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Progestagenic Aquatic Contaminants Act as Potent Androgens in Fish

Experimental Studies in Three-spined Stickleback and Zebrafish

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2016

ISSN 1651-6214
ISBN 978-91-554-9587-9
urn:nbn:se:uu:diva-286535

Dissertation presented at Uppsala University to be publicly examined in Lindahlsalen, Evolutionsbiologiskt Centrum, Norbyvägen 18 A, Uppsala, Friday, 10 June 2016 at 10:00 for the degree of Doctor of Philosophy. The examination will be conducted in English. Faculty examiner: Professor Ian Mayer (Norwegian University of Life Sciences).

Abstract
Svensson, J. 2016. Progestagenic Aquatic Contaminants Act as Potent Androgens in Fish. *Experimental Studies in Three-spined Stickleback and Zebrafish*. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 1379. 45 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9587-9.

The extensive use of pharmaceuticals and their poor removal by wastewater treatment plants has led to the emergence of pharmaceutical compounds as global aquatic contaminants. Progestins, the synthetic analogues to progesterone (P4), are receiving increasing attention as contaminants and have been shown to impair reproduction in fish and amphibians at low ng L^{-1} concentrations. Certain progestins have androgenic properties and are several orders of magnitude more potent in terms of reproductive impairment in fish than non-androgenic progestins. To characterize the androgenic effects of progestins in fish, adult three-spined sticklebacks (*Gasterosteus aculeatus*) and zebrafish (*Danio rerio*) larvae were exposed to progestins via the ambient water. In female sticklebacks, the androgenic progestins levonorgestrel (LNG) and norethindrone (NET) induced production of the androgenic biomarker protein spiggin and reduced production of the egg yolk protein vitellogenin. Comparison with well-known environmental androgens showed that LNG and NET, with regard to spiggin induction and vitellogenin induction, are among the most potent environmental androgens known. In male sticklebacks, LNG inhibited the post-breeding regression of secondary sex characters and spiggin production, as well as the resumption of spermatogenesis, functionally inhibiting the natural transition from breeding into non-breeding condition. Exposure of zebrafish larvae to LNG caused all fish to develop into males, whose sexual development was also significantly accelerated. P4 had no effect on the sex ratio, while slightly accelerating sexual development at high concentrations. Suppression of vitellogenesis in females, disruption of the male reproductive cycle, male-biased sex ratios and precious male puberty could all entail severe fitness costs and severely affect fish populations. Most of the effects of androgenic progestins in this thesis occurred at levels within the range of reported environmental levels, and may therefore occur in progestin-contaminated waters. In conclusion, the present results establish LNG and NET as highly potent androgenic pollutants of environmental concern, and provide strong support to the contention that the reproductive impairment in fish caused by progestins is chiefly mediated by their androgenic properties.

Keywords: Pharmaceuticals as contaminants, Progestins, Androgenic properties, Fish

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ISSN 1651-6214
ISBN 978-91-554-9587-9
urn:nbn:se:uu:diva-286535 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-286535)

For Sven

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Svensson, J.**, Fick, J., Brandt, I., Brunström, B. (2013) The Synthetic Progestin Levonorgestrel is a Potent Androgen in the Three-spined Stickleback (*Gasterosteus aculeatus*). *Environmental Science & Technology*, 47(4):2043-2051

II **Svensson, J.**, Mentor, A., Fick, J., Brunström, B. (2016) Androgenic and anti-estrogenic potencies of progestins and other environmental androgens in female three-spined stickleback (*Gasterosteus aculeatus*). Manuscript

III **Svensson, J.**, Fick, J., Brandt, I., Brunström, B. (2014) Environmental concentrations of an androgenic progestin disrupt the seasonal breeding cycle in male three-spined stickleback (*Gasterosteus aculeatus*). *Aquatic Toxicology*, 147:84-91

IV **Svensson, J.**, Mustafa, A., Fick, J., Schmitz, M., Brunström, B. (2016) Developmental exposure to progestins causes male bias and precocious puberty in zebrafish (*Danio rerio*). Submitted manuscript

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Contents

Introduction

Pharmaceuticals in the environment

The global use of pharmaceuticals is extensive. In the European Union alone there are nearly 3000 active pharmaceutical ingredients (APIs) used in human medicine (Fent et al., 2006). In sheer mass, consumption of the most common APIs is in the order of hundreds of tons per year. High economic growth and increased life expectancy in many parts of the world will likely lead to increased quantity and diversity of pharmaceuticals consumed (Arnold et al., 2014). The most commonly used classes of pharmaceuticals are non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics, β-blockers, hypolipidemic agents, steroids and steroid-related hormones (Christen et al., 2010). Following consumption, pharmaceuticals are metabolized to varying extents, excreted and eventually emitted into a sewage system. However, removal of pharmaceutical compounds by wastewater treatment plants (WWTPs) is often incomplete. Veterinary pharmaceuticals enter the environment more directly, mainly from livestock and aquaculture. Consequently, pharmaceuticals have over the past 20 years become recognized as global environmental contaminants and are detected in WWTP effluents, surface and ground water, sediments and biota.

Although pharmaceuticals are generally detected in low concentrations (ng-μg L^{-1} range) in the environment, the inherent properties of pharmaceuticals have raised concern about possible effects on aquatic wildlife. Pharmaceuticals are carefully designed with the specific purpose of affecting physiological systems, and to do so at the lowest possible dose. Furthermore, many drug targets are evolutionarily conserved across animal phyla and are present in non-target aquatic organisms such as fish and amphibians (Gunnarsson et al., 2008). Numerous studies have shown adverse effects of pharmaceuticals on aquatic organisms, occurring within the ranges of concentrations found in the environment (Overturf et al., 2015). By far the most extensively studied pharmaceutical pollutant is 17α-ethinylestradiol (EE_2). This synthetic steroidal estrogen is used in oral contraceptives, and has been shown to impair reproduction in fish even at concentrations below 1 ng L^{-1} (Caldwell et al., 2008). Relatively recently, another class of contraceptive pharmaceuticals has emerged into focus in ecotoxicology: the progestins.

Progestins

As functional analogues to mammalian progesterone (P4), progestins are steroidal hormones which induce progestagenic changes in the mammalian uterus, i.e. transforming the endometrium from the estrogen-induced proliferative state to the secretory state (Stanczyk, 2002). Natural progestins are important reproductive hormones in all vertebrates. In female mammals, P4 is involved in ovulation, implantation and maintenance of pregnancy (Graham and Clarke, 1997). In fish, the main natural progestin is 17α,20β-dihydroxy-4-pregnen-3-one (DHP). In females, DHP is responsible for final maturation of oocytes (Nagahama and Yamashita, 2008), while in males it is involved in spermiation, milt production and sperm motility (Tubbs and Thomas, 2009). Furthermore, DHP is known to function as a female pheromone in several fish species, triggering reproductive behavior and milt production in males (Scott et al., 2010).

There are about 20 different synthetic progestins used in human and veterinary medicine, which all activate progesterone receptors (PRs) (Sitruk-Ware and Nath, 2010). However, progestins are relatively unspecific and also interact with other steroid hormone receptors, exerting combinations of progestagenic, (anti) androgenic, (anti) estrogenic, glucocorticoidogenic and anti-mineralocorticoidogenic effects (Africander et al., 2011). These off-target interactions depend on the parent compound from which the progestin is derived (P4, testosterone or spironolactone), where for example the 19-nortestosterone derived progestins are the ones which interact most with the androgen receptor (AR) (Schindler et al., 2008). An overview of all progestins presently in use and of the parent compounds from which they are derived is given in Table 1. Progestins are sometimes also divided into four “generations”, depending on when they were developed. The specificity for PRs generally increases from first- to fourth-generation progestins (Sitruk-Ware, 2008).

Table 1. *Classification and parent compounds of progestins (Schindler et al., 2008)*

	Structural derivation	Progestin	Abbreviation	CAS #
Structurally related to progesterone		Progesterone	P4	57-83-0
	Retroprogesterone	Dydrogesterone	DGS	152-62-5
	17α-hydroxyprogesterone	Chlormadinone acetate	CMA	302-22-7
		Cyproterone acetate	CPA	427-51-0
		Medroxyprogesterone acetate	MPA	71-58-9
		Medroxyprogesterone	MPE	520-85-4
		Megestrol acetate	MGA	595-33-5
		Melengestrol acetate	MNA	2919-66-6
	19-norprogesterone	Nestorone	NES	7759-35-5
		Nomegestrol acetate	NGA	58652-20-3
		Trimegestone	TRI	74513-62-5
Structurally related to testosterone	Testosterone	Ethisterone	ETH	434-03-7
	19-nortestosterone	Desogestrel	DES	54024-2-5
		Dienogest	DIE	65928-58-7
		Ethynodiol diacetate	EDA	297-76-7
		Etonogestrel	ENG	54048-10-1
		Gestodene	GES	60282-87-3
		Levonorgestrel	LNG	797-63-7
		Norelgestromin	NGM	53016-31-2
		Norethindrone acetate	NEA	38673-38-0
		Norethindrone	NET	68-22-4
		Norgestimate	NTE	35189-28-7
		Norgestrel	NGT	6533-00-2
	Spironolactone	Drospirenone	DRO	67392-87-4

In human medicine and healthcare, P4 and synthetic progestins are used in e.g. contraceptive pills, hormone replacement therapy and cancer treatment. In contraceptive pills, progestins are used either singly in so-called minipills, or in combination with an estrogen, e.g. EE_2. In combination pills, the progestins are chiefly responsible for the contraceptive effect by inhibiting ovulation and inducing a cervical mucus plug (Erkkola and Landgren, 2005). In contraceptive medicines, the progestin content is usually 3-100 times higher than the estrogen content, depending on the formulation. Consequently, human consumption and environmental emission of progestins are generally substantially higher than the consumption and emission of estrogens. Progestins are also used as growth promoters in livestock, and in certain countries like China, animal excretion contributes substantially to the environmental load of progestins (Zhang et al., 2014). Natural excretion of P4 by women and livestock is also an important source for P4 in the environment (Fent, 2015, Kumar et al., 2015).

Unlike estrogens, less than half of all progestins have been monitored in aquatic environments. Nevertheless, the ones that have been analyzed for have almost always been detected, and progestins are likely wide-spread aquatic contaminants. However, that relatively few measurements that have been conducted makes it difficult to assess whether the reports are from especially contaminated sites or if they are representative of the general level of contamination. The technical difficulty in analyzing the trace concentrations of progestins likely also contributes to the highly variable concentrations reported. Generally, progestin levels are highly dependent on what type of water that is sampled. The very highest concentrations are reported in animal farm flush water, and associated runoff and lagoons in China and the U.S. Measurements have been made in such waters of CPA, DGS, MGA, MNA, NGT and P4 concentrations ranging from a few to often hundreds or thousands of ng L^{-1} (Bartelt-Hunt et al., 2012, Liu et al., 2012a, Liu et al., 2012b, Liu et al., 2014, Yost et al., 2014). Lower concentrations are reported in WWTP influents and effluents. As WWTP influent water comes mainly from municipal sewage systems, it is mainly P4 and progestins used in contraception which are monitored and detected. In WWTP influent water, DGS, LNG, MEP, MPA NET and P4 have been measured in concentrations ranging from a few to more than a hundred ng L^{-1} (Kuch and Ballschmiter, 2001, Kolodziej et al., 2003, Vulliet et al., 2008, Chang et al., 2011, Liu et al., 2014). In effluent water, concentrations are generally an order of magnitude lower, but NET has been reported to reach as high as 188 ng L^{-1} (Al-Odaini et al., 2010). In surface waters, which are most relevant from an ecotoxicological point of view, the reported progestin concentrations are surprisingly similar to WWTP effluent concentrations. In rivers, streams and ground water, DGS, LNG, MGA, MPA, NET, NGT and P4 are detected at concentrations of typically a few ng L^{-1} (Vulliet et al., 2008, Chang et al., 2011, Vulliet and Cren-Olivé, 2011, Liu et al., 2012b, Liu et al.,

2014). There are, however, a few reports of substantially higher concentrations, several tens of ng L^{-1}, in rivers in China (Chang et al., 2009), Malaysia (Al-Odaini et al., 2010) and France (Vulliet and Cren-Olivé, 2011).

Being steroids, progestins are hydrophobic compounds (Log K_{OW} values between 3.1 and 5.4 (Kumar et al., 2015)) and thus have the potential for bioconcentration. Based on the log K_{OW} values, the predicted fish plasma bioconcentration factors (BCFs) for most progestins are in the range of 7-1756 (Kumar et al., 2015). Plasma BCFs have been measured for NET and MPA to 10.6 and 8.9, respectively (Nallani et al., 2012, Steele et al., 2013). An exceedingly high measured plasma BCF of 12000 was reported for LNG, which has a predicted plasma BCF of 46 (Fick et al., 2010). Further studies are however needed to confirm such a level of bioconcentration.

There is an increasing volume of literature on endocrine-disrupting effects of progestin exposure, in several species of fish and amphibians. The reported effects are many and include, e.g. decreased sex hormone levels, transcriptional alterations, impaired oocyte development and development of male secondary sex characters in females (Kvarnryd et al., 2011, Fent, 2015, Kumar et al., 2015) . Most attention has, however, been given to the effect which is the intended therapeutic effect of progestins in women, i.e. decreased fecundity. Decreased fecundity in fish has been reported to follow from exposure to DES, DGS, DRO, GES, LNG, MGA, MPA, NET and P4 (Zeilinger et al., 2009, Paulos et al., 2010, DeQuattro et al., 2012, Runnalls et al., 2013, Han et al., 2014, Zhao et al., 2015). The decrease in fecundity is arguably the most ecologically relevant effect, as it directly translates into a decrease in fitness.

The mechanisms underlying the reproductive effects of progestins in fish are incompletely understood, but the general consensus is that they are mainly mediated by PR activation (Zeilinger et al., 2009, Paulos et al., 2010, Runnalls et al., 2013, Overturf et al., 2015). It is apparent from previous studies that allow direct comparison, i.e. the fecundity studies, that the concentrations required to cause reduced fecundity in fish differ substantially between different progestins, ranging from < 1 ng to several µg L^{-1}. Considering the concentrations of progestins measured in surface waters, this indicates that certain progestins should be of much higher environmental concern than other. The progestins which cause decreased fecundity at concentrations comparable to environmental levels are LNG, NET and GES (Zeilinger et al., 2009, Paulos et al., 2010, Runnalls et al., 2013). In accordance with the PR hypothesis, these progestins all strongly bind to and activate human nuclear PRs (Philibert et al., 1999, Runnalls et al., 2013). However, these three progestins are also the ones that, out of all progestins, most strongly bind to and active human ARs (Philibert et al., 1999, Runnalls et al., 2013). Furthermore, both non-aromatiz-

able (e.g. 17β-trenbolone, Tb; and dihydrotestosterone, DHT) and aromatizable (e.g. methyltestosterone, MT) androgens have effects in fish highly similar to those caused by progestins (Ankley et al., 2001, Ankley et al., 2003, Kang et al., 2008, Margiotta-Casaluci and Sumpter, 2011). These relationships constitute the rationale for the overall hypothesis of this thesis: that the androgenic properties of certain progestins significantly contribute to their reproductive effects in fish. This was investigated in the four experimental studies of this thesis, using the three-spined stickleback and zebrafish models.

Three-spined stickleback (*Gasterosteus aculeatus*)

Living in fresh, estuarine and coastal waters in temperate regions, the three-spined stickleback is one of the most common fish species in the northern hemisphere. This small species is widely used in biological research and its behavior, ecology and evolution have been extensively studied. Sticklebacks are iteroparous seasonal breeders and have a marked and well-characterized reproductive cycle. In early summer, the male stickleback develops secondary sexual characters such as conspicuous breeding colors with a red belly and bright blue irises. At the same time, the kidney of the male hypertrophies and produces a sticky glycoprotein named spiggin (Jones et al., 2001). Using spiggin as a glue, the male builds a nest from filamentous plant material. The male courts a female which deposits her eggs inside the nest, after which the male fertilizes them. Following fertilization, the male chases away the female and proceeds to guard the eggs and fan water over them until hatching. The breeding status of male sticklebacks is governed by plasma androgen levels which peak during the breeding season (Mayer et al., 1990), induced by long photoperiods and a subsequent increase of gonadotropin secretion (Hellqvist et al., 2006). Unlike in most vertebrates, spermatogenesis in sticklebacks is inhibited by androgens, and is thus quiescent throughout the breeding period (Borg, 1982, Hellqvist et al., 2006). Instead spermatogenesis takes place immediately after the breeding season as a result of the post-breeding drop in plasma androgen levels, which also causes regression of secondary sexual characteristics and cessation of spiggin production (Borg, 1982, Hellqvist et al., 2006). The stickleback is uniquely useful in ecotoxicology as it is the only fish species which possesses quantifiable biomarkers for both androgenic and estrogenic effects. Sticklebacks are easily maintained in laboratory facilities, and their full genome is sequenced and available at www.ensemble.org.

The spiggin biomarker

Spiggin induction is the only known quantitative, molecular biomarker for androgens in fish. Spiggin production is considered to be governed directly and

specifically by AR activation, and is efficiently blocked by the model AR antagonist flutamide (Katsiadaki et al., 2002, Jolly et al., 2006, Katsiadaki et al., 2006). Female sticklebacks do not produce spiggin under normal conditions but its production is readily induced by exposure to exogenous androgens, serving as the androgenic equivalent to the estrogen biomarker vitellogenin, described below. Spiggin production can be measured indirectly by measuring kidney hypertrophy, which occurs naturally when the kidney transforms from a metabolic and excretory organ to a secretory gland. Kidney hypertrophy is measured as relative kidney weight and as the height of the proximal tubule epithelium, the site of spiggin synthesis. The spiggin protein can be measured using enzyme-linked immunosorbent assay (ELISA) (Katsiadaki et al., 2002) and the transcript using quantitative real-time PCR (qPCR) analysis (Hogan et al., 2008). Based on previous studies it appears as though measurement of *spiggin* gene transcripts is a more sensitive method than measurement of the actual protein (Katsiadaki et al., 2002, Hogan et al., 2008). Furthermore, *spiggin* transcript levels seem to correspond well to spiggin protein levels as both basal spiggin mRNA and protein levels are 5 to 6 orders of magnitude higher in males than in females (Katsiadaki et al., 2006, Hogan et al., 2008). In this thesis, the spiggin biomarker was therefore measured as *spiggin* transcript levels, complemented by kidney morphology and/or kidney histology.

The vitellogenin biomarker

Vitellogenin is a plasma protein produced in the liver of all oviparous species and is the major precursor of yolk proteins in the developing oocyte, for which it is essential for normal development. Vitellogenin production is regulated mainly by plasma estrogens like 17β-estradiol (E_2). The *vitellogenin* genes are present in both males and females, but are normally only expressed in mature females, which have high enough plasma E_2 levels. However, males also produce vitellogenin if exposed to estrogenic substances. Thus, vitellogenin expression in male fish is a widely used biomarker for exposure to estrogenic substances (Sumpter and Jobling, 1995). Conversely, inhibition of vitellogenin in female fish serves as a biomarker for anti-estrogenic effects, which are known to occur following exposure to non-aromatizable androgens (Shilling and Williams, 2000, Ankley et al., 2003, Miracle et al., 2006).

CYP1A

Cytochrome P450 1A (CYP1A) is a subfamily of the CYP superfamily of monooxygenase enzymes. CYP1As catalyze oxidations of a plethora of endogenous and exogenous substances and are highly involved in, e.g. lipid, steroid and fatty acid metabolism, as well as in the metabolism of many xenobiotic compounds (Zeldin and Seubert, 2007). CYP1As are regulated by the aryl hydrocarbon receptor (AhR) and their catalytic activity level and tissue

expression are widely used as biomarkers for exposure to AhR agonists in fish, birds and mammals. CYP1A is highly expressed in the proximal tubule epithelium of the kidney (Stegeman et al., 1991, Abrahamson et al., 2007), the same tissue where spiggin is produced in sticklebacks. As CYP1A is not directly regulated by the AR, a change in *CYP1A* transcripts could be indicative of a change in kidney function.

LH-β

In teleost fish, reproduction is governed by the hypothalamic-pituitary-gonadal (HPG) axis. Production of sex steroids and gonadal development are governed by the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are secreted by the pituitary. The secretion of gonadotropins is in its turn governed by gonadotropin-releasing hormone (GnRH), released from the hypothalamus. The HPG axis is regulated by feedback from sex hormones which can either inhibit (negative feedback) or induce (positive feedback) secretion of GnRH and gonadotropins (Shao et al., 2013). LH is chiefly responsible for controlling sex hormone production in the gonads of both sexes, and transcript levels of the β subunit of the LH peptide (LH-β) in the pituitary in males are indicative of the plasma androgen levels and/or their regulation (Shao et al., 2013).

Zebrafish (*Danio rerio*)

The small cyprinid zebrafish is a tropical fresh-water fish found naturally in streams and rivers in the Himalayan region of India, Pakistan, Nepal and Bangladesh. Although initially used as a popular aquarium species, the zebrafish has now become one of the most important vertebrate model species in genetics, developmental biology and biomedicine. The whole zebrafish genome is sequenced and is available at www.zfin.org. Zebrafish possess a number of attributes which make them highly suitable for scientific experimentation (Hutchinson et al., 2006). Their small size and robustness make them very easy to maintain in large numbers in laboratory facilities. They are continuous breeders, with females spawning several hundreds of eggs every 2-3 days, and the generation time is only 3-4 months (Spence et al., 2008). Zebrafish development is rapid and well characterized, making the species particularly suitable for developmental studies.

Sex differentiation

In gonochoristic fish, sex differentiation can be described as the molecular and cellular processes which cause a bipotential gonad primordium to develop into

testes in males, and ovaries in females, including the development of primordial germ cells into spermatogonia and oogonia, respectively (Devlin and Nagahama, 2002). In zebrafish, sex differentiation occurs between about 20 and 35 days post fertilization (dpf) (Chen and Ge, 2012, 2013). The process takes longer to be completed in males, up to 45 dpf, than in females (Chen and Ge, 2013). This is likely due to the fact that zebrafish exhibit juvenile hermaphroditism, where both genotypic males and females first develop ovary-like gonads (Uchida et al., 2002). Testicular differentiation consequently requires additional processes, to remove oocyte-like cells by apoptosis (Uchida et al., 2002). Sex differentiation is governed by a network of genes, most of which are relatively conserved across vertebrate species (Leet et al., 2011). Two of the genes that are highly upregulated during testis differentiation in zebrafish are *anti-Müllerian hormone* (*amh*) and *CYP11B* (Wang and Orban, 2007). Expression of *amh* is located to Sertoli cells, but its function in fish is not fully understood. *CYP11B* is expressed mainly in Leydig cells and the enzyme is required for synthesis of 11-ketotestosterone, the main endogenous fish androgen. Gonadal aromatase, *CYP19a1a*, is highly expressed during ovarian differentiation in zebrafish and is responsible for the conversion of testosterone to E_2 (Wang and Orban, 2007).

In many fish species, sex differentiation is relatively plastic and can be influenced by external factors like temperature, pH and social interactions (Leet et al., 2011). This is likely a reason why early development, including sex differentiation, is one of the periods most sensitive to effects of endocrine disrupting chemicals (EDCs). There are many published studies showing that exposure to EDCs during sex differentiation can skew the sex ratio in fish, where exposure to estrogens or anti-androgens causes female bias (Antonopoulou et al., 1995, Nimrod and Benson, 1998, Parrott and Blunt, 2005, Örn et al., 2006) and exposure to androgens causes male bias (Örn et al., 2006, Morthorst et al., 2010, Baumann et al., 2013).

Puberty

After sex differentiation, animals stay sexually immature for different time periods, depending on species. It is not until the process of puberty completes, that an animal for the first time acquires the capability for reproducing. During puberty, germs cells in males and females mature into fully functional sperm and eggs, respectively. Puberty initiates as a consequence of activation of the HPG axis, and is marked by increased pituitary expression and circulating levels of FSH and LH (Chen and Ge, 2012, 2013). The time of puberty onset is different in different species, and is often reflective of life histories. Long-lived fish like salmonids often do not reach puberty until one to two years of age (Chen and Ge, 2013). In zebrafish, with its short life cycle, puberty initiates at around 45 dpf in females, and around 53-55 dpf in males (Chen and

Ge, 2012, 2013). However, the timing of puberty shows considerable plasticity and is highly influenced by external factors like food availability, salinity, temperature and photoperiod (Chen and Ge, 2013, Melo et al., 2014). It has also been shown that treatment with androgenic steroids and aromatase inhibitors can advance sexual maturation in fish, both with regard to gonadotropin production and gonad maturation (Antonopoulou et al., 1995, Melo et al., 2015).

Objectives

The overall objective of this thesis was to investigate the *in vivo* androgenic effects of certain progestins in fish, and how these effects might contribute to the reproductive impairment of aquatic organisms. The specific aims of this thesis were to:

- Acquire a quantitative measurement of the *in vivo* androgenic effect of LNG in fish, using the female stickleback model (**paper I**).

- Compare the androgenic and anti-estrogenic potencies of LNG and NET with those of other well-known androgenic pollutants (**paper II**).

- Investigate whether LNG could disrupt the highly androgen-dependent seasonal breeding cycle of male sticklebacks (**paper III**).

- Compare the effects of an androgenic progestin (LNG) and a non-androgenic progestin (P4) on sexual development of zebrafish (**paper IV**).

Materials and methods

Animals

The sticklebacks in **papers I-III** were caught in the harbors of Skanör and Trelleborg on the southwestern coast of Sweden. The fish in **paper I** and **II** were maintained at the Evolutionary Biology Centre, Uppsala, Sweden. The fish in **paper III** were maintained at the Department of Zoology, Stockholm University. During maintenance, the fish in both papers were held under short photoperiod in flow-through water tanks. Two months prior to the experiment, the male fish in **paper III** were put in long photoperiod, inducing their maturation into breeding condition. After two months, approximately the length of a natural breeding period, the fish were brought to the Department of Environmental Toxicology, Uppsala University, and were put in short photoperiod. The zebrafish (AB wild type strain) in **paper IV** were obtained as fertilized eggs from the SciLife Lab zebrafish facility at the Evolutionary Biology Centre, Uppsala, Sweden. All studies were approved by the Uppsala Ethical Committee on Animal Research.

Experiments

In **paper I**, female sticklebacks were exposed to LNG for 21 days after which effects on spiggin biomarkers and *vitellogenin* transcript levels were evaluated. In **paper II**, female sticklebacks were exposed to either LNG, NET, MT, Tb or androstenedione (A4). For each compound, the androgenic and anti-estrogenic potency was evaluated and compared using spiggin biomarkers and *vitellogenin* transcript levels, respectively. In **paper III**, males were exposed to LNG for 45 days during their transition from breeding to non-breeding condition. After exposure, breeding status was evaluated based on breeding coloration, spiggin production and spermatogenic activity, and was compared to the breeding status of initial controls representing males still in breeding condition. In **paper IV**, zebrafish larvae were exposed to either LNG or P4 from 20 to 80 dpf. Effects on sex differentiation and puberty were evaluated using histological analyses and gonadotropin gene transcript levels at different times during the exposure. All exposures were aqueous in Uppsala tap water and were conducted in plastic aquaria (Ferplast, Castelgomberto, Italy). Exposure

renewal was semi-static, with 50% of the water volume being replaced daily. Exposure concentrations and experimental setups are summarized in Table 2.

Table 2. *Overview of the exposure scenarios in papers I-IV*

Paper	Experimental animal	Exposure length	Compound(s)	Concentrations (ng L^{-1})
I	Adult female stickle-backs	21 days	LNG	5.5, 40 and 358
II	Adult female stickle-backs	21 days	LNG	35, 125 and 1292
			NET	39, 169 and 1582
			MT	32, 131 and 1634
			Tb	19, 102 and 809
			A4	33, 138 and 1403
III	Adult male stickle-backs	45 days	LNG	6.5, 65 and 750
IV	Zebrafish larvae	60 days (20-80 dpf)	LNG	5.5, 79 and 834
			P4	3.7, 77 and 1122

Chemical analysis

Water samples were taken for chemical analysis in all aquaria at least once a week during all experiments. In short, the water concentrations of LNG, NET, P4, MT, Tb and A4 were measured using an in-line solid phase extraction column coupled to liquid chromatography-tandem mass spectrometry. For detailed descriptions of the chemical analyses, see **paper I** and **III**. The mean recoveries of LNG in **papers I**, **III** and **IV** were similar, and ranged between 36 and 83%. The mean recoveries of P4 in **paper IV** ranged between 37 and 112%. In **paper II**, however, the measured concentrations were markedly higher than the nominal, with mean recoveries for LNG, NET, MT, Tb, and A4 ranging between 190 and 390%. The reasons for this are unknown. The mean measured concentrations were used in the analysis and presentation of all results.

Gross morphology and histology

Relative stickleback kidney weight in **papers I-III** was expressed as nephrosomatic index (NSI), calculated as: (kidney weight/body weight) × 100. Male breeding coloration in **paper III** was classified as present if the fish had coincident blue irises and red belly. Stickleback kidneys (**paper I** and **III**) and testes (**paper III**), and zebrafish trunk sections (**paper IV**), were fixed in phosphate buffered formaldehyde (4%, Histolab Products AB, Gothenburg, Sweden), dehydrated in increasing concentrations of ethanol and embedded in hydroxyethyl methacrylate (Leica Historesin, Heidelberg, Germany). Kidney sections were stained with hematoxylin-eosin (HE) or periodic acid/Schiff, which strongly stains carbohydrate-rich molecules (e.g. spiggin). Testis sections and trunk sections were stained with toluidine blue. Measurement of the KEH was carried out in HE-stained sections using ImageJ software (Rasband, W.S., ImageJ, U.S. National Institute of Health, Bethesda, Maryland, USA); for full description, see **paper I**. In **paper III**, presence of spermatogenic activity in the testes was evaluated across whole testis sections. The testes were classified as either quiescent or active. The gonads in the zebrafish trunk sections were classified as male, female, intersex or undifferentiated; maturational stage of the gonads were assessed using a numerical staging system (Johnson et al., 2009) based on relative proportions of cells at different stages of gametogenesis (see **paper IV**).

Quantitative real-time PCR

The relative transcript levels of *spiggin* (**papers I-III**), *vitellogenin* (**paper I** and **II**), *CYP1A* (**paper I**) and *LHβ* (**paper III**) in stickleback, and *amh*, *CYP11B*, *CYP19a1a*, *LHβ* and *FSHβ* in zebrafish (**paper IV**), were measured using real-time qPCR analysis. Total RNA was isolated using the Aurum Total RNA Fatty and Fibrous Tissue kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in **paper I**, and the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA, USA) in **papers II-IV**. RNA purity and quantity were determined spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and quality was verified by agarose gel electrophoresis. Reverse transcription in all papers was conducted using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc.), except for pituitary samples in **paper IV**, where reverse transcription was made using random primers, dNTPs and SuperScript III Reverse Transcriptase (Invitrogen, USA). All qPCR analyses were conducted on a Rotor-gene 6000 DNA amplification system (Qiagen, Hilden, Germany) using the iQ SYBR Green Supermix kit (Bio-Rad Laboratories, Inc.); the only exception was in **paper IV**, where qPCR of pituitary samples was conducted on an Mx3000 real-time PCR machine (Stratagene, La Jolla, CA, USA) using the Brilliant III Ultra-fast SYBR

Green QPCR Master Mix (Agilent Technologies, Stratagene). PCR reaction efficiencies (E) were calculated from standard curves of serial dilutions of pooled cDNA (**paper I, II** and **IV**) or using the LinRegPCR software (Ruijter et al., 2009) (**paper III**). Relative transcript levels in all papers were calculated using the Pfaffl method (Pfaffl, 2001), employing *ubiquitin* (**paper I** and **II**) and *elongation factor 1 α* (*EF1-α*, **paper IV**) as reference genes. In **paper III** no stable reference gene could be found in the kidney samples, whereby the transcript levels of *spiggin* and *CYP1A* were normalized only to total RNA input. Transcript levels in these samples were calculated as E^{-Ct}. Transcript levels of pituitary genes in **paper IV** were calculated from the standard curve of each gene using the MxPro software version 4.10 (Stratagene, La Jolla, CA, USA) using *40S ribosomal RNA* (*40S*) as reference gene.

Statistics

All graphical illustrations and statistical analyses were made using GraphPad Prism version 5.01 (GraphPad Software Inc., CA, USA). Normality and homoscedasticity were tested for using Shapiro-Wilk and Bartlett's tests, respectively. If needed, data were log transformed prior to parametric analyses. Differences in NSI (**papers I-III**), KEH (**paper I** and **III**), mean transcript levels (**papers I-IV**) and gonadal maturity distribution (**paper IV**) were tested for using one-way ANOVA (**paper I** and **III**) or Kruskal-Wallis analysis of variance (**paper II** and **IV**), followed by t-tests corrected using the sequential Bonferroni-Holm method (Holm, 1979) (**paper I**), Mann-Whitney U tests corrected using the sequential Benjamini-Hochberg method (Benjamini and Hochberg, 1995) (**paper II**), Tukey's multiple comparison test (**paper III**) or Dunn's post hoc test (**paper IV**). Differences in frequencies of spermatogenic status (**paper III**) and sex ratio (**paper IV**) were compared using the Chi-square test, followed by pair-wise comparisons using Fisher's exact test, whose significance levels were Bonferroni-Holm (**paper III**) or Benjamini-Hochberg (**paper IV**) corrected. Differences were considered significant if $p \leq 0.05$, unless Bonferroni-Holm or Benjamini-Hochberg corrections were used. Quantitative data are presented as mean ± standard error of the mean (SEM), unless otherwise stated.

Results

Paper I

The androgenic and anti-estrogenic effects of LNG were analyzed in adult female sticklebacks, after 21 days of exposure. There was no effect of LNG at the lowest concentration of 5.5 ng L^{-1}. At 40 and 358 ng L^{-1}, however, there was massive induction of *spiggin* transcripts, with mean levels being ~ 150 000 and 250 000 times higher than in the control group, respectively. This was concurrent with pronounced kidney hypertrophy in both these groups. The transcript levels of *CYP1A* in the kidneys were reciprocal to those of *spiggin*, being lower in the groups exposed to 40 and 358 ng L^{-1}. *Vitellogenin* transcript levels in the liver were decreased by LNG exposure at 40 and 358 ng L^{-1}, with mean levels being ~ 9 and 142 times lower than in the controls in the groups, respectively.

Paper II

The androgenic and anti-estrogenic potencies of the progestins LNG and NET were compared to those of the well-known androgenic pollutants MT, Tb and A4 in female sticklebacks, after 21 days of exposure. Exposure to LNG caused an increase in mean *spiggin* transcript levels at 125 and 1292 ng L^{-1}, as did NET at 169 and 1582 ng L^{-1} and MT at 131 and 1634 ng L^{-1}. The mean *spiggin* transcript levels were not affected by exposure to Tb or A4 at any of the tested concentrations. *Vitellogenin* transcript levels were reduced by NET and MT at all concentrations tested, while by LNG at 125 and 1292 ng L^{-1} and by A4 at 138 and 1403 ng L^{-1}. The mean transcript levels of *vitellogenin* were not affected by Tb at any of the tested concentrations.

Paper III

The effect of LNG on the seasonal breeding cycle of adult male sticklebacks was investigated in fish that were exposed during a period when transition from breeding condition into non-breeding condition normally occurs. Before start of exposure, visual inspection confirmed that most fish were in breeding

condition, as indicated by the prevalence of breeding coloration. This was confirmed by histological examination of the kidneys and testes in fish that were sampled at the start of the experiment (initial controls). The initial controls had high levels of *spiggin* transcripts in the kidneys, which were markedly hypertrophied. Pituitary *LHβ* transcript levels were high and spermatogenesis was quiescent, the testes being filled with only mature spermatozoa. After six weeks in short photoperiod, the unexposed controls had, as expected, transitioned into the non-breeding condition as shown by loss of breeding coloration, background *spiggin* transcript levels, normal kidney size and histology, lowered *LHβ* transcript levels as well as active spermatogenesis in the testes, which were filled with cysts of spermatogonia, spermatocytes and spermatids.

In the groups that were exposed to LNG concurrently with short photoperiod, the transition into non-breeding condition was impaired. In the group exposed to the lowest concentration tested, 6.5 ng L^{-1}, KEH was maintained higher than in the control group. In the LNG exposures at 65 and 750 ng L^{-1}, the fish were similar to the initial controls in terms of all variables studied. What this essentially means, is that LNG at ≥ 65 ng L^{-1} maintained the fish in full breeding condition when they should have transitioned into the non-breeding condition. As in **paper I**, kidney *CYP1A* transcript levels were reciprocal to *spiggin* transcript levels.

Paper IV

To investigate the developmental effects of progestin exposure, zebrafish larvae were exposed to either LNG or P4 for 60 days during the critical developmental period of sex differentiation and puberty. The control fish displayed normal sexual development, with sex ratios and pubertal timing as expected at all examined time points. Exposure to LNG caused all fish to develop into males, at all concentrations tested. P4, by contrast, did not affect the sex ratio at any of the concentrations tested. Transcript levels of *amh*, *CYP11B* and *CYP19a1a* measured at different time points, showed that the masculinizing effect by LNG was initiated very rapidly, as it was clearly visible after only 3 days of exposure.

In the control fish, pituitary transcription of the gonadotropin genes *FSHβ* and *LHβ* in the pituitary was still low at 44 dpf, but had increased by 55 dpf, indicating that puberty had been initiated. In fish exposed to LNG and P4, gonadotropin transcript levels at 44 dpf were nearly as high as in the control fish at 80 dpf. The effects on gonadotropin transcript levels were corroborated by the histological staging of gonad maturity, which showed that at all exposure concentrations of LNG, the testes of nearly all males were fully mature at 50 dpf. P4 did also cause an increase in testis maturity at concentrations ≥ 77 ng L^{-1},

although the effect was not as pronounced as that by LNG. P4 did not have any effect on ovarian maturity. Taken together, the early increase in gonadotropin expression and the advanced testicular maturity show that both LNG and P4 caused precocious puberty in male fish, although LNG was much more potent.

Discussion

This thesis has shown that the 19-nortestosterone-derived progestins LNG and NET have strong endocrine-disrupting effects in both adult and developing fish. The effects seen were most likely mediated via AR activation. Spiggin production is well-characterized to be unambiguously governed by AR activation (Katsiadaki et al., 2002, Jolly et al., 2006, Katsiadaki et al., 2006). There are numerous previous studies where model androgens (Borg, 1981, Andersson et al., 1988, Katsiadaki et al., 2002, Allen et al., 2008, Hogan et al., 2008) and aromatase inhibitors (Bornestaf et al., 1997) cause similar effects in sticklebacks as LNG and NET in **paper I-III**. It has previously been shown that P4 does not induce spiggin production, neither *in vitro* (Jolly et al., 2006) nor *in vivo* (Jones et al., 2001, Nagae et al., 2007). Furthermore, plasma levels of DHP are constantly low in male sticklebacks (Mayer et al., 1990). This is in stark contrast compared with plasma levels of endogenous androgens, which fluctuate extensively over the year, co-varying with the physiological changes during the breeding cycle. This indicates that DHP, and therefore PR activation, plays at most a minor role in the seasonal male reproductive cycle in this species, which is a further indication that the effects of LNG on the male stickleback breeding cycle in **paper III** were due to AR activation. It is well-known that exposure to non-aromatizable androgens can decrease vitellogenin production in female fish like in **papers I** and **II** (Shilling and Williams, 2000, Ankley et al., 2003, Ankley et al., 2004, Jensen et al., 2006, Miracle et al., 2006). There are indications that this suppression is due to lowered plasma E_2 levels as a consequence of AR activation in the pituitary (negative feedback) (Ankley et al., 2003, Ankley et al., 2004). Decreased plasma sex steroid levels is a common effect of exogenous androgen exposure (Ankley et al., 2003, Jensen et al., 2006, Margiotta-Casaluci and Sumpter, 2011), and has been shown after exposure of female fish to the androgenic progestins GES, LNG and NET (Paulos et al., 2010, Runnalls et al., 2013) but not the non-androgenic DES and DRO (Runnalls et al., 2013). Consequently, it seems likely that LNG in **paper I** and **II** and NET in **paper II** reduced the expression of *vitellogenin* transcripts by causing decreased plasma concentrations of E_2. The effect of LNG on sex differentiation in **paper IV** is also most certainly AR-mediated, as P4 had no effect on the sex ratio, even at a concentration of > 1 µg L^{-1}. Several previous studies have shown that AR agonists cause male-biased sex ratios in fish exposed during early development (Örn et al., 2003, Holbech et al., 2006, Morthorst et al., 2010, Baumann et al.,

2013). Furthermore, a recent study similar to that in **paper IV** showed that skewing of sex ratios toward more males by LNG exposure was counteracted by co-exposure to flutamide (Hua et al., 2015). The potent induction of precocious puberty in zebrafish by LNG in **paper IV** conforms to previous studies where AR agonists caused accelerated gonadal maturation, particularly in male fish (Örn et al., 2003, Morthorst et al., 2010, Baumann et al., 2013, Melo et al., 2015). Since P4 is essentially devoid of any affinity for ARs (Schindler et al., 2008), it seems likely that its effect on male puberty, although relatively weak, could be mediated via PR activation. It can therefore not be excluded that the effect of LNG on the timing of puberty could be due to PR- as well as AR activation, as LNG has strong binding affinities for both mammalian PRs and ARs (Schindler et al., 2008). However, as discussed below, LNG does apparently not interact with fish PRs.

There are several previous *in vitro* studies which provide corroborating evidence that the effects of progestins in the present thesis likely were AR-mediated. Neither CMA nor NET was able to displace radiolabeled DHP from spotted seatrout (*Cynoscion nebolusus*) membrane PRs (Thomas and Das, 1997). In the same species, the binding affinity to nuclear PRs, relative to DHP, was less than 0.5% for CMA, CPA, MGA, NET and NGT (Pinter and Thomas, 1997). Fathead minnow (*Pimephales promelas*) nuclear PRs were slightly activated by P4 and DRO, but not at all by ENG, GES, LNG, NET, MNA, MPA and NGA (Ellestad et al., 2014). However, the 19-nortestosterone-derived ENG, NET, LNG and GES strongly activated fathead minnow ARs, and were at least as potent as DHT (Ellestad et al., 2014). The 17α-hydroxyprogesterone-derived MNA and MPA did not activate fathead minnow ARs, and P4 and DRO did so only weakly (Ellestad et al., 2014). In a similar study, neither ENG, LNG, GES, NET nor NTE activated Murray-Darling rainbowfish (*Melanotaenia fluviatilis*) nuclear PRs, and P4 and DRO did so only weakly (Bain et al., 2015). However, again the androgenic ENG, LNG, GES, NET and NTE activated ARs more strongly and potently than DHT, while P4 and DRO had no activating effect (Bain et al., 2015). In summary, what these studies indicate is that, in spite of being strong agonists of human PRs, P4 and synthetic progestins interact with fish PRs only very weakly. Furthermore, the androgenic 19-nortestosterone derived progestins, which are the very strongest human PR agonists (Schindler et al., 2008), do not appear to activate fish PRs at all. They also appear to be even stronger AR agonists in fish than in humans.

The agonism of fish ARs was confirmed in **paper II**, where LNG and NET caused induction of *spiggin* transcripts and reduction of *vitellogenin* transcripts in female sticklebacks, at levels similar to effect levels of the known androgenic contaminant MT, and at levels much lower than the effect levels of A4 and Tb. Previous findings show that the model androgen DHT also appears to be much less potent than LNG and NET, when it comes to spiggin

induction (Katsiadaki et al., 2002). Thus this thesis not only shows that the 19-nortestosterone-derived progestins act as androgens in fish, but that they are highly potent androgens as well. Considering their androgenic potency and their frequent global use and contamination of inland waters, the 19-nortestosterone-derived progestins may well be the most significant of the few androgenic contaminants identified thus far.

Several of the effects of progestin exposure presented in this thesis can be argued to be adverse, with the potential of affecting the structure and viability of wild fish populations, should they occur in nature. While there is no conclusive evidence of adverse effects consequent to spiggin induction in female sticklebacks, there is one report of severe kidney inflammation in female sticklebacks exposed to MT for 2 months, and that this kidney inflammation was the cause of increased mortality in the MT-exposed fish (Hahlbeck et al., 2004). Chronic production of spiggin in females, a process which normally occurs in males for only about three months each year, would likely at least be energetically costly (Chellappa et al., 1989, FitzGerald et al., 1989). During natural spiggin production in male sticklebacks the normal functions of the kidney, e.g. excretion and osmoregulation, largely cease. It has been shown that androgen-induced transformation of the proximal tubular epithelium into a glandular, spiggin-secreting state leads to markedly reduced ion reuptake and excess water retention (de Ruiter and Mein, 1982, Ruiter et al., 1985). The reciprocal reduction of *CYP1A* following spiggin induction in **paper I** and **III** likely reflects this downregulation of normal kidney function. In breeding-state males and androgen-treated females, there is increased intestinal fluid production and an upregulation of ion- and water-transporting membrane structures in enterocytes (Ruiter et al., 1985), suggesting that the intestine at least partly takes over osmoregulation in spiggin-producing sticklebacks. The long-term efficiency of this normally temporary process is not known.

Suppression of *vitellogenin* transcript levels as shown in **paper I** and **II** is more likely than spiggin induction to lead to ecologically relevant adverse effects. There is a correlation between suppressed vitellogenin levels in female fish and decreased fecundity (Miller et al., 2007). Importantly, this correlation holds irrespective of the causative mechanism (Miller et al., 2007), indicating that impaired vitellogenesis *per se* leads to decreased fecundity. Reduced fecundity is a highly ecologically relevant effect, as it can directly translate into a reduction in fitness. Cumulative fecundity is considered vital to overall population trajectories (Kramer et al., 2011), and there are both predictive modeling (Miller and Ankley, 2004) and empirical (Kidd et al., 2007) evidences that decreased fecundity in fathead minnows caused by exposure to androgenic or estrogenic steroids leads to population declines. It must be noted, however, that the impact of an incomplete decrease in fecundity on population status

might differ substantially between species with different life histories and reproductive strategies (Wester and Vos, 2003).

Of possible ecological relevance is also the finding in **paper III**, that exposure to LNG inhibited the natural transition of male sticklebacks from the breeding into the non-breeding condition. Should this occur in nature, it could have a direct negative impact on the fitness of sticklebacks. This would be largely due to the complete inhibition of spermatogenesis but also to the high energetic cost (Chellappa et al., 1989, FitzGerald et al., 1989) and increased risk of predation (Whoriskey and Fitzgerald, 1985, Magnhagen, 1991) that are consequential to breeding condition, and likely reasons for the high mortality rates observed in post-breeding male sticklebacks. The three-spined stickleback is one of the most common zooplanktivorous fish in the northern hemisphere and population declines could have considerable effects on the structure of aquatic food webs (Lefébure, 2012). Disruption of the reproductive cycle in a similar way as reported for stickleback in this thesis would likely also adversely affect other aquatic organisms with seasonal breeding cycles, as the timing of spawning is essential for reproductive success (Rowe and Hutchings, 2003).

Skewed sex ratios, especially of the magnitude shown after LNG exposure in **paper IV**, clearly have the potential to affect the structure of wild fish populations. Furthermore, male-biased sex ratios may have a higher potential for affecting population structures and viability than female-biased sex ratios, as the reproductive potential of many fish communities is mainly dependent on the number of females available for egg production (Ospina-Alvarez and Piferrer, 2008). Interestingly, in spite of the many reports of skewed sex ratios in fish exposed to EDCs in the laboratory, there are surprisingly few reports of this from field surveys (Mills and Chichester, 2005).

Negative effects of precocious puberty in farmed fish are well-known from aquaculture. These include reduced somatic growth, immunosuppression and induction of agonistic or spawning behavior at inappropriate times (Taranger et al., 2010). It is plausible that precocious puberty could adversely affect wild fish populations as well. As previously stated, the timing of puberty in fish is highly sensitive to environmental conditions (Chen and Ge, 2013, Melo et al., 2014). This is likely because reproductive success depends on reproductive behaviors being temporally synchronized with the proper external environment (Rowe and Hutchings, 2003). Consequently, as reproductive behaviors are directly dependent on sexual maturity (Söffker and Tyler, 2012), precocious puberty can lead to reproductive behaviors at unfavorable environmental conditions. The ecological importance of this effect will likely differ among species with different life histories and reproductive strategies. For example, in fish species with short generation times and only a few reproductive events,

the timing of puberty might be of greater importance than in long-lived species with multiple reproductive cycles. Another aspect of possible concern is the fact that only the male zebrafish in **paper IV** were affected by precocious puberty. In many fish species, reproduction follows a temporal pattern and is sequential and synchronized between the sexes (Söffker and Tyler, 2012). In such species, differences in the timing of puberty between the sexes could therefore further impair reproductive success. Precocious puberty induced by xenobiotics, out of sync with both the environment and between the sexes, could therefore conceivably entail serious fitness costs, should it occur in nature.

This thesis showed that LNG affected all endpoints investigated, with some of the effects occurring at concentrations as low as 6.5 (**paper III**) and 5.5 (**paper IV**) ng L^{-1}. NET also affected all endpoints investigated, with the lowest effective concentration being 39 ng L^{-1} (**paper II**). This means that the effects of LNG occurred within the range of reported surface water concentrations of 3.6 to 38 ng L^{-1} (Fent, 2015). As the range of reported surface water concentrations of NET is 1 to 7.5 ng L^{-1} (Fent, 2015), the reported effective concentration of 39 ng L^{-1} is higher than environmental concentrations. However, as this concentration was the lowest tested, it is possible that effects would occur at even lower concentrations. The only effect of P4 in this thesis was on male zebrafish puberty, which occurred at concentrations ≥ 77 ng L^{-1} (**paper IV**). As the range of P4 levels measured in surface waters is 0.4 to 17.8 ng L^{-1} (Fent, 2015), the effect of P4 in this thesis occurred at higher than environmental concentrations. The results of this thesis are therefore in concordance with the previous studies, that it is the 19-nortestosterone-derived progestins that have effects in fish at environmentally relevant concentrations, and pose a threat to wild fish populations living in progestin-contaminated waters. However, still very little is known about the typical levels of progestin contamination in rivers, lakes and streams. Much more data is needed to determine whether the reported levels of progestins represent general or especially contaminated situations.

The origin of the overall hypothesis of this thesis was the conspicuous relationship between impairment of fish fecundity by certain progestins and their binding to mammalian ARs. The androgenic, 19-nortestosterone-derived progestins GES, LNG and NET had lowest observed effect concentrations (LOECs) for reduced fecundity at around 1 ng L^{-1} (Zeilinger et al., 2009, Paulos et al., 2010, Runnalls et al., 2013). The non-androgenic DGS, DRO, MGA and P4 had LOECs for reduced fecundity of 1263, 6500, 606 and 100 ng L^{-1}, respectively (Zeilinger et al., 2009, DeQuattro et al., 2012, Han et al., 2014, Zhao et al., 2015); concentrations far higher than those expected to occur in the aquatic environments. The non-androgenic MPA did not affect fecundity even at the highest tested concentration of 342 ng L^{-1} (Zhao et al.,

2015). The only exception to the pattern is the 19-nortestosterone-derived DES, of which was required 10000 ng L^{-1} to reduce fecundity (Runnalls et al., 2013). Furthermore, DES lacked a masculinizing effect on females (Runnalls et al., 2013). DES is an inactive prodrug, which in humans is converted to the androgenic ENG (Schindler et al., 2008). As ENG is highly similar to GES and LNG in fish *in vitro* systems with regard to PR and AR activation (Ellestad et al., 2014, Bain et al., 2015), the extremely high concentration needed to reduce fecundity and the absence of female masculinization suggest that the activation of DES into ENG is very minor, if not absent, in fish. The evidence of the *in vivo* androgenic activity of progestins presented in this thesis, especially together with the aforementioned *in vitro* data (Ellestad et al., 2014, Bain et al., 2015), corroborates the contention that the difference seen in effective concentrations on fish fecundity is due to the androgenic effect of the 19-nortestosterone-derived progestins. There is a proposed Adverse Outcome Pathway (AOP) named "Androgen receptor agonism leading to reproductive dysfunction" (available at https://aopwiki.org) approved by the OECD Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST). This AOP is valid for adult female fish and non-aromatizable androgens, like the 19-nortestosterone-derived progestins. The outline of the AOP is as follows: AR agonism → reduced gonadotropin production in the pituitary (negative feedback) → reduced testosterone production in theca cells → reduced E_2 production in granulosa cells → reduced plasma E_2 levels → reduced vitellogenin production in hepatocytes → reduced plasma vitellogenin levels → impaired oocyte development → reduced fecundity. The results of this thesis and several previous studies provide evidence that 19-nortestosterone-derived progestins can cause most of these events: AR agonism (**papers I-IV**) (Ellestad et al., 2014, Bain et al., 2015), reduced testosterone production (Runnalls et al., 2013), reduced E_2 levels (Paulos et al., 2010, Runnalls et al., 2013), reduced vitellogenin production (**papers I-II**) and reduced fecundity (Zeilinger et al., 2009, Paulos et al., 2010, Runnalls et al., 2013). Thus, it is hereby proposed that androgenic progestins decrease egg production in fish via this AOP.

Conclusions and perspectives

In conclusion, this thesis provides solid *in vivo* evidence that certain progestins act as androgens in fish. **Papers I-II** showed that the 19-nortestosterone-derived LNG and NET induced production of the androgenic biomarker spiggin and suppressed expression of the estrogenic biomarker vitellogenin in female sticklebacks. Furthermore, **paper II** indicates that the androgenic potencies of LNG and NET rank among the very highest of all known environmental androgens. **Paper III** showed that LNG exposure functionally maintained male sticklebacks in breeding condition when they should have transitioned into the non-breeding state. In **paper IV**, exposure of zebrafish larvae to LNG or P4 during sexual development showed that LNG, but not P4, caused all-male populations to develop. Both progestins, but only LNG at low concentrations, caused precocious puberty in males.

Suppressed vitellogenesis in females, prolongation of the breeding condition in males, male-biased sex ratios and precocious puberty in larvae are all to be considered as adverse effects with the potential for affecting the structure of fish populations. Most of the effects occurred within the range of reported environmental concentrations, indicating that the androgenic potencies of certain progestins may be high enough to cause adverse androgenic effects in wild fish living in progestin-contaminated waters.

Progestins were initially considered as one coherent group of environmental contaminants, defined according to medical literature as agonists to human and mammalian PRs. However, this thesis and recent literature strongly suggest that when considering progestins in ecotoxicology, their capacity as androgens might be of much greater importance and relevance than their capacity as progestagens. Simply put, nearly all available research indicates that when it comes to ecologically relevant effects in fish, like reduced fecundity and skewed sex ratios, it is the androgenic 19-nortestosterone-derived progestins that are of environmental concern. This observation provides a highly useful identification of the androgenic testosterone-derived progestins as constituting the environmental risk, while the P4- and spironolactone-derived progestins may be considered more “safe” for the environment. The development of new progestins (i.e. of the fourth generation) for contraception is highly focused on PR specificity, to reduce androgenic side effects in women. The identification of androgenic progestins as reproductive and developmental

toxicants in fish provides a further incentive for substitution of the androgenic first- and second-generation progestins with new, non-androgenic progestins in human medicine.

Considering the extensive global use of progestins, and their frequent detection in the environment, there is a dire need for further studies of their ecotoxicological effects. For most of the progestins in use, there is a complete lack of ecotoxicological data. Obviously, more progestins need to be investigated, first and foremost the ones that are highly consumed and are found in the environment. Most studies so far have used short-term exposures. More realistic exposure scenarios are also needed, e.g. multi-generational exposures. The identification of progestins as a new class of environmental androgens raises the question what effects a realistic simultaneous exposure of fish to androgens and estrogens would have. As many contraceptive pharmaceuticals are combinations of progestins and EE_2, it is important to assess the combined effects of these highly potent steroidal hormones in fish. For example, EE_2 is known to induce female-biased sex ratios at a concentration similar to that of LNG inducing a male-biased sex ratio in **paper IV**. Would the net effect of combined exposure to, e.g., LNG and EE_2 be estrogenic, androgenic, or would wholly new effects appear? Of absolute essence is that further analyses of the environmental levels of progestins in water, sediments and biota are conducted, to enable environmental risk assessment of this group of contaminants. For most progestins, the level of environmental contamination is completely unknown. Environmental analyses need to be expanded to include more compounds, especially the newer progestins, e.g. DRO, which have not been analyzed for at all. Proper environmental risk assessments of progestins should be highly prioritized considering the severity of the effects in aquatic organisms shown in many laboratory studies, including those presented in this thesis.

Swedish summary/svensk sammanfattning

Den omfattande användningen av läkemedel och den dåliga förmågan hos vattenreningsverk att rena bort många läkemedelssubstanser har lett till att man runt om i världen hittar dessa substanser i akvatiska miljöer. Kort sagt, läkemedel har blivit globala akvatiska föroreningar. Stor oro finns nu för hur detta kan komma att påverka akvatiska organismer och deras ekosystem. En av de mest använda grupperna av läkemedel är p-piller. De vanligaste, och i högst koncentration förekommande ingredienserna i p-piller är progestiner, vilka är syntetiska varianter av gulkroppshormonet progesteron (P4). Progestiner hittas i vattendrag i hela världen och experimentella studier har visat att progestiner kan påverka fortplantningsförmågan hos fisk och groddjur redan vid de låga halter som uppmätts i naturen. Den allvarligaste effekten av progestiner som tidigare visats i fisk är minskad äggläggning. Alla tjugotalet progestiner som används har samma effekter i människokroppen som progesteron har, men de vars kemiska struktur är baserad på det manliga könshormonet testosteron, har även androgena bieffekter. Av de progestiner som tidigare visats ge minskad äggläggning hos fisk är det just de med androgena effekter som påverkar äggläggningen vid de låga koncentrationer av progestiner som påträffas i naturen. Syftet med det arbete som presenteras i denna avhandling var att karaktärisera den androgena effekten av vissa progestiner i fisk.

För att studera detta usattes både honor och hanar av storspigg (*Gasterosteus aculeatus*) via vattnet för de androgena progestinerna levonorgestrel (LNG) och noretisteron (NET). Under fortplantningssäsongen producerar storspiggshanar ett klibbigt protein i njuren. Detta protein kallas spiggin, och används som ett klister när hanarna bygger sitt bo av döda växtdelar. Produktionen av spiggin i njuren styrs helt av halten androgener i blodet, vilken ökar markant under fortplantningssäsongen. Storspiggshonor producerar i normala fall aldrig spiggin, eftersom deras androgenhalter i blodet är för låga. Spigginproduktion kan dock induceras i honor om de utsätts för androgener via det omgivande vattnet. På så sätt fungerar spigginproduktion i storspiggshonor som en så kallad biomarkör för exponering för androgena substanser. För att undersöka effekter av progestiner på könsutvecklingen hos fisk, utsattes yngel av zebrafisk (*Danio rerio*) för det androgena LNG eller P4, som inte är androgent, via vattnet under den del av den tidiga utvecklingen då könsdifferentiering och pubertet infaller.

Spigghonor som utsattes för de androgena progestinerna LNG och NET började producera stora mängder spiggin i njuren och uppvisade en minskad produktion av ägguleproteinet vitellogenin i levern. Produktionen av vitellogenin är avgörande för äggens normala utveckling i fisk. Jämförelse med välkända androgena miljögifter som till exempel metyltestosteron och trenbolon visade att LNG och NET, med avseende på spiggin-induktion och vitellogenin-sänkning, är bland de mest potenta androgener man känner till. Spigghanar utsattes för LNG i slutet av en lekperiod och resultaten visade att LNG förhindrade den normala förlusten av lekfärger och spigginproduktion samt blockerade återupptagandet av spermieproduktionen. LNG bibehöll helt enkelt spigghanarna i reproduktivt stadium när de skulle återgått till sin normala fysiologi utanför fortplantningssäsongen. Exponering av zebrafiskyngel för LNG gjorde att alla fiskar utvecklades till hanar. Dessa hanar uppvisade dessutom kraftigt påskyndad mognad av testiklarna, och kom i puberteten snabbare än normalt. P4 hade ingen effekt på könskvoten, men vid högre koncentrationer så hade även P4 en svag påskyndande effekt på könsmognaden hos hanar.

Minskad vitellogeninproduktion och störning av den hanliga fortplantningscykeln är allvarliga effekter som skulle kunna påverka storspiggar och andra fiskar i naturen mycket negativt. En skev könskvot, speciellt av den magnitud som orsakades av LNG, utgör en uppenbar fara för vilda fiskpopulationer. För tidig pubertet kan också påverka fisk negativt, då det är av stor vikt för fortplantningens framgång att parningsbeteende sammanfaller med gynnsamma yttre förhållanden. Huvuddelen av effekterna av androgena progestiner i denna avhandling uppkom vid halter som ligger inom det spann av progestinhalter som uppmätts i vattenmiljön, vilket ger en signal om att dessa effekter kan uppkomma i progestinkontaminerade vatten.

Sammanfattningsvis visar resultaten i denna avhandling att de progestinerna som har en androgen bieffekt är kraftfulla androgena miljögifter som kan utgöra ett hot mot vilda fiskpopulationer som lever i progestinkontaminerade vatten. De ger också starkt stöd till hypotesen att störningen av fiskars fortplantning orsakad av progestiner till en betydande del medieras via substansernas androgena egenskaper, och att det är de testosteronbaserade progestinerna som utgör ett hot mot fiskars utveckling och reproduktion. De allvarliga reproduktionsstörande effekter av progestiner hos fisk som visats i många studier, inklusive de i denna avhandling, belyser det skriande behovet av analys av progestiner i olika vatten, för att kunna bedöma hur stort hot dessa läkemedel utgör för den akvatiska miljön.

Acknowledgements

The work in this thesis was carried out at the Department of Environmental Toxicology, Evolutionary Biology Centre, Uppsala University, Sweden. Financial support was provided by the Swedish Foundation for Strategic Environmental Research (Mistra), through the MistraPharma research programme.

Many thanks to **Björn Brunström** and **Ingvar Brandt**, for excellent supervision, guidance and inspiration throughout this project.

Thank you **Jerker Fick**, Umeå University, for your indispensable chemical analyses.

Thank you **Margareta Mattson** for your excellent and tireless work with all histological sections.

Thank you **Monika Schmitz** for fun and exciting collaboration, and for improving my knowledge of fish physiology.

Thank you **Bertil Borg**, Stockholm University, for kindly providing sticklebacks, organizing fishing trips and general stickleback advice.

Thank you also everyone else who has worked on or otherwise helped me during this work: **Anna Mentor**, **Arshi Mustafa**, **Erika Jansson**, **Gaëtan Philippot** and **Mattias Hogvall**.

A final thanks to everyone at the Department of Environmental Toxicology, for a positive and helpful research environment, and for making the last five years a great time!

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