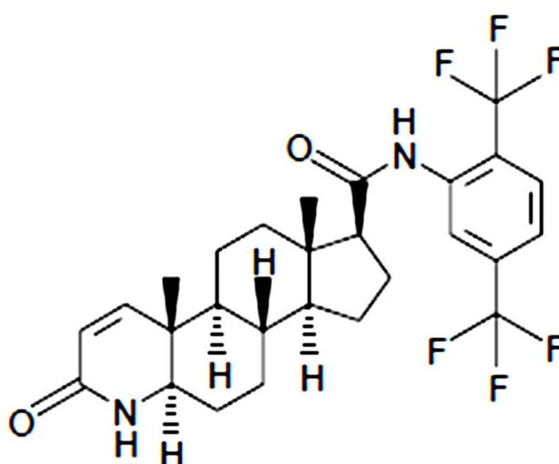


Figure 1.7. *Chemical structure of dutasteride.*

The crystallized form of dutasteride is a white to pale yellow powder with a very low solubility in water (solubility < LOD = 0.038 ng/ml). However, it is soluble in solvents like ethanol (44 mg/mL), methanol (64 mg/mL) and polyethylene glycol 400 (3 mg/mL). The drug is lipophilic, as indicated by the high octanol/water partition coefficient of 5.09 (logP), and is formulated as hard capsules or soft gelatine capsules containing 0.5 mg of dutasteride, alone or combined with tamsulosin hydrochloride (0.4 mg).

During treatment, the recommended dose of Avodart is one capsule (0.5 mg) taken orally once a day. The official therapeutic use is the treatment of BPH, however it is also used, without official approval, for the treatment of androgenic alopecia (male baldness).

All the data and information described in the following sections were provided by GSK (2001) during the drug approval process at US FDA (Application Number: 21-319), including pre-clinical and clinical toxicity data, and entirely reflect GSK interpretation of the results.

1.8.1 Biopharmaceutics

Absorption of dutasteride following oral administration of soft gelatin capsules was rapid, with T_{max} of 1-4 hours (T_{max} is defined as the time after administration of a drug when the maximum plasma concentration is reached). Food reduced the total amount of drug absorbed by the body by approximately 10-15%. The drug exhibits a large volume of distribution (300 to 500 L) and was highly bound to plasma proteins (99.0%). The terminal elimination half-life of dutasteride was approximately 5 weeks; therefore, bioaccumulation of the drug is expected after daily administration. Following repeated doses of 0.5 to 5 mg, the dutasteride plasma level, after 28 days, wass about 14 to 16-fold higher than after a single dose (GSK, 2001).

1.8.2 Metabolism

The only data concerning *in vivo* metabolism of dutasteride are based on a preliminary (pilot) metabolism study *in vivo* (ARIA10012) with unlabeled dutasteride. The results showed that the drug was extensively metabolized to approximately 10 metabolites (4 major and 6 minor). Overall, approximately 5% of the dose was excreted unchanged in the faeces and approximately 40% excreted as unidentified pooled metabolites. Approximately 55% of the dose was unaccounted for, confirming the hypothesis of high potential of bioaccumulation for this drug. No traces of metabolites related to dutasteride were detected in urine.

The *in vivo* findings of extensive metabolism in humans were not in agreement with the results obtained *in vitro*, showing that the isoenzyme CYP 3A4 was the only active enzyme, accounting for only 5% of the metabolism, but none of the other tested isoenzyme or enzymes (CYP1A2, CYP2A6, CYP2C9, CYP2C19,CYP2D6 and CYP2E1, glucuronyltrasferase and sulphotraspherase) were found to be responsible for the metabolism of the drug, using either cDNA-expressed CYP450 enzymes, liver microsomes, or hepatocytes. Hence, the metabolic pathways of dutasteride remain unclear.

Based on a US FDA request, the sponsor (GSK) submitted a proposed metabolic pathway in animals and human, which did not include any information on the potentially involved isoenzymes (Fig. 1.8). The main metabolites were 4-hydroxydutasteride and 6-hydroxidutasteride, and the former was the major circulating metabolite in humans. The biological activity of these metabolites is mostly unknown.

The average serum concentration of unchanged dutasteride was approximately 40 ng/ml, compared to the average concentration of 10 ng/ml of the major metabolite 4-hydroxydutasteride (Table 1.2).

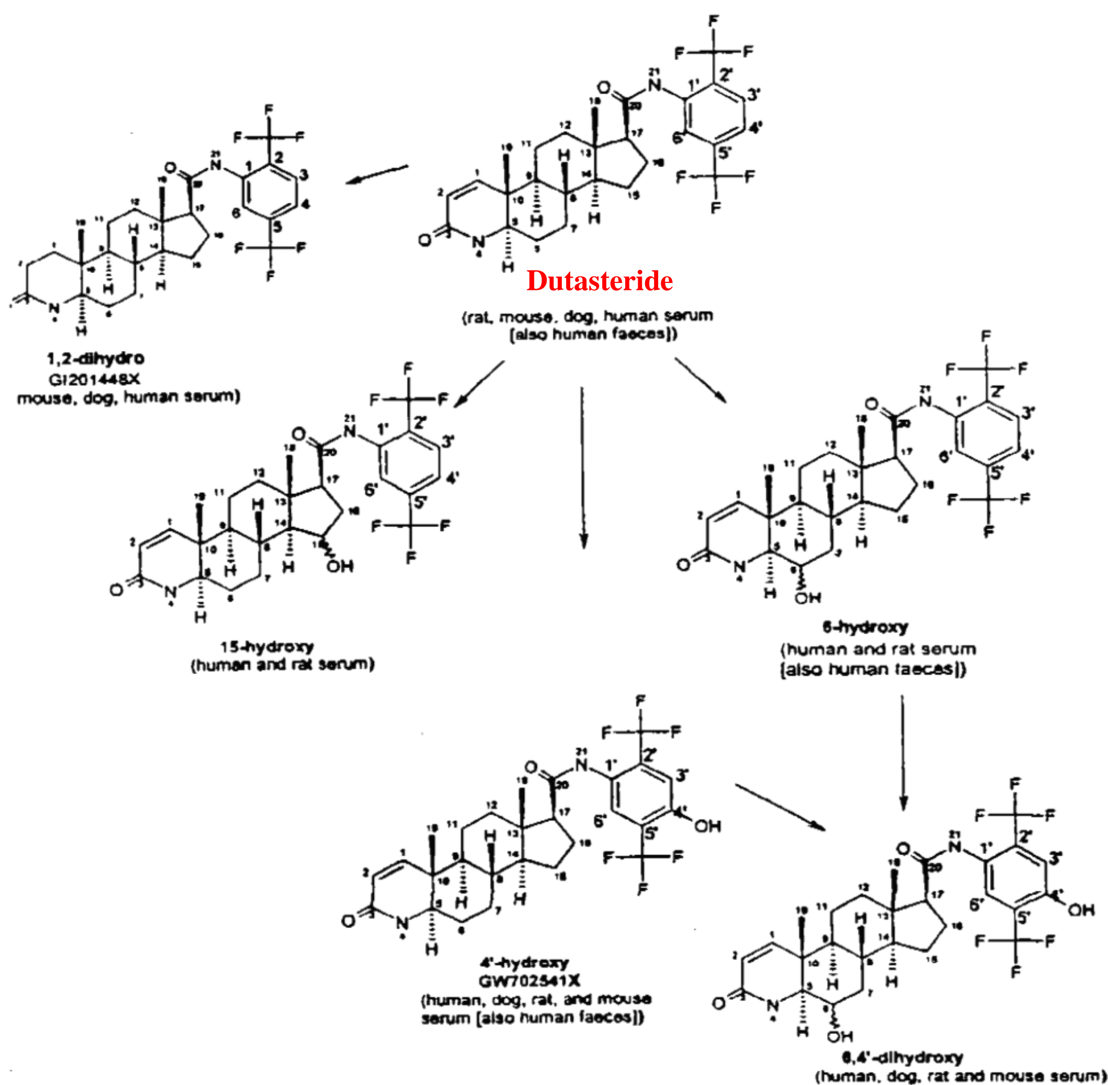


Figure 1.8. *Proposed metabolic pathway of dutasteride in humans and animals (GSK, 2001).*

Table 1.2. *Summary data for dutasteride metabolism (GSK, 2001).*

Matrices (Fluids)	Dutasteride	4-hydroxydutasteride	% Dose
Serum (n = 8)	40 ng/mL (16-78 ng/mL)	10 ng/mL (2-30 ng/mL)	-
Urine (n = 8)	< 0.1 ng/mL	-	< 0.06% (0-0.06%)
Faeces (n = 8)	80 µg (15-230 µg)	-	5% (1-15%)
Faeces (parent + all metabolites)			45% (6-97%)
Note: 4-hydroxydutasteride concentration was not reported in urine or faeces. The total is for parent and all dutasteride-related materials.			

1.8.3 Efficacy biomarkers and pharmacokinetics/pharmacodynamics relationship

In terms of pharmacokinetics/pharmacodynamics (PK/PD) relationships relevant to DHT inhibition (the biochemical marker), the maximum effect caused by dutasteride was dose and time dependent. Daily administration of a 0.5 mg dose produced about 80% reduction in circulating concentrations of DHT after 7 days of treatment, and the maximum effect was observed within 1 to 2 weeks. The administration of a 5 mg dose caused almost complete inhibition of circulating DHT. Additional repeats of dose-response studies showed that the highest dose produced 80% reduction in DHT within one day, 95% reduction within one week and 98% reduction in 6 months. Steady-state dutasteride concentration (~40 ng/mL) was completely achieved within 6 months.

1.8.4 Side effects and adverse reactions

Approximately 19% of the 2167 patients who received dutasteride in the Phase III placebo-controlled trials developed adverse reactions. The majority of events were mild to moderate and occurred in the reproductive system. No change to the adverse event profile was apparent over a further 2 years in open-label extension studies. Table 1.3 shows adverse reactions from controlled clinical trials and post-marketing experience. The listed adverse events from clinical trials are investigator-judged drug-related events reported with a higher incidence in patients treated with dutasteride compared with placebo during the first year of treatment. Adverse events from post-marketing experience were identified from spontaneous post-marketing reports; therefore the true incidence is unknown.

Table 1.3. *List of adverse reactions caused by treatment with dutasteride and their incidence.*

Organ system	Adverse reaction	Incidence from clinical trial data	
		Incidence during year 1 of treatment (n = 2167)	**Incidence during year 2 of treatment (n = 1744)**
Reproductive system and breast disorders	Impotence	6.0 %	1.7 %
	Altered (decreased) libido	3.7 %	0.6 %
	Ejaculation disorders	1.8 %	0.5 %
	Breast disorders (includes breast enlargement and/or breast tenderness)	1.3 %	1.3 %
Immune system disorders	Allergic reactions including rash, pruritus, urticaria, localised oedema, and angioedema	**Incidence estimated from post-marketing data**	
		Unknown	

1.8.5 Dutasteride toxicity - Preclinical safety data

The available studies of general toxicity, genotoxicity and carcinogenicity did not show any particular risk posed by dutasteride administration to humans. Reproduction toxicity studies in male rats showed a decreased weight of the prostate and seminal vesicles, decreased secretion from accessory genital glands and a reduction in fertility indices (caused by the pharmacological effect of dutasteride). The clinical relevance of these findings is unknown.

As with other 5αR inhibitors, feminisation of male fetuses in rats and rabbits occurred when dutasteride was administered during gestation. Dutasteride was found in the blood of female rats after mating with dutasteride treated males. When dutasteride was administered to primates during gestation, no feminisation of male fetuses was seen at blood exposure levels sufficiently in excess of those likely to occur via human semen. It is considered unlikely that a male fetus will be adversely affected following seminal transfer of dutasteride.

1.8.5.1 *Effects on male fertility*

The effects of dutasteride 0.5 mg/day on semen characteristics were evaluated in healthy volunteers aged 18 to 52 (n = 27 dutasteride, n = 23 placebo) throughout 52 weeks of treatment and 24 weeks of post-treatment follow-up. At 52 weeks, the mean percent reduction from baseline in total sperm count, semen volume and sperm motility in the dutasteride group were 23%, 26% and 18%, respectively. Sperm concentration and sperm morphology were unaffected. After 24 weeks of follow-up, the mean percent change in total sperm count in the dutasteride group remained 23% lower than baseline. While mean values for all parameters at all time points remained within the normal ranges and did not meet the predefined criteria for a clinically significant change (30%), two subjects in the dutasteride group had decreases in sperm count of greater than 90% from baseline at 52 weeks, with partial recovery at the 24 week follow-up. The possibility of reduced male fertility cannot be excluded. In another study, Amory *et al.* (2007) demonstrated that healthy men receiving dutasteride (0.5 mg) (n = 33) once a day for 1 year, showed a decrease of total sperm count compared to baseline at 26 weeks (-28.6%) but not at 52 weeks (-24.9%) nor at the 24 week follow-up (-23.3%). At 52 weeks, semen volume was decreased (-29.7%) as was sperm concentration (-13.2%). There was a significant reduction of 6–12% in sperm motility during treatment with dutasteride and at follow-up. The treatment did not have any effect on sperm morphology.

In rats, effects on male fertility and reproductive organs were observed at 0.5 mg/kg/day (corresponding to 0.04-0.11 times the steady state clinical blood levels in humans) after 12 weeks of treatments. Decreased weight of the prostate, seminal vesicles and epididymides

were observed at all doses, along with decreased secretory activity of the prostate and seminal vesicles and vacuolation of the epidydimal tubular epithelium.

1.8.5.2 ***Dutasteride in semen***

Dutasteride was partially excreted in semen; about 12% of the serum concentration was present in semen following a 0.5 mg dose. In the study ARA10009, the average semen concentration at steady state was 3.3 ng/mL, while the highest concentration was 14 ng/mL. Based on this study, exposure of pregnant women to dutasteride appeared to be minimal; however, this depends on the degree of the toxicity of the drug and its metabolites. It should be noted that based on animal studies, the “no effect dose” on embryo fetal development of the male primate is 260 ng/kg; hence, exposure of the fetus to high levels of 5αR inhibitor may be associated with high risk of inadequate development of male genitalia.

1.8.5.3 ***Effect on testosterone and other endocrine endpoints***

In healthy volunteers, 52 weeks of treatment with dutasteride (0.5 mg/day; n = 26) resulted in no clinically significant change compared with placebo (n = 23) in plasma concentrations of sex hormone-binding globulin, E2, luteinizing hormone, follicle-stimulating hormone, thyroxine (free T4), and dehydroepiandrosterone. Statistically significant, baseline-adjusted mean increases compared with placebo were observed for total T at 8 weeks (97.1 ng/dL, $p < 0.003$) and thyroid-stimulating hormone at 52 weeks (0.4 mcIU/mL, $p < 0.05$). The median percentage changes from baseline within the dutasteride group were 17.9% for T at 8 weeks and 12.4% for thyroid-stimulating hormone at 52 weeks. After stopping dutasteride for 24 weeks, the mean levels of T and thyroid-stimulating hormone returned to baseline in the group of subjects for which there were available data. In patients with BPH treated with dutasteride in a large randomized, double-blind, placebo-controlled study, there was a median percent increase in luteinizing hormone of 12% at 6 months and 19% at both 12 and 24 months.

1.8.5.4 ***Feminization of the male fetus***

No effects on feminization of the male fetus were observed in monkeys after exposure to 0.07 and 0.28 ng/ml of dutasteride throughout gestation (4 to 16-fold the estimated blood levels of a partner-exposed woman).

In a rabbit embryo-fetal study doses of 30, 100, and 200 mg/kg (28 to 93-fold the expected clinical exposure in men) were administered orally on days 7 to 29 of pregnancy to encompass the late period of external genitalia development. Histological evaluation of the genital papilla of fetuses revealed evidence of feminization of the male fetus at all doses. A

second embryo-fetal study in rabbits at doses of 0.05, 0.4, 3.0, and 30 mg/kg/day (0.3 to 53-fold the expected clinical exposure) also produced evidence of feminization of the genitalia in male fetuses at all doses. It is not known whether rabbits or rhesus monkeys produce any of the major human metabolites.

In an oral pre- and post-natal development study in rats, dutasteride doses of 0.05, 2.5, 12.5, or 30 mg/kg/day were administered. Unequivocal evidence of feminization of the genitalia (i.e., decreased anogenital distance, increased incidence of hypospadias, nipple retention) of F1 generation male offspring occurred at doses ≥ 2.5 mg/kg/day (14 to 90-fold the expected clinical exposure in men). At a daily dose of 0.05 mg/kg/day (0.05-fold the expected clinical exposure), evidence of feminization was limited to a small, but statistically significant, decrease in anogenital distance. Doses of 2.5 to 30 mg/kg/day resulted in prolonged gestation in the parental females and a decrease in time to vaginal patency for female offspring and a decrease in prostate and seminal vesicle weights in male offspring. Effects on the newborn startle response were noted at doses greater than or equal to 12.5 mg/kg/day. Increased stillbirths were noted at 30 mg/kg/day.

In an embryo-fetal development study in female rats, oral administration of dutasteride at doses 10 times less than the maximum recommended human dose (MRHD) resulted in abnormalities of male genitalia in the fetus, and nipple retention, hypospadias, and distended preputial glands in male offspring. An increase in stillborn pups was observed at 111 times the MRHD, and reduced fetal body weight was observed at doses ≥ 15 times the MRHD. Increased incidences of skeletal variations, considered to be delays in ossification associated with reduced body weight, were observed at doses ≥ 56 times the MRHD. Abnormalities of male genitalia were also observed in an oral pre- and post-natal development study in rats and in 2 embryo-fetal studies in rabbits at one-third the MRHD.

In an embryo-fetal development study, pregnant rhesus monkeys were exposed intravenously to a dutasteride blood level comparable to the dutasteride concentration found in human semen. The development of male external genitalia of monkey offspring was not adversely affected; however, reduction of fetal adrenal weights, reduction in fetal prostate weights, and increases in fetal ovarian and testis weights were observed.

1.8.5.5 *Neurotoxicity and effects on other organs*

In rats, after multiple administration of dutasteride resulting in a blood level higher than 17,000 ng/ml, centrally mediated neurotoxicity was observed (hypoactivity, dilated pupils, uncoordinated behaviour), with no histopatological correlation. Adrenal effects were observed

in dogs and mice at relatively high dose. Effects on the thyroid, pituitary, female reproductive organs, mammary glands, spleen, bone marrow, lungs and liver were also observed at high doses in dogs.

1.8.5.6 *Genotoxicity*

Dutasteride showed no potential genotoxicity in standard test batteries.

1.8.5.7 *Other Effects*

The plasma lipid profile and bone mineral density were evaluated following 52 weeks of dutasteride 0.5 mg once daily in healthy volunteers. There was no change in bone mineral density as measured by dual energy x-ray absorbtiometry compared with either placebo or baseline. In addition, the plasma lipid profile (i.e., total cholesterol, low density lipoproteins, high density lipoproteins, and triglycerides) was unaffected by dutasteride. No clinically significant changes in adrenal hormone responses to ACTH stimulation were observed in a subset population (n = 13) of the 1-year healthy volunteer study.

1.8.5.8 *Dutasteride concentrations in tissues*

After daily exposure (7 days) of mice, dutasteride was found in the following organs: adrenals, gall bladder, Harderian glands, liver > pancreas, peri-renal fat, brown fat, inguinal fat, preputial gland, kidney > epididymis, lachrymal glands, thyroid, pituitary, brain, salivary glands > gastrointestinal system, nasal mucosa, prostate > blood. After 14 days: adrenals >> nasal mucosa > epididymis. Concentrations were higher in adrenal cortex than in medulla.

1.9 The hypothalamic-pituitary-gonadal axis in teleost fish

One of the key controlling systems of the teleost fish endocrine homeostasis - as in all the vertebrates - is the hypothalamic-pituitary-gonadal (HPG) axis. Its structure and function have been described in great detail in several reviews, books and book chapters (e.g., Ankley and Johnson, 2008; Kime 1998; Norris 2010; Van Der Kraak *et al.* 1998; WHO 2002), therefore here it will be provided only a brief and general description of the axis.

The HPG axis consists of three components, physiologically connected by a complex multi-level system of signalling molecules and their transducers. The anatomical components of the axis are hypothalamus, pituitary gland (or hypophysis) and gonads (testis and ovary). The cascade of signalling molecules can be basically described in the following way. Neurosecretory neurons in the hypothalamus directly innervate the pituitary gland and secrete several isoforms of gonadotropin-releasing hormone (GnRH) (Fernald and White, 1999;

Gothilf *et al.*, 1996; Lethimonier *et al.*, 2004; Powell *et al.*, 1994), which act on specific gonadotropin secreting cells - the gonadotrophs – often located in the proximal pars distalis of the pituitary gland, stimulating the secretion of the pituitary tropic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These tropic hormones are released into the circulatory system and this carries them to specific cells in peripheral endocrine tissues (i.e. gonads), which express key components of the steroidogenic pathways. Gonadotropins stimulates synthesis and secretion of steroid hormones (e.g. sex steroids), which are subsequently released into the intercellular spaces or into the circulatory system. Steroid hormones then affect many non-endocrine targets and induce negative or positive feedbacks on the hypothalamus and/or the pituitary (Fig. 1.9) (Norris, 2010; Schulz *et al.*, 2010).

One of the main differences between the HPG axis of mammals and fish is the peculiar and unique anatomy of the hypothalamus/pituitary gland complex that characterizes fish. In fact, different from the situation in mammals, where the hypothalamus and pituitary gland are anatomically separate units connected by a vascular portal system, in teleost fish these two components are anatomically connected, and the hypothalamic neurons directly innervate the pituitary gland, secreting the GnRHs directly into the intercellular spaces of the pituitary (Norris, 2010).

Communication within vertebrates can follow three dynamic patterns: 1) the signal (typically a neurohormone), is secreted by a neuron directly onto or near the surface of the cells receiving the signal, 2) the signal, a hormone, is secreted by specialized cells within the tissue into the extracellular space, and consequently into the blood stream, by which is transported and relocated throughout the body to specific cells within target tissues, and 3) the signal is synthesized and secreted by one cell type into the extracellular space where it diffuses to adjacent cells of a different type that respond to the signal (paracrine communication) (Schulz *et al*., 2010).

The target cells respond to the signalling molecules through specific molecular receptors, which are the apical factor of a complex molecular response that leads to the final physiological effect. The dynamic of the system is capable of maintaining the homeostasis of the organism, defined by Cannon (1929) as “balanced physiological systems operating in the organism to maintain a dynamic equilibrium”, and when new external stimuli are received (e.g. temperature, light, presence of a potential sexual partner), the system responds by shifting toward a new dynamic state, necessary to mediate specific physiological processes such sex maturation, gamete development, reproductive behaviour, and reproduction.

The importance of the HPG axis in the environmental toxicology context is due to the potential interference caused by exogenous chemicals present in the environment, including some human pharmaceuticals, which can alter the normal functional equilibrium of the HPG axis, by disrupting the synthesis of one (or more) signalling molecules, or the functional interaction between signalling molecule and its receptor, and consequently the feedback responses. The disruption of the endocrine homeostasis could lead to the incapability of the system to respond in the appropriate way to normally occurring environmental stimuli that regulate critical processes such as sexual maturation or reproduction.

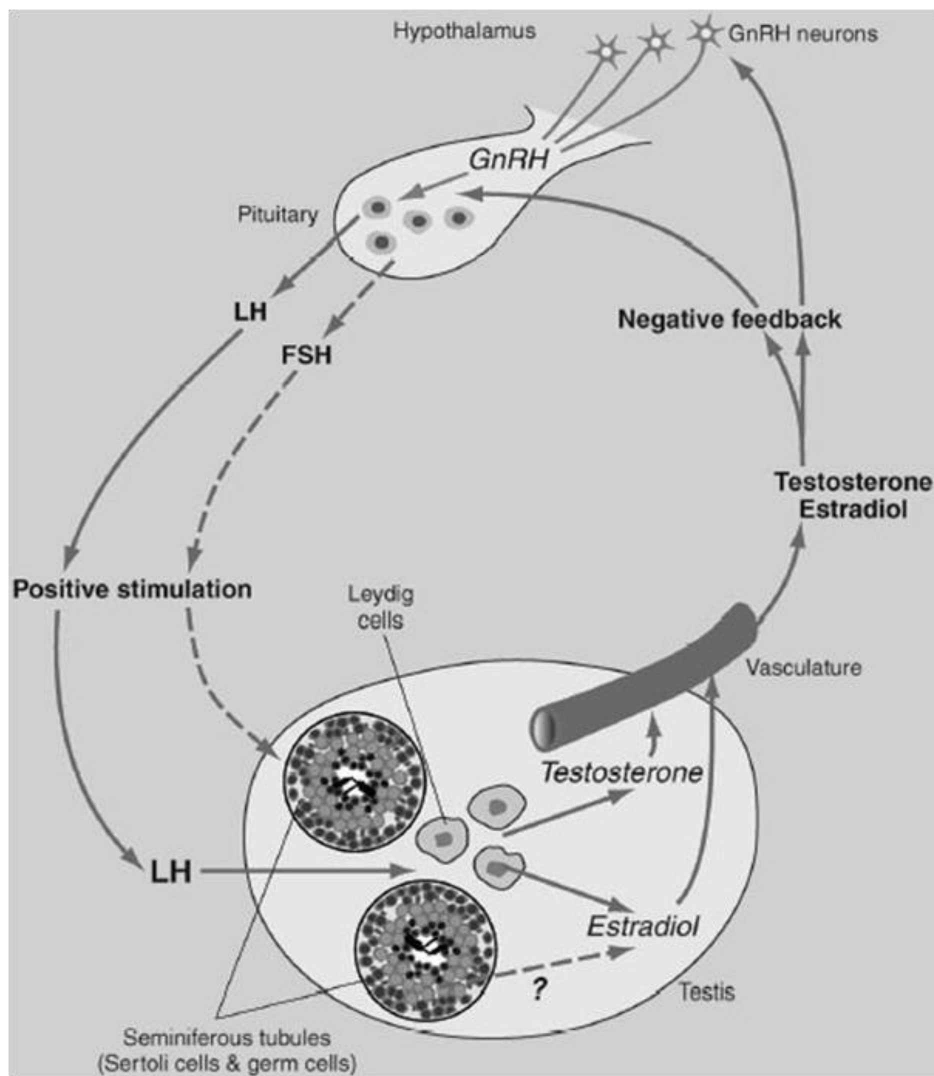


Figure 1.9. *Basic overview of the male teleost hypothalamic-pituitary-gonadal axis. The linkages between components of the axis illustrate how the system maintains a dynamic equilibrium. GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone. (from Ankley and Johnson, 2004).*

1.9.1 HPG axis and sex differentiation

Endocrine control of sex differentiation involves a complex bi-directional link between brain and gonad, along the HPG axis. This link is estabilished through signal molecules such as gonadotropins, produced by gonadotrope cells of the pituitary gland, and steroids produced in gonads and brain (Le Page *et al.*, 2010; Nagahama, 1994; Nagahama, 2005). Sex steroids exert a local and direct action on germ-cell development, but also act by influencing other cell types and organs involved in sex differentiation. A large number of biochemical, neurological, and physiological pathways are involved in this complex multilevel control process, and this complexity is of critical importance to provide the necessary plasticity to the developing gonad, which allows it to proceed in a dynamic way with the influence of intrinsic and environmental factors, but at the same time, provides many levels at which the system can be disrupted by chemicals with endocrine interfering properties (Devlin and Nagahama, 2002; Penman and Piferrer, 2008; Piferrer and Guiguen, 2008).

A broad debate is occurring on whether sex steroids play a fundamental role in the sex determination mechanism of teleost fish, or if the process is only regulated by intrinsic genetic processes, independently from the surrounding endocrine environment. This issue will be described in detail in Chapter 5, which summarizes the great number of studies supporting the hypothesis that the action of sex steroids affects definitive sex determination of fish during a short period of the early developmental stage.

1.9.2 HPG axis, sexual maturation and reproduction

The three small fish species classically used as experimental models in toxicology (fathead minnow, medaka, zebrafish) are all asynchronous spawners, being able to spawn successively for a relatively long period when experiencing the appropriate stimuli and environmental conditions. Each spawning cycle also involves a number of hormonal, molecular, biochemical and physiological responses. The frequency of spawning is species-specific, when not dependent on the environmental conditions, and for fathead minnows kept in standard laboratory conditions is generally between 3 and 6 days (Ankley and Johnson, 2004).

Detailed reviews of fish spermatogenesis and oogenesis have recently been published by Schultz *et al.* (2010) and Lubzens *et al.* (2010), respectively, beside which there are a large number of papers and books focusing on the same subject. Therefore, the following section will describe only the key events of gametogenesis, focusing on the endocrine control of these processes.

1.10 General structure of the teleost testis

The testis of vertebrates, including fish, is composed of two main compartments, the germinal (or tubular) and the interstitial (or inter-tubular) compartments. The latter is composed of steroidogenic Leydig cells, macrophages, blood/lymphatic vessels, and connective tissue, all enclosed by the tunica albuginea, which form the testis's wall (Koulish *et al.*, 2002; Schulz *et al.*, 2010). The germinal compartment contains the germinal epithelium, formed by somatic Sertoli cells, and germ cells at different stages of development. *In vivo*, germ cells survival is strictly dependent on the interaction with Sertoli cells; the latter are an important target of the hormonal cascade regulating spermatogenesis (Matta *et al.* 2002).

Teleost fish present a typical cystic type of spermatogenesis (Callard, 1996). The spermatogenic cyst represents the basic functional unit of spermatogenesis, and is formed by a group of Sertoli cells surrounding one developing germ cell clone. Germ cell development occurs synchronously in each cyst, and different cysts host clones in different stages of development (Leal *et al.*, 2009; Norbega *et al.*, 2010; Schulz *et al.*, 2010). Sertoli cells are essential for germ cell survival, development, and physiological functioning. Furthermore, they secrete the fluid that fills the tubular lumen, and present a highly active phagocytic activity toward apoptotic germ cells, residual bodies left by spermatids maturation, and residual sperm (Almeida *et al.*, 2008; Billard, 1969; Grier and Taylor, 1998; Schulz *et al.*, 2010; Vilela *et al.*, 2003).

1.11 General structure of the teleost ovary

The ovary, like the testis, is enclosed in the tunica albuginea. It shows asynchronous gametogenesis, and hence contains oocytes at all stages of development. An inner spherical layer of cells, called granulose cells, surround each developing oocyte, and mediate the incorporation of vitellogenin into the developing oocyte, whereas the outer layer contains another cell type, the thecal cells. Both cell types are involved in the synthesis of steroid hormones (Ankley and Johnson, 2004).

1.12 Cell types involved in sex steroid production

In both testis and ovary, the main roles of somatic cells are to sustain developing germ cells and to synthesize sex steroids, providing the correct hormonal environment to support either spermatocyte or oocyte development (Devlin and Nagahama, 2002).

In testis, Leydig cells represent the main site of androgen synthesis, whereas in ovaries, the two somatic cell layers of the follicle play complementary roles in steroid biosynthesis (Hoar and Nagahama, 1978; Nagahama *et al.*, 1982). Generally, thecal cells, after stimulation by LH, produce T and other androgens, whereas granulosa cells convert T to estrogen via the enzyme aromatase, whose activity is induced by FSH (Nagahama, 1997).

Immunohistochemical studies with trout testes showed that the steroidogenic enzymes P450scc, P450c17, 11β-hydroxylase, and 3β-hydroxysteroid dehydrogenase are active in Leydig cells, but not in germ or Sertoli cells (Kobayashi *et al.*, 1998; Morrey *et al.*, 1998), suggesting that Leydig cells are the major site of steroid synthesis in fish testis. Although aromatase levels in the testis are often undetectable, there is evidence for E2 biosynthesis (Pasmanik and Callard, 1988), suggesting the involvement of estrogen in the normal functioning of the testis (Devlin and Nagahama, 2002).

The brain also contains active steroidogenic components, such as aromatase, the enzyme responsible for the conversion of T into E2, which has been identified in brain and pituitary tissues (Melamed *et al.*, 1999; Pasmanik and Callard, 1988). Brain aromatase activity is induced by *in vivo* treatment with T and E2, and its activity showed a good correlation with seasonal changes in gonadal activity and growth in goldfish (Pasmanik and Callard, 1988; Pasmanik *et al.*, 1988), suggesting its involvement in the mediation of feedback responses induced by sex steroids in the brain.

The presence of aromatase activity and estrogen production in testis (Pasmanik and Callard, 1988) may be involved in the mediation of feedback responses between the gonad and brain. Both E2 and its receptor have been found in fish brain tissue, supporting the hypothesis of a functional role for brain estrogens (Begay *et al.*, 1994; Fine *et al.*, 1990; Pakdel *et al.*, 1990; Zhang *et al*., 2008).

1.13 The spermatogenic process – an overview

Spermatogenesis starts with diploid spermatogonia that proliferate and progress toward a series of differentiation steps until the formation of mature haploid spermatozoa. The process can be divided in three different phases: 1) the mitotic or spermatogonial phase, characterized by the occurrence of different generations of spermatogonia in different differentiation stages; 2) the meiotic phase, during which primary and secondary spermatocytes are produced; and 3) the spermiogenic phase with the meiosis process generating spermatids, which in turn

differentiate – without further proliferation – into motile spermatozoa (Fig. 1.10) (Nóbrega *et al.*, 2009; Schulz *et al.*, 2010).

The spermatogenic process progresses with the sequential appearance of undifferentiated type A spermatogonia, differentiated type A spermatogonia, and then type B spermatogonia, which increase in number in the maturation process by exhibiting several generations. The number of generations is species-specific (Ando *et al.*, 2000) and genetically determined. For example, 9 generations were observed in zebrafish (Leal *et al.*, 2009), and 14 in the guppy (Billard, 1969). Considering a signle cyst, after the first spermatogonial division cytoplasmic bridges appear between germ cells, generating a clonal syncytium (Loir *et al.*, 1995). The type B spermatogonia divide more rapidly than the type A spermatogonia (Schulz *et al.* 2005), and after the final mitosis, they differentiate into primary (pre-leptotene) spermatocytes. The following developmental stages are primary spermatocytes (1st meiotic division), secondary spermatocytes (2nd meiotic division), spermatids (differentiation without proliferation), and spermatozoa (Schulz *et al.*, 2010).

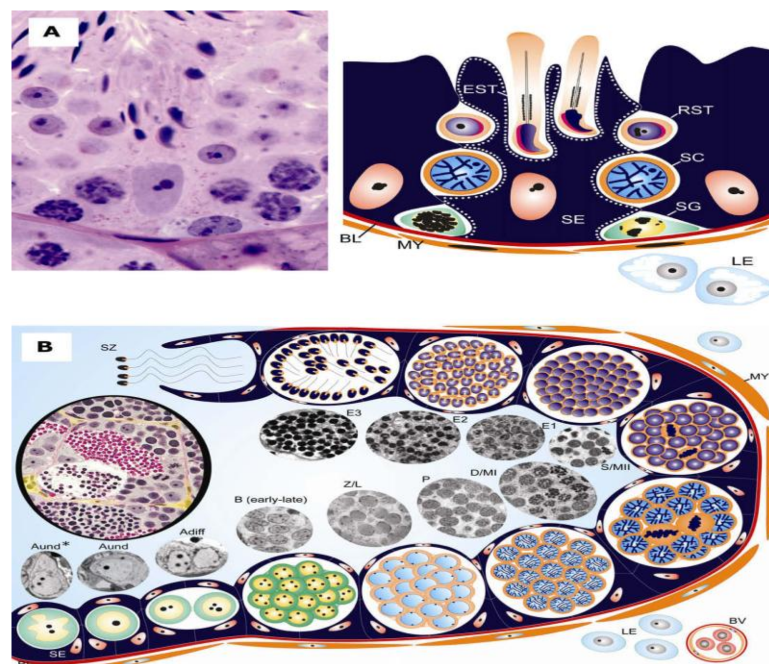


Figure 1.10. *Comparison of mammalian (A, mouse) and fish (B, zebrafish) testis. Segments of spermatogenic tubules are shown to illustrate the differences in Sertoli/germ cell relation between cystic (B) and non-cystic (A) spermatogenesis. The germinal epithelium contains Sertoli (SE) and germ cells, delineated by a basal lamina (BL) and peritubular myoid cells (MY). The interstitial Leydig cells (LE) and blood vessels (BV) are shown. (A) spermatogonia (SG); spermatocyte (SC); round spermatid (RST); and elongated spermatid (EST). (B) Type A undifferentiated* spermatogonia (Aund*); type A undifferentiated spermatogonia (Aund); type A differentiated spermatogonia (Adiff); spermatogonia type B [B (early–late)]; leptotenic/zygotenic primary spermatocytes (L/Z); pachytenic primary spermatocytes (P); diplotenic spermatocytes/metaphase I (D/MI); secondary spermatocytes/metaphase II (S/MII); early (E1), intermediate (E2) and final spermatids (E3); spermatozoa (SZ) (from Schulz et al., 2010).*

1.13.1 Extrinsic regulation of spermatogenesis

1.13.1.1 *Pituitary hormones and growth factors*

The gonadotropins, LH and FSH, play key roles in the regulation of testicular physiology. In mammals FSH and LH, at physiological concentrations, interact with their receptors (FSHR and LHR, respectively) with high specificity. LH mediates Leydig cell steroidogenesis, while FSH regulates Sertoli cell activities, including nutritional and regulatory support of germ cell development (Huhtaniemi and Themmen, 2005). The described dichotomy of functions is less clear in teleost fish. In fact, in the majority of the studies where the binding characteristics of FSHR and LHR have been characterized using hormones from the same species (African catfish: Garcia-Lopez *et al.*, 2009; Channel catfish: Kumar *et al.*, 2001; Japanese eel: Kazeto *et al.*, 2008; zebrafish: So *et al.*, 2005), FSHR showed a higher affinity for FSH, but was also activated by LH, even if the concentrations of LH required to observe the cross-activation were relatively high, but nonetheless similar to the ones detected during the spawning season. On the other hand, LHR can be cross-activated by FSH, but at concentrations much higher than the maximum plasma FSH concentrations, so that the LHR can be considered specific for LH (Schulz *et al.*, 2010).

Leydig cells in fish testis express the FSHR gene (Garcia-Lopez *et al.*, 2008), and FSHR has also been detected in both Leydig and Sertoli cells of eel testis using immunological techniques (Ohta *et al.*, 2007). Combining the available data it is possible to hypothesize that LH and FSH regulate Leydig cell steroidogenesis, while FSH mainly mediates Sertoli cell functions, although LH could also play a role in the FSHR activation when circulating at high concentrations, such as during the spawning season (Schulz *et al.*, 2010). Other evidence suggests that FSH-mediated steroidogenesis stimulates the early stages of spermatogenesis. The other roles played by FSH in mammals, such as stimulation of Sertoli cell proliferation/terminal differentiation during puberty, support of terminal phases of spermiogenesis and spermiation (Huhtaniemi and Themmen, 2005), have not been confirmed yet in fish.

Sertoli cells also produce several growth factors that regulate germ cell proliferation, for example activin, growth and differentiation factors (GDFs), anti-mullerian hormone (AMH) and its receptor (Miura and Miura, 2001; Miura *et al.*, 2002; Morinaga *et al.*, 2007; Sawatari *et al.*, 2007). It is currently not known if FSH can regulate the expression of these growth factors in fish.

Other than LH and/or FSH, other pituitary hormones can modulate testicular steroidogenesis, in particular growth hormone (GH) (Singh *et al.*, 1988); indeed, cross-talk between the reproductive and growth axes occurs via the modulation exerted by sex steroids on the release of GH from the pituitary (Melamed *et al.*, 1995). In some fish species, like in the Chinook salmon (*Oncorhynchus tshawytscha*), the start of spermatogonial proliferation occur simultanously with the elevation of FSH, androgens, and insulin-like growth factor 1 (IGF1) (Campbell *et al.*, 2003); the latter is released by the liver in response to GH stimulation. Moreover, GH receptors have been detected in trout testis and Sertoli cell (LeGac *et al.*, 1993; Gomez *et al.*, 1998), the expression of IGF1 mRNA and IGF-I receptors occurs in germ and Sertoli cells in trout (LeGac *et al.*, 1996) and tilapia testis (Berishvili *et al.*, 2006), and IGF1 stimulates the proliferation and/or differentiation of spermatogonia (Loir and LeGac, 1994; Vinas and Piferrer, 2008). Therefore it is possible to hypothesize that GH can modulate testicular steroidogenesis and/or germ cell proliferation directly and/or through locally produced IGF1 (LeGac *et al.*, 1996; Loir, 1999).

1.13.1.2 ***Steroid hormones***

Concentrations of sex steroids show important variations during sexual maturation. In male teleosts, E2 plasma concentrations are generally low (Amer *et al.* 2001; Chaves-Pozo *et al.*, 2007; Miura *et al.* 1999), and show a transitory increase at the beginning of the reproductive cycle in trout (Gomez *et al.*, 1999). Concentrations of androgens (T, KT) increase with the proceeding of spermatogenesis and decrease at spermiation. During the reproductive cycle of salmonid fish, two peaks of the progestin 17α,20β-dihydroxy-4- pregnen-3-one (DHP) levels have also been observed. A big peak occurs during the spawning season, and a small peak during the progression of spermatogonial proliferation (Depeche and Sire, 1982; Scott and Sumpter, 1989; Vizziano *et al.*, 1996).

Estrogens

Three ER subtypes (α, β1 and β2) are expressed in several tissues of teleost fish, including in the testis, where ER expression has been detected in the somatic testicular cells and in haploid male germ cells (Bouma and Nagler, 2001; Chang *et al.* 1999; Ito *et al.*, 2007; Menuet *et al.*, 2002; Miura *et al.*, 1999; Wu *et al.*, 2001).

A number of studies indicate that estrogens may play an important role in regulating gene expression in the testis, for example by regulating the expression of genes involved in the steroidogenic pathway or in its regulation (i.e. Star, 3βHSD, aromatase), and in the spermatogenic process (Alsop *et al.*, 2008; Song and Gutzeit, 2003).

Androgens

One or two AR subtypes (α and β) have been described in several fish species, and the testis is a major site of expression (Takeo and Yamashita, 1999; Todo *et al.*, 1999; Ikeuchi *et al.*, 2001). Differently from germ cells, Sertoli and interstitial cells express AR (Ikeuchi *et al.*, 2001), suggesting a functional interaction with androgens (Schultz *et al*., 2010). Androgens strongly influence the expression of testicular genes involved in spermatogenesis and steroidogenesis (LeGac *et al.*, 2007), and distinct androgens (e.g. T, KT) up/down regulate gene expression in a steroid-specific fashion (Schulz *et al.*, 2010). This suggests that different steroids may regulate different processes during testis maturation and spermatogenesis (Schulz *et al.*, 2010).

Androgens are capable of inducing the whole spermatogenic process, or at least some critical steps such as spermatogonial multiplication and spermatocyte formation or maturation (Billard *et al.*, 1982; Billard, 1986; Borg, 1994; Fostier *et al.*, 1983; Nagahama, 1994). They may also play an important role in the initiation of puberty (Cavaco *et al.*, 1998; Miura *et al.*, 1991), and in the induction of spermiation in some species, although they are less effective than progestins (Ueda *et al.*, 1985).

Progestins

Both nuclear and membrane progestin receptors are expressed in the testis of fish (Hanna *et al.*, 2010; Hanna and Zhu, 2009; Todo *et al.*, 2000; Tubbs *et al.*, 2011; Zhu *et al.*, 2003). Circulating progestin concentrations have a peak during the entire spermiation process and these hormones can regulate several testicular functions. In some species it has been shown that progestins such as 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) or, in certain species, 17,20β,21-trihydroxy-pregn-4-en-3-one (17,20β,21-P), can induce spermiation in Salmonidae and Cyprinidae (Ueda *et al.*, 1985), increase milt production (Baynes and Scott, 1985; Yueh and Chang, 1997), stimulate spermatozoa motility (Miura *et al.*, 1992; Tubbs and Thomas, 2008), and initiate meiosis (Miura *et al.*, 2006). For a detailed review of the roles of progestins in male fish, see Scott *et al.*, (2010).

1.14 Oogenesis in teleost fish

1.14.1 From primordial germ-cells to mature oocytes

During the advancement of oogenesis, the developing oocyte acquires the capability of forming a viable embryo after fertilization. This process occurs with significant structural and functional changes (Lubzens *et al.*, 2010).

Major stages of oocyte development include formation of primordial germ cells (PGCs), the transformation of PGCs into oogonia and subsequently, with the beginning of meiosis, their transformation into primary oocytes (Fig. 1.11) (Patiño and Sullivan, 2002). The first meiotic division remains arrested at the end of the prophase in the diplotene stage, for a few days or months in most teleost fish (Babin *et al.*, 2007). During this arrest, vitellogenesis occurs and the oocyte exhibits a dramatic growth, during which it accumulates nutritional (e.g. vitellogenin, lipids, vitamins) and informational (maternal mRNA) reserves needed for the development of the embryo, and completes the differentiation of its cellular and non-cellular envelopes. At maturation the accumulation process progressively slows down or stops, meiosis restarts, the nucleus (called germinal vesicle) breaks down (GVBD), cortical alveoli form a monolayer under the oocyte plasmatic membrane, and the hydration of the oocytes occurs (Lubzens *et al.*, 2010). The first meiotic division generates two cells of different size: one small cell (first polar body), which degenerates, and one large secondary oocyte which continues the maturation process, and undergoes the second meiotic division, which is arrested in the metaphase until fertilization (Babin *et al.*, 2007). At the end of the maturation process, ovulation takes place by enzymatic degradation and rupture of the follicle, and expulsion of the oocyte from the surrounding follicular cell layers into the ovarian lumen or abdominal cavity (depending on the species). At this stage the female gamete is haploid and ready to be fertilized (Lubzens *et al.*, 2010).

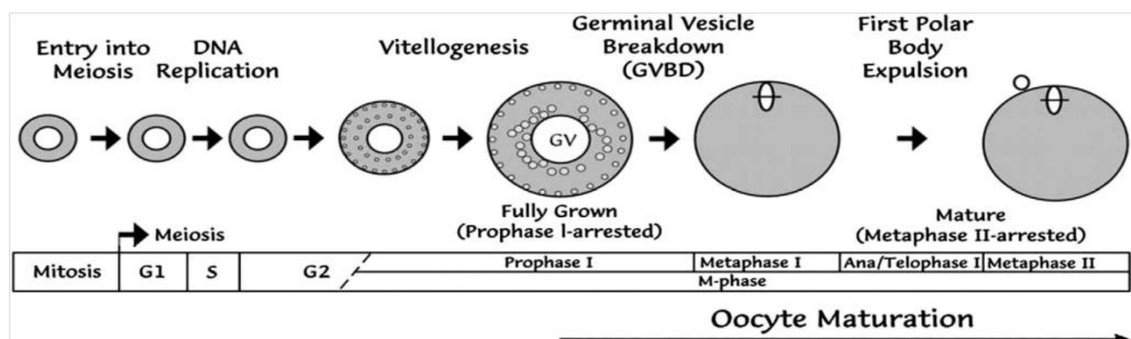


Figure 1.11. *A schematic description of the development stages of oocytes in relation to meiosis in teleost fish. From left to right: from primary oocytes, to vitellogenic and mature oocytes (from Lubens et at.,2010).*

1.14.2 Endocrine control of oocyte growth

Early vitellogenesis is characterized by increases in the plasma concentrations of FSH and E2, and increased expression of ovarian FSH receptor (e.g. Breton *et al.*, 1998; Kwok *et al.*, 2005; Kobayashi *et al.*, 2008, 2009; Oba *et al.*, 2001; Santos *et al.*, 2001; Tyler *et al.*, 1997). Thecal cells synthesize the androgen substrate used by the ovarian granulosa cells to produce E2, *via* the enzymatic activity of the cytochrome P450 aromatase (Nagahama, 1994; Senthilkumaran *et al.*, 2004; Young *et al.*, 2005). In turn, E2 stimulates hepatic synthesis of VTG, while FSH increase VTG uptake by ovarian follicles, as demonstrated *in vitro* by Tyler *et al.* (1991). GH enhances the effects of E2 in stimulating VTG synthesis (Peyon *et al.*, 1996), and also plays a role in stimulating E2 synthesis by ovarian tissue (Singh *et al.*, 1988; Singh and Thomas, 1993).

Also IGF-1 seems to be involved in the regulation of ovarian steroidogenesis, although its mechanisms of action are unclear (Berishivili *et al.*, 2006; Kagawa *et al.*, 1995; Perrot *et al.*, 2000; Schmid *et al.*, 1999). For example IGF-1 inhibited steroid production in thecal cells of coho salmon ovarian follicles, and at the same time stimulated steroid production in granulosa cells (Maestro *et al.*, 1997).

Other growth factors are involved in the control of oogenesis, like GDF9 (Halm *et al.*, 2008), and activins (β_A and β_B) (Wang and Ge, 2004). Activin β_A seems to be involved in promoting ovarian follicle growth, while activin β_B appears to play an important role in the processes of oocyte maturation and/or ovulation. In zebrafish, the expression of both activin β_A and β_B has been shown to be stimulated by the "epidermal growth factor" (EGF) produced in the oocytes (Lubzens *et al.*, 2009; Wang and Ge, 2004).

1.14.3 Endocrine control of oocyte maturation

The last phase of oocyte maturation is characterized by termination of vitellogenesis and resumption of the first meiotic division, and is associated with increased plasma LH concentrations, and increased expression of LH receptor. During this process there is a change in the dynamics of the ovarian steroidogenic pathways, resulting in a switch from E2 synthesis to maturation-inducing steroids (MIS) synthesis (Nagahama and Yamashita, 2008). MIS are species-specific derivatives of progesterone and act by binding to oocyte membrane-specific receptors, activating the maturation promoting factor (MPF), which in turn causes the breakdown of the germinal vesicle and the resumption of meiosis (Lubzens *et al.*, 2010). The synthesis of MIS by the follicle is coupled with the acquisition of sensitivity of the oocyte to respond to the MIS, thanks to the increases expression of membrane MIS receptors and to the

enhanced cell-cell communication through gap junctions. This process is known as maturational competence, and is induced by gonadotropins (Babin *et al*., 2007; Patiño and Sullivan, 2002; Weber *et al.*, 2007; Yamamoto and Yoshizaki, 2008). Also at this stage, a number of other ovarian factors are involved in the maturation processes, such as activin, follistatin (Ge, 2000; Pang and Ge, 1999, 2002), EGF and TGF-b (Pang and Ge, 2002).

1.15 Test organism: the fathead minnow (*Pimephales promelas*)

The fathead minnow is a small freshwater teleost fish belonging to the Cyprinidae family, with a ubiquitous distribution across North America (Fig. 1.12). Together with zebrafish and Japanese medaka, fathead minnow is one of the most commonly used fish species in ecotoxicological research. Its basic biology has been described by several authors, including Scott and Crossman (1974), Jensen *et al*. (2001), and Watanabe *et al*. (2007). The species is gonochoristic and sexually dimorphic; sexually mature males are easily distinguished from the females for the larger body size (3-7 g versus 2-3 g respectively), and in particular for the presence of several male secondary sexual characteristics (SSCs), including vertical dark bands on the body, nuptial tubercles on the snout, and prominent fatpad on the dorsal area of the body, between the head and the dorsal fin (Fig. 1.13). Male fish are also characterised by the presence of dark spots on the dorsal fin and at the base of the tail fin. These SSCs can be expressed with variable intensity, reaching the peak during the breeding period. On the other hand, females are olive/silver in colour, have a more pointed head, and present an ovipositor, a female egg laying organ, just anterior to the anal fin (Fig. 1.13).

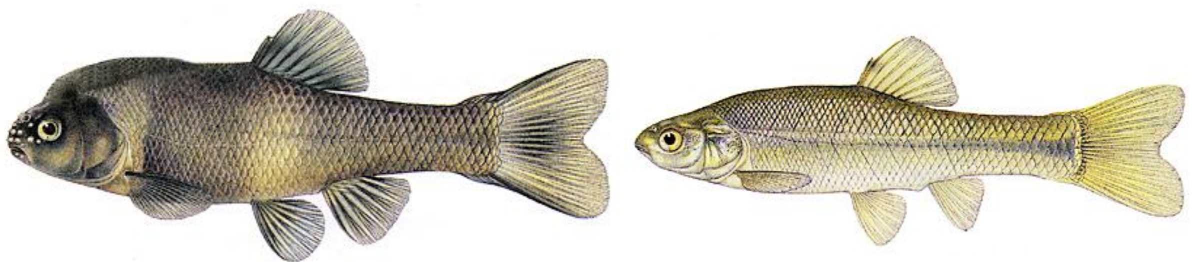


Figure 1.12. *Illustration of sexually mature male (left) and female (right) fathead minnows (Pimphales promelas) (from Yonkos et al., 2000).*

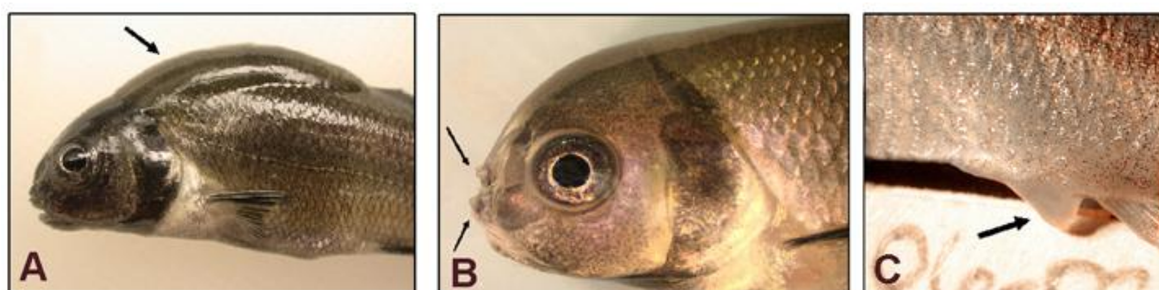


Figure 1.13. *Fathead minnow is a sexually dimorphic species. Males are characterized by specific SSCs, including (A) dorsal fatpad (arrow), and (B) nuptial tubercles on the snout (arrows). On the other hand, females show (C) ovipositor just anterior to anal fin (from the Behavioral Studies Laboratory, University of Maryland, USA, http://aquaticpath.umd.edu/behavior).*

Under standard laboratory conditions the fathead minnow reaches sexual maturity at four to six months post-hatch. The sexually mature males select and prepare the nest site, which is typically located on the under-surface of a U-shaped PVC tile, and exhibit territorial behaviour. Females spawn every 2-12 days (typically 3-4 days) (Watanabe *et al.*, 2007), deposing clutches of eggs (approximately 50-400 eggs per spawning event) that stick on the surface of the nesting site. The males show territorial behaviour and protect the eggs from cannibalism; moreover, using their caudal and dorsal fins enhance water circulation in proximity of the egg mass, increasing oxygen availability. The spongy dorsal fatpad is used to scrub the nest site to remove parasites (e.g. fungi). Embryos hatch within 4-5 days depending on temperature (Harries *et al*, 2000; Yonkos *et al*, 2000; Jensen *et al*, 2001). A detailed description of sexual differentiation and development of fathead minnow will be provided in Chapter 4.

1.15.1 Role of the fathead minnow in ecotoxicological research

The fathead minnow is used as a model test species in routine regulatory toxicity tests by several environmental or regulatory agencies including the American environmental protection agency (US EPA), the European environment agency (EA) and the organisation for economic cooperation and development (OECD).

In particular, this species represent one of the most important models for determining the reproductive and developmental effects of exposure to endocrine disrupting chemicals (e.g. Ankley *et al.*, 2001; Ankley & Villeneuve, 2006; Harries *et al*, 2000; Leino *et al*, 2005; Panter *et al.*, 2002; Paulos *et al.*, 2010; Zerulla *et al*, 2002). The reasons justifying its utility include:

- a body mass larger than that of zebrafish or medaka, which allow to collect a volume of blood large enough to perform the analytical quantification of several circulating

steroid hormones (e.g. T, KT, E2), allowing a deeper understanding of the effects of chemicals on steroid hormone dynamics;

- the expression of SSCs driven by sex-steroids (e.g. androgens stimulate the expression of male SSCs);
- a rapid spawning rate which, in optimal condition, can remain constant for several weeks;
- a good characterization of it basic biology and endocrinology, and the growing quantity of –omic information available in the bioinformatics databases, which can improve the design of mechanistic studies;
- the high degree of evolutionary similarity to zebrafish, which in turn presents a high degree of evolutionary conservation of human drug targets.

For all these reasons, the fathead minnow was considered an ideal test species for the experiments described later in this thesis.

1.16 Aim of the Thesis

The aim of my Ph.D. project was to test the hypothesis that the human pharmaceutical dutasteride induces in fish effects similar to those caused in men taking the drug to treat prostatic diseases, by specifically inhibiting the activity of 5αRs, the enzymes which convert T into DHT. The experimental studies performed, using fathead minnow as the model species, had the following objectives:

- Determine whether or not 5αRs are expressed and functional in fish;
- Determine if the product of 5αRs biosynthetic activity, the androgen DHT, circulates in the plasma of fish, and at what concentrations;
- Determine whether or not DHT has any role in regulating spermatogenesis in fish;
- Determine if dutasteride affects embryo development, and survival and growth of fish exposed to the drug during early life stages;
- Determine if dutasteride affects sexual maturation of adult fish exposed during early life stages;
- Determine if dutasteride affects the reproductive performances of fish.

CHAPTER 2 : CLINICAL USE AND ECOTOXICOLOGICAL RELEVANCE OF STEROIDS AND ANTI-STEROIDS IN THE UK

2.1 Introduction

Many human pharmaceuticals have been shown (or predicted) to occur in aquatic environments; the question arising is, then, what are the consequences of the exposure to these substances in non-target species?

Modes of action and mechanisms of toxicity of any chemical compound are generally defined by the interaction between the toxicant and one or more targets, according the so called "toxicant-target paradigm". Targets can be represented by receptors, enzymes, genetic material, and transport molecules. Differences in the toxicological profile of different chemicals acting through the same mode of action may derive from differences in the interaction properties with their targets (Rabinowitz *et al*., 2009).

Considering the importance of the interaction between toxicant and target, analysis of the phylogenetic and functional conservation of molecular targets across taxa should be a key procedure to apply in the risk assessment of environmental pollutants (Ankley *et al*., 2007). This approach is relatively straightforward when applied to the group of human pharmaceuticals, since the mechanism of action, and the metabolism, of each active pharmaceutical ingredient (API) is extensively studied in several species (Wishart *et al*., 2006). Information on target conservation could help in elucidating the sensitivity of different taxa, or species, to each API, and, as suggested by several authors, represent a key aspect in the development of more efficient testing strategies (Ankley *et al*., 2007; Christen *et al*., 2010; Gunnarsson *et al*, 2008; Kostich and Lazorchak, 2008; Seiler, 2002).

Gunnarsson *et al*. (2008) predicted the presence of orthologous genes coding for 1318 human drug targets in sixteen species, from frogs to bacteria, including two fish species, zebrafish and stickleback (Fig. 2.1). Orthologs, or orthologous genes, are genes in different species that are similar to each other because they originated from a common ancestor, and represent an estimate of the degree of conservation of a target among different species. Their results showed how fish and frog were predicted to have the greatest number of human drug target orthologs, with the highest degree of similarity, suggesting a very high sensitivity of these two taxa to low APIs in the environment. The authors also highlighted the fact that the presence of a conserved drug target in a species does not guarantee that a functional interaction with the drug will occur. On the other hand, functional interactions between a drug and other non-orthologous (or non-target) proteins are also possible. A classic example is represented by the ER. Its ortholog has been described in several mollusc species, but it is not activated by 17β-estradiol (E2) or other vertebrate steroids (Kohler *et al*., 2007; Thornton *et al*., 2003). However, EE2 can induce reproductive responses in molluscs at low ng/L concentrations

(Jobling *et al*., 2003), suggesting that "EE2 could act via either a non-ortholog receptor or possibly via a non-characterized ortholog" (Gunnarsson *et al*., 2008).

It is important to note that, in some species, conserved pathways of action can be involved in the mediation of physiological effects different than the ones occurring in humans. For example, thyroid hormones regulate the timing of metamorphosis in amphibians (Tata, 1998), a physiological process without counterparts in humans. In other cases, APIs may activate pathways that in humans lead to side-effects; while in other species these pathways mediate critical physiological effects, which may assume significant ecotoxicological relevance, as in the case of vulture populations decimated by diclofenac (see Chapter 1). However, despite the potential variability of functions, the presence in a species of conserved molecular targets should still indicate if a potential response to an API exposure is likely to occur (Gunnarsson *et al*., 2008; Searls, 2003), and should have a significant influence in the prioritization process for toxicity testing.

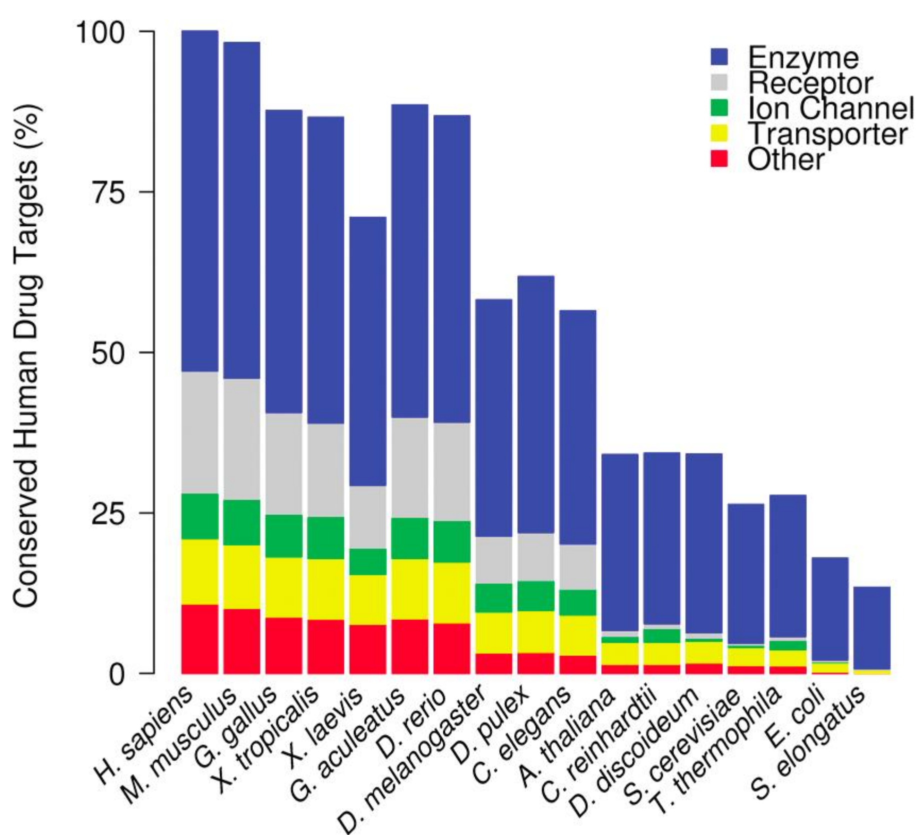


Figure 2.1. *Overview of the predicted conservation of drug targets in humans and in other vertebrate/invertebrate species, classified according to the gene ontology in five categories (enzyme, receptor, ion cannel, transporter, other). Note the high similarity of the targets in H. sapiens and fish species (G. aculeatus, D. rerio) (from Gunnarsson et al., 2008).*

As discussed in Chapter 1, from an ecotoxicological perspective, EE2 is the steroid on which the scientific community has focused the greatest attention, but it is not the only steroidal pharmaceutical in widespread use. The contraceptive "Pill" also contains a synthetic progestogen, which is present at a considerably higher dose than EE2. Various synthetic androgens and androgen antagonists are used in the treatment of some common diseases, such as androgen deficiency and prostate cancer, respectively. A wide range of synthetic corticosteroids are in use to treat the symptoms of very common illnesses such as inflammation and asthma. All these pharmaceutical steroid agonists and antagonists act via their respective receptors. Fish possess these same receptors, and hence it is possible that, if these pharmaceuticals reached the aquatic environment, they could cause effects to fish, just as EE2 can (Table 2.1). Given this fairly logical and straightforward line of argument, it is perhaps surprising that no systematic search for the presence of any steroidal pharmaceuticals (agonists or antagonists) other than estrogens in the aquatic environment appears to have been conducted.

In this Chapter I will provide information on the clinical use of synthetic progestogens, androgens, glucocorticosteroids, and mineralocorticosteroids and also their antagonists (including antagonists that do not operate via a receptor, but instead inhibit synthesis of the natural steroid). It will be also discussed in general terms what effects we would expect these pharmaceuticals to have on fish, should they reach the aquatic environment at high enough concentrations to cause effects, highlighting the fact that this group of pharmaceuticals should be a high priority for research.

Table 2.1. *Gene similarity between human and fish steroid receptors. Multiple values derive from multiple sequences available in Gene Bank classified as "complete coding sequences", obtained by different laboratories and characterized by different lengths (Table modified from Christen et al., 2010).*

Human receptor	Species	Similarity to human sequence (%)
Estrogen receptor 1 (alpha)	Zebrafish (*Danio rerio*)	53/56
	Fathead minnow (*Pimephales promelas*)	58
	Rainbow trout (*Oncorhynchus mykiss*)	57.1/48.3
	Salmon (*Salmo salar*)	56
	Killifish (*Fundulus heteroclitus*)	50.4
Estrogen receptor 2b (beta)	Zebrafish (*Danio rerio*)	56.2/68.8
	Fathead minnow (*Pimephales promelas*)	52.5
	Rainbow trout (*Oncorhynchus mykiss*)	54.8/62.5/58.9
	Japanese medaka (*Oryzias latipes*)	53.8
Progesterone receptor	Zebrafish (*Danio rerio*)	64
Androgen receptor	Zebrafish (*Danio rerio*)	69.3/71.3/73.5/69.3
	Fathead Minnow (*Pimephales promelas*)	68.8
	Rainbow-trout (*Oncorhynchus mykiss*)	70.6/68.5
	Japanese medaka (*Oryzias latipes*)	68.5
Glucocorticoid receptor	Zebrafish (*Danio rerio*)	49
	Fathead minnow (*Pimephales promelas*)	50
	Rainbow trout (*Oncorhynchus mykiss*)	53/52
Mineralcorticoid receptor	Zebrafish (*Danio rerio*)	58.8/50
	Rainbow trout (*Oncorhynchus mykiss*)	57.6

2.2 Materials and methods

The data in this chapter were, for the most part, obtained from a publicly accessible and free database (http://www.ic.nhs.uk/) maintained by the National Health Services (NHS) of the United Kingdom (U.K). Occasionally, specific information on one particular pharmaceutical was obtained directly from the manufacturer or distributer of that drug. Prescriptions Cost Analysis (PCA) data for England, Wales, Scotland, and Northern Ireland were obtained from the regional NHS websites. All regional data referred to the year 2006, except the data from Scotland, which referred to the year ending on 31 March 2007. According to the NHS, the PCA data 'were based on information obtained from prescriptions sent to the Prescription Pricing Division of the Business Services Authority (PPDBSA) for payment'. PCA data covered all prescriptions dispensed to the community; that is, by community pharmacists, appliance contractors, dispensing doctors, and items personally administered by doctors.

As the NHS provides healthcare for the vast majority of citizens of the UK, it is generally considered that most medicines prescribed in the UK will be prescribed within the NHS. Medicines prescribed in hospitals, by private doctors, or purchased via the internet will not feature on the database we used. Nor will drugs taken illegally (e.g., anabolic steroids taken by some athletes). Although it is not possible to know with a high degree of accuracy what proportion of pharmaceuticals used in the UK are prescribed or otherwise obtained from outside the NHS (and hence are not covered by the database we used), we consider that it will be relatively low. Thus, although the information on amounts prescribed in this paper will be under-estimates of actual use, we do not consider that our data are compromised to any significant extent.

The PCA data are based on the therapeutic grouping used in the British National Formulary (September 2005; edition 50). For each macro-region, the following data were considered (the NHS definition is provided for each element):

- ***Drug name***: The drug was shown by individual preparation name, which may be proprietary or generic, followed by form and strength.
- ***Items dispensed (PXS)***: A prescription item referred to a single item prescribed by a doctor (or dentist/nurse) on a prescription form.
- ***Quantity (QTY)***: The quantity of a drug dispensed was measured in units specified on the information supplied with the product.
- ***Standard quantity unit (SQU)***: The code indicated the form of the drug and the units in which quantity is measured:

Code 1 – a unit (e.g., one tablet, capsule, pack, aerosol, etc.)

Code 3 – millilitres

Code 6 – grammes

Code 0 – individually formulated (unit varies)

The total amount of active principal prescribed was calculated for each individual preparation, multiplying the value in the QTY column by the amount of active principal in each unit (according to the class). For example, 129,213 tablets of 'Arimidex Tab 1 mg' were prescribed by the NHS in England during 2006. Each tablet contained 1 mg of Anastrozole (an aromatase inhibitor). According to our methodology, 129.213 g of Anastrozole were dispensed.

If the active principal was conjugated with further chemical groups, the parental active principal was considered. For example, Novaldex D Tab 20 mg contained 30.4 mg of tamoxifene citrate, equivalent to 20 mg of tamoxifen. When the drug was dispensed as a gel or topical cream, the percentage of active principal was calculated. In the case of multiphase drugs, containing units with different strengths and compositions in each pack, the different compositions were taken into account. When the value in the QTY column referred to a blister or a pack, that value was multiplied by the number of tablets or patches contained in each blister or pack, according to the British National Formulary 2006. Similarly, the value was multiplied by the number of doses in a single prescription for the inhaler drugs. By combining the data obtained for each active principal, the total amount of active principal prescribed during 2006 was obtained, and expressed in kilograms.

Experimental Log P values for each chemical were obtained from the Physical Properties Database (PHYSPROP, On-Line Interactive Demo Version, http://www.srcinc.com/what-we-do/databaseforms.aspx?id=386). When an experimental value was not available, the estimated Log P was calculated using ALOGPS (v2.1) software (Tetko *et al*. 2005; VCCLAB 2005).

2.3 Results

2.3.1 Annual Usage in the UK

The amounts of all steroid and anti-steroid pharmaceuticals dispensed by the NHS of the UK in year 2006 were determined. Data were grouped by class (e.g., estrogens/anti-estrogens, androgens/anti-androgens), and each class considered in turn below.

2.3.2 Estrogens/anti-estrogens

Both natural and synthetic estrogens are widely used. Estrogens are prescribed alone or in combination with progestogens in hormone replacement therapy (HRT), and as a treatment for estrogens deficiency symptoms in post and perimenopausal women. Estrogens are also used for oral contraception (in conjunction with a progestogen), and for treatment of several conditions including hypogonadism, primary ovarian failure, and acne. The most prescribed estrogen in the UK is the natural estrogen 17β-estradiol (Table 2.2). In comparison, a relatively small amount of the very potent synthetic estrogen EE2 is used: about 23 kg per year, as opposed to 320 kg of E2.

Selective estrogen receptor modulators (SERMs) are used in large amounts (more than 1 ton in total). SERMs are distinguished from pure agonists and antagonists in that their actions are different in different tissues, thereby providing the possibility to selectively inhibit or stimulate estrogen-like action in various tissues. They are used in the treatment of breast cancer (e.g., tamoxifen), in HRT (e.g., raloxifene), and to treat anovulation (e.g., clomifene).

Aromatase inhibitors (which inhibit the synthesis of estrogens) are used principally in the treatment of breast and ovarian cancer. Significant quantities of these pharmaceuticals (more than 100 kg) are prescribed annually (Table 2.2). All the SERMs and Aromatase inhibitors are synthetic compounds.

Table 2.2. *Estrogens/Antiestrogens prescribed in 2006 in the UK for clinical use, classified by Mode of Action.*

Mode of Action	*Principal Name*	*CAS Number*	*Log P*	*Amount Prescribed (kg)*
Agonist	Estradiol	50-28-2	4.01	320.60
Agonist	Conjugated Estrogens†	438-67-5§	2.75§*	66.01
Agonist	Tibolone	5630-53-5	2.69*	64.16
Agonist	Ethinylestradiol	57-63-6	3.67	22.99
Agonist	Estrone	53-16-7	3.13	7.19
Agonist	Estriol	50-27-1	2.45	6.33
Agonist	Diethylstilbestrol	56-53-1	5.07	2.11
				Total 488.79
SERM	Tamoxifen	10540-29-1	5.93*	720.57
SERM	Raloxifene	84449-90-1	5.45*	486.74
SERM	Clomifene	911-45-5	6.08*	33.36
SERM	Toremifen	89778-26-7	5.65*	1.65
				Total 1242.32
Aromatase Inhibitor	Exemestane	107868-30-4	2.67*	68.07
Aromatase Inhibitor	Anastrozole	120511-73-1	2.31*	19.87
Aromatase Inhibitor	Letrozole	112809-51-5	1.86*	12.79
				Total 100.73

(†) Conjugated estrogens containing the sodium sulphate forms of estrone, equilin, 17α-estradiol, 17α-dihydroequilin, 17β-dihydroequilin, 17α-dihydroequilenin, 17β-dihydroequilenin, equilenin, 17β-estradiol, and $\Delta^{8,9}$-dehydroestrone (CAS Numbers: 438-67-5, 16680-47-0, 16680-48-1, 56050-04-5, 4999-79-5, 56050-05-6, 16680-49-2, 56086-66-9, 16680-50-5). (§) Data referred to the sodium estrone sulphate, which represents 52.5% - 61.5% of the mixture. (*) Estimated Log P value (by ALOGPs). Abbreviations: SERM, Selective Estrogen Receptor Modulator.

2.3.3 Androgens/anti-androgens

As with estrogens/anti-estrogens, this group includes agonists, receptor antagonists, and compounds that inhibit the synthesis of androgens (Table 2.3). Fairly similar amounts of these three groups of androgens/anti-androgens were used as the same groups of estrogens/anti-estrogens. As with the estrogen agonists, the androgen agonists used in the greatest amount was the main natural androgen, testosterone. Synthetic androgens were used in considerably smaller amounts, although the most widely prescribed synthetic androgen, stanozolol, was prescribed in approximately the same amounts as the main synthetic estrogen, EE2 (17 vs. 23 kg, respectively). Androgen agonists are prescribed mainly as replacement therapy in males with a deficiency or absence of endogenous testosterone associated with hypogonadism. Much of the testosterone prescribed is in the form of esters. The aim of esterification is to improve the lipophilicity of the molecule, which slows the release of testosterone from the site of entry into the body.

Anti-androgenic pharmaceuticals are prescribed in large amounts – more than 1 ton – each year (Table 2.3). Two different sub-classes of anti-androgenic pharmaceuticals are available:

AR antagonists (e.g., Bicalutamide) and inhibitors of the enzyme 5-alpha reductase, which converts T into DHT (e.g., Finasteride). The use of large amounts of anti-androgenic pharmaceuticals reflects the high incidence of prostatic diseases amongst the male population; in fact, prostate cancer is the most common cancer of men in the UK (ONS, 2007), and it is dependent on endogenous androgen for development, growth and survival (Culig and Bartsch 2006). All anti-androgenic pharmaceuticals on the market are synthetic.

Table 2.3. *Androgens/Antiandrogens prescribed in 2006 in the UK for clinical use.*

Mode of Action	*Principal Name*	*CAS Number*	*Log P*	**Amount Prescribed (kg)**
Agonist	Testosterone	58-22-0	3.32	280.98
Agonist	Stanozolol	10418-03-8	4.06*	17.00
Agonist	Mesterolone	1424-00-6	3.75*	8.15
Agonist	Prasterone	53-43-0	3.23	0.39
Agonist	Nandrolone	434-22-0	2.62	0.08
Agonist	5α-Dihydrotestosterone	521-18-6	3.55	0.02
				Total 306.62
AR Antagonist	Bicalutamide	90357-06-5	2.70*	606.37
AR Antagonist	Cyproterone	2098-66-0	3.81*	401.17
AR Antagonist	Flutamide	13311-84-7	3.35	258.61
				Total 1266.15
5αR Inhibitor	Finasteride	98319-26-7	3.03	242.21
5αR Inhibitor	Dutasteride	164656-23-9	5.45*	5.07
				Total 247.28

(*) Estimated Log P value (by ALOGPs). Abbreviations: AR, Androgen Receptor; 5αR, 5-alpha Reductase.

2.3.4 Progestogens

Only progesterone receptor (PR) agonists were prescribed by the NHS; no PR antagonists or pharmaceuticals that target the synthesis of progesterone were prescribed, as far as we can ascertain. Many different progestogens were prescribed. The 10 most widely prescribed progestogens (by weight) are listed in Table 2.4. Nine of these are synthetic progestogens. In total, 1700 kg of progesterone agonists were prescribed, which is considerably more than both estrogen agonists (less than 500 kg) and androgen agonists (300 kg). This dominance is considerably more pronounced if the synthetic hormones alone are compared; the amounts of synthetic estrogens, androgens, and progestogens prescribed were approximately 70, 25, and 1560 kg, respectively.

Progestogens are most commonly and widely used for contraception, either alone or in combination with estrogens. They are also used for treatment of a number of other conditions,

including HRT, menstrual problems, endometriosis, anorexia, cancer, and assisted reproduction. They are administered via a variety of routes, including pills, patches, injection, implant, gels, creams, and suppositories.

At least one PR antagonist, Mifepristone, is used in the UK, as an abortifacient during the first 2 months of pregnancy. It is also used in 'morning-after treatment', to prevent possible pregnancy. This pharmaceutical is not prescribed through the NHS, and hence no information on its use was available via the NHS website that we used as the main source of our information. However, it has been established through other sources (Exelgyn, personal communication, 2008) that in 2006 about 18.6 kg of Mifepristone was used in the UK (Table 2.4), and in 2007 this increased to about 21 kg.

Table 2.4. *The major progestogens prescribed in 2006 in the UK for clinical use.*

Mode of Action	*Principal Name*	*CAS Number*	*Log P*	**Amount Prescribed (kg)**
Agonist	Medroxyprogesterone	520-85-4	3.52*	529.70
Agonist	Norethindrone	68-22-4	2.97	440.16
Agonist	Dydrogesterone	152-62-5	3.27*	209.02
Agonist	Drospirenone	67392-87-4	2.36*	153.19
Agonist	Megestrol	3562-63-8	3.71*	146.69
Agonist	Progesterone	57-83-0	3.87	141.56
Agonist	Norgestimate	35189-28-7	3.80*	24.12
Agonist	Levonorgestrel	797-63-7	3.25*	19.59
Agonist	Desogestrel	54024-22-5	4.30*	13.87
Agonist	Ethynodiol Diacetate	297-76-7	3.99*	13.85
Agonist	Others**	/	/	12.90
				Total 1704.65
PR Antagonist	Mifepristone	84371-65-3	5.33*	18.6
				Total 18.6

(*) Estimated Log P value (by ALOGPs). (**) Others include: Norelgestromin, 56016-31-2; Norgestrel, 6533-00-2; Etonogestrel, 54048-10-1; Gestodene, 60282-87-3; Gestrinone, 16320-04-0; 17α-Hydroxyprogesterone, 630-56-8.

2.3.5 Corticosteroids

Corticosteroids are either glucocorticosteroids (interacting primarily with the glucocorticosteroid receptor, or GR) or mineralocorticosteroids (interacting principally with the MR). Synthetic corticosteroids are used to treat a wide variety of conditions including adrenocortical insufficiency, hypersensitivity, asthma, rheumatic disease, inflammatory skin disorders such as eczema and psoriasis, contact dermatitis, inflammatory eye and ear conditions, immunosuppression in patients with lymphomas and leukemia, allergic rhinitis, Cushing's disease, and adrenal replacement therapy.

The large number of conditions treated with corticosteroids, together with the increasing prevalence of some of these illnesses (e.g., asthma), explains the high usage of corticosteroids (Tables 2.5 and 2.6). In total, more than 7000 kg of corticosteroid agonists and antagonists were prescribed in 2006. The wide range of conditions that are treated with corticosteroids also explains why so many (about 30) different glucocorticosteroids are in use (c.f. synthetic estrogens, only two of which were prescribed).

Almost all use of corticosteroids refers to synthetic compounds. In most of the synthetic corticosteroids, H on the 9th carbon is substituted by F. In others it is substituted by Cl. These substitutions increase the stability of the compounds in the human body, so that frequent administration is avoided.

Only GR agonists were prescribed (Table 2.5); no glucocorticosteroid antagonists, or pharmaceuticals that inhibit synthesis of endogenous glucocorticosteroids (e.g., cortisol, corticosterone), were prescribed. The total use of synthetic glucocorticosteroids prescribed – more than 4,300 kg – was far in excess of the amounts of synthetic estrogens, androgens, and progestogens prescribed (or the combined total of these three groups).

Only a single mineralocorticosteroid was prescribed, and this in a very small amount (2 kg). In contrast, a very significant amount of mineralocorticosteroid antagonist (more than 2500 kg) was prescribed, which was accounted for almost entirely by a single pharmaceutical, spironolactone (Table 2.6).

Table 2.5. *Glucocorticosteroids prescribed in 2006 in the UK for clinical use.*

Mode of Action	*Principal Name*	*CAS Number*	*Log P*	Amount Prescribed (kg)
Agonist	Hydrocortisone	50-23-7	1.61	1810.91
Agonist	Prednisolone	50-24-8	1.62	1488.30
Agonist	Betamethasone	378-44-9	1.94	305.33
Agonist	Beclometasone	4419-39-0	2.12*	273.96
Agonist	Fluticasone	90566-53-3	2.69*	176.29
Agonist	Budesonide	51333-22-3	2.42*	89.46
Agonist	Mometasone	105102-22-5	2.81*	51.81
Agonist	Clobetasone	54063-32-0	2.61	44.72
Agonist	Methylprednisolone	83-43-2	2.06*	28.98
Agonist	Dexamethasone	50-02-2	1.83	27.26
Agonist	Clobetasol	25122-41-2	2.48	21.60
Agonist	Triamcinolone	124-94-7	1.16	16.61
Agonist	Cortisone	50-06-5	1.47	15.72
Agonist	Others**	/	/	16.77
				Total 4367.72

(*) Estimated Log P value (by ALOGPs). (**) Others include: Deflazacort, 14484-47-0; Flumetasone, 2135-17-3; Fluocinolone, 807-38-5; Diflucortolone, 2607-26-9; Fluocinonide, 356-12-7; Fluocortolone, 152-97-6; Rimexolone, 49697-38-3; Alclometasone, 66734-13-2; Fluorometholone, 426-13-1; Flunisolide, 3385-03-3; Fludroxycortide, 1524-88-5; Ciclesonide, 141845-82-1; Fluprednidene, 1255-35-2; Desoximetasone, 382-67-2; Halcinonide, 3093-35-4.

Table 2.6. *Mineralcorticosteroids prescribed in 2006 in the UK for clinical use, classified by mode of action.*

Mode of Action	*Principal Name*	*CAS Number*	*Log P*	**Amount Prescribed (kg)**
Agonist	Fludrocortisone	127-31-1	1.67	2.18
				Total 2.18
MR Antagonist	Spironolactone	52-01-7	2.78	2536.78
MR Antagonist	Eplerenone	107724-20-9	1.67*	33.46
				Total 2570.24

(*) Estimated Log P value (by ALOGPs). Abbreviation: MR, Mineralcorticosteroid Receptor.

2.3.6 Environmental Concentrations

A representative summary of current knowledge about the environmental concentrations of natural and synthetic steroid hormones and their antagonists is provided in Table 2.7. Effluent concentrations are reported much more frequently than surface water (river) concentrations. In both cases, variability in concentrations would be expected. In the case of effluents, the efficiency of the STW will be a major factor in determining effluent concentrations of "down-the-drain" micropollutants (Johnson *et al*. 2007). In the case of river water concentrations, the degree of dilution of the WWTP effluent in the river, which can be highly variable, will play a major role in determining concentrations. Yet despite these variables, the general message is a

very consistent one: concentrations of both natural and synthetic steroid hormones are in the low ng/L range, or even in the pg/L range.

The estrogens can serve as an example because much more is known about them than any other group of steroid hormones. In the case of the synthetic estrogen EE2, the annual usage of around 23 kg in the UK leads to effluent concentrations in the low ng/L range (Johnson *et al*. 2005; Kanda and Churchley 2008), with river water concentrations, even in rivers heavily impacted by effluent, less than this (Williams *et al*. 2003). Such low concentrations can be difficult to measure reliably, which has led to some erroneous (high) concentrations being reported (discussed in Johnson *et al*. 2008). A very comprehensive recent analysis of both measured and modelled concentrations of EE2 in rivers (Hannah *et al*. 2009) demonstrates convincingly that the maximum likely concentration is 1ng/L, with average concentrations being one or even two orders of magnitude below this.

There are many less data available on measured concentrations of androgens, progestogens, glucocorticoids and mineralocorticoids, but those which there are fit well with the data on estrogens. All published data suggest that, in both effluents and rivers, concentrations of individual steroids are in the low ng/L, or sub-ng/L, range (Table 2.7). However, it should be kept in mind that quite a few (possibly even ten or more) different progestogens or glucocorticoids will probably be present simultaneously, and hence the total concentration, certainly in effluents, may reach a hundred ng/L, or more. Nevertheless, concentrations in the μg/L range, even in effluents, seem extremely unlikely, except possibly in unique locations.

Environmental concentrations of steroid hormone antagonists are currently very poorly documented. In most cases (e.g., antagonists of androgens, progestogens and mineralocorticoids) no data are available, either for effluents or rivers. A very limited amount of data is available for the ER antagonist Tamoxifen (Roberts and Thomas 2006), which suggests that the environmental concentration is in the low hundreds of nanograms/L range. Although this concentration is possible, because a considerable amount of this pharmaceutical is in use (720 kg annually), further work is needed to confirm this value.

In summary, aquatic wildlife are probably exposed, albeit continuously, to very low ng/L, or even sub-ng/L, concentrations of both natural and synthetic steroid hormones and their antagonists: these are the “environmentally-relevant” concentrations that need to be assessed for potential effects.

2.3.7 Effect Concentrations

Summarized in Table 2.8 is a representation of current knowledge on the effects of natural and synthetic steroid hormones and their antagonists on fish. Fish have been chosen as the target species because almost all available data relate to effects on fish. When data on other groups of organisms are available, they seem to be considerably less sensitive to these pharmaceuticals than fish (Caldwell *et al*. 2008).

Effect concentrations of the different steroids and their antagonists appear to be reasonably variable, although all are relatively low (in the μg or ng/L ranges). Fish are extremely sensitive to EE2, with effects occurring in the very low ng/L range. For example, a number of comprehensive, thorough studies have shown that concentrations as low as a few ng/L prevent fish reproducing (Länge *et al*. 2001; Kidd *et al*. 2007) , and even lower concentrations can produce biochemical changes. Similarly, recent data on synthetic progestogens (Paulos *et al*., 2010; Zeilinger *et al*. 2009) shows that at least some representatives of this class of pharmaceutical will inhibit egg laying of fish at extremely low concentrations, possibly less than 1 ng/L. Androgens may be somewhat less potent, but are still having an effect in the range of a few tens of ng/L (see Chapter 4). There are no published effects data for synthetic mineralocorticoids at environmentally relevant concentrations, and only one publication is available for synthetic glucocorticoids (Kugathas and Sumpter, 2011).

A reasonable amount of information is available on the effects of both estrogen and androgen antagonists on fish. Both types of estrogen antagonists (receptor blockers and aromatase inhibitors) have been studied in well executed studies (e.g., Williams *et al*. 2007; Sun *et al*. 2007), and in both cases it has been found that μg/L concentrations are required to produce measurable responses in fish, with the LOEC being in the low μg/L, but not ng/L, range. The AR receptor blocker flutamide has also been reasonably well studied, and appears to be relatively inactive in fish, with the LOEC being in the high μg/L range (Table 2.8). In contrast, the AR antagonist Cyproterone Acetate appears quite potent, and has anti-androgenic effects in the low ng/L range. The only data available on the effects of 5α-reductase inhibitors (anti-androgens) in fish are presented in this Ph.D. Thesis, and confirm a LOEC in the μg/L range. Currently no data are available or on the mineralocorticoid inhibitor spironolactone, despite the very high annual usage (more than 2500 kg) of this drug.

In summary, although there is a significant degree of variability in effect concentrations between the different steroid hormones, and also between different antagonist (even within one class of pharmaceutical), plenty of high quality data shows that μg/L concentrations will always cause major effects, ng/L concentrations often will (especially those of the synthetic

steroids), and even sub-ng/L concentrations of some steroids can cause major adverse effects (for example, lack of reproduction).

Table 2.7. *Representative concentrations of natural and synthetic steroid hormones and their antagonists in the aquatic environment.*

	Mode of Action	***Name***	***Effluent (ng/L)***	***Surface water (ng/L)***	***Reference***
ESTROGENS	**Agonist**	Ethinyl Estradiol	0.8-2.8		Johnson *et al.* (2005)
		Ethinyl Estradiol		4.6 max	Williams *et al.* (2003)
		Estradiol	< 0.4-4.3	ND	Williams *et al.* (2003)
		Estrone	< 0.4-12.2	0.32-2.12	Williams *et al.* (2003)
		Conjugated Estrogens	0.07-2.6	Not measured	Tyler *et al.*(2009)
	Antagonists	Tamoxifen	146	27-212	Roberts and Thomas (2006)
	Aromatase Inhibitor		No data	No data	
ANDROGENS	**Agonist**	Testosterone	0.3 - 8	Not measured	Kolodziej *et al.* (2003)
		Testosterone	Not measured	2.8 - 6.0	Vulliet *et al.* (2008)
	Antagonists		No data	No data	
	5α-Reductase Inhibitor		No data	No data	
PROGESTOGENS	**Agonists**	Norethindrone	5.2-41	Not measured	Vulliet *et al.* (2007)
		Levonorgestrel	0.9-17.9	Not measured	Vulliet *et al.* (2007)
	Antagonists	Mifepristone	No data	No data	
GLUCOCORTICOIDS	**Agonists**	Triamcinolone	14 ± 1	Not measured	Schriks *et al.* (2010)
			Not measured	< 0.63	Tölgyesi *et al.* (2010)
		Cortisone	0.13-0.58	0.06-4.2	Chang *et al.* (2007)
		Prednisolone	0.47-0.72	0.03-0.64	Chang *et al.* (2007)
MINERALOCORTICOIDS	**Agonists**		No data	No data	
	Antagonists		No data	No data	

Table 2.8. *Representative effect concentrations of natural and synthetic steroid hormones and their antagonists. All reliable data concerns effects on freshwater fish LOEC = Lowest Observed Effect Concentration.*

	Mode of Action	*Name*	*Effects*	*LOEC (μg/L)*	*Species*	**Reference**
ESTROGENS	Agonist	Ethinyl Estradiol	Secondary sexual characteristics / Altered sex ratio / feminisation / VTG	0.004	*Pimephales promelas*	Länge *et al*. (2001)
		Estradiol	Reduction in reproductive output	0.78	*Pimephales promelas*	Thorpe *et al*. (2003)
		Estrone	VTG / Sex ratio / Reproduction / secondary sexual characteristics	0.10 0.005-0.025 (VTG)	*Danio rerio*	Brion *et al*. (2004)
	Antagonist	Tamoxifen	Inhibition of Reproductive output/ VTG / Gonadal histology	5.60	*Pimephales promelas*	Williams *et al*. (2007)
	Aromatase inhibitor	Fadrozole	GSI / VTG	24.80	*Pimephales promelas*	Panter *et al* (2004)
		Letrozole	Fecundity / fertility / VTG	5	*Oryzias latipes*	Sun *et al*. 2007
ANDROGENS	Agonist	Testosterone	-	-	-	-
		5α-Dihydrotestosterone	Growth increase in males/T increase in males and decrease in females/ovary histopathology/ masculinisation of females	0.02	*Pimephales promelas*	Margiotta-Casaluci and Sumpter (2011)
			Growth increase in females/stimulation of spermatogenesis	0.20		
			Masculinisation of females/ Vtg induction in females	6.00	*Pimephales promelas*	Panter *et al*. (2004)
	AR Antagonist	Cyproterone	Plasma T and KT reduction in males	0.10	*Fundulus heteroclitus*	Sharpe *et al*. (2004)
			Plasma T reduction in females	0.01		
		Flutamide	Spiggin inhibition	500	*Gasterosteus aculeatus*	Sebire *et al*. (2008)
			Nest building behaviour / Male courtship behaviour	100		
			Testis histopathology/ Ovary histopathology	62.70	*Pimephales promelas*	Jensen *et al*. (2004)
			Plasma E2 increase in males / Vtg induction in males and females / T increase in females / fecundity / hatching	651		
	5αReductase Inhibitor	Dutasteride	Decreased larval growth/Long term retardation of oogenesis/plasma T and KT decrease in males/ovary histopathology	32	*Pimephales promelas*	Margiotta-Casaluci and Sumpter (in prep.)
			Decreased larval survival/Long term retardation of spermatogenesis/decreased egg production/decreased male SSCs/plasma E2 decrease and T increase in females	100		
PROGESTOGENS	Agonist	Norethindrone	Inhibition of reproduction/ masculinisation of females / steroid levels	25 1-10	*Oryzias latipes* *Pimephales promelas*	Paulos *et al*, (2010)
		Levonorgestrel	Inhibition of reproduction/ masculinisation of females	0.0008	*Pimephales promelas*	Zeilinger *et al*. (2009)
GLUCOCORTICOID	Agonists		Plasma glucose increase/VTG gene downregulation/Linphocytes reduction	1	*Pimephales promelas*	Kugathas and Sumpter (2011)
MINERALOCORTICOID	Agonists		No Data			
	Antagonists		No Data			

2.4 Discussion

This Chapter deals with the (potential) ecotoxicological problems caused by the presence of human pharmaceuticals in the environment, particularly the aquatic environment.

2.4.1 Clinical use of steroid/anti-steroids in the UK and their environmental concentrations

The results show that steroid hormones and antagonists of endogenous steroids are widely used as pharmaceuticals, although the amounts used are much lower than the amounts of some other pharmaceuticals in widespread use, such as some analgesics, cardiovascular drugs, anti-inflammatories, and antibiotics. Nevertheless, it should not be forgotten that although the annual use of EE2 in the UK is less than 25 kg, this pharmaceutical is of ecotoxicological concern (Caldwell *et al*. 2008); this pharmaceutical demonstrates that founding environmental risk assessment strategies on amounts of drugs used is not wise or defensible. Even amongst the synthetic estrogens in use as pharmaceuticals, EE2 is not the one used in the greatest amount in the UK: Tibolone occupies that position. When use of synthetic estrogens is compared to the use of synthetic steroids in other classes (e.g., androgens, progestogens and glucocorticosteroids), it is found that the amount of estrogen used is small compared to these other groups of steroids. For example, about 20-times more Medroxyprogesterone acetate and Norethindrone, more than 80-times as much Hydrocortisone, and more than 50-times as much Prednisolone, is used.

As demonstrated in Table 2.7, although much is still to be learnt about concentrations of steroid hormones in the aquatic environment, the picture that is emerging is a reasonably consistent one. Effluent concentrations are likely to be in the low ng/L range, which in most cases would give rise to sub-ng/L concentrations in rivers. Only in situations where the degree of dilution of effluent is low (as it can be in densely-populated areas with relatively small rivers) are river concentrations of steroid hormones likely to be in the ng/L range (Sumpter and Johnson 2005). However, the total concentration (of the different progestogens or glucocorticoids, for example) is probably the most relevant exposure parameter, because pharmaceuticals with similar mechanisms of action are likely to act additively (Brian *et al*. 2005). Thus, environmentally-relevant concentrations of some steroid hormones may be in the tens of ng/L range in heavily impacted locations.

A key parameter controlling the concentrations of synthetic steroids in the environment is their degradation rate in WWTPs. If they are readily degraded, then that will lead to much

lower concentrations in rivers than would otherwise occur. In the case of estrogens, natural ones (especially E2) appear to be relatively readily biodegraded in WWTPs, whereas EE2 is much less easily removed (Kanda and Churchley 2008). It seems likely that the ethinyl group (C2H5) attached to carbon 17 of EE2 (Figure 2.2A) very effectively blocks degradation in WWTPs (and probably the wider environment). The ethinyl group is attached specifically to do just that in the human body; it dramatically reduces the rate of metabolism and excretion, hereby increasing the half-life of EE2 in the body, and its potency. Unsurprisingly, the same or similar 'chemical tricks' have been used to increase the half-life of synthetic progestogens and glucocorticosteroids, hereby making them more effective pharmaceuticals. The addition of chlorine or fluorine atoms to a pharmaceutical has the same effect (Muller *et al*. 2007) of stabilizing the molecule: this 'trick' is especially prevalent in the case of many of the synthetic glucocorticosteroids in widespread current use.

In Figure 2.2 the structures of a few of the synthetic steroids used in significant amounts are illustrated, to show how various atoms and functional groups have been added to the basic structure of that particular class (e.g., estrogens, androgens, etc) in order to produce useful pharmaceuticals. Hence, it can be hypothesized that many synthetic steroids will be poorly removed in WWTPs, and hence they will enter rivers in effluents.

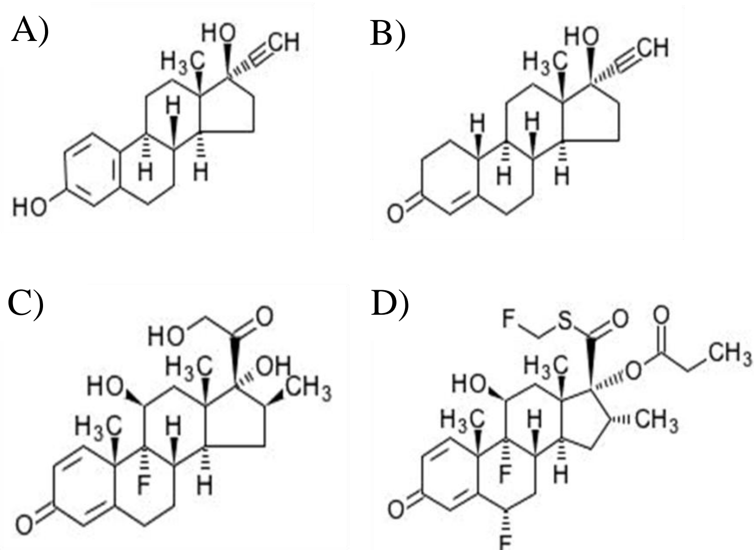


Figure 2.2. *Chemical structure of (A) Ethinylestradiol, (B) Norethindrone, (C) Betamethasone, and (D) Fluticasone propionate. Note the ethinyl group attached to the carbon 17 of the synthetic estrogen Ethinylestradiol and the synthetic progestogen Norethindrone, and the single or multiple fluorine atoms attached to the glucocorticosteroids Betamethasone and Fluticasone propionate. Chemical structures obtained using KEGG 47.1 (Goto et al. 2002).*

This view is supported by some thorough studies of Labadie and Budzinski (2005), who assessed the stability of various natural and synthetic steroids in effluent using a spiking

approach. They found that whereas natural steroids (e.g., E1, E2, progesterone) were reasonably rapidly removed (especially progesterone), presumably by biodegradation, synthetic steroids (EE2, Levonorgestrel, Norethindrone) were fairly recalcitrant. Even after 7 days, little or no loss of these synthetic steroids occurred (Labadie and Budzinski 2005). But in contrast, Chang *et al*. (2007) report high removal rates of 5 of the 6 natural and synthetic glucocorticosteroids they studied. These apparently conflicting results only serve to illustrate how little is established about removal rates of natural and synthetic steroids in WWTPs. More research on this issue is urgently needed.

Very little information is available presently regarding the presence or absence of steroid hormone antagonists in either WWTP effluents or the wider aquatic environment. These pharmaceuticals vary considerably in structure: some are based on the basic steroid nucleus (e.g., the aromatase inhibitor Exemestane), but others have more complex structures and are not steroidal in nature (e.g., Tamoxifen, Raloxifene). This variability, combined with a complete lack of data on ease of biodegradation, makes it very difficult, if not impossible, to predict likely environmental concentrations. Furthermore, a significant technical challenge is the accurate determination of these compounds by analytical chemistry, yet without that information, it is impossible to know if these particular pharmaceuticals merit ecotoxicological attention.

2.4.2 Ecotoxicological relevance of steroids/anti-steroids in the environment

Even if biologically extremely active chemicals such as pharmaceuticals are present in the aquatic environment, this does not mean that they pose a threat to aquatic organisms. To do so, they must first get into those organisms, and then reach (internal) concentrations high enough to elicit effects. Extremely little is known about how readily the pharmaceuticals mentioned in this paper can get into the aquatic organisms; only a little information on a few of these pharmaceuticals is available. However, some general 'rules' (McKim and Schmieder 1985) are available that can be used to predict whether or not a chemical will readily cross the gills of a fish. Key characteristics include the hydrophobicity of a chemical, its charge, and its size. In general, the more hydrophobic a chemical (which is usually expressed as log Kow or log P), the more readily it bioconcentrates in aquatic organisms. As far as size is concerned, if the molecular weight is less than 600, the chemical will cross the cell membrane unhindered; above this value, and its passage could be hindered to varying degrees (the larger the molecule is, the less easily it passes through cell membranes; e.g. Zitko and Hutzinger, 1976).

These ‘rules’ would suggest that most of the pharmaceuticals covered in this paper should readily pass across the gills of fish, and get into the blood stream. However, recent research has shown that other factors besides degree of hydrophobicity and size affect the rate of uptake of chemicals from the water into fish. Scott *et al*. (2005) were the first to show that the rate of uptake of sex steroids (estrogens and androgens) from the water into fish was dramatically affected by their affinity for sex steroid binding protein (SHBG). The rate of uptake of sex steroids can be extremely fast (Maunder *et al*. 2007). The mechanism responsible for this very rapid uptake of sex steroids was recently elucidated: fish gills contain very high amounts of SHBG (Miguel-Queralt and Hammond 2008). That latter study also demonstrated that fish SHBG also has a high affinity for synthetic sex steroids, such as EE2 and some progestogens. Collectively, the results in these papers probably explain why sex steroids, including synthetic ones, are so potent, and affect fish physiology at extremely low environmental concentrations (Länge *et al*. 2001; Purdom *et al*. 1994; Paulos *et al*. 2010; Zeilinger *et al*. 2009).

Once these pharmaceuticals get into aquatic organisms and reach concentrations high enough to cause effects, it seems likely that the effects will be similar to those occurring in people taking those pharmaceuticals. This is because the targets of these drugs (such as receptors and enzymes) are well conserved phylogenetically, at least across all the vertebrates (Gunnarsson *et al*. 2008). Thus, for example, the synthetic estrogen EE2 binds to the estrogen receptor in fish, just as it does in woman, and thereby causes estrogenic effects, including adversely affecting reproduction (Länge *et al*. 2001). Taking a comparative molecular approach, in which genomic and proteomic databases are used to look for conservation of targets, seems sensible. However, even if some potential effects can be predicted, many important unknowns remain.

It is likely that some unique effects, that do not occur in people taking the drug, will manifest in aquatic organisms: vitellogenin synthesis in response to estrogens is an example. Others will undoubtedly occur. Many pharmaceuticals have multiple modes of action: for example, some synthetic progestogens are also androgenic, and hence may induce androgenic effects as well as progestogenic effects (Paulos *et al*. 2010), and SERMs can be both agonists and antagonists, and hence have multiple modes of action (Smith and O’Malley 2008). A range of complex issues are raised by the fact that all these pharmaceuticals are present simultaneously in the aquatic environment, and hence aquatic organisms are exposed to mixtures of pharmaceuticals. It is very likely that pharmaceuticals with similar mechanisms of action (for example, a mixture of the many glucocorticosteroids in use) will act in an additive manner, as

has already been demonstrated for a mixture of estrogenic chemicals (Brian *et al*. 2005). However, it is much more difficult to predict whether or not antagonists might attenuate the effects of agonists when both groups are present simultaneously, or whether androgen agonists might neutralise the effects of estrogen agonists.

There is one further biological issue that merits consideration when attempting to rank pharmaceuticals based on the degree of environmental hazard they present. Different groups of steroids and steroid antagonists will produce a range of effects with different ecotoxicological relevance. For example, preventing reproduction would undoubtedly be considered a catastrophic effect, whereas reducing inflammation would probably not be considered a serious, adverse effect. Thus it is possible to argue that pharmaceuticals such as estrogens and progestogens, which can prevent fish reproducing at low environmental concentrations (Länge *et al*. 2001; Zeilinger *et al*. 2009; Paulos *et al*. 2010), are of more concern, and merit greater attention from environmental scientists, especially ecotoxicologists, than do pharmaceuticals such as many glucocorticosteroids, which (probably) primarily target the immune system.

Recent evidence strongly supports the argument that amongst the pharmaceuticals in use today, steroid hormones merit particular attention from ecotoxicologists. Both Johnson *et al*. (2008) and Besse and Garric (2009) have identified progestogens as requiring hazard assessment for the aquatic environment. Their concerns are confirmed by two recently published independent studies, which have demonstrated that some synthetic progestogens can inhibit fish reproduction at very low (ng/Litre, or even sub-ng/L) environmental concentrations (Paulos *et al*. 2010; Zeilinger *et al*. 2009).

Another recently published paper suggests that environmentally-relevant concentrations of glucocorticoids may cause effects in fish (Kugathas and Sumpter, 2011), and even the very well researched “estrogens in the aquatic environment” issue continues to produce surprises; such as Tyler *et al*. (2009) showing that equine estrogens used in hormone replacement therapy are present in the aquatic environment, and very potent in fish.

2.5 Conclusions

The information provided in this Chapter suggests that when attempting to prioritize pharmaceuticals for potential environmental impact, and hence research, then synthetic steroid hormones merit serious attention. This is based on the fact that they are present in the aquatic environment, and that they can affect fish at low concentrations. Currently,

knowledge of the risk posed by steroid hormones (and possibly also their agonists) is limited; a lot is known about EE2 (e.g., Caldwell *et al*. 2008), but very little, if anything, is known about the other groups of steroid hormones. Research on potential chronic effects of these pharmaceuticals is urgently required, in order that an informed, balanced assessment of their (potential) ecotoxicological effects can be formed.

CHAPTER 3 : OCCURRENCE AND FUNCTIONALITY OF 5α-REDUCTASE IN THE FATHEAD MINNOW

3.1 Introduction: 5-alpha reductase and teleost fish

Mode of action and mechanism of toxicity of any chemical compound are generally defined by the interaction between the toxicant and one or more targets, according to the so-called "toxicant-target paradigm" (Rabinowitz *et al.*, 2009). In the assessment of (eco)toxicological risk of APIs to target species, the presence of drug-specific functional targets in those species should be considered a key factor for the prediction of potential adverse effects caused by exposure to the drug, and for the design of *ad hoc* toxicological test which maximize the chances of detecting predicted effects (Ankley *et al.*, 2007; Christen *et al.*, 2010; Gunnarsson *et al*, 2008; Kostich and Lazorchak, 2008; Seiler, 2002; Winter *et al.*, 2010).

The API object of studied in the present Thesis was dutasteride, which exerts its clinical effect by inhibiting the activity of the two isoforms of 5αR, the enzyme that convert T into DHT, causing a sharp decrease of circulating concentration of DHT (>80%). At the beginning of this Ph.D. project, a significant theoretical issue came to light, since a general assumption in fish endocrinology is that DHT is not synthesized in teleosts, or if it is, it has a modest or no physiological relevance (Borg, 1994). Hence, the question arising was: "why study the effects of dutasteride, a 5αR inhibitor, in fathead minnow, if this species seems to lack the biochemical apparatus targeted by the drug?"

In order to find an objective answer to this question, the preliminary phase of the project was aimed at clarifying if 5αRs are present and functional in the fathead minnow. The occurrence and functionality of the enzyme 5αR in teleost fish has been scarcely investigated so far; however, sparse information available in the scientific literature, based on studies carried out mainly during the 1980s, indicated that 5αR activity can be detected in the tissues of several teleost species, such as the testes of the urohaze-goby (*Glossogobius olivaceus*) (Asahina *et al.*, 1985), the brain of male and female goldfish (*Carassius auratus*) and toadfish (*Opsanus tau*) (Pasmanik and Callard, 1988, 1985), the ovary of European eel (*Angiulla anguilla*), common carp (*Cyprinus carpio*) and grey mullet (*Mugil cephalus*) (Eckstein and Azoury, 1979; Querat *et al.*, 1987), and the skin of rainbow trout (*Oncorhynchus mykiss*) (Latz and Reinboth, 1993). After injection of labelled T in flounder (*Pseudopleuronectes americanus*) and Atlantic salmon (*Salmo salar*), metabolites in the bile were primarily forms of DHT (Truscott, 1983), which was also a major metabolite in a similar experiment performed with fathead minnow (Parks and LeBlanc, 1998), indicating that 5αR activity appears to be involved in androgen metabolism in these species. Also, the results of some *in vitro* experiments suggested that teleost fish can synthesise DHT (Joss *et al.*, 1996; Lee *et al.*, 1995), even if the relevance of these *in vitro* results to the *in vivo* situation is unclear.

3.2 Aim of the study

Considering the poor knowledge about presence and functions of 5αR in the fathead minnow, the preliminary phase of this Ph.D. project focused on determining if 5αR is present and functional in this species. This aim was pursued by using two different approaches:

- Detection of 5αR gene expression in the testis of male adult fathead minnows;
- Quantification of the biosynthetic product of 5αR activity, the androgen DHT, in fathead minnow plasma by analytical chemistry (Ultrasensitive GC-MS/MS).

3.3 Approach 1: Detection of 5αR gene expression in the testis of fathead minnow

The major site of DHT production in male vertebrates is the testis. In men, about 25% of circulating DHT is synthesised by testicular tissue, and the remainder by other tissues, such as liver, kidney, muscle, prostate, and skin. Therefore, the testis represents a major target tissue for dutasteride. Considering the low levels of gene expression expected in fish, in order to maximize the chances of detecting the presence of 5αR mRNA, attention was focused on male fish, and the testis was chosen as the target organ to assess if 5αR genes (*srd5a1* and *srd5a2*) were expressed in the fathead minnow.

3.3.1 Materials and Methods

The following methodological steps were performed in order to confirm *srd5a1* and *srd5a2* gene expression in the testis:

1) Screening of bioinformatics databases of teleost fish looking for genes, expressed sequence tags (ESTs), and proteins showing medium to high degree of similarity to mammals 5αRs;

2) Identification of two fathead minnow ESTs similar to zebrafish 5αRs, and experimental assessment of gene expression by RT-PCR;

3) Confirmation of the identity of the amplified DNA targets by direct sequencing;

4) RACE PCR in order to amplify, isolate and sequence the full length of the genes;

5) Computational and phylogenetic analysis.

3.3.1.1 ***Tissue acquisition***

Testis tissues were dissected from adult male fathead minnow. Fish were sacrificed with 500 mg/L Ethyl 3-aminobenzoate methanesulfonate salt (MS-222) (Sigma), buffered with 200 mg/L NaHCO3. Testes were dissected and transferred into individual clean microcentrifuge tubes and snap frozen in liquid nitrogen. All microcentrifuge tubes were subsequently stored at -80 °C to prevent RNA degradation.

3.3.1.2 ***RNA extraction***

Total Ribonucleic Acid (RNA) was isolated from testes of three different fathead minnow males using the RNeasy Kit (Qiagen Ltd.). The testes were homogenized in lysis buffer and β-mercaptoethanol using an electric Rotor Homogenizer (Fisher Scientific Ltd.) for 20-40 seconds. The tissue homogenate was centrifuged, and the supernatant was collected and mixed with 70% ethanol in order to provide appropriate binding conditions. Samples were subsequently applied to RNeasy Mini spin columns, which were washed and eluted according to the kit instructions. The extracted RNA was solubilised in 20µL of RNase-free H2O; quantification and purity of each RNA sample were determined by spectrophotometry (Nanodrop, Fisher Scientific Ltd.), and the RNA integrity was visually checked by agarose gel electrophoresis. All samples were stored at – 80 °C.

3.3.1.3 ***Generation of complementary DNA by reverse transcription***

Complementary cDNA was synthesised using the SuperScript III First-Strand synthesis system for reverse transcription-PCR kit (Invitrogen). For each sample, 1µL of 50 µM oligo(dT) primer, 1 µL of 10 mM deoxyribonucleotide triphosphate (dNTP) mix and 5 µg of mRNA were combined together, briefly centrifuged, incubated at 65 °C for 5 minutes and then placed on ice for 1 minute. This step allowed the annealing of the oligo(dT) primer to the mRNA poly-A tail. Successively, 2 µL of 10X RT buffer, 4 µL of 25 mM MgCl2, 2 µL of 0.1M DTT, 1 µL of RNase (40 U/µL) and 1 µL of SuperScriptTM III RT (200 U/µL) were added, mixed and briefly centrifuged. The mixture was subsequently incubated at 50 °C for 50 minutes to allow the synthesis of the cDNA. The reaction was terminated by incubation at 85 °C for 5 minutes. Residual RNA was removed by adding 1 µL of RNase H (2 U/µL) to the reaction and incubating it at 37 °C for 20 minutes.

cDNA samples were stored at -20 °C until further analysis. To verify successful cDNA synthesis, RT-PCR for β-actin was run for each sample. β-actin primers were designed by Dr. Tamsin Runnalls (Brunel University) (Forward 5' – GAT ATG GAG AAG ATC TGGC- 3' and Reverse 5' – GTT GGC TTT GGG GTT CAG G – 3').

3.3.1.4 *RT-PCR*

Neither *srd5a1* nor *srd5a2* mRNA sequences for fathead minnow were available in the NCBI databases; however, the screening of NCBI Expressed Sequence Tags (ESTs) database lead to the identification of two ESTs, DT087910 and DT25933, which showed a high similarity, respectively, with *srd5a1* and *srd5a2* expressed in other species. These sequences were subsequently used to design the primers for the amplification of fathead minnow putative *srd5a1* and *srd5a2*.

Primers were designed using PRIMER3 web software (www.biotools.umassmed. edu/bioapps/primer3_www.cgi). The general rules for primer design were followed. All primers were between 16 and 30 nucleotides long and were purchased from Sigma Genosys. Stock primer solutions were made in autoclaved MilliQ water (100µM). Each primer was designed to have a guanine and cytosine (G and C) content of 40-60%, G and/or C nucleotide at the 3' end, and melting point between 55-64 °C.

Each primer pair was designed to have the same, or similar, melting point ($\Delta T < 1$ °C); the characteristics of each primer, including melting temperature, likelihood to form hetero-dimers, self-dimers and hairpin, were assessed using the oligo-calculator available on the Sigma Genosys website (www.sigmaaldrich.com/life-science/custom-oligos.htmL), and the java tool OligoAnalyzer 3.1 (www.eu.idtdna.com/analyzer/Applications/ OligoAnalyzer). The primer sets used for the amplification of fathead minnow putative *srd5a1* and *srd5a2* are shown in Table 3.1.

The AccuPrime Taq DNA Polymerase System (Invitrogen) was used in the RT-PCR reactions. All the reactions were performed according to the basic RT-PCR protocol, as indicated by the Kit's manifacturer. A typical 25 µL reaction mixture was prepared by mixing:

- 10.0 µL 10X AccuPrime PCR Buffer I
- 0.25 µL Forward primer (10 µM)
- 0.25 µL Reverse primer (10 µM)
- 1.0 µL cDNA
- 0.5 µL AccuePrime Taq DNA Polymerase
- 8.0 µL autoclaved Millipore H2O

Table 3.1. *Primer sets used to isolate srd5a1 and srd5a2 from the fathead minnow.*

	Gene	
Primer ID	***srd5a1***	***srd5a2***
	First round of amplification	
Forward	5' AATGTCAAGTTCGCCTGGTTC 3'	5' GAAACACCTGATGCTCTGGAC 3'
Reverse	5' AGGTATCTTATAGCCCGTCTCTCC 3'	5' CTGTGATGGTGATAGGCTCTTG 3'
	5' RACE	
Forward	Kit Anchor Primer	
Reverse1	5' CCATCCCAGGAACCACATACA 3'	5' CACGTACTCAAATAAGCCGCC 3'
Reverse 2	5' TTAGCCTTCCTCGAAATAAGCA 3'	
	5' RACE NESTED	
Forward	Kit Anchor Primer	
Reverse	5' CCAGAGCAAACGAGATGAATG 3'	
	3' RACE	
Forward 1	5' CCAATGTATTAGTGTCCAACTC 3'	
Forward 2	5' ATTAGCCTTCCTCGAAATAAGC 3'	
Forward 3	5' TCCAATGTATTAGTGTCCAACT 3'	5' CCAAGAGCCTATCACCATCAC 3'
Reverse	Kit Anchor Primer	
	3' RACE NESTED	
Forward	5' CAGGGTATCACTGTTTATTGCT 3'	
Reverse	Kit Anchor Primer	

The optimal annealing temperature for each primer pair was determined by performing RT-PCR reactions with a gradient of temperatures (Δ = 0.8 °C). After gel electrophoresis of the PCR products, the temperature at which the amplification of the target fragment had an apparent better yield and specificity was chosen for the subsequent reactions.

Generally, PCR amplifications were performed as follow:

- Denaturation : 94 °C for 15-30 seconds
- Annealing: 55-64 °C for 15-30 seconds
- Extension: 68-72 °C for 1 minute per kb

} 25-35 cycles

- Final elongation: 68-72 °C for 5 minutes
- Final hold: 15 °C

} 1 cycle

3.3.1.5 ***Rapid amplification of cDNA ends (RACE) PCR***

After the successful amplification of fragments of both *srd5a1* and *srd5a2*, it was possible to attempt the amplification of the entire sequences by RACE PCR.

The SMART RACE cDNA amplification kit (Clonetech, Cat # 634914) was used to amplify both the 5' and 3' ends of the genes. This kit integrates Marathon® cDNA Amplification Kit (Chenchik *et al.*, 1995; 1996) with SMART (Switching Mechanism At 5' end of RNA Transcript) cDNA synthesis technology. A detailed illustration of the procedure is illustrated in Figure 3.1 and 3.2. The cDNA synthesis starts by the annealing of a modified oligo dT primer to the poly-A tale at the 3' end of mRNA. The Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV RT) synthesizes the cDNA, and upon reaching the end of a RNA template, exhibits terminal transferase activity, adding 3–5 residues (predominantly dC) to the 3' end of the first-strand cDNA.

The SMART oligonucleotide contains a terminal stretch of G residues that anneals to the dC-rich cDNA tail and serves as an extended template for RT. MMLV RT switches templates from the mRNA molecule to the SMART oligo, generating a complete cDNA copy of the original RNA with the additional SMART sequence at the end. Since the dC-tailing activity of RT is most efficient if the enzyme has reached the end of the RNA template, the SMART sequence is typically added only to complete first-strand cDNAs.

Following reverse transcription, the first-strand cDNA is used directly in 5'- and 3'-RACE PCR reactions, where the amplification is achieve by using gene specific primers and 5' or 3' end-specific primers. 5' and 3' RACE were also performed using nested primers, specifically designed to amplify a shorter fragment. This analysis help to determine if any multiple bands

are a result of correctly primed PCR or non-specifically primed PCR. If bands are real (i.e., the result of correct priming), they should be slightly shorter in the reaction using the nested gene specific primers.

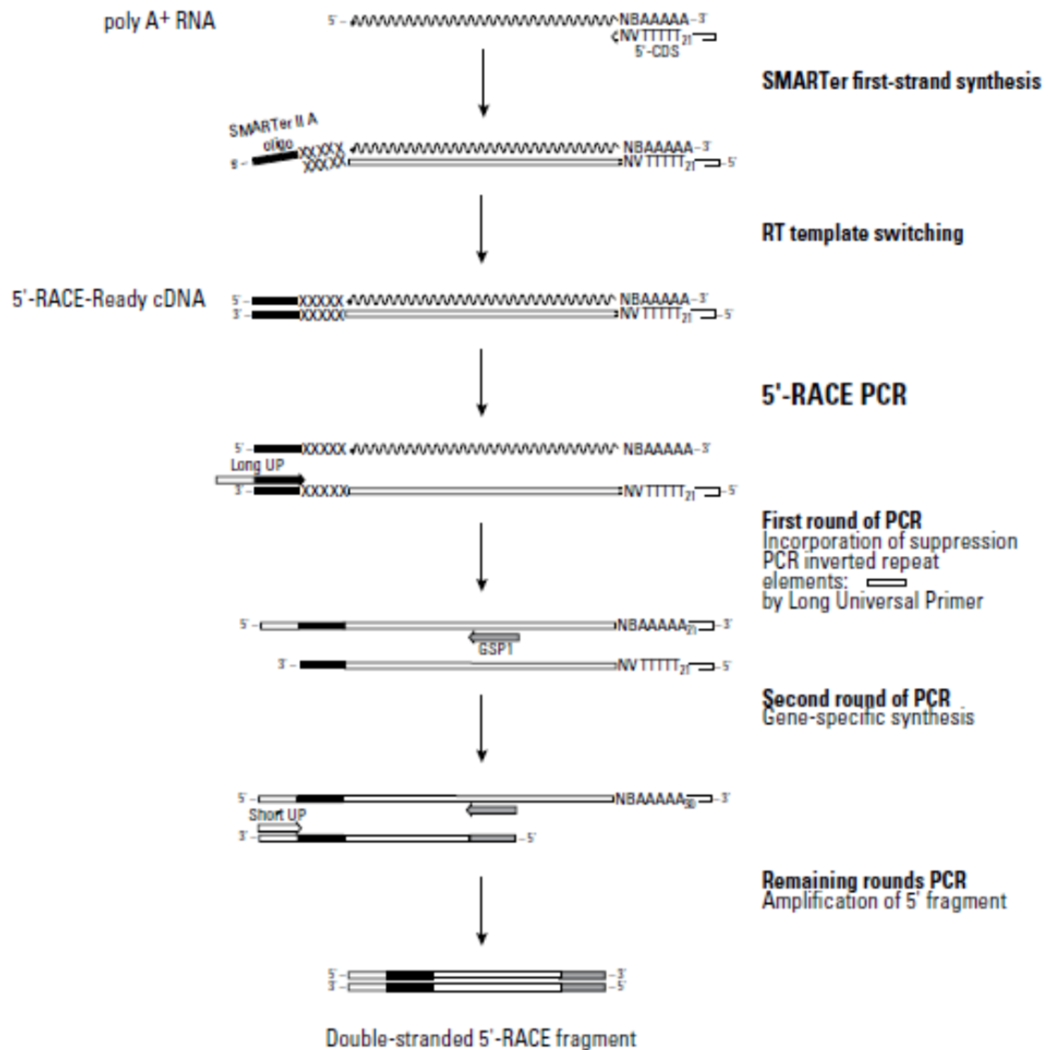


Figure 3.1. *Detailed mechanism of the 5’ RACE reaction.*

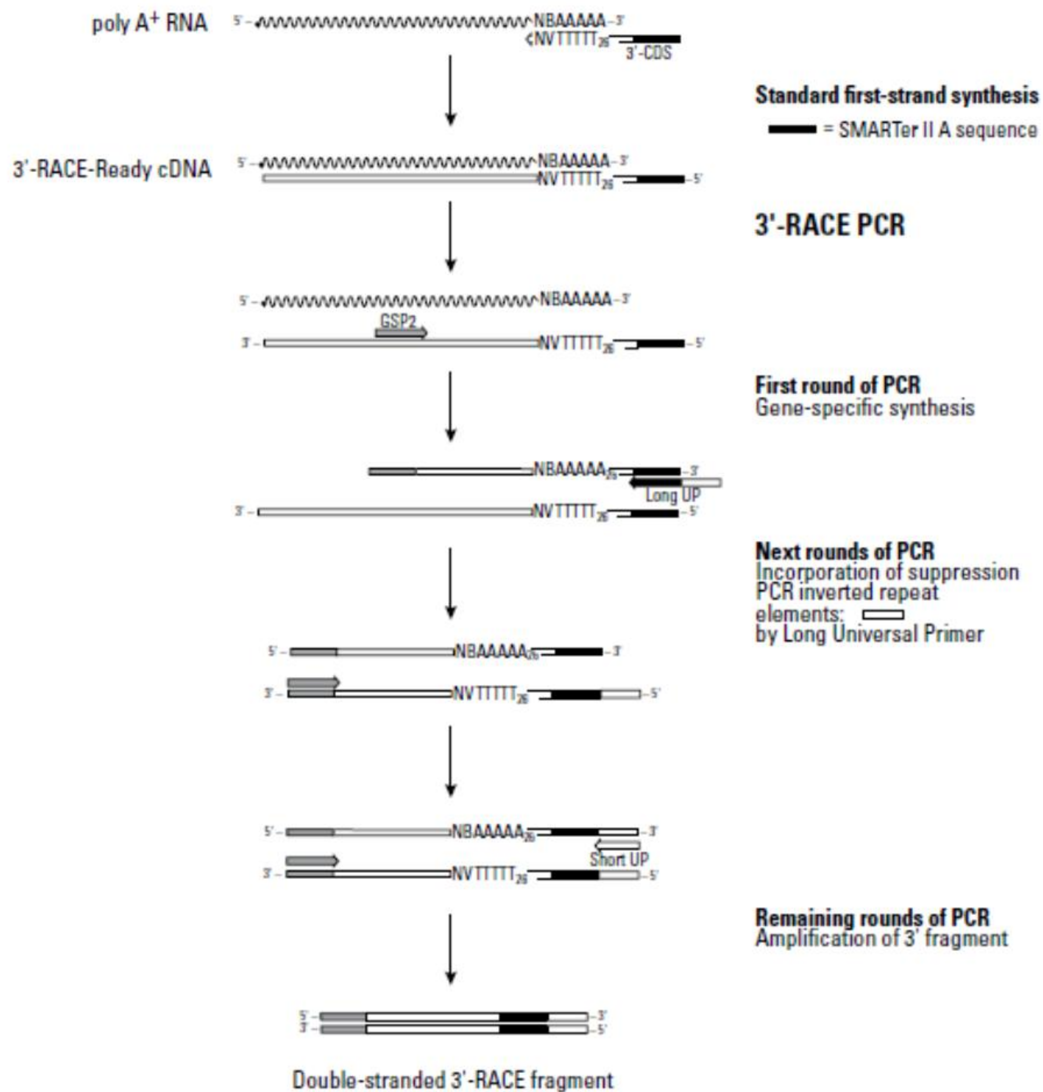


Figure 3.2. *Detailed mechanism of the 3' RACE reaction.*

3.3.1.6 *Gel electrophoresis*

The DNA fragments amplified by PCR were visualized by gel electrophoresis. The gel was made by adding 1.5 g of certified biological agarose (Bio-Rad) to 100 mL of 1X Tris borate ethylenediaminetetraacetic acid (EDTA) (TBE) buffer. The latter was prepared by adding 9 parts of autoclaved deionized H_2O to 1 part of autoclaved 10X TBE stock containing:

- 108 g Tris Base
- 55 g Boric acid
- 9.3 g Na2EDTA.H2O (pH 8.3)

made up in 1 L deionized water

The mixture was prepared in conical flasks (250 mL) and heated in a microwave for approximately 2 minutes until it reached its boiling point. The conical flask was subsequently removed from the microwave, shaken and reheated in order to ensure a full dissolution of the agarose powder. Once cooled at room temperature, 5 μL of ethidium bromide (Sigma) were added and mixed to the solution (0.005%). The gel was decanted into a plastic mould with a comb inserted, in order to create empty wells. The gel was left to cool and solidify for approximately 30 – 45 minutes.

2.5 μL of gel-loading buffer were added to 25μL of each sample. The gel-loading buffer was prepared by mixing:

- 750 μL 40% Ficol (Fisher Scientific)
- 100 μL 10 x TBE
- 150 μL dH_2O
- 8 μL 0.1% xylene cyanol (Sigma)

The gel-loading buffer increased the density of the sample, preventing the settlement of the PCR product in the gel's well. The dye (xylene cyanol) is negatively charged in neutral buffers and migrates in the gel in the same direction as the DNA during electrophoresis. This allows the monitoring of the progress of the electrophoresis.

12 μL of each sample were loaded into the gel. According to the size of the gel, one or two wells were also loaded with 5 μL of 1 Kb DNA ladder (Fermentas Life Sciences Ltd.). The gels were typically run at 80 Volts for 40-60 minutes, and successively examined under UV illumination. Visualisation was achieved by ultraviolet (UV) illumination. Each sample was analyzed for the presence of amplified fragments having the expected size, as calculated during the phase of primers design.

3.3.1.7 *Extraction of PCR product from the gel*

If a band of the correct size was observed, it was excised from the agarose gel under UV light using a clean, sharp scalpel (Swan-Morton, Sheffield) and transferred into a clean 2.0 mL microcentrifuge tube. The gel slice was weighed and the DNA extracted from the gel using the MinElute gel extraction kit (Qiagen), following the protocol indicated by the manufacturer.

Three volumes of digestion buffer were added to 1 volume of gel. The mixture was then incubated at 50 °C for 10 minutes to enhance the dissolution process. One gel volume of isopropanol was added to the sample and mixed by inverting the tube several times. The mixture was then transferred to a MinElute column placed inside a 2 mL collection tube,

which was centrifuged at 17,900 x g for 1 minute at room temperature. The flow-through was discarded and 500 µL of a wash buffer were added to the spin column. A further step of centrifugation was performed at 17,900 x g for 1 minute at room temperature. The flow through was again discarded and 750 µL of a second wash buffer were added to the column, which was left standing for 5 minutes and centrifuged at 17,900 x g for 1 minute at room temperature. The flow through was discarded and the spin column was centrifuged for an additional minute. Finally, the MinElute column was placed into a clean 1.5 mL microcentrifuge tube, 10 µL of sterile autoclaved Millipore H_2O were added to the column, which was centrifugated at 17,900 for 1 minute at room temperature. The eluted DNA was collected, quantified by spectrophotometry, and stored at -20 °C until further analysis.

3.3.1.8 ***Sequencing***

The purified DNA products of the correct size were diluted to a concentration of 30-120 ng in 90 µL of autoclaved Millipore H_2O. Forward and reverse primers were also prepared at a concentration of 32 picomoles in 10 µL of autoclaved Millipore H2O. Both diluted samples and primers were sent to the Sequencing Service at Dundee University (www.dnaseq.co.uk) for direct sequencing analysis. The results from the sequencing were provided as a chromas plot indicating the nucleotide sequence of the DNA product, as shown in Figure 3.3. The quality of the sequencing at the beginning of the process is usually low, as indicated by the modest height and definition of the peaks. A sequence considered reliable was taken from the point where the nucleotide peaks had become higher, sharp and well defined. The sequences obtained were analyzed by computational methods, including Blast and alignment with the EST sequences used to design the specific primers.

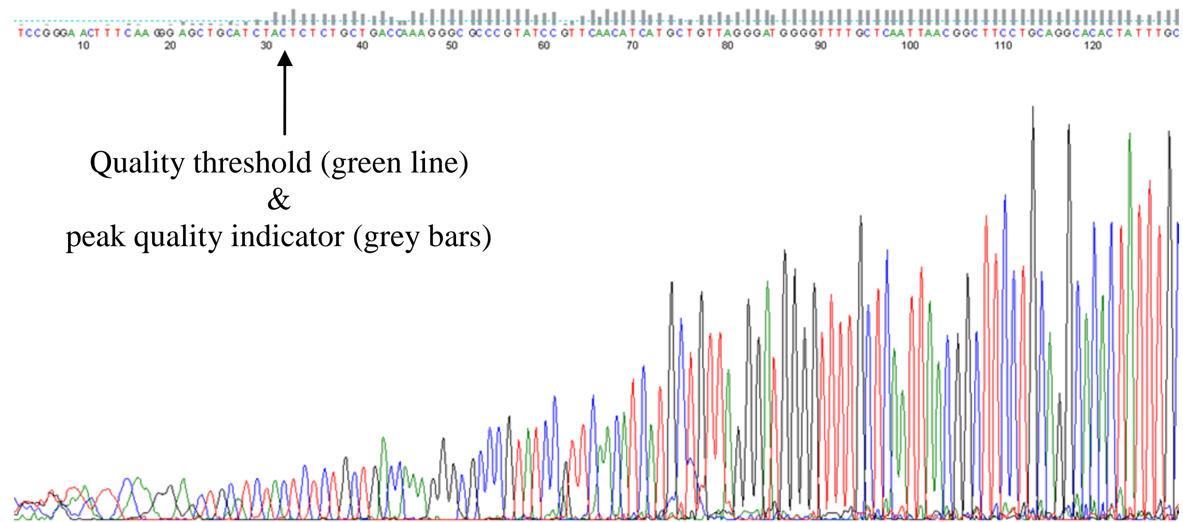


Figure 3.3. *Example of a chromatogram file indicating the nucleotide sequence of a fragment of srd5a2 expressed in fathead minnow testis analysed without cloning. Note the increasing quality of the peaks, indicated by the quality bar at the top of the graph going above the green line threshold. The arrow indicates where the strength of sequence peaks was judged to be satisfactory. The direct sequencing of the fragment causes a loss of information at the beginning of the process, at the primer annealing site. Typically the first 20-40 sequenced bases show low quality peaks. For this reason, to obtain a high quality full sequence of the isolated nucleotides it is necessary to insert the fragment in a plasmid, so that the sequencing process will start outside the target sequence. Due to the cloning method being very time demanding, the direct sequencing method is only used to confirm that the amplified DNA is the target of interest.*

3.3.1.9 *Computational analysis*

Sequence alignments and database screening

Nucleotide and aminoacid sequences were analysed for similarity to other 5αR sequences from other species using the software ClustalW2, available at http://www.ebi.ac.uk/Tools/msa/clustalw2/. A number of online bioinformatic databases and tools were used to collect information on 5αR genes and proteins, and to analyze the nucleotide sequences obtained by RACE PCRs, including:

- ✓ NCBI databases and tools:

  -nucleotide, protein, expressed sequence tags (ESTs) databases;

  -nucleotide basic local alignment search tool (blast) (search a nucleotide database using a nucleotide query);

  -blastx (search protein database using a translated nucleotide query);

  -tblastx (search translated nucleotide database using a translated nucleotide query);

  -conserved domain database (CDD), which include a number of external source databases (Pfam, SMART, COG, PRK, TIGRFAM).

- ✓ Kyoto Encyclopedia of Genes and Genomes (KEGG)
- ✓ Ensembl Genome Browser (EMBL-EBI)
- ✓ BRENDA, the Comprehensive Enzyme Information System

When an aminoacid sequence was required for the computational analysis, the related nucleotide sequence was translated into protein sequence using the ExPASy proteomics server of the Swiss Institute of Bioinformatics (www.expasy.ch/tools/ dna.htmL). The translation process normally generated six possible reading frames. Frame 1 was obtained from translating from the first nucleotide, frame 2 from the second and frame 3 from the third. Frames 4 to 6 were the same as frames 1 to 3 but in the reverse direction. The frame that produced the longest reading frame with no stop codons was selected, and its correctness was double checked by blast analysis.

Phylogenetic analysis

Phylogenetic trees were constructed using the software CustalX, using as source data the multiple sequence alignment file obtained by ClustalW2, and applying the neighbour-joining (NJ) method. Trees were visualized using the software NJplot (Perriere and Gouy, 1996). The results were presented in the form of a rectangular tree. To evaluate the reliability of the inferred tree, a bootstrap analysis was performed and bootstrap values were presented on each node of the tree, providing the degree of confidence in the node. The bootstrap values were provided as percentages, and were calculated by re-sampling each alignment 1000 times.

3.3.2 Results

3.3.2.1 *Database screening*

The screening of several online bioinformatic databases provided both full length or partial mRNA sequences coding for several isoforms of 5αR in teleost fish, including zebrafish, Atlantic salmon (*Salmo salar)*, stickleback (*Gasterosteus aculeatus*), and medaka (*Oryzias latipes*) (Table 3.2). No mRNA sequences were available for fathead minnow. However, the screening of the NCBI ESTs database lead to the identification of two ESTs, DT087910 and DT25933, which showed a high similarity, respectively, with *srd5a1* and *srd5a2* of other species. The BLAST of the EST similar to *srd5a1* highlighted a significant evolutionary conservation of the *srd5a1* and *srd5a2* genes, not only in vertebrates, but also in invertebrates like the nematode (*Caenorhabditis elegans*), in unicellular eukaryotes like trypanosome

(*Trypanosoma cruzi*), and in bacteria, fungi and plants (*Arabidopsis taliana*). According to the InterPro database (EMBL-EBI), the conserved C-terminal domain characteristic of 5αRs protein sequences also followed the same distribution.

Table 3.2. *Access numbers for srd5a1 and srd5a2 of the species used in the multiple alignment analysis and in the phylogenetic analysis.*

<table>
<tr><th>Accession number</th><th>srd5a1</th><th colspan="2">srd5a2</th></tr>
<tr><td>Human
(Homo sapiens)</td><td>NM_001047</td><td colspan="2">NM_000348</td></tr>
<tr><td>House mouse
(Mus musculus)</td><td>NM_175283</td><td colspan="2">NM_053188</td></tr>
<tr><td>Rat
(Rattus norvegicus)</td><td>NM_017070</td><td colspan="2">NM_022711</td></tr>
<tr><td>Cow
(Bos taurus)</td><td>NM_001099137</td><td colspan="2">NM_001205937</td></tr>
<tr><td>Frog
(Xenopus tropicalis)</td><td>NM_001006840</td><td colspan="2">NM_001017113</td></tr>
<tr><td>Fathead minnow
(Pimephales promelas)</td><td>DT087910</td><td colspan="2">DT259333</td></tr>
<tr><td>Zebrafish
(Danio rerio)</td><td>NM_001076653</td><td>NM_001017703</td><td>NM_001017693</td></tr>
<tr><td>Medaka
(Oryzias latipes)</td><td>EF537018</td><td colspan="2">EF537017</td></tr>
<tr><td>Stickleback
(Gestreotus aculeatus)</td><td>ENSGACG00000008961</td><td colspan="2">/</td></tr>
<tr><td>Atlantic salmon
(Salmo salar)</td><td>/</td><td colspan="2">BT125262</td></tr>
<tr><td>Fugu
(Tetroaodon nigroviridis)</td><td colspan="3">CR711491</td></tr>
<tr><td>Nematode
(Caenorhabditis elegans)</td><td colspan="3">NM_077670</td></tr>
<tr><td>Trypanosome
(Trypanosoma cruzi)</td><td colspan="3">XM_814384</td></tr>
<tr><td>Plant
(Arabidopsis taliana)</td><td colspan="3">U53860</td></tr>
</table>

3.3.2.2 *Amplification, isolation and sequencing of fathead minnow srd5a1 and srd5a2*

Using the two fathead minnow ESTs, similar to *srd5a1* and *srd5a2*, as reference sequences for the design of primers, a first PCR round was carried out, leading to the amplification of two fragments of 475 pb and 474 pb for *srd5a1* and *srd5a2*, respectively (Fig. 3.4). The sequencing of the fragments performed at the University of Dundee confirmed a 100% identity between the amplified fragments and the ESTs identified in the database. Successively a new cDNA from testicular tissue was synthesised for RACE PCR, and new sets of primers were designed in order to attempt the amplification of the 5’ and 3’ ends of the genes.

Targeting *srd5a1*, 5'RACE produced a fragment of 627 pb, and the specificity of the reaction was confirmed by a Nested RACE PCR performed with a new set of primers designed to produce a fragment 150 pb shorter than the first one (Fig. 3.5). The fragments obtained were sequenced and the result showed the presence of the 5' tale anchor sequence added during the synthesis of cDNA with the SMART RACE cDNA amplification kit, plus 7-8 bases, mainly G and C, indicating the successful amplification of the 5'end of *srd5a1* (Fig. 3.6). On the other hand, 3'RACE produced a high number of amplified fragments, and a "smear" of amplification, which made further isolation of specific bands difficult (Fig. 3.5).

Targeting *srd5a2*, both 3' and 5' RACE did produced low quality reactions, with a high amount of unspecific smear. Both reactions did not produce fragments of the expected size; two fragments of approximately 700 pb and 300 pb were isolated, purified and sequenced, but once the results were analyzed by BLAST, they did not show any similarity to any of the 5αR gene isoforms available in the NCBI databases. This result was confirmed also by the very low similarity value obtained aligning the sequenced fragments with the fathead minnow EST used as the starting reference sequence, confirming that the amplified fragments were a product of non-specific amplification.

In conclusion, *srd5a1* and *srd5a2* were both expressed in the testis of fathead minnow, a major target for the action of dutasteride. Isolation attempts lead to the sequencing of 627 bp of *Srd5a1* gene (Fig. 3.6), including the 5'end of the gene, and 474 pb of *srd5a2* (Fig. 3.7).

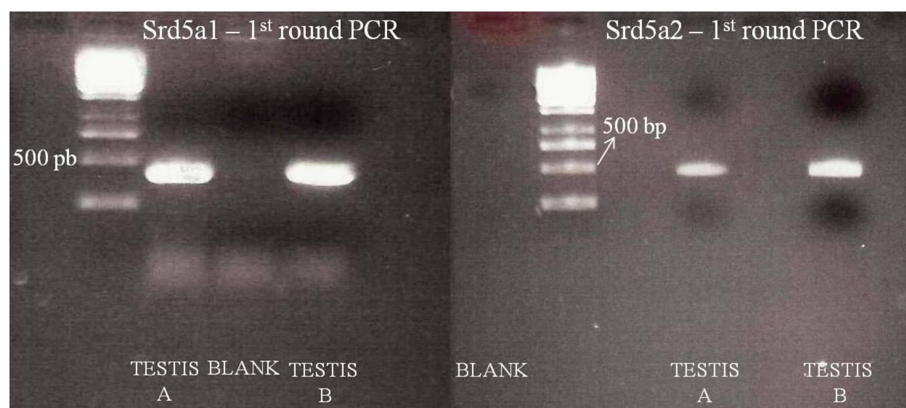


Figure 3.4. *Example of UV image of the products of 1st round PCR performed to target fathead minnow srd5a1 and srd5a2 in testes of two different fish. The product bands obtained are clean, clear and at approximately the correct size of 475 bp. Both products were successively extracted from the gel and sequenced.*

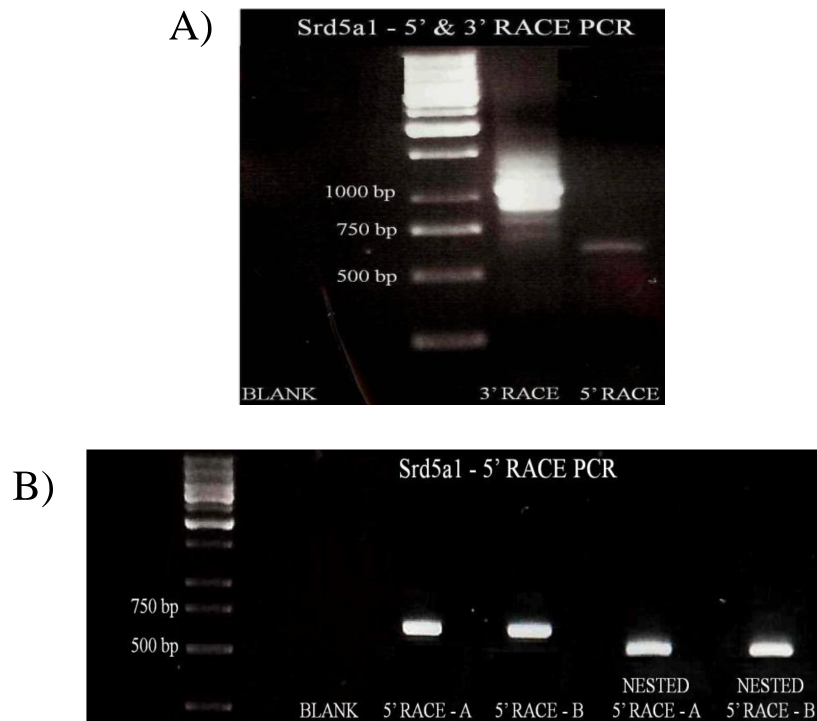


Figure 3.5. *Example of UV image of the products of (A) 3'RACE PCR and 5' RACE PCR performed to target fathead minnow srd5a1 in the testes of two different fish. The 3'RACE PCR produced a smear plus several bands of different size. On the other hand, 5' RACE PCR produced a product of the expected size (approx. 650 bp). (B) Further 5'RACE PCRs were performed using nested primers designed to produce a fragment of approx 500 bp. The results confirmed the specificity of the longer 5'RACE PCR product. Both products were successively extracted from the gel and sequenced.*

5'CTAATACGACCCCTTTTAGGGCAAGCAGTGGTATCAACGCCGAGTACGCGCGGGGAGT
CCGTTTGTGCACGAGAATGAAGTTACACACTTTCTTCTGCTCATAAGTATAATTTCCATAA
GCAACTATGGACGCTATTCTGAAGATATTATTTTCTTCGGAGGAGGAAGAACGTACGTTC
TGGATTGTTTATCGTACTTGATGATGGTGATGGCGTTCATAACTTTTGTGACTTTACTCTTT
GAGCACGTGCCATACGGCAGATATGCATCCAGCAGGTATGGATTCCCTGTAAATGTCAAG
TTCGCCTGGTTCGTTCAAGAATTGCCGGCTTTTCTGGTGCCTTTGTGTTTGGTACTATGGA
GTCCATGTGCAAAGCTAATGCACCTGCCCAACCAATTGCTTCTTCTCATGTTCTGTTGCCA
TTACGCGCAAAGGTCCCTCATCTACCCATTTTTAATTCGAGGAGGAAAATCAAC**ACCATT**
CATCTCGTTTGCTCTGGCCTTTGTCTTCTGCATCTATAATGGGTATCTGCAGGCAAGATA
CCTGAGTCACTATGCAGATTACCCACCTGATTGGGTTACACATCCTATTTCATCACAGG**TT**
CTTGTATGTGGTTCCTGGGATGG **3'**

Figure 3.6. *Partial sequence (627 bp) of fathead minnow srd5a1. The red highlighted sequences represent the primers used to confirm the sequence in the 5' direction by the Sequencing service. Despite the fact that the fragment was not inserted in a plasmid and cloned, and the sequencing quality at the ends of the fragment was not very high, there is a high probability that the amplification reached the 5' end of the gene. In fact, at the beginning of the sequence it is possible to recognize the "Adaptor" added by the Clonetech RACE Kit to the 5'end during the cDNA synthesis (highlighted in blue), and a sequence of 8-10 bases (mainly G or C) added by the MMLV reverse transcriptase (highlighted in green).*

5'**GAAACACCTGATGCTCTGGACC**TTCTGCTTGCATTACTTCCAAAGGAGCTGCATCTGA
GCTGCATCTACTCTCTGCTGACCAAAGGGCGCCCGTATCCGTTCAACATCATGCTGTTAGG
GATGGGGTTTTGCTCAATTAACGGCTTCCTGCAGGCACACTATTTGCTGCATTGTGCTACA
TATAACAACACATGGTTTTCCGATGCCTGTTTGCTAGCCGGCCTGATCATATTCTTCATAG
GAATGGGAGTCAATATTCACAGTGACCACATTCTTCGCAGCCTGAGAAAACCTGGAGAA
ATTACCTATAAAATCCCGAGAGGCGGCTTATTTGAGTACGTGTCTGGTGCAAATTTCTTTG
GGGAGATAGTACAGTGGCTGGGTTACGCGGTTGCTACCTGGTCCTTACCCACATTTGCTTT
TGCTTTTTTCACAATTTGCTTCATAGGCCC**AAGAGCCTATCACCATCACAGG** **3'**

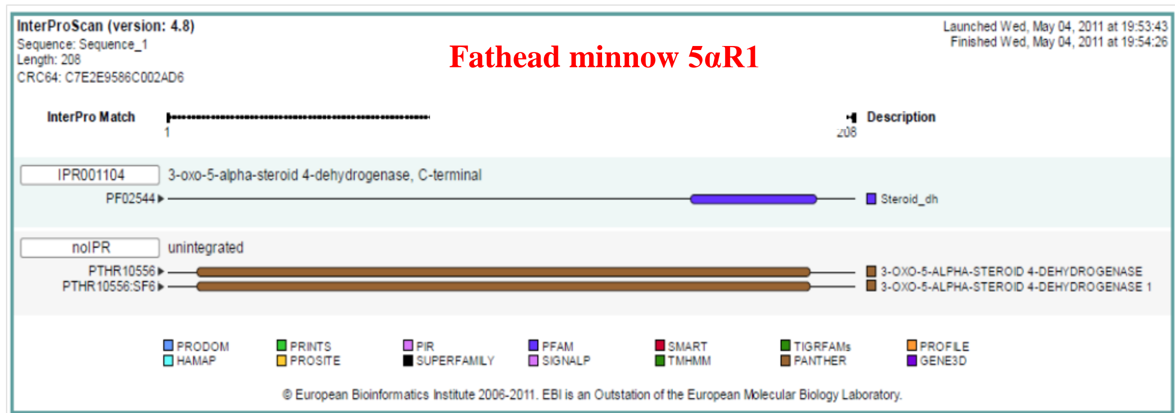
Figure 3.7. *Partial nucleotide sequence (474 bp) of fathead minnow srd5a2. The sequences highlighted in red represent the primers used to confirm the sequence in the 5' and 3' direction by the Sequencing service.*

3.3.2.3 *Computational and phylogenetic analysis of fathead minnow srd5a1 and srd5a2*

After translating the sequenced fragments into estimated aminoacid sequences, the latter were analysed by InterPro, a database able to identify protein "signatures" used for the classification and automatic annotation of proteins and genomes. InterPro classifies sequences at superfamily, family and subfamily levels, predicting the occurrence of functional domains, repeats and important sites. Both fathead minnow putative *srd5a1* and *srd5a2* were characterised by the presence of a functional domain typical of "steroid oxidoreductase (Steroid_dh) superfamily" (PFAM database), which includes several enzymes, such as steroid dehydrogenases and isoprenylcysteine carboxyl methyltransferase enzymes (Fig. 3.8). These proteins also contain a varying number of transmembrane regions. Furthermore, the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System recognized specifically the domains typical of 3-oxo-5-alpha steroid 4 dehydrogenase (5αR) isoforms 1 and isoform 2, respectively, in the fathead minnow putative 5αR Type 1 and 5αR Type 2.

Only in the sequence of fathead minnow putative 5αR Type 2, the ProfileScan database identified the domain "5α-reductase", and the TMHMM database, which predicts transmembrane helices in proteins, identified three transmembrane regions.

All the InterPro results confirmed that the isolated and sequenced fragments coded for fathead minnow 5αR Type 1 and 5αR Type 2.



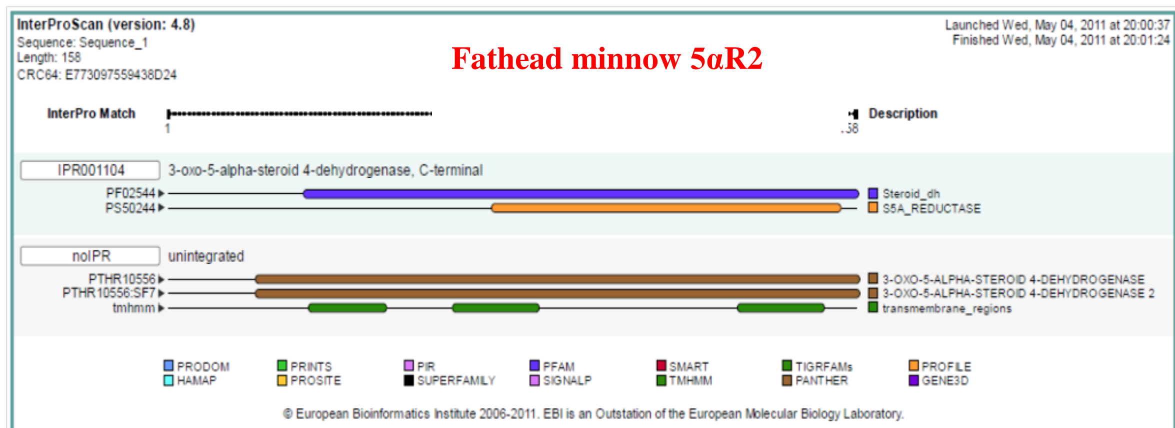


Figure 3.8. *The DNA fragments amplified by targeting fathead minnow ESTs were isolated, and their nucleotide sequences were translated into aminoacid sequences. The latter were analyzed using InterPro, an online bioinformatic tool able to identify protein "signatures", such as functional domains, repeats and important sites. The analysis identified the functional domains characteristic of 5αR in both fathead minnow putative* 5αR Type 1 *and* 5αR Type 2.

Considering the perfect identity match between the isolated fragments and the fathead minnow ESTs used as reference sequence, the latter were used for the phylogenetic analyses. Fathead minnow sequences were first aligned to *srd5a1* and *srd5a2* present in other teleost fish species, showing a medium to high degree of interspecies similarity (Fig. 3.9 & 3.10; Table 3.3). The highest degree of similarity was found when the sequences were aligned to the zebrafish sequences (*srd5a1*: 81%; *srd5a2*: 89%). Zebrafish also had a third isoform of 5αR gene, classified as *srd5a2b*, which showed only a medium degree of similarity to fathead minnow *srd5a2* (61%).

A phylogenetic analysis was performed in order to broaden the range of comparisons to other taxa, including human, domestic mouse, rat, frog, nematode, trypanosome, and plant. The analysis of the nucleotide sequences lead to the phylogenetic tree shown in Figure 3.11. The tree was characterized by low bootstrap values, indicating a modest reliability of the result. However, the analysis did give a good classification of the two isoforms of srd5a, with a clear

dichotomy that separated *srd5a1* from *srd5a2*. Furthermore, the fish sequences were evolutionary closer to each other than to the mammalian ones. The low bootstrap values were probably due to the fact that the length of the analyzed sequences was not the same in all the species. In fact, some sequences represented full-length genes, while others where only partial sequences, with a consequent loss of evolutionary information.

The reliability of the result was strongly improved by the use of the aminoacid sequences, which are typically more conserved than the nucleotide ones. The phylogenetic tree obtained for the protein sequences showed, in fact, highly significant bootstrap values, confirming the good degree of similarity among 5αRs in different species (Fig. 3.12).

Interestingly, the putative 5αR of trypanosome and plant were classified in a separate subgroup, while the nematode 5αR was evolutionary closer to the vertebrate ones.

```
CLUSTAL 2.1 multiple sequence alignment


Medaka          ------------------TACCTGCCACTCGCT---------AAATGTCTCCGGTTTCTC 33
Stickleback     ------------------------------------------------------------
Zebrafish       AGATTTCTCCATGGTGTCAGACTGTTTCGTGCATCTACTGTAAACTACGTGTAAGTTGCC 60
Fathead-minnow  -----------------GAGTCCGTTT-GTGCA-CGAGAATGAAGT---TACACACTTTC 38


Medaka          GCCTTTTTCTTTGT-----CATTTTGTGCGTTTGTAGCTTTTGCTGCAATGACGTCATTC 88
Stickleback     ------------------------------TCGCGGCTCTT------------------- 11
Zebrafish       GTCTTATTCTTAAT-CGGTTTTGATTAGCAACTATGGACGCTCTTCTGAATACGTTATT- 118
Fathead-minnow  TTCTGCTCATAAGTATAATTTTCATAAGCAACTATGGACGCTATTCTGAAGATATTATT- 97
                                                    *    *

Medaka          CTTCGTGTGATCTCATCGGAGGAAGAGGAGCTGTACTTGCTGGACTGCATGGCTTATCTC 148
Stickleback     -----------CTCCTCGGAAGAAGAGGAGCGCTACGCGTTGGACTGCATGGCTTACCTC 60
Zebrafish       -----------TTCTTCTGAGGAGGAAGAATTGTACGTTTTGGATTGCTTATCGTATCTG 167
Fathead-minnow  -----------TTCTTCGGAGGAGGAAGAACGGTACGTTCTGGATTGTTTATCGTACTTG 146
                            ** ** ** ** ** **    ***    **** **  *  * **  *

Medaka          ATGCTTTTCATGGCCGTCTGCACTTTAGTTAGTTTGCGGTTTGAAAACGTCCCTTATGGC 208
Stickleback     ATGCTGGTCATGGCAGCCTGCACGTTTGTGACTCTGCTCTTTGAAAACGTCCCGTACGGG 120
Zebrafish       ATGATGGCCATGGCTCTGATAACTTTTGTTGCTTTACTCTTTGAGCATGTGCCCTATGGC 227
Fathead-minnow  ATGATGGTGATGGCGTTCATAACTTTTGTGACTTTACTCTTTGAGCACGTGCCATACGGC 206
                *** *    *****        ** ** **   * * *  *****  * ** ** ** **

Medaka          CGTTACGCCTCCAGCAAATATGGTTTCCCGGTGAACGTGAAGCTCGCCTGGTGCGTTCAG 268
Stickleback     CGCTATGCAACCAGCAGGTACGGATTCCCGGTGAACGCCCGGTTGGCGTGGTTCATTCAG 180
Zebrafish       AGGTATGCATCCAGCAGGTTTGGATTCCCTGTAAATGTCAAGTTGGCTTGGTTCGTTCAA 287
Fathead-minnow  AGATATGCATCCAGCAGGTATGGATTCCCTGTAAATGTCAAGTTCGCCTGGTTCGTTCAA 266
                 * ** **  ******  *  ** ***** ** ** *    * * ** **** * ****

Medaka          GAGCTGCCGTCCCTCCTGATCCCGTTATATCTGACGGTCTCAGCGACCTCCGTCAAAACC 328
Stickleback     GAGCTGCCTGCCTTCCTGGTGCCGCTGTGTCTGGTAGTGTGGACGTCCTCTGGTAAAACC 240
Zebrafish       GAGCTGCCTTCTTTTTTGTTGCCTTTGAGTTTAGCTTTATGGA-GTTCAAGTTCAAAAAT 346
Fathead-minnow  GAATTGCCGGCTTTTCTGGTGCCTTTGTGTTTGGTACTATGGA-GTCCATGTGCAAAGCT 325
                **  ****  *  *  ** * **  *    * *     *  *    *       ***

Medaka          --TCCGTCCTGCCAAACCAGCTCCTCATCGCCATGTTCGTGTGTCACTACATCCAGAGAG 386
Stickleback     --TCCCTGCTGCCCAACCGGCTGCTCATTGCCATGTACTTCTGCCACTATGTCCACAGAT 298
Zebrafish       AATCCA-CCTGCCCAACCAACTCCTCCTGCTCATGTTCGTTTGCCATTATATGCAAAGGT 405
Fathead-minnow  AATGCA-CCTGCCCAACCAATTGCTTCTTCTCATGTTCTGTTGCCATTACGCGCAAAGGT 384
                  * *   ***** ****    * **   *    ***** *    ** ** **    ** **

Medaka          CCATCATTTATCCCTTTCTGATCCGGGGTGGGAAAGGGACGCCGTTTGTGTCCTTCGCCT 446
Stickleback     CTCTTATTTATCCTTTTCTAATCCGAGGAGGTAAACCAACGCCATTCGTCTCATTCGCCC 358
Zebrafish       CTCTCATCTACCCGTTTTTAATTAGAGGAGGGAAATCAACACCGTTCATCTCCCTAGTTC 465
Fathead-minnow  CCCTCATCTACCCATTTTTAATTCGAGGCGGAAAATCAACACCATTCATCTCGTTTGCTC 444
                *  * ** ** ** *** * **   * ** ** ***    ** ** **  * **   * *

Medaka          TGGCCTTGCTTTTCTGCTTGTATAACGGCTACATGCAGGTCAGATATCTGAGCCATTATG 506
Stickleback     TGGCCTTTGTTTTCTGCATCTATAATGGGTACATGCAGATCAGGTACCTGAGCCATTACG 418
Zebrafish       TAGCCTTTGTCTTCTGCATCTATAATGGGTACCTCCAAGGTAGATACCTGAGTCACTATG 525
Fathead-minnow  TGGCCTTTGTCTTCTGCATCTATAATGGGTACCTGCAGGCAAGATACCTGAGTCACTATG 504
                * *****  * ****** * ***** ** *** * **    ** ** ***** ** ** *

Medaka          CCGACTATCGTCCGCACTGGGTCACACATCCACTCTTCATCCTTGGGTCTGTTCTGTGGC 566
Stickleback     CCGAGTACCCTGCAGACTGGGTTACACATCCACGCCTCATCGCAGGTTCTCTGCTGTGGC 478
Zebrafish       CTGATTACCCAGCTGATTGGGTTACACACCCCTGCTTCATCATAGGGTCTTGTATGTGGT 585
Fathead-minnow  CAGATTACCCACCTGATTGGGT-ACAC---------------------------------- 530
                * ** ** *   *  * ***** ****

Medaka          TGGTCGGCTGGCTGTTGAACTTGCATTCTGACCACATCCTGAGAAACCTGAGAAAGCCTG 626
Stickleback     TGCTCGGCTGGGTGGTGAATGTGCACTCTGACCACATCCTGAGGAACCTGAGGAAGCCCG 538
Zebrafish       TCCTGGGATGGATTATTAATATGCACTCTGACCATATCCTCAGGAACCTCCGTAAACCTG 645
Fathead-minnow  ------------------------------------------------------------
```

Figure 3.9. *ClustalW alignment of the fathead minnow srd5a1 sequence versus the one of zebrafish, medaka, and stickleback. Sequences similarity are showed in Table 3.3. * indicates identical nucleotide in all four species of fish.*

```
CLUSTAL 2.1 multiple sequence alignment

Zebrafish_srd5a2a      ------------------TCACATCAAGACGGAGAAGACAGCAGGGCACTGAGAAACACG 42
Atlantic_salmon        ----------------------------ATGGAG-------------------------- 6
Fathead_minnow         ---------------GACTTG------GACAGATA---------------------CTCA 18
Zebrafish_srd5a2b      ATTAGGCTCATACCTGGTTTGAAAACGGACGGATA---------------------CTCG 39
                                                   *  **

Zebrafish_srd5a2a      CACAGCAATGCTCTGTCAGGAAAACACTGTTCACTTTGGCAGCTGGGCTTTTGTAGTCGG 102
Atlantic_salmon        -------------TGTAAGGAGAATGTGATCCACTACCTCAGCTGGGGTTCATCGTCAG 53
Fathead_minnow         ACCTCCA-TGCAGTGCCAGGAGCTCCTCGTGCAGGGCCTGAGTTTGGGGATGATCCTGGG 77
Zebrafish_srd5a2b      ACCTCCAATGCAGTGTCAGGAGCTCCTCGTGCGGGGCCTGAGTTTAGGGATGATCCTGGG 99
                                    **  ****        * *        ** *  *   *  *  *  *

Zebrafish_srd5a2a      TGGATTGCTTTACCTTCTGAAACAACTGACCACG---CAAACGGCGTACGGACGCTATGT 159
Atlantic_salmon        TGGGGTGGCGTATCTCTTTTGGCA---GACCAGGTTCCAGACCCCATACGGACGCTACGT 110
Fathead_minnow         TGGGGTAATGCACTTTATATGTCT---CAGGAAGTCTCACGCTGCCTATGGCCGCTACGT 134
Zebrafish_srd5a2b      TGGGGTAATGCACTTTATTTGTCT---CATGAAGTCTCACGCTGCCTATGGCCGCTACGT 156
                       ***  *     *  *  *    *     *  * *   **  *  * ** ** ***** **

Zebrafish_srd5a2a      GGACAC---AAA---ATCCCCTGGGTTGATGGTCCCAGCCAGGGCAGGCTGGTTTATACA 213
Atlantic_salmon        GACCTC---GGATGGGACCCCAGGGAGGACCTGCCCTGCCAAGCTGGCCTGGTTCCTCCA 167
Fathead_minnow         GGACACCTCGGGC--AGCTCC----ATGATGGTGCCCGCAAGGCTAGCCTG-TTCCTCCA 187
Zebrafish_srd5a2b      GGACACCTCGGGC--AGCTCC----ATGATGGTGCCCGCAAGGCTAGCGTGGTTCCTCCA 210
                       *  * *           * **      **     ** ** * *   *  ** **  * **

Zebrafish_srd5a2a      GGAACTTCCTTCATTCTTAGTGCCCGTGCTGTTGTTTTTTGCAACGGAAAGTTTGCCAGG 273
Atlantic_salmon        GGAACTGCCTTCTTTCCTGGTGCCCATGCTCCTTCTGCTCTCCACTGACACACAGCCCAG 227
Fathead_minnow         GGAGCTTCCCGCTCTCTTGGTGCCTCTGCTTTTGATGCTGATCACGGATGGAAAACAAAG 247
Zebrafish_srd5a2b      GGAGATTCCAGCTTTACTGGTGCCCTTGCTTTTGATGGTGACCACCGATGGAAACCACGG 270
                       ***  * **  *  *  * *****  ****  *  *  *    ** **       *   *

Zebrafish_srd5a2a      CGTTGGAAAACACATACTGTTCTGGACATTTTGTTTGCACTACTTTCAAAGGACATTTGT 333
Atlantic_salmon        CCTGGGCAAAGACCTGCTTCTCTGGACCTTCTGTCTGCACTACTTCCAAAGAACATTCAT 287
Fathead_minnow         CACTGGGAAACACCTGATGCTCTGGACCTTCTGCTTGCATTACTTCCAAAGGAGCTGCAT 307
Zebrafish_srd5a2b      CACTGGGAAACACCTGATGCTCTGGACCTTCTGCCTGCACTACTTCCAAAGGAGCTGTAT 330
                       *   ** *** ** *  *  ******* ** **  **** ***** ***** *  *   *

Zebrafish_srd5a2a      GTATTCACTGCTGACTAAAGGCCGTCCATCTCCTCTCCATATTGTGGTCAGTGCGGTGGT 393
Atlantic_salmon        CTACTCCTTGCTGACCAAAGGCCGCCCTTCCCCTCTCTACATTGTCTTCTCTGCGGTAGT 347
Fathead_minnow         CTACTCTCTGCTGACCAAAGGGCGCCCGTATCCGTTCAACATCATGCTGTTAGGGATGGG 367
Zebrafish_srd5a2b      CTACTCTCTGCTGACCAAAGGACGACCGTATCCATTTAACATCATGTTAACAGGGGTGGT 390
                        ** **  ******* ***** ** ** *  **  *  * **  *  *    * * * *

Zebrafish_srd5a2a      CTTCTGCTCGATCAATGGCTTTCTGCAGGGTCACTACATGCTGCACTGCACACAATACAG 453
Atlantic_salmon        CTTCTGCTCTGTCAACGGTTTTCTCCAAGGCCACTACATGCTCCACTGTGCCACGTACGA 407
Fathead_minnow         GTTTTGCTCTATTAACGGCTTCCTGCAGGCACACTATTTGCTGCATTGTGCTACATATAA 427
Zebrafish_srd5a2b      GGTTTGCTCTATTAACGGCTTCCTGCAGGCACACTATCTGCTCCACTGTGCTACATTTCA 450
                         * *****  * ** ** ** ** ** *  *****  **** ** **  *    *

Zebrafish_srd5a2a      CAGCGACTGGCTCACAGACACACGCTTCATCACTGGTTTGTTGGTGTTTTTGTGTGGAAT 513
Atlantic_salmon        TGATGCGTGGCAGACCGACATTCGTCTGTACACCGGTTTGCTGATGTTTTTCCTGGGAAT 467
Fathead_minnow         CAACACATGGTTTTCCGATGCCTGTTTGCTAGCCGGCCTGATCATATTCTTCATAGGAAT 487
Zebrafish_srd5a2b      CAACACATGGTTTTCTGATGCCTGTTTACTGACTGGTCTGATCATATTTTTCCTAGGAAT 510
                              ***    * **     *  *     * **  ** *  * ** **    *****

Zebrafish_srd5a2a      GGCCATCAATATCCACAGTGACTATATTTTACGAAACCTGAGGAAACCCGGAGAGGTCAG 573
Atlantic_salmon        GGCCATCAACATTCACAGCGACCACATTCTTCTAAATTTAAGAAAACCCGGAGAAGTTGT 527
Fathead_minnow         GGGAGTCAATATTCACAGTGACCACATTCTTCGCAGCCTGAGAAAACCTGGAGAAATTAC 547
Zebrafish_srd5a2b      GGGAATCAATATCCACAGTGACCACATTCTTCGAAACCTGAGAAACCCTGGAGAAATATC 570
                       **   **** ** ***** *** * *** * *  *   * ** ** ** *****  *

Zebrafish_srd5a2a      CTATAAAATCCCAAGAGGAGGGCTGTTTGAGCTGGTGTCCGGCGCCAACTTCTTCGGGGA 633
Atlantic_salmon        TTACAAGATTCCCAAAGGGGGAATGTTTGAGTACGTCTCCGGAGCAAACTTCTTTGGAGA 587
Fathead_minnow         CTATAAAATCCCGAGAGGCGGCTTATTTGAGTACGTGTCTGGTGCAAATTTTTT-GGGGA 606
Zebrafish_srd5a2b      CTATAAAATCCCGAGAGGCGGCATGTTCGAGTACGTGTCTGGAGCAAATTTCTTCGGAGA 630
                        ** ** ** ** * *** **  * ** ***   ** ** ** ** ** ** ** ** **

Zebrafish_srd5a2a      GATTGTTGAATGGTGTGGATACGCCGTGGCCAGCTGGTCATTTCCTGCTTTCTCCTTCGC 693
Atlantic_salmon        GATTCTGGAGTGGTGCGGGTATGCCCTAGCAACGTGGTCCCTGCCGACGTTCTCCTTTGC 647
Fathead_minnow         GATAGTACAGTGGCTGGGCTACGCGGTTGCTACCTGGTCCTTACCCACATTTGCTTTTGC 666
Zebrafish_srd5a2b      GATAGTGGAGTGGCTGGGATACGCTGTTGCTACATGGTCTTTACCAACGTTTGCTTTTGC 690
                       ***  *  * ***   ** ** ** ** *  *****  * **  *  ** *  ** **

Zebrafish_srd5a2a      CCTGTTCACCATCTGCTCCATCGGGCCTCGAGCCTACCATCATCACA------------- 740
Atlantic_salmon        TCTTTTCACTATGTGCTCGATTGGACCGCGGGCCTTCCACCACCACA------------- 694
Fathead_minnow         TTTTTTCACAATTTGCTTCATAGGCCCAAGAGCCTATCACCATCACA------------- 713
Zebrafish_srd5a2b      TTTTTTCACAATCTGCTTCATAGGCCCCAGAGCCTATCATCATCACAAGTAAACCGAAAT 750
                         * ***** ** ****  ** ** **  * ****  ** ** ****
```

Figure 3.10. *ClustalW alignment of the fathead minnow srd5a2 sequence versus the one of zebrafish (2 isoforms), and Atlantic salmon. Sequences similarities are showed in Table 3.3. * indicates identical nucleotide in all four species of fish.*

Table 3.3. *Percentage similarity scores of srd5a1 and srd5a2 gene sequences between fathead minnow, human and other teleost fish.*

Species	***srd5a1***	***srd5a2***
Human	79	65
Rat	81	66
Frog	69	70
Zebrafish	81	67 (a)* 89 (b)*
Medaka	63	72
Stickleback	61	/

* *Two isoforms of srd5a2 have been characterized in zebrafish.*

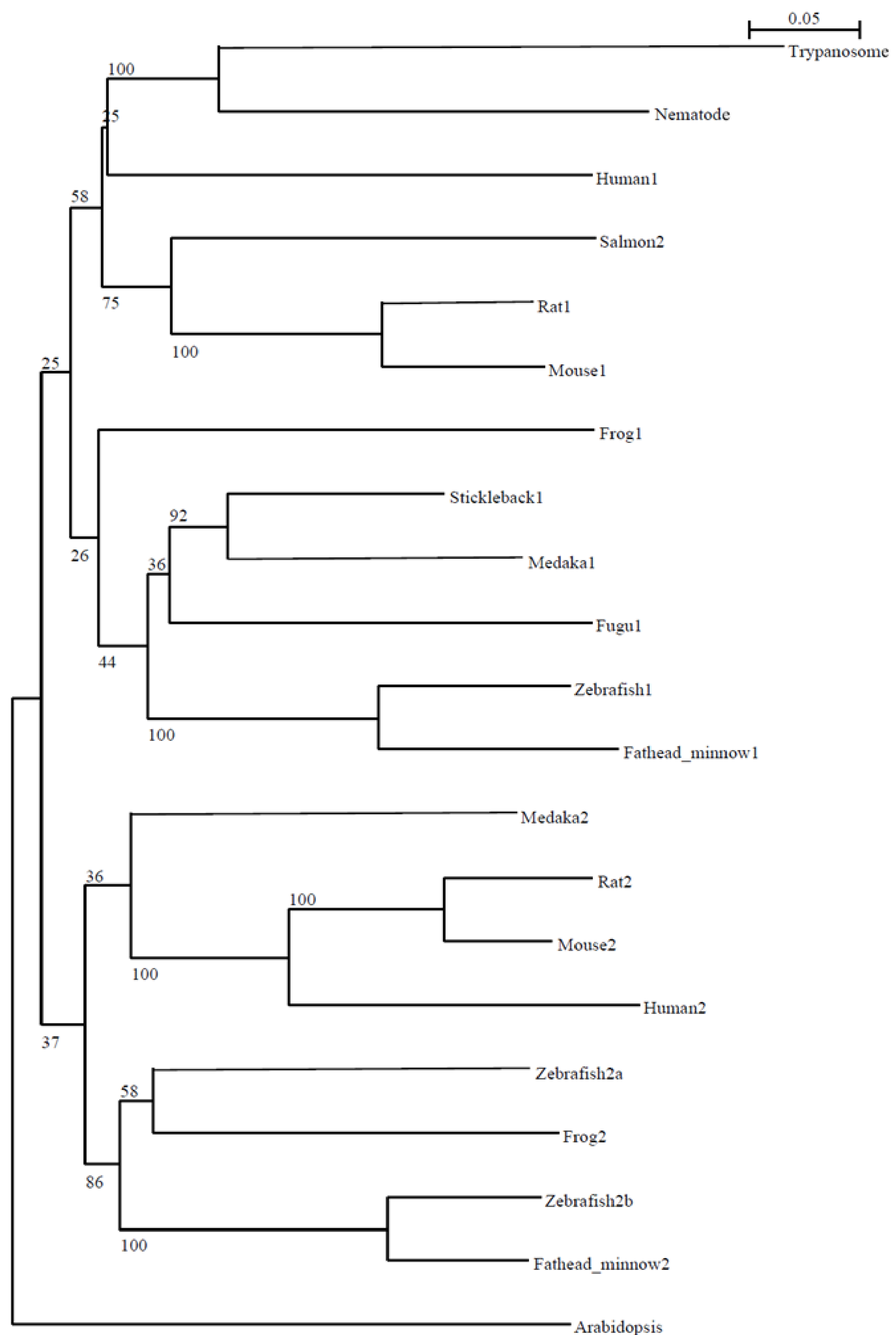


Figure 3.11. *Phylogenetic tree of the genes srd5a1 and srd5a2 coding, respectively, for the enzymes* 5αR Type 1 *and* 5αR Type 2. *The tree was generated using ClustalX (neighbour joining method). The out-group used in the analysis was Arabisposis. Percentage bootstrap values are provided for each node (n = 1000). The number at the end of the name of the species indicates the specific isoforms (1 or 2). The absence of a number indicates that only one isoforms was available for that species. The database accession ID for each sequence is shown in Table 3.2. 5αR genes were classified into two main categories, srd5a1 and srd5a2. Considering the non-vertebrate taxa, the gene sequences of trypanosome and nematode were evolutionary closer to vertebrate srd5a1 than the gene sequence present in the plant Arabidopsis.*

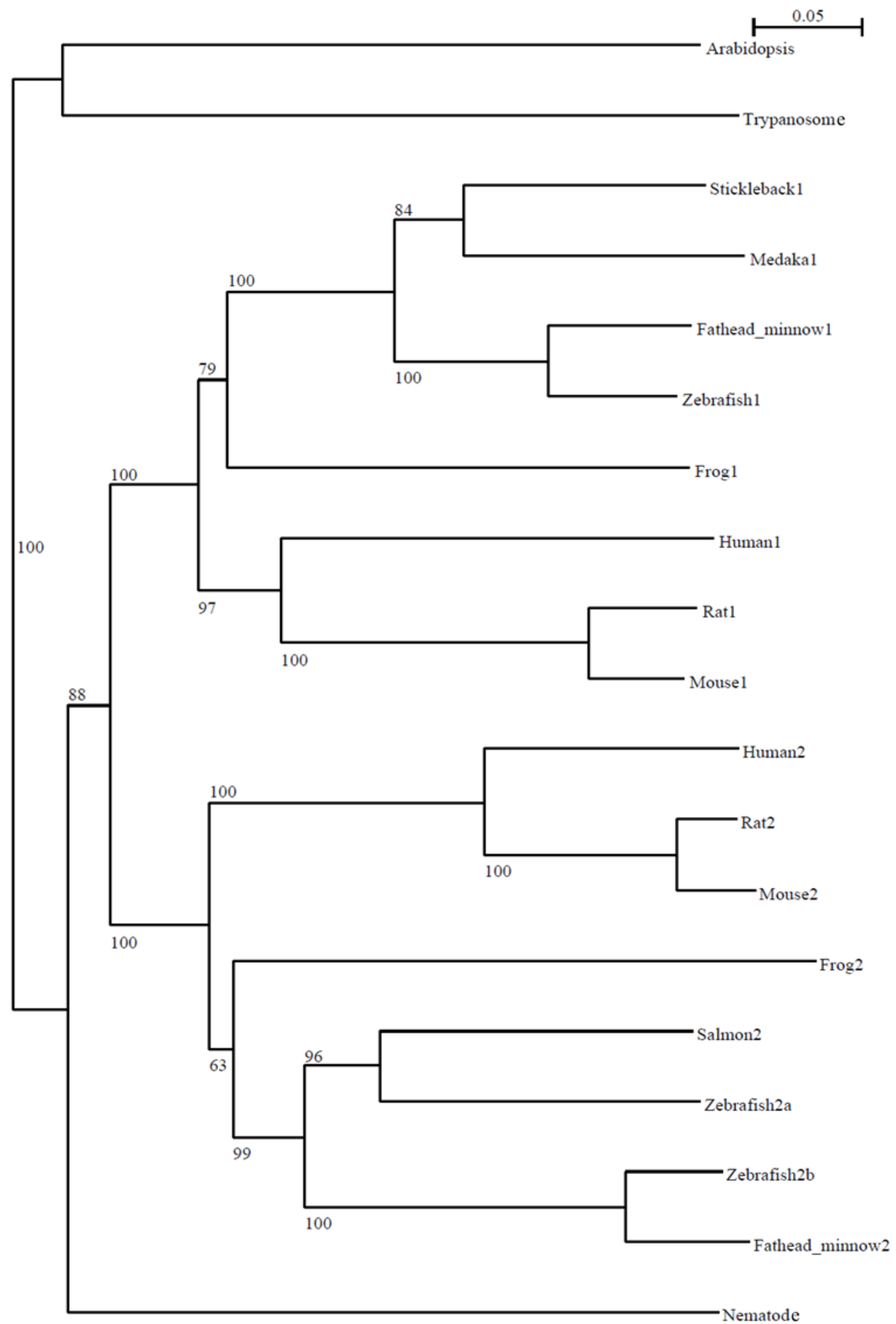


Figure 3.12. *Phylogenetic tree of the proteins* 5αR Type 1 *and* 5αR Type 2. *The protein sequences were obtained from NCBI and were estimated. The tree was generated using ClustalX (neighbour joining method). The out-group used in the analysis was Arabisposis. Percentage bootstrap values are provided for each node (n = 1000). The number at the end of the name of the species indicates the specific isoforms (1 or 2). The absence of a number indicates that only one isoforms was available for that species. The enzyme 5αR was classified into two main categories,* 5αR Type 1 *and* 5αR Type 2. *Considering the non-vertebrate taxa, the protein sequence of nematode was evolutionary closer to the vertebrate isoforms than the protein present in the trypanosome and in the plant Arabidopsis.*

3.4 Approach 2: Detection of DHT in fathead minnow plasma by ELISA and Ultrasensitive GC-MS/MS

In order to confirm the occurrence of a functional 5αR in fathead minnow, attention was focused on the detection of the product of its enzymatic activity - the androgen DHT - in fish plasma. A preliminary assay was performed testing the plasma of 3 mature males and 3 mature females with a DHT ELISA kit (Demeditec, Germany, Cat No. DE2330). The assay detected the presence of DHT at mean concentrations of 1.96 ± 0.09 ng/mL in male fish and 1.26 ± 0.12 ng/mL in female fish (data not shown). However, despite the fact that the cross-reactivity values provided by the Kit's manufacturing company (Demeditec) were very low (T: 8.7%; 5β-DHT: 2.0%; Androstenedione: 0.2%), some concerns remained about the specificity of the assay to detect DHT in fish plasma. In order to obtain an incontrovertible quantitative result, fish plasma was analyzed using analytical chemistry techniques instead of immunoassay. In particular, the aim was achieved by quantifying the concentrations of circulating DHT, T, and KT in adult and juvenile fathead minnows by Ultrasensitive GC-MS/MS, in collaboration with the Laboratory for the Study of Residues and Contaminants in Food (LABERCA) group, located in Nantes, France.

LABERCA's group developed a highly sensitive analytical method to quantify circulating androgens in pre-pubertal boys and girls, who normally have very low concentrations of circulating sex steroids (Courant *et al.*, 2010). The high sensitivity of the method was considered of great potential utility to clarify if DHT circulates in fathead minnow plasma, even at very low concentrations.

3.4.1 Materials and Methods

The steroids analysed by GC-MS/MS were specifically DHT, T and KT. For simplicity, in the following sections the same steroids will be defined respectively as DHT, T and KT.

3.4.1.1 *Plasma samples*

Blood samples were collected from the caudal peduncle of fish using heparinised capillary tubes and transferred into eppendorf tubes containing the enzyme inhibitor Aprotinin (Sigma). Blood samples were kept on ice until plasma was separated by centrifugation at 14000 × g for 5 min, removed and stored at −20 °C until steroid analyses. Two different experiments were performed. In the first preliminary experiment, several blood samples were collected from juvenile male fathead minnows (80-90 dph) and from sexually mature male and female fathead minnows (150-180 dph), obtained from a stock maintained at Brunel University,

London (UK). Fish at both ages were kept in stock tanks (150 L) containing a number of mixed sex individuals, between 150-200 for juveniles and 50-100 for adult fish. A resume of the morphometric data and male SSCs of the sampled fish is shown in table 3.4. Samples were pooled at LABERCA according to the sex, and the first results indicated that a minimum of 200 µL were required for the analysis; hence in the second experiment plasma samples were directly pooled at Brunel University, in order to obtain the required 200 µL.

In the second experiment, plasma samples were taken from adult fish (160-200 dph) kept at two different densities and breeding conditions:

1) Mixed sex fish were kept in stock tanks (150 L) containing between 50 and 100 fish (mixed sex);

2) Fish were transferred from highly populated stock tanks into single 30 L tanks containing 2 males and 4 females, and allowed to actively breed for 3 weeks before the blood sampling.

The aim of the second experiment was to assess if an active breeding behaviour was correlated with a potential increase of circulating DHT. A resume of morphometric data and male SSCs of fish used in the second experiment is shown in Table 3.4.

The final number of analyzed samples, obtained from pooling 3-5 different plasma samples from different individuals, was:

1 Juvenile Males

4 Adult males from stock tanks

3 Adult Breeding Males

3 Adult Breeding Females

In order to exclude the possibility that the food used to sustain fathead minnow stock at Brunel University was the source of DHT detected in fish plasma, two samples of food were processed and steroid extraction was performed in ethyl-acetate, as described by Beresford *et al*. (2011). Steroid extraction was performed on both adult brine shrimp (Tropical Marine Centre, Gamma irradiated) and flake food (King British Tropical flake food, Lillico, Surrey).

Additionally, DHT was quantified in two flake food samples spiked with 100 ng of DHT, and on one blank sample.

Table 3.4. *Morphometric data and male SSCs of fish used in Experiment 1 and Experiment 2.*

Experiment 1							
Group & Sex	**Length (mm)**	**Weight (g)**	**No. of Tubercles**	**Tubercles Grade**	**Fatpad Grade**	**Plasma volume per sample (µL)**	**No. of Samples**
Immature Males	54.1±0.3	2.7±0.6	/	/	/	16.5±2.7	4
Mature Males	64.7±4.2	6.3±0.9	17±1.7	4.5±0.5	3.2±0.8	55.2±17.2	6
Mature Females	55.3±0.3	2.7±0.6	/	/	/	42.2±14.3	6

Experiment 2							
Group & Sex	**Length (mm)**	**Weight (g)**	**No. of Tubercles**	**Tubercles Grade**	**Fatpad Grade**	**Plasma volume per sample (µL)**	**No. of Samples**
Adult Breeding Males	69.1±6.5	7.4±2.1	18±1.2	4.7±0.3	3.8±0.4	65.2±27.9	12
Adult Stock Males	64.8±3.0	6.1±1.3	17±1.9	4.2±0.4	3.1±0.6	53.3±13.7	12
Adult Breeding Females	55.8±5.1	3.2±0.7	/	/	/	36.6±18.1	16

All the following experimental steps were performed at LABERCA by its staff, according to the methods described by Courant *et al*. (2010).

3.4.1.2 *Reagents and chemicals*

All solvents and reagents were of analytical or grade quality and purchased from Solvent Documentation Synthesis (SDS, Peypin, France). Solid phase extraction cartridges (SPE) (ChromP, SiOH) were also provided by SDS. Purified *Helix pomatia* enzymatic preparation was used for steroid deconjugation (Sigma Chemical Co., St. Louis, MO). Derivatization reagents included *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide, *N*,*O*-bis (trimethylsilyl)-trifluoroacetamide, and pentafluorobenzylbromide were purchased from Fluka (Buchs, Switzerland). Dithiothreitol and trimethyliodosilane were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Standard reference steroids were purchased from Sigma (St.

Louis, MO). The deuterated internal standard 17α- methyltestosterone-d_3 was provided by Steraloids (Wilton, NY).

3.4.1.3 ***Sample preparation***

The sample preparation procedure (extraction and purification) used for the isolation of target steroids has previously been described by Courant *et al*. (2007). Quantification was performed by isotopic dilution. Plasma samples (200 μL) were spiked with 17α-methyltestosterone-d_3 (200 pg in Experiment 1; 500 pg in Experiment 2), which was used as the internal standard. An enzymatic hydrolysis (with *H. pomatia* enzymes) was performed before the extraction of non-polar compounds with diethylether. The quantification of plasma steroids is commonly conducted by the analysis of the un-conjugated steroids, since conjugated metabolites (glucuronidated and sulfated) are generally considered biologically inactive. In this study, an enzymatic hydrolysis was performed before measurement of steroid levels to quantify total steroid concentrations (unconjugated plus conjugated).

Measuring the total steroid levels increases the chance of obtaining measurable values (higher than LOQ) in samples with extremely low concentrations. Furthermore, although circulating conjugated metabolites may not be active, their presence is nevertheless evidence of sex steroid production. The role of unconjugated steroids is not fully understood, in fact it has been shown that local deconjugation and conversion into more active compounds may occur in certain hormone-responsive tissues (Thijssen, 2004).

A first purification step was performed on a ChromP SPE cartridge. Then, androgens were separated by a liquid/liquid partitioning with *n*-pentane. Extracts were finally purified on a silica SPE cartridge.

3.4.1.4 ***Derivatization reaction***

Derivatization of the androgen fraction was carried out with a *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide/trimethyliodosilane/dithiothreitol mixture (1000:5:5; vol/vol/wt) for 40 min at 60ºC.

3.4.1.5 ***GC-MS/MS measurement***

GC-MS/MS involves two steps: a separation phase (gas chromatography or GC) and an analytical phase (tandem mass spectrometry or MS/MS). The gas chromatographer separates the different chemicals in a sample based on their physical and/or chemical properties (e.g. volatility). The sample containing a mixture of compounds, including the target ones, is injected in the machine, vaporized and carried by a mobile phase (e.g. helium) through a capillary column, having on its internal surface a stationary phase, which, in our case, was a

special arylene polymer (Arylene Matrix Technology™). GC is based on partition equilibrium of analyte between the solid stationary phase and the mobile gas. Chemicals with high volatility travel through the column more quickly than chemicals with low volatility. Once the sample has travelled through the GC, the separated chemicals reach the mass spectrometer. A mass spectrometer is a machine which ionizes and fragments different molecules by blasting them with electrons. As the ions continue into the mass spectrometer, they travel through a magnetic field toward a detector, which detects the charge or current created by each ionized particle, quantifying them as mass-to charge ratios (*m/z*). Each fragment has a specific *m/z*.

A tandem mass spectrometer (MS/MS) is one capable of multiple rounds of mass spectrometry, usually separated by some form of molecule fragmentation. In particular, the triple quadrupole mass spectrometer, as the one used in this study, is a tandem mass spectrometer consisting of two quadrupole mass spectrometers in series, with a (non mass-resolving) radio frequency (RF) only quadrupole between them to act as a collision cell for collision-induced dissociation. The first (Q1) and third (Q3) quadrupoles serve as mass filters, whereas the middle (q2) quadrupole serves as a collision cell. This collision cell uses an inert gas such as Ar, He, or N2 gas to provide collision-induced dissociation of a selected precursor ion that is selected in Q1. Subsequent fragments are passed through to Q3 where they may be filtered or scanned. This configuration is often abbreviated QqQ, here Q1q2Q3.

The MS also quantifies the amount of each chemical detected in a sample as a peak. Based on the peak area, the mass spectrometer determines the concentration of each chemical in the sample by comparing it to the peak areas of the standards run at the beginning and end of each GC-MS/MS run.

In this study, measurements were carried out by GC-EI-MS/MS with positive electrical ionization. Electron energy was set at 70 eV. An Agilent 7890A gas chromatograph coupled to a 7000 GC triple-quadrupole device (Agilent, San José, USA) was used for analyte detection and quantification. Injector and transfer line temperatures were set at 250°C and 280°C, respectively. Source and analyzer temperatures were set to 220°C and 150 °C, respectively. GC column was a 15 m x 0.25 mm id., film thickness 0.1 µm (Restek). Temperature programme was set as follows: 120°C (1 min), 10°C min 1 until 220°C (1 min), 2°C min 1 until 225°C (0 min) and 15°C min 1 until 320°C (2 min). The mass spectrometer was operated in the selected reaction monitoring (SRM) acquisition mode (Table 3.5). Argon was used as the collision gas.

Table 3.5. GC-MS/MS acquisition parameters used for the measurement of androgens and estimated LOD and LOQ.

Steroid	**Transition**	**Collision energy (eV)**	**LOD (pmol/L)**	**LOQ (pmol/L)**	**RT (min)**
T	432.4 > 417.4	5	0.03	0.07	27.73
	432.3 > 209.2	12			
DHT	434.4 > 405.4	8	0.05	0.10	27.08
	434.4 > 195.1	12			
17α-Methyltestosterone-d_3 (IS)	449.3 > 301.2	15			29.48

For conversion purposes, 1 pmol/L = 0.288 ng/L for 17β-testosterone; 1 pmol/L = 0.290 ng/L for DHT. IS, internal standard; RT, retention time.

3.4.1.6 *Performance of the method*

The limit of detection (LOD) is defined as the lowest concentration or amount of an analyte that can be reliably identified as being quantitatively present in the sample. The limit of quantification (LOQ) is defined as the lowest concentration or amount of analyte that can be reproducibly quantified in a sample. In this experiment, LOD and LOQ for each analyte were determined as the concentration inducing a signal to noise ratio of 3 and 6, respectively (Table 3.5). The repeatability was calculated on the basis of methyltestosterone-d_3 internal standard signals for 20 replicates.

3.4.2 Results

3.4.2.1 *Experiment 1*

Identification was performed according to European analytical criteria (2002/657/CE). Standard extracted calibration curves were established by adding fixed amount of 17α-methyltestosterone-d_3 (200 pg) to 200 µL water and increasing quantities of the target analytes from 40 to 1000 pg (i.e. 0.2 to 5 ng/mL). The response linearity (R^2 values) was higher than 0.99 for both T and DHT.

T was identified and quantified in all the 3 pooled samples, while DHT was identified and quantified in pooled samples from males (Fig. 3.13) and females. Conversely, this compound was not detected in pooled sample from juveniles, but this result may be due to the very restricted sample size (i.e. 70 µL). A summary of these quantitative results is presented in Table 3.6.

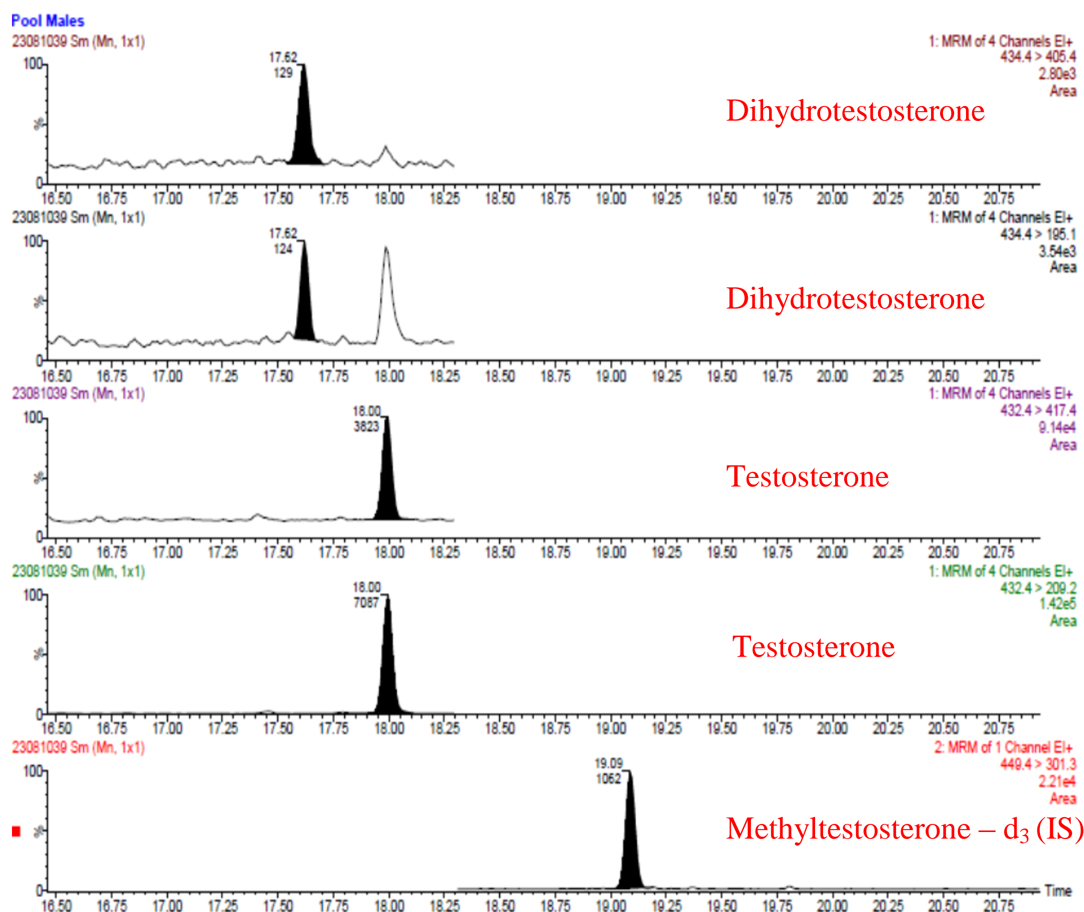


Figure 3.13. *Example of diagnostic ion chromatograms of DHT (434.4 > 405.4 and 434.4 > 195.1), T (432.4 > 417.4 and 432.4 > 209.2), and methyltestosterone –d$_3$ detected in the plasma of male fathead minnows sampled from fish stock tanks.*

3.4.2.2 *Experiment 2*

The objective of this second study was to measure T, DHT, and KT in fish plasma and to confirm the absence of DHT in the food used to feed the fathead minnow stocks at Brunel University. Standard extracted calibration curves were established by adding fixed amount of 17α-methyltestosterone-d$_3$ (500 pg) to 200 µL water and increasing quantities of the target analytes from 40 to 1000 pg (i.e. 0.2 to 5 ng/mL). The response linearity (R^2 values) was higher than 0.99 for DHT and 17β-T. However, the R^2 value was much lower for KT. Indeed, this compound showed significant different chemical properties than DHT and T and required a different labelled internal standard. Therefore, only semi-quantitative observations were made on KT.

DHT and T were identified and quantified in all samples (Fig. 3.14; Table 3.6). DHT concentrations were approximately 8% of the circulating T concentrations in both males and females. The average DHT concentration in males was 0.59 ± 0.17 ng/mL versus 7.53 ± 2.80

ng/mL of T. In juvenile males, the concentration of T (3 ng/mL) was lower than the concentration measured in adult fish. DHT was below the LOQ for this sample, probably due to the small volume of plasma available (70 μL). On the other hand, in female fathead minnow, both T and DHT were measured at concentrations of 5.18 ± 3.31 ng/mL and 0.42 ± 0.17 ng/mL, respectively. A summary of the quantitative results is presented in Table 3.6.

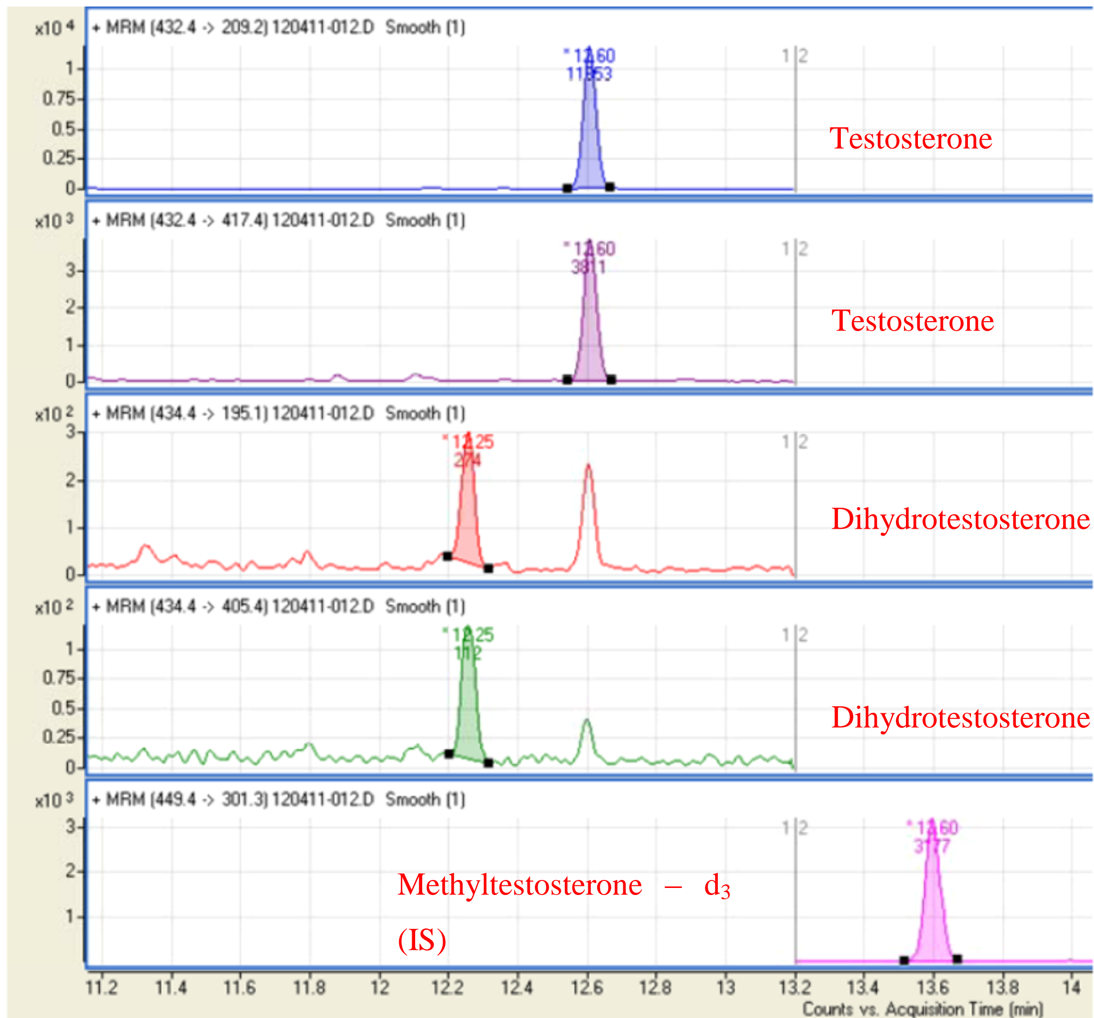


Figure 3.14. *Example of diagnostic ion chromatograms of T (432.4 > 417.4 and 432.4 > 209.2) and DHT (434.4 > 405.4 and 434.4 > 195.1) detected in the plasma of breeding male fathead minnows.*

Table 3.6. *Quantification of the concentrations of DHT and T in fathead minnow plasma samples.*

Experimental group	Sample ID & Experiment No.	Testosterone (ng/mL)	Dihydrotestosterone (ng/mL)
Mature males	M1 - Exp. 1	7.79	0.78
	M2 - Exp. 2	6.16	0.28
	M3 - Exp. 2	4.63	0.59
	M4 - Exp. 2	5.28	0.65
Average ± SD		**5.97±1.37**	**0.58±0.21**
Mature breeding males	M5 - Exp. 2	13.29	0.57
	M6 - Exp. 2	6.31	0.46
	M7 - Exp. 2	7.04	0.57
	M8 - Exp. 2	9.71	0.83
Average ± SD		**9.09±3.16**	**0.61±0.16**
All mature males Average ± SD		**7.53±2.80**	**0.59±0.17**
Juvenile males	M9 – Exp. 1	3.00	< LOD
Mature females	F10 – Exp. 1	2.77	0.40
Mature breeding females	F11 – Exp. 2	8.96	0.48
	F12 – Exp. 2	22.69	0.61
	F13 – Exp. 2	3.81	0.20
Average ± SD		**6.39±3.64**	**0.43±0.21**
All mature females Average ± SD		**5.18±3.31**	**0.42±0.17**

KT was identified in all samples from male fathead minnows (between 2 and 60 ng/mL). It was not detected in any female samples (Fig. 3.15).

Two food samples, one spiked food sample, and one blank sample were analysed using the same protocol. DHT was identified at high levels only in the food spiked with 100 ng of DHT, but was not identified in any of the other samples (Fig. 3.16).

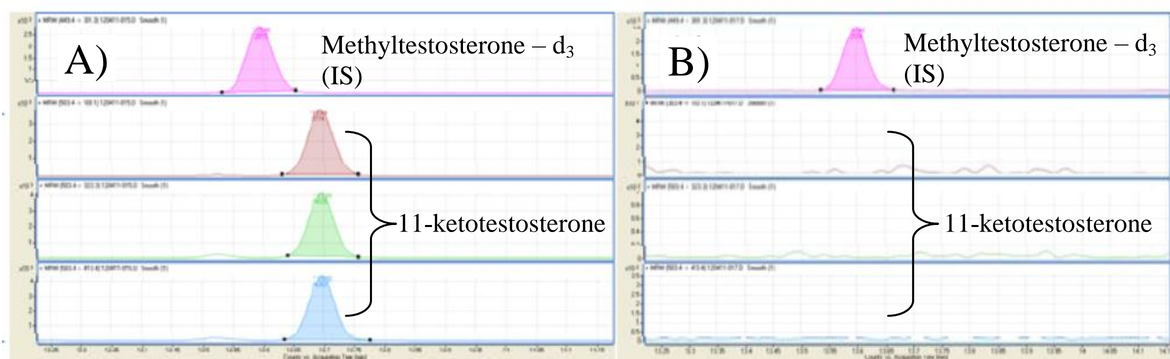


Figure 3.15. *Example of diagnostic ion chromatograms of KT (503.4 > 169.1, 503.4 > 323.3 and 503.4 > 413.4) detected in (A) the plasma of a male fathead minnow (sample M7) and (B) the plasma of female fathead minnow (sample F1).*

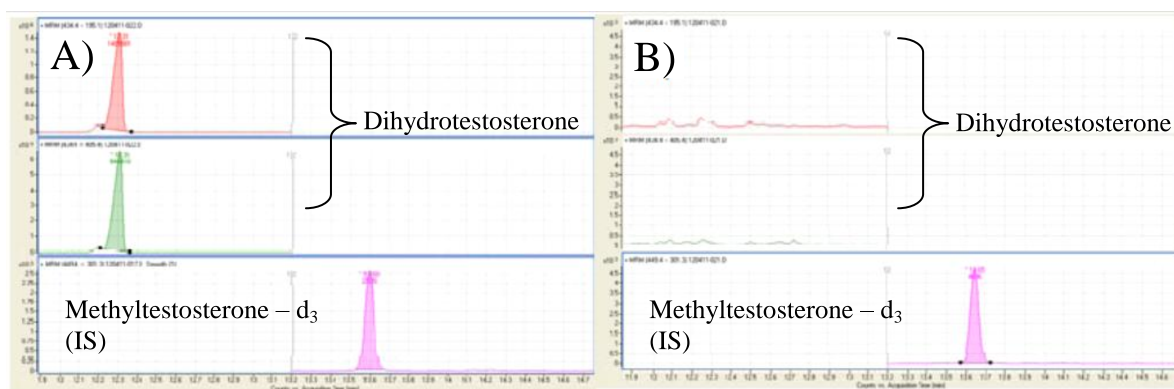


Figure 3.16. *Example of diagnostic ion chromatograms of DHT (434.4 > 405.4 and 434.4 > 195.1) detected in (A) a flake food sample spiked with DHT (100 ng), and (B) a unspiked flake food sample.*

3.5 Discussion

The demonstration that the genes coding for 5αR Type 1 and Type 2 were expressed in mature testis of fathead minnow provided strong evidence of the presence of a functionally active 5αR in this species. This hypothesis was supported by the strong correlation found between 5αR enzymatic activity and its mRNA level, for both Type 1 and Type 2, in human prostate tissue (Shirakawa *et al*., 2004; Soderstrom *et al*., 2001).

From an evolutionary persepective, an important finding was that 5αR is conserved in a broad range of phyla, including bacteria, plants, fungi, and humans. Although plants and animals diverged more than 1 billion years ago, it is remarkable that poly-hydroxylated steroidal molecules are used as hormones in both of these kingdoms. Brassinosteroids are a class of plant-specific steroid hormones, and control many developmental and physiological processes, including regulation of gene expression, cell division, differentiation, programmed

cell death, photomorphogenesis, and homeostasis (Basse *et al*, 2002; Clouse, 2011; Thummel and Cory, 2002).

Recombinant Arabidopsis steroid 5αR, encoded by the *det2* gene, can be expressed in human embryonic kidney 293 cells, where it is able to reduce several mammalian steroids with a 3-oxo structure, including T, androstenedione, and progesterone (Li *et al*. 1997). Arabidopsis 5αR and the mammalian enzyme show similar affinities for animal steroids, with similar K_m values (Li *et al*. 1997). Therefore, both the structural and functional conservation between Det2 and mammalian steroid 5αR suggest that they evolved from a common ancestor. Chory *et al*. (1991) isolated Arabidopsis with a *det2-1* mutation, which caused defective brassinolide biosynthesis. The mutant plants were characterized by dwarfism, increased number of chloroplasts in a reduced cell volume (causing the appearance of dark green leaves), reduced male fertility, and delayed senescence and flowering (Chory *et al*., 1991; Li *et al*., 1996; Altmann, 1998; Clouse and Sasse, 1998).

Fathead minnow *srd5a1* showed a high degree of similarity to the homologous gene in humans, rat and zebrafish; on the other hand, *srd5a2* showed a high degree of similarity only when compared to the zebrafish enzyme (89%), while this value was lower when considering the human and rat *srd5a2* (respectively 65% and 66%). When considering only teleost fish, the zebrafish gene was evolutionary closest to the fathead minnow gene, whereas, for example, the medaka and stickleback gene showed only a medium degree of similarity (~60%).

The second approach used to determine if 5αR was functional in fathead minnow focused on the detection of plasma circulating DHT. This approach was considered to be more suitable than the analysis of 5αR enzymatic activity, since it could provide definitive evidence of the presence of DHT in fish plasma.

T and DHT are crucial to the development of male reproductive organs and male characteristics in adults, as described in Chapter 1. From a clinical point of view, the measurement of serum T is an important endpoint in the diagnosis and treatment of male hypogonadism and pubertal delay, while measurements of serum DHT and the T/DHT ratio are used to monitor androgen replacement therapies, 5αRs deficiency, 5αR inhibitor treatment of BPH, and for the prevention of prostate cancer (Shiraishi *et al*., 2008; Mohler *et al*., 2004; Wang *et al*., 2000).

Historically, several radioimmunoassays (RIAs) have been developed to measure concentrations of circulating sex steroids in fish (Scott *et al*., 1984). The same technique,

together with other immunoassay methods (e.g. ELISA), has also been used in the clinical field to measure steroids concentrations in human plasma. However, the majority of these assays were designed to measures relatively high levels of target steroids; this, together with the potential cross-reactivity of the antibodies employed with non-target steroids, may make these assays unsuitable to detect very low concentrations of hormones in biological matrixes, like plasma.

From a clinical point of view, the development of accurate and sensitive steroid assays is important to define exact reference ranges in women and men for the diagnosis of endocrine dysfunctions, like hormone deficiency or excess states (e.g. hyperandrogenism), or to monitor the effectiveness of hormonal therapy, like hormone replacement therapies. Several studies have compared the concentrations of steroids measured by RIAs or other immunoassay, with the ones obtained using analytical techniques, like LC or GC coupled with mass-spectrometry (Hsing *et al*., 2007; Santen *et al*., 2008; Wang *et al*., 2008; Rothman *et al*., 2011). The general conclusion arising from these studies was that immunoassays tend to over-estimate steroid concentrations in biological matrixes, such as plasma. For example, Santen *et al*. (2008) compared E2 levels measured by RIA and LC-MS/MS or GC-MS/MS, showing that when samples contain very low concentrations of E2 (close to the LOD of RIA), estrogen metabolites may interfere with RIA, elevating the measured E2 concentration, which in turn could lead to wrong clinical interpretations (e.g. in cases of breast cancer). Also Rothman *et al*. (2011) concluded that serum levels of sex hormones - including T, DHT and E2 - were lower when measured with LC-MS/MS compared with RIA, and LC–MS/MS provided more accurate and precise measurements, in particular when the measured concentration where close to the RIA's LOD. This technical issue assumed an important relevance in clinical endocrinology, hampering the understanding of the physiological role of the low concentrations of DHT circulating in women (typically below 0.2 ng/mL) (Rothman *et al*., 2011).

According to the preliminary analysis performed on fathead minnow plasma using a commercial DHT ELISA Kit, the concentrations of circulating DHT in this species were expected to be very low, in the low ng/L range or even below 1 ng/L. Therefore highly sensitive technique was required in order to obtain a definitive result. In this study, our objective was to demonstrate that DHT circulates in fathead minnow, even if at low concentrations; therefore, the Ultrasensitive GC-MS/MS available at LABERCA represented an optimal methodology, eliminating the concerns of non-specificity arising from the use of ELISA. Indeed, the methodology used in this study was developed by Courant *et al*. (2010) to

detect the low concentrations of androgens and estrogens circulating in pre-pubertal boys and girls, and it could detect T and DHT with LODs of 0.288 pg/mL and 0.290 pg/mL, respectively.

The results demonstrated that DHT is present in fish plasma at a concentration comparable to the human one (Table 3.7), and in the case of male fish, DHT concentrations are in the same range as in male humans, providing a good comparative background for the study of the effects of dutasteride in fathead minnow. On the other hand, DHT concentrations in female fish were approximately between 2 and 15 times higher than the ones measured in women, leading to the hypothesis that DHT has an important physiological role also in female fathead minnow, and that the inhibition of its synthesis would cause adverse effects. The higher concentration of DHT in female fathead minnows when compared to humans is probably due to the high concentration of T circulating in female fathead minnow (median value 3.25 ng/mL, according to Watanabe *et al*., 2007), very different from pre-menopausal human females, where T plasma concentrations are in the range of 0.1-0.6 ng/mL (Table 3.7). A further significant result was that circulating DHT concentrations in both sexes of fathead minnow were approximately 7-8% of circulating T concentrations, and this value is very close to the typical 10% observed in humans (Becker, 2001).

In men, about 25% of circulating DHT is synthesised by the testicular tissue, and the remainder by other tissues, such as liver, kidney, muscle, prostate, and skin. However, circulating concentrations of sex steroids are not the only parameter to take into consideration, since the local concentration of the same hormones in tissues where there is an active biosynthesis can be higher than the circulating values. Indeed, in human prostate, the DHT concentration is 5 to 10-fold higher than in the peripheral blood (Becker, 2001; McConnell *et al.*, 1992).

The downside of the technique employed in this study was that the minimum volume of plasma required for the analysis was 200 μL, versus the few μL required for RIAs. The volume of plasma that it is possible to obtain from an individual fathead minnow is about 20-100 μL for adult males, and 15-60 μL for adult females. In order to obtain the 200 μL of plasma required, plasma samples from different individual fish (3-5 individuals) were pooled. This approach probably hid the variability of DHT concentrations among different individuals, making this technique, as it was used in this study, unsuitable for routine analysis of plasma steroid concentrations in small laboratory fish (e.g. fathead minnow, zebrafish, medaka) used in (eco)toxicological studies; however, this experiment represented the first attempt, to our knowledge, to quantify circulating plasma DHT concentrations in fathead

minnow, and in teleost fish in general, by analytical chemistry, so it should be seen as a first step to confirm the presence of circulating DHT in fish, whose biosynthesis is inhibited by the drug studied in this Thesis.

Table 3.7. *Concentrations of T and DHT in plasma of humans and fathead minnows.*

Species	Testosterone (ng/mL)		Dihydrotestosterone (ng/mL)		Reference
	♂	♀	♂	♀	
Human	3.82±1.0		0.33±0.90		Hsing *et al.*, 2007
	2.60-9.70	0.09-0.58 Pre-menopausal	0.14-0.77	0.03-0.26 Pre-menopausal	Shiraishi, 2008
		0.15-0.23 Pre-menopausal		0.08-0.09 Pre-menopausal	Rothman *et al.*, 2011
		0.10-0.11 Post-menopausal		0.026-0.036 Post-menopausal	Rothman *et al.*, 2011
	4.57±0.05				Labrie *et al.*, 2009
Fathead minnow	7.53±2.80	5.18±3.31	0.59±0.17	0.42±0.17	

When plasma steroids are quantified by GC- or LC-MS/MS, a common strategy is to analyze only the un-conjugated steroids, since conjugated metabolites (glucuronidated and sulfated) are considered biologically inactive. In this study, an enzymatic hydrolysis was performed before measurement of steroid levels, in order to quantify total steroid concentrations (un-conjugated plus conjugated), with the aim of increasing the chance to detect and quantify with high confidence (concentrations higher than LOQ) even very low concentrations of DHT (Courant et al., 2007, 2010). The de-conjugation step performed in this work was justified not only by methodological reasons, but also by biological ones. In fact, despite conjugated steroids being considered inactive, their role is not fully understood, and it has been shown that in certain tissues these metabolites can be de-conjugated and converted into active steroids (Thijssen, 2004), suggesting that the pool of steroids in an organism should be considered as a dynamic system, rather than as a static one (conjugated or un-conjugated) (Courant et al., 2010). Several authors have suggested that the conjugation of steroids (e.g. with glucuronic acid) may play a critical role in the regulation of the intracellular ratio between un-conjugated and conjugated steroids, determining their biological activity in tissues (Belanger et al., 2003; Labrie et al., 1997; Rittmaster, 1993), and that the quantification of both specific androgens and their metabolites appears to be the most suitable approach to accurately estimate the total androgen pool in an individual (Vandenput et al., 2007). Finally, independently from their potential activity, the presence of conjugated steroids represents evidence of active sex steroid production (i.e. DHT) (Courant et al., 2010).

In conclusion, a number of pieces of evidence collected during the preliminary phase of the project – both experimentally and from the literature - strongly suggest that DHT may have a physiological role in the fathead minnow. The evidence includes the expression of 5αR genes in tissues such as the testis and the occurrence of circulating DHT at concentrations similar to the ones detected in humans (as previously described in this Chapter), the high affinity of DHT for the fathead minnow AR (Wilson *et al.*, 2007), the high potency of DHT to induce the transcription of teleost fish AR (Ikeuchi *et al.*, 1999; Katsu *et al.*, 2007; Olsson *et al.*, 2005; Takeo and Yamashita, 2000; Todo *et al.*, 1999), and the paracrine action of DHT in specific tissues (Griffiths *et al.*, 1997). This conclusion constitutes the rationale for testing the effects of dutasteride, a dual 5αR inhibitor, in the fathead minnow.

CHAPTER 4 : COMPARATIVE STUDY OF THE *IN VIVO* ANDROGENIC POTENCY OF 11-KETOTESTOSTERONE AND 5α-DIHYDROTESTOSTERONE IN THE FATHEAD MINNOW

4.1 Introduction

Androgens regulate a wide range of physiological processes in male vertebrates, mediating their action through the androgen receptor (AR) (Brinkmann *et al.*, 1999; Heinlein and Chang, 2002; Li and Al-Azzawi, 2009). DHT is one of the most physiologically important androgens in many male vertebrates (George and Wilson, 1994), with the exception of teleost fish, in which T and KT are generally considered the major circulating male androgens, as well as the most potent ones (Borg, 1994). KT, in particular, shows a low affinity to fish sex hormone binding globulins (SHBGs) (Miguel-Queralt *et al.*, 2004, 2005), and higher levels of free circulating hormone in the blood when compared to other androgens (Borg, 1994). KT is involved in fish gonad differentiation (Piferrer *et al.*, 1993), regulation of development of secondary sex characteristics (SSCs) (Borg *et al.*, 1993; Borg, 1994), and modulation of behaviour (Brantley *et al.*, 1993a, 1993b; Kobayashi and Nakanishi, 1999), and was the only sex steroid able to induce all stages of spermatogenesis in species such as the Japanese eel (*Anguilla japonica*) and the African catfish (*Clarias gariepinus*) (Borg, 1994; Cavaco *et al.*, 1998, 2001; Miura *et al.*, 1991).

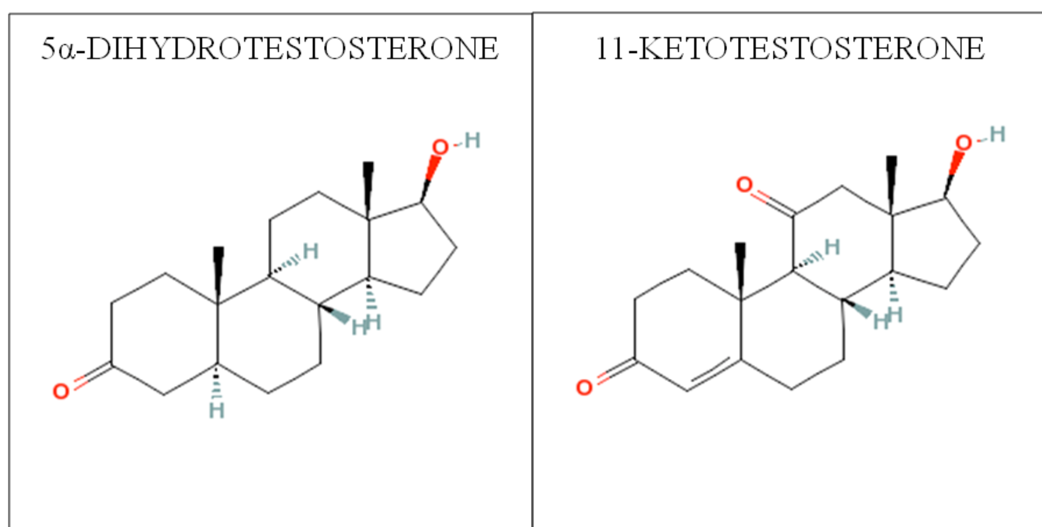


Figure 4.1. *Chemical structures of 5α-dihydrotestosterone and 11-ketotestosterone.*

ARs have been isolated and characterized from a number of teleost species; however, none of these receptors are specific for KT (Ikeuchi *et al.*, 1999; Olsson *et al.*, 2005; Sperry and Thomas, 1999; Takeo and Yamashita, 2000; Todo *et al.*, 1999; Touhata *et al.*, 1999; Wells and Van der Kraak, 2000). Therefore, the molecular mechanisms explaining the high androgenic potency of KT in teleost fish are not understood. Furthermore, the presence, in some species, of multiple AR isoforms with different binding specificities and tissue

distributions suggest that androgens different than KT may be involved in the mediation of the androgenic response in different target tissues (Sperry and Thomas, 1999, 2000).

The roles played by DHT in teleost fish have scarcely been investigated, and the general assumption seems to be that DHT does not have any major physiological role. For example, DHT didn't induce any spermatogenic activity in the eel (Borg, 1994), and inhibited testicular growth and spermatogenesis in the African catfish, without stimulating male SSCs (Cavaco *et al.*, 1998). However, all the components involved in DHT biosynthesis and action are present and evolutionary conserved in teleost fish. In particular, the genes coding for the enzymes that convert T into DHT, namely 5-alpha reductase 1 and 5-alpha reductase 2 (5αR1, 5αR2), have been sequenced in zebrafish and medaka (GeneID: 767715, 550398, 550388, 100125491, 100125532), and are expressed in several tissues in the fathead minnow (see Chapter 3); 5αR activity has been detected in the testes of the urohaze-goby (Asahina *et al.*, 1985), the brain of male and female goldfish and toadfish (Pasmanik and Callard, 1988, 1985), and the skin of rainbow trout (Latz and Reinboth, 1993), and 5α-reduced androgen metabolites were found in gonads and peripheral tissues of both male and female teleosts (Kime, 1993). After injection of labelled T in flounder and Atlantic salmon , metabolites in the bile were primarily forms of DHT (Truscott, 1983), which was also a major metabolite in a similar experiment performed with fathead minnow (Parks and LeBlanc, 1998), indicating that steroid 5αR activity appears to be involved in androgen metabolism in these species. The results of some *in vitro* experiments suggest that teleost fish can synthesise DHT (Joss *et al.*, 1996; Lee *et al.*, 1995), even if the relevance of these *in vitro* results to the *in vivo* situation is unclear. Lastly, an androgen receptor with high binding affinity to DHT is present in many teleost species, and DHT is often one of the most potent inducers of AR transcriptional activity *in vitro* (Fitzpatrick *et al.*, 1994; Olsson *et al.*, 2005; Sperry and Thomas, 1999a; Todo *et al.*, 1999).

4.2 Aim of the study

The fathead minnow is one of the most frequently used fish models in aquatic toxicology; its basic biology and endocrine system are well characterized, and the presence of discrete SSCs make this species very useful in the study of mechanisms of action and effects of endocrine disrupting chemicals (Ankley and Villeneuve, 2006). Since all the components required for DHT synthesis are present in this species, and the fathead minnow AR has a high binding affinity to DHT, we investigated the effects of KT and DHT exposure *in vivo* on fathead minnow juveniles, with the aim of clarifying the comparative effects of the two androgens on critical physiological processes, such as gonad maturation, growth, and SSCs development.

4.3 Materials and methods

4.3.1 Test species

Juvenile fathead minnows were obtained from a stock maintained at Brunel University, London (UK). Fish age at the beginning of the experiment was between 60 and 65 days post-hatch (dph), and wet weight was approximately 140 ± 50 mg (Fig. 4.2). The aim was to expose fish to KT and DHT during the sexual development and gonad maturation phases, which are very susceptible to endocrine interference (van Aerle *et al.*, 2002). The choice of age, weight and exposure duration were based on results obtained from a study of the sexual development of the fathead minnow stock maintained at Brunel University (van Aerle *et al.*, 2004). A lab-specific choice was required, since fathead minnows maintained at different laboratories can have different rates of sexual maturation and growth. The period of exposure from 60 to 105 dph was driven by the hypothesis that both androgens would stimulate fish spermatogenesis, and terminating the experiment with control fish at an early stage of gonad maturation (i.e. testis with only spermatogonia) would allow us to more easily distinguish any stimulating effects caused by androgens from the ones due to normal sexual maturation (i.e. presence of more advanced stages of germ cells). Fish was acclimated to experimental conditions for 7 days prior to the start of the exposures.

Figure 4.2. *Fathead minnow juveniles (60 dph) at the beginning of the experiment.*

4.3.2 Test substances and dilution water

KT (CAS number 564-35-2) was purchased by Sigma-Aldrich, Gillingham (UK), and DHT (CAS number 521-18-6) from Steraloids Inc., Newport (USA). Concentrated stock solutions were prepared in HPLC grade N,N-Dimethilformamide (DMF) 99+% (Fisher Scientific, UK; CAS number 68-12-2).

The dilution water was monitored daily for general parameters (e.g. pH, temperature, dissolved oxygen). During the exposure studies, mean water pH ranged from 7.5 to 8.2, temperature from 23.9 to 25.1ºC, and dissolved oxygen from 6.4 to 8.9 mg/L.

4.3.3 Experimental design and protocol

The experiment was carried out using a continuous flow-through system. Thermostatically heated (25 ± 1°C) dechlorinated carbon filtered (5 and 10 µm) tap water, from a header tank, flowed through 12 flow-meters into 12 mixing chambers via medical grade silicon tubing (VWR, UK) at a rate of 300 mL/min. Test chemical stock solutions dissolved in DMF (300 µg/L and 3000 µg/L for both KT and DHT) were pumped by a Watson Marlow (Cornwall, UK) Multi channel peristaltic pump into the respective mixing chambers at a rate of 0.02 mL/min, in order to achieve nominal concentrations in the fish tanks of 20 and 200 ng/L for both androgens. The solvent control (SC) tank received DMF at the same rate, such that the water in all tanks contained DMF at 0.0067% (OECD suggested limit: 0.0095%). From each mixing chamber, the water was distributed into the fish tank through silicone rubber tubing. In total there were 12 glass fish tanks with a working volume of 30 L. Each tank was aerated and received approximately 14 tank volume renewals per day. During the exposure period, dilution water and chemical stock solutions flow rates were checked daily. Stock solutions were replaced every 4 days to avoid any potential chemical degradation. The stability of the stock solutions of KT and DHT over different times and temperatures was established prior to the commencement of the study (data not shown).

The 45 days androgen exposure experiment consisted of six treatments: dilution water control (DWC), SC, 20 ng KT/L, 200 ng KT/L, 20 ng DHT/L, and 200 ng DHT/L. Each treatment was tested in duplicate, each fish tank contained 14 randomly allocated juvenile fathead minnows (28 fish per treatment), where the sex of the juvenile fish was unknown. Fish were fed *ad libitum* twice a day with a combination of pellet and brine shrimp and were maintained in a photoperiod of 16 hours of light followed by 8 hours of dark with a 20 minutes dawn/dusk transition.

At 105 dph, fish were humanely sacrificed according to UK Home Office procedures, using an overdose of ethyl 3-aminobenzoate methanesulfonate (500 mg MS222/L, adjusted to pH 7.5 using 1 M NaOH), followed by trans-spinal severance. Blood samples were collected from the caudal peduncle using heparinised capillary tubes and transferred into eppendorf tubes containing the enzyme inhibitor Aprotinin (Sigma). Blood samples were kept on ice until plasma was separated by centrifugation at 14000 × g for 5 min, removed and stored at −20 °C until steroid analyses. Fish were weighed and measured (fork length), and the condition index (CI) was calculated (CI = body weight (g) / fork length (cm) × 100). The presence of secondary sexual characteristics (SSC) was recorded for every fish, including number and prominence of nuptial tubercles, black dorsal fin spot occurrence, fatpad presence and weight (Smith, 1978). Fish were then cut ventrally in order to allow penetration of the fixative into the body cavity, fixed *in toto* in Bouin's solution for 24h and subsequently transferred to 70% industrial methylated spirits (IMS) and stored for histopathological analyses.

4.3.4 Analysis of water samples

Water samples (200 mL) were collected from each tank on day 0, 4, 7 and subsequently every seven days until the end of the experiment. The target steroids were extracted from the water by solid-phase-extractions (SPE). Briefly, the Sep-Pak Plus C18 extraction cartridges (Waters Ltd., UK), pre-conditioned with 5 ml methanol and 10 ml deionized water (Milli-Q), were placed on a vacuum Manifold, and the water samples were drawn through methanol-washed Teflon-lined tubes, and through the cartridges at an approximate rate of 3-9 mL/min. Once all the water samples had passed through the SPE cartridges, they were dried with air for 30 minutes, and then eluted with 5 ml HPLC-grade methanol (Fisher Scientific, UK) into clean glass vials. The methanol was successively evaporated under a stream of nitrogen at room temperature. Once dry, each sample was reconstituted with 1 ml of ethanol, sealed with parafilm, and stored at 4ºC until further analysis.

KT concentrations were determined by a specific radioimunassay (RIA) (Scott *et al*., 1984), while DHT concentrations were measured using a DHT ELISA kit (Demeditec, Germany, Cat No. DE2330). For the latter analysis, the samples were prepared according to the methods used by Swart and Pool (2007), and the analysis was performed following the instructions provided by the manufacturer. The extraction efficiency for each androgen was tested in 4 independent experiments performed using tank water samples spiked with known amounts of KT and DHT (20 and 200 ng/L), before processing and analyzing the experimental samples.

4.3.5 Measurement of sex steroids in the plasma by radioimmunoassay

The concentrations of sex steroids in the plasma of fathead minnow were measured by radioimmunoassay (RIA). The principle of RIA, illustrated in Figure 4.3, is based upon the equal competition between a radiolabelled ligand (e.g. tritiated T) and an unlabelled ligand (the target steroid; e.g. T in the plasma sample) for binding to a limited amount of specific antibody over 24h. The amount of radiolabelled ligand and antibody used in the assay are kept constant, so that the amount of radiolabelled ligand bound to the antibody will be inversely proportional to the amount of unlabelled ligand in the mixture. At the end of the RIA, the free unbound label is separated from the antibody-ligand complex, by the use of activated charcoal, to which small molecules will be adsorbed. Unlabelled ligand will thus be adsorbed and this complex will be precipitated by centrifugation. The antibody (with bound labelled or unlabelled hormone attached) remains in the supernatant, which is decanted directly into beta scintillation vials and retained for counting. The radioactivity, or beta rays, emitted from the samples are measured using a scintillation counter.

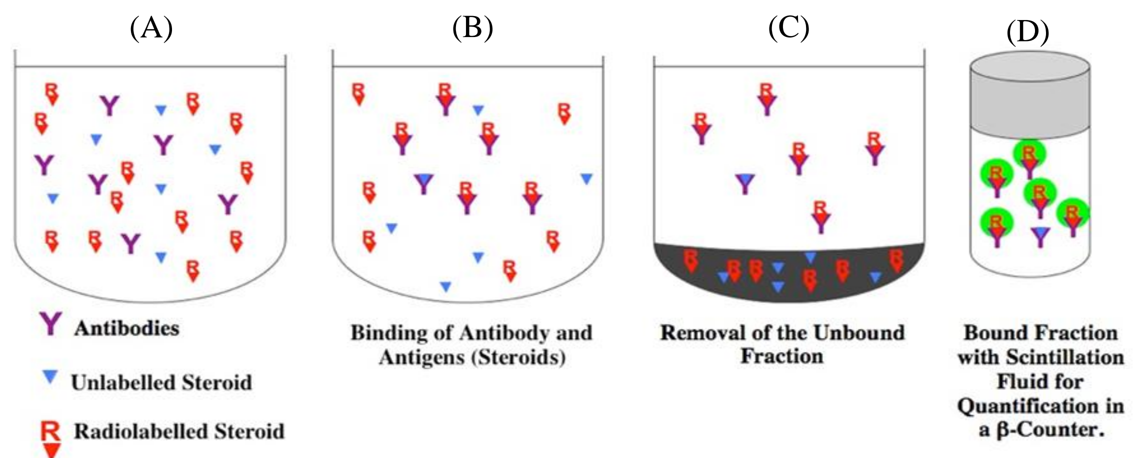


Figure 4.3. *Depiction of the principle of radioimmunoassay. (A & B) Over time the antigens bind to the limited amount of antibody in proportion to the amounts of each that are present. (C) The unbound steroids are removed with activated charcoal, and (D) the sample is placed into a vial with scintillation fluid which fluoresces when β-particles are emitted by the radiolabelled steroid; the fluorescence is quantified by a β-counter (from Aoki, 2010).*

Materials and components:

- ***Steroid Assay Buffer (SAB)***:

1 litre dH20 made to a concentration of 0.05 M phosphate buffered saline (PBS, 10 tablets, Dubelco Ltd.), 1 g gelatine (Type A from porcine skin; Sigma-Aldrich Inc.), 1 g sodium azide (Sigma-Aldrich Inc.).

Using a heated stirrer, gelatine and PBS tablets were dissolved in the water. Once cooled sodium azide was added and mixed. The buffer was then stored at 4ºC.

- **Antibody**:

Dilutions for each steroid of interest were prepared in SAB as follows:

T antibody was diluted 1:50,000

KT antibody was diluted 1:50,000

E2 antibody was diluted 1: 40,000

- **Radiolabel**:

Tritiated steroids (T, KT, E2) were prepared purchased from Amersham International. Working aliquots were prepared in SAB at radioactivities of approximately 6000 dpm per 100 µl, and stored at -20ºC.

- **Standard:**

Solutions of T, KT, and E2 were prepared at concentrations of 10 ng/L and stored at –20ºC.

- **Activated Charcoal Solution**:

200 mL prepared at 0.5% activated charcoal (Sigma-Aldrich Ltd.) with 0.05% dextran (Sigma-Aldrich Ltd.) at least 15 minutes prior to use.

- **Liquiscint Scintillation Fluid** (National Diagnostics Ltd.)

Radioimmunoassay method: Day 1

Radiolabelled steroid, antibody and steroid standard were removed from the freezer and defrosted on ice. Tubes were prepared in duplicate in batches of no more than 50 tubes (25 samples). An entire assay generally consisted of no more than 4 batches. Steroids were extracted from each plasma sample through the addition of ethyl acetate. According to the standard protocol 40-100 µL of plasma were placed into a polypropylene tube (LP3P, Luckham) followed by 0.4-1 mL of ethyl acetate. The tube was capped, shaken thoroughly, and centrifuged at 2,500 g for 5 minutes, in order to separate the ethyl acetate (containing the extracted steroids; upper phase) from the plasma (lower phase). The ethyl acetate (upper phase) was carefully separated from the lower phase, and duplicate of extractions (100 µL each) were placed into clean tubes. The aliquots of extracted samples that were not used on the same day were stored at -20ºC until further analysis. Modifications to this steroid extraction method were required when the volume of plasma available for the analysis was very low (e.g. plasma collected from juvenile fish). If applied, these modifications will be described in the correspondent methods section in each chapter.

The ethyl acetate was then evaporated using a concentrator (miVac, Genevac Ltd.), and the extracted steroids were re-dissolved in 100 µL SAB.

The standard curve samples were prepared by serially diluting the steroid standard 11 times to yield nominal concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.04, 0.02, 0.01, and 0.005 ng/ml.

Other than standard and unknown samples, in each assay additional tubes were set up in order to determine:

Maximum Binding (MB), by incubating the labelled ligand and the antibody alone;

Non Specific Binding (NSB), some small amount of label will also act as bound ligand, even when there is no antibody, due to binding of label to other assay components, trapping of label to the bound complex, impurities in the label, absorption of free tracer to the reaction tube, or incomplete aspiration of the unbound fraction from the tube.

Specific volumes of SAB, antibody and label were also added to the test tubes according to their identity (e.g. sample, standard, Non Specific Binding, Maximum Binding, Total Counts), as shown in Table 4.1. Tubes were whirl mixed and briefly centrifuged to ensure that all the reagents were at the bottom, covered with a clean paper towel, and incubated overnight at 4ºC.

Table 4.1. *Volumes of various components added to the different tubes in the steroid RIA.*

Tube	Volume (μL)				
	SAB	**Antibody**	**Label**	**Standard**	**Sample**
Total Counts	200		100		
Non-Specific Binding	200		100		
Maximum Binding	100	100	100		
Standards	100 (not in the first tube)	100	100	100 (1st and 2nd tubes only)	
Samples	/	100	100		100 (in SAB)

Radioimmunoassay method: Day 2

After 24 hours incubation, the tubes were removed from the fridge and placed on ice; 500 µl SAB was added to the Total Counts Tubes. Then, 500 µl activated charcoal solution (0.5%) was added as quickly as possible to each tube of one batch. All tubes were vortexed, and incubated on ice for 10 minutes (counted from the addition of the charcoal solution to the first tube), prior to centrifuging at 2,500 g for 15 minutes at 4ºC. The supernatant was carefully removed, and decanted into a clean scintillation vial. Four millilitres of scintillation fluid were added to each vial, and the vials were shaken vigorously.

Measurement and analysis

Radioactivity in the samples was counted for 5 minutes using a Liquid Scintillation Counter (Packard Bioscience Co.). The amount of radiolabel which binds to the antibody in the standards and sample tubes is expressed as a percent of the normalized Maximum Binding for each batch. This was determined using the following calculation:

$$\%\ \text{Binding} = \frac{(\text{mean sample decays per minute} - \text{mean Non-Specific Binding})}{(\text{mean Maximum Binding} - \text{mean Non-Specific Binding}).} \times 100$$

The standard curve was plotted on log-normal scale, and the unknown values were read from the linear part of the curve, usually between 20% and 80% binding.

Plasma testosterone measurement

An optimised RIA protocol for T measurement (Scott *et al.*, 1984) was required for the small volume of plasma available from fathead minnow juveniles (6-20 µl/fish) (Jensen *et al*, 2001). Frozen plasma samples were thawed on ice, and reconstituted to a working volume of 100 µl with 0.1 M phosphate buffered saline (PBS; 0.1 M $NaPO_4$, 0.15 M NaCl, pH 7.6). Sex steroids were extracted through the addition of ethyl acetate, and T concentrations were measured by specific RIA. Detection limit, EC50, and intra-assay variations (CV%) were respectively 0.04 ng/mL, 0.17 ng/mL, and 2.3% (n = 20).

4.3.6 Histological processing and quantitative analysis of gametogenesis

Samples were stored in Bouin's solution, which was replaced by 70% industrial methylated spirits (IMS) after 24 hours, and again after 48 hours. Whole bodies of each fish were cut transversally in three transverse sections ~0.5 cm in order to separate the gonad into anterior, median and posterior regions. These sections were then processed according to standard histological methods, and slices were prepared for histopathology analyses according to the methods briefly detailed hereinafter.

Tissues were processed according to the protocol in Table 4.2, using the automatic tissue processor (TP 1020, Leica Inc.), and subsequently embedded into wax blocks. Tissues were sectioned to 3 µm thickness using the RM 2235 microtome (Leica Inc.), and placed onto microscope slides coated with Histobond (RA Lamb, UK). They were dried for ~48 h before staining with Eosin and Haematoxylin according to the protocol in Table 4.3. Finally, the stained sections were mounted using Histomount (National Diagnostics, USA) and covered with coverslips. The slides were examined under an Olympus BX51 compound light microscope and photographs were taken using a digital camera and the Q-Capture Pro v. 5.1.1.14 program (Media Cybernetics Inc.). All gonads were examined to identify the developmental stage, for the presence of cells in all stages of gametogenesis, and for any evidence of intersex (spermatogenic cells in the ovaries, or oogenic cells in the testes). The evaluation of the histopathological alterations was conducted according to the EPA guidelines (EPA, 2006), and the diagnostic criteria used for the analysis are described in the following Section.

Table 4.2. *Summary of the tissue processing method of the Leica Tissue Processor, indicating the various solutions used and the time of each step.*

Order	Solution	Time (hours)
1	70% IMS	3
2	90% IMS	2.5
3	95% IMS	1.5
4	100% IMS	1.5
5	100% IMS	1.5
6	100% IMS	1.5
7	100% IMS	1.5
8	Histoclear (National Diagnostics, USA)	1.5
9	Histoclear	1.5
10	Histoclear	1.5
11	Paraffin Wax (RA Lamb Inc.)	1.25
12	Paraffin Wax	1.25

Table 4.3. *The staining protocol for histological slides of whole-body cross sections of fish.*

Solution	Time	Function
Histoclear	15 minutes	Dissolves wax
100% IMS	2 minutes	Rehydration
90% IMS	2 minutes	
70% IMS	2 minutes	
Water	2 minutes	
Haematoxylin (VWR Inc.)	15 minutes	Stains nuclear material
Water	15 minutes	Wash
Acid Alcohol [HCl/70% IMS (1:99)]	5-30 seconds	Resolves stain
Water	20 seconds	Wash
Saturated Solution of Li_2CO_3 (Sigma-Aldrich, UK)	20 seconds	Raises pH and removes any Bouin's residue
Eosin (RA Lamb, UK)	5-20 seconds	Counter-stains
Water	5 minutes	Wash
70% IMS	2 minutes	Tissue dehydration
90% IMS	2 minutes	
100%IMS	5 minutes	
Histoclear	5 minutes	Helps mounting coverslip

4.3.6.1 *Diagnostic Criteria*

Males:

- ***Altered proportion of spermatogenic stages;***

- *Change in gonadal staging:*

Criteria for Staging Testes:

- **Juvenile**: gonad consists of spermatogonia exclusively;
- **Stage 0** – Undeveloped: entirely immature phases (spermatogonia to spermatids) with no spermatozoa;

- **Stage 1** – Early spermatogenic: immature phases predominate, but spermatozoa may also be observed; the germinal epithelium is thinner than it is during Stage 2;
- **Stage 2** – Mid-spermatogenic: spermatocytes, spermatids, and spermatozoa are present in roughly equal proportions; the germinal epithelium is thinner than Stage 1 but thicker than Stage 3;
- **Stage 3** – Late spermatogenic: all stages may be observed, however, mature sperm predominate; the germinal epithelium is thinner than it is during Stage 2;
- **Stage 4** – Spent: loose connective tissue with some remnant sperm.

- Presence of intersex (both ovo-testis and testis-ova): The presence of one or more individualized or clustered oogenic cells within the testis, or of spermatogenic tissue into the ovary, showing multiple stages of spermatogenesis.

- Increased testicular degeneration: Testicular degeneration is characterized by individual or clustered apoptotic germ cells, vacuolated germ cells, and/or multinucleated (syncytial) cells in the germinal epithelium or testicular lumen. Apoptotic germ cells are characterized by cell shrinkage, nuclear condensation, and fragmentation into spherical, membrane-bound bodies, which are often phagocytized by neighboring cells. There is no inflammation associated with these cells.

- Interstitial cell (Leydig cell) hyperplasia/hypertrophy: An increase in number and/or size of the interstitial cells responsible for producing androgens. Interstitial cells may have larger, more rounded nuclei, and interstitial cell aggregates may occupy and expand some interstitial spaces.

Females:

- Increased oocyte atresia: An increase in degradation and resorption of oocytes at any point in development. Atresia is characterized by clumping and perforation of the chorion, fragmentation of the nucleus, disorganization of the ooplasm, and/or the uptake of yolk materials by perifollicular cells.

- Perifollicular cell hyperplasia/hypertrophy: Increase in the size or number of granulosa, theca, and/or surface epithelium cells involved in a developing follicle. Abnormal perifollicular cell hypertrophy must be distinguished from the normally enlarged granulosa and theca cells of a post-ovulatory follicle.

- Decreased yolk formation: A decrease in the amount of vitellogenic/yolk material that is deposited in the developing oocyte. Decreased vitellogenesis is characterized by the presence of oocytes in which yolk material is not present despite their relatively large size.

- Change in gonadal staging*:*

Criteria for Staging Ovaries:

- **Juvenile**: gonad consists of oogonia exclusively;
- **Stage 0** – Undeveloped: entirely immature phases (oogonia to perinucleolar oocytes); no cortical alveoli;
- **Stage 1** – Early development: vast majority (e.g., >90%) are pre-vitellogenic follicles, predominantly perinucleolar through cortical alveolar;
- **Stage 2** – Mid-development: at least half of observed follicles are early and mid-vitellogenic;
- **Stage 3** – Late development: majority of developing follicles are late vitellogenic;
- **Stage 4** – Late development/hydrated: majority of follicles are late vitellogenic and mature/spawning follicles; follicles are larger as compared to Stage 3;
- **Stage 5** – Post-ovulatory: predominately spent follicles, remnants of theca externa and granulose;

- Interstitial fibrosis**:** The presence of increased fibrous connective tissue (collagenous fibers and fibrocytes or fibroblasts) within the ovarian interstitium (stroma).

Each section of testis or ovary was analyzed in toto; sex was assigned according to the presence of male or female germ cells, the occurrence of sex specific gonad morphology (i.e. ovarian cavity), and number of mesenteric attachments of the gonad to the peritoneal wall, since a double attachment is characteristic of the female reproductive tract, while a single attachment is the typical condition in males (van Aerle *et al.*, 2004). When spermatogenic tissue was present in an ovary, the gonad was classified as intersex (Jobling *et al.*, 1998). The dominant germ cell and the most advanced stage of gametogenesis were also recorded. In order to evaluate the effect of KT and DHT on oocyte growth, the cross sectional area of each oocyte was calculated using the software ImageTool (v 2.00), and the final measurements were pooled by oocyte class and maturation stage. The following classes were considered: perinucleolar oocyte (PNO), corticoalveolar oocyte (CAO), early vitellogenic oocyte (EVO), and mid-late vitellogenic oocyte (MLVO).

4.3.7 Statistical analysis

The data for wet weight, standard length, condition factor, SSC and plasma T concentrations (log transformed) were analysed by analysis of variance (ANOVA). Where assumptions of normality and homogeneity were met, ANOVA was followed by the Dunnett's test to compare the treatment means with the respective controls. Where the assumptions were not met, data were analysed using a non-parametric test (Kruskal–Wallis), followed by Dunn's post-hoc test (Zar, 1999). Effects of the treatments on gonadal maturation stage and dominant germ cell type were analysed using a variation of the chi-squared test, the randomization test of goodness of fit with Montecarlo simulation (n = 10,000) (McDonald, 2009). The results are reported as means ± standard deviations, or percentages (gonadal sex ratio). Ovaries presenting an intersex condition were grouped with female fish for the purpose of the statistical analyses, except for the analysis of ovary maturation stage and oocyte cross-section area. Oocyte cross section area was statistically compared and analyzed between similar ovary maturation stages, in order to avoid the effect of gonad maturation on oocytes dimensions.

4.4 Results

4.4.1 Measured water concentrations of KT and DHT

Water analysis showed that fish were exposed to the test substances in the expected range of concentrations and that there was no contamination of control tanks with steroids. KT and DHT concentrations ranged, respectively, from 74.8% to 148.5% and from 68.45% to 153.5% of the nominal concentrations, except on day 28, when a minor problem with the peristaltic pump occurred, accounting for the unexpected high concentrations in all the treatment tanks (Table 4.4); however, the problem was quickly fixed, and fish were exposed to the elevated concentrations for no more than 9-12 hours. The extraction efficiency was above 90% for both steroids, thus no correction was applied (KT: 91.1 ± 12.0%; DHT: 93.4 ± 17.9%).

Table 4.4. *Measured water concentrations (ng/L) of KT and DHT during the exposure period. Steroids concentrations were measured by RIA and ELISA, respectively.*

Exposure Day	Replicate	Nominal concentrations of 11-ketotestosterone (KT) and dihydrotestosterone (DHT) (ng/L)					
		Controls		KT		DHT	
		*DWC	*SC	20	200	20	200
0	A	ND	ND	28.1	192.5	25.0	210.0
	B	ND	ND	25.6	191.5	26.0	220.0
3	A	ND	ND	29.7	230.0	24.1	207.6
	B	ND	ND	21.9	258.1	27.3	220.4
7	A	ND	ND	26.6	203.3	26.8	136.9
	B	ND	ND	29.7	225.1	29.0	196.9
14	A	ND	ND	24.8	211.7	14.5	210.0
	B	ND	ND	23.8	266.0	22.0	241.0
21	A	ND	ND	20.1	191.9	19.0	151.0
	B	ND	ND	21.8	221.6	25.0	155.0
28	A	ND	ND	40.1	328.0	46.0	456.0
	B	ND	ND	57.0	348.4	52.0	429.0
35	A	ND	ND	22.8	202.0	28.3	191.0
	B	ND	ND	28.7	217.9	29.0	199.0
42	A	ND	ND	21.1	148.2	22.6	267.0
	B	ND	ND	22.2	165.2	31.0	307.0
Mean Values (ng/L)		ND	ND	27.7 ± 9.2	225.1 ± 53.3	28.3 ± 9.9	240.7 ± 96.7
Mean Values (excluding Day 28) (ng/L)		ND	ND	24.8 ± 3.3	210.2 ± 32.8	24.9 ± 4.7	207.1 ± 48.7

ND = Not Detected.

4.4.2 Somatic growth (standard length, wet weight, condition factor)

Male fish exposed to 20 and 200 ng/L of both KT and DHT had significantly higher wet weights and fork lengths when compared to the fish in DWC and SC groups ($p < 0.01$), while female fish showed a significant increase of the same endpoints only when exposed to 200 ng/L of both androgens ($p < 0.01$) (Fig. 4.4). Thus, both fork length and wet weight data showed a dose-related response in both sexes, with males responding at lower concentrations than females. This trend was not seen in the condition factor data for both sexes, where there were no statistically significant differences between treatment groups.

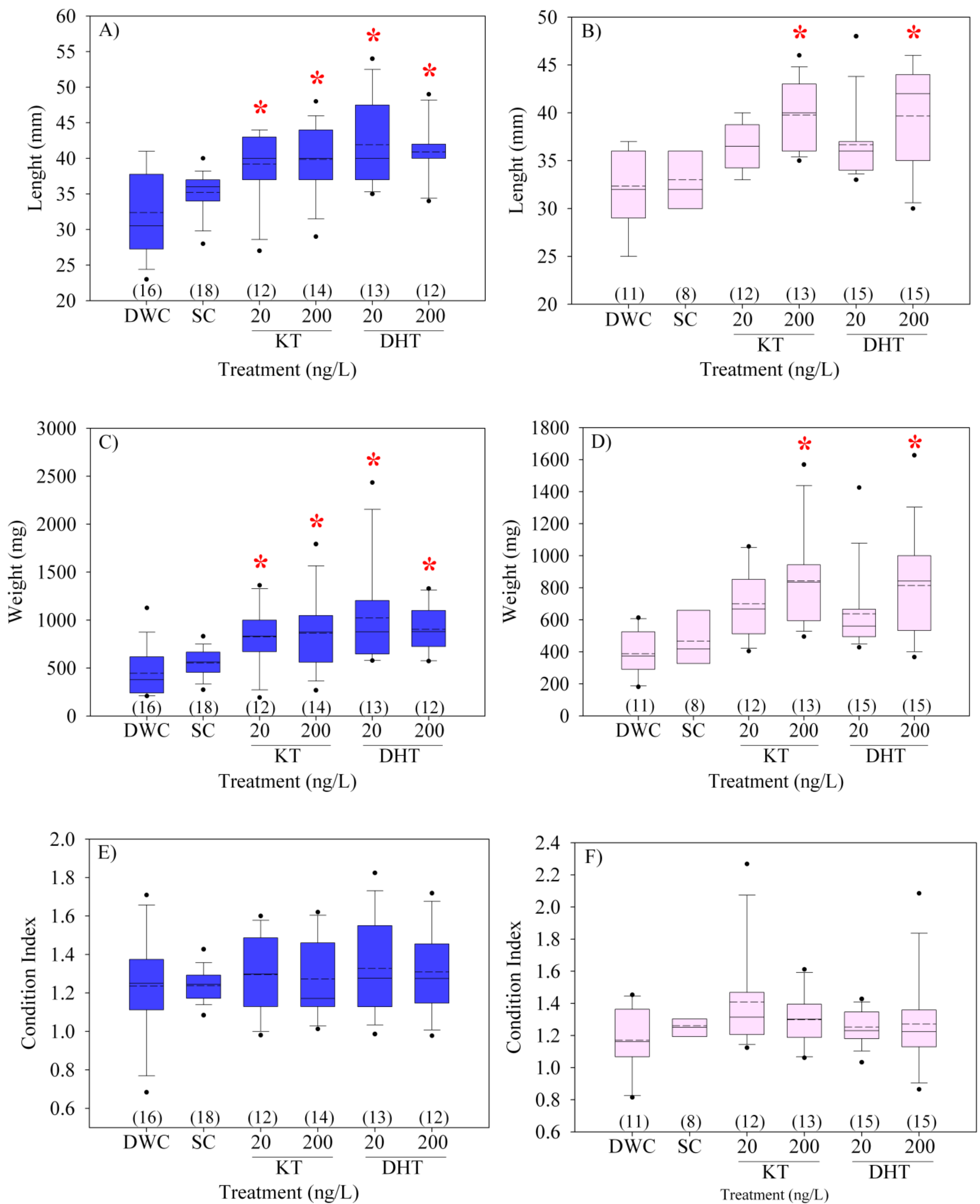


Figure 4.4. *Box plots of (A,C,E)length, weight and condition index of fathead minnow males and (B,D,F) females exposed to KT and DHT between 60 and 105 dph. Boxes represent median (full line), and mean (dashed line) with 25th and 75th percentiles. The bars extend to the 10th and 90th percentiles, with outliers represented as dots. * $p < 0.05$ compared to the Solvent Control group. Sample size is indicated above each treatment label on the x-axis.*

4.4.3 Secondary sexual characteristics

Both KT and DHT caused the appearance of male SSCs in both males and females (Table 4.5); fish in the DWC and SC groups had no male SSCs, with the exception of one male in the DWC group, which had a dorsal fin spot. The latter endpoint was particularly affected by DHT, indicated by the fact that 92% of males and 75% of females exposed to 200 ng/L developed this characteristic. Nuptial tubercles were present on some of the fish exposed to the androgens and fatpad presence was recorded only in one male exposed to KT (200 ng/L) and one male exposed to DHT (20 ng/L).

Table 4.5. *Expression of secondary sexual characteristics in fish exposed to KT and DHT between 60 and 105 dph. For each treatment the number of fish with nuptial tubercles (NT) and dorsal fin spot (FS) is indicated.*

Endpoint	**Sex**	**Treatment**					
		DWC	SC	KT 20 ng/L	KT 200 ng/L	DHT 20 ng/L	DHT 200 ng/L
Fish with NT	M	0	0	0	3	1	2
	F	0	0	1	0	0	2
Fish with FS	M	1	0	4	4	2	11
	F	0	0	1	0	1	11
Sample Size	M	16	18	12	14	13	12
	F	11	8	12	13	15	15

4.4.4 Plasma testosterone concentrations

Effects on plasma T were different in males and females (Fig. 4.5). Males exposed to KT showed an increase in plasma T at both exposure concentrations, although the increase was statistically significant only at 20 ng/L ($p < 0.01$), whereas males exposed to DHT had significantly higher plasma T concentrations at both 20 and 200 ng/L ($p < 0.01$). In contrast, females showed a general decrease of circulating plasma T when exposed to the androgens. The decrease was statistically significant ($p < 0.01$) in females exposed to the highest dose of KT and to both doses of DHT. Furthermore, females in the DWC group had significantly lower plasma T level than the SC ($p < 0.05$).

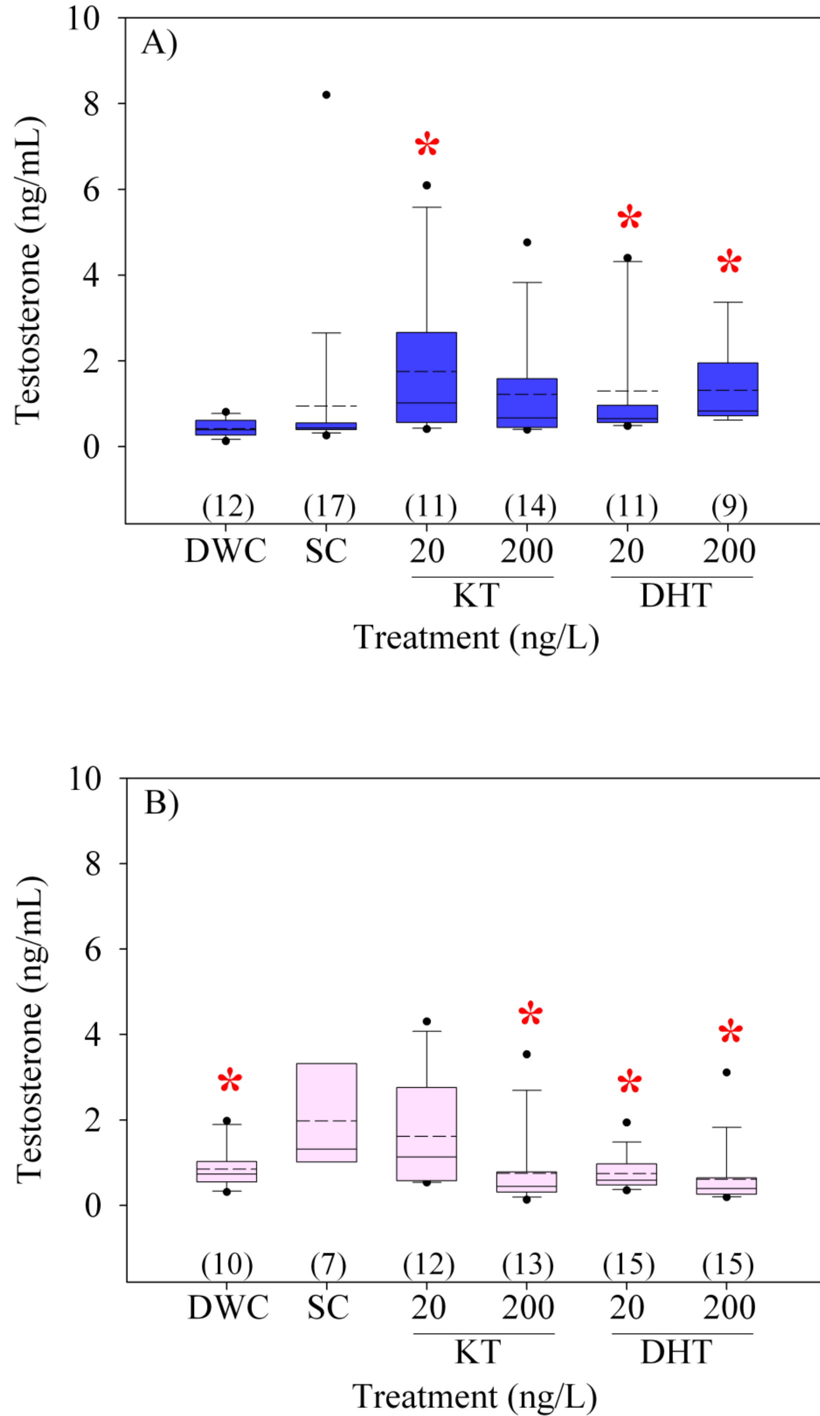


Figure 4.5. *Plasma testosterone concentrations of (A)male and (B) female fathead minnows exposed to KT and DHT between 60 and 105 dph. Boxes represent median (full line), and mean (dashed line) with 25th and 75th percentiles. The bars extend to the 10th and 90th percentiles, with outliers represented as dots.* * *$p < 0.05$ compared to the Solvent Control group. Sample size is indicated above each treatment label on the x-axis.*

4.4.5 Histopathology

4.4.5.1 *Males*

At the end of the study, the majority of males in the DWC and SC groups were in the early stages of sexual maturation (Fig. 4.6). All the fish presenting only gonia-stage cells were classified as juvenile males, based on the pattern of distribution of somatic and germ cells, the absence of an ovarian cavity, the presence of the efferent duct (sperm duct) and the single attachment of each gonad to the peritoneal wall (Fig. 4.7). Additionally, at 106 dph, fathead minnow ovaries normally contain perinucleolar oocytes (PNOs) or Corticoalveolar oocytes (CAOs), and have two attachments to the peritoneal wall (van Aerle *et al.*, 2004).

Immature testis (Juvenile and Stage 0) were present in 93.75% and 77.8% of the fish in the DWC and SC groups, respectively. Both KT and DHT stimulated maturation of the testis and progression toward advanced stages of spermatogenesis. This effect was only significant at 200 ng/L for both androgens ($p < 0.01$). KT caused an increase of the percentage of fish where spermatozoa were the most advanced stage of spermatogenesis, from 22.2% in the SC group to 36.36% and 35.71% in the 20 and 200 ng/L groups, respectively. At the same time, spermatocytes represented the most advanced stage of spermatogenesis in 16.67% of fish in the SC group, and in 36.36% and 50% of fish in the 20 and 200 ng/L groups, respectively, with a correspondent decrease in the proportion of fish with only spermatogonia in their testis (Fig. 4.6).

Males exposed to DHT followed the same pattern; however, at the highest dose (200 ng/L), there was a stronger effect on the spermatogenic process, where 80% of fish exposed to this concentration had spermatozoa in their testis (Fig. 4.6 & 4.7).

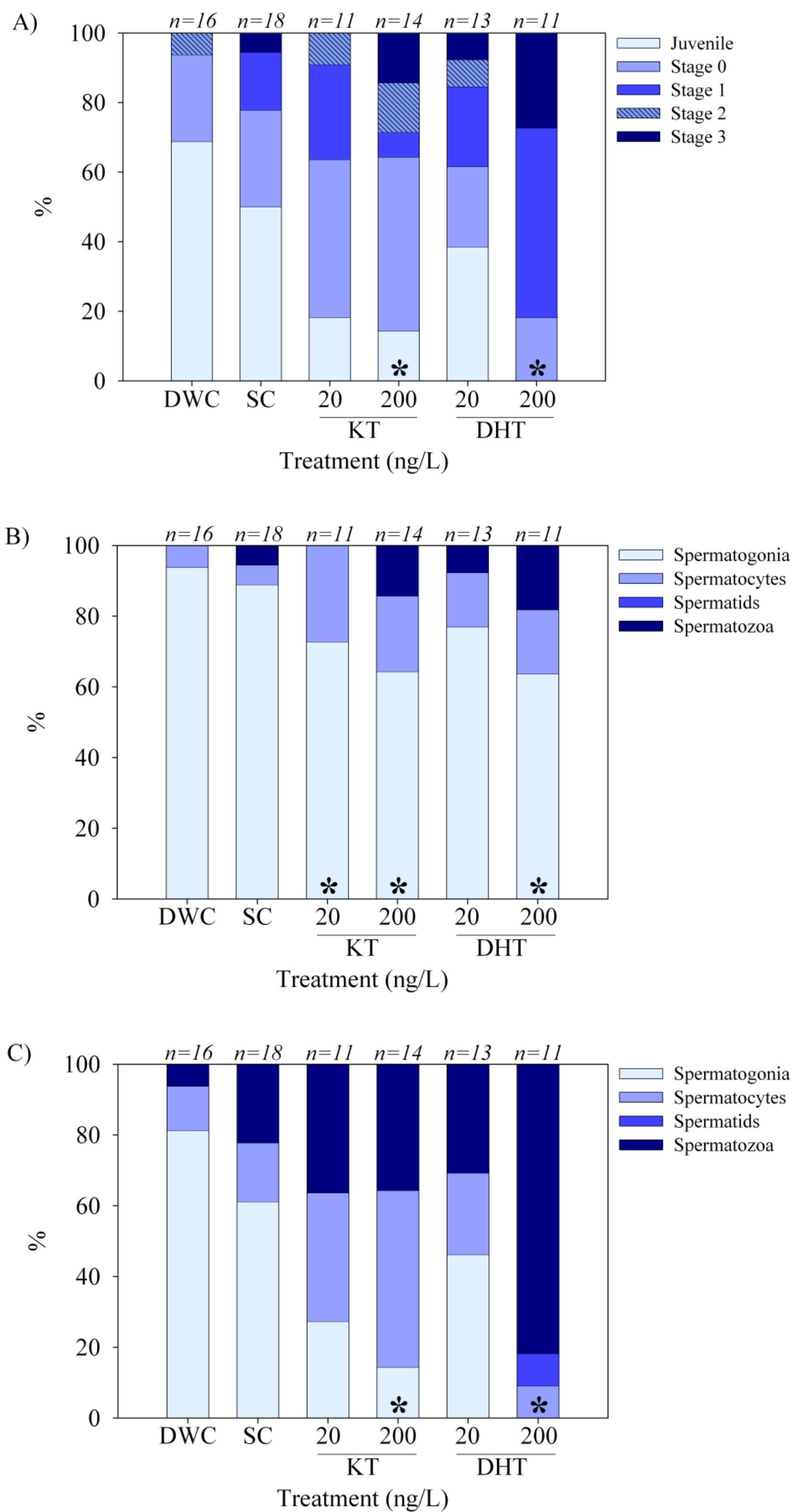


Figure 4.6. *Effect of KT and DHT exposure on (A) testis maturation stage, (B) dominant germ cell, and (C) most advanced stage of spermatogenesis. Juvenile (Juv): gonad consists of exclusively spermatogonia; Stage 0: Undeveloped, entirely immature phases (spermatogonia to spermatids) with no spermatozoa; Stage 1: Early spermatogenic: immature phases predominate, but spermatozoa may also be observed; Stage 2: Mid-spermatogenic: spermatocytes, spermatids, and spermatozoa are present in roughly equal proportions; Stage 3: Late spermatogenic, mature sperm predominate (US EPA, 2006). * $p < 0.05$ compared to the Solvent Control group. Sample size is indicated on the top of each treatment stacked bar.*

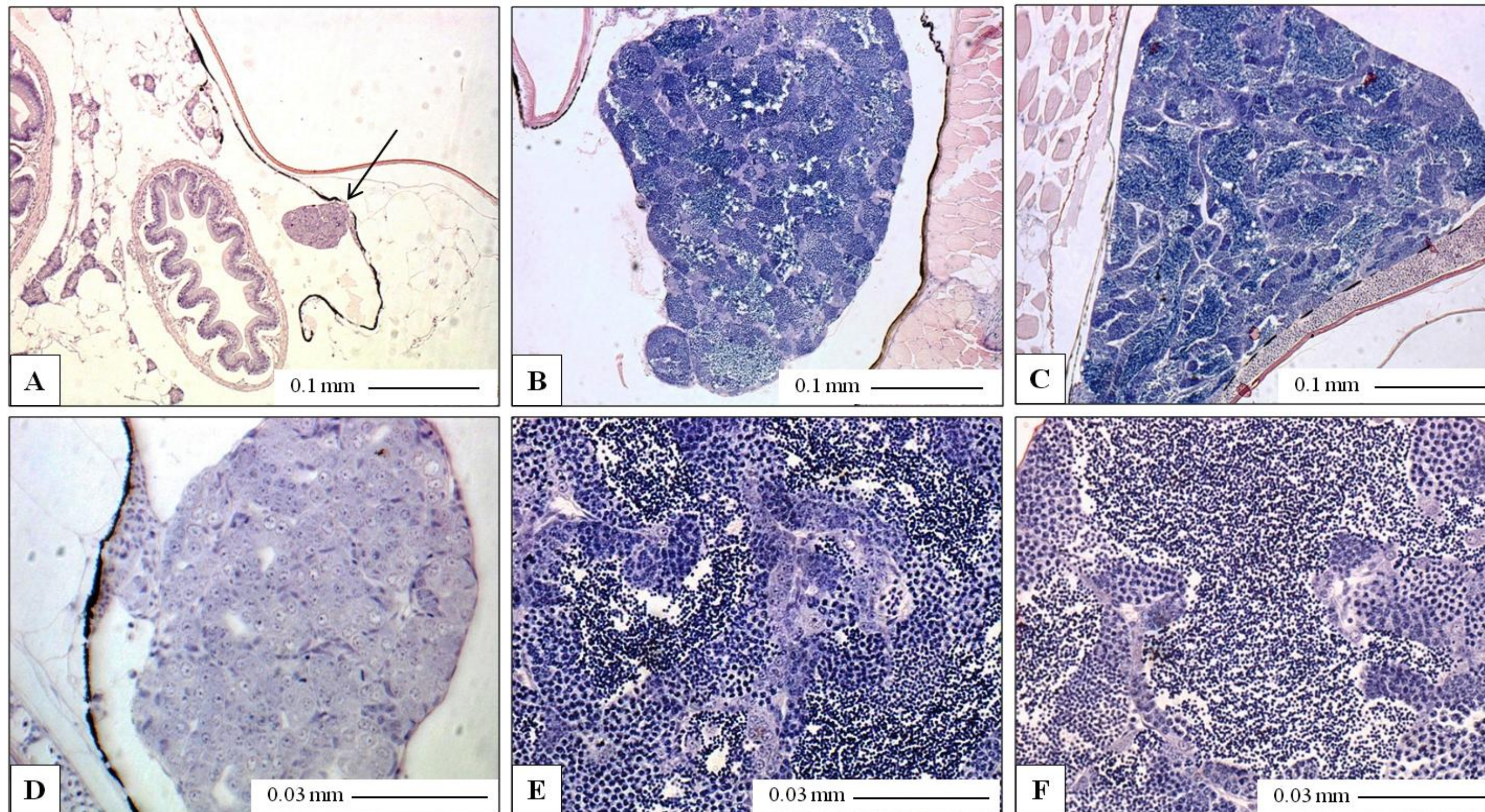


Figure 4.7. *Transverse sections of testes from male fathead minnows from the (A, D) Solvent Control, (B,E) KT (200 ng/L), and (C, F) DHT (200 ng/L) groups. The gonads in figures A (indicated by the arrow) and D had only spermatogonia distributed in cystic structures, somatic cells scattered among germ cells, and only one attachment to the peritoneal wall; all these characteristics are typical of developing testis (Van Aerel et al., 2004). Gonads presenting this pattern of development were classified as male juveniles, and this maturation stage was present in 68.8% and 50.0% of all male gonads, respectively, in the DWC and SC groups. The testes of fish exposed to 200 ng/L of KT and DHT (B,C,E,F) typically contained spermatogenic cell types at more advanced developmental stage (spermatocytes through to spermatozoa). In particular, 81.8% of fish exposed to 200 ng DHT/L had spermatozoa in their testis.*

4.4.5.2 *Females*

KT at 20 ng/L did not induce any significant effect on the stage of maturation of the ovary or on the most advanced stage of oogenesis. On the other hand, fish exposed to 200 ng/L of KT showed a significant regression ($p < 0.01$) of the stage of maturation of the ovary, with an evident increase of females at stage 1 (early development), from 12.5% in the SC group, to 53.90% in the group exposed to KT. The same treatment caused a significant decrease in the proportion of fish with vitellogenic oocytes as the most advanced stage of oogenesis (Fig. 4.8).

DHT had a severe effect on the ovarian tissue at both concentrations. The most evident effect was the occurrence of testicular tissue in the ovaries (Fig. 4.9). Most (86.7%) of females exposed to DHT at 200 ng/L developed the intersex condition (Fig. 4.8), and one female with a masculinised ovary was also found at 20 ng/L. The extent of the testicular tissue varied from 10% to 95% of the ovary in longitudinal cross section, and in some samples, a concomitant proliferation of macrophages and interstitial tissue was found. In fish with a lower severity intersex condition, the disruption was typically found around the area where the gonads attach to the body wall.

It is important to note that histological sections taken from left or right side ovary, or at different places along the ovary longitudinal axis, sometimes showed totally different conditions, ranging from totally normal to high severity intersex. This variability along the longitudinal axis of the ovary is probably due to the fact that the intersex condition develops as a local disruption. This underlines the importance of screening the ovary at different places along the longitudinal axis when looking for this kind of disruption in juvenile fish.

The occurrence of testicular tissue in the ovaries influenced all the histopathological endpoints analyzed, with a general regression toward the early stages of oogenesis, characterized by previtellogenic oocytes rather than vitellogenic ones. In particular, the incidence of previtellogenic oocytes (PNO+CAO) increased from 60.0% and 57.2% in the DWC and SC groups, to 80% and 86.7% after DHT treatment at 20 and 200 ng/L, respectively.

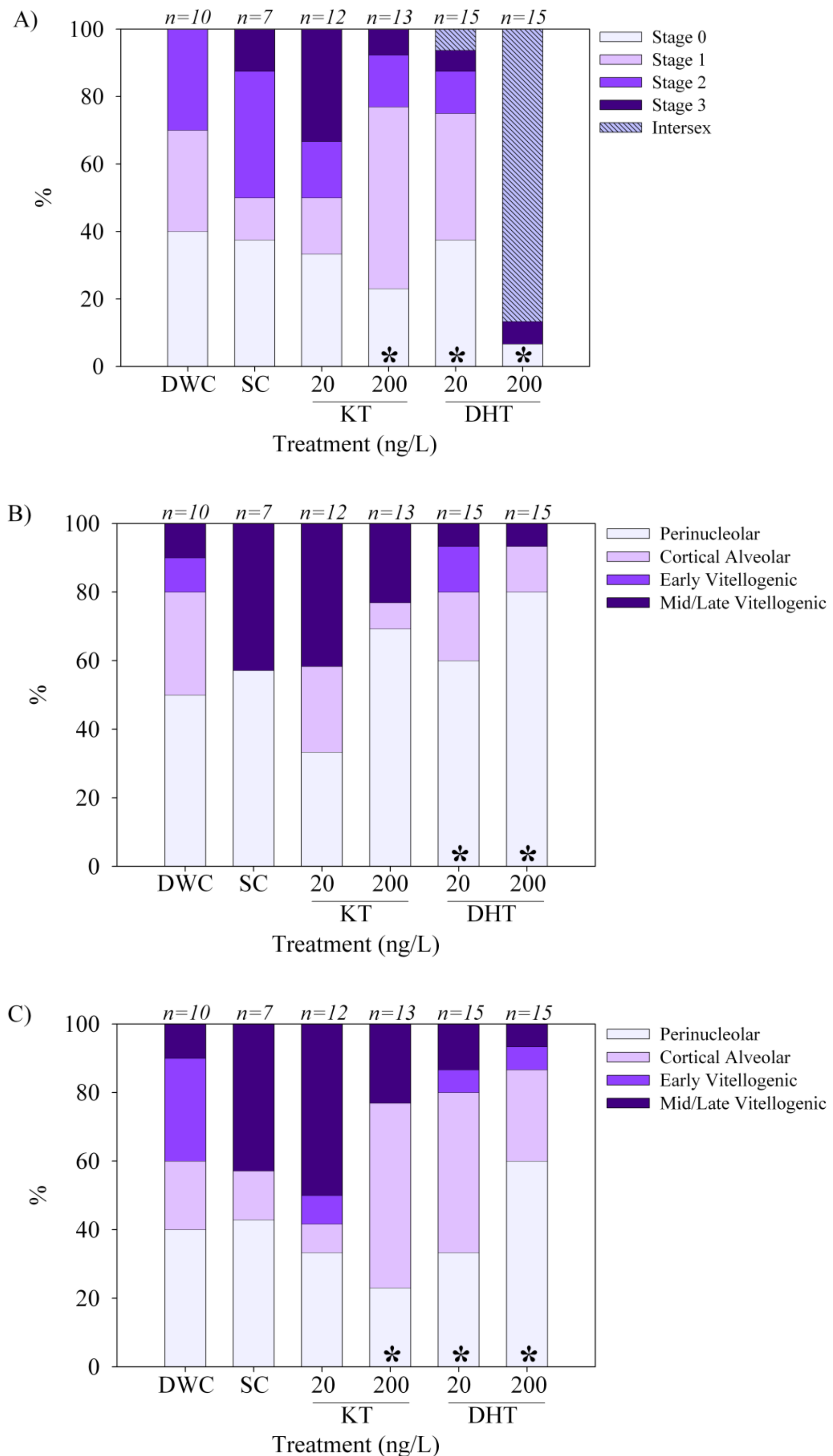


Figure 4.8. *Effect of KT and DHT exposure on (A) maturation stage of the ovary, (B) dominant germ cell, and (C) most advanced stage of oogenesis. Stage 0: Undeveloped, entirely immature phases (oogonia to Perinucleolar oocytes), and no cortical alveoli; Stage 1: Early development, vast majority (e.g., >90%) were pre-vitellogenic follicles, predominantly perinucleolar through cortical alveolar; Stage 2: Mid-development, at least half of observed follicles were early and mid-vitellogenic; Stage 3: Late development, majority of developing follicles were late vitellogenic; Intersex: ovary showing presence of spermatogenic tissue (US EPA, 2006).*

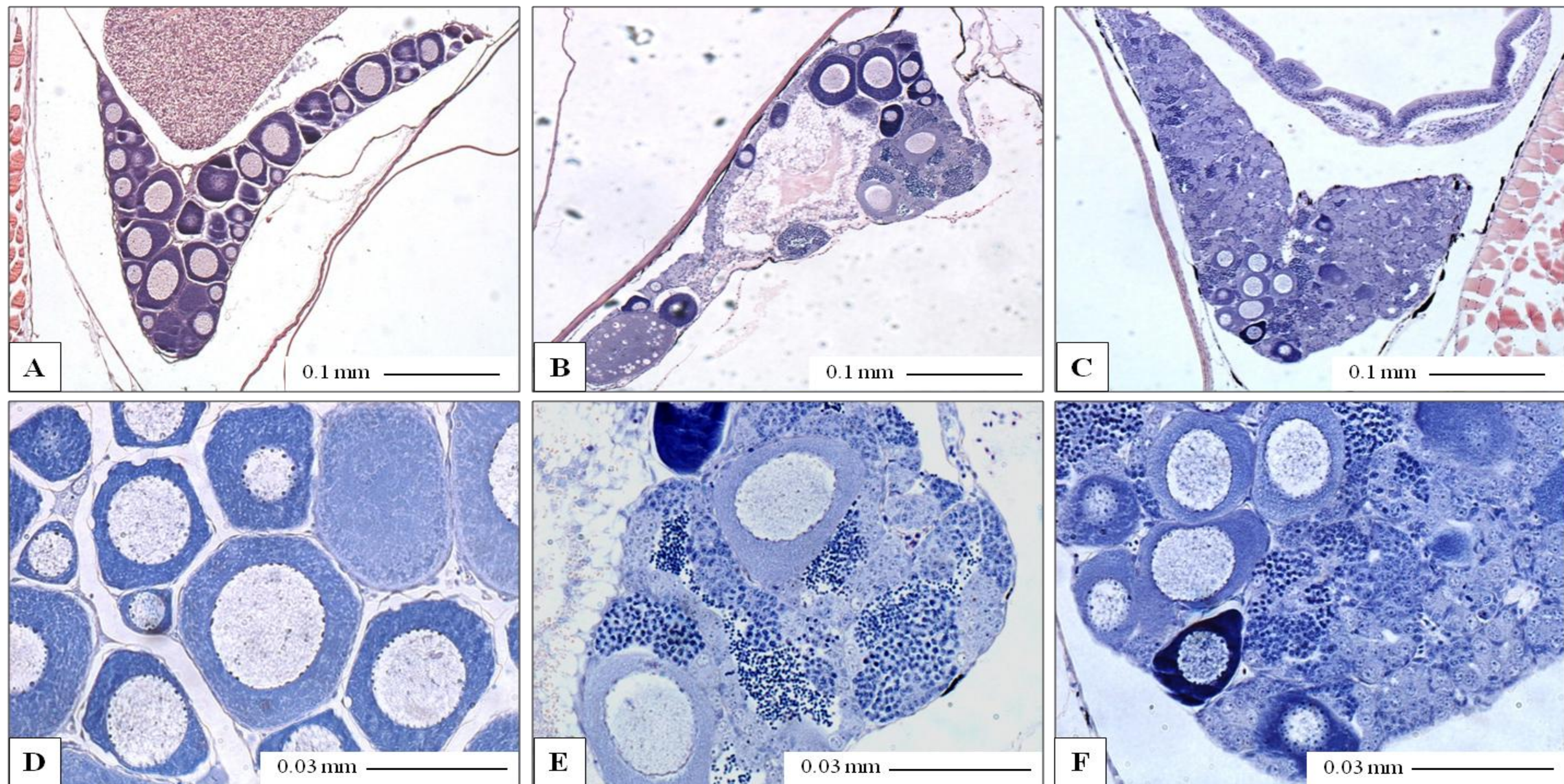


Figure 4.9. *Transverse sections of fathead minnow ovaries (A, D) from the Solvent Control group and (C, D, E, F) from the DHT group (200 ng/L), showing the presence of masculinised tissue within the gonad, characterized by the presence of multiple stages of spermatogenesis, from spermatogonia to spermatozoa. On the other hand, KT did not induce any appearance of spermatogenic tissue in any of the ovaries of exposed female (figure not shown).*

4.4.6 Quantitative assessment of the size of oocytes

Analysis of the cross-sectional area of oocytes indicated a complex pattern of response (Table 4.6). KT at 20 ng/L produced a significant increase in the dimensions of all the classes of oocytes ($p < 0.01$). While the same steroid at 200 ng/L induced a significant size increase in PNOs and mid-late vitellogenic oocytes (M-LVOs), a decrease in early vitellogenic oocytes (EVOs), and a contrasting pattern of response in CAOs, with a size increase in 2 out of 3 maturation stages, and a decrease in 1 out of 3 ($p < 0.01$).

Exposure to DHT at the low dose (20 ng/L) did not have any effect on the cross-sectional area of PNOs and EVOs, but induced a significant increase in M-LVOs size, and a complex response in CAOs, with different responses in different maturations stages ($p < 0.01$). Intersex samples were analyzed separately, and did not show any significant variation in the sizes of PNOs and CAOs (the only two classes present). The exposure to the highest dose of DHT (200 ng/L) caused a significant decrease in size in EVOs, and a increase in M-LVOs ($p < 0.01$), while in the fish with intersex gonads, DHT induced a significant size decrease in PNOs and an increase in CAOs ($p < 0.01$).

Table 4.6. *Effect of KT and DHT exposure on the cross-sectional area of oocytes according to class and ovary maturation stage. The arrows ↑ and ↓ represent, respectively, a statistically significant (p < 0.01) increase or decrease when compared to the Solvent Control group. The cross-sectional area of oocytes was statistically compared and analyzed among ovaries at the same stage of maturation, in order to avoid the confounding effect of gonad maturation stage on oocyte dimensions.*

Treatment	**Germ Cell Type**															
	PNO				CAO				EVO				M-LVO			
	Maturation stage															
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
KT 20 ng/L	↔	↑	↑	↑	-	↔	↔	↑	-	↑	↔	↔	-	-	↔	↑
KT 200 ng/L	↔	↔	↑	↔	-	↓	↑	↑	-	-	↔	↓	-	-	↑	↔
DHT 20 ng/L	↔	↔	↔	↔	-	↔	↓	↑	-	-	↔	↔	-	-	↔	↑
DHT 200 ng/L	↔	-	-	↔	-	-	-	↔	-	↔	-	↓	-	-	-	↑
DHT 200 ng/L - IS	↓	↔	-	↓	-	↑	-	↑	-	-	-	↔	-	-	-	-

PNO: Perinucleolar Oocytes; CAO: Cortico Alveolar Oocytes; EVO: Early Vitellogenic Oocytes; M-LVO: Mid-Late Vitellogenic Oocytes.IS: Intersex; "↔ ": no significant difference; "-": Not Available.

4.5 Discussion

In the present study, we investigated and compared the androgenic effects of KT and DHT on juvenile fathead minnows. The first steroid is considered the most important androgen in teleost fish, while the second one is the most active in higher vertebrates, but is not generally considered present or active in fish.

As hypothesized by a comparison of the properties of several teleost fish ARs, DHT caused effects on fathead minnow juveniles, showing an *in vivo* androgenic potency comparable to KT in males and higher than KT in females. Exposure to 20 and 200 ng/L of both androgens during the juvenile phase (60-105 dph) accelerated fish growth, induced the appearance of male SSCs in both males and females, and stimulated spermatogenesis and testis maturation in males. However, only DHT exposure caused the appearance of spermatogenic tissue in ovaries of females.

Experiments conducted on African catfish and Japanese eel showed that DHT did not have any effect on spermatogenic processes and KT was the only androgen capable of inducing full spermatogenesis (Borg, 1994; Cavaco *et al.*, 1998, 2001; Miura *et al.*, 1991). However, our results show a different response in fathead minnow, suggesting a species-specific response, presumably related to the AR characteristics, in particular AR/steroid binding affinities and the abilities of steroids to induce AR transcription.

The AR is a nuclear receptor characterized by the ability to bind androgens and to mediate androgen-induced male specific behaviour, sexual development, and maturation (Lindzey *et al.*, 1994; Zhou *et al.*, 1994). DHT showed higher AR binding affinity than KT in several teleost species, such as fathead minnow (Wilson *et al.*, 2007), zebrafish (Jorgensen *et al.*, 2007), rainbow trout (Takeo and Yamashita, 2000), kelp bass (*Paralabrax clathratus*) (Staub and DeBeer, 1997), Atlantic croacker (*Micropogonias undulatus*) (Sperry and Thomas, 1999, 2000), stickleback (Olsson *et al.*, 2005) and Japanese eel (Ikeuchi *et al.*, 2001). A more complex situation arises from an analysis of the abilities of steroids to induce transcriptional activity of the AR. DHT was a stronger inducer than KT in Japanese eel (AR subtype 1) (Ikeuchi *et al.*, 1999), and in the rainbow trout (Takeo and Yamashita, 2000), while KT was the most potent inducer in stickleback (Olsson *et al.*, 2005), mosquitofish (*Gambusia affinis*) (Katsu *et al.*, 2007), and Japanese eel (AR subtype 2) (Ikeuchi *et al.*, 1999; Todo *et al.*, 1999).

This variability in the properties of the AR, together with the potential presence of multiple AR isoforms with different characteristics in some species, as shown in Atlantic croacker, kelp bass and Japanese eel (Ikeuchi *et al.*, 2001; Sperry and Thomas 1999a, 1999b), may

suggest that different androgens could mediate the androgenic response in different tissues, according to the species and to the differential expression and abundance of AR isoforms in each tissue (Sperry and Thomas, 2000).

Table 4.7. *Comparison of log effective concentrations of various steroids at 50% displacement (EC50) and relative binding affinity (RBA) for the androgen receptor in human (hAR), rainbow trout (rtAR) and fathead minnow (fhAR). KT and DHT values are highlighted in red (from Wilson et al., 2007). Note the differences in the DHT/KT RBA ratios in the three species, in particular between rainbow trout (0.48) and fathead minnow (1.70).*

	hAR		rtAR		fhAR	
Test chemical	Log EC50	RBA[a]	Log EC50[b]	RBA	Log EC50	RBA
Inert R1881	−8.75 ± 0.05	100.00	−9.42 ± 0.07	100.00	−8.88 ± 0.07	100.00
Methyltestosterone	−8.20 ± 0.12	28.2	−8.65 ± 0.09	16.9	−8.74 ± 0.05	71.7
17α-Trenbolone	−8.16 ± 0.20	26.2	−7.86 ± 0.06	2.8	−8.08 ± 0.04	15.5
17β-Trenbolone	−8.04 ± 0.14	19.7	−7.78 ± 0.08	2.3	−7.75 ± 0.10	7.3
Dihydrotestosterone	−7.60 ± 0.08	7.09	−7.12 ± 0.15	0.49	−7.55 ± 0.09	4.6
11-Ketotestosterone	−7.22 ± 0.09	2.97	−7.43 ± 0.29	1.03	−7.31 ± 0.05	2.7
Progesterone	−7.21 ± 0.27	2.93	−6.97 ± 0.15	0.35	−7.38 ± 0.05	3.1
Androstenedione	−7.04 ± 0.09	1.96	−6.55 ± 0.10	0.14	−7.04 ± 0.08	1.42
Testosterone	−6.92 ± 0.17	1.50	−6.44 ± 0.11	0.11	−6.97 ± 0.06	1.21
17β-Estradiol	−6.46 ± 0.13	0.514	−6.13 ± 0.06	0.052	−5.99 ± 0.03	0.129
M2	−6.09 ± 0.22	0.222	−6.36 ± 0.08	0.089	−6.05 ± 0.13	0.145
M1	−5.62 ± 0.12	0.075	−5.13 ± 0.19	0.0051	−4.72 ± 0.14	0.008
Hydroxyflutamide	−5.60 ± 0.24	0.072	−6.39 ± 0.11	0.0932	−6.40 ± 0.06	0.331
Vinclozolin	−5.58 ± 0.09	0.067	−5.45 ± 0.09	0.0106	−5.23 ± 0.14	0.022
Flutamide	−5.45 ± 0.07	0.0500	−4.98 ± 0.07	0.0036	−5.26 ± 0.07	0.024
Linuron	−4.71 ± 0.07	0.0092	−4.28 ± 0.12	0.0007	−4.41 ± 0.13	0.003
p,p'-DDE[c]	−4.45 ± 0.09	0.0055	−4.21 ± 0.15*[d]	0.0063	−4.47 ± 0.05	0.0038
Ketoconazole	−4.49 ± 0.16	0.0055	−4.20 ± 0.11	0.0006	−4.23 ± 0.24	0.002
Dibutylphthalate	−3.89 ± 0.09*	0.0004	−3.5 ± 0.09*	0.00024	−3.58 ± 0.09*	0.0005
Diethylhexyl phthalate	NA[e]	NA	NA	NA	NA	NA
Atrazine	NA	NA	NA	NA	NA	NA

[a] RBA for each AR = (EC50 of R1881/EC50 of test chemical)·100.
[b] * = data for log EC50 are presented as the mean ± standard error of three replicate assays.
[c] *p,p'*-DDE = 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene.
[d] Estimated EC50. A full displacement curve could not be achieved, so these results are considered to be equivocal.
[e] NA = 50% displacement never achieved at any concentration tested.

In the present study, both KT and DHT accelerated somatic growth processes, increasing both length and weight, and exerted an anabolic effect, as already observed in several studies involving the exposure of fish to androgens. For example, addition of KT (10 mg/Kg) to the diet stimulated the growth of juvenile tilapia (*Oreochromis mossambicus*) (Davis *et al.*, 2010); T and KT exposure caused an increase of muscle size in the plainfin midshipman fish (*Porichthys notatus*) (Brantley *et al.*, 1993a), and an increase of length and weight was recorded in juvenile goldfish and juvenile guppy (*Poecilia reticulata*) exposed to the synthetic androgen trenbolone acetate (TBA) (Herrera *et al.*, 2008; Zamora *et al.*, 2008). The presence of AR in muscles and bones suggest a direct effect of androgens on these tissues (Hofbauer and Khosla, 1999; Hossain *et al.*, 2008; Wiren *et al.*, 1997), causing, for example, muscle hypertrophy and formation of new muscle fibers (Griggs *et al.*, 1989; Kadi, 2000), and

inducing proliferation, differentiation, and mineralization of osteoblastic lineage cells, as shown *in vitro* (Kearns and Khosla, 2004).

The different somatic growth response of males and females to different androgen doses could be due to differential and sex-specific expression of AR in tissues such as muscles, skeleton, and brain. Such differences could account for why 20 ng/L of both androgens significantly stimulated growth of males, but not females, in this study. Although no data are available for fathead minnow, a sex-specific abundance of the AR has been demonstrated in zebrafish tissues, including muscles (de Waal *et al.*, 2008; Hossain *et al.*, 2008), with higher concentrations in males.

SSCs in male fathead minnows are affected by circulating levels of androgens, and females exposed to androgenic compounds can develop male SSCs (Ankley *et al.*, 2003; Panter *et al.*, 2004). In our study, SSCs were induced by both KT and DHT, including the appearance of nuptial tubercles in some treated male and female fish. The same effect was observed on adult pre-spawning fathead minnow (135-150 dph) exposed to 6-8.6 μg/L of DHT for 14 days (Panter *et al.*, 2004), whereas in female sticklebacks DHT (>2μg/L) has been shown to induce the synthesis of spiggin, a male-specific glue protein produced by the kidney and used as cementing substance for the building of the nest (Katsiadaki *et al.,* 2002). Interestingly, the two androgens showed different potencies at inducing the dorsal fin colouration, such that 92% of males and 75% of females exposed to DHT (200 ng/L) had a black spot on the dorsal fin, a typical male characteristic (Parrot *et al.*, 2004; Paulos *et al.*, 2010), whereas only 27% of males and no females exposed to the same concentration of KT had fin spots. The variability in the potency of the two androgens on this specific endpoint could indicate that the process of colouration of the dorsal fin in fathead minnow may not be controlled by KT. In other fish species, plasma levels of T and KT reflect colour pattern, colour intensity and dominance status (Dijkstra *et al.*, 2007; Korzan *et al.*, 2008; Pottinger and Pickering, 1985). Adachi *et al.* (2010) showed that in juvenile red seabream (*Pagros major*), eumelanin accumulation was induced by oral treatment with the synthetic androgen methyl-testosterone (MT), while females bitterlings (*Rhodeus ocellatus*) treated with KT or MT showed male-type nuptial coloration in skin and fins (Kobayashi *et al.*, 2009). Androgen regulation of skin pigmentation is also common in higher vertebrates (Slominski *et al.*, 2004); for example, human genital melanocytes are androgen target cells, expressing the AR in their nuclei, and they also had high levels of 5αR activities, the enzyme that converts T into DHT (Tadokoro *et al.*, 1997). Furthermore, prepubertal male Syrian hamsters treated with DHT exhibited

increased pigmentation on their dorsal costovertebral spots and scrotal skin (Diaz *et al.*, 1986).

One of the most important outcomes of the present study was that DHT stimulated spermatogenesis in immature testes of fathead minnow in a comparable way to KT, which is different from what has been shown in other teleost species. In fact, DHT failed to stimulate spermatogenesis in juvenile African catfish *in vivo* (Cavaco *et al.*, 1998), and showed no effect on spermatogonia mitotic activity of Japanese eel testis *in vitro* (Miura *et al.*, 1991). The latter result does not have a clear explanation, since Japanese eel ARs have higher affinity to DHT than KT, and their transcriptional activity is strongly induced by DHT (Ikeuchi *et al.*, 2001). So far, the effect of DHT on fish spermatogenesis has been investigated only in a limited number of teleost species, but it is evident that currently there is a discrepancy between the molecular data (AR binding and activation by DHT) and physiological data (stimulation of spermatogenesis). In our study, both KT and DHT had a positive effect on the spermatogenic processes, in particular inducing a dose-dependent advancement from the juvenile stage (gonad with only spermatogonia) to Stage 0 (spermatogonia to spermatids) and Stage 1 (early spermatogenic, containing spermatozoa). It is important to note that only DHT (200 ng/L) induced the appearance of spermatozoa in more than 80% of male fish, versus 35.7% of fish exposed to the same concentration of KT. These results suggest that the influence of DHT on fathead minnow spermatogenesis is at least comparable, or even stronger, to the one induced by the putative physiological androgen KT.

A surprising effect was the development of spermatogenic tissue in more than 80% of the ovaries of juvenile females exposed to DHT (200 ng/L) and even in one individual exposed to 20 ng/L. On the contrary, KT did not induce any similar disruption. This severe effect could be linked to the different potencies of the two androgens at inducing androgenic molecular and physiological responses. Ovarian masculinisation has been recorded in several studies involving exposure to androgens. TBA induced the appearance of spermatogenic tissue in ovaries of female juvenile mosquitofish (Sone *et al.*, 2005), and irreversible masculinisation of female zebrafish exposed between 0 and 60 dph (Morthost *et al.*, 2010). Furthermore, an irreversible masculinisation was observed in zebrafish exposed to the aromatase inhibitor fadrozole during gonad differentiation, probably due to an accumulation of androgens during a critical process, such as gonad differentiation (Fenske and Segner, 2004).

The mechanisms of action by which androgens masculinise ovaries are not clearly understood. Some evidence suggests that androgen-induced masculinisation acts through the suppression of steroidogenic enzyme expression, including P450-aromatase, and that the

action is probably mediated by the AR. In fact, several studies have observed an androgen-induced suppression of P450-aromatase gene/protein expression (Bhandari *et al.*, 2006; Devlin and Nagahama, 2002; Govoroun *et al.*, 2001; Vizziano *et al.*, 2008). Furthermore, the 5'-flanking region of the ovarian form of P450-aromatase contains an androgen responsive element (ARE) (Tong and Chung, 2003). The consequent inhibition of estrogen production probably leads to the down-regulation of key genes involved in differentiation of the ovary (foxl2a, fst, fshb), and at the same time the over-expression of some testicular genes, such as nr0b1 and dmrt1 (Vizziano *et al.*, 2008), which show distinct expression patterns during testicular differentiation in the rainbow trout (Marchand *et al.*, 2000), tilapia (Guan *et al.*, 2000), medaka (Kobayashi *et al.*, 2004), and black porgy (Wu *et al.*, 2005).

The variability along the longitudinal axis of the ovaries, or between left and right ovary, is probably due to the fact that the intersex condition develops as a local *in situ* disruption, with exogenous steroids acting locally and directly on germ cell development (Devlin and Nagahama, 2002), and underlines the importance of screening the ovary at different points along the longitudinal axis when looking for this kind of disruption in juvenile fish.

The failure of KT to induce severe disruption in the ovary, such as the intersex condition, suggests that KT and DHT could have different interaction characteristics with the ovary AR. The presence of AR in the female reproductive tissues of many vertebrates, including fish, suggests that androgens could play an important role in the regulation of female physiology (Nitta *et al.*, 1991; Staub and DeBeer, 1997; Tetsuka and Hiller, 1996). The androgen KT has also been found in several female fish (Lokman *et al.*, 2002); however, its physiological role, if any, in females is still unclear. There is some evidence showing that KT plays a role in controlling growth of pre-vitellogenic oocytes in immature female fish (Kortner *et al.*, 2008; Matsubara *et al.*, 2003), and these data are confirmed in our study. At 20 ng/L, KT caused an increase in the size of all classes of oocytes, but showed mixed responses at 200 ng/L in different oocyte classes, with both size increases and decreases. This effect could be explained assuming that, at low dose, KT plays a stimulating role, while at high dose the androgenic action appears to be more relevant, causing the regression of ovaries toward previtellogenic stages. DHT, on the other hand, showed only limited effects on oocyte growth, such as an increase in the dimensions of M-LVOs; however, caution is needed when interpreting this result, because there were only a few ovaries not containing spermatogenic tissue that were available for analysis.

The feedback caused by KT and DHT on plasma circulating T was positive in males, and negative in females. The increase of plasma T levels in males exposed to both androgens

could be a consequence of a positive feedback on the HPG axis, or of an increase of T producing cells (Leydig cells) in the stimulated testis. In females, a similar reduction of plasma T was recorded by Ankley *et al.* (2003) after exposure of reproductively active fathead minnow to the androgen TBA for 21 days, and by Sharpe *et al.* (2004), who exposed mummichog to MT. This result is probably a direct consequence of negative feedback on the HPG axis, but is not clearly linked with the masculinisation of the ovaries.

In conclusion, the results described in this Chapter show for the first time, to our knowledge, that DHT has an *in vivo* androgenic potency comparable to that of KT in juvenile male fathead minnows, acting on somatic growth, SSCs expression, and spermatogenesis; whereas in females, DHT caused completely different effects than KT, inducing severe disruption of ovarian physiology and morphology, including the development of spermatogenic tissue. These results strengthen the hypothesis that the concentrations of DHT quantified in the plasma of both male and female fathead minnows during the experiments described in Chapter 3 are physiologically relevant.

CHAPTER 5 : EFFECTS OF DUTASTERIDE ON THE EARLY LIFE STAGES OF THE FATHEAD MINNOW

5.1 Introduction

Fish embryo development and early life stages represent a critical window of sensitivity for the action of endocrine disruptors (van Aerle *et al.*, 2002). The fathead minnow is commonly employed in partial life-cycle tests to determine lethal and sub-lethal effects both of single chemicals and complex mixtures (Ankley and Villeneuve, 2006). In particular, in recent years, increasing attention has been paid to the early life-stages toxicity tests, as a growing number of studies suggest that data obtained exposing fish to toxicants during early development can be predictive of chemical effects in adults (McKim, 1977).

Several regulatory agencies have developed standardized toxicity tests in order to assess the potential toxicity of chemicals on fish early life stages (ASTM, 2000; OECD, 1992; USEPA 1989; USEPA, 2002). A summary of fathead minnow life-stages, protection status, and duration of standard OECD toxicity tests is represented in Figure 5.1 (Embry *et al.*, 2010). In particular, OECD Test 210 has been used as the reference test for the experiments described later in this chapter. During the test, fish early-life stages are exposed to a range of concentrations of a test substance dissolved in water, preferably under flow-through conditions. At the beginning of the test, fertilised eggs are placed in the test vessels and exposed at least until all the control fish are capable of external feeding (often until 30 dph). Lethal and sub-lethal effects are assessed, and the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) are determined (OECD, 1992).

Exposure of fish to endocrine disruptors during the embryo development phase and early life stages could affect critical processes such as gonadal sex determination (the genetic or environmental process by which the sex of an individual is established) and the associated sex differentiation (the process of gonad development after sex has been determined). The genetic, molecular, biochemical and environmental factors regulating these important processes are extensively described by several reviews (Baroiller *et al.*, 2009; Devlin and Nagahama, 2002; Nakamura, 2010; Penman and Piferrer, 2008; Piferrer and Guiguen, 2008; Sandra and Norma, 2010), thus in the following sections, only an overview of the main developmental processes occurring during fish early life stages will be provided.

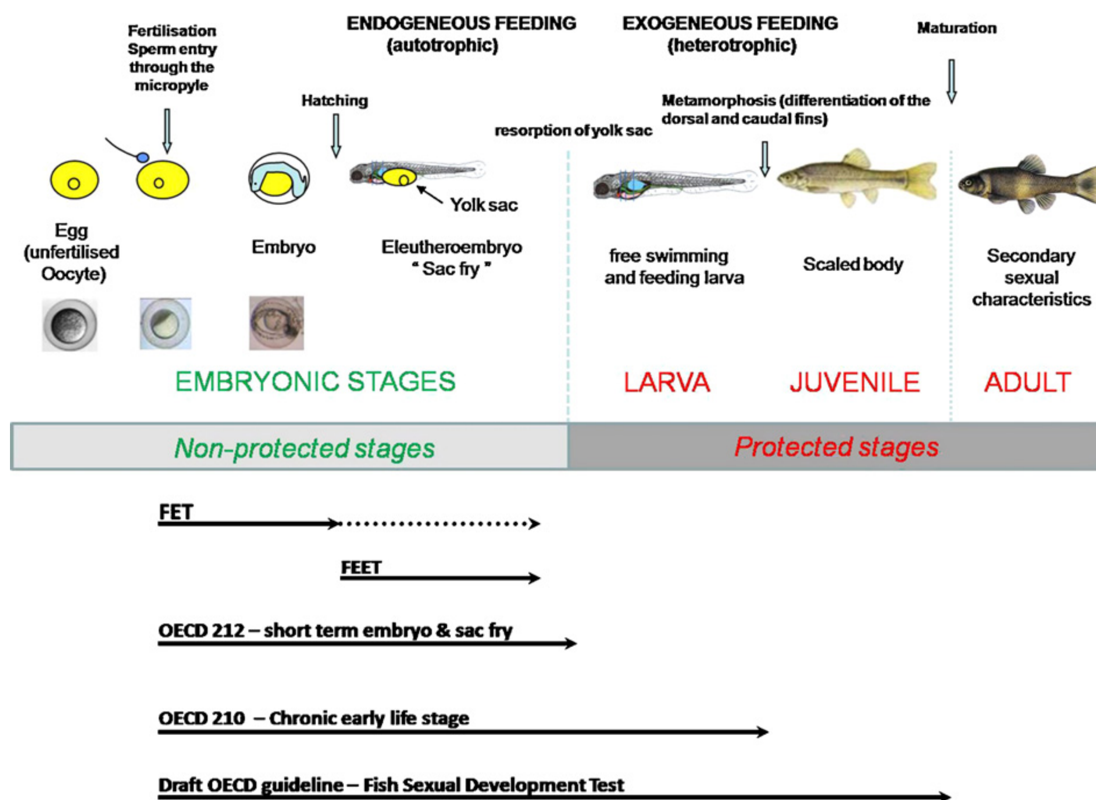


Figure 5.1. *Fish life-stages, protection status, and duration of standard toxicity tests. Solid arrows indicate the duration of the referenced tests; the dashed arrow indicates the additional duration of the "extended" fish embryo test (FET), which includes the eleutheroembryo stage. FEET stands for fish eleutheroembryo toxicity test (from Embry et al., 2010).*

5.1.1 Fathead minnow embryo development

The development of fathead minnow is basically similar to that of zebrafish and other teleost fish (Manner and Dewese, 1974); some differences are present in the timing of development, and are probably due to the different temperatures set as optimal for each species. The main embryo developmental stages are represented in Figure 5.2, and include blastulation, gastrulation, completion of somites and optic cup formation, spontaneous contraction and tail detachment, retinal pigmentation, hearth beating, body pigmentation and commencement of hatching.

5.1.2 Endocrine and molecular control of sex differentiation

Endocrine control of sex differentiation involves a complex bi-directional link between brain and gonad, along the HPG axis. This link is estabilished through signal molecules such as gonadotropins, produced by gonadotrope cells of the pituitary gland, and steroids produced in gonads and brain (Le Page *et al.*, 2010; Nagahama, 1994; Nagahama, 2005). Sex steroids exert a local and direct action on germ-cell development, but also act by influencing other cell types and organs involved in sex differentiation. A large number of biochemical, neurological,

and physiological pathways are involved in this complex multilevel control process, and this complexity is of critical importance to provide the necessary plasticity to the developing gonad, which allows it to proceed in a dynamic way with the influence of intrinsic and environmental factors, but at the same time, provides many levels at which the system can be disrupted by chemicals with endocrine interfering properties (Devlin and Nagahama, 2002; Penman and Piferrer, 2008; Piferrer and Guiguen, 2008).

A broad debate is occurring on whether sex steroids play a fundamental role in the sex determination mechanism of teleost fish, or if the latter is only regulated by intrinsic genetic processes, independently from the surrounding endocrine environment. Medaka is one of the few species of teleost fish in which a clear genetic mechanism of sex determination has been discovered, with a sex-specific gene linked to the Y-chromosome, called *dmy*, which is required for testicular development, as happens in mammals with the *SRY* gene (Matsuda *et al.*, 2002). Iwamatsu *et al.* (2006) demonstrated that genetic XX females without *dmy* can be sex-reversed to functional males if exposed to androgens during the pre-hatch phase (0-10 dpf), long before the beginning of gonadal sex differentiation. Furthermore, several pieces of evidence are available indicating that a few hours of exposure of embryos at the morula stage to sex steroids (androgens and estrogens) can respectively induce sex reversal of genotypic females into phenotypic males (Kobayashi and Iwamatsu, 2005), and the contrary (Iwamatsu *et al.*, 2005). A very large literature on the manipulation of sex differentiation in fish with exogenous steroids is available and it has been reviewed by many authors (Nakamura, 2010; Pandian and Sheela, 1995; Piferrer, 2001). Moreover, the exposure to an aromatase inhibitor during early developmental stages can lead to the masculinisation of female fish as a consequence of the inhibition of E2 biosynthesis (Afonso *et al.,* 2001; Guiguen *et al.*, 1999; Kitano *et al.*, 2000; Piferrer *et al.*, 1993). These results seem to confirm that the action of sex steroids appears to affect definitive sex determination of embryos during a short period of the early developmental stage. In particular, the mechanism proposed by Iwamatsu *et al.* (2006) suggests that independently from the expression of Y chromosomal *dmy*, sex steroids alter the cascade of expression of gonad-specific genes (e.g. *dmrt*1) involved in the differentiation of the somatic cells surrounding primordial germ cells, acting as regulatory elements in the sex-determining pathway.

5.1.3 Ontogenesis of steroid production in fish

It is currently unknown how sex-determination mechanisms influence the gonadal developmental processes leading to steroid production and gonad differentiation (Devlin and Nagahama, 2002). Generally, developing fish embryos have relatively high levels of sex

steroids just after fertilization, declining levels during yolk sac absorption, and increasing levels during the fry stage (Feist and Schreck, 1990; Feist *et al.*, 1990; Rothbard *et al.*, 1987; Yeoh *et al.*, 1996). Feist and Schreck (1996) suggested that the temporal changes in steroid levels during development reflect an initial metabolism of maternal steroids by embryos, followed by endogenous steroid synthesis by the newly hatched individuals.

Changes in the levels of endogenous steroids during development are due to ontogenetic changes in the enzymes involved in steroid biosynthesis and metabolism (Antila, 1984; Goldstone *et al.*, 2010; Hsu *et al.*, 2009; Yeoh *et al.*, 1996). Molecular, histochemical and ultrastructural studies have shown that steroidogenic enzymes and steroid producing cells occur at specific times during early development in several teleost species (Hsu *et al.*, 2009; Nakamura and Nagahama, 1993; Takahash and Iwasaki, 1973; Trant *et al.*, 2001). Changes in the levels of steroids in developing larvae before histological evidence of gonadal differentiation suggest as well that sex steroids may play a role in the sex differentiation process (Feist and Schreck, 1990; Feist and Schreck, 1996; Feist *et al.*, 1990; Yeoh *et al.*, 1996). A related theory has been proposed by Bogart (1987), suggesting that the local ratio androgens:estrogens determines the gonadal sex. However, other authors argue that sex steroid production in teleosts is possible only after the occurrence of gonadal differentiation mechanisms (Antila, 1984; Rothbard *et al.*, 1987; Takahash and Iwasaki, 1973; Takahash and Iwasaki, 1973; Vandenhurk *et al.*, 1982), and that steroids are not sex determining factors, but only the result of sexual differentiation (Wilson *et al.*, 1981). Experiments involving exposing medaka to aromatase inhibitors and antiestrogens showed that ovary development may occur independently from the action of estrogens (Kawahara and Yamashita, 2000). Further support for this hypothesis is the fact that steroid producing cells are generally not histologically evident before testicular or ovarian differentiation (Nakamura and Nagahama, 1985), as demonstrated in medaka (Satoh, 1974), even if there are some exceptions, like some poeciliid species, where differentiation of somatic tissues precedes the germ cell differentiation (Kramer and Kallman, 1985).

Independently from the exact initiation mechanisms of the sex determination and differentiation, an objective fact is that steroids are strictly correlated with all the early steps of sex determination and gonadal differentiation, and if steroid synthesis is disrupted, these processes can be disregulated.

5.1.4 Hormonal control of sexual maturation

During sexual maturation, the dramatic changes occurring in steroid biosynthetic activity have profound effects on both somatic and germinal tissues (Devlin and Nagahama, 2002; Kagawa, 2010; Kramer and Kallman, 1985; Piferrer *et al.*, 2005; Villeneuve *et al.*, 2010). In males, plasma levels of steroid hormones show important variations during gonad maturation. Generally, estrogens, like E2, are present in blood at low concentrations (Amer *et al.*, 2001; Chaves-Pozo *et al.*, 2008; Miura *et al.*, 1994). E2 plasma levels show a temporary increase during the spawning period in male roach (Geraudie *et al.*, 2010). Androgens (T, KT) increase gradually with the progression of spermatogenesis, and then decrease at spermiation (Schulz *et al.*, 2010). Androgens are critically important in the spermatogenic process, in particular during some specific events, such as spermatogonial multiplication, spermatocytes formation and maturation (Borg, 1994; Nagahama *et al.*, 1994; Schulz *et al.*, 2010; Schulz and Miura, 2002). They may also have a key role in the initiation of puberty (Cavaco *et al.*, 1998; Goos and Schulz, 1997; Schulz and Goos, 1999), and in the induction of spermiation in some species, even if they seem to be less effective than progestins (Miura *et al.*, 2006; Miura and Miura, 2008). In fact, high levels of circulating progestins are observed during the spermiation phase, and these hormones appear to regulate several testicular functions (Schulz *et al.*, 2010; Miura and Miura, 2008). For example, 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) is able to stimulate and induce spermiation (Miura *et al.*, 1991), increase milt production (Baynes and Scott, 1985; Yueh and Chang, 1997), and stimulate spermatozoa motility (Miura *et al.*, 1992; Tubbs and Thomas, 2008).

In females, the production of T and E2 during sexual maturation is generally reduced, and production of 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) is highly enhanced (Nagahama, 1994; Nagahama, 1997). The rise in 17,20β-P concentration during the final phases of sexual maturation is responsible for inducing final maturation of oocytes (Kobayashi *et al.*, 1987; Nagahama, 1987; Nagahama, 1997). Other steroids capable of inducing oocyte maturation (e.g. 17,20β, 21-trihydroxy-4-pregnen-3-one) have also been identified (Scott and Canario, 1992; Trant *et al.*, 1986), suggesting that different steroids may play the role of ovarian maturation hormones in different fish species (Devlin and Nagahama, 2002).

5.1.5 Fathead minnow histological sex differentiation

A detailed histological description of the timing and morphology of testicular and ovarian development in the fathead minnow has been published by van Aerle *et al.* (2004), and is represented in Figure 5.3. The beginning of ovarian differentiation was first seen at 10 dph, when gonads of some of the fry contained cysts of pre-meiotic cells located in the centre of

the gonad. The ovaries developed rapidly and at 25 dph the ovaries contained germ cells of all stages up to the primary oocyte stage.

On the other hand, germ cell differentiation in the testis occurred only after 90-120 days post-hatch. This gap in the timing of gonad development, where the ovary in the female develops earlier than the testis in males, has been observed in many gonochoristic fishes (Nakamura *et al.*, 1998).

In teleost fish, the spatial distribution of somatic cells in the gonad during the period of sex differentiation is often gender-specific, and this factor can be used to identify the sex of the gonad (Nakamura *et al.*, 1998). This spatial specificity was also observed by van Aerle *et al.* (2004) in the fathead minnow prior to the presence of definitive sex cells; in ovaries, pre-meiotic cells occurred in the centre of the gonad with somatic cells at the gonad periphery, whereas in males somatic cells were scattered throughout the gonad amongst the germ cells. The spatial organization of germ and somatic cells, together with the development of sex-specific morphological characters of the gonad, such as the sperm duct, can indicate the occurrence of a male gender before germ cell differentiation is histologically evident.

5.2 Aim of the study

Fish embryo development and early life stages represent a critical window of sensitivity for the action of endocrine disruptors (van Aerle *et al.*, 2002). The majority of the scientific literature on the action of endocrine disruptors on fish early life stages is based on the effects caused by steroid agonists, and only sparse information is available on the effects of anti-steroids on these critical life stages.

The major aim of the study was to assess the potential effects of dutasteride on fathead minnow embryo development, larval growth and survival. Furthermore, we designed an experiment in order to clarify if an early life stage exposure to the drug could have long term effects on adult fish, for example impairing gonad differentiation processes. Moreover, thanks to the highly specific mechanism of action of the drug (i.e. inhibition of 5αR Type 1 and Type 2), a further aim was to obtain precious information about the potential role of DHT in fathead minnow sex differentiation.

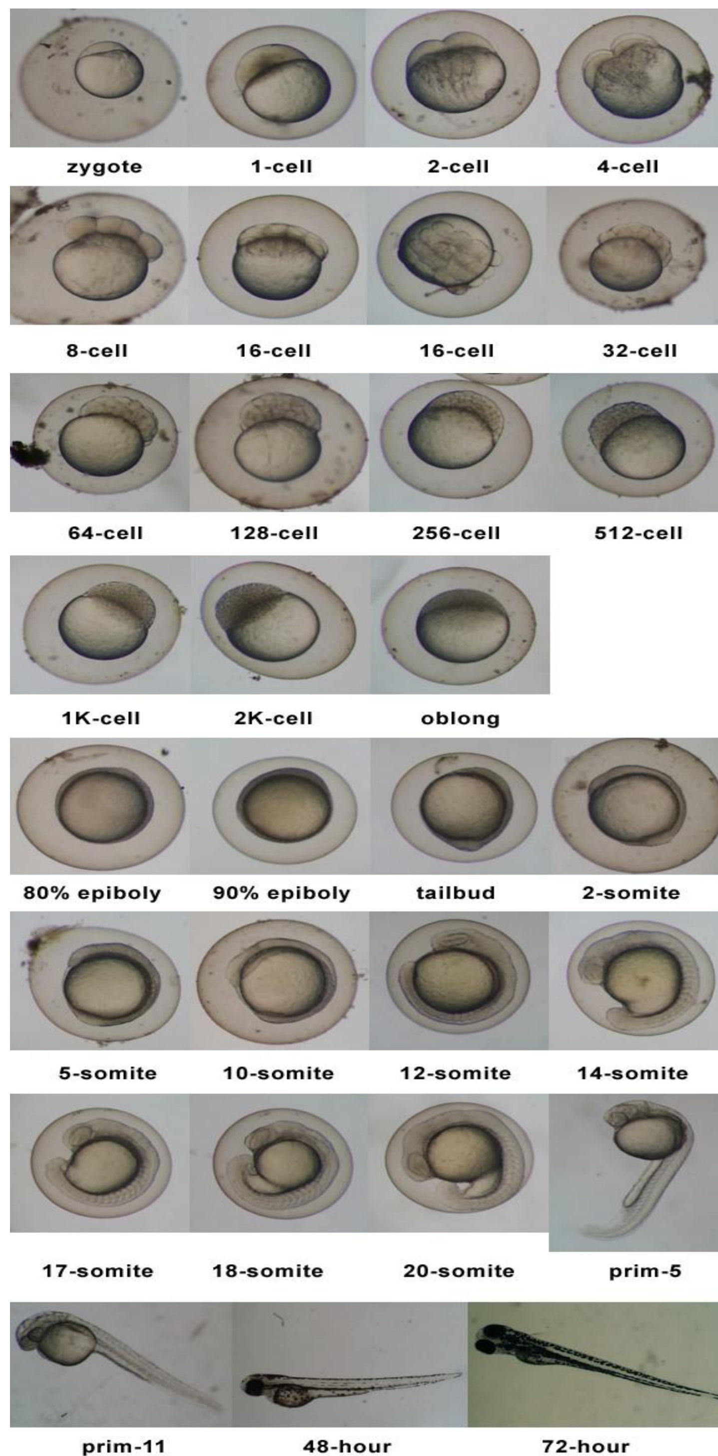


Figure 5.2. *Photographs showing specific stages in the early development of zebrafish (from Kimmel et al., 1995).*

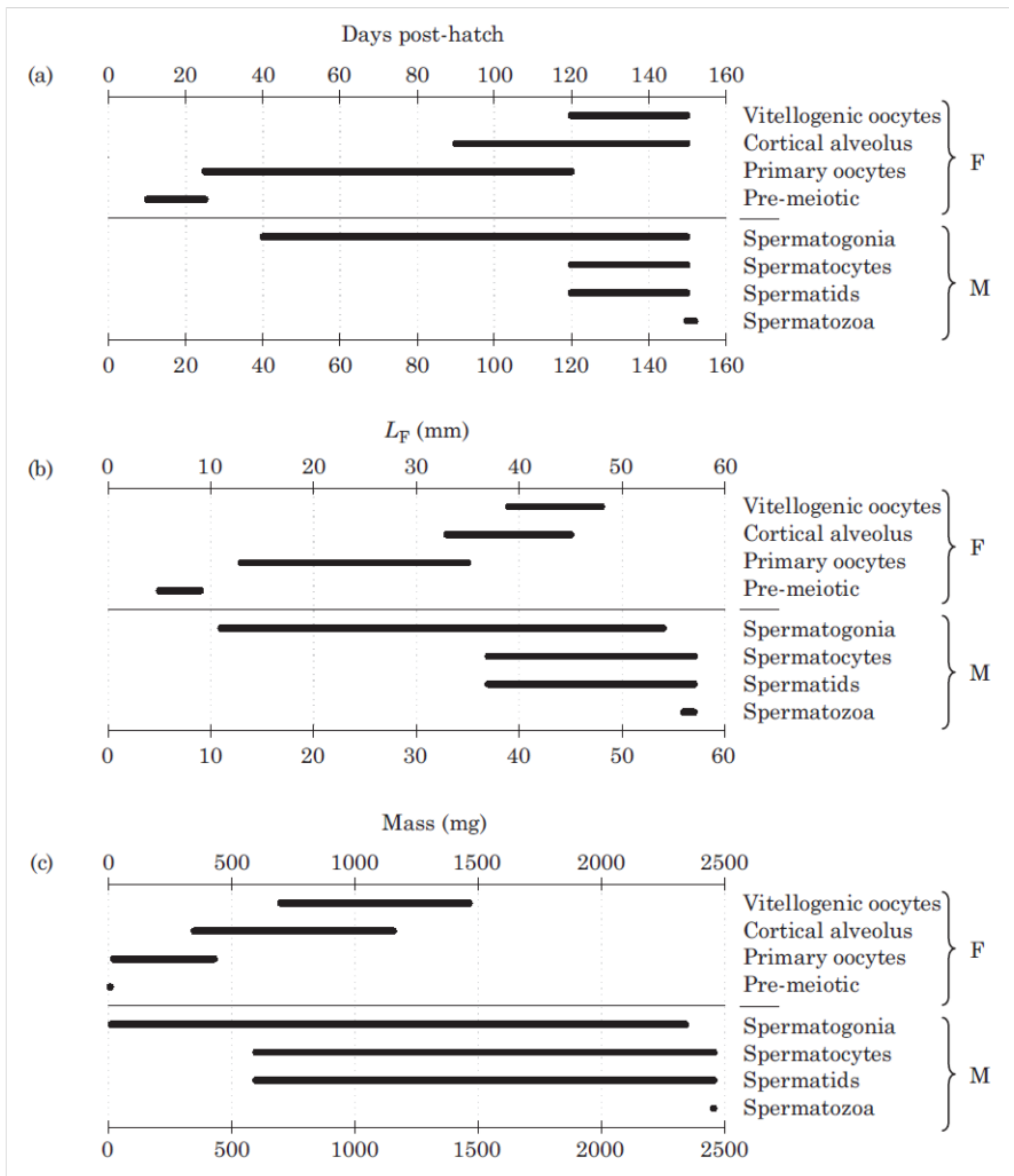


Figure 5.3. *Presence of the various sex cell types in gonads of fathead minnow females (F) and males (M) in relation to (a) age, (b) fork length and (c) mass (from van Aerle et al., 2004).*

5.3 Methods

5.3.1 Test species

Fathead minnow newly fertilized embryos (collected at < 6 h post fertilization) were obtained from a stock held at Brunel University, London, UK. During the early life stages tests, fry were fed twice a day with a combination of pelleted fish food SDS 100 (80-200 µm particles) (Special Diet Service, UK) and *Artemia salina* nauplii, and as the fry developed, *A. salina* were supplemented with increasing size of pelleted fish food SDS 200-300-400 *ad libitum* (150-800 µm particles) (Special Diet Service, UK). After 40 dph, fish were fed twice a day with a combination of flake food and frozen (thawed before use) brine shrimp *ad libitum*.

5.3.2 Test substances and dilution water

Dutasteride (CAS number 164656-23-9) was obtained from GlaxoSmithKline (GSK) as 99.9% pure. Concentrated stock test solutions were prepared in N,N-Dimethylformamide (DMF, CAS number 68-12-2, ≥99%) (Sigma, Poole, UK). One master concentrated stock solution (5.0 g/L) was made in DMF, stored at +4 °C, and used weekly to make up fresh dosing stock solutions at 0.089, 0.28, 0.89, and 2.78 g/L.

The dilution water was monitored for general parameters (e.g. pH, hardness, conductivity). During the exposure studies, mean water pH ranged from 7.6 to 8.2, temperature from 24.91 to 26.32 ºC, and dissolved oxygen from 6.79 to 7.92 mg/L.

5.3.3 Test conditions and exposure protocol

5.3.3.1 *Fish early life test 1 (ELS Test 1)*

The fish ELS Test protocol was based on the OECD Test Guideline 210 (OECD, 1992). The experiments were carried out using a continuous flow-through system. The test apparatus comprised glass fish tanks (4 per treatment) with a working volume of 10.5 L, and two embryo incubation mesh baskets (Cadisch Ltd, UK) suspended in each test vessel. Thermostatically heated (25 ± 1°C) dechlorinated tap water from a header tank flowed through 6 flow-meters into 6 mixing chamber at a rate of 333.3 mL/min. The same chambers also received the test chemical stock solution in DMF at a rate of 0.012 mL/min, in order to achieve the desired nominal concentrations. Separate lines from each mixing chambers supplied about 9 tank volume changes per day to each of the four replicate tanks. The final experimental set up included six groups: one dilution water control group (DWC), one solvent control group (SC), and four dutasteride exposed groups (3.2, 10, 32, 100 µg/L). Each SC

tank received DMF at the same rate as the exposed groups, such that the water in all test vessels contained DMF at 0.0036% (OECD suggested limit: 0.0095%). The described experimental set up was run for 4 days before the beginning of the embryos exposure, in order to allow the system to equilibrate. The test was run at a nominal water temperature of 25 ± 1 °C, with a photoperiod of 16 hours light: 8 hours of dark, and with 20 minute dawn/dusk transition periods. The light intensity was measured by a lux meter (Lutron, LX-101) once a week during the study.

Fertilized eggs from a minimum of six adult pairs were gently removed from the PVC tiles, collected and pooled in a Petri dish. Under light microscopy observation, groups of 10 embryos were randomly selected and transferred to each incubation basket until reaching a sample size of 60 embryos in each replicate tank (30 embryos in each incubation basket). All the embryos were no older than 6 hours post fertilization (early cleavage to blastula stage). The numbers of dead and hatched embryos were recorded daily, and any dead embryo was discarded to avoid fungi contamination. When hatching was at least 90% complete, or 24 hours after the first hatch (approximately after 4-6 days), larvae were released in the tank, hatching time recorded, and baskets removed. Daily observations of mortality, behaviour and appearance were made and recorded.

The exposure period was terminated after 28 dph, when 8 fish from each replicate (32 fish per treatment) were sacrificed by exposure to an overdose of anaesthetic (500 mg/L of MS222, adjusted to pH 7.5 using 1 M NaOH), measured for fork length and wet weight, followed by trans-spinal severance. The CI was calculated as described in Section 4.3.3. Whole fish were then placed in sterile tubes and stored at -80ºC for future molecular analysis.

5.3.3.2 *Fish recovery study in clean water*

Remaining fish from the ELS Test 1 were transferred to 30 L test vessels and kept in clean dilution water until the end of the study. A schematic picture of the dosing regime and sampling time is shown in Figure 5.4.

At 120 dph, 10 fish per tank (40 per treatment) were randomly sacrificed by exposure to an overdose of anaesthetic. Due to the lower number of surviving individuals, only 8 fish per replicate (32 per treatment) were sampled from the group exposed to 100 µg dutasteride/L. Blood was collected, and after trans-spinal severance, fork length and wet weight were measured, and CI calculated as described in Section 4.3.3. Since at this stage only a few fish had developed external SSCs in each treatment group, it was not possible to externally

distinguish males and females, and SSCs assessment was not applied. The body cavity was opened and sex was assigned according to visual inspection of the appearance of the gonads.

Since exposure to the high dose of dutasteride (100 µg/L) in the ELS Test 1 caused decreased fish survival (see Results, Section 5.4.2), at 120 dph, only for this group, two (rather than four) new replicate tanks were created, pooling fish in replicate tanks 1 and 2, in pool 100A, and fish in replicate tanks 3 and 4, in pool 100B. This protocol modification was required since it is known that fish density in the test vessel can cause variation in the growth of individuals, because of the consequent competition for food and space (an effect known as "growth depensation" or "size hierarchy effect") (Brown, 1957; Woltering, 1984).

A further sampling was performed at 160 dph, when 8 males and 8 females per treatment were randomly collected, and sacrificed by exposure to an overdose of anaesthetic. Blood was collected from the caudal vein, standard length and wet weight recorded, and CI calculated as described in Section 4.3.3. After SSCs assessment, each fish was slit ventrally in order to allow the fixative to penetrate the body cavity, prior to fixation in Bouin's and subsequent storage in 70% IMS, for histopathological analyses.

5.3.3.3 ***Fish early life stage test 2 (ELS Test 2)***

A repeat of an ELS Test was performed with the same test conditions and experimental procedure, as described in Section 5.3.3.1, with the following modifications. Only three dutasteride concentration were tested (10, 32 and 100 µg/L), the number of embryos allocated in each test vessel was 25 (6 replicates for the groups DWC, SC and 100 µg/L; 4 replicates for the groups 10 and 32 µg/L), and the exposure period was terminated at 14 dph, when fish were sacrificed by exposure to an overdose of anaesthetic, and 15 randomly selected fish per replicate were measured for fork length and wet weight. Considering the small size of the sampled fish, pools of three fish were used for the wet weight determination, in order to obtain a more accurate measurement (to 0.010 mg). CI calculated as described in Section 4.3.3.

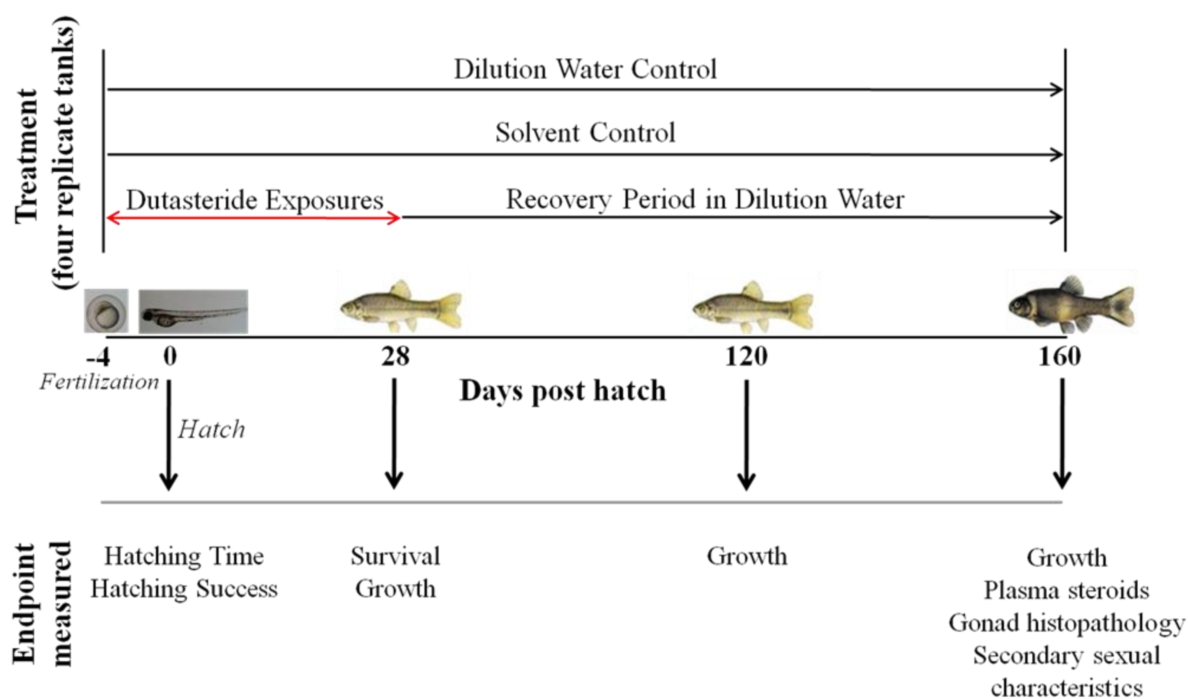


Figure 5.4. *Experimental design for exposure of early life stage fathead minnows to dutasteride. For each control and treatment group there were four replicate tanks. Endpoints measured were: Day 0 - hatching time, hatching success; Day 28 – survival, growth; Day 120: growth; Day 160: growth, plasma steroids, gonad histopathology, and secondary sexual characteristics.*

5.3.4 Gonadal histopathology

Three slices of 3-4 mm thickness were transversally cut from the each gonad, dividing it in anterior, median, and posterior parts. Slices were prepared for the histopathology analyses following the method detailed in Section 4.3.6, and analysed according to the EPA guideline described in the same section (EPA, 2006).

5.3.5 Determination of circulating plasma steroid concentrations

Circulating plasma levels of T and KT in fish sampled at 160 dph were quantified by specific radioimmunoassays, as described in Section 4.3.5.

5.3.6 Chemical analyses of water concentrations of dutasteride

Water samples were randomly collected from two out of four replicates per treatment on day 0, 4, 7, and then weekly for the duration of the study. Water samples were diluted with acetonitrile (50:50 *v/v*) and analysed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The samples were quantified against standards of dutasteride.

The LC-MS/MS technique is based on the same principles described in section 4.4.1.4., with the only difference that the mobile phase used during the separation phase is a liquid instead of a gas.

The LC-MS/MS apparatus included an HPLC Agilent 1050 Series (Agilent Technologies, Waldbronn, Germany), equipped with a Perkin Elmer autosampler 200 Series. The LC separation was achieved using an Ascentis C18 column (3 µm, 50 mm × 3 mm). The mobile phase used was a mixture of 10 mM ammonium formate buffer/acetonitrile (15/85 *v/v*, pH adjusted to 3.0 with formic acid), which was pumped at a flow-rate of 0.2 mL/min. Mass spectrometric detection was performed on an API 365 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) with a turbo electrospray interface in positive ionization mode. The main working parameters of the mass spectrometer are summarized in Table 5.1. Data processing was performed on Analyst 1.3 software package (SCIEX).

Table 5.1. *Tandem mass-spectrometer main working parameters used for the quantification of dutasteride in the water samples.*

Parameter	**Value**
Source temperature (ºC)	300
Dwell time per transition (ms)	1000
Curtain gas (psi)	8
Collision gas (psi)	4
Ion spray voltage (V)	4800
Entrance potential (V)	10
Declustering potential (DP) (V)	54
Collision energy (V)	50
Collision cell exit potential (V)	15
Mode of analysis	Positive
Ion transition for dutasteride (*m/z*)	529.1/461.3

5.3.7 Statistical analyses

Statistical analyses were conducted using SigmaStat software (version 3.5, Systat Software Inc., Germany). Data for hatching success, survival, wet weight, fork length, condition factor, SSCs and plasma steroids concentrations were analysed for normality (Kolmogorov-Smirnov

test) and variance homogeneity (Levene's test). Where assumptions of normality and homogeneity were met, one-way analysis of variance (ANOVA) was followed by the Dunnett's test to compare the treatment means with respective controls. Where the assumptions were not met, data were analysed using a non-parametric test, Kruskal–Wallis ANOVA on Ranks, followed by Dunn's post-hoc test (Zar, 1999). Survival was analyzed by Kaplan-Meier Survival Analysis (Log-Rank). Statistical comparisons were made to determine if there were significant differences between the DWC and SC groups, by t-test or, in case of non-normal and non-homogeneous data, by Mann-Whitney Rank Sum test; all treatment comparisons were made against the SC group. At 160 dph, all the data were analysed according to the sex of the fish.

Effects of treatments on gonadal developmental stage, most advanced stage of gametogenesis, and dominant germ cell were analysed using a variation of the chi-squared test, the randomization test of goodness of fit with Montecarlo simulation (n = 10,000) (McDonald, 2009).

Where necessary, data were log-transformed for normalization and to reduce variance heterogeneity; percentage data were arc sine transformed. The results are reported as means ± standard error of the mean to indicate the precision of the mean estimate. Statistical significance was set at a level of $p = 0.05$, unless otherwise indicated.

5.4 Results

5.4.1 Water concentrations of dutasteride

Water analysis showed that fish were exposed to the test substance in the expected range of concentrations and that there was no contamination of control tanks with dutasteride. In ELS Test 1, concentrations ranged from 70% to 156% of the nominal values (Table 5.2), and in ELS Test 2 from 68% to 138% (Table 5.3).

5.4.2 Hatching success, hatching time and survival.

Dutasteride didn't have any significant effect on hatching success and hatching time in both ELS Test 1 and ELS Test 2 (Figure 5.5). The detailed results for each replicate tank are shown in Table 5.4. Considering the effects of dutasteride on larvae survival and growth (see below), it is plausible that the embryo chorion acted as a barrier to the penetration of the drug.

After the embryos hatched, the highest dutasteride concentration (100 µg/L) had a significant negative effect on larval survival (Figure 5.6). The same survival dynamics were recorded in both ELS Test 1 ($p < 0.0001$) and ELS Test 2 ($p = 0.0004$). In the first experiment, at the end of the exposure period (28 dph) only 55.20% ± 10.3 of fish had survived in the highest concentration group, while in the second experiment, at 14 dph, only 65.44% ± 7.38 of fish survived with the same treatment (100 µg/L), compared to a mean survival value higher than 80% in all the other groups, in both experiments (Table 5.4).

Table 5.2. *Measured water concentrations (µg/L) of dutasteride during the fathead minnow early-life-stage exposure study 1.*

Exposure Day	Replicate	Nominal concentrations of dutasteride (µg/L)					
		*DWC	*SC	3.2	10	32	100
0	A	< 0.5	< 0.5	3.84	14.25	32.11	96.33
	B	< 0.5	< 0.5	4.03	5.32	12.12	75.24
3	A	< 0.5	< 0.5	4.22	12.58	32.58	129.77
	B	< 0.5	<0.5	4.47	10.83	29.26	125.59
7	A	< 0.5	< 0.5	3.91	11.31	24.13	123.50
	B	< 0.5	< 0.5	4.28	12.43	33.06	78.09
14	A	< 0.5	< 0.5	3.67	14.16	34.01	87.59
	B	< 0.5	< 0.5	5.02	11.34	29.26	60.99
21	A	< 0.5	< 0.5	3.80	11.12	35.15	87.78
	B	< 0.5	< 0.5	5.51	12.85	27.74	91.96
32	A	< 0.5	< 0.5	3.25	8.63	34.39	93.67
	B	< 0.5	< 0.5	4.20	9.39	41.23	101.08
Mean Values (µg/L)		**< 0.5**	**< 0.5**	**4.18 ± 0.33**	**11.18 ± 1.45**	**30.43 ± 5.09**	**95.97 ± 19.12**

** The analysis detection limit is quoted.*

Table 5.3. *Measured water concentrations (µg/L) of dutasteride during the fathead minnow early-life-stage exposure Study 2.*

Exposure Day	Replicate	Nominal concentrations of dutasteride (µg/L)				
		*DWC	*SC	10	32	100
0	A	< 0.1	< 0.1	13.36	31.35	98.59
	B	< 0.1	< 0.1	13.89	34.77	91.80
	C	--	--	--	--	101.52
7	A	< 0.1	< 0.1	11.95	23.75	91.20
	B	< 0.1	< 0.1	9.69	25.08	123.01
	C	--	--	--	--	76.40
14	A	< 0.1	< 0.1	11.70	30.40	74.80
	B	< 0.1	< 0.1	9.29	24.70	68.40
	C	--	--	--	--	71.60
Mean Values (ng/L)		**< 0.1**	**< 0.1**	**7.76 ± 5.59**	**28.34 ± 4.38**	**88.59 ± 14.71**

** The analysis detection limit is quoted.*

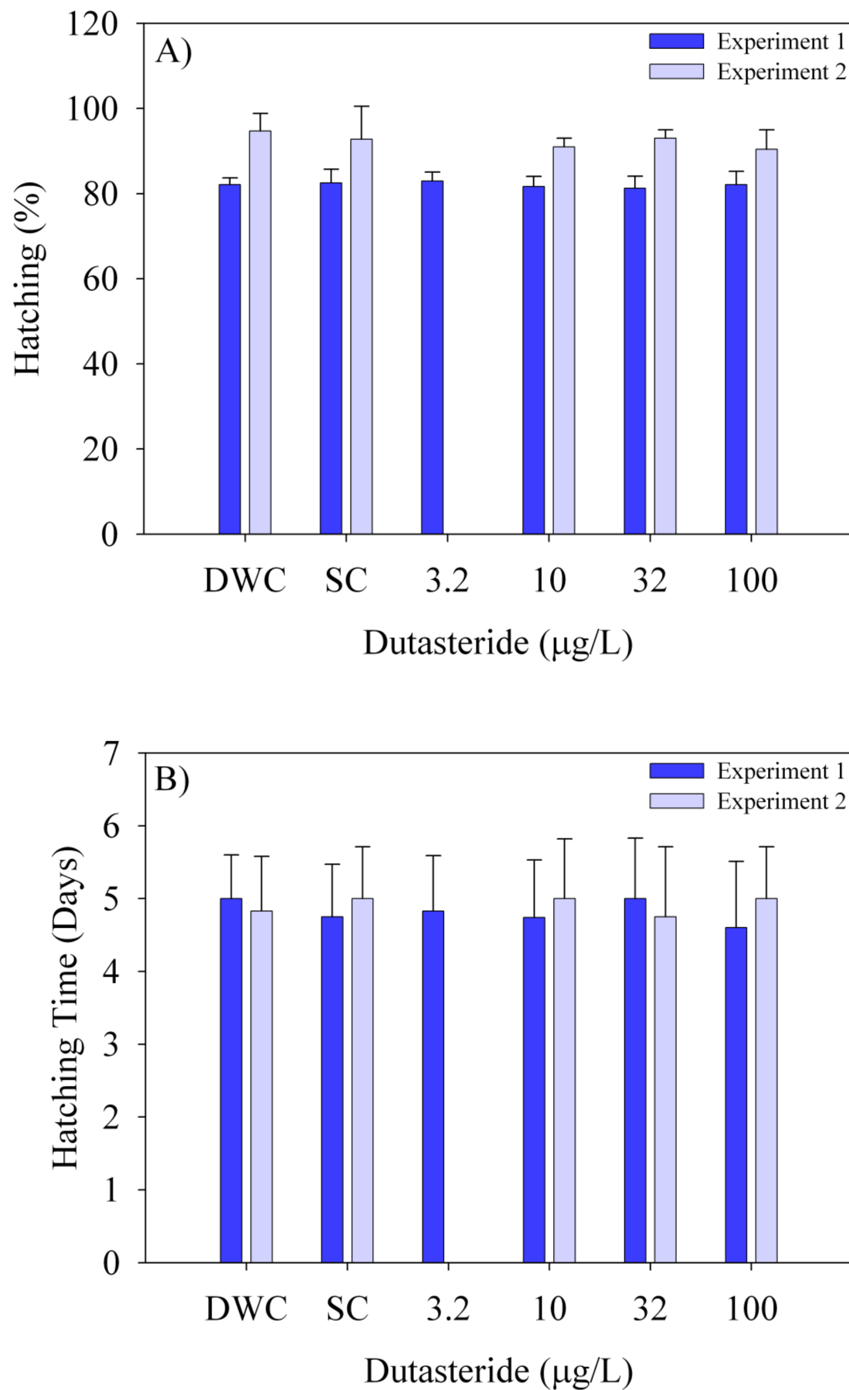


Figure 5.5. *Effect of different concentrations of dutasteride on (A) the hatching success and (B) hatching time of fathead minnow embryos exposed from 4-6 hours post fertilisation to hatch. Data were obtained from two independent experiments, and are expressed as means ± SD. The number of starting embryos per treatment was n = 240 for experiment 1, and n = 125 for experiment 2.*

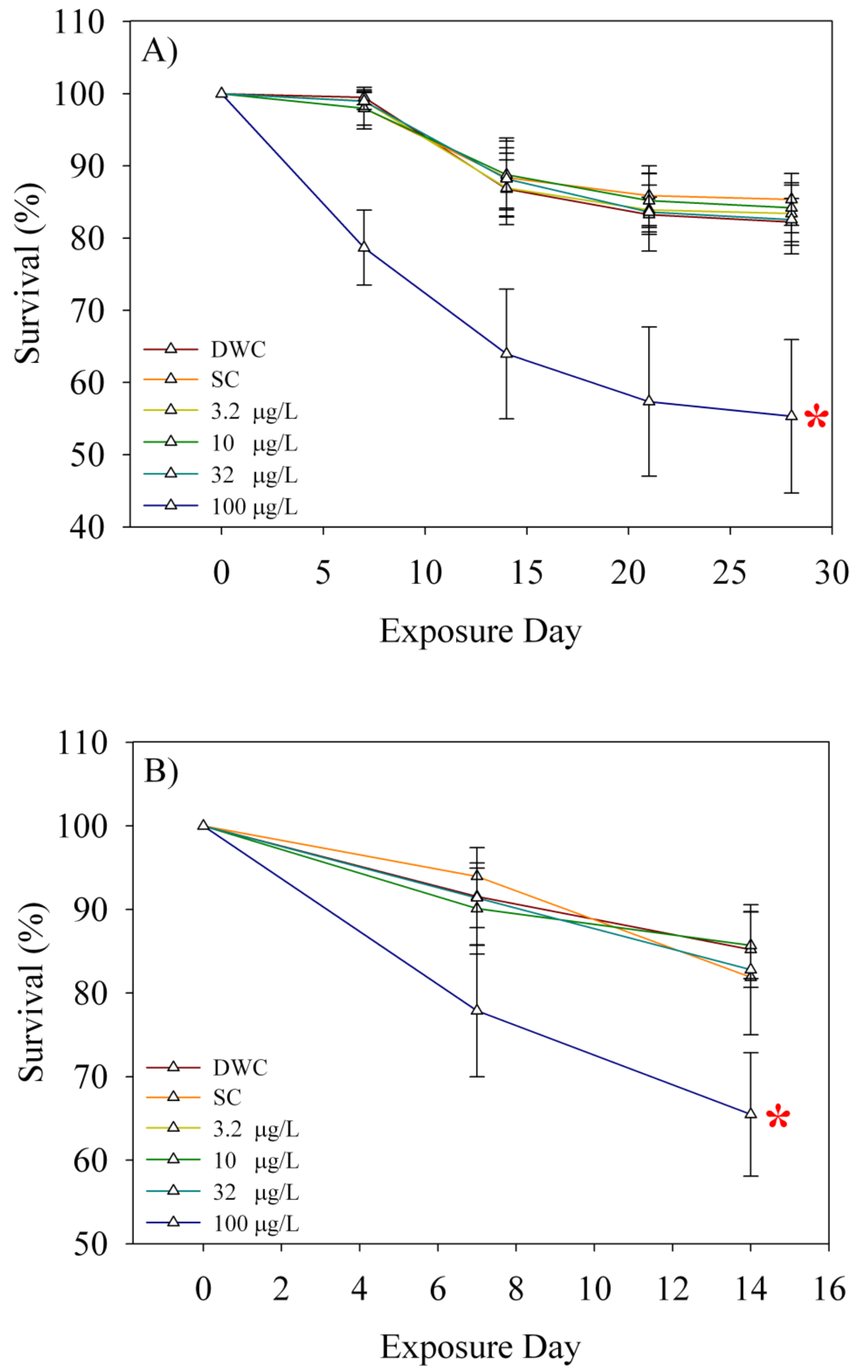


Figure 5.6. *Effect of different concentrations of dutasteride on the survival of fathead minnow exposed from 4-6 hours post fertilisation to (A) 28 or (B) 14 days post hatching. Data were obtained from two independent experiments, and are expresses as means ± SD. The asterisk (*) indicates a significant difference (Kaplan-Meier Survival Plus Log Rank Mantel-Cox Analysis;: Study 1: $p < 0.0001$; Study 2: $p = 0.0004$).*

Table 5.4 *Hatching success and survival of fathead minnows exposed from embryo stage to 28 dph to dutasteride (3.2, 10, 32 and 100 µg/L).*

Dutasteride conc. (µg/L)	Tank number	Replicate	Number of eggs at start	Number of larvae		Percentage hatch			Survival at 7 dph				Survival at 14 dph				Survival at 21 dph				Survival at 30 dph			
				Hatched	Released	Indiv. Repl.	Pooled repl.	SD	Number of fish	%	Average %	SD	Number of fish	%	Average %	SD	Number of fish	%	Average %	SD	Number of fish	%	Average %	SD
DWC	1	R1	60	48	48	80.00	82.08	1.60	47	97.92	99.48	1.04	42	87.50	86.83	4.95	40	83.33	83.26	2.39	40	83.33	82.26	3.23
	2	R2	60	49	49	81.67			49	100.00			45	91.84			42	85.71			42	85.71		
	3	R3	60	50	50	83.33			50	100.00			40	80.00			40	80.00			39	78.00		
	4	R4	60	50	50	83.33			50	100.00			44	88.00			42	84.00			41	82.00		
SC	17	R1	60	51	51	85.00	82.50	3.19	51	100.00	97.94	2.89	47	92.16	88.46	5.47	45	88.24	85.88	4.15	45	88.24	85.37	3.59
	18	R2	60	49	49	81.67			46	93.88			44	89.80			44	89.80			43	87.76		
	19	R3	60	51	51	85.00			51	100.00			41	80.39			41	80.39			41	80.39		
	20	R4	60	47	47	78.33			46	97.87			43	91.49			40	85.11			40	85.11		
3.2	5	R1	60	50	50	83.33	82.92	2.10	50	100.00	98.98	1.18	44	88.00	87.00	3.89	44	88.00	83.95	3.42	44	88.00	83.45	3.91
	6	R2	60	50	50	83.33			49	98.00			43	86.00			41	82.00			40	80.00		
	7	R3	60	51	51	85.00			51	100.00			42	82.35			41	80.39			41	80.39		
	8	R4	60	48	48	80.00			47	97.92			44	91.67			41	85.42			41	85.42		
10	13	R1	60	50	50	83.33	81.67	2.36	50	100.00	97.98	2.33	46	92.00	88.82	4.64	44	88.00	85.22	3.72	44	88.00	84.17	3.46
	14	R2	60	49	49	81.67			47	95.92			44	89.80			43	87.76			42	85.71		
	15	R3	60	50	50	83.33			48	96.00			41	82.00			40	80.00			40	80.00		
	16	R4	60	47	47	78.33			47	100.00			43	91.49			40	85.11			39	82.98		
32	9	R1	60	48	48	80.00	81.25	2.85	48	100.00	98.98	1.18	43	89.58	88.29	4.30	41	85.42	83.73	5.39	41	85.42	82.69	4.76
	10	R2	60	49	49	81.67			49	100.00			41	83.67			41	83.67			40	81.63		
	11	R3	60	51	51	85.00			50	98.04			44	86.27			39	76.47			39	76.47		
	12	R4	60	47	47	78.33			46	97.87			44	93.62			42	89.36			41	87.23		
100	21	R1	60	48	48	80.00	82.08	3.15	40	83.33	78.71	5.19	33	68.75	63.88	8.99	28	58.33	57.23	10.32	26	54.17	55.20	10.63
	22	R2	60	48	48	80.00			39	81.25			31	64.58			29	60.42			29	60.42		
	23	R3	60	52	52	86.67			41	78.85			37	71.15			35	67.31			34	65.38		
	24	R4	60	49	49	81.67			35	71.43			25	51.02			21	42.86			20	40.82		

5.4.3 Somatic growth at the end of the ELS Tests

At the end of ELS Test 1 (28 dph), significantly reduced length and weight were recorded in fish exposed to 32 and 100 μg/L ($p < 0.001$), as well as in the DWC group ($p < 0.001$). A significant decrease of the condition index was observed in fish exposed to 100 μg dutasteride/L ($p = 0.005$), and in the DWC group ($p < 0.001$) (Figure 5.7).

The second ELS Test was carried out for a shorter period, until 14 dph, and it replicated the results obtained with the Experiment 1, with a significant decrease of length and weight in the DWC, 32 μg/L, and 100μg/L ($p < 0.001$) groups, except for the condition index, which was significantly higher ($p = 0.032$) in fish exposed to the high dutasteride concentration (100μg/L) (Figure 5.7). However, the condition index calculated for ELS Test 2 is based on weight values measured in triplicate, while the length was measured for each individual. This is probably the cause of the unexpected results (CI increase), making this result unreliable.

5.4.4 Fish recovery in clean water. Somatic growth and SSCs.

After the end of the exposure period, surviving fish from ELS Test 1 were transferred in clean water, and kept until sexual maturation. The first sampling time was set at 120 dph. Fish showed a full recovery in growth parameters, with no significant differences among groups in length, weight or condition index (Figure 5.8). At 120 dph, only a very small number of fish had developed secondary sexual characteristics, and also visual inspection of the gonads didn't always give reliable results. For these reasons, an additional sampling was performed at 160 dph, when 8 males and 8 females were randomly sampled from each treatment. Data obtained from the last sampling confirmed the full recovery of growth parameters in fish exposed to dutasteride during the early life stages (Figure 5.9). Sampled females showed a non significant increasing trend in length and weight, and females exposed to 3.2 μg/L during the early life stages showed a significant increase of condition index ($p = 0.048$), which is probably a random effect not linked to the dutasteride exposure.

No significant differences were observed among treatments in number and grade of nuptial tubercles, fatpad index, and fatpad height in males, as well as in the ovipositor length in females (data not shown).

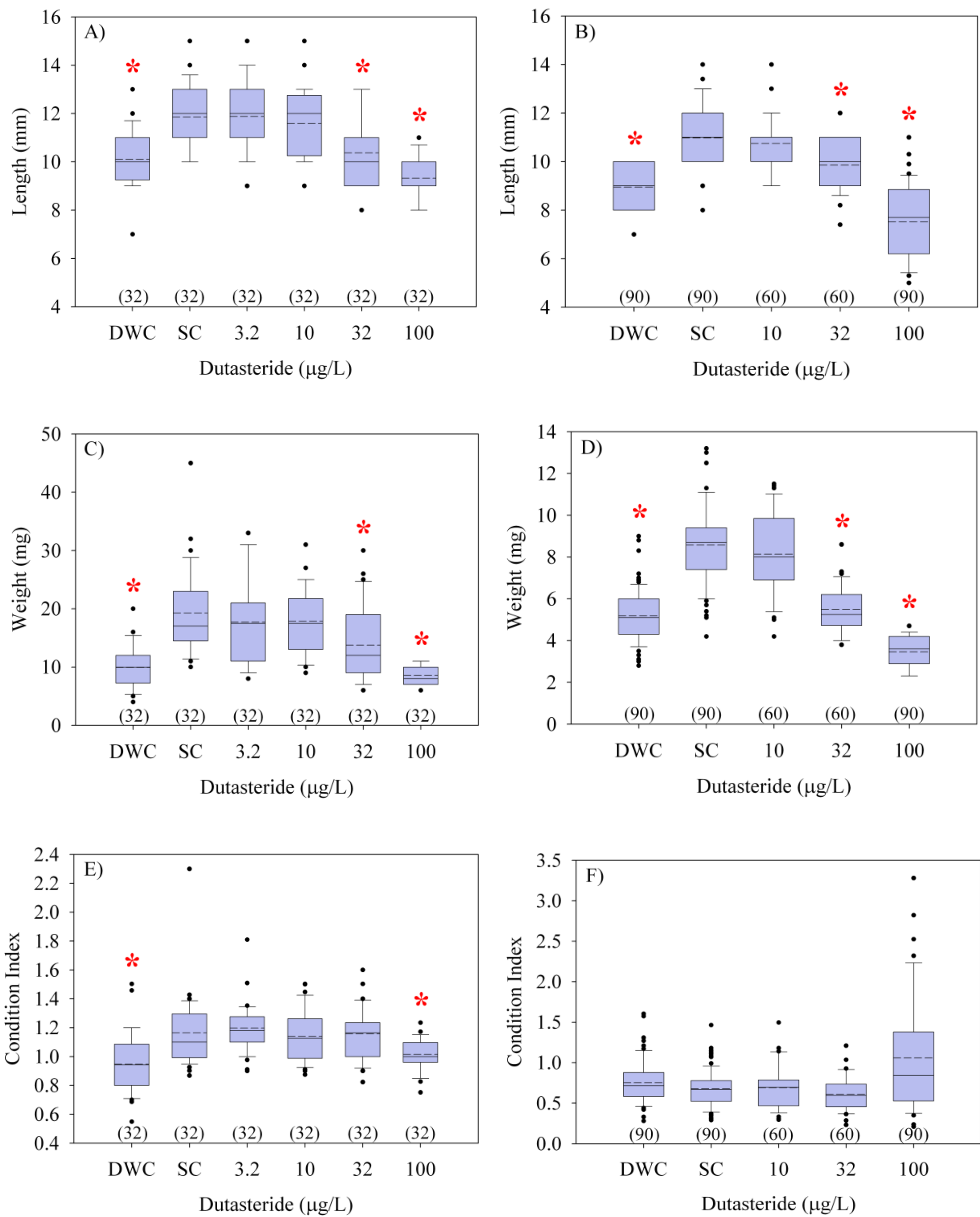


Figure 5.7. *Box plots of (A,B) length, (C,D) weight and (E,F) condition index of fathead minnow juveniles exposed to different concentrations of dutasteride. Data were obtained from two independent experiments. Fish were exposed to the drug from 4-6 hours post fertilisation to 28 days post hatching in experiment 1 (A,C,E), and from 4-6 hours post fertilisation to 14 days post hatch in experiment 2 (B,D,F). Boxes represent median (full line), and mean (dashed line) with 25th and 75th percentiles. The bars extend to the 10th and 90th percentiles with outliers represented as dots. Sample size is indicated above each treatment label on the x-axis. * $p < 0.05$ compared to the Solvent Control group.*

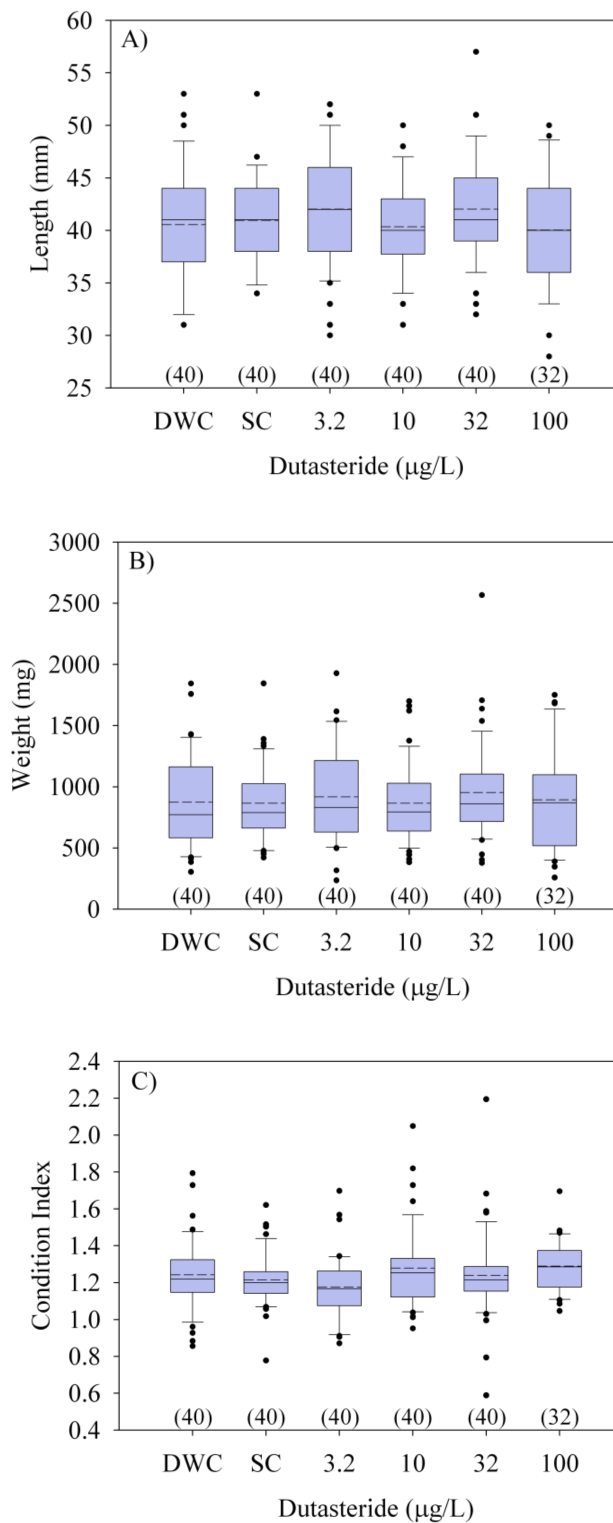


Figure 5.8. *Box plots of length (A), weight (B), and condition index (C) of fathead minnow juveniles exposed to different concentrations of dutasteride from 4-6 hours post fertilisation to 28 days post hatching, and then kept in clean water until the sampling time (120 dph). Boxes represent median (full line), and mean (dashed line) with 25th and 75th percentiles. The bars extend to the 10th and 90th percentiles, with outliers represented as dots. Sample size is indicated above each treatment label on the x-axis.*

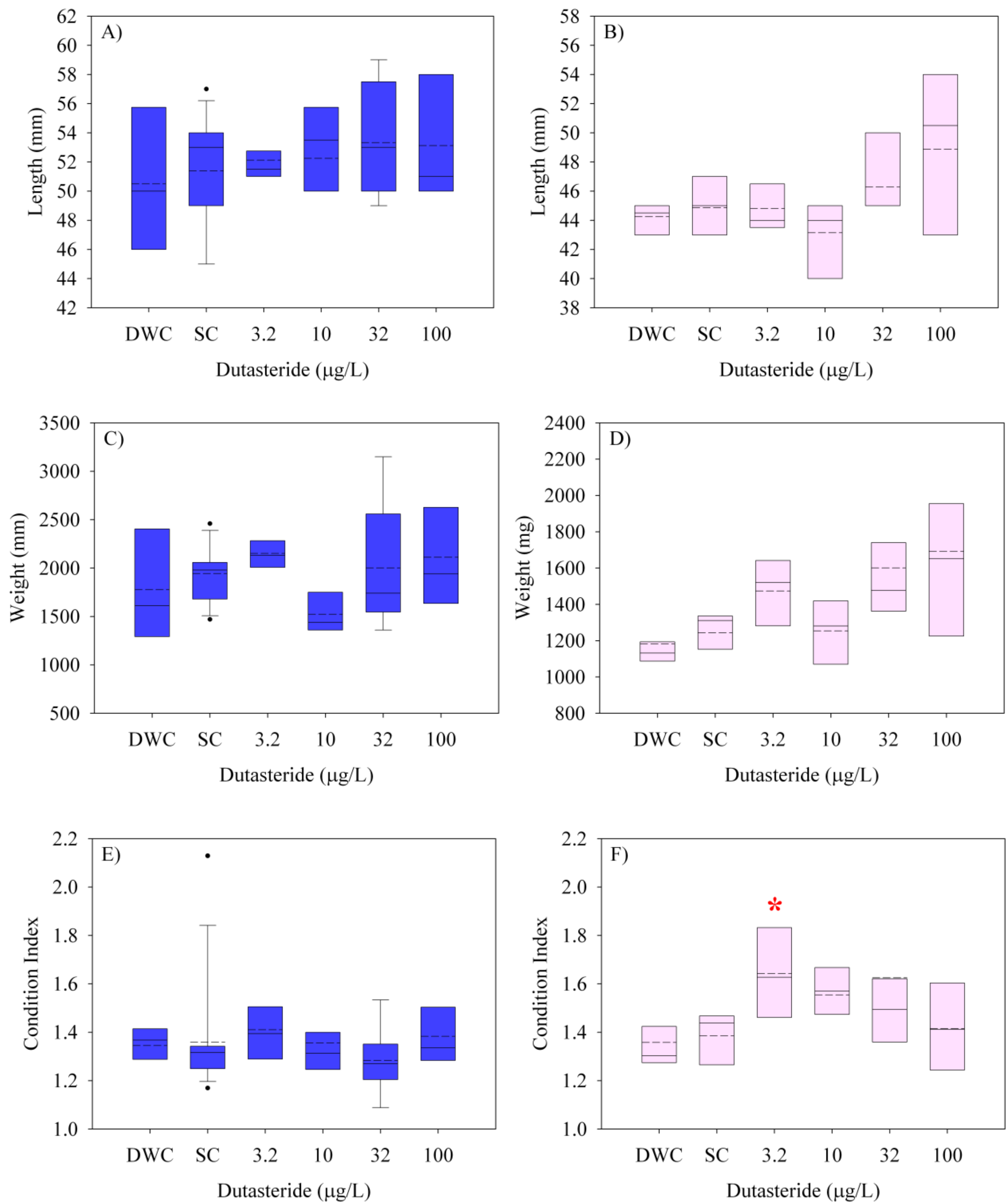


Figure 5.9. *Box plots of length, weight and condition index of fathead minnow males (A,C,E) and females (B,D,F) exposed to different concentrations of dutasteride from 4-6 hours post fertilisation to 28 days post hatching, and then kept in clean water until the sampling time (160 dph). Boxes represent median (full line), and mean (dashed line) with 25th and 75th percentiles. The bars extend to the 10th and 90th percentiles, with outliers represented as dots.* * *$p < 0.05$ compared to the Solvent Control group. Sample size: $n = 8$.*

5.4.5 Fish recovery in clean water. Gonad histopathology.

Histopathological analyses were performed to assess the effect of an early life stage exposure to dutasteride on gonadal differentiation and maturation processes in adult fish occurring after a relatively long period of time from the end of the exposure.

5.4.5.1 *Males*

Male fathead minnows sampled at 160 dph presented testes at different maturation stages, with a general dominance of early spermatogenic stages (Stage 0 + Stage 1), but also with individuals with late spermatogenic gonads (Figures 5.10 & 5.11). Males in the 100 µg/L group showed a significant retardation of the spermatogenic process (p = 0.005), presenting only undeveloped or early spermatogenic testes, with no individuals with testes at mid or late spermatogenic stages. This result was confirmed by the analysis of both dominant germ cell and most advanced stage of spermatogenesis, which showed how fish exposed to the highest concentration of dutasteride during early life spresented a "slower" gonad maturation process compared with other groups; in particular, spermatozoa were never observed as the dominant germ cell (p = 0.015), and represented the most advances stage of spermatogenesis only in 33% of samples versus 100% and 87.5% in DWC and SC groups, respectively (p = 0.005).

5.4.5.2 *Females*

All the control females sampled at 160 dph had ovaries at the vitellogenic stage (Early, Mid-Late or Hydrated), while treated fish were characterized by the occurrence of undeveloped ovaries, with an absence of cortical alveolar or vitellogenic oocytes (Figures 5.12 & 5.13). The proportion of fish at Stage 0 of maturation was significantly higher than control groups in fish exposed to 32 (p = 0.045) and 100 (p = 0.040) µg/L of dutasteride during the early life stages. In the same groups, previtellogenic oocytes represented the dominant germ cell respectively in 83.3% (p = 0.045) and 68.2% (p = 0.025) of samples. Furthermore, the same groups showed a significant reduction in the percentage of samples having late vitellogenic oocytes as the most advanced stage of oogenesis, from 100% in DWC and SC groups, to 16.7% ($p < 0.001$) and 44.4% ($p < 0.001$), respectively, in the groups exposed to 32 and 100 µg/L.

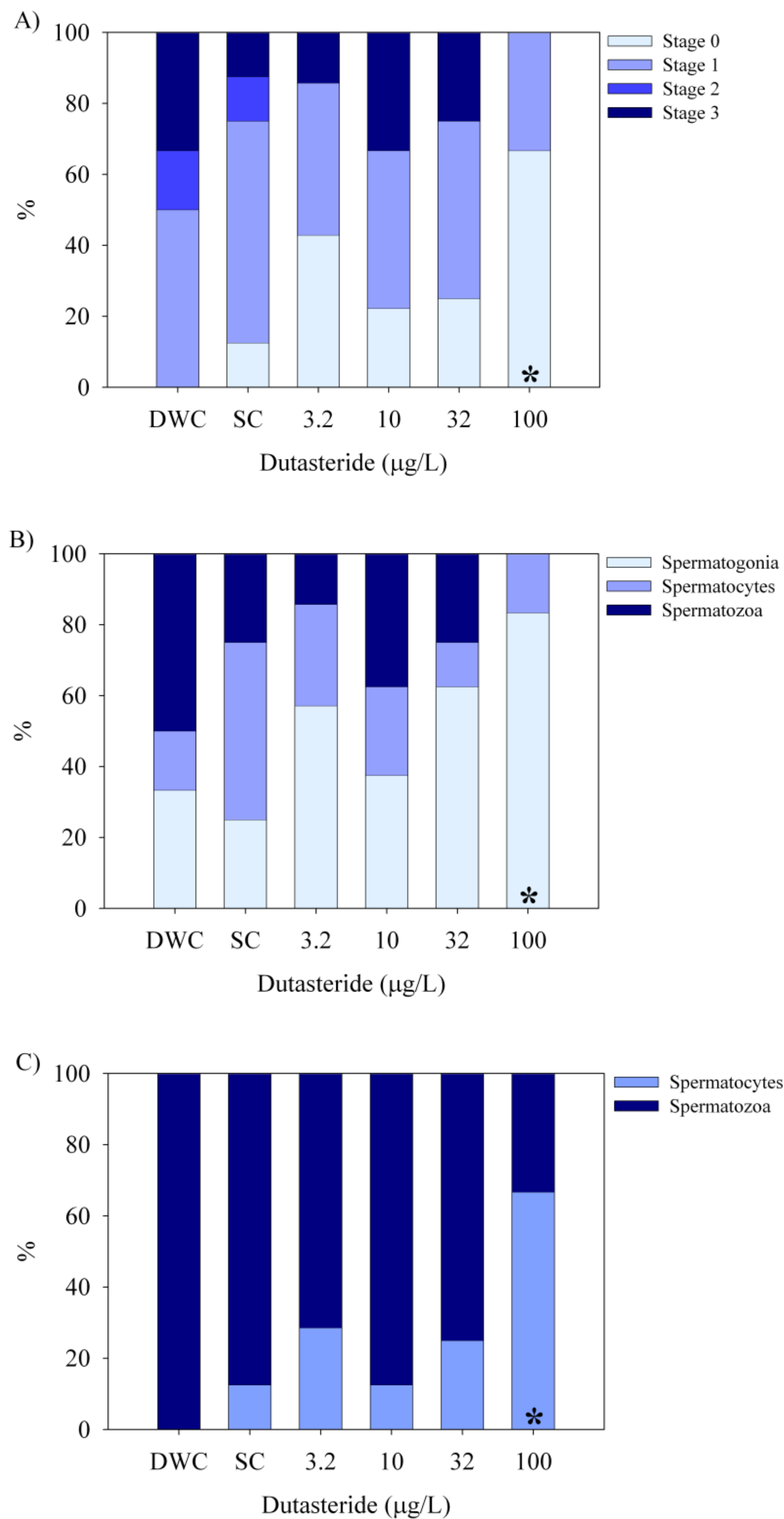


Figure 5.10. *Effect of an early life stage exposure to dutasteride on the testis of adult fathead minnows. Fish were exposed to different concentrations of the drug from 4-6 hours post fertilisation to 28 days post hatching, and then kept in clean water until the sampling time (160 dph). (A)Maturation stage of the testis, (B) dominant germ cell, and (C) most advanced stage of spermatogenesis are presented. Stage 0: Undeveloped, entirely immature phases (spermatogonia to spermatids) with no spermatozoa; Stage 1: Early spermatogenic: immature phases predominate, but spermatozoa may also be observed; Stage 2: Mid-spermatogenic: spermatocytes, spermatids, and spermatozoa are present in roughly equal proportions; Stage 3: Late spermatogenic, mature sperm predominate. Asterisks (*) represent significant differences compared to the SC group ($p < 0.05$). Sample size: $n = 8$.*

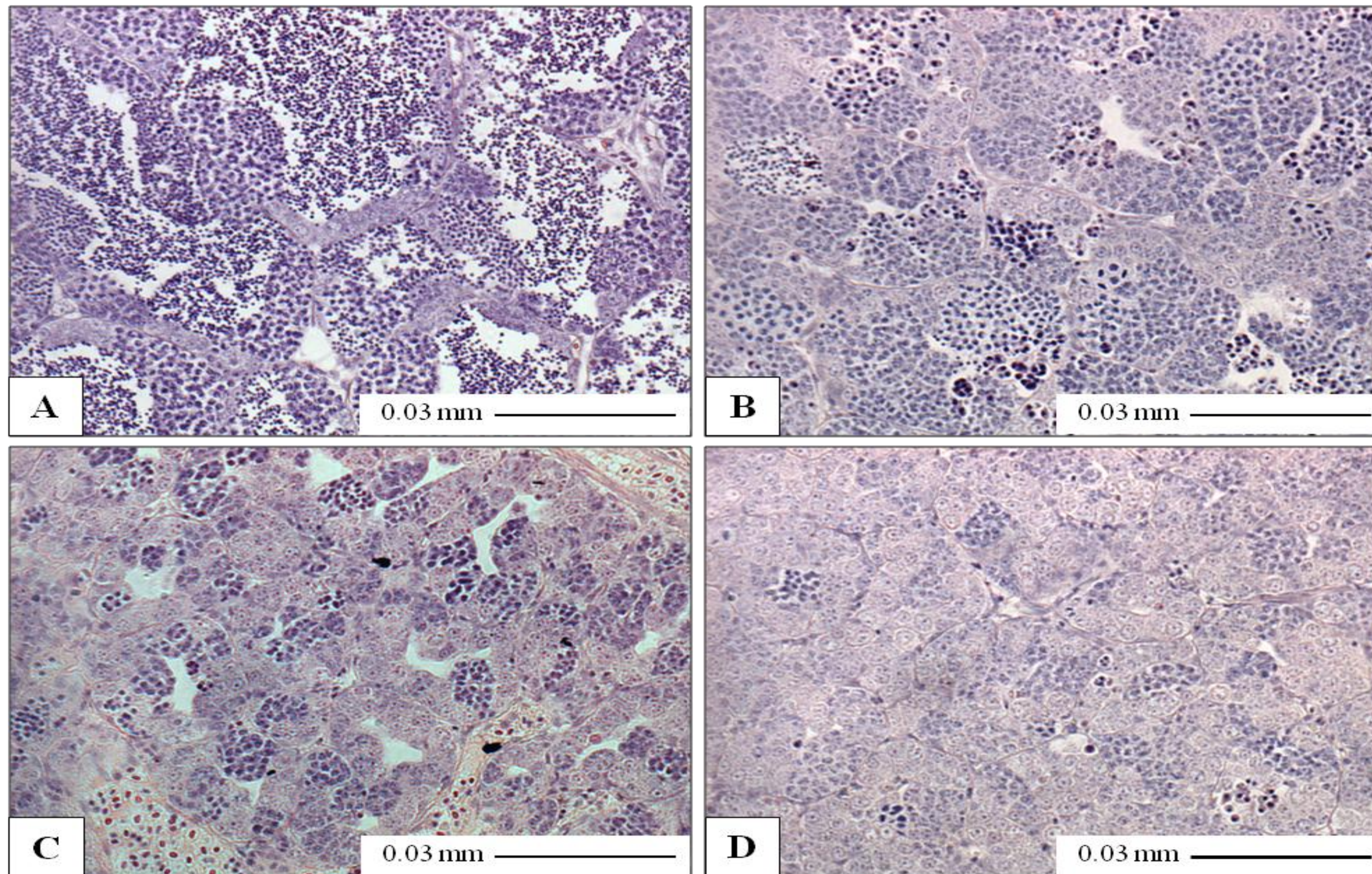


Figure 5.11. *Transverse sections of fathead minnow testes from (A,B) the Solvent Control group and (C,D) from the group exposed to the highest concentration of dutasteride (100 µg/L) during the period "fertilised egg-28 dph". After the end of the treatment, fish were transferred into clean dilution water, and sampled at 160 dph. The testes in figures A and B represent examples of, respectively, advanced and early spermatogenic activity found in the testes of fish in the Solvent Control group. In the same group, spermatozoa were the most advanced stage of spermatogenesis in 87.5 % of samples, versus 33% in the group exposed to 100µg/L of dutasteride.*

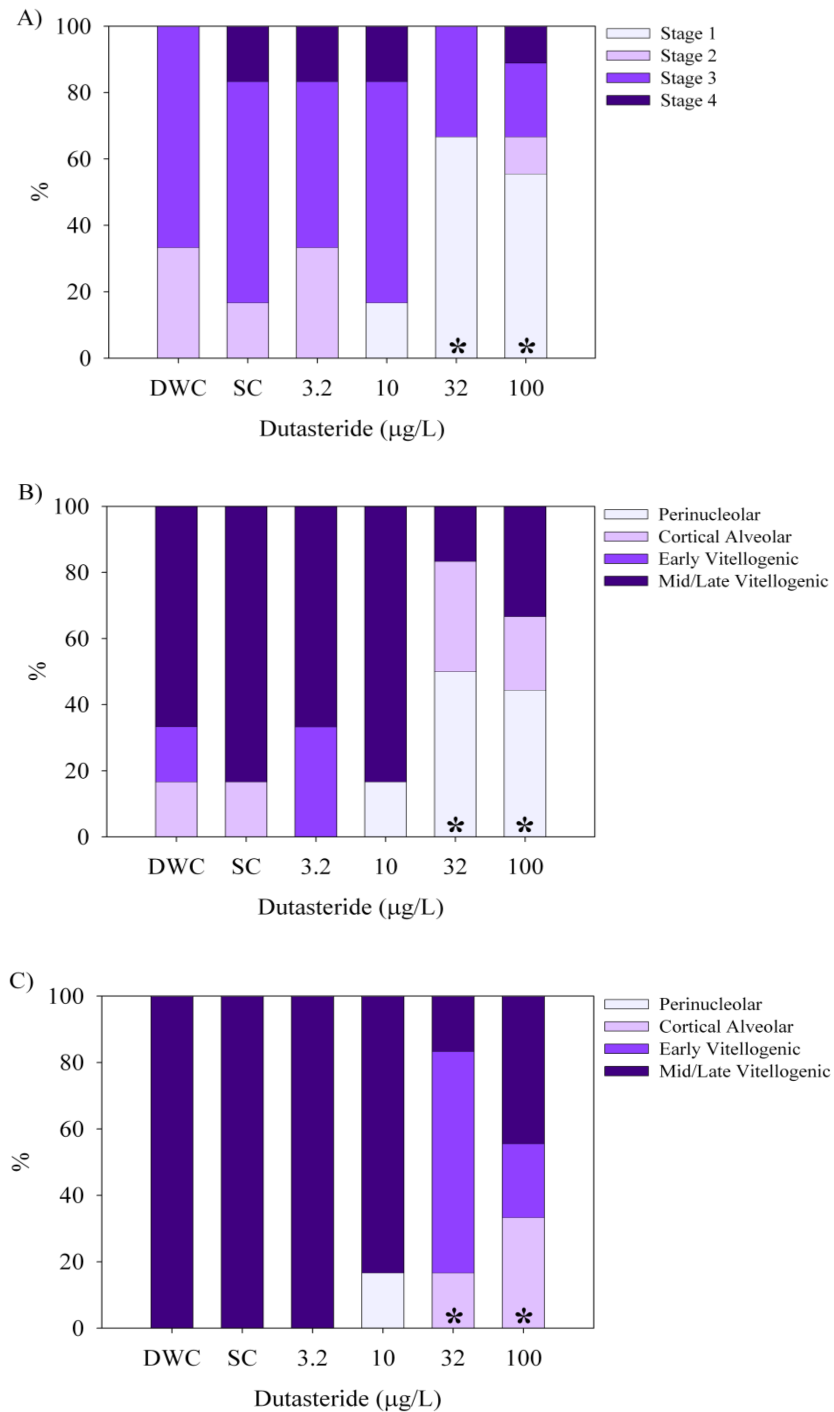


Figure 5.12. *Effect of an early life stage exposure to dutasteride on the ovaries of adult fathead minnows. Fish were exposed to different concentrations of the drug from 4-6 hours post fertilisation to 28 days post hatching, and then kept in clean water until the sampling time (160 dph). (A) Maturation stage of the ovary, (B) dominant germ cell and (C) most advanced stage of oogenesis are presented. Stage 0: Undeveloped, entirely immature phases (oogonia to Perinucleolar oocytes), and no cortical alveoli; Stage 1: Early development, vast majority (e.g., >90%) were pre-vitellogenic follicles, predominantly perinucleolar through to cortical alveolar; Stage 2: Mid-development, at least half of 520 observed follicles were early and mid-vitellogenic; Stage 3: Late development, majority of developing follicles were late vitellogenic; Stage 4: Late development/hydrated: majority of follicles are late vitellogenic and mature/spawning follicles. In figures b) and c) the classes of oocytes are indicated. Sample size: n = 8. Asterisks (*) represent significant differences compared to the SC group (p < 0.05).*

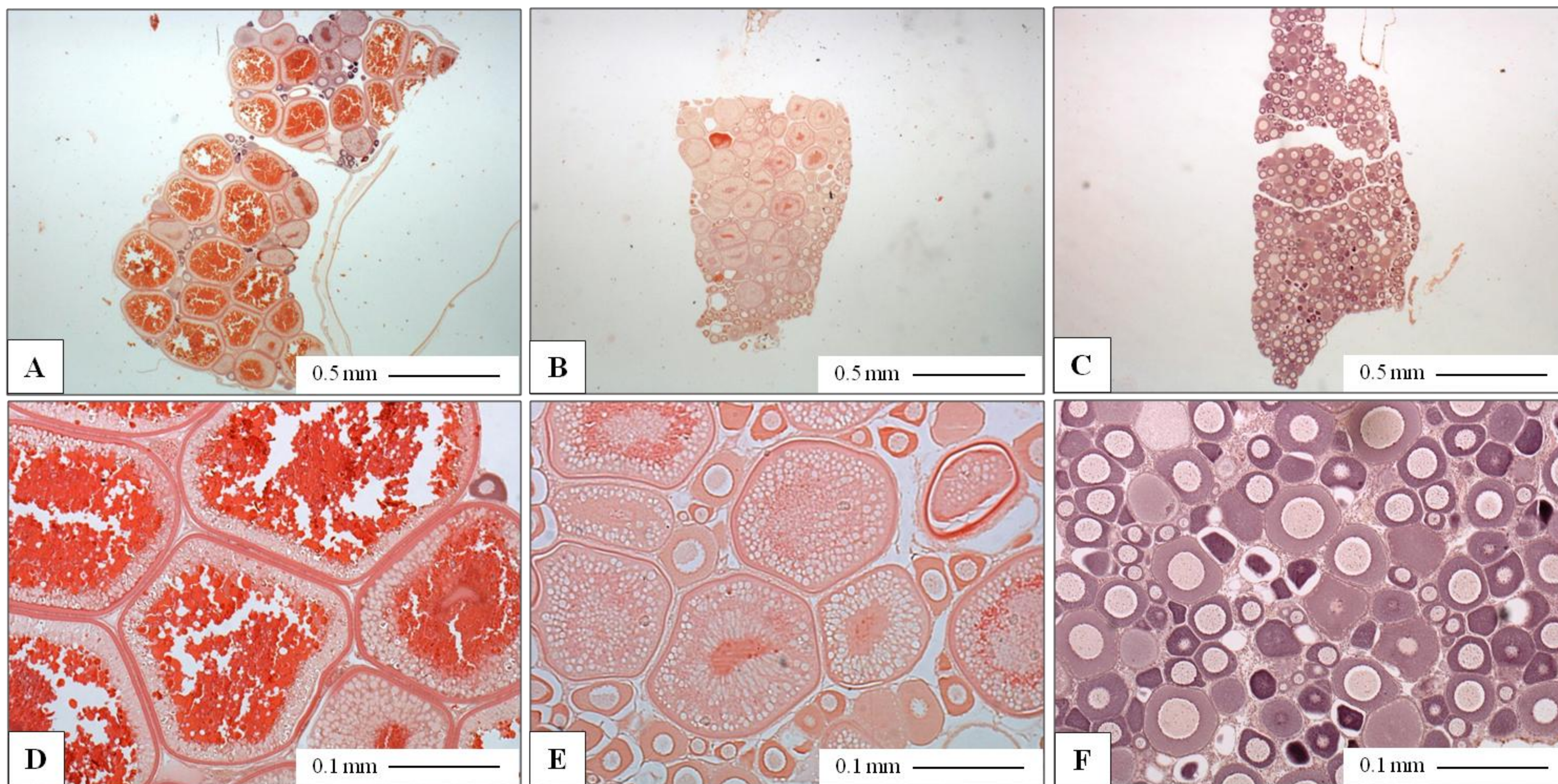


Figure 5.13. *Transverse sections of fathead minnow ovaries (A,D) from the Solvent Control group and from the groups exposed to (B, E) 32 µg/L and (C,F) 100 µg/L of dutasteride during the period "fertilised egg-28 dph". After the end of the treatment, fish were transferred into clean dilution water, and sampled at 160 dph. The ovary in figures A and D represents an example of ovaries containing late vitellogenic oocytes, which were present in 100% of fish in the Solvent Control group, versus 16.7% and 44.4%, respectively, in the groups exposed to 32 and 100µg/L of dutasteride. Figures B,C,D,F show examples of ovaries with reduced or completely absent vitellogenesis, found in fish exposed to 32 and 100 µg/L of dutasteride.*

5.4.6 Fish recovery in clean water. Plasma steroid concentrations.

No significant differences were observed in plasma T concentrations of males and females, and in plasma KT concentrations of males, sampled at 160 dph (Fig. 5.14).

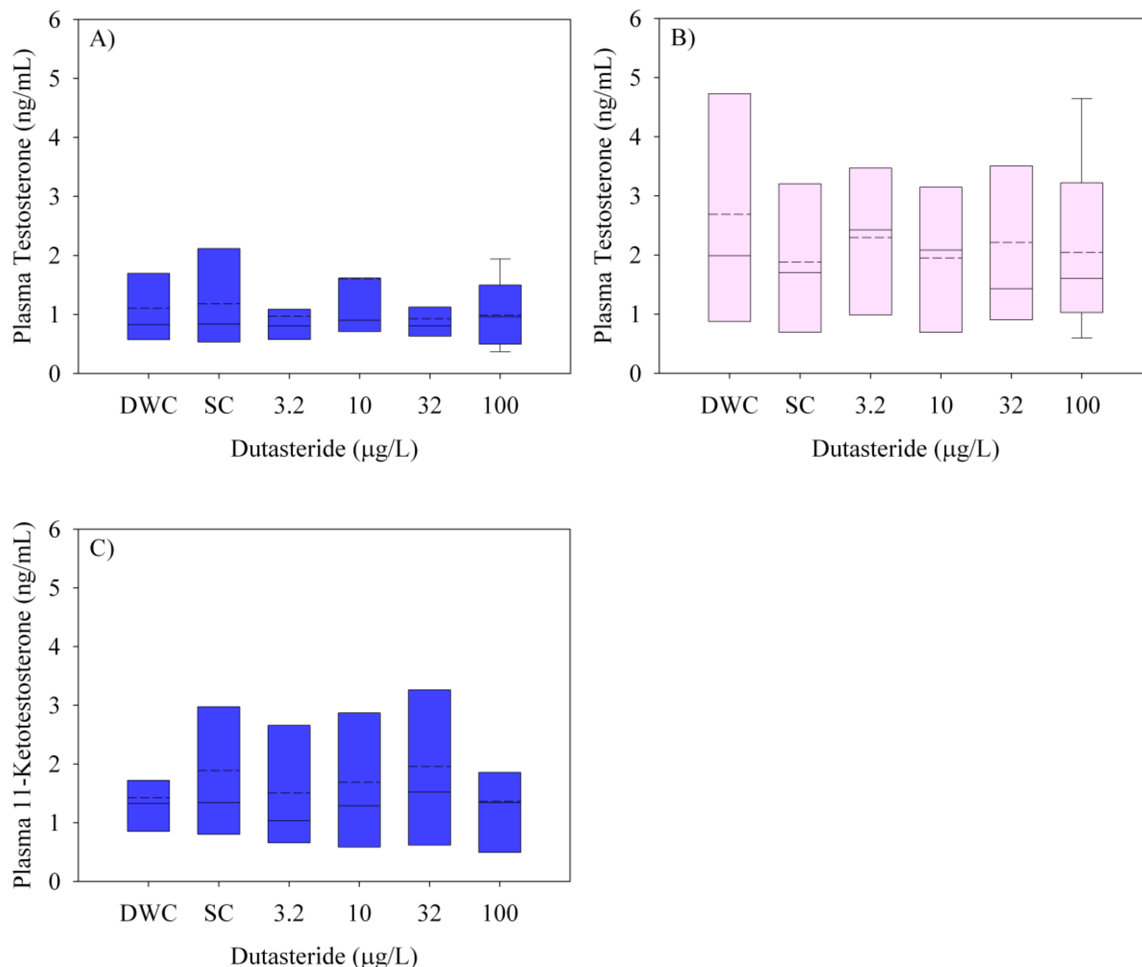


Figure 5.14. *Effect of an early life stage exposure to dutasteride on plasma concentrations of testosterone and 11-ketotestosterone in adult (A,C) male and (B) female fathead minnows. Fish were exposed to different concentrations of the drug from 4-6 hours post fertilisation to 28 days post hatching, and then kept in clean water until the sampling time (160 dph). Boxes represent median (full line), and mean (dashed line) with 25th and 75th percentiles. The bars extend to the 10th and 90th percentiles, with outliers represented as dots. Sample size: n = 8.*

5.5 Discussion

In the present study we investigated the effects of dutasteride on the early life stages of fathead minnow (egg-28 dph), and the consequences of the exposure on the maturation of the gonads in the adult stage. Exposure to dutasteride did not cause any effect on embryos hatching rate and hatching time; however, significant effects on growth and survival were

recorded in the groups exposed, respectively, to ≥32 and 100 µg/L. Furthermore, fish exposed to 32 and 100 µg/L presented histologically-evident alterations of gametogenesis in the adult stage (160 dph).

The effects caused by exposure to dutasteride arose only after the embryos hatched; in fact, neither hatching time nor hatching rate was affected by exposure to the drug. The difference of sensitivity between pre-hatch and post-hatch life stages was probably linked to the inability of the dutasteride to penetrate the chorion which surrounds the developing embryos (Manner *et al.*, 1977). The teleost fish egg chorion is an acellular multilayered envelope composed mainly of polysaccharides, proteins and glycoproteins. The exact biochemical composition varies during different stages of development, from the unfertilized egg up to hatching time, and both the number of layers and thickness present species-specific variability (Bonsignorio *et al.*, 1996; Brivio *et al*, 1991). The chorion provide a protective barrier between the developing embryo and the external environment, however, the isolation of the embryo is not complete, since a large number of pores traverse the chorion structure (Hart and Donovan, 1983; Zhang and Rawson, 1996). The chorion of zebrafish contains approximately 7 million pores, with an outer diameter of 0.2 and 0.7 µm (Hart and Donovan, 1983; Rawson *et al.*, 2000), and is freely permeable to water, electrolytes and a range of cryoprotectants such as methanol, dimethyl sulfoxide (DMSO), ethylene glycol, propane-1,2-diol (Zhang and Rawson, 1996; Harvey *et al.*, 1983), but also to E2 and polyethylene-glycols up to a molecular weight of 40,000 (Lillicrap, 2010).

A number of factors can determine if a substance is able to penetrate the chorion, such as molecular weight, steric properties, electrical charge, hydrophylicity, and osmotic pressure. The molecular weight of dutasteride is 528.5, and its molecular dimensions are smaller than those of other molecules able to penetrate into the embryo (Lillicrap, 2010), hence dimensional and steric factors can't explain the inability of the drug to penetrate the chorion. However, dutasteride has a very high lipophylicity (logP: 5.45, ALOGPS; 5.09, GSK), which may cause a dimensional increase of the molecule, by binding organic matter dissolved or suspended in the water and forming aggregates, which in turn would prevent dutasteride from crossing the pores. Furthermore, other studies indicated that the barrier function of the chorion increases with the lipophilicity of the target compound (Braunbeck *et al.*, 2005). Other potential explanations for the insensitivity of fathead minnow embryos to dutasteride are that the drug penetrated the chorion only partially, without reaching an

intra-embryonic concentration high enough to exert physiological effects, or that the drug did fully penetrate the chorion, but it did not have any effect on hatching.

In our study the growth of juvenile fish exposed to dutasteride was significantly reduced at concentrations ≥ 32 µg/L; a similar effect has been reported when fish were exposed, at early life stages, to other anti-androgens. For example, the AR antagonists vinclozolin and cyproterone acetate caused reduced growth in fathead minnow (at 1200 µg/L) (Makynen *et al.*, 2000) and Japanse medaka (at 1 µg/L) (Kiparissis *et al.*, 2003). No data are available in the literature concerning the effects of 5α-reductase inhibitors (finasteride or dutasteride) on other fish species. Dutasteride has been tested on other animal species, in order to assess its toxicity on the early life stages exposed *via* maternal administration, and in particular, in an embryo-fetus development study with the rat, oral administration of dutasteride at doses of 0.05. 2.5, 12.5, and 30 mg/kg/day resulted in a feminization of male fetuses and male offspring at all doses, along with a decreased fetal body weight, and delay of ossification process at 2.5 and 30 mg/kg/day (15 to 111-fold the expected clinical exposure) (GSK, 2001). It should be noted that the cross-species comparison (teleost fish *vs* other vertebrates) of the effects caused by dutasteride may not necessarily be informative, since in vertebrates, except teleost fish, DHT is generally the major circulating androgen, while in fish this role is played by KT (Borg, 1994).

One of the most obvious effects caused by dutasteride (100 µg/L) was the decrease of larval survival at 28 dph in the ELS Test 1. In order to assess the repeatability of this result, and to exclude the possibility that the mortality was linked to other accidental factors, we performed a second ELS Test, which terminated at 14 dph (instead of 28 dph), confirming the effects on both growth and survival. In a recent study, Owen *et al.* (2010) highlighted the importance of replication when performing toxicological tests, showing how two independent studies performed with the aim to assess the effects of clofibric acid on growth rates of the rainbow trout, lead to completely contrasting results (significant effect at all the concentration in the first study; no effect at all the concentrations in the second study). The second study was statistically more robust than the first one; however, both of them had enough statistical power to meet the criteria set by the OECD protocol 215. Within this perspective, our study showed a very good repeatability between ELS Test 1 and ELS Test 2, so we can argue with confidence that the effects of dutasteride on larval growth and survival rate were caused by the exposure to dutasteride, and not by other environmental or accidental factors.

At the end of the exposure period, fish were moved into clean water, grown up and sampled at 120 and 160 dph, with the aim to assess the persistence of the effects caused by the early life stages exposure to dutasteride. At both sampling times, the fish that at the end of the exposure period had shown decreased growth, recovered completely all the growth parameters (length, weight and condition index), so that there were not any significant differences among groups. This response could be explained by the concept of catch-up growth, which is usually defined as an accelerated growth observed during recovery by a stress condition that caused the growth inhibition, such as food deprivation, non-optimal temperature, hypoxia (Ali *et al.*, 2003; Kajimura *et al.*, 2005; Wootton, 2004), and chemical exposure (Moens *et al.*, 2007). This phenomenon has been observed not only in fish, but also in many animal species, including humans (Ali *et al.*, 2003; Kay's *et al.*, 2006). Interestingly, male rats exposed in utero to the 5α-reductase inhibitor finasteride, exhibited catch-up growth after birth (Clark *et al.*, 1990).

The sensitivity of fish early life stages to endocrine disruptors is well known (van Aerle *et al.*, 2002), and short periods of exposure during these critical life stages can cause disruption of the normal sexual development and maturation processes, which can persist into adulthood, even if the exposure to the chemical had been terminated. The histopathological analyses of the gonads represent a very powerful tool to assess these effects, as well as being one of the most sensitive endpoints of endocrine disruption. For example, in our study, fish sampled in the adult stage (160 dph), after an initial 32 days exposure to dutasteride (egg-28 dph), presented significant histopathological alterations, despite both morphometric data and plasma concentrations of T and KT not showing any significant differences among treatment groups. In particular, all the histopathological parameters employed in the analyses (gonad maturation stage, dominant germ cell, most advanced stage of gamatogenesis) confirmed the retardation in gonad maturation and sex cell progression, in both males and females. However, whereas males were affected only when exposed to 100 µg/L of dutasteride, females showed a significant delay in maturation when exposed to 32 µg/L. This result suggests that in the fathead minnow the ovary may be more sensitive to disruption of DHT biosynthesis than the testis. A more extended discussion on the sensitivity of ovaries to disruption of androgen biosynthesis disruption will be discussed in Chapter 7.

Unfortunately, as with the growth data, it was not possible to compare our histopathology results with similar studies, since no published information concerning the effects of 5α-

reductase inhibitors (finasteride or dutasteride) on fish is available in the literature so far. Furthermore, even broadening the comparison to other anti-androgens, such as flutamide or viclozolin, only very few studies involved exposing fish during early life stages (Bayley *et al.*, 2002; Kiparissis *et al.*, 2003; Makynen *et al.*, 2000), and in the only one which employed histopathological endpoints (Kiparissis *et al.*, 2003), the exposure period (egg-100 dph) was much longer than the one used in our study. Results from the latter study showed both an inhibition of spermatogenesis in the testes of medaka, caused by vinclozolin (2500 µg/L) and cyproterone acetate (1 and 10 µg/L), and a retarded oogenesis in females exposed to cyproterone acetate (1 and 10 µg/L), with a decrease in the proportion of females with ovaries at advances stages of oogenesis.

Interestingly, the observed retardation of gametogenesis, in particular in the ovary, occurred when adult fish were exposed to several anti-androgens, such as vinclozolin (Makynen *et al.*, 2000), cyproterone acetate (Kiparissis *et al.*, 2003; Rouse *et al.*, 1977), and flutamide (Jensen *et al.*, 2004; Kinnberg and Toft, 2003). These results may indicate that during the early life stage exposure (egg-28 dph), dutasteride disrupted some key processes regulating the maturation of the gonad, and that disruption was strong enough to be present even 5 months after the end of the exposure; furthermore, even if the identity of the disrupted process is unknown, it would be possible that the disruption of the androgenic system, caused both by 5α-reductase inhibitors and AR antagonists, may affect a shared set of molecular/physiological process that regulate the normal gonad maturation.

Despite the identity of the exact disrupted processes being unknown, the possibility of inducing long-term effects by exposing fish only during the early life stages to endocrine disruptors has been broadly demonstrated by exposing fish to sex steroids. In particular, van Aerle *et al.* (2001) determined the window of sensitivity for the estrogenic effects of EE2 on early-life stages of fathead minnow, showing how short exposures (egg-20, egg-5, 5-10, 10-15, 15-20 dph) to environmental concentrations of EE2 (10 ng/L) lead to disruption in gonad development in adult fish, causing vitellogenin synthesis induction, formation of ovarian-like cavity and spermatogenesis retardation in males. Furthermore, three-spined stickleback exposed to EE2 (1.75 and 27.7 ng/L) for the first 4 weeks post-hatch, exhibited alterations of adult reproductive performances, even in fish without histopathological alteration of the gonad (e.g. intersex) (Maunder *et al.*, 2007).

A further factor to take into consideration is the potential bioaccumulation of the drug in the fish; in fact, dutasteride is a highly lipophilic molecule, and excretion studies performed in humans showed 55% of the administered dose is subject to bioaccumulation in the patient's body (range 5% to 97%); furthermore, terminal elimination half-life of dutasteride was approximately 5 weeks at steady state, and serum concentrations of dutasteride remained detectable for up to 4 to 6 months after termination of the treatment. If a similar bioaccumulation pattern happened in fish, dutasteride may have exerted its effects even weeks after the termination of the exposure. However, the weight of the fish at the end of the exposure (28 dph) was approximately 100 times lower than the weight of fish sampled at 160 dph, suggesting that the potentially bio-accumulated dutasteride would have been subject to "dilution" with the passing of time.

5.6 Conclusions

In this study we have demonstrated that the exposure to dutasteride during early life stages of fathead minnow can negatively affect larval/juvenile growth and survival. However, the drug exerted these effects only after the embryos hatched, suggesting that the chorion surrounding the developing embryo may act as a barrier to the penetration of dutasteride into the embryo. The $LOEC_{survival}$ and $LOEC_{growth}$ were respectively of 100 and 32 µg/L. Interestingly, the exposure of the fish to dutasteride from egg to 28 dph produced long term effect on the gametogenic processes of adult fish, providing new interesting details on the consequences of the exposure of fish to anti-androgens, and raising new questions on their mechanisms of action.

CHAPTER 6 : EFFECTS OF DUTASTERIDE ON FATHEAD MINNOW REPRODUCTION AND F1 GENERATION HATCHABILITY

6.1 Introduction

6.1.1 Vertebrate reproduction and endocrine disrupting chemicals

The development of a reliable testing system able to identify chemical compounds with the capability to interfere with reproductive functions is one of the highest priorities among scientists and environmental/regulatory agencies, because of the growing awareness of the potential long-term effects of EDCs on wildlife and human reproductive health.

Reproduction of vertebrates is regulated by the hypothalamic-pituitary-gonadal (HPG) axis through a cascade of hormones and feedback control mechanisms, as described in Chapter 1. Any interference with this complex, multi-organ, biochemical/molecular network could negatively affect vertebrate reproduction, with a consequent alteration of population dynamics. Furthermore, since human reproduction is controlled by the same signalling system as in other vertebrates, the same interference (e.g. environmental pollutants) could also influence human reproduction, for example, by causing a decrease of fertility, or, more generally, playing an important role in the aetiology of human pathologies strictly correlated to alterations of the endocrine homeostasis (e.g. cancer of prostate, testis, breast).

During the last two decades, human fertility rates – defined as births per woman - have been showing a general declining trend all over the world (Skakkebaek *et al.*, 2005; World Bank, 2011). However, it is generally assumed that these trends are driven by social and economic changes, such as the spread of contraception systems, women's emancipation and participation to the work force, or increased education level (Skakkebaek *et al.*, 2005). The role of biological/environmental factors in the process of declining fertility rates has been poorly considered, even in cases where the drop of the fertility rates is so dramatic to suggest an alternative or additional explanation to the more classic ones, like the increased use of contraceptive systems (Andersen and Erb, 2006; Rosenfield and Schwartz, 2005).

It is nevertheless true that there has been a continuous increase in the amount of epidemiological evidence that human reproductive health, particularly in the Western world, is declining. A striking example is the case of Denmark, where the reproductive status of the population has been monitored and deeply studied for decades. Sperm counts in the infertile range are present in more than 10% of Danish men, and up to 30% of them have sperm counts in the sub-fertile range (Jorgensen *et al.*, 2006). The decline of fertility is parallel to the significant increase in the number of couples undergoing infertility treatments in Western Countries, and particularly in Denmark, where during 2002–2004 more than 6% of children were born thanks to assisted reproduction (Andersen and Erb, 2006). It is important to

highlight that among the various treatments to cure infertility, a significant increase in demand for intra-cytoplasmatic sperm injection (ICS) has occurred, which suggest an increasing number of couples with male fertility problems, in particular poor semen quality and testicular failure (Andersen and Erb, 2006; Skakkebaek *et al.*, 2005). In the same country, epidemiological studies showed how, during the last century, there has been an increase in pathological conditions like congenital genital abnormalities (e.g. cryptorchidism and hypospadias), testis cancer, and infertility (Moller, 1993; Richiardi *et al.*, 2004; Toppari *et al.*, 2001), which taken altogether constitute the pathology named as "testicular dysgenesis syndrome" (TDS) (Sharpe & Skakkebaek, 2003; Skakkebaek *et al.*, 2001). There is growing evidence that these conditions are related to an impaired fetal gonadal development and function, due to maternal exposure to environmental chemicals (Hardell *et al.*, 2003; Fisher *et al.*, 2003; Foster *et al.*, 2001; Skakkebaek *et al.*, 1987).

Other data suggest that the female reproductive system, in some Western countries, is also facing important changes which could be caused by environmental factors, including chemicals in the environment, but their impact on fertility/fecundity is difficult to quantify. For example, in Europe and United States, median age at menarche and first breast development are steadily decreasing, and estrogenic chemicals present in the environment could have a role in this trend (Aksglaede *et al.*, 2009; Herman-Giddens *et al.*, 1997; Parent *et al.*, 2003; Partsch and Sippell, 2001; Proos *et al.*, 1991). However, there are many controversies about the use of the endocrine disruption hypothesis to explain the decrease of human fertility (in particular in males), and a considerable debate is currently ongoing in the scientific community. For example, Joffe (2010) argued that the influence of environmental endocrine disruptors on male fertility is very minor compared to other factors, like DNA damage of germ cells (spermatids) via oxidative stress caused by a number of etiological agents present in the environment or in the food, like tobacco smoking, cis-unsaturated fatty acids, glycidamide - a metabolic product of acrylamide especially present in starchy food cooked at high temperature (including chips and crisps) (Wang *et al.*, 2010) - and heat (e.g. increase of intra-scrotal temperature caused by sedentary life-style) (Aitken *et al.*, 2006; Bujan *et al.*, 2000; Mieusset *et al.*, 2007).

A further issue is the difficulty in proving that one or a few specific chemicals present into the environment are directly linked with a negative health effect, since humans, like wildlife, are exposed to hundreds or thousands of chemical compounds simultaneously, so isolating the effects caused by one or few of them will always be very difficult. However, this multi-exposure should increase the concern, and the critical question is whether or not wildlife and

human health is at risk from chronic exposure to low doses of these compounds, either alone or in mixtures (Brian *et al.*, 2005; Sumpter and Johnson, 2008).

Despite all the controversies, characterizing the toxicity profile of chemicals introduced into the environment or in the food chain has a critical importance in terms of human and wildlife health preservation; in particular, when these chemicals have the potential ability to disrupt critical processes, such as reproduction.

6.1.2 Teleost fish reproduction in the regulatory context

From the regulatory point of view, the OECD has recently approved two Test Guidelines (TGs) for the testing and assessment of endocrine active chemicals on fish reproduction: TG229 and TG230, known also as "Fish Short Term Reproduction Assay", and "21-day Fish Assay", respectively (OECD, 2009). TG230 is designed to screen only substances with estrogenic, androgenic, and aromatase inhibitor activity, while TG229 is designed to detect the action of substances that impact reproduction of fish by different mechanisms of action, including endocrine interference. TG229 includes a higher number of endpoint compared to TG230, including fecundity and gonad histopathology.

6.1.3 Fish reproduction and anti-androgens

The importance of androgens in fish sex differentiation, development, puberty and sexual maturation was described in Chapter 5. Androgens also have a critical role during the reproductive phase, acting both directly and indirectly, as precursors in the synthesis of other steroids, on many physiological processes. In adult males, testicular androgens are required for the maintenance of many reproductive functions, including sperm production, sex accessory gland secretion, muscle mass and strength, and behavior. The latter is of critical importance, since the disruption of processes like courtship, territoriality, and aggression could indirectly affect reproductive activity (Borg, 1994; Breton and Sambroni, 1996; Trudeau *et al.*, 1991). The presence of the AR in the reproductive tissues of many female vertebrates, including fish, suggests that androgens could play an important role in the regulation of female physiology (Nitta *et al.*, 1991; Staub and DeBeer, 1997; Tetsuka and Hiller, 1996). The androgen KT has also been found in several female fish (Lokman *et al.*, 2002); however, its physiological role, if any, in females is still unclear. There is some evidence showing that KT plays a role in controlling growth of pre-vitellogenic oocytes in immature female fish (Kortner *et al.*, 2008; Matsubara *et al.*, 2003), and these data were confirmed in our experiments, as described in Chapter 4.

Several EDCs have been tested for reproductive toxicity in fish short-term reproduction assays using fathead minnow, zebrafish, and medaka. The majority of these studies conducted up to 2009 are listed in the review published by Dang *et al.* (2011), and the list is continuously growing. Among the various anti-androgens, the human pharmaceutical flutamide has been proposed as a model AR antagonist for EDCs research, on the basis of studies in mammalian systems (Ankley *et al.*, 2004). Flutamide and/or its 2-hydroxylated metabolite bind with high affinity to mammalian ARs *in vitro* and is able to block the expression of AR-mediated responses *in vivo* (Katchen and Buxbaum, 1975; Neri, 1989). Flutamide has been tested on fish with the hypothesis being that it exerts its effects via the same MoA as it displays in mammals, and some *in vivo* studies have partially confirmed this hypothesis (Bayley *et al.* 2002; Jensen *et al.*, 2004; Kang *et al.*, 2006; OECD, 2006; Sebire *et al.*, 2008). Considering the binding affinity to fish ARs, some studies indicate that flutamide and it metabolite, hydroxyflutamide, bind fathead minnow AR, and that the metabolite has higher affinity than the parent compound (Ankley *et al.*, 2004; Makynen *et al.*, 2000), while other studies indicate little specific binding of flutamide to ARs present in cytosolic fractions of rainbow trout and goldfish (Wells and Van der Kraak, 2000).

Another AR antagonist tested on fish was vinclozolin, a fungicide commonly used in vineyards. It has been shown that exposure to this compound can alter fish reproduction at different organisational levels, for example, courtship behavior of male guppy (Baatrup and Junge, 2001), or fecundity and gonadal condition (GSI, histology) of female fathead minnows (Makynen *et al.*, 2000; Martinovic *et al.*, 2008). However, Makynen *et al.* (2000) found little competitive binding affinity of vinclozolin or its metabolites to the fathead minnow AR at the concentrations tested *in vivo*.

Other than the chemicals directly interacting with the androgen receptor, few other compounds with different mechanisms of androgen inhibition have been tested in fish. Inhibitors of steroidogenic cytochrome P450s (CYPs), including fadrozole, prochloraz, and ketoconazole, caused reduced fecundity of fathead minnows over the course of a 21-day reproduction test (Ankley *et al.*, 2002, 2005, 2007). The reproductive impact of this class of inhibitors is consistent with the important roles played by their enzymatic targets. For example, CYP11A (cholesterol side chain cleavage) and aromatase (CYP19a, CYP19b) are involved in the production of steroids and E2, respectively. CYP17 α-hydroxylase/17, 20-lyase is a key element in the steroidogenic pathway that produces progestin, which is needed to support final germ cell maturation (Miller, 1988, 2005; Pandey and Miller, 2005; Patiño *et al.*, 2001; Scott *et al.*, 2010; Villeneuve *et al.*, 2008).

Up to date, only one study is available in the scientific literature investigating the effects of the inhibition of hydroxysteroid dehydrogenases (HSD) on fish reproduction (Villeneuve *et al.*, 2008), which involved exposing fathead minnow to trilostane, a human pharmaceutical acting through specific inhibition of 3β-hydroxysteroid dehydrogenase (3β-HSD), and which is mainly used for treatment of Cushing's disease and breast cancer. The HSD family play critical roles in the steroidogenic process (Miller, 1988, 2005), and in particular, 3β-HSD/Δ5-Δ4 isomerase converts Δ5-3β-hydroxysteroids (e.g., pregnenolone, 17α-hydroxypregnenolone) into Δ4-3-ketosteroids (e.g., progesterone, 17α-hydroxyprogesterone, androstenedione) (Norris, 2007). The potential relevance of inhibition of other steroidogenic enzymes is less clear.

6.2 Aim

Chemical interference with the vertebrate hypothalamic-pituitary-gonadal (HPG) axis can cause several adverse developmental and reproductive effects. To date, the majority of the work with HPG-disrupting chemicals in fish has focused on those interacting with the ER (agonists and antagonists), and to a lesser extent, the AR. However, alterations in the synthesis of key molecules within the axis, including DHT, can also perturb the HPG function. Dutasteride is a pharmaceutical used as inhibitor of the 5α-reductases, the enzymes converting T into DHT, and is usually employed in the treatment of prostatic diseases (e.g. prostate enlargement, prostate cancer). While it is unlikely to be environmentally relevant as a contaminant, dutasteride is well suited as a model chemical since it is a highly specific inhibitor of 5α-reductases (GSK, 2001). Given the specificity of the mode of action, this study represents the first robust test of the hypothesis that interference with the synthesis of DHT, through the inhibition of 5α-reductases, could cause reproductive dysfunction in the teleost fathead minnow.

6.3 Methods

6.3.1 Test species

Fathead minnows were supplied from breeding stocks maintained at Brunel University, London, UK. Two weeks before the beginning of the study, sexually mature males and females were separated to prevent any spawning activity and acclimated to the test conditions. Fish were fed three times per day, once with adult brine shrimp (Tropical Marine Centre,

Gamma irradiated) and twice with flake food (King British Tropical flake food, Lillico, Surrey). Fish were not fed on the sampling day.

6.3.2 Test substances and dilution water

The test substance, dutasteride (CAS number 164656-23-9), was obtained from GlaxoSmithkline (GSK) as 99.9% pure. Concentrated stock test solutions were prepared in N,N-Dimethylformamide (DMF, CAS number 68-12-2, ≥99%) (Sigma, Poole, UK). One master concentrated stock solution (10.0 g/L) was made in DMF, stored at $+4^{\circ}$C, and used to make up fresh dosing stock solutions at 0.4, 1.28, and 4 g/L. Fresh dosing stock solutions were made up weekly, in order to avoid potential degradation in the stock bottle. The dilution water was monitored for general parameters (e.g. pH, hardness, conductivity). During the exposure studies, mean water pH ranged from 7.6 to 8.1, temperature from 24.79 to 26.12 ºC, and dissolved oxygen from 7.06 to 7.95 mg/L.

6.3.3 Test conditions – Fish short term reproduction assay

The 21-days reproduction study was performed using a protocol proposed by Harries *et al* (2000) and Ankley *et al* (2001), and subsequently adapted by Winter *et al* (2008). The experiment was carried out using a continuous flow-through system, as described in Chapter 4. The test apparatus was comprised of glass fish tanks (8 per treatment) with a working volume of 10.5 L. A spawning U-shaped PVC tile and a collection tray were placed in each tank. The collection tray consisted of a rectangular glass tray (110mm×110mm×20mm) covered with a 0.5 cm^2 stainless steel mesh. The tile was used as the nest-site for spawning, while any eggs that fell off the tile were collected in the underneath tray; the mesh allowed eggs to be trapped into the tray, without them being affected by the fish (Fig. 6.1). Thorpe *et al.* (2007) demonstrated that this type of system of egg collection is highly robust and reduces the variability of egg numbers produced per couple per batch. Screens were placed between all test vessels to prevent fish visually interacting with those in neighbouring tanks and to minimize disturbance due to operator movements.

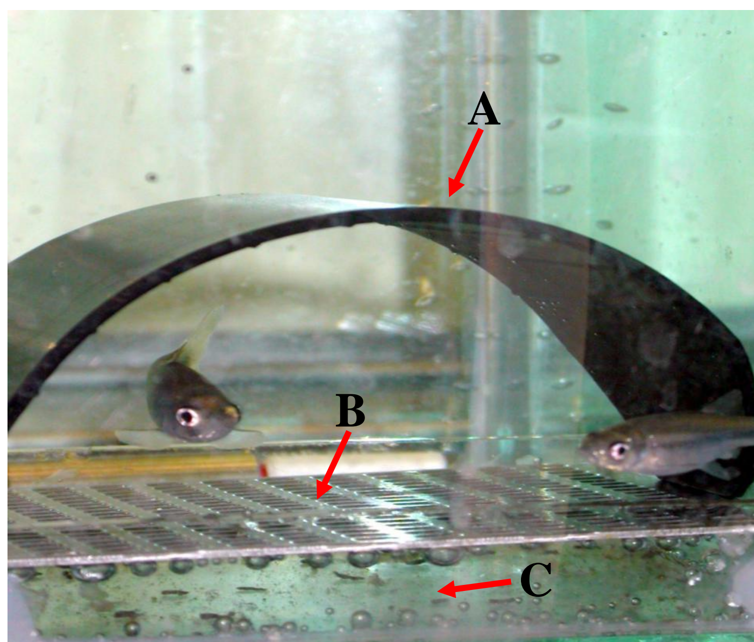


Figure 6.1. *Spawning tank with male and female fathead minnow. Following male courtship behaviour, females lay adherent eggs on the undersurface of the tile. Males guard the spawning site and are strongly territorial during the spawning period. The spawning site is made of (A) spawning tile, (B) metal mesh and (C) glass tray. The base is designed to contain any eggs that do not adhere to the tile surface and would therefore fall to the bottom of the tank (or those eggs laid directly onto the flat metal base).*

Thermostatically heated (25 ± 1°C) dechlorinated tap water from a header tank flowed through 6 flow-meters into six glass mixing chambers at a rate of 600 mL/min. The same chambers also received test chemicals stock solution in DMF at a rate of 0.015 mL/min, in order to achieve the desired nominal concentrations. Separate lines from each mixing chamber supplied about 12 tank volumes changes per day to each of eight replicate test vessels. The final experimental set up included five groups: one dilution water control group (DWC), one solvent control group (SC), and three dutasteride exposed groups (10, 32, 100 µg/L). The SC vessel received the same rate of addition of DMF, such that the water in all test vessels contained DMF at 0.0025% (OECD suggested limit: 0.0095%). The test was run at a nominal water temperature of 25 ± 1 °C, with a photoperiod of 16 hours light: 8 hours of dark, and with 20 minute dawn/dusk transition periods. The light intensity at the surface of the test vessels was measured by a lux meter (Lutron, LX-101) once a week during the study, and was 740 ± 120 lux (n = 6).

6.3.4 Exposure protocol

To initiate the test, one male and one female fish were randomly selected and placed in each tests vessel; there were eight pairs for each concentration of dutasteride and for the control groups. The fish were then left to acclimate with minimal disturbance for 3 d, during which the fish were just fed (no cleaning or measurement of any reproductive parameters were undertaken). After 3 d, the fish were then left for a further 11 d to assess breeding compatibility via observations of normal breeding behaviour, egg number, egg quality (e.g. viability and the presence of abnormalities) and spawning success (e.g. whether the female laid eggs on the spawning tile). During this pre-acclimation period, "spare" pairs of fish were held under identical conditions as the others, until successful breeding was confirmed in all test vessels. Any fish that did not meet these requirements was replaced by one of the spare fish, with all movements being recorded. The spawning data from this period, although recorded, were not used in the final analysis to assess the effect of the test chemical on reproductive performance.

After the acclimation period of 14 days, fish reproductive performances were assessed during a 21-day pre-exposure period, a 3-day transition (when dosing of dutasteride was started to allow the system equilibration), and a further 21 days of exposure to dutasteride. Each day the spawning tiles and the collection trays were removed from each tank, and if eggs had been laid, the apparatus was replaced with a clean set. Under microscope observation, the number of fertilized, non-fertilized, and dead eggs were counted, with the help of a ticker counter. Furthermore, fish behaviour was daily monitored (e.g. feeding behaviour, male aggressiveness), and any abnormal situation recorded. A schematic representation of the experimental protocol is represented in Figure 6.2.

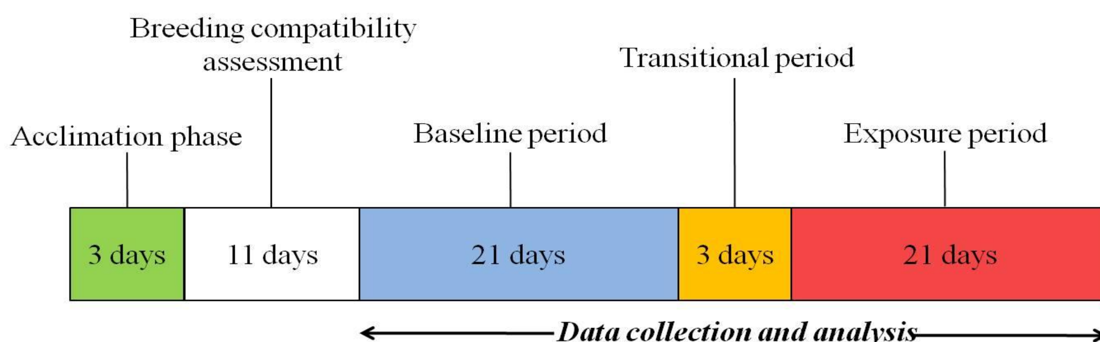


Figure 6.2. *Schematic representation of the fish short term reproduction assay, showing the different phases of the experiment, including the acclimation phase, breeding compatibility assessment, baseline, transitional and exposure periods. Only data from the baseline and exposure period were analysed. Data from the transitional period were collected, but not used for the statistical analyses.*

6.3.5 Fish sampling

After 21 days of exposure to dutasteride, fish were terminally anaesthetized using Ethyl 3-Aminobenzoate Methanesulfonate salt (MS-222, 0.5 g/L; adjusted to pH 7.5 with 1M NaOH) (Sigma, Poole, UK; CAS No: 144-55-8). Once the male appeared to be listing and hence under anaesthesia, sperm samples were collected using a 5 µl graduated capillary tube (Sigma Aldrich, Z611239-250EA) with the volume collected noted, using the protocol described by Kime *et al.* (2001) and Hala *et al.* (2010). Briefly, the fish was removed from the anaesthetic solution and its ventral surface dried by using absorbent paper, in order to avoid potential activation of sperm motility by inadvertently contaminating the milt sample obtained with water. The male was subsequently placed onto its dorsal surface and held firmly within a slit cut into a normal domestic dish-washing sponge. The lower abdomen surrounding the urogenital opening was once again dried of excess moisture, and milt was obtained by gently compressing the abdomen uni-directionally from just below the pectoral fins toward the uro-genital opening. The collected milt was expelled into a microfuge tube and diluted 1:50 in cold Catfish extender (94mM Sodium Chloride, 27 mM Potassium Chloride, 15mM Trizma Hydrochloric Acid and 50 mM Glycine pH to 7.5) and then stored at +4 ^{o}C until density and motility analyses were conducted (22 hours later).

After trans-spinal severance, blood samples were collected from each fish via the caudal peduncle using 75 µl heparinized capillary tubes and decanted into eppendorfs containing aprotinin (Sigma), then stored on ice until centrifugation at 7,000 × *g* for 5 min at 4°C. The resulting plasma samples were subsequently stored at −20 °C until analysis for steroid levels by radioimmunoassay (RIA) (Scott *et al.*, 1984), and determination of VTG protein levels by ELISA (Biosense Laboratories AS, Bergen, Norway). After blood collection, all fish were measured for wet weight (g) and fork length (mm), and these parameters were used to calculate the CI as described in Section 4.3.3. The liver was immediately extracted for molecular analyses, promptly weighed and then frozen in liquid nitrogen before being stored at -80°C. Its weight was used to calculate the hepatosomatic index (HSI = liver weight (g) / body weight (g) × 100). The gonads were subsequently extracted, weighed to determine the GSI (GSI= gonad weight (g) / body weight (g) × 100), fixed in Bouin's solution for 6 hours, and stored in IMS 70% until histopathological analyses. Finally, the expression of SSCs was recorded for every fish, including number and prominence of nuptial tubercles, black dorsal fin spot occurrence, fatpad presence and weight (Smith, 1978).

6.3.6 F1 generation hatchability trials

In addition to assessing adult fish reproductive performance, specific batches of eggs were subjected to hatchability trials to assess early embryo-larval development, during both baseline and exposure periods. The protocol used was a modification of the one described by Winter *et al.* (2008). During the baseline period, all the fish pairs were assessed in order to define the quality of the spawning batches (e.g. number of dead or non-fertilized eggs) and embryos hatchability success. After the first two spawning assessment, the 3 best couples in each treatment group were selected, and the following hatchability trials were performed only on batches of egg from those fish pairs until the end of the study. This strategy was required because a great variability was observed in the quality of embryos spawned by different fish pairs.

During the baseline period, for each hatchability trial, 60 viable embryos were randomly selected from the tile, and these were placed in 3 replicate wells of a sterile 6 well plate (Greiner Bio-One Ltd., UK) with a total volume of 16 mL/well, and incubated at 25 °C (20 embryos per well). Embryos were incubated in the water taken from the parental tank, and the incubation solution in each well was replaced daily. If the number of eggs on the tile was insufficient to provide 60 fertilized eggs, they were taken from the tile and/or the mesh. For each fish pair, a gap of one spawning event was included between two hatchability trials. Embryos were observed daily for mortalities and abnormalities, and dead ones were removed in order to prevent fungal infections. Embryos were incubated until complete hatching, and when at least 90% of embryos were hatched, the hatching time was recorded. The newly hatched larvae were assessed for morphological abnormalities, and survival until hatching was complete (normally within 2 days from the first hatching event). Following the assessment of hatchability, all larvae were sacrificed using an overdose of anaesthetic (0.5 g/L MS222 in dilution water, adjusted to pH 7.5 with 1M NaOH).

During the exposure period, the hatching trials were performed as described before, with only one modification of the protocol. During this phase, and only for the pairs exposed to dutasteride, 120 eggs were collected from each spawning event, and randomly allocated in 6 replicate wells containing 20 embryos each. Three replicate were incubated in clean dilution water, while the other three were incubated in parental tank water (containing dutasteride), in order to evaluate the effect of the drug on the hatching ability. All the incubation solutions were replaced daily.

6.3.7 Sperm analyses

6.3.7.1 *Sperm samples preparation and motility video-recording*

A video-camera mounted Olympus BH-2 microscope (Olympus, Tokia, Japam), with negative phase contrast and heated stage, was attached to a monitor and a compact-disc (CD) recorder was used to observe and record the motility of fathead minnow spermatozoa. Diluted milt samples (1:50) in microfuge tubes were resuspended, and a 10 µl aliquot of the sperm suspension was diluted a further 1:2 with 10 µl of aquaria tank water (maintained at 25°C) in order to induce motility. Immediately after this dilution step, a 1 µl aliquot was taken and placed onto a single well of a dipped multi-well slide (12- Well Multi-Test Slide, ICN Biomedicals Inc., Cat. No. 6041205E) and covered with a Bovine Serum Albumin (BSA, Sigma, Cas. No. 9048-46-8) coated coverslip (prepared by washing in 1% BSA). Motile sperm cells visible within the frame of view (10X objective with a 2.2 column) were recorded for 5 minutes. The heated stage of the microscope was maintained at 25°C throughout the duration of the sperm cell motility recordings.

6.3.7.2 *Sperm Tracking and the CASA System*

The sperm tracking was carried out using The Hobson Sperm Tracker (Hobson Vision Systems, Sheffield, UK). The computer-assisted sperm analysis (CASA) system was used to record the movement of sperm. The recordings were replayed and tracked for set time increments using tracking software which tracks individual spermatozoa and calculates various motility parameters (Kime *et al.* 2001). Sperm quantity and quality parameters included milt volume (µL), sperm concentration (cells/ml) and sperm motility parameters (Fig. 6.3), including:

VCL (µm s^{-1}) = Curvilinear Velocity, which constitutes the sum of incremental distances moved by a spermatozoa along a trajectory for each time frame divided by the time taken to cover that particular trajectory.

VAP (µm s^{-1}) = Average Path Velocity, which is a velocity over a calculated, smoothed path, i.e. a shorter distance than that used for calculating VCL.

VSL (µm s^{-1}) = Straight Line Velocity, the straight-line distance between the start and end of a trajectory divided by the total track time.

LIN (%) = Percentage Linearity of motion, expressed as a percentage, is described as the straight-line distance between the start and endpoints of a track divided by the sum of the incremental distances along the path of trajectory (VSL/VCL×100).

STR (%) = Straightness, the straightness of the average path (VSL/VAP×100).

MOT (%) = percent motility expressed as a percentage, it is the number of motile cells divided by the total numbers of cells visible in the analysis field (motile and immotile).

The recorded video sequences of motile sperm cells were tracked in 15 second time increments up to 2 minutes (Kime *et al.* 2001).

6.3.7.3 *Calculation of sperm concentration*

Sperm counts were conducted on a Neubauer haemocytometer by diluting the already diluted milt (1:50) a further 1:2 in cold catfish extender (10 μl diluted milt + 10 μl extender) in a microfuge tube. A 15 μl aliquot of this diluted milt was allowed to flood one chamber of the Neubauer haemocytometer. Spermatozoa were counted after allowing 1-2 minutes for the cells to settle. Sperm cells were counted on an Olympus (BX50) microscope (with a 40X objective) coupled to a camera and JVC TV monitor. Sperm cells occupying the central grid of 25 hatched squares (5x5 squares) of the flooded chamber were observed. Within this grid of 25 squares, spermatozoa were firstly counted in the first square of the grid (top/left corner of the 25 hatched squares) using the following rules: if < 10 spermatozoa were counted in this first square, then a total cell count was conducted in all 25 squares and averaged; if 10-40 spermatozoa were counted then total counts in 10 squares were averaged; if >40 spermatozoa were counted, then total counts of 5 squares were averaged. The average number of spermatozoa counted within the volume of the 25 square counting grid (0.1 μl) were expressed as counts x 10^4 cells/ml (average spermatozoa counts in 0.1 μl x 10 000 = cells in a 1 ml volume). This count was finally corrected for the 1:50 dilution of milt in extender and then the 1:2 dilution prior to conducting the cell counts (Fig. 6.4).

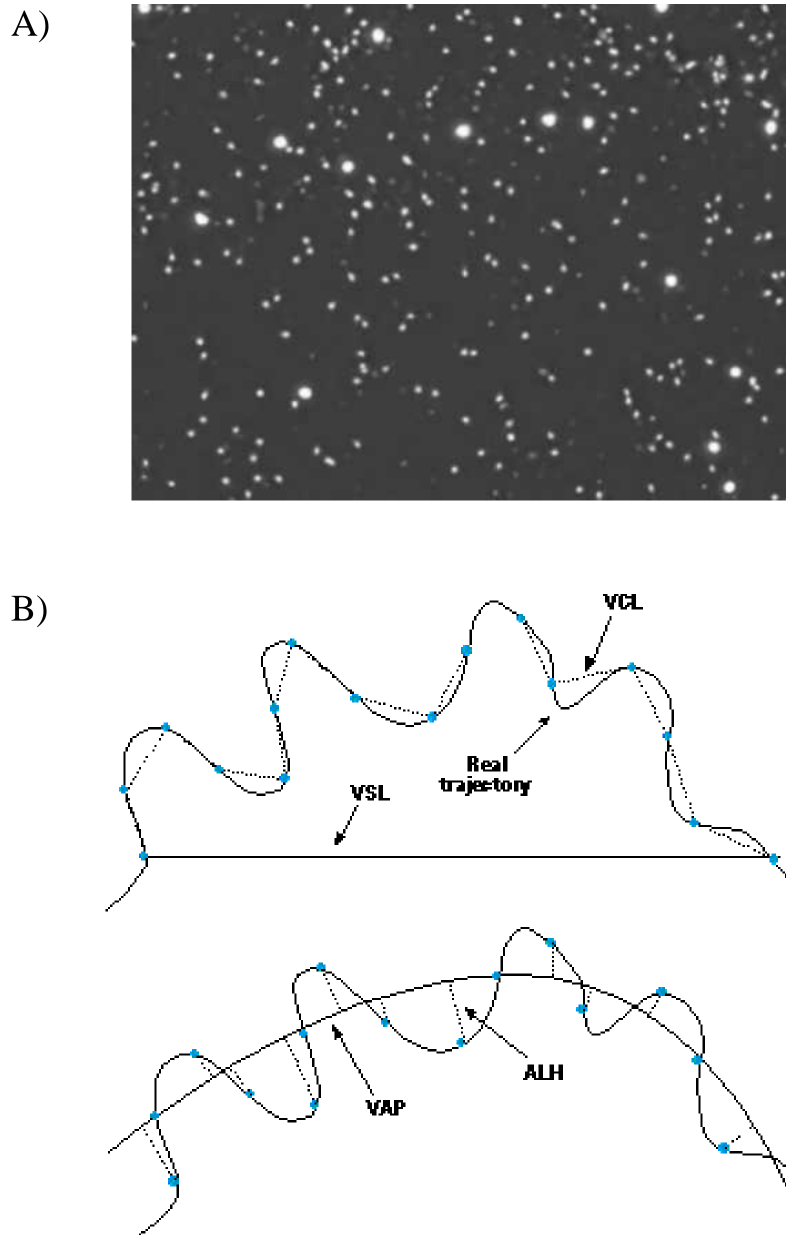


Figure 6.3. *Quantification of sperm motility using computer-assisted sperm analysis (CASA). a) Example of fish sperm observed using an Olympus BH-2 microscope equipped with a negative phase contrast objective (10X). b) Definition of sperm tracks and related parameters. VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity. The blue circles show the position of the sperm head centroid in each frame (from Tablado et al., 1996).*

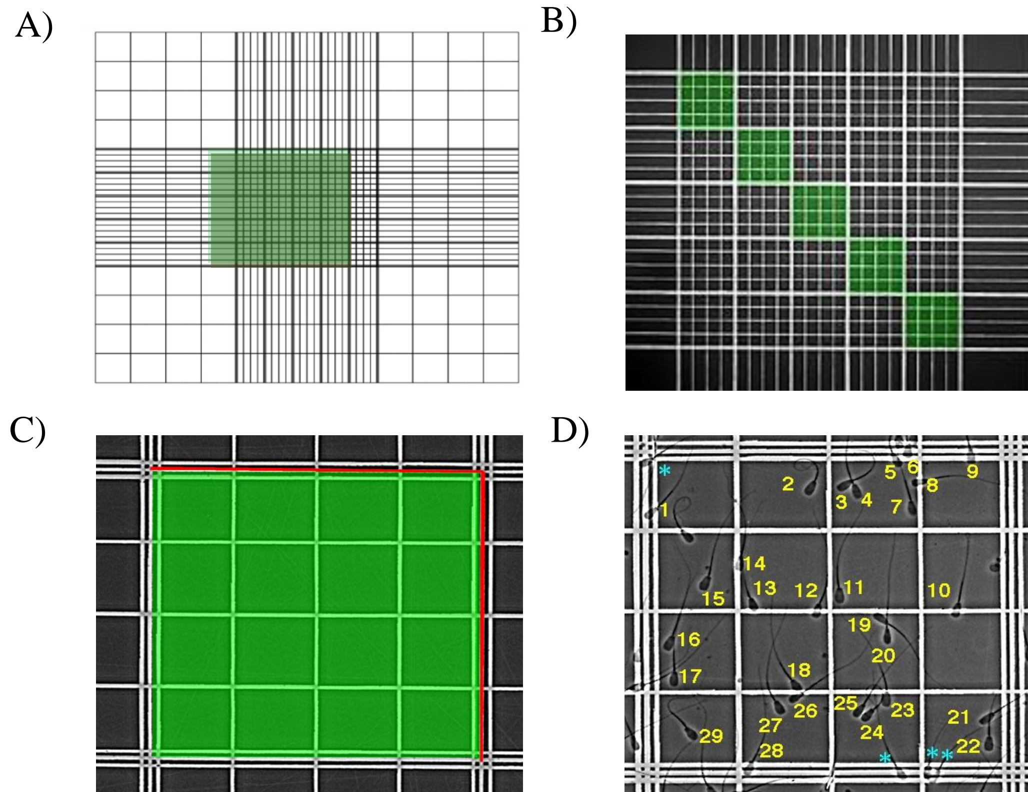


Figure 6.4. *View of the haemocytometer grid system, with a progressive zoom on the area if interest. The green colour indicates the area of the grid used for the sperm count. (A) The haemocytometer is 0.1 mm deep and the 25 large squares represent an area of 1 square mm; the volume above the 25 squares shown is 0.1 μl.(B) Assuming that the count of the sperm was performed in 5 squares, it gives an estimate of the sperm present in 0.02 μl (0.1/5 = 0.02). (C) Each of the 5 squares is divided in 16 further squares. This value multiplied by 50,000 gives the number of spermatozoa present in 1.0 ml. A visual example of sperm counted in one of the 5 squares is illustrated in (D).*

6.3.8 Gonad histopathology

Three slices of 3-4 mm thickness were transversally cut from the each gonad, dividing it in anterior, median, and posterior parts. Slices were prepared for the histopathology analyses following the method detailed in Section 4.3.6, and analysed according to the EPA guideline described in the same section (EPA, 2006).

6.3.9 Determination of circulating plasma steroid concentrations

Circulating plasma levels of T, KT and E2 in males and females were quantified by specific radioimmunoassays, as described in Section 4.3.5.

6.3.10 Plasma vitellogenin quantification

VTG protein levels were quantified using a specific fathead minnow VTG ELISA kit (Biosense Laboratories AS, Bergen, Norway), following the instruction of the manufacturer.

6.3.11 Chemical analyses of water concentrations of dutasteride

Water samples were randomly collected from four out of eight replicates per treatment on day 0, 4, 7, 14 and 21. The concentrations of dutasteride were measured by LC-MS/MS.

6.3.12 General statistical analyses

Statistical analyses were conducted using SigmaStat software (version 3.5, Systat Software Inc., Germany). Data for sperm cell count, wet weight, standard length, condition factor, GSI, HIS, SSCs, VTG in females, and plasma steroid concentrations were analysed for normality (Kolmogorov-Smirnov test) and variance homogeneity (Levene's test). Where assumptions of normality and homogeneity were met, one-way analysis of variance (ANOVA) was followed by the Dunnett's test to compare the treatment means with respective controls. Where the assumptions were not met, data were analysed using a non-parametric test, Kruskal–Wallis ANOVA on Ranks, followed by Dunn's post-hoc test (Zar, 1999). Effects of treatments on the most advanced stage of gametogenesis, and dominant germ cell, were analysed using a variation of the chi-squared test, the randomization test of goodness of fit with Montecarlo simulation (n = 10,000) (McDonald, 2009). Statistical comparisons were made to determine if there were significant differences between the DWC and SC, by t-test or, in case of non-normal and non-homogeneous data, by Mann-Whitney Rank Sum test.

Reproductive performances, in particular total and mean (both per pair and per treatment) egg production, number of batches of eggs, fertilization success, F1 hatchability and larval fish survival (% of hatched fish that survive to 48 h post-hatch), were analyzed to compare pre-exposure versus exposure data within and among treatment groups. Where assumptions of normality and homogeneity were met, t-test and One-Way ANOVA followed by a Dunnett's test were performed to compare, respectively, two or multiple groups; if the assumptions were not met, data were analysed using non-parametric Mann-Whitney Rank Sum test and Kruskal–Wallis ANOVA on Ranks followed by Dunn's test for pairwise or multiple comparisons respectively.

Where necessary, data were log-transformed for normalization and to reduce variance heterogeneity and percentage data were arc sine transformed. The results are reported as

means ± standard error of the mean to indicate the precision of the mean estimate. Statistical significance was set at a level of p = 0.05, unless otherwise indicated.

6.3.13 Statistical analyses of sperm motility data

6.3.13.1 ***Analysis of motility parameters (VCL, VAP, VSL, LIN, STR, MOT).***

Approach 1: Sperm populations from all the replicates in each treatment were considered and analysed as a single group (n = 8).

Approach 2: Mean values of each parameter was calculated for the sperm population of each single fish, and combined according to the treatment (n = 8).

Approach 3: Mean values of each parameter was calculated for the sperm population of each single fish, and combined according to the treatment (n = 8). In each treatment group, samples presenting a number of tracks < 35 were removed (DWC = 0, SC = 1, D10 = 2, D32 = 2, D100 = 2).

All the data were tested for normality and equal variance (percentage values were log transformed). Where assumptions of normality and homogeneity were met, one-way analysis of variance (ANOVA) was followed by the Dunnett's test to compare the treatment means with respective controls. Where the assumptions were not met, data were analysed using a non-parametric test, Kruskal–Wallis ANOVA on Ranks, followed by Dunn's post-hoc test.

6.3.13.2 ***Pattern Analysis: PATN***

A detailed description of the analyses is given by Abaigar *et al.* (1999) and Holt at al. (2001). The motility data from all replicates were merged and subjected to multivariate analysis using the software PATN (CSIRO, Canberra, Australia) (Belbin, 1993). The program analyzes and compares the motility parameter values associated with each spermatozoon and identifies subgroups within the sperm population ("patterns"), which are successively subject to hierarchical classification. All the procedure is carried out by the program entirely independently of the investigator. In the present study, the groups were distinguished on the basis of multivariate combinations of the following motility parameters: VCL, VAP, VSL, LIN, and STR.

After completion of the PATN analysis, each individual spermatozoon is categorized as belonging to one of the sub-groups, and the relative percentages of spermatozoa belonging to each group were calculated.

6.3.13.3 *Statistics of PATN results.*

Approach 1: Analysis was applied to the whole dataset, considering the sperm populations obtained from all the replicates as a single group (n = 8). Data were analyzed using a chi-square test for goodness-of-fit (McDonald, 2009).

Approach 2: Relative percentages of sperm classified into different groups were calculated for each individual sample. The single values were combined by treatment, log transformed, checked for normality and homogeneity, and analyzed by One-Way ANOVA (n = 8) followed by Dunnett's Test.

6.3.13.4 *Analysis of sperm populations in a 2-dimensional graph (LIN by VAP).*

Sperm populations from all the replicates in each treatment were considered as a single group (n = 8).

6.4 Results

6.4.1 Water concentrations of dutasteride

Water analysis showed that fish were exposed to the test substance in the expected range of concentrations and that there was no contamination of control tanks with dutasteride (Table 6.1).

6.4.2 Morphometric endpoints

At the end of the exposure phase, the somatic endpoints (length, weight, CI, GSI, HSI) were not significantly affected in male and female fish exposed to dutasteride, when compared to the SC, except the GSI of females exposed to the highest concentration of the drug (100 μg/L), which showed a significant increase (p = 0.034) (Table 6.2).

Table 6.1. *Measured water concentrations (µg/L) of dutasteride during the exposure period, measured by LC-MS-MS.*

Exposure Day	Replicate	Nominal concentrations of dutasteride (µg/L)				
		DWC	SC	10	32	100
0	A	< 0.5	< 0.5	14.4	37.8	116.9
	B	< 0.5	< 0.5	13.0	35.3	125.5
	C	< 0.5	< 0.5	13.9	39.5	125.6
	D	< 0.5	< 0.5	--	37.1	104.2
7	A	< 0.5	< 0.5	12.6	44.8	108.0
	B	< 0.5	< 0.5	12.0	34.0	100.6
	C	< 0.5	< 0.5	15.3	40.7	134.8
	D	< 0.5	< 0.5	12.2	39.1	112.9
14	A	< 0.5	< 0.5	12.3	40.7	109.2
	B	< 0.5	< 0.5	11.2	39.9	130.6
	C	< 0.5	< 0.5	16.0	37.6	126.2
	D	< 0.5	< 0.5	9.8	39.5	120.4
21	A	< 0.5	< 0.5	6.9	22.4	83.2
	B	< 0.5	< 0.5	13.2	20.2	61.8
	C	< 0.5	< 0.5	15.0	30.9	57.6
	D	< 0.5	< 0.5	11.2	--	54.8
Mean Values (µg/L)		**< 0.5**	**< 0.5**	**12.6 ± 0.9**	**35.3 ± 7.2**	**104.3 ± 26.9**

"--" = Sample Not Available.

Table 6.2. *Mean values (±SD) of fish fork length, wet weight, condition factor (CI), gonadosomatic index (GSI), and hepatosomatic index (HSI) for male and female fathead minnows exposed to dutasteride (10, 32, 100 µg/L) during a short term reproduction assay.*

Dutasteride (µg/L)	Sex	Morphometric endpoints				
		Length (mm)	Weight (g)	CI	GSI	HSI
DWC	M	68.00±4.28	5.97±1.28	1.88±0.25	1.79±0.27	2.38±0.58
	F	51.13±3.04	2.16±0.69	1.59±0.33	12.70±3.44	3.18±0.67
SC	M	67.25±5.23	6.04±1.51	1.96±0.25	1.90±0.51	2.65±0.58
	F	51.00±1.85	2.05±0.19	1.56±0.25	13.13±3.12	3.28±0.73
10	M	64.50±5.88	4.78±1.02	1.77±0.20	1.87±0.47	2.33±0.35
	F	53.63±2.72	2.27±0.42	1.47±0.15	13.08±3.45	3.21±0.96
32	M	68.50±6.67	5.87±1.93	1.76±0.18	1.73±0.40	2.24±0.53
	F	51.00±3.16	2.21±0.48	1.59±0.28	14.94±1.92	3.43±0.83
100	M	65.13±3.36	4.75±0.80	1.72±0.22	2.34±0.59	2.60±0.73
	F	52.88±2.90	2.27±0.31	1.55±0.24	16.57±2.69*	3.08±0.90

Asterisks (*) represent a significant difference compared to the Solvent Control (SC) (: $p < 0.05$).*

6.4.3 Effects on reproductive performance

Cumulative egg production during both the baseline (pre-exposure) and exposure periods of 21 days each are shown in Fig. 6.5. Egg production was similar in all five treatments in the baseline period. In the exposure period, the control groups – both SC and DWC - continued to lay eggs at the same rate as before. In contrast, egg production was significantly reduced in the groups exposed to dutasteride. A very pronounced effect was produced by 100 µg dutasteride/L ($p < 0.001$), with egg production slowing very markedly within the first 7 days of exposure. Interestingly, within this exposure group, fish responses presented a range of severity: two pairs had the last spawning event during the transitional period (3 days) (when dosing of the chemical began and its concentration was allowed to stabilize in the dosing system), completely ceasing spawning activity thereafter, while the remaining pairs presented a more progressive slowing down of egg production, but still ceasing completely egg production after 14 days of exposure, except one pair, which spawned on the 19th day of exposure. Dutasteride produced a statistically significant reduction in egg production ($p = 0.038$) also at 32 µg/L. Also within this group, fish responses presented a range of severity, and 3 out of 8 pairs did not show any significant reduction of total egg production. The lowest concentration of dutasteride (10 µg/L) had no effect on egg production.

At the same time, there was a significant reduction in the mean number of spawning events per pair in the group exposed to 100 µg dutasteride/L ($p < 0.001$) when compared to the pre-exposure phase (from 6.3 ± 0.7 during the baseline period to 2.6 ± 1.7 in the exposure period) (Fig. 6.6A). A similar significant reduction was recorded in the mean number of eggs per spawning event in the groups exposed to both 32 µg/L ($p = 0.019$) and 100 µg/L ($p = 0.008$) (Fig. 6.6B). The mean number of eggs produced per pair in the pre-exposure and exposure phases was 387 ± 106 versus 313 ± 130, respectively, in the 32 µg dutasteride/L treatment group, while in the group exposed to 100 µg dutasteride/L it was 373 ± 88 *versus* 252 ± 154.

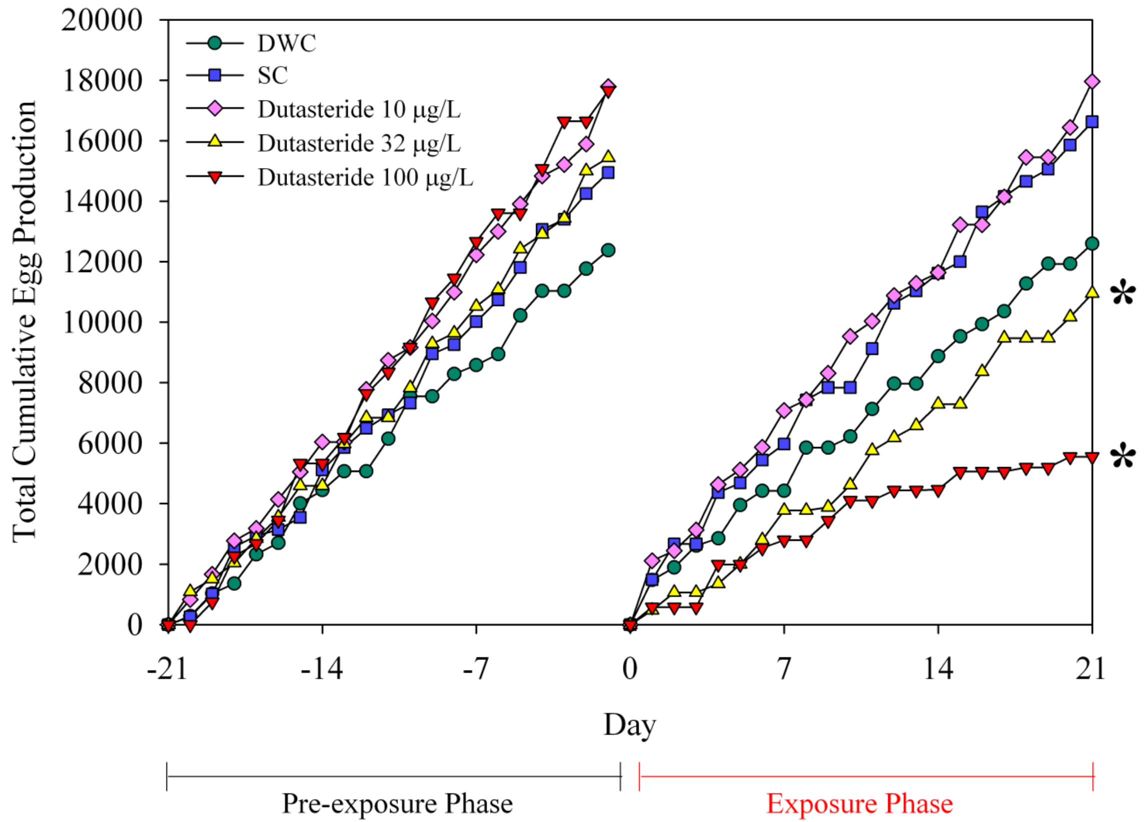


Figure 6.5. *Effect of different concentrations of dutasteride on cumulative total egg production during the fathead minnow short term reproduction assay. During the pre-exposure period, the rate of egg production was similar across each treatment group. Concentration-dependent effects on egg production became apparent within a few days of chemical exposure. *: $p < 0.05$ compared to the SC group ($n = 8$).*

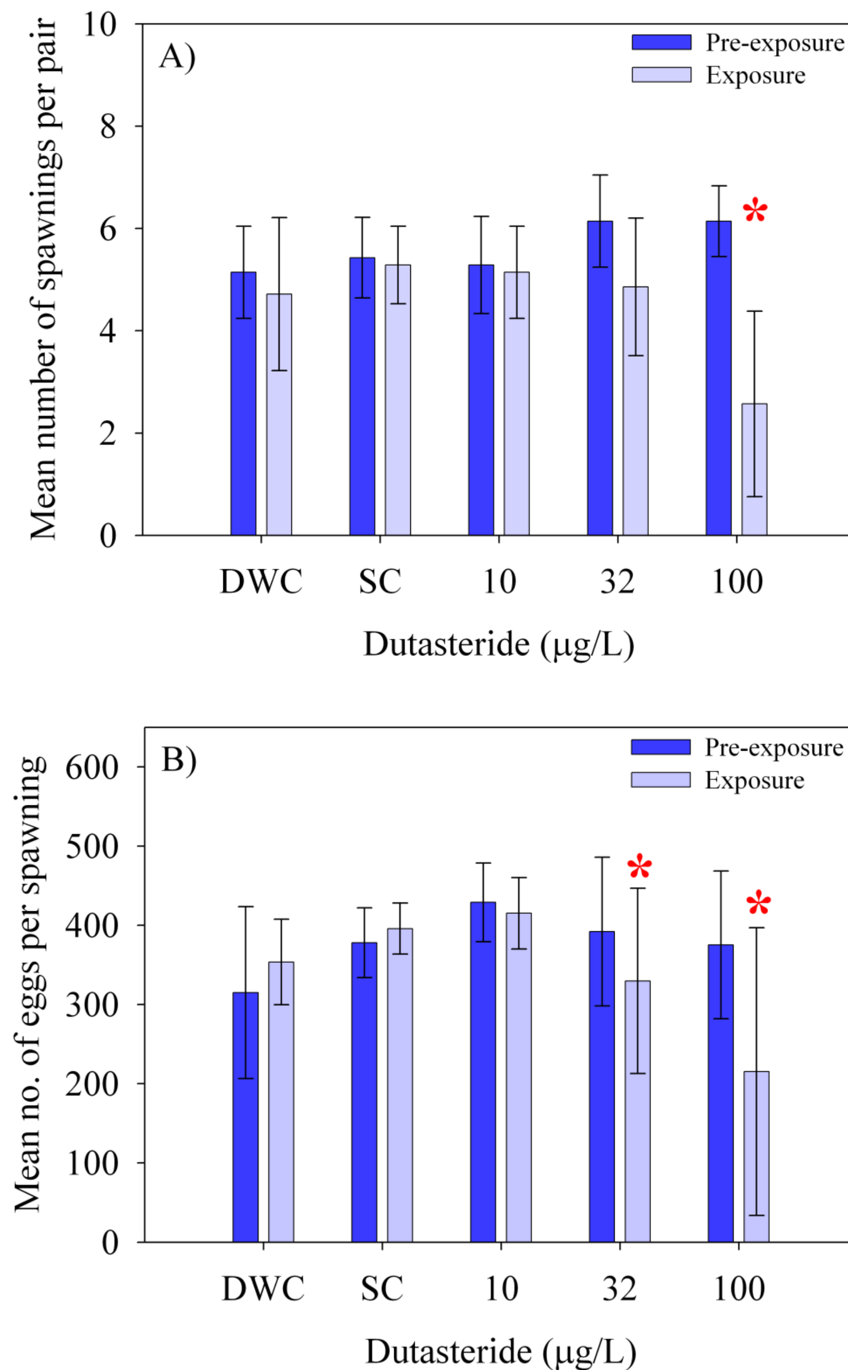


Figure 6.6. *(A) Number of spawning events and (B) mean number of eggs spawned per pair of fathead minnows (mean ± SD) during the pre-exposure and exposure phases. The asterisks (*) denote a significant difference in each treatment group between the exposure phase and the pre-exposure phase ($p < 0.05$; $n = 8$).*

The exposure to dutasteride did not cause any significant effect on the percentage of non-fertilized eggs and dead embryos in each spawning event (Fig. 6.7). In the group exposed to 100 µg/L of dutasteride, a non-significant increase of both endpoints during the exposure phase was recorded, when compared with the baseline period; however, this apparent increase was due to the fact that in this group the size of the egg batches spawned during the exposure period was much smaller than their size in the baseline period, and the spawning events with

a very small number of eggs, in the statistical analysis, acted as outliers, producing the large standard deviation illustrated in Figure 6.7 in the 100 µg/L group (exposure period) in both endpoints.

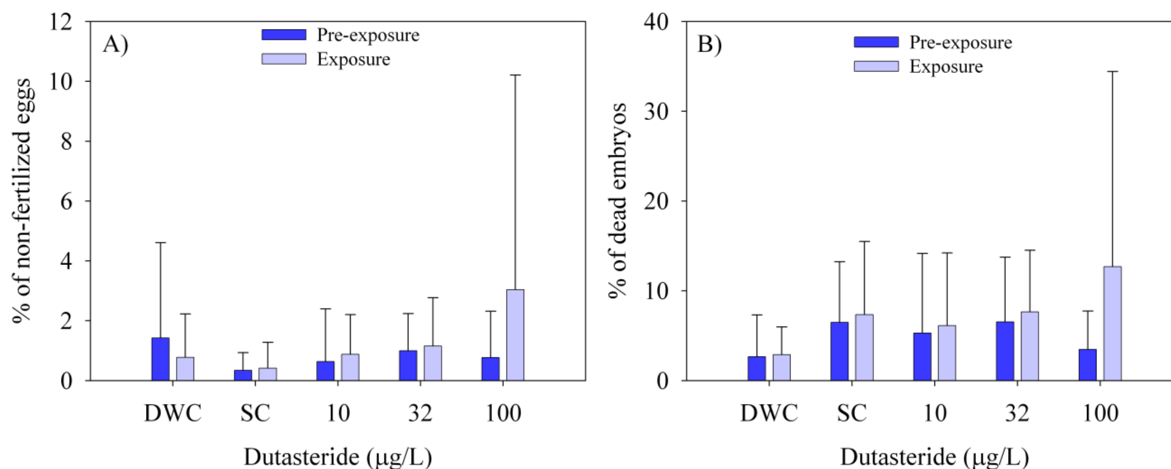


Figure 6.7. *(A) Percentage of non-fertilized eggs and (B) dead embryos per spawning event (mean ± SD) during the pre-exposure and exposure phases (n = 8).*

6.4.4 Secondary sexual characteristics

Among the SSCs used as endpoints in this study, the expression of nuptial tubercles in males was the most sensitive to the exposure to dutasteride, which caused a significant decrease of both tubercle prominence grade ($p < 0.001$) and tubercles number ($p < 0.001$) at 100 µg/L. A non-significant decreasing trend was already evident at 32 µg/L. This result was reinforced by the procedure employed in this study to assess SSCs, involving two independent operators, one of whom performed a blind analysis, without knowing the identity of the samples. The results obtained by the two operators were compared, and the statistical analysis did not show any significant difference (data not shown). On the other hand, fatpad height and index in males, and ovipositor length in females, did not show any significant variation after the exposure to dutasteride (Fig. 6.8).

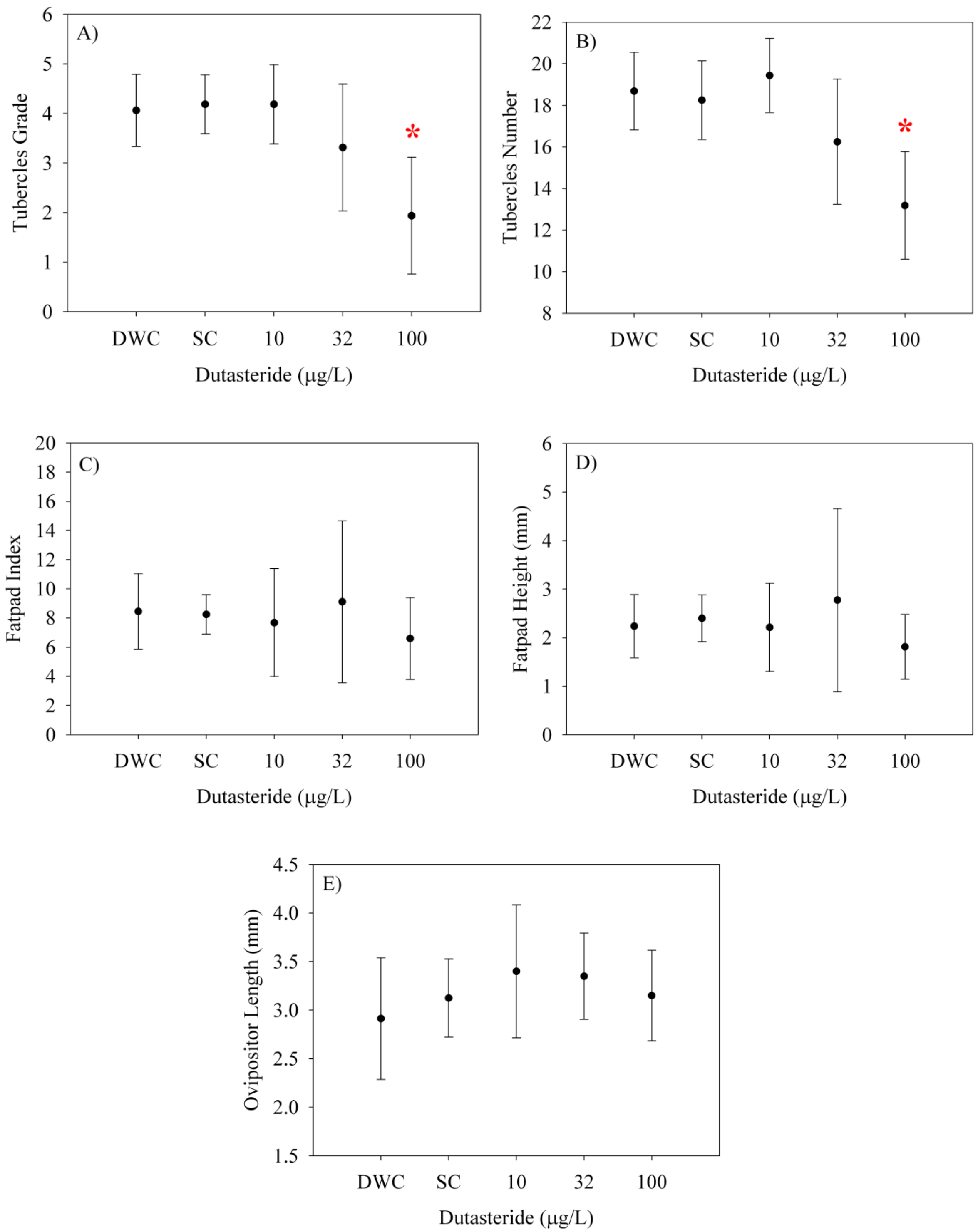


Figure 6.8. *Expression of secondary sexual characteristics in fish exposed to dutasteride during a 21-days reproduction test. (A) The grade of prominence of nuptial tubercles, (B) tubercle number, (C) fatpad index and (D) fatpad height were assessed in male fish, while the ovipositor length (E) was assessed in females (n = 8). *: p < 0.05 compared to the SC group (n = 8).*

6.4.5 Plasma steroid and vitellogenin concentrations

Dutasteride induced different responses in males and females (Fig. 6.9). In males, plasma concentrations of T were significantly reduced by the exposure to 100 µg dutasteride/L (p = 0.016); while KT concentration was reduced at both 32 µg/L (p = 0.025) and 100 µg/L (p = 0.002). The exposure to 100 µg dutasteride/L produced a reduction of both androgens concentrations of 50% *circa* compared to the concentrations detected in the SC group. Concentrations of plasma E2 were not affected by the exposure to the drug in males, even if a non-significant decrease was observed in fish exposed to 32 and 100 µg/L.

In females, the exposure to 100 µg dutasteride/L caused a significant decrease of circulating E2 (p = 0.042) and increase of T (p = 0.011), while VTG concentrations in the plasma were not affected by any of the dutasteride concentrations used in this study.

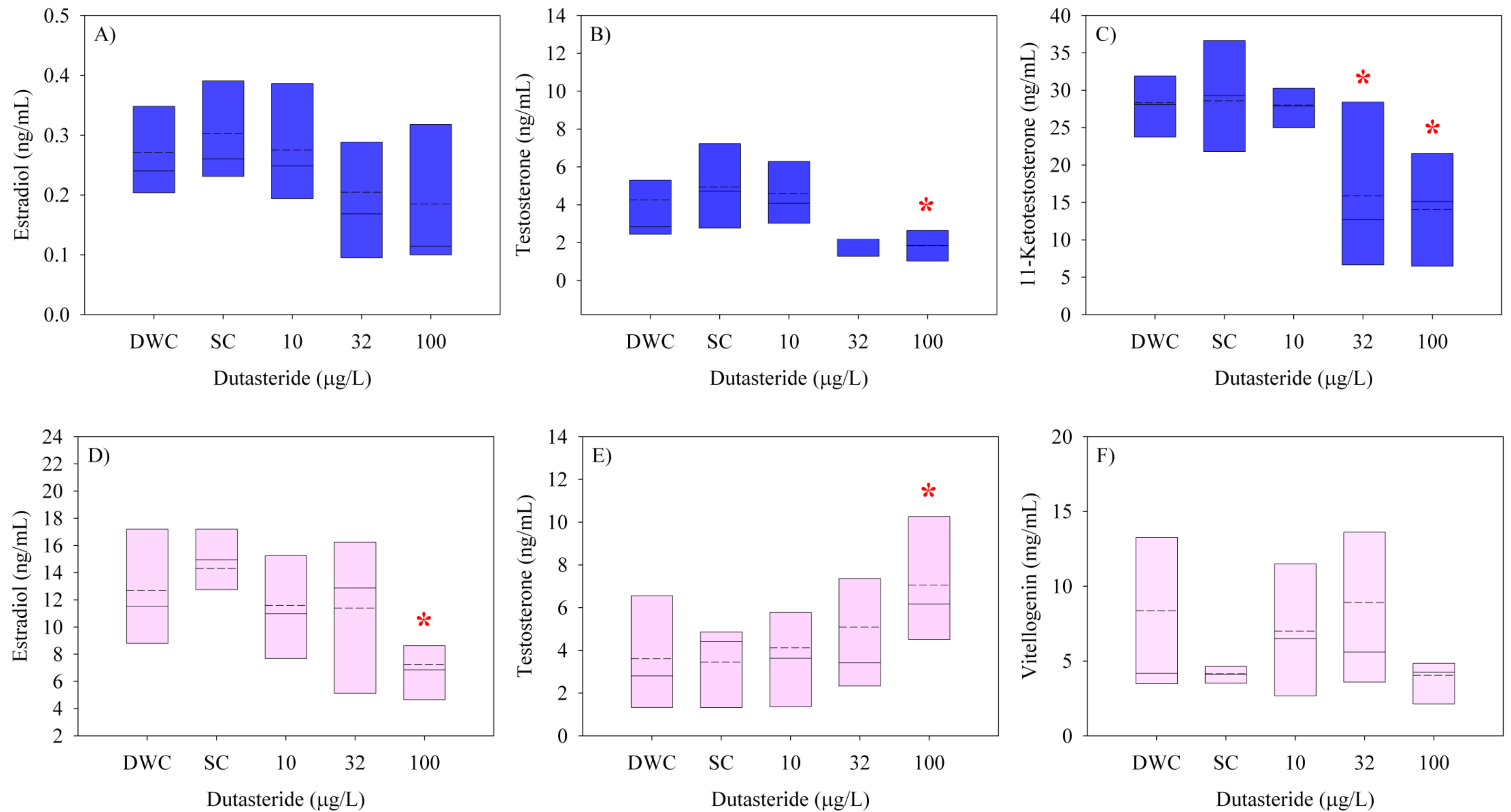


Figure 6.9. *Effect of dutasteride exposure on plasma concentrations of (A) E2, (B) T and (C) KT in males and on plasma concentrations of (D) E2, (E) T and (F) VTG in females at the end of a fathead minnow short term reproduction assay. Boxes represent median (full line), and mean (dashed line) with 25th and 75th percentiles. *: $p < 0.05$ ($n = 8$).*

6.4.6 Gonad histopathology

6.4.6.1 *Males*

Histological assessment of the testes did not show any significant differences among treatments. All the testes were at an advanced stage of spermatogenesis, spermatozoa were abundant and represented the dominant germ cell in all the samples (Fig. 6.10). No signs of pathological conditions were present in any treatment group. The thickness of the germinal epithelium, consisting of multiple spermatocysts in various phases of development, showed similar variability range among treatments; furthermore, no significant differences were observed in the proportional distribution of spermatogenic cell types.

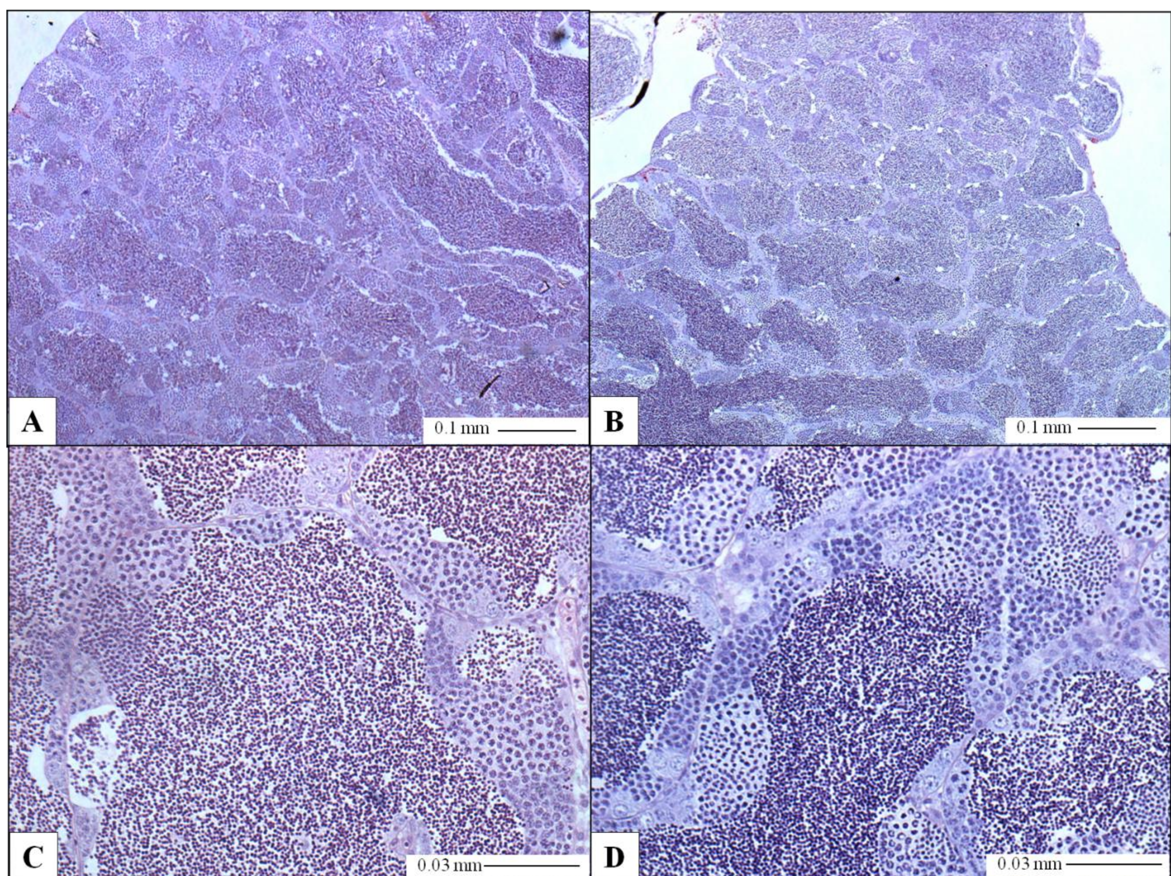


Figure 6.10. *Transverse sections of fathead minnow testes (A,C) from the Solvent Control group and (B,D) from the group exposed for 21 days to the highest concentration of dutasteride (100 µg/L). At the end of the exposure phase, the males in all the treatment groups had testes in an advanced stage of spermatogenesis; spermatozoa were abundant and represented the dominant germ cells in all the samples. No signs of pathological conditions were present in any treatment group.*

6.4.6.2 *Females*

The ovaries of female fathead minnow exposed to 100 µg dutasteride/L exhibited a significantly decreased proportion of vitellogenic oocytes when compared with fish in the SC group ($p = 0.048$) or DWC group ($p = 0.007$) (Fig. 6.11 & 6.12). The ratio between the number of vitellogenic and pre-vitellogenic oocytes ranged between 0.93-4.64% in the SC group *versus* 0.03-1.65% in the group exposed to the highest concentration of dutasteride; this decrease was mainly due to a significant reduction in the proportion of early vitellogenic oocytes ($p < 0.001$), and an increase in the number of perinucleolar oocytes ($p = 0.047$). The proportions of cortical alveolar and mid-late vitellogenic oocytes were not affected by the treatment, even if the latter showed a significant decrease when compared to the DWC group ($p = 0.046$) (Fig. 6.12).

Exclusively in the group exposed to 100 µg dutasteride/L, several histopathological conditions were observed (Fig. 6.13 & 6.14). All the exposed fish showed the presence of atretic follicles, characterised by clumping, perforation or degeneration of the chorion (or vitelline envelope), fragmentation of the nucleus, disorganization of the ooplasm, abnormal proliferation of interstitial/somatic tissue, and vacuolar hypertrophy of the surrounding granulose cells due to phagocytic processes (EPA, 2006). In vitellogenic follicles, one of the first morphological signs of atresia was a change in the appearance of the yolk (Saidapur, 1978), due to proteolysis of yolk proteins by cathepsins within the oocyte (Carnevali *et al.*, 2003; Eykelbosh and Van der Kraak, 2010; Wood and Van Der Kraak, 2003).

The resorption activity was also characterised by the proliferation of interstitial tissue containing macrophage aggregates. These cell clusters presented with small condensed eccentric or peripheralized nuclei, and their physiological presence has been shown to be enhanced by exposure to environmental toxicants or infectious agents (Blazer *et al.*, 1987). The presence of mitotically dividing oogonia was also observed, appearing as packets of cells resembling spermatocytes at first sight, but with a smaller nucleus. Accumulation of lipidic tissue was observed in seven out of eight fish from the same group, and interstitial proteinaceous fluid in three out of eight samples. The latter was visible as homogenous dark pink material, presumably VTG, in interstitial spaces among follicles.

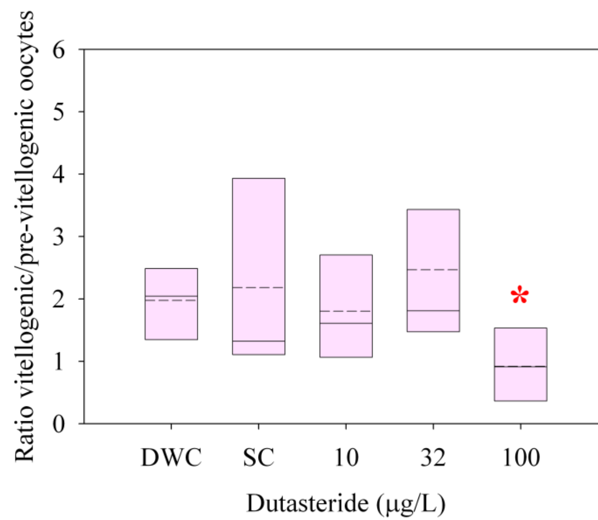


Figure 6.11. *Ratio between the proportions of vitellogenic and pre-vitellogenic oocytes in female fathead minnows exposed to different concentrations of dutasteride for 21 days. Boxes represent median (full line), and mean (dashed line) with 25th and 75th percentiles.* *: $p < 0.05$ *(n = 8).*

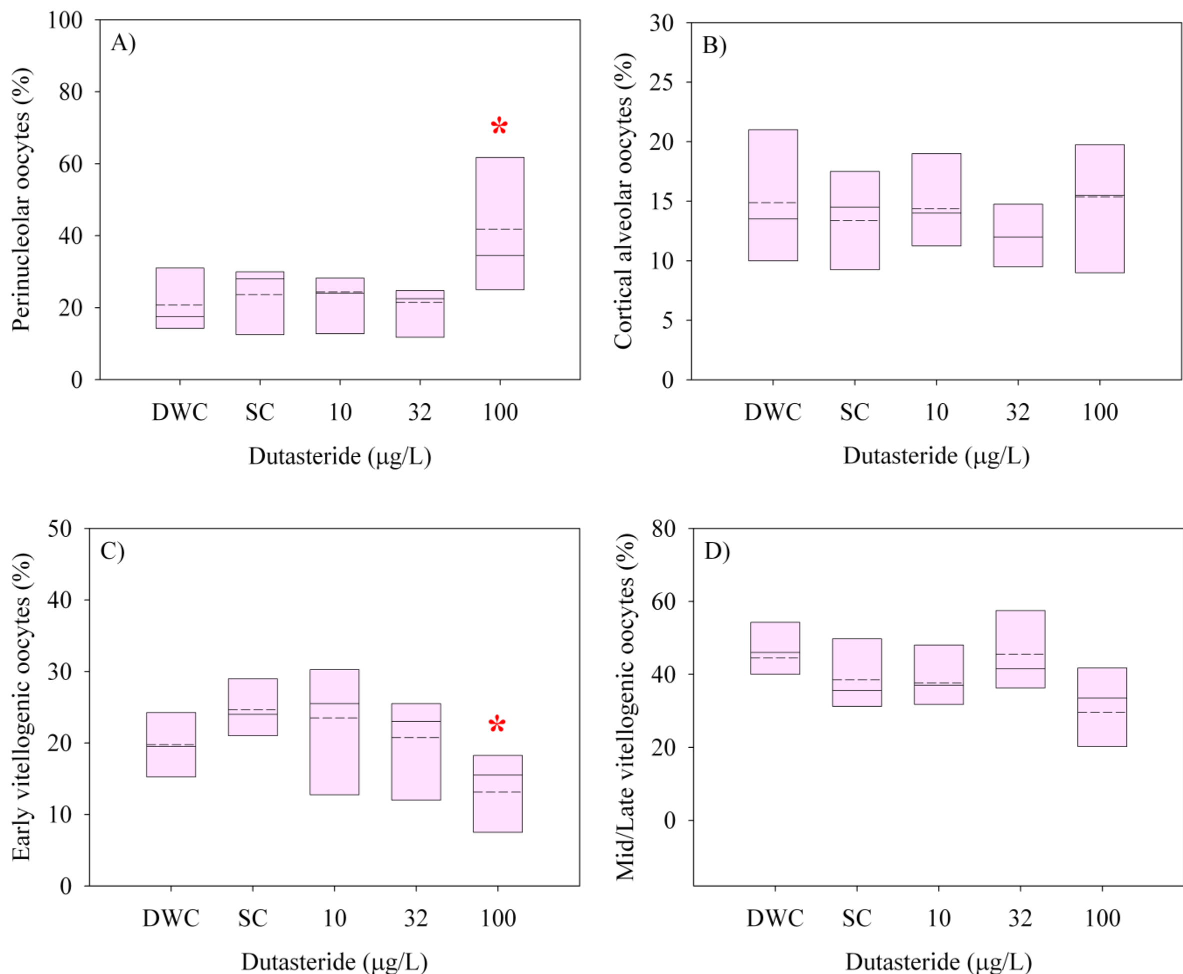


Figure 6.12. *Proportions of oocytes at different stages of maturation in female fathead minnows exposed to different concentrations of dutasteride for 21 days; in detail, perinucleolar oocytes (A), cortical-alveolar oocytes (B), early vitellogenic oocytes (C) and mid-late vitellogenic oocytes. Boxes represent median (full line), and mean (dashed line) with 25th and 75th percentiles.* *: $p < 0.05$ *(n=8).*

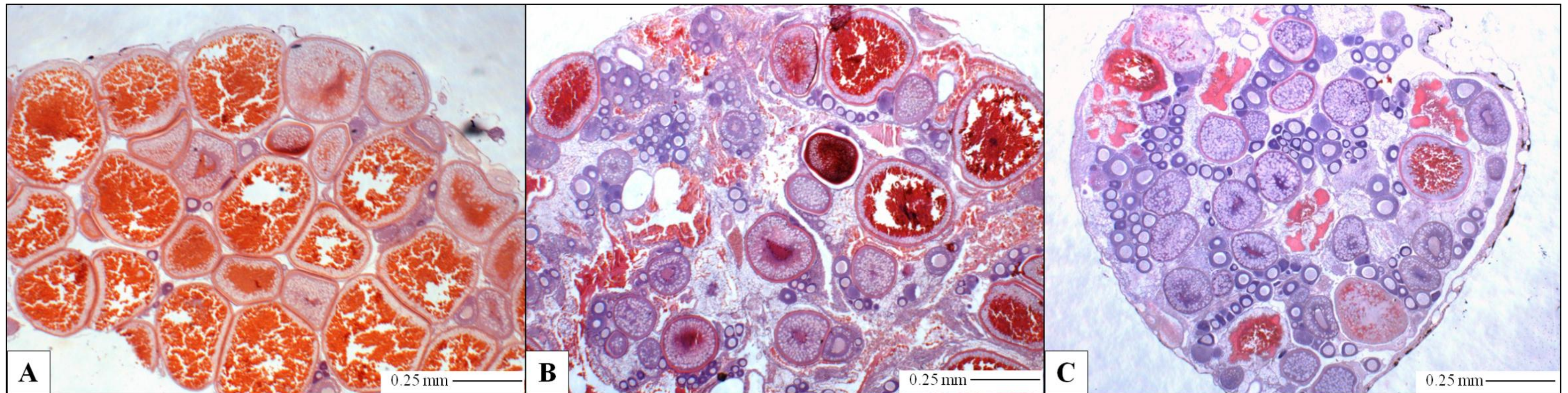


Figure 6.13. *Transverse sections of fathead minnow ovaries (A) from the Solvent Control group and (B, C) from the group exposed to 100 µg/L of dutasteride for 21 days during a short term reproduction assay. The ovary in figure A represents a typical example of an ovary from an actively spawning female, mainly containing late vitellogenic oocytes. Figures B and C show examples of ovaries with significant histopathological alterations found in fish exposed to 100 µg/L of dutasteride. Note the decreased number of vitellogenic oocytes, the presence of atretic oocytes and proteinaceus fluid, and the increased presence of interstitial tissue (somatic or adipose) in (B) and (C), compared to the ovary from the female in the Solvent Control group (A).*

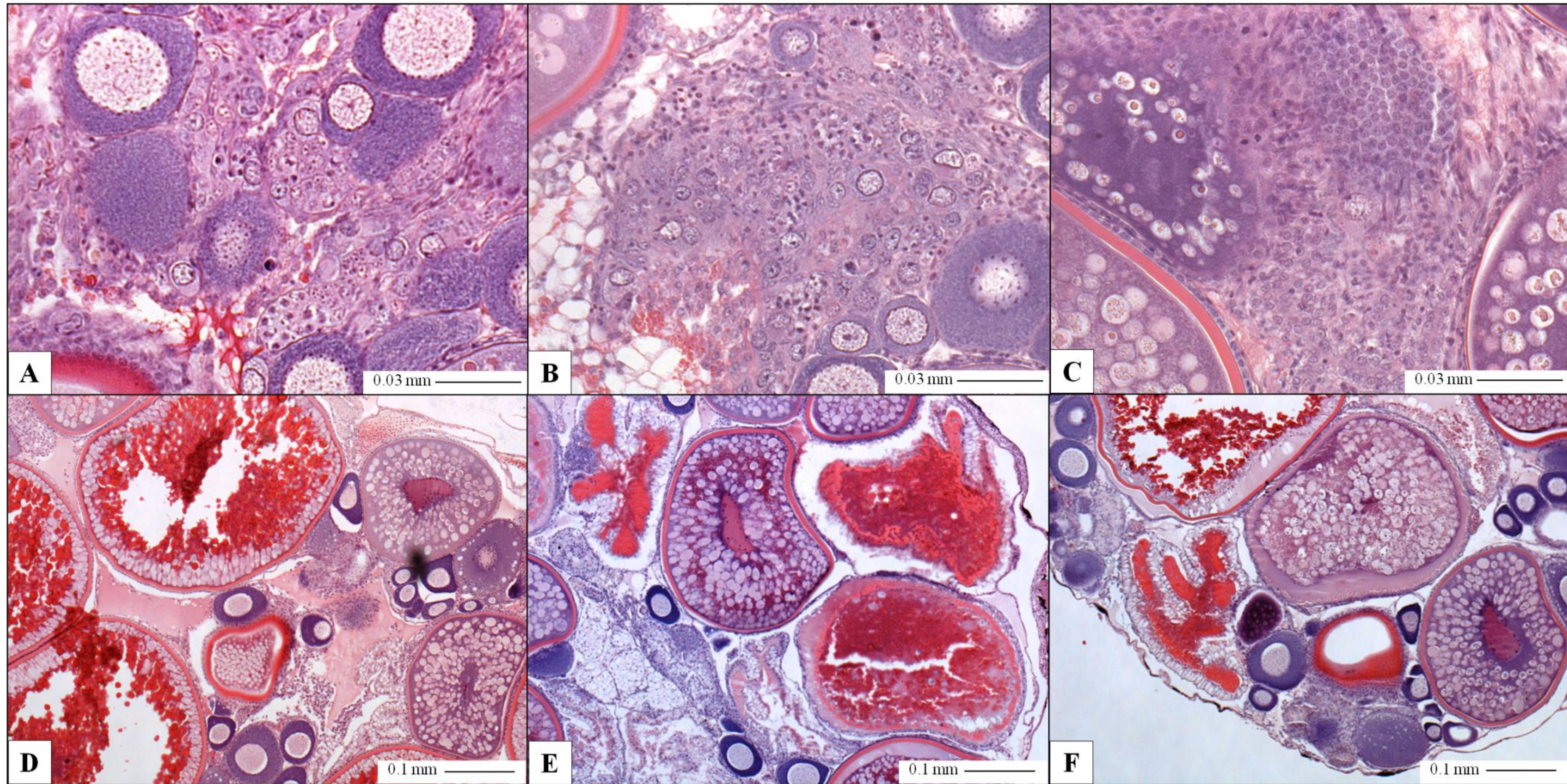


Figure 6.14. *Examples of histopathological conditions found in ovaries of female fathead minnow exposed to 100 µg dutasteride/L for 21 days. The observed alterations included (A,B,C) abnormal tissue resorption activity characterised by the proliferation of interstitial tissue containing macrophage aggregates, (D) presence of interstitial proteinaceus fluid, (E) increased presence of lipidic tissue, (D,E,F) increased occurrence of atretic follicle, characterized by clumping, perforation or degeneration of the chorion (or vitelline envelope), fragmentation of the nucleus, disorganization of the ooplasm, change in the appearance of the yolk.*

6.4.7 Effects of dutasteride on fathead minnow sperm quality

6.4.7.1 *Sperm count, motility and viability*

The exposure to dutasteride for 21 days did not cause any significant effect on sperm cell count and volume of milt produced on the sampling day; however, a significant decrease of sperm motility was recorded in fish exposed to 32 and 100 µg dutasteride/L (p = 0.006 and p = 0.01, respectively) (Fig. 6.15) Furthermore, the highest exposure concentration caused a significant increase of non-viable sperm (p = 0.004) up to *circa* 6-fold the baseline levels recorded in the control groups (SC: 1.2 ± 0.6%; 100µg/L group: 6.8 ± 6.7%).

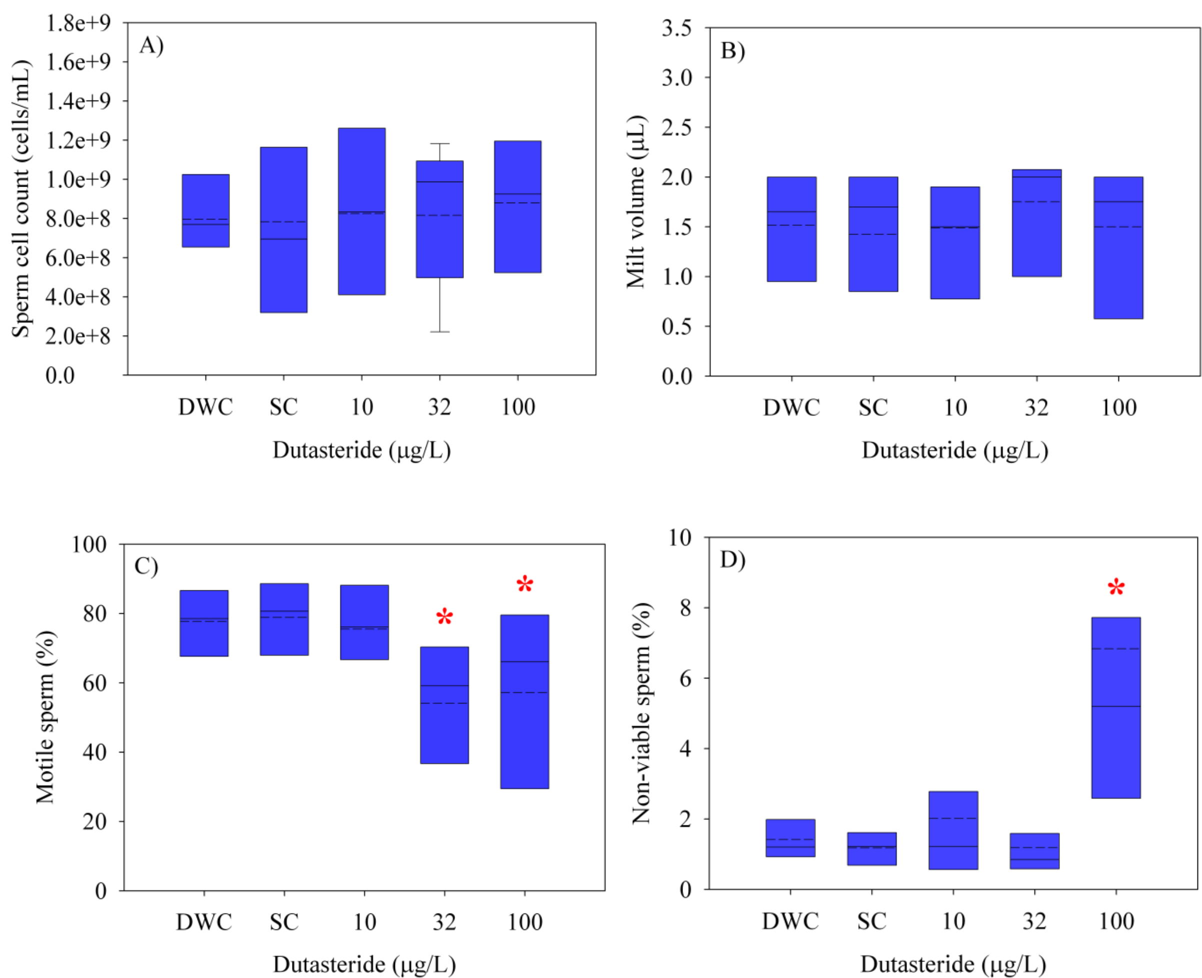


Figure 6.15. *Changes in (A) sperm cell concentration, (B) milt volume, (C) % of motile sperm, and (D) % of non-viable sperm, of breeding male fathead minnows exposed to different concentrations of dutasteride for 21 days. Boxes represent median (full line), and mean (dashed line) with 25th and 75th percentiles.* *: $p < 0.05$ ($n = 8$).

6.4.7.2 ***Sperm motility parameters***

The exposure to dutasteride for 21 days caused some effects on the sperm motility parameters; however, the significance of these effects was dependent on the statistical approach used to analyze the data.

When sperm populations from all the replicates in each treatment were considered as a single group, highly significant results were obtained (Fig. 6.16). In particular, the highest concentration of dutasteride caused an increase of all the velocity parameters: VCL ($p < 0.001$), VAP ($p < 0.001$), VSL ($p < 0.001$). Furthermore, both sperm linearity and straightness were reduced in fish exposed to 32 and 100 µg dutasteride/L (LIN: $p < 0.05$; STR: $p < 0.001$).

In the second approach, mean and median values of the parameters were calculated for the sperm population of each single fish, then combined according to the treatment, and statistically analyzed. This method produced only some apparent trends, but none of the results were significant (Figure 6.17).

Finally, to assess the influence of the few samples with the number of tracked spermatozoa < 35, these samples were excluded from the analysis (DWC = 0, SC = 1, D10 = 2, D32 = 2, D100 = 2). After this step, mean and median values of the parameters were calculated for the sperm population of each single fish, and analyzed as described in the second approach. As a result, the non-significance persisted, except for the VAP of sperm in the group exposed to 100 µg dutasteride/L, which showed a significant increase ($p = 0.025$) (data not shown).

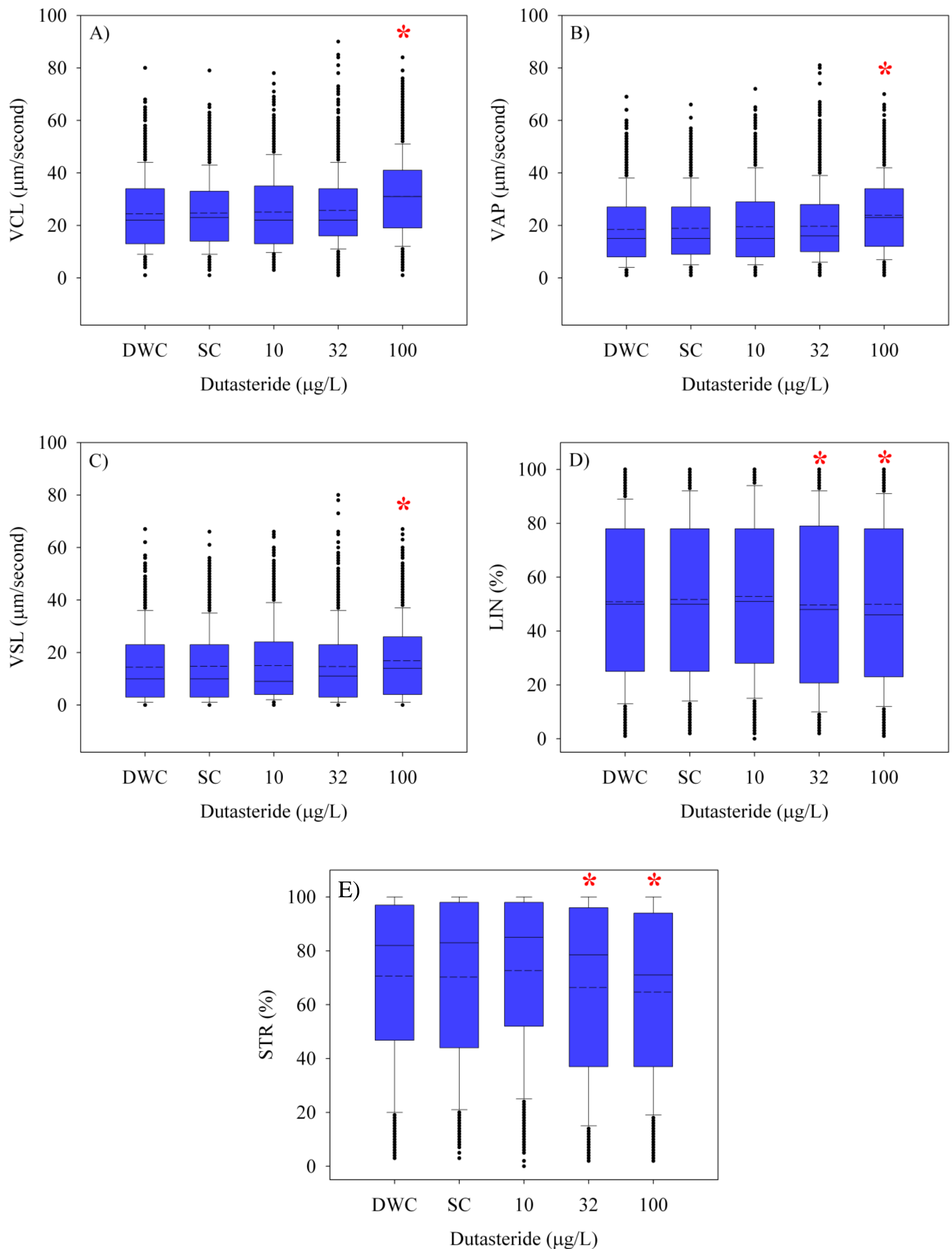


Figure 6.16. *Effect of dutasteride on fathead minnow sperm motility parameters after 21 days of exposure. Boxes represent median (full line), and mean (dashed line) with 25th and 75th percentiles. The bars extend to the 10th and 90th percentiles, with outliers represented as dots. (A) VCL, Curvilinear Velocity; (B) VAP, Average Path Velocity; (C) VSL, Straight-Line Velocity; (D) LIN, Linearity; (E) STR, Straightness.*: p < 0.05. (DWC: n = 1318; SC: n = 1747; Dutasteride 10 µg/L: n = 1346; Dutasteride 32 µg/L: n = 1378; Dutasteride 100 µg/L: n = 1985).*

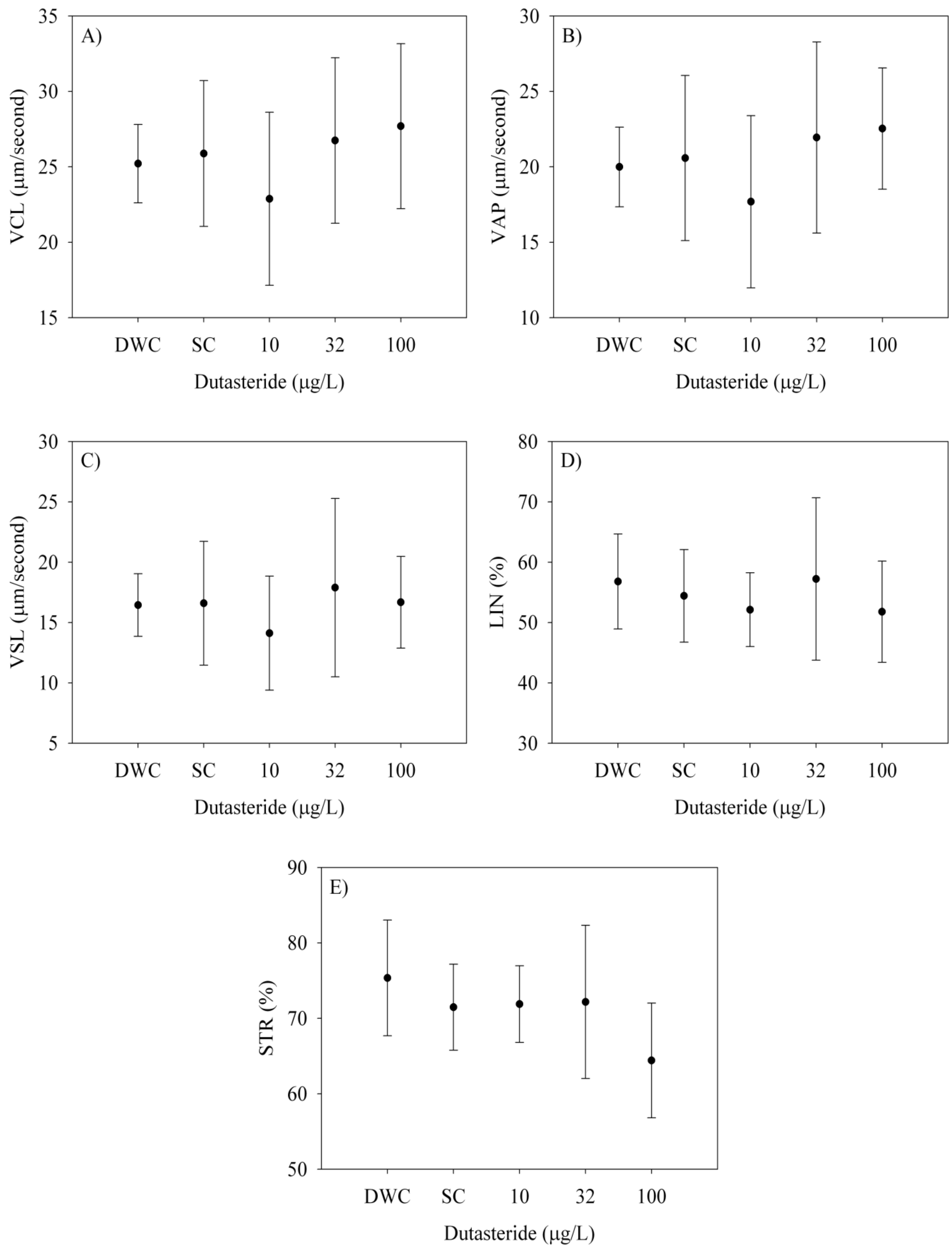


Figure 6.17. *Effect of dutasteride on fathead minnow sperm motility parameters after 21 days of exposure. Data are presented as mean ± SD. (A) VCL, Curvilinear Velocity; (B) VAP, Average Path Velocity; (C) VSL, Straight-Line Velocity; (D) LIN, Linearity; (E) STR, Straightness; (n = 8).*

6.4.7.3 *PATN analysis – Group allocation frequency*

The effects caused by the exposure to dutasteride on fathead minnow sperm were investigated using the PATN technique, which identified four subgroups of spermatozoa across the whole dataset. The characteristics of the four subgroups are shown in Table 6.3, while the proportion of spermatozoa in each group is illustrated in Figure 6.18. PATN Group 1 was the most represented group in the sperm populations of all the treatments, with *circa* 80% of spermatozoa of fish in DWC and SC treatments classified in this group. Spermatozoa in Group 1 were characterised by high velocities (VCL, VAP, VSL), medium/high linearity and high straightness, indicating the occurrence of highly active and progressive spermatozoa (Holt *et al.*, 2010). The remaining spermatozoa of fish in the SC group were classified as, Group 2 (5.8 ± 2.6%), Group 3 (13.4 ± 4.9%), and Group 4 (5.3 ± 3.1%). These three groups were all characterised by low linearity, respectively 22.4 ± 4.1%, 17.9 ± 4.8%, and 8.7 ± 0.37, indicating a lower grade of sperm activity. The low percentages of spermatozoa falling outside Group 1 suggested an overall good quality of the sperm populations, even after 22 hours from the sampling time, reinforcing the reliability of the results.

Exposure to dutasteride caused moderate and non-significant effects on the group allocation frequency, using both statistical approaches described in the methodological section. The highest concentration of dutasteride (100 μg/L) caused a non-significant decrease of the proportion of spermatozoa allocated to Group 1 (p = 0.103), and consequently, a slight non-significant increase in the proportion of spermatozoa allocated to Group 2 (p = 0.455) and Group 4 (p = 0.144). The same statistically non-significant result was confirmed when performing a G-goodness-of-fit test (a modification of the classic Chi-Squared test), with the extrinsic hypothesis that the proportion of sperm population allocated in each of the four groups would follow the pattern observed in the sperm populations from the fish in the SC group. Comparing the SC group with the group exposed to the highest concentration of dutasteride (100 μg/L), the statistical significance was p = 0.102.

6.4.7.4 *Analysis of sperm populations in a 2-dimensional graph (LIN by VAP)*

Figure 6.19 presents two-dimensional plots of average path velocity (VAP) *versus* linearity, in which individual spermatozoa appear as single dots. The majority of spermatozoa are situated in the left part of the graphs, curving toward the right at the top of the distribution (spermatozoa with high velocity and high linearity).

If arbitrary thresholds are imposed on the graphs (linearity>70% and VAP>20μms^{-1}), then the most rapid, but less progressive, spermatozoa can easily be identified as being within the

boxes representing the thresholds. Indeed, using this approach, the sperm population of fish exposed to 100 µg dutasteride/L demonstrate an enrichment of faster but less progressive spermatozoa, moving following more circular pathways, which were classified by PATN as Group 2. This graphical, two-dimensional treatment complemented the simple use of means and standard deviations to describe the PATN results, enhancing the visualization of the results.

Table 6.3. *Summary of fathead minnow sperm motility parameters for the groups identified by PATN analysis.*

Parameter & Groups	Mean	Minimum	Maximum	St. Dev.
VCL (µm/sec)				
Group 1	27.62	13.86	44.35	12.33
Group 2	37.16	27.19	47.12	9.96
Group 3	12.80	9.50	19.23	4.55
Group 4	20.15	13.57	26.74	6.58
VAP (µm/sec)				
Group 1	20.45	5.91	39.58	12.65
Group 2	25.50	17.69	33.30	7.80
Group 3	8.281	7.00	9.34	0.97
Group 4	15.00	11.79	18.21	3.21
VSL (µm/sec)				
Group 1	16.30	3.28	37.67	11.40
Group 2	7.56	6.86	8.25	0.70
Group 3	1.80	1.18	2.21	0.45
Group 4	1.44	0.99	1.88	0.44
LIN (%)				
Group 1	54.86	26.99	87.43	20.54
Group 2	22.36	18.21	26.50	4.14
Group 3	17.90	13.56	24.59	4.80
Group 4	8.74	8.366	9.10	0.37
STR (%)				
Group 1	78.46	60.13	95.06	14.97
Group 2	33.39	25.97	40.81	7.42
Group 3	26.20	17.22	33.32	6.70
Group 4	11.54	10.46	12.62	1.08

VCL, curvilinear velocity; VAP, velocity along the average path; VSL, straight line velocity; LIN, Linearity of track; STR, Straightness along the average path.

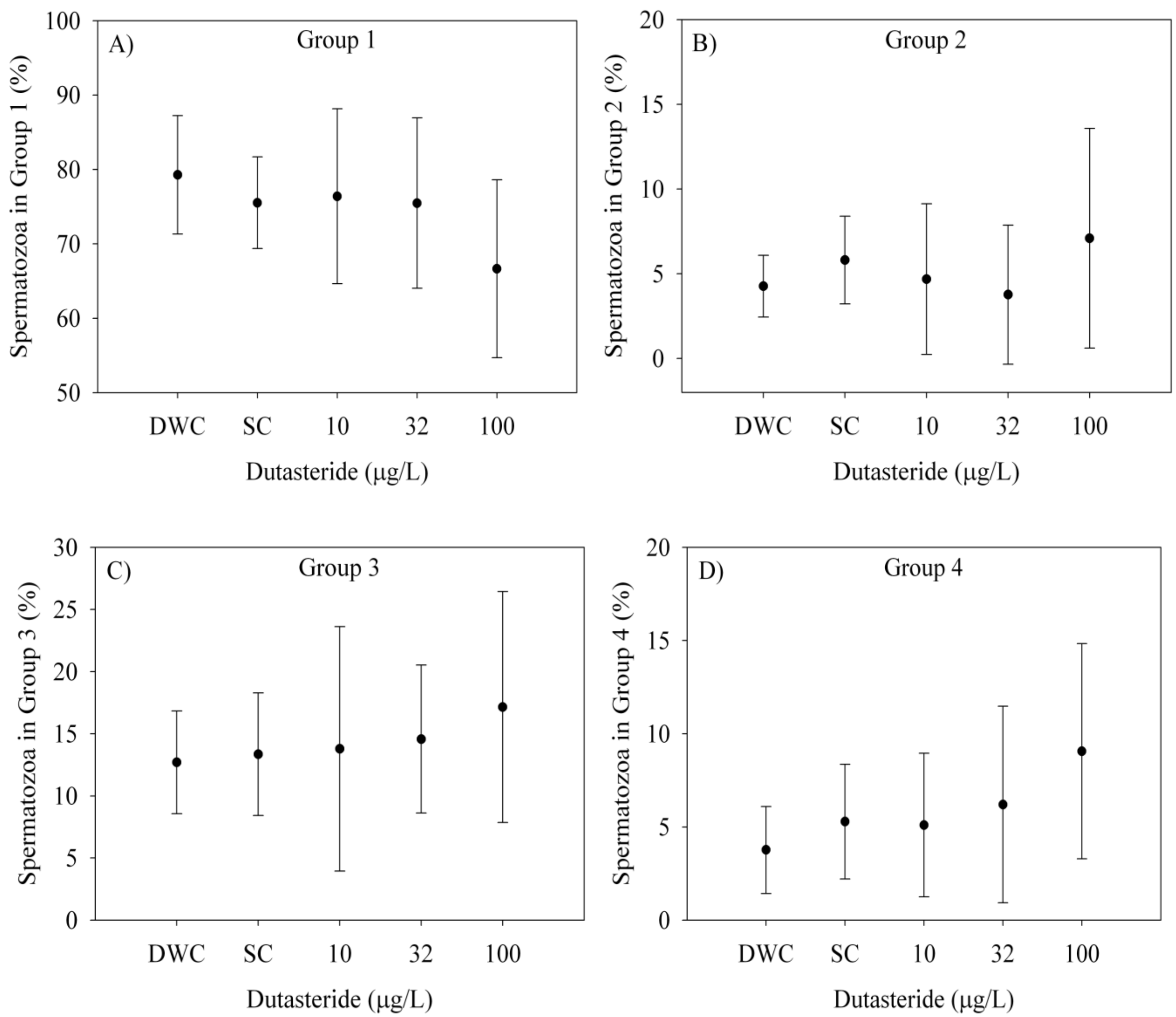


Figure 6.18. *The PATN analysis clustered the spermatozoa in each population in four different groups, characterised by the set of parameters described in Table 6.3. The graphs represent the effects of dutasteride on the proportion of sperm population allocated to each group (Mean ± SD; n = 8).*

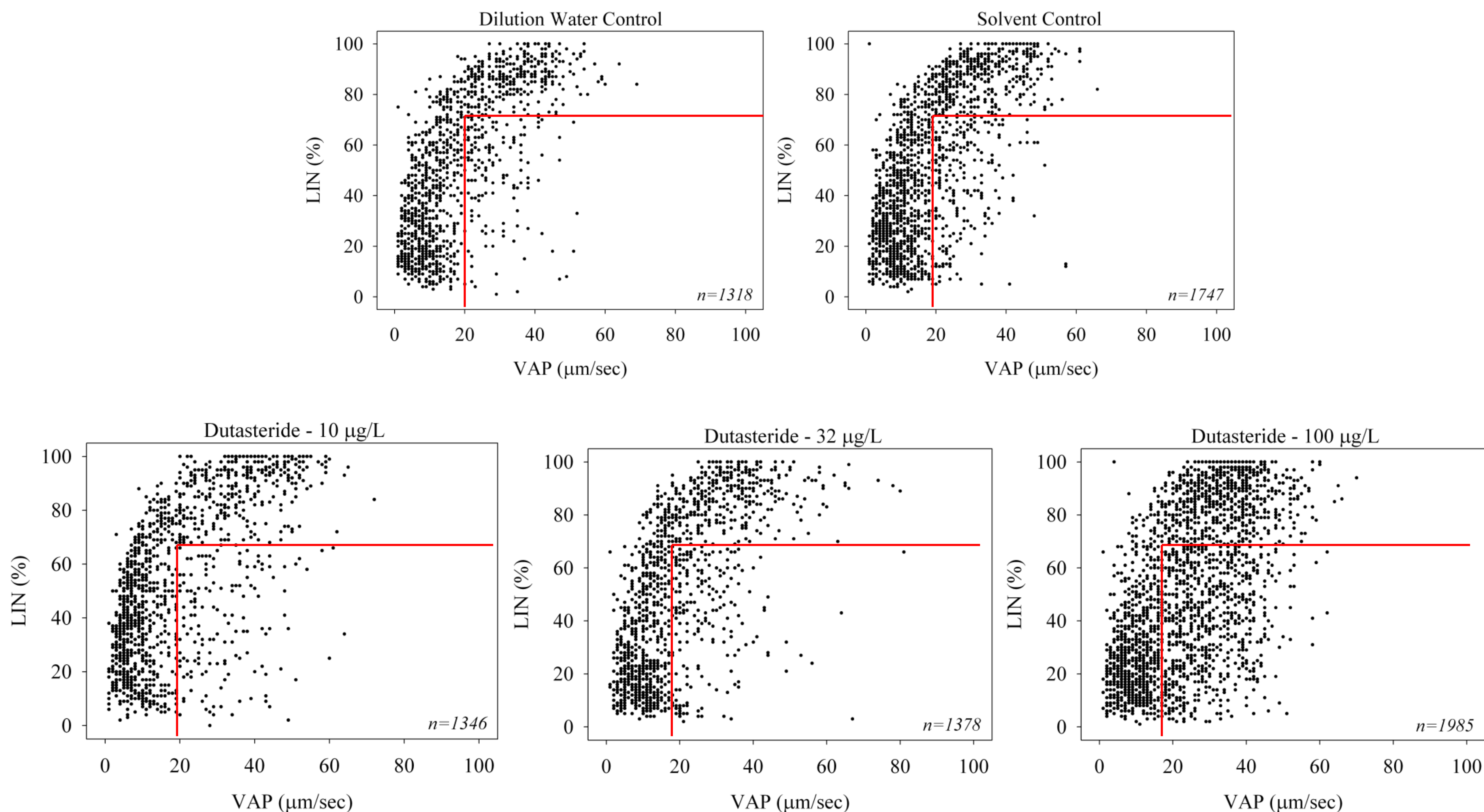


Figure 6.19. *Dot-plots showing the effect of dutasteride exposure on the distribution of fathead minnow sperm motility characteristics within each treatment. Path linearity (LIN: a reflection of progressive motility) is plotted against mean path velocity (VAP: a reflection of tail beating) for each individual sperm track analyzed. The two-dimensional representation reveals the presence of sperm subpopulations with different behaviours. Dots with low average path velocity (VAP) and linearity of track (LIN) values (bottom left of graphs) represent slow and non-linear sperm tracks, whereas dots plotted in the upper right portion of the graphs represent fast and highly progressive spermatozoa. The red lines are plotted at arbitrary thresholds to emphasise that the treatment with 100 µg dutasteride/L enriched the population of faster but less progressive spermatozoa (shown as dots within the boxes).*

6.4.8 Effect of dutasteride on F1 hatchability

A high variability was observed in the quality of the F1 generation produced by different fathead minnow pairs, in terms of F1 hatching success, and stability of the hatching rates during the baseline period. In each group, the three fathead minnow pairs which produced the highest quality F1 generation were followed until the end of the study, and only these pairs were analyzed for statistically significant effects (Table 6.4).

All the selected pairs, in each treatment, produced batches of fertilized eggs with high hatching rates (between 78% and 93%) during the baseline period. There was no evidence that the exposure of fathead minnow breeding pairs (F0) to dutasteride affected the F1 generation hatching success (Fig. 6.20 & 6.21) or hatching time (Fig. 6.22), when the hatching trials were performed by transferring the fertilized eggs from parental tank water to clean dilution water.

However, when the incubation of the embryos was performed using parental tank water, the hatching success of embryos in the highest concentration group (100 μg/L) was strongly reduced (A: $p = 0.043$; B: $p < 0.001$; C: $p = 0.002$). The hatching success of embryos produced by pairs A and C in two different testing times along the exposure phase is shown in Figure 6.21. In the same treatment group, the embryos produced by only one of the three replicates showed an increase of the time required to hatch during the exposure period, both when incubating the embryos in clean dilution water and parental tank water ($p = 0.049$). However, the statistical significance was just below the set significance limit (0.05), and the lower number of spawning events recorded during the exposure period compared to the baseline one (4 versus 9) - on which an hatching trial was performed – would suggest a lack of relevance for this specific result.

Table 6.4. *Number of hatching trials undertaken for each fathead minnow pair during the study.*

Concentration of dutasteride (µg/L)	Baseline or exposure period	Pair identity and number of trials undertaken		
		A	**B**	**C**
DWC	Baseline	9	9	9
	Exposure - DW	9	9	9
	Exposure - PTW	9	9	9
SC	Baseline	9	9	9
	Exposure - DW	12	9	9
	Exposure - PTW	9	9	9
10	Baseline	9	9	9
	Exposure - DW	12	15	9
	Exposure - PTW	12	15	9
32	Baseline	9	9	9
	Exposure - DW	9	9	9
	Exposure - PTW	9	9	9
100	Baseline	9	9	9
	Exposure - DW	4	3	6
	Exposure – PTW	3	3	6

DW, Dilution Water; PTW, Parental Tank Water.

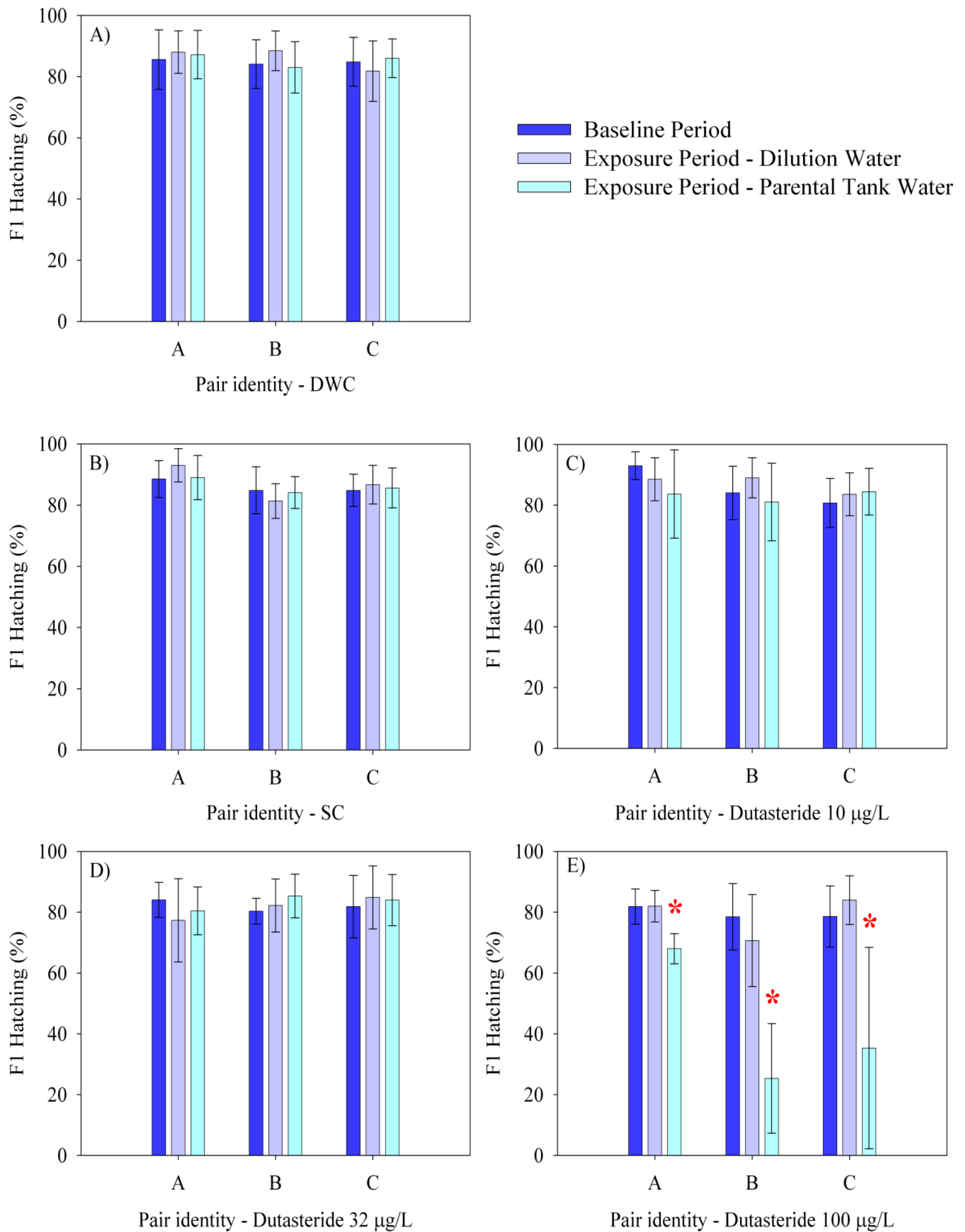


Figure 6.20. *Effect of dutasteride on F1 hatchability. In each treatment (indicated at the bottom of each graph) three fathead minnow pairs were selected and followed until the end of the study. At every spawning event, F1 hatching success was estimated by transferring the fertilized embryos into 6 wells plates, containing as incubation medium either clean dilution water or parental tank water. The medium was replaced every 24 hours, and each trial was performed in triplicate. The number of embryos was n = 20 for each replicate.*

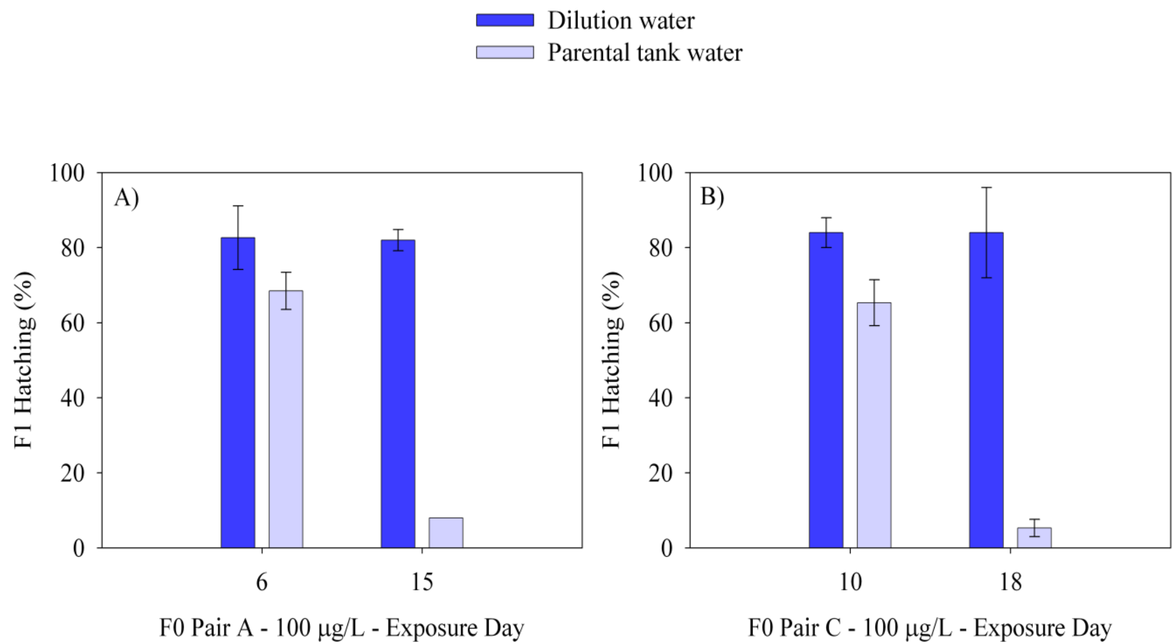


Figure 6.21. *Hatching rates of fathead minnow embryos obtained from fish pairs exposed to 100 μg dutasteride/L for 21 days, during two different times along the exposure phase: (A) day 6 and 15 for Pair A, and (B) day 10 and 18 for Pair C. As described in the legend, embryos from the same source were incubated in clean dilution water, and also in parental tank water containing the drug. Note the drop of hatching success between the first and second trial, as the study progressed. Each bar represent the mean of 3 trials, except in "Day 15 – Parental tank water" (n = 1). In each trial the number of embryos was n = 20 for each replicate.*

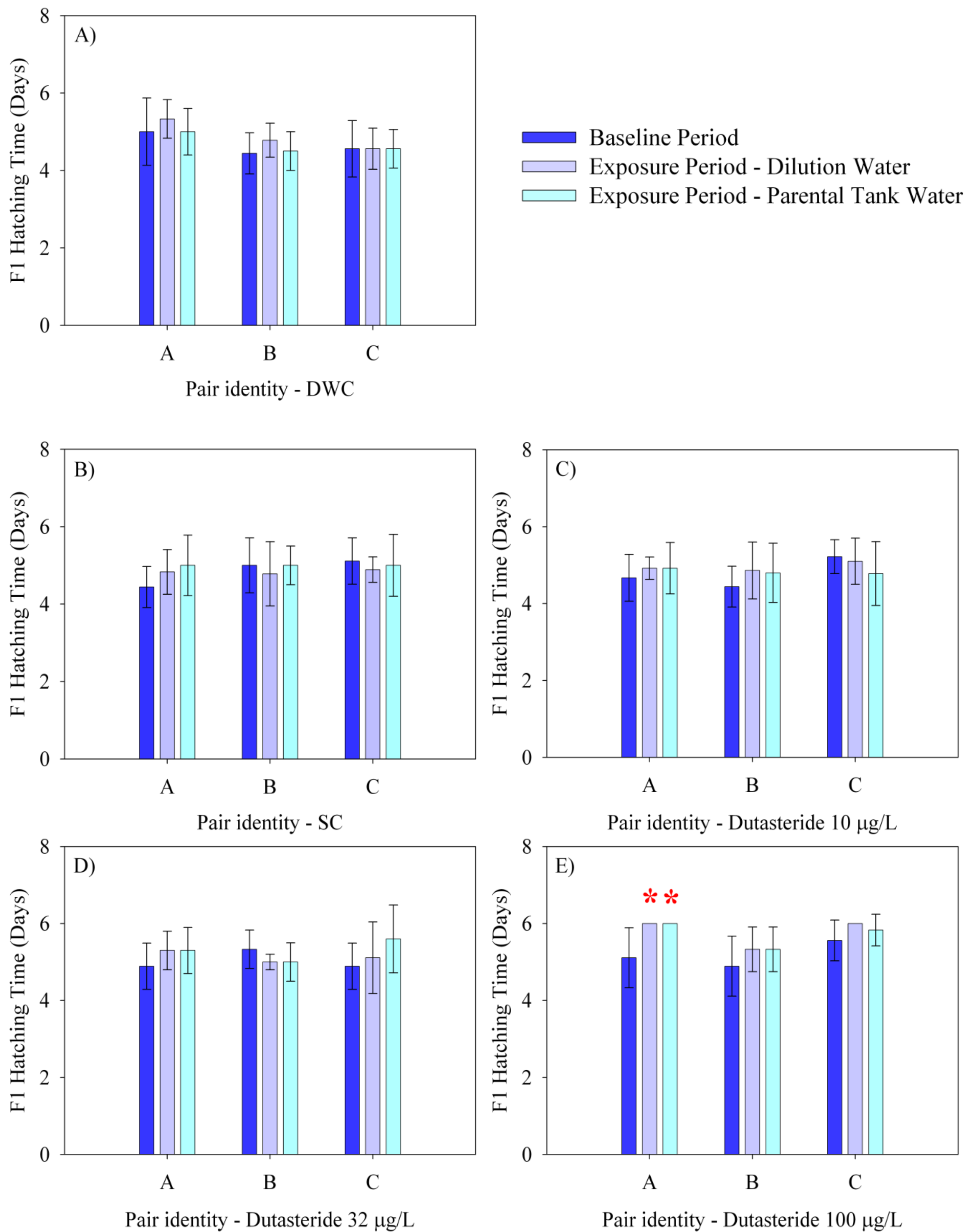


Figure 6.22. *Effect of dutasteride on F1 hatching time. In each treatment (indicated at the bottom of each graph) three fathead minnow pairs were selected and followed until the end of the study. At every spawning event, F1 hatching time was estimated by transferring the fertilized embryos into 6 wells plates, containing as incubation medium either clean dilution water or parental tank water. The medium was replaced every 24 hours, and each trial was performed in triplicate. The number of embryos was n = 20 for each replicate.*

6.5 Discussion

The objective of the study described in this Chapter was to evaluate the effects of a non-model anti-androgen, dutasteride, on reproductive biology of fathead minnow using a short term reproduction assay specifically designed to detect chemicals that affect pathways controlled by estrogens and/or androgens (Ankley *et al.*, 2001; Harries *et al.*, 2000; OECD, 2006). Exposure to 32 and 100 µg dutasteride/L for 21 days significantly reduced fecundity of fish and affected different aspects of reproductive endocrine function in both males and females. For example, plasma steroids showed a sex-specific response; at the tissue level, spermatogenesis in males was not affected by dutasteride exposure, but there were significant alterations of female ovary histology, characterized by a decreased proportion of vitellogenic oocytes and increased ovarian atresia. Despite the absence of histological alterations in the testis, sperm quality was negatively affected by dutasteride, in terms of the proportions of motile sperm and non-viable sperm. Alterations of motility parameters were also observed, even if their biological significance remained unclear. Finally, F1 generation hatching success and time were not affected by the parental exposure to the drug; however, there was an apparent increase of sensitivity to dutasteride exposure in embryos produced by pairs exposed to the highest dose of dutasteride (100 µg/L). Based on these results, it is possible to conclude that reproductive endocrine function in the fathead minnow can be affected by chemicals that in mammalian models inhibit the enzymatic activity of 5α-reductases.

Since there was a lack of published information concerning the effects of 5α-reductases inhibitors (dutasteride and finasteride) on fish, in order to better understand if the effects caused by dutasteride could be due to its anti-androgenic mechanism of action, this Chapter will provide a comparative perspective of the effects caused by selected anti-androgens in sexually mature fish, preferably during 21 days fish reproduction tests. The rationale to compare only experiments involving adult reproductively mature fish in was because the molecular and physiological status of breeding fish is different from that of juvenile or non-breeding fish, so the same compound may cause effects on non-breeding fish different than the ones observed in a 21 days fish reproduction test.

In previous works, the effect of anti-androgenic chemicals on the fathead minnow reproductive axis has mainly been studied by exposing fish to flutamide, a pharmaceutical considered as a classic androgen receptor antagonist in higher vertebrates. Other tested anti-androgens have included cyproterone acetate (AR antagonist), vinclozolin (AR antagonist), trilostane (3β-hydroxysteroid dehydrogenase inhibitor), and ketoconazole (CYPs inhibitor and weak AR antagonist). A comparative perspective of their effects on fish is summarised in

Table 6.5. The listed compounds were selected for a comparative discussion, since all of them, except vinclozolin, are human pharmaceuticals; furthermore, they cover a broad range of modes of action, from AR antagonism to inhibition of steroidogenic enzymes. According to the scientific literature, flutamide is currently the most tested drug (13 studies), followed by vinclozolin (2 studies). The remaining compounds have been tested on fish reproduction only in one study.

The overview of the effects caused by the selected compounds on reproduction of teleost fish shows how, according to the LOECs for fecundity, the potency rank appeared to be ketoconazole (25 μg/L) > dutasteride (32 μg/L) > vinclozolin (60 μg/L) > flutamide (651 μg/L) > trilostane (1500 μg/L). Hence, the drug classically considered as a model anti-androgen caused adverse effects on fecundity at concentrations about 20-fold higher than some of the inhibitors of steroidogenic enzymes, like ketoconazole or dutasteride, and about 10-fold higher than another AR antagonist, vinclozolin, which is not a human drug but an agricultural fungicide. According to OECD (2006), medaka was most sensitive to flutamide, than fathead minnow and zebrafish, with LOECs for reproduction of 100, 1000 and >1000 μg/L, respectively. However, OECD's results showed a very low replicability (the effect was detected only in 1 out of 4 independent experiments), and were not confirmed by other studies, like that of Kang *et al.* (2006), who recorded a LOEC for reproduction of 1560 μg/L using medaka as the experimental species. No published information was available in the scientific literature concerning the effects of cyproterone acetate on fish reproductive performance, but it should be noted that this drug showed a very high potency in fish, causing alteration of the levels of circulating plasma steroids at concentration as low as 10 ng/L (Sharpe *et al.*, 2004).

The effect of dutasteride on plasma steroids concentrations was sex-specific, however the response was different from the prediction made on the basis of the mammalian response. According to GSK (2001), in humans the daily administration of 0.5 mg of dutasteride produced a great reduction of serum DHT concentrations, up to 94% compared to the pre-exposure levels. As a consequence, it was possible to observe an increase in serum T of *circa* 18-22%. Furthermore, in healthy male volunteers, 52 weeks of treatment with 0.5 mg dutasteride/day resulted in no clinically significant change compared with placebo in plasma concentration of sex hormone-binding globulin, E2, luteinizing hormone, follicle-stimulating hormone, thyroxine (free T4), and dehydroepiandrosterone. No data were available on the effects of dutasteride on sex steroids in females.

Based on this information, the hypothesis was that exposing male fathead minnows to dutasteride would cause an increase of circulating T, followed by a potential increase of E2; however, in the present study there was a general negative feedback on steroid production, which led to a significant decrease of circulating T (at 100 µg/L) by 63% and KT (at 32 and 100 µg/L) by 43% and 50%, respectively, and a non-significant decrease of circulating E2. Interestingly, the predicted increase of circulating T was observed in females exposed to the highest concentration of dutasteride; the same group also showed a decrease of circulating E2. Both steroids increased or decreased, respectively, by 50% compared to the levels recorded in the SC group. These results indicated a sex-specific induction of the hormonal feedback loops.

The decrease in the concentration of circulating T, KT and E2 in males was observed with exposure to both dutasteride and cyproterone acetate (Sharpe *et al.*, 2004), two drugs that act through two different mode of actions, 5α-reductases inhibition and AR antagonism, respectively. The response caused by the two drugs diverged when considering the response of T in females, which increased with dutasteride and decreased with cyproterone acetate. It should be noted that the described effects have been recorded in two different species, fathead minnow and mummichog; it is possible that the previously described similarity could be accidental, and due to a different species-specific feedback on the HPG axis.

Other than cyproterone acetate, only flutamide affected circulating T levels, causing an increase in female fathead minnows exposed to 651 µg/L, but no effects were observed in male fish (Jensen *et al.*, 2004). When considering the AR antagonists, the increase of plasma T can be explained by the ability of these anti-androgens to block negative feedback of T at the hypothalamic and pituitary level, leading to an increase in luteinizing hormone secretion, and consequently increased T concentrations (O'Connor *et al.*, 2002), while in the case of dutasteride the mechanism that lead to T increase was probably different, since the drug does not act through the AR, hence the classical explanation would be that the increase of T derives from the inhibited conversion of T into DHT. However, some theoretical gaps make this explanation unclear; for example, circulating DHT concentrations in females are unlikely to be high enough to produce, once its synthesis is inhibited, an increase of T by 50%. Moreover, the concomitant decrease of circulating E2 does not fit with the simple explanation of the equilibrium of steroid ratios. It is possible that unknown feedback mechanisms produced the effects observed in females.

The observed decrease of circulating concentrations of androgens in fathead minnow males also explain the phenotypic effect of dutasteride on male SSCs expression, characterized by a

significant decrease of both number and prominence of nuptial tubercles. The effect was statistically significant only in fish exposed to 100 μg dutasteride/L, but the decreasing trend was already detectable at 32 μg dutasteride/L. Fatpad properties were not affected by dutasteride exposure. The higher sensitivity of nuptial tubercles to androgens/anti-androgens, compared to the fatpad, was also confirmed in other studies (Martinovic *et al.*, 2008). Other than dutasteride, the only previously listed anti-androgen that caused effects on male SSCs was vinclozolin (Martinovic *et al.*, 2008). Flutamide, in one study (OECD, 2006), caused a decrease of tubercle number in fathead minnow exposed to 754 μg/L, but this result was equivocal since this effect was not observed in the other two laboratories replicating the experiment (OECD, 2006).

It is known that VTG production is controlled by E2 (Sumpter and Jobling, 1995), but in this study, despite the highest concentrations of dutasteride causing a significant decrease of circulating E2 (by 50%) in females, plasma concentrations of VTG were not affected. Considering the experimental set up of the fish short term reproduction assay, which involves exposure of fish for 21 days, the sensitivity of VTG as biomarker of exposure to anti-androgens is unclear, since only few studies detected significant alterations of its plasma concentrations; in particular, a significant VTG response in females has been detected only in 2 out of 12 studies testing flutamide (LOEC: 651 μg/L for fathead minnow; 95.5 μg/L for medaka) (OECD, 2006), in 1 out of 2 studies testing vinclozolin (LOEC: 255 μg/L for fathead minnow) (Martinovic *et al.*, 2008), and in one study testing trilostane (LOEC: 1500 μg/L for fathead minnow) (Villeneuve *et al.*, 2008).

An important consideration is that circulating VTG is a very stable protein in the plasma, as shown in male fathead minnows by Thorpe *et al.* (2007); in fact, male fish exposed to E2 for 14 days showed a significant dose-related increase of plasma VTG concentrations, which remained elevated 70 days after cessation of exposure. In females in the active reproductive phase, one of the main mechanism of VTG elimination by the plasma is the uptake into the developing oocytes, rather than elimination through the kidneys (Thorpe *et al.*, 2007). To explain the non-significant VTG response of fish to the exposure to dutasteride, it is possible to hypothesize that the reduced reproductive activity (e.g. reduced egg production and spawning frequency) caused a decreased excretion of VTG via deposition into the eggs; therefore, the decrease of VTG caused by decreased E2 concentrations could have been hidden by the high stability of basal plasma VTG levels, which cannot be cleared anymore by the process of uptake into developing oocytes. However, other studies recorded a decrease of the plasma VTG concentration in females concomitant with a decreased fecundity, and

sometimes decreased circulating E2, when exposing female fathead minnows to fadrozole (aromatase inhibitor) (Ankley *et al.*, 2002), trilostane (β-HSD inhibitor) (Villeneuve *et al.*, 2008), and fenarimol (aromatase inhibitor & ER antagonist) (Thorpe *et al.*, 2007).

With regards to this comparison, the review of the literature revealed that the concentrations of circulating E2 in the females used in our study were higher when compared to the ones measured in other studies involving exposure of actively spawning fathead minnows to various chemicals. In particular, despite the fact that the E2 circulating in the females of our control groups (5-20 ng/mL) was in the "normal" range defined by Watanabe *et al.* (2007) for actively spawning fathead minnows (0.2-21.4 ng/mL), the mean values (DWC: 12.7 ng/mL; SC: 14.3 ng/mL) were higher than the ones recorded in other studies, even ones previously performed in our laboratory, which were in the range of 3-7 ng/mL (Ankley *et al.*, 2002; Ankley *et al.*, 2005; Makynen *et al.*, 2000; Martinovic *et al.*, 2008; Runnalls, personal communication; Paulos *et al.*, 2010). The reason for these differences is not known, but after a deep review of all the technical and mathematical steps performed for the assay, it was possible to conclude that the measured values are likely to be real; moreover, the same assay was performed to measure E2 in both male and female samples, and the values obtained for males agreed perfectly with all the other published studies. The "higher" than normal E2 concentration in females could represent an additional factor that led to a non-significant result for the VTG data, and a drop in plasma concentration of E2 from 14.3 ng/mL (SC) to 7.2 ng/mL (100 μg dutasteride/L) may not be enough to produce a negative effect on *vtg* gene expression.

In male fish, despite the observed decrease of circulating T and KT concentrations, exposure to dutasteride did not cause any histopathological alterations of the testis. Toxicity studies conducted in rats and dogs showed how daily administration of high doses of dutasteride (up to 500 mg/kg/day in males rats and up to 50 mg/kg/day in male dogs respectively) induced treatment-related alterations in male reproductive organs, including a decrease in the size of the prostate of rats and dogs, epithelial atrophy and decreased secretion of the seminal vesicles in rats, decreased epididymis weight in the rat and histopathological changes consistent with atrophy in the epididymis in dogs. However, the effects on the testis were limited to an increase in testis weight in rats, and there were no significant changes in spermatogenesis in either species (GSK, 2001).

Among the anti-androgen listed at the beginning of the Chapter, only flutamide and ketoconazole caused histopathological alteration of the testes in male fish during the 21 days experiment, including the appearance of clusters of pycnotic and degenerating cells in the

seminiferous tubule epithelium (the reliability of this result is unclear, due to the small number of fish used for the analyses, n = 3) (Jensen *et al*; 2004), decreased number of spermatogenic cysts (Kinnberg and Toft, 2006), increased proportion of spermatogonia, and increased number of interstitial cells (Leydig cells) (Ankley *et al.*, 2007; OECD, 2006). In addition, inhibition of the late spermatogenic stages has been observed in mature male sticklebacks exposed to cyproterone acetate (Rouse *et al.*, 1977).

On the other hand, dutasteride (100µg/L) caused obvious histological alterations in the ovary, including a decreased proportion of early vitellogenic oocytes, an increased proportion of perinucleolar oocytes, an increased occurrence of atretic follicles, an abnormal proliferation of interstitial/somatic tissue, vacuolar hypertrophy of the surrounding granulose cells due to phagocytic processes, proliferation of interstitial tissue containing macrophages aggregates, the presence of mitotically dividing oogonia, an accumulation of adipose tissue, and the presence of interstitial proteinaceus fluid. These results confirmed the higher sensitivity of the ovary to dutasteride compared to the testis. It is important to remember than the affected fish also had a significant increase of the concentration of circulating T, coupled with a decrease of E2, which could explain the previously described histopathological alteration.

Toxicity studies on rats and dogs showed treatment-related effects on female reproductive organs, including decreased ovary and uterus/cervix weights, increased incidence of dioestrus or increased occurrence of ovarian (follicular) cysts in rats, microscopic changes in the uterus and shifts in the oestrus cycle to the luteal phase in dogs; however, there was no available information specifically concerning the histopathological effects of dutasteride in higher female vertebrates (GSK, 2001).

Interestingly, the increase of atretic oocytes, and the decreased proportion of vitellogenic oocytes caused by dutasteride, were also observed in fish exposed to flutamide and vinclozolin (Jensen *et al.*, 2004; Martinovic *et al*, 2008; OECD, 2006), supporting the hypothesis of a high sensitivity of the ovary to the disruption of the androgenic pathways. A further factor should be taken into account to explain the increased occurrence of atresia in fish exposed to 100 µg dutasteride/L; in fact, it is known that atresia can be also induced by decreased or absent spawning activity (McCormick *et al.*, 1989), due to alterations of male behaviour, such as a decrease of male aggressiveness and lack of interest in the female or in spawning. Therefore, the observed histological alterations of fathead minnow ovaries could be due to a combination of factors, directly or indirectly induced by the exposure to dutasteride, and mediated by the alteration of T and E2 biosynthesis.

The sensitivity of the ovary to disruption of androgen biosynthesis has been confirmed in all the studies presented in this Thesis; moreover, the histopathological endpoints were found to be the most sensitive to detect the effects of anti-androgens, and also the most reliable; in fact, obvious histological alterations were detected in 11 out of 12 studies on AR antagonists and in 2 out of 3 studies on enzymatic inhibitors. The role of androgen in female fish is still unclear; however, androgens such as KT, seem to be involved in the growth of perinucleolar oocytes (Chapter 4, Kortner *et al.*, 2008), and AR-mediated signalling is known to affect expression of several regulators of folliculogenesis (Drummond, 2006). For example, it has been shown that the disabling of AR-mediated androgen signalling caused the down-regulation of several genes involved in folliculogenesis, which consequently produced an increased occurrence of atretic follicles and infertility in that species (fish or mammals) (Shiina *et al.*, 2006).

Exposure to dutasteride did not affect sperm cell count or milt volume; however, it reduced the percentage of motile sperm by 32% and 27%, respectively, at 32 and 100 µg/L, and lowered the percentage of viable sperm slightly but significantly, from 98.8% in the SC group to 93.2% in the group exposed to 100 µg dutasteride/L. The analysis of the sperm motility parameters gave different levels of statistical significance depending on the statistical approach used. The treatment caused significant alteration only if the data were treated in order to keep the high variability, which is characteristic of sperm populations. In contrast, when the variability was summarised by mean values and standard deviations, all the significance was lost. This observation was confirmed by Holt *et al.* (2007), who noticed that although it is common practice to use the mean and standard errors provided by CASA systems as inputs to statistical analyses, a deeper examination of a typical set of sperm motility data reveals that this is not an appropriate approach, since there is a great amount of information that is ignored when means are derived. This observation is exemplified by the great variability recorded for velocity parameters, such as VSL. In fact, Holt *et al.* (2007), analysing the dynamics of VSL in sperm populations, found that there is no simple linear relationship between the mean VSL value and the maximum value, and that the difference between the two values is often large, up to 3 to 4-fold. The maximum values may carry important information; for example, the subpopulation of sperm showing very fast movement (e.g. high VSL) could also have a competitive advantage over the slower subpopulations in the fertilization process, and this is one of the reasons why it is important to keep the whole sperm population variability in the statistical analysis. The authors also presented interesting examples of how the persistent presence in the scientific literature of inadequate statistical analyses, presented in support of various hypotheses, led to erroneous conclusions.

Within this perspective, the biological significance of the result obtained in our study is not clear. The analysis of the single motility parameters showed a significant increase of all the velocity parameters (at 100 μg/L), and a decrease of linearity and straightness (at 32 and 100 μg/L), suggesting that dutasteride induced a faster but more circular movement of the spermatozoa. This deduction was only partially confirmed by the PATN analysis, which showed only non-significant trends, suggesting a decrease in the proportion of fast and highly linear sperm, and a consequent increase of frequency of fast/circular and slow/circular sperm. It has been proven that CASA parameters are well correlated with fertilization and hatching rates in fish (Gage *et al.*, 2004; Lahnsteiner *et al.*, 1998; Rurangwa *et al.*, 1998); however, the complete absence of negative effects caused by dutasteride on fertilization rates and proportion of dead embryos suggest that the alteration recorded in the sperm motility parameters did not have consequences on male fertility.

Particular attention was given to the study of the sperm quality, since the decrease of male fertility and sperm quality is one of the side-effects of the daily administration of dutasteride in mammals. For example, in humans the therapeutic dose of 0.5 mg/day caused a sharp drop in sperm count, sperm concentration, sperm motility, sperm morphology and semen volume after 24-28 week of treatment. Similar effects were also observed with administration of 5 mg/day of finasteride (GSK, 2001). However, in a fertility study performed on male rats treated orally with dutasteride (0.05 to 500 mg/kg/day) for up to 31 weeks, no effects were noted in the testis, and sperm concentration and motility were unaffected by treatment (GSK, 2001). It is important to note that the results described in this Chapter were obtained from a study involving only 21 days of exposure to dutasteride.

In order to test the effects of dutasteride on the F1 generation hatching processes, the three F0 pairs in each group producing the best quality F1 batches of eggs during the baseline period were chosen and followed until the end of the study. During the exposure phase, embryos were incubated in two different medium. Firstly, in clean dilution water, in order to assess the effect of any potential parental transfer of dutasteride from the breeding pair to the fertilized embryos. This hypothesis was supported by the fact that dutasteride is a highly lipophilic drug (LogP 5.45), which can accumulate into the body, and has been shown to be present in sperm samples of men treated with dutasteride. The semen dutasteride steady-state concentration has been shown to be about 10% of that of the serum concentration (*circa* 3.3 ng/mL of semen); the highest concentration recorded in the semen was 14 ng/mL (GSK, 2001). Different from humans or other mammals, in fathead minnow the fertilization of the eggs is external, so that there is no milt potentially containing dutasteride transferred into the female body; however,

female fathead minnows exposed to the drug could show a potential accumulation of the drug into the ovary and into the developing eggs. However, the hatching trials did not show any evidences of any alteration in the hatchability success and hatching time of the F1 generation.

Other than clean dilution water, F1 embryos were also exposed to parental tank water, containing dutasteride. In these trials, there was a significant decrease of hatchability success for the embryos produced by pairs exposed to 100 μg dutasteride/L. The effect was consistent in all three replicates. It is important to remember that dutasteride did not affect the hatchability of embryos in the two early-life stages studies described in Chapter 5. The result obtained with the F1 generation could be interpreted in two ways. Firstly, the decreased hatchability could be caused by factors unrelated to the drug exposure, such as oxygen concentration in the medium, temperature, pH. However, the incubation temperature was controlled by an automatic incubator, and it was similar to the water temperature recorded in the fish tanks (25 ± 1ºC), the medium was replaced every 24 hours, and even if neither oxygen concentration nor pH in the incubation wells were measured, they were tested in the source tank just before the transfer of the parental tank water from the tank to the incubation wells, without showing any significant variation from the SC parameters. Although is not possible to completely exclude the possibility that some environmental factors affected embryos hatching, there was evidence that the hatchability success decreased with the progress of the study, strongly supporting the hypothesis that dutasteride progressively accumulated in the F0 females, and was subsequently transferred to the developing eggs, and hence to the fertilized embryos. The presence of dutasteride in the F1 embryos could explain the increased sensitivity to a second exposure to the drug during the F1 hatching trials.

In conclusion, the results of the short term reproduction study with fathead minnow demonstrated that dutasteride, a mammalian anti-androgen and 5α-reductase inhibitor, can affect reproductive endocrine function in fish, and that the observed effects are consistent with an anti-androgenic mode-of-action. Furthermore, its potency to negatively affect fathead minnow reproduction was about 20-fold higher than that of flutamide, a human pharmaceutical considered as a model anti-androgen. These results very strongly suggest that DHT could have an important physiological role in this species.

Table 6.5. *Effects of anti-androgenic chemicals, acting through different mode of action, on fish reproductive activity. For each effect, the LOEC is indicated (measured concentrations).*

Drug and MoA	LogP	Species	Nominal Tested Conc.	Fecundity	Fertilization	Vitellogenin	E2	T	KT	SSCs	Gonad histopathology	Reference
Flutamide: AR antagonist	2.6	Fathead minnow	50,500	651 - ↓	No effect	♂: 651 - ↑ ♀: 651 - ↑	♂: No effect (651, non sig. ↑) ♀: No effect (651, non sig. ↑)	♂: No effect ♀: 651 - ↑	♂ : 651, KT:T - ↑	No effects	♀: Atresia - 651 - ↑ Vitellogenic follicles - 651 - ↓	Jensen *et al.*, 2004
		Fathead minnow	100,500,1000	754 - ↓ (3/3)		♂: No effect ♀: No effect				Tubercles: 754 - ↓ (1/3)	♂: Spermatogonia - 464 - ↑ (1/3) Spermatocytes – 940 - ↓ (1/3) Testicular stage - 754 - ↑ (3/3) ♀: Atresia – 88.8 - ↑ (1/3) Ovarian stage – 354 ↓ (1/3)	OECD, 2006
		Medaka	100,500,1000	95.5 - ↓ (1/4)		♂: 95.5 - ↑ (1/4) ♀: 95.5 - ↑ (1/4)				No effect	♂: Testicular stage - 95.5 -↑(3/3) ♀: Post-ovulatory follicles - 97.4 - ↓ (1/3)	OECD, 2006
		Medaka	101,202,397, 787,1560	1560 - ↓	1560 - ↓	No effect					♂: Testis-ova - 202	Kang et l., 2006
		Zebrafish	100,500,1000	No effect		♂: No effect ♀: No effect					♂ Interstitial cells -250 - ↑ (2/3) Spermatogonia – 788 - ↑ (2/3) Testicular stage - 949 - ↓ (1/3) ♀: No effect	OECD, 2006
		Stickleback	100,500,1000			♂: No effect ♀: No effect				Spiggin: 500 - ↓		Sebire *et al.*, 2008
Vinclozolin: AR antagonist	3.0	Fathead minnow	200,700	Problems in the control group			♂: 700 - ↑ ♀: No effect				♂: No effect ♀: Pre-vitellog. oocytes - 700 - ↑	Makyenn *et al.*, 2000
		Fathead minnow	100,400,700	60 - ↓	No effect	♂: No effect ♀: 255 – ↑		♂: No effect ♀: No effect		Tubercles: 255 - ↓ Fatpad: 450 - ↓	♂: No effect (GSI – 255 -↑) ♀: Atresia - 60 - ↑	Martinovic *et al.*, 2008
Cyproterone acetate AR antagonist & weak PR & GR agonist	3.8	Mummichog					♂: 0.25 - ↓	♂: 0.1 - ↓ ♀: 0.01 - ↓	♂: 0.1 - ↓			Sharpe *et al.*, 2004

Drug & MoA	LogP	Species	Nominal Tested Conc.	Fecundity	Fertilization	Vitellogenin	E2	T	KT	SSCs	Gonad histopathology	Reference
Dutasteride: 5αRs inhibitor	5.5	Fathead minnow	10,32,100	32 - ↓	No effect	No effect	♂: No effect (32 – Non. Sig. ↓) ♀: 100 - ↓	♂: 100 – ↓ ♀: 100 - ↑	♂: 32– ↓	Tubercles: 32 - ↓	♂: No effect ♀: Pre-vitellogenic oocytes – 100 - ↑ Atresia - 100 - ↑ Interstitial tissue - 100 - ↑ Proteinaceus fluid - 100 - ↑ Lipidic tissue - 100 - ↑ Macrophages - 100 - ↑	Chapter 6 of this Thesis
Trilostane 3β-HSD inhibitor	3.0	Fathead minnow	60,300,1500	1500 - ↓	No effect	♀: 1500 - ↓	♂: 1500 - ↑ ♀: No effect (1500, non sig. ↓)			No effect	♂: No effect (GSI - 60 -↑) ♀: No effect	Villeneuve *et al.*, 2008
Ketaconazole CYPs inhibitor Weak AR antagonist	4.0	Fathead minnow	6-25-100-400	25- ↓	No effect	♂: No effect ♀: No effects	♂: No effect ♀: No effects	♂: No effect ♀: No effect	♂: No effect ♀: No effect	No effect	♂: Interstitial cells - 7 -↑ ♀: No effect	Ankley *et al.*, 2008

MoA, Mode of Action; Conc., Concentration; E2, Estradiol; T, Testosterone; KT, 11-Ketotestosterone; SSCs, Secondary Sexual Characteristics AR, Androgen Receptor; PR, Progesterone Receptor; GR. Glucocorticoid Receptor; Non Sig., Non Significant. The fractions in brackets (e.g.1/4) indicate that the effect has been recorded only in some of the replicate studies performed. (e.g 1 out of 4).

CHAPTER 7 : GENERAL DISCUSSION

7.1 General considerations on the environmental risk assessment of steroids and anti-steroids

The analysis of the clinical use of the human pharmaceuticals classified as steroids and anti-steroids discussed in Chapter 2 showed that steroid hormones and antagonists of endogenous steroids are widely used as pharmaceuticals, although the amounts used are much lower than the amounts of some other pharmaceuticals in widespread use, such as some analgesics, cardiovascular drugs, anti-inflammatories, and antibiotics. However, it should not be forgotten that although the annual use of EE2 in the UK is less than 25 kg, this pharmaceutical is of ecotoxicological concern (Caldwell *et al.* 2008), demonstrating that basing environmental risk assessment strategies on amounts of drugs used is not wise or defensible. Similar to EE2, some synthetic progestogens, which are prescribed in amounts up to approximately 500 kg/year, can inhibit fish reproduction at very low (ng/Litre, or even sub-ng/L) environmental concentrations (Paulos *et al.* 2010; Zeilinger *et al.* 2009).

Although much is still to be learnt about concentrations of steroid hormones in the aquatic environment, the picture that is emerging is a reasonably consistent one. Effluent concentrations are likely to be in the low ng/L range, which in most cases would give rise to sub-ng/L concentrations in rivers. Only in situations where the degree of dilution of effluent is low (as it can be in densely-populated areas with relatively small rivers) are river concentrations of steroid hormones likely to be in the ng/L range (Sumpter and Johnson 2005). However, the total concentration (of the different progestogens or glucocorticoids, for example) is probably the most relevant exposure parameter, because pharmaceuticals with similar mechanisms of action are likely to act additively (Brian *et al.* 2005). Thus, environmentally-relevant concentrations of some steroid hormones may be in the tens of ng/L range in heavily impacted locations.

The information provided in Chapter 2 suggests that when attempting to prioritize pharmaceuticals for potential environmental impact, and hence research, then synthetic steroid hormones merit serious attention. This is based on the fact that they are present in the aquatic environment, and that they can affect fish at low concentrations. Currently, knowledge of the risk posed by steroid hormones (and possibly also their antagonists) is limited; a lot is known about EE2 (e.g., Caldwell *et al.* 2008), but very little, if anything, is known about the other groups of steroid hormones. Research on potential chronic effects of these pharmaceuticals is urgently required, in order that an informed, balanced assessment of their (potential) ecotoxicological effects can be formed.

In general, despite the fact that human pharmaceuticals are regularly detected in the aquatic environment, there is still no evidence of any significant effects on aquatic wildlife species at the population level. The vast majority of the effects caused by pharmaceuticals on fish have been observed in laboratory studies at concentrations exceeding the environmentally relevant ones. The only two cases where pharmaceuticals have been directly linked to a disruption of wildlife populations dynamics are (a) the fish sexual disruption caused by steroid agonist, in particular estrogens and EE2, that have been shown to contribute to the feminisation of wild roach populations in UK Rivers (Jobling *et al.*, 2006); (b) the high mortality of vultures caused by diclofenac in Asia (Oaks *et al.*, 2004). However, the effect of intersex - the most evident exposure-related disruption - on fish population dynamics and reproductive success is still unclear, since a large study performed by Harris *et al.* (2011) with wild roach demonstrated that the majority of intersex fish were able to breed, albeit with varying degrees of success, suggesting that if severely intersex fish are present in a river, there could be implications for the fish population dynamics, whereas if only mildly intersex fish are present, the effects (if there are any) will be less severe.

For all other pharmaceuticals, there is no evidence for adverse effects caused by their occurrence in the environment on wild fish populations, even if there are many experimental and scientific aspects that have to be improved before drawing a definitive conclusion; for example, the need for long-term chronic studies in order to increase the confidence for the absence of harmful effects, or the potential effect of bioaccumulated pharmaceuticals on both human health and wildlife. Moreover, the vast majority of the toxicity studies involving human pharmaceuticals have been performed on a limited group of fish species, but it is known that the inter-species sensitivity to a specific API can be significant; therefore, some precautions and awareness should be used in the attempt to extrapolate toxicity information from one fish species, with the aim to apply it to other species, or even to species in other phyla. The growing availability of -omic information for an increasing number of species could provide a fundamental help to understand the issue of species sensitivity, playing an important role in the risk assessment process.

The physiological relevance of the endpoints assessed during toxicity studies should also be considered in the process of environmental risk assessment, since retarded sexual maturation or an interruption of reproductive functions will have a different influence on the population dynamics than the alteration of the expression of a specific gene or of the activity of specific enzymes, which could be only transitional responses.

Finally, one of the most important issues to face in the future is the toxicity of mixtures of pharmaceuticals. Additive effects of chemicals have been observed in fish, demonstrating the capacity for chemicals to act together to affect reproductive performance of fathead minnow, even when individual components were present below the threshold of detectable effects (Brian *et al.*, 2007).

7.2 Use of integrated testing strategies to assess the potential adverse effects caused by dutasteride in the fathead minnow

The work presented in this Thesis addressed the question of whether or not dutasteride, a human pharmaceutical mainly used for the treatment of benign prostatic hyperplasia, may cause adverse effects in the fathead minnow by inhibiting the activity of both isoforms of 5αR, the enzyme which converts T into DHT. At the beginning of the Ph.D. project, a significant theoretical issue came to light, since a general assumption in fish endocrinology is that DHT is not synthesized in teleost fish, or if it is, it has a modest or no physiological relevance (Borg, 1994). Hence, the question arising was: "why study the effects of dutasteride, a 5αR inhibitor, in fathead minnow, if this species seems to lack the biochemical apparatus targeted by the drug?"

In order to avoid random testing strategies, which carry the risk of producing only partial results which can be difficult to interpret, and to enhance the scientific reliability of the whole study, an integrated testing strategy was adopted. The theoretical framework used to guide the design of the experimental studies described in this Thesis was based on the combination of several conceptual approaches, including the study of the evolutionary degree of conservation of the drug targets, the study of the conservation of functionality of the drug target in the experimental model chosen for the experimental studies, and the cross-species extrapolation of pharmacological and toxicological information generated during pre-clinical and clinical studies in mammals during drug development.

These approaches have been recently suggested by some authors as key factors that should be taken in account in order to enhance the reliability of environmental risk assessment (Ankley *et al.*, 2007; Berninger and Brooks, 2010; Christen *et al.*, 2010; Gunnarsson *et al*, 2008; Kostich and Lazorchak, 2008; Owen *et al.*, 2007; Seiler, 2002; Winter *et al.*, 2009), and have been extensively applied by Owen *et al.* (2007) for the study of the effects of β-blockers in fish.

7.3 Utility of bioinformatics tools to fill the gaps in the background knowledge

A number of pieces of evidence collected during the preliminary phase of the Ph.D. project – both experimentally and from the literature - strongly suggested that DHT has a physiological role in the fathead minnow. The evidence includes the expression of 5αR genes in tissues such as the testis, and the occurrence of circulating DHT at concentrations similar to the ones detected in humans, and represented the rationale for testing the effects of dutasteride, a dual 5αR inhibitor, in the fathead minnow.

The use of bioinformatics tools not only allowed the identification of putative genes coding for 5αRs in the fathead minnow, but gave a surprisingly wide overview of the evolutionary degree of conservation of 5αR in many phyla, including plants, bacteria and fungi. Interestingly, plants (Arabidopsis sp.) carrying a mutation in the gene coding for 5αR, which in turn causes defective brassinolide biosynthesis, were characterized by several abnormal conditions including dwarfism, reduced male fertility, and delayed senescence and flowering (Chory *et al.*, 1991; Li *et al.*, 1996; Altmann, 1998; Clouse and Sasse, 1998). These results allow us to speculate that if dutasteride was able to enter into the plant (by uptake from water or ground), and exert its inhibitory effects on the activity of 5αR, it may affect key physiological processes, such as male fertility.

Generally APIs will be pharmacologically active in organisms in which the molecular targets (primary or secondary) are expressed and functional (Gunnarsson *et al.*, 2008; Seiler, 2002). The high degree of functional conservation of 5αR in such diverse phyla strongly suggests that this factor should be taken in account in the environmental risk assessment of dutasteride.

In particular, within this perspective, a further key element arising from this Thesis is the issue of species-sensitivity. In fact, even inside the teleost fish taxon, the degree of conservation of 5αR showed a wide range of variability, with zebrafish and fathead minnow having a higher degree of similarity to human 5αRs than other fish species, such as medaka and stickleback. It is not known if this variability in the degree of similarity – at nucleotide and protein level - also reflects variability of function (i.e. 5αR expression, activity rates, kinetics). However, it is possible to hypothesise that the effects caused by dutasteride will have different levels of severity according to the species used as the experimental model, and that the study of the evolutionary conservation of functionality of the target would help to identify the potentially more sensitive species.

5αRs and the product of their activity (DHT) represent a good example of the issue of species sensitivity. In fact, not only 5αRs do show a significant variability in the degree of

evolutionary conservation among teleost fish, but, as described in Chapter 3, such variability is also present in the fish AR, the component which transduces the presence of DHT into molecular and physiological responses. Hence, the degree of evolutionary and functional conservation should be evaluated not only for the specific target of the drug, but, if possible, also for other key components of the pathways affected by the drug.

7.4 Effects of dutasteride on the fathead minnow

The collection of toxicity data and side effects occurring in mammals, including humans, suggested that exposure to dutasteride may affect mainly the following physiological processes: sex determination and sex maturation; male fertility; steroid hormone dynamics. In order to test the hypothesis that similar physiological processes could also be affected in fathead minnows exposed to dutasteride, the following studies were performed:

- Early life stage toxicity tests to determine the effects of dutasteride on hatching, growth, and survival (Chapter 5);
- Early life stage toxicity test plus recovery period in clean water to determine the potential long term effects of an early life stage exposure to dutasteride (e.g. sex determination, sex maturation; sex steroid hormone dynamics in the plasma) (Chapter 5);
- 21 days reproduction test to determine the effects of dutasteride on reproductive functions (e.g. fertility, fecundity, sperm quality; sex steroid hormone dynamics in the plasma) (Chapter 6);
- F1 generation hatchability trials to determine the potential susceptibility of the F1 generation to dutasteride after parental (F0) exposure (e.g. through maternal transfer of dutasteride to embryos) (Chapter 6).

Dutasteride caused significant adverse effects in fathead minnow in all the *in vivo* studies performed during the project, and all the tested life stages were sensitive to the inhibition of 5αRs activity. A resume of the NOEC and LOEC for each endpoint is provided in Table 7.2. All the results obtained during the project strongly suggest that dutasteride exerts its effect, both in the fathead minnow and in humans, by acting by a highly specific mechanism of action, the inhibition of 5αRs activity. In order to exclude any potential interaction of dutasteride with the fathead minnow AR, a Yeast Androgen Screen (YAS) was kindly performed by Satwant Kaur at Brunel University. No interaction between AR and dutasteride was detected at concentrations ranging between 0.0005 and 1 mM.

None of the observed adverse effects occurred at concentrations of exposure lower than 32 μg/L, with the NOEC at 10 μg/L.

When possible, the response of the endpoints used in the fish studies was compared with similar endpoints in humans. For example, particular attention was given to the study of sperm quality, since the decrease of male fertility and sperm quality is one of the side-effects of the daily administration of dutasteride in mammals. In fathead minnow, dutasteride caused some alteration of sperm motility parameters, and decreased sperm motility and viability; however, the biological significance of these results was not clear, since no negative consequence on fertilization rates and occurrence of dead embryos was observed, thus excluding the occurrence of negative alterations of male fertility even when fish are exposed to high concentrations of dutasteride (100 μg/L).

Interestingly, the effect of dutasteride on plasma steroids concentrations was sex-specific; however, the response was different from the prediction made on the basis of the mammalian response. According to GSK (2001), in men the daily administration of 0.5 mg of dutasteride produced a major reduction of serum DHT concentrations, up to 94%, a consequent increase in serum T of *circa* 18-22%, and no clinically significant changes in plasma concentrations of sex hormone-binding globulin, E2, LH, and FSH. Based on this information, the hypothesis was that exposing male fathead minnows to dutasteride would cause an increase of circulating T, followed by a potential increase of E2; however, in the present study there was a general negative feedback on steroid production in male fish, which led to a significant decrease of circulating concentrations of T and KT, and a non-significant decrease of circulating E2. Notably, the predicted increase of circulating T was observed in females exposed to the highest concentration of dutasteride; the same group showed also a decrease of circulating E2. These differences suggest that the system of hormonal feedback mechanisms may work differently in humans and fathead minnow.

According to the so-called "read-across hypothesis" (Gunnarsson *et al.*, 2008; Winter *et al.*, 2009), human pharmaceuticals cause the same effects in fish as they do in humans, and these effects occur only if plasma concentration of the pharmaceuticals in fish reach approximately the same concentration required in humans to exert their therapeutic effects.

During this Ph.D. project, the plasma concentration of dutasteride in fathead minnows exposed to the drug were not experimentally quantified; however, one of the approaches employed to test the read-across hypothesis involved the use of a mathematical model developed by Huggett and co-workers (Huggett *et al.*, 2003), and known as the "fish plasma

model" (FPM). This model aims to predict the uptake of pharmaceuticals from the surrounding water into fish plasma. Conceptually, the FPM estimates the "fish steady-state plasma concentration" ($F_{SS}PC$) of a drug, on the basis of a given water concentration (e.g. predicted environmental concentration, PEC). $F_{SS}PC$ is successively compared with the human/mammalian therapeutic plasma concentration (H_TPC) (known to give a pharmacological response), in order to obtain an "effect ratio" ($ER = H_TPC/F_{SS}PC$), that represents an indication of risk. In other words, if the model predicts that a specific pharmaceutical, at environmentally relevant concentrations, can accumulate in fish plasma, reaching concentrations similar or higher than the H_TPC ($ER \leq 1$), there is an obvious risk that the compound could exert its pharmacological effect in exposed fish.

The parameters and the equations used in the model are showed in Table 7.1. In the FPM, the major parameter that drives the uptake of a compound from the water into the blood of fish is its lipophilicity, expressed by a partitioning factor (e.g. Log K_{ow}). This parameter is not used directly by the FPM, but it is first converted into a more specific estimation of partitioning between the aqueous phase and the arterial blood (Log $P_{blood:water}$), as described by Fitzsimmons *et al.* (2001):

$$\text{Log } P_{blood:water} = 0.73 \times \text{Log } K_{ow} - 0.88$$

The latter coefficient is then used to estimate the $F_{SS}PC$ (Huggett *et al.*, 2003):

$$F_{SS}PC = PEC \times P_{blood:water}$$

A large variability in the Log K_{ow} of dutasteride was observed when comparing estimated and experimental values; therefore, in order to take in account this uncertainty, values from both sources were used to run the FPM. Furthermore, the polar nature of many pharmaceuticals, including dutasteride, indicates that Log K_{ow} may not represent the most realistic option. Several authors suggested that the distribution coefficient Log D, measured at physiological pH (7.4), is probably a more realistic indicator of uptake potential in fish than Log K_{ow}, at least for compounds that can have variable degrees of ionization at different pH conditions (Cunningham, 2004; Owen *et al.*, 2009; Winter *et al.*, 2008). Therefore, taking into account these suggestions, Log $D_{7.4}$ was the third partitioning factor used to run the model.

The second important parameter required for the application of the FPM is the H_TPC of the drug that is taken into consideration. This value (or range of values) is usually available in the reports published by the manufacturing company and are obtained during the drug development process. The H_TPC is usually expressed as "maximum concentration" (C_{max}) or

“area under the curve” (AUC) values, describing the presence of a drug in the systemic circulatory system at either a single point in time (C_{max}) or as a function of time (AUC) (Hardman and Limbird, 1996). C_{max} values for dutasteride were obtained from the official documentation published by GSK (2001). Since the H_TPC depends on the duration of the treatment, I considered two different values obtained from two different clinical studies: a long-term treatment (12 months) and a short-term treatment (28 days). Parameters such as C_{max} are often available as a range of values, and the lowest value is chosen in order to obtain a conservative estimate, according to the precautionary principle.

In this section I describe the application of the FPM with the aim to estimate if the concentrations of dutasteride used in the experiments described in Chapter 6 would produce a concentration of drug in the plasma of fathead minnow similar to the H_TPC, providing a theoretical justification for the biological effects observed during the toxicity studies performed during this Ph.D. project. In order to achieve this aim, the equations of the model were inverted to calculate 1) the water concentration of dutasteride required to produce $F_{SS}PC$ = H_TPC, and 2) to estimate the concentrations of dutasteride in the plasma of fathead minnow exposed to 10, 32 and 100 µg dutasteride/L.

The FPM indicated that the water concentration of dutasteride required to obtain $F_{SS}PC$ = H_TPC varies significantly as a consequence of the partition coefficient used to run the model. In fact, the bioconcentration coefficient “$P_{blood:water}$”, calculated by the equation of Fitzsimmons *et al.* (2004), was 12589, 1259, and 89, respectively, if calculated using experimental Log K_{ow}, predicted Log K_{ow} or Log $D_{7.4}$. It is evident that there is a large difference (~140-fold) between the first and the last value.

When the model was run using Log K_{ow} (both experimental and predicted), the concentrations of dutasteride required to obtain $F_{SS}PC$ = H_TPC were in the range of 2-32 ng/L, more than 1000-fold lower than the LOEC (32 µg/L) obtained in the experiments described in this Thesis, suggesting that a significant inhibition of the 5αR activity would occur at those very low concentrations. On the other hand, using Log $D_{7.4}$, this value goes up to 265-500 ng/L, which is still 64-107 times lower than the LOEC. Furthermore, according to the model (based on Log $D_{7.4}$), the latter would produce a $F_{SS}PC$ approximately 69-fold and 121-fold higher than, respectively, the H_TPC at 12 months and 28 days.

Apparently, these predictions do not fit with the biological effects observed during the experiments previously described in this Thesis, suggesting that the FPM could overestimate the bioaccumulation process. However, it is important to remember that the model does not

predict if the potentially occurring pharmacological effect is an adverse effect. It is possible to hypothesise that an increase in the incidence of the adverse effects would be correlated with the increase of the blood concentration of the drug. For example, histopathological alteration of the ovary were present in the 12.5% and 87.5% of the female fathead minnows exposed, respectively, to 32 and 100 µg dutasteride/L, while egg production was affected, respectively, in the 50% and 100% of the females in the same treatment groups. Since the endpoints considered during the study can be considered as severe adverse effect, they are likely to appear at plasma concentrations higher than the therapeutic ones.

The discrepancy between the prediction made by the FPM and the experimental observations of the adverse effects could also be partially explained by the high lipophilicity of dutasteride. It is possible to hypothesise that a significant fraction of dutasteride was not bioavailable, due to adsorption to any organic matter present into the fish tanks, including excrements, food breakdown products, suspended bacterial population, and bacterial biofilm. Although many efforts were made to minimize the presence of organic matter in the exposure set-up (e.g. by daily cleaning and aspiration of the sedimented organic fraction), the use of a solvent for the delivery of the drug (i.e. DMF) promoted bacterial growth, which became visible after only a few days of exposure, and even if three days of dosing were allowed to "chemically" saturate the system, daily inputs of "fresh" organic matter were represented by food administration and excretion processes.

It should also be noted that the FPM does not take into account the factor "time" and other processes that prevent, decrease or increase the bioaccumulation rate, such as drug hepatic metabolism, elimination, and binding to plasma proteins (e.g. steroids binding to SSBG), which, taken all together, contribute to the complexity of the real bioaccumulation dynamics.

In conclusion, the FPM is currently in an early stage of development and incorporates major assumptions that still require further experimental validation. Among the few studies which experimentally tested the model, Owen *et al.* (2009) and Winter *et al.* (2008) found a reasonably good degree of agreement between predicted and measured plasma concentrations of two β-blockers (propranolol and atenolol). The Log K_{ow} values of these two compounds are, respectively, ~ 3.0 and ~ 0.5, much lower than the 5.5 - 6.8 of dutasteride. Brown *et al.* (2007) compared the modelled and measured concentrations of five human pharmaceuticals (ibuprofen, naproxen, diclofenac, ketoprofen, gemfibrozil) in the blood of rainbow trout exposed for 48 hours in a laboratory set-up, or for 14-23 days to sewage treatment plant effluent. The five drugs had Log K_{ow} values ranging between 3.0 and 4.8. In the short-term laboratory exposure, the concentrations predicted by the FPM were between 6.6 and 17.15-

fold higher than the measured ones, except for ketoprofen (166-fold). No clear correlation was observed between the Log K_{ow} and the ratio modelled:measured concentration; for example, the concentration of gemfibrozil predicted by the FPM had the same degree of over-estimation as naproxen, despite a difference of 1.7 in their Log K_{ow} values. It is unclear if the short length of the exposure (48 hours) could have played a role in the lack of the previously mentioned correlation. Finally, in the same experiment, all the pharmaceuticals, except ibuprofen, had equal, lower or considerably lower Bioaccumulation Factors (BCF) than predicted by the FPM, supporting the hypothesis previously described in this Chapter that the FPM could overestimates the bioaccumulation process, at least in some cases. A further confirmation of this hypothesis was provided by Fick *et al.* (2010), who highlighted how the FPM overestimated the rainbow trout plasma BCF for 11 out of 21 pharmaceuticals analyzed after exposure of the fish to sewage effluents.

It is clear that in order to define the reliability of the FPM applied to pharmaceuticals, a larger number of compounds with a larger range of physicochemical characteristics needs to be experimentally tested. The development of correction factors (e.g. accounting for pH, concentration of organic matter in the system, temperature, affinity to plasma binding proteins, etc.) is probably needed to improve reliability of the FPM. In fact, if it is true that the overestimation features of the FPM could not be considered negative in general terms, in the perspective of the “precautionary principle” used in the environmental risk assessment, the same model limitations could lead to an underestimation, with all the relative negative consequences, as demonstrated by Fick *et al.* (2010) for levonorgestrel (a synthetic progestogen). The authors obtained an experimental fish plasma BCF 185 to 260-fold higher than the predicted one, probably due to the high affinity of this compound to the SSBG present into the gills of fish. In this case, it is likely that the affinity of the drug to the binding proteins present in the plasma, and not the Log K_{OW}, represented the major factor driving the uptake dynamics, suggesting the need for more complex mathematical models.

Table 7.1. *Parameters and equations used for the calculation of the predicted concentration of dutasteride in the water that would give a concentration in the plasma of fish equal to the human therapeutic concentration in humans, according to the fish plasma model developed by Hugget et al. (2003). A description of the method is provided in Section 7.4.*

Parameters

H_TPC following administration of 0.5 mg/d for different periods of time:

H_TPC (A) = Steady State after 12 months = 40 ng/mL

H_TPC (B) = C_{max} after 28d of treatment with 0.5 mg.day = 23.6 ng/mL

Partitioning Coefficients:

Log K_{ow} = 6.8 (Experimental: PhysProp)

Log K_{ow} = 5.45 (Predicted: ALOGPS)

Log $D_{7.4}$ = 3.87 (Cunningham *et al.*, 2009)

Equations

1) Log $P_{Blood:Water}$ = 0.73 × Log K_{ow} – 0.88 (Fitzsimmons *et al.*, 2004)

$P_{Blood:Water}$ = 12589 (based on Experimental Log K_{ow})
$P_{Blood:Water}$ = 1259 (based on Predicted Log K_{ow})
$P_{Blood:Water}$ = 89 (based on Log $D_{7.4}$)

2) $F_{SS}PC$ = EC × ($P_{Blood:Water}$) (Hugget *et al.*, 2003)

The environmental concentration required to obtain $F_{SS}PC = H_TPC$:

With H_TPC = 40.0 ng/mL and	1) Experimental Log K_{ow}	→ EC = 3.1	ng/L
	2) Predicted Log K_{ow}	→ EC = 32	ng/L
	3) Log $D_{7.4}$	→ EC = 500	ng/L
With H_TPC = 23.6 ng/mL and	1) Experimental Log K_{ow}	→ EC = 1.9	ng/L
	2) Predicted Log K_{ow}	→ EC = 18.7	ng/L
	3) Log $D_{7.4}$	→ EC = 265	ng/L

$F_{SS}PC$ of dutasteride estimated at different exposure concentrations:

With EC = 10 µg/L and	1) Experimental Log K_{ow}	→ $F_{SS}PC$ = 125890	ng/mL
	2) Predicted Log K_{ow}	→ $F_{SS}PC$ = 12590	ng/mL
	3) Log $D_{7.4}$	→ $F_{SS}PC$ = 890	ng/mL
With EC = 32 µg/L and	1) Experimental Log K_{ow}	→ $F_{SS}PC$ = 402845	ng/mL
	2) Predicted Log K_{ow}	→ $F_{SS}PC$ = 40288	ng/mL
	3) Log $D_{7.4}$	→ $F_{SS}PC$ = 2848	ng/mL
With EC = 100 µg/L and	1) Experimental Log K_{ow}	→ $F_{SS}PC$ = 1258900	ng/mL
	2) Predicted Log K_{ow}	→ $F_{SS}PC$ = 125900	ng/mL
	3) Log $D_{7.4}$	→ $F_{SS}PC$ = 8900	ng/mL
With EC = 0.03* ng/L and	1) Experimental Log K_{ow}	→ $F_{SS}PC$ = 0.3777	ng/mL
	2) Predicted Log K_{ow}	→ $F_{SS}PC$ = 0.0378	ng/mL
	3) Log $D_{7.4}$	→ $F_{SS}PC$ = 0.0027	ng/mL

* = Predicted Environmental Concentration (Cunningham *et al.*, 2009)

7.5 Female fish are highly susceptible to the disruption of androgenic pathways

At the beginning of the project, the use of female fathead minnows to test the effects caused by exposure to dutasteride was underestimated. In fact, it was not clear if this species would express functional 5αR at all; therefore, the attention was focused on males. However, after the first *in vivo* study to assess the potency and effects of DHT and KT on sexual maturation of juvenile fathead minnow, the high sensitivity of female fish to the disruption of the androgenic pathways came to the light, confirming the high androgenic potency of DHT in this species, which in females was higher than the potency of KT (e.g. only the females exposed to DHT had masculinised ovaries). This sensitivity was confirmed also by all the following *in vivo* studies involving exposing fish to dutasteride, (i.e. inhibiting the biosynthesis of DHT). The histopathology of the ovary was one of the most sensitive endpoints, and the physiology of this organ was affected even months after termination of an early life stage exposure (e.g. retardation of oogenesis) (Chapter 5). A potential explanation for the sensitivity of females could be that DHT is a physiological important hormone in female physiology, as confirmed by the analytic detection of DHT circulating in female fish plasma at concentrations 2 to 10-fold higher than in humans. On the other hand, KT was not detected in any of the female plasma samples analysed by Ultrasensitive GC-MS/MS. Moreover, it is important to note that the sensitivity of female fish to the disruption of the androgenic pathways was also confirmed by other studies testing various anti-androgens, as discussed in Chapter 6.

In this case, the utility of mammalian toxicity studies to predict the effects of dutasteride in female fish was limited. In fact, studies on rats and dogs showed treatment-related effects in female reproductive organs, including decreased ovary and uterus/cervix weights, increased incidence of dioestrus or increased occurrence of ovarian (follicular) cysts in rats, microscopic changes in the uterus and shifts in the oestrus cycle to the luteal phase in dogs; however, there was no information available specifically concerning the histopathological effects of dutasteride in higher female vertebrates (GSK, 2001).

Despite the lack of knowledge of the effect of dutasteride on ovarian histopathology in vertebrates, some speculations can be made on the similarity between the human pathological condition named Polycystic Ovary Syndrome (PCOS) and the alterations recorded in the ovaries of female fish exposed to dutasteride during a 21 d reproduction test (Chapter 6). The factor shared between these two conditions is the hyperandrogenism. In fact, PCOS - the most common cause of anovulatory infertility in women - is characterized by hyperandrogenism, oligo-/anovulation, cessation of oocyte growth, and follicle dysfunction (Shi *et al.*, 2009).

Affected ovaries present ovarian hypertrophy, and a number of histopathological conditions, including an increased number of primary growing follicles, thickened follicle capsule, hyperplasia and fibrosis of ovarian stroma, decreased thickness of granulose layer, increased thickness of the internal theca, and atresia. It has been proposed that these morphological alterations are driven by an excess of androgens (Azziz *et al.*, 2007; Chang and Razer, 2009; Goodarzi *et al*., 2011).

Some of these histological alterations were also observed in female fathead minnows exposed to dutasteride, in particular a decreased proportion of early vitellogenic oocytes, an increased proportion of perinucleolar oocytes, an increased occurrence of atretic follicles, abnormal proliferation of interstitial/somatic tissue, vacuolar hypertrophy of the surrounding granulose cells due to phagocytic processes, proliferation of interstitial tissue containing macrophage aggregates, the presence of mitotically dividing oogonia, accumulation of lipidic tissue, and presence of interstitial proteinaceus fluid. Interestingly, the same female fish had decreased concentrations of circulating E2 and increased concentrations of T. A similar concomitance (ovary histological alteration and increased plasma T concentration) was also observed by Jensen *et al.* (2004) when they exposed fathead minnows to flutamide. In conclusion, it is possible to hypothesise that, as happens in humans, the excess of circulating or *in situ* androgens caused by the exposure to dutasteride could be the causal factor that explains the histological alterations of the ovary in the fathead minnow.

The results presented in the present Thesis suggest that female fish would be of great utility for the evaluation of potential adverse effects caused by anti-androgens in the fathead minnow, and quite possibly other species of fish as well.

7.6 General considerations on the environmental risk assessment of dutasteride

No information is available on the occurrence of dutasteride in surface waters or WWTP's effluents; however, according to Cunningham *et al.* (2009), the predicted environmental concentration (PEC) of dutasteride in surface water is 0.02 ng/L in Europe, and 0.03 ng/L in US. These values were derived using the PhATE™ model (Pharmaceutical Assessment and Transport Evaluation) for North America and the GREAT-ER (Geo-referenced Regional Exposure Assessment Tool for European Rivers) model for Europe.

The list of "Environmentally Classified Pharmaceuticals" published every year by the Stockholm County Council (2011) expresses the environmental hazard of each API using the so called PBT index, composed of three factors:

- **Persistence** – ability to resist degradation in the aquatic environment;

- **Bioaccumulation** – accumulation in adipose tissue of aquatic organisms;

- **Toxicity** – the potential to poison aquatic organisms;

To each of these characteristics is assigned a numerical value (0–3), and the total of these values constitutes the PBT Index for the substance (0-9). According to this list, dutasteride has a PBT index equal to 8, and its environemntal risk, defined as the risk of toxicity to the aquatic environment and based on the ratio between PEC and the highest concentration of the substance that does not have a harmful effect in the environment (PNEC), is classified as "Cannot be excluded", meaning that the information provided by the manyfacturer was insufficient for the assessment of the risk.

Cunningham *et al.* (2009) performed a human risk assessment for 44 APIs marketed by GlaxoSmithKline (GSK), focusing attention on the potential risk deriving from drinking water and fish consumption. The assessment considered the highest dose of pharmaceutical derived from fish consumption, in conjunction with the highest possible acceptable dose deriving from drinking water, and compared it to the acceptable daily intake (ADI), defined as "the level of daily intake that should not result in an adverse human health effect from direct exposure in a population, including particularly sensitive individuals". Considering children as the most sensitive group of the general population, the ADI for dutasteride was 0.0016 μg/kg/day, the predicted no effect concentration for drinking water (PNEC) was 23 ng/L, the PNEC for fish consumption was 19 ng/L (taking into account the fish BCF), and the combined PNEC (drinking water + fish consumption) was 10 ng/L.

In conclusion, the low volume of drug prescribed every year (5.07 kg in the UK in 2006), and the relatively high concentration required to elict any significant adverse effect on fathead minnow (approximately 32 μg/L) suggest that, at present, the potential presence of dutasteride in the environment does not represent a threat to either wild fish populations or humans. However, a potentially significant reason of concern is the high bioaccumualtion factor of the compound (BCF_{fish} = 190.5; Log P = 5.45), exemplified by the fact that approximately 55% of the daily dose (0.5 mg) is potentially bioaccumulated in humans. This suggest that further studies should be performed in order to elucidate the potential risk arising from the bioaccumualtion process in both humans and fish populations.

7.7 Limitations of the project and recommendations for future work

7.7.1 Analytical determination of circulating DHT in fathead minnow plasma

The use of Ultrasensitive GC-MS/MS allowed the detection of circulating DHT in plasma of both male and female fathead minnows. Due to time and resources limitiations, only a relatively limited number of samples was analysed, in particular for juvenile fish. One of the main technical limitation of GC-MS/MS was the high volume of plasma required for the analysis (200 µL), which is much higher than the average volume normally collected from individual fish (20-80 µL). The necessity to pool several plasma samples for the analysis lead to a loss of information usually provided by inter-individual variability. This method seems at the moment unsuitable for routine analysis of plasma steroids in small fish species, used as the model species in toxicological tests. However, the concentrations of DHT detected in the plasma of fathead minnow suggest that a specific RIA could be suitable for this kind of purpose.

Additional experiments should be performed in order to evaluate the onthogenesis of DHT biosynthesis, since DHT was not detected in the single sample of plasma collected from juvenile fish (pool of 4 individual samples). Furhtermore, future experimental designs should try to detect any difference between the plasma DHT concentration of adult fish in active reproduction phase and adult fish kept without sexual stimuli, in order to clarify if DHT synthesis is stimulated by reproductive activity. Finally, plasma samples collected from fish exposed to dutasteride would represent an ideal negative control, as well as providing a potential estimate of the rate of DHT inhibition following an exposure to dutasteride.

7.7.2 Gene expression study

The study of srd5a1 ans srd5a2 gene expression in different tissues of the fathead minnow at different life stages was planned but not performed, due to the higher priority given to *in vivo* studies aimed at assessing the effects of dutasteride on fish. The hypothesis behind the planned study was that knowledge of the sites of expression of the two genes could have been of high predictive value for the the assessment of the effects of dutasterie in the following *in vivo* toxicity studies. For example, it is possible to hypothesise that the detection of high levels of expression of the two target genes in the ovary of fathead minnow would have helped to predict the high sensitivity of female fish to dutasteride, in particular at the level of the ovary. This approach could also give precious information on the onthogenesis of DHT biosynthesis in the fathead minnow.

7.7.3 Bioaccumulation of dutasteride in the fathead minnow

The high BCF of dutasteride in fish suggest that the bioaccumulation of this compound in specific tissues, such as testis or ovary, could play an important role in conditions of chronic exposure. A bioaccumulation test was not performed during the project; however, some indications of potential maternal transfer of dutasteride from adult (F0) females to the F1 generation were collected during the F1 hatchability trials preformed during the 21 days reproduction test (Chapter 6). In order to clarify the possible occurrence of maternal transfer, future studies should include a more robust experimental desing, involving an higher number of breeding couples, together with the analytical determination by LC-MS/MS of the concentrations of dutasteride potentially bioaccumulated in F1 fertilized embryos.

7.7.4 Concentration of dutasteride in the plasma of fathead minnow

One of the key paradigms of the "read-across" hypothesis is that human pharmaceuticals will cause the same effects in fish as they do in humans when they are present at similar plasma concentrations. The measurment of dutasteride concentration in fathead minnow plasma was not performed during the project, and the application of the fish plasma model (Hugget *et al.*, 2003), previously described in this Chapter, estimated that water concentrations between 2 and 500 ng dutasteride/L would be enough to obtain a concentration of drug into the plasma of fish similar to the human therapeutic plasma concentration. These estimates are much lower than the LOEC obtained in the toxicity studies described in this Thesis, allowing to hypothesise that the FPM applied to dutasteride produced an over-estimation of the bioaccumulation process. Using dutasteride as target drug, an experimental comparison between modelled and measured concentrations would represent a significant test case for the assessment of the the reliability of the FPM applied to highly lipophilic human pharmaceuticals.

Table 7.2. *Resume of LOECs and NOECs for each endpoint used during the in vivo studies carried out in this Ph.D. project.*

Study	Life stage & Sex	Endpoint	LOEC (µg/L)	NOEC (µg/L)
Early life stage test 1 + evaluation of long term effects	Embryos	Hatching success	>100	100
		Hatching time	>100	100
	28 dph Mixed sex	Survival	100	32
		Length	32	10
		Weight	32	10
		CI	100	32
	120 dph Mixed sex	Length	>100	100
		Weight	>100	100
		CI	>100	100
	160 dph Males	Length	>100	100
		Weight	>100	100
		CI	>100	100
	160 dph Female	Length	>100	100
		Weight	>100	100
		CI	>100	100
	160 dph Male	Testis histology	100	32
	160 dph Male	N° of tubercles	>100	100
		Tubercles grade	>100	100
		Fatpad index	>100	100
		Fatpad height	>100	100
	160 dph Female	Ovipositor Length	>100	100
	160 dph Female	Ovary histology	32	10
	160 dph Male	Testosterone	>100	100
	160 dph Male	11-Ketotestosterone	>100	100
	160 dph Female	Testosterone	>100	100
Early life stage test 2	Embryos	Hatching success	>100	100
		Hatching time	>100	100
	14 dph Mixed sex	Survival	100	32
		Length	32	10
		Weight	32	10
		CI	>100	100
Short term reproduction test	180 dph Male	Length	>100	100
		Weight	>100	100
		CI	>100	100
		GSI	>100	100
		HSI	>100	100
	180 dph Female	Length	>100	100
		Weight	>100	100
		CI	>100	100
		GSI	100	32
		HSI	>100	100

Short term reproduction test		Cumulative egg production	32	10
	180 dph Male+Female	N° of spawnings per pair	100	32
		N° of eggs per spawning	32	10
	180 dph Male	Fertilization	>100	100
	F1 Embryos	% of dead embryos	>100	100
	180 dph Male	N° of tubercles	100	32
		Tubercles grade	100	32
		Fatpad index	>100	100
		Fatpad height	>100	100
	180 dph Female	Ovipositor Length	>100	100
	180 dph Male	Testosterone	100	32
		11-Ketotestosterone	32	10
		Estradiol	>100	100
	180 dph Female	Testosterone	100	32
		Estradiol	100	32
		Vitellogenin	>100	100
	180 dph Male	Testis histology	>100	100
	180 dph Female	Ovary histology	100	32
	180 dph Male	Sperm cell count	>100	100
		% of motile sperm	32	10
		% of viable sperm	100	32
		Milt volume	>100	100
		Sperm motility (by CASA)	32	10
F1 Hatching trials in dilution water	F1 Embryos	F1 Hatching success	>100	100
		F1 Hatching time	>100	100
F1 Hatching trials in parental tank water		F1 Hatching success	100	32
		F1 Hatching time	100	32

CI, Condition Index; GSI, Gonadosomatic Index; HIS, Hepatosomatic Index.

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