Review

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A review of studies on androgen and estrogen exposure in fish early life stages: effects on gene and hormonal control of sexual differentiation

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ABSTRACT: Teleost fish are unique among vertebrates in that phenotypic sex or onset of sex inversion can be easily manipulated by hormonal treatments. In recent years, researchers have begun reporting concentrations of synthetic and natural hormones in the environment. Although concentrations are very low (in the parts per trillion to low parts per billion), they are still of concern because of the high potency of synthetic hormones and the enhanced susceptibility of teleost fishes, especially early life stages, to hormonal exposures. In this review, we will focus on sex differentiation in teleost fishes and how these processes in fish early life stages may be impacted by environmental hormones which are known to contaminate aquatic environments. We will start by reviewing information on sources and concentrations of hormones in the environment and continue by summarizing the state of knowledge of sex differentiation in teleost gonochoristic fishes, including information on genes involved (e.g. *cyp19*, *dmrt1*, *sox9* and *foxl2*). We will end our review with a summary of studies that have examined the effects of androgens and estrogens on fish sex differentiation after exposure of fish embryos and larvae and with ideas for future research. **Copyright © 2011 John Wiley & Sons, Ltd.**

Keywords: sex differentiation; sex determination; teleosts; endocrine disrupting chemicals; hormones; gene expression; dmrt1; cyp19; sox9; foxl2

INTRODUCTION

There is growing concern about hormones in the environment. This stems from the fact that both natural and synthetic hormones have been detected in aquatic environments. Main sources of hormones are runoff from lands in which animal manure has been applied as a fertilizer and discharge of treated municipal wastewater into bodies of water. Even at low concentrations (parts per trillion to low parts per billion) synthetic estrogens and androgens can have high potency to teleost fishes, especially in sensitive early life stages. Fish have been used as model organisms to study the effects of androgen and estrogen exposure; however, much of this research has been conducted in adult fish. There is much less literature available on the effects of these hormones on fish at early life stages, especially during periods of enhanced sensitivity such as sex determination and differentiation.

The purpose of this review is to determine what is currently known about the effects of environmental hormones on early life stage fishes, including potential sources of hormones and modes of hormone action. We begin the review with an overview of the state of hormones in the environment and their potential sources, including human and animal sources. We continue with an overview of the current state of knowledge of sex determination and sex differentiation in teleost gonachorist (unisexual) fishes, as these processes can be greatly influenced by environmental hormones. We then present a summary of studies that have examined the effects of androgens and estrogens on fish sex determination and differentiation after exposure of fish embryos and larvae, and conclude with ideas for future research.

NATURAL AND SYNTHETIC HORMONES IN THE ENVIRONMENT

Human Sources

A summary with information regarding steroidal hormones reviewed in this article is presented in Tables 1 and 2. One of the major sources of hormones to the environment is effluent from sewage wastewater treatment plants (SWWTP). Women excrete a range of natural estrogens, with pregnant, premenopausal and postmenopausal women excreting 3115, 6.7 and 3.6 μg 17β-estradiol (17β-E_2) equivalence per day, respectively (Liu *et al.*, 2009). Men excrete testosterone (T), dihydrotestosterone, androstenedione and androsterone at rates of 81 μg T equivalence

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Table 1. List of steroidal estrogens and androgens that have been detected in the environment. See Table 2 for specific sources and environmental concentrations reported

Hormone	Class	CAS no.	Chemical structures[a]	Log K_{ow}/MW
Estrogens				
17*α*-Ethinylestradiol	Semisynthetic alkylated estradiol estrogen/human use (contraceptive)	57-63-6		4.12/296
17*β*-Estradiol	Reproductive hormone/all vertebrate animals	50-28-2		3.94/272
Estrone	An aromatized C18 steroid/ converted from androstendione directly or from testosterone via estradiol/all vertebrate animals	53-16-7		3.43/270
Estriol	Hydroxylated metabolite of estradiol or estrone/all vertebrate animals	50-27-1		2.81/288
Androgens				
17*β*-Trenbolone	Metabolite of trenbolone acetate, an anabolic synthetic steroid used to promote growth in animals	10161-33-8		2.35^{b}/270.37
17*α*-Trenbolone	Metabolite of 17*β*-trenbolone	10161-33-8		2.35^{b}/270
Testosterone	Androgenic steroid/can be further converted to dihydrotestosterone or estradiol/all vertebrate animals	58-22-0		3.3/288
Dihydrotestosterone	Metabolite of testosterone/ cannot be aromatized to estradiol (considered a 'pure' androgenic steroid)/ all vertebrate animals	521-18-6		NA/290[c]

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Table 1. *(Continued)*

Hormone	Class	CAS no.	Chemical structures[a]	Log K_{ow}/MW
Androstendione	Androgenic steroid/precursor of testosterone, estrone, or estradiol/all vertebrate animals	63-05-8		NA/286
Androsterone	Metabolite of testosterone or androstenedione all vertebrate animals	53-41-8		NA/290

[a]From the National Center for Biotechnology Information (NCBI) PubChem Compound; http://www.ncbi.nlm.nih.gov
[b]From Lee *et al.* (2007).
[c]NA, not available.

per day (Liu *et al.*, 2009). Although studies have shown that aqueous phase removal efficiencies in treatment plants can be as high as 99%, effluents may still contain environmentally unsafe hormone concentrations (Chimchirian *et al.*, 2007). The results obtained by Chimchirian *et al.* (2007) suggest that the treatment processes that employ suspended growth media (e.g. activated sludge) are more effective at removing estrogens from influent than static biofilms (e.g. packed beds). Liu *et al.* (2009) conducted a review of estrogens and androgens detected in SWWTP influent and effluent in several countries. Their review found that the USA had the greatest effluent concentrations of androstenedione (up to 7720 ng l^{-1}), T concentrations similar to those in Canada (up to 20 ng l^{-1}) and estrogen concentrations as high as 49, 20 and 140 ng l^{-1} for estrone (E_1), 17β-E_2 and estriol (E_3), respectively. Healthcare facilities can contribute significant hormone loads to SWWTP due to patient excretion and pharmaceutical disposal. Nagarnaik *et al.* (2010) found that a hospital generated hormone mass loadings of 92 mg per day, with androgens constituting more than 65% of the total load. The potential estrogenicity of the hospital wastewater effluent was found to be 130 ng l^{-1} as 17β-E_2. Chimchirian *et al.* (2007) reported significantly higher 17β-E_2 concentrations in influent to a SWWTP receiving hospital wastewater compared with two others only receiving municipal wastewater; however, the samples were not analyzed for androgens.

In addition to the contribution of hormones to the environment from treated sewage, hormones also are released to the environment from untreated sewage by combined sewer overflow (CSO) events and the application of biosolids to agricultural fields. Pailler *et al.* (2009) investigated the contribution of CSO events to hormone loads in a stream in Germany by sampling downstream of nine CSO locations and a SWWTP. They collected stream samples over hydrographs during 11 flood events and calculated 17α-ethinylestradiol (EE_2), 17β-E_2 and E_1 loads of up to 122, 78, and 274 mg per storm event, respectively. The average estrogen concentrations observed in the stream during these events were *ca* 5–6 ng l^{-1} each for EE_2, 17β-E_2 and E_1. Langdon *et al.* (2010) reviewed the literature to identify ranges of concentrations of chemicals in biosolids. The estrogens detected in biosolids were EE_2, 17β-E_2 and E_1 with ranges of 0.42–17, 0.31–49 and nondetected–150 µg kg^{-1}, and means of 4.01, 13.5 and 10.9 µg kg^{-1}, respectively. In 1998, the US Environmental Protection Agency (USEPA) estimated that 2.8 million dry tons of biosolids were land applied, and estimated that, by 2010, as much as 8.2 million dry tons could be applied (USEPA, 1999). Therefore, in 2010, an average of 0.23 tons of estrogens were potentially introduced into the environment from biosolids applications. The predicted maximum runoff concentrations from soils amended with biosolids were estimated to be 0.01, 0.03 and 0.24 µg l^{-1}, respectively, with E_1 expected to be the most mobile and EE_2 the least mobile.

Animal Sources

Owing to the growing number of concentrated animal feeding operations (CAFOs) in the USA, manure is increasingly being viewed as a potential threat to nearby aquatic ecosystems. Each year, over 100 billion pounds of manure are produced in the USA, with the majority being land-applied (USEPA, 2000). This manure contains both natural and synthetic hormones, which are inadvertently introduced into the environment when manure is applied to agricultural fields as a nutrient source. Lange *et al.* (2002) estimated that, in the USA, the annual production of manure contains over 50 tons of natural androgenic and estrogenic hormones, with nearly all of the estrogens excreted by pregnant cattle and about half of the androgens coming from cattle (calves and bulls) and chickens (broilers and laying hens). Synthetic hormone excretion is primarily from the cattle industry, as the US Food and Drug Administration has approved the use of five steroids in implants (17β-E_2, progesterone, T propionate, trenbolone acetate and zeranol), of which one or two are used in each implant (Kolok and Sellin 2008).

The presence of hormones detected in surface waters impacted by CAFOs has been reviewed by Lee *et al.* (2007) and Kolok and Sellin (2008), and is summarized here (see Table 2). Irwin *et al.* (2001) sampled water from ponds on cattle farms known to contain fish and turtle populations. All four ponds located on the farm contained average 17β-E_2

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Table 2. Hormone concentrations in surface water and their sources

Compound	Average and Standard Deviations[a] (ng l^{-1})	Source	References
Estrogens			
17α-Ethinylestradiol	28.6 (3.1)	SWWTP	Duong *et al.* (2010)
	6 (—)	CSO/SWWTP	Pailler *et al.* (2009)
	4.3 (0.6)	SWWTP	Duong *et al.* (2010)
	0.04 (—)	SWWTP	Baronti *et al.* (2000)
17β-Estradiol	84.3 (117)	CAFO*	Chen *et al.* (2010)
	5 (1)	CSO/SWWTP	Pailler *et al.* (2009)
	3.0 (NR)	SWWTP	Borch *et al.* (2009)
	1.8 (0.13)	Cattle feedlot	Irwin *et al.* (2001)
	1.7 (0.3)[b]	SWWTP	Kolok *et al.* (2007)
	0.52 (NR)	Dairy cattle and ewes	Matthiessen *et al.* (2006)
	0.4 (NR)	SWWTP	Borch *et al.* (2009)
	0.29 (0.15)	Cattle feedlot	Irwin *et al.* (2001)
	0.11 (—)	SWWTP	Baronti *et al.* (2000)
Estrone	393 (452)	CAFO*	Chen *et al.* (2010)
	46.4 (53.8)	CAFO*	Chen *et al.* (2010)
	22.6 (NR)	SWWTP	Borch *et al.* (2009)
	9.31 (NR)	Dairy cattle and ewes	Matthiessen *et al.* (2006)
	7.4 (2.2)[b]	SWWTP	Kolok *et al.* (2007)
	6 (5)	CSO/SWWTP	Pailler *et al.* (2009)
	1.7 (NR)	Cattle feedlot	Soto *et al.* (2004)
	1.5 (—)	SWWTP	Baronti *et al.* (2000)
	1.4 (NR)	SWWTP	Borch *et al.* (2009)
	0.10 (NR)	Pregnant beef cattle	Matthiessen *et al.* (2006)
Estriol	82.5 (69.6)	CAFO*	Chen *et al.* (2010)
	11.1 (NR)	SWWTP	Borch *et al.* (2009)
	2.7 (52.5)	CAFO*	Chen *et al.* (2010)
	2.4 (NR)	SWWTP	Borch *et al.* (2009)
	0.8 (NR)	SWWTP	Borch *et al.* (2009)
	0.33 (—)	SWWTP	Baronti *et al.* (2000)
Androgens			
17β-Trenbolone	7 (NR)	Cattle feedlot	Durhan *et al.* (2006)
	5 (NR)	Cattle feedlot	Durhan *et al.* (2006)
	0.002 (NR)	Cattle feedlot	Soto *et al.* (2004)
	0.001 (NR)	Cattle feedlot	Soto *et al.* (2004)
17α-Trenbolone	50 (NR)	Cattle feedlot	Durhan *et al.* (2006)
	5 (NR)	Cattle feedlot	Durhan *et al.* (2006)
	0.035 (NR)	Cattle feedlot	Soto *et al.* (2004)
	0.005 (NR)	Cattle feedlot	Soto *et al.* (2004)
Testosterone	1.8 (1.7)[b]	Cattle feedlot	Kolok *et al.* (2007)
	0.9 (0.3)[b]	SWWTP	Kolok *et al.* (2007)
	0.6 (0.3)[b]	Cattle feedlot	Kolok *et al.* (2007)
Dihydrotestosterone	3 (NR)[c]	SWWTP	Thomas *et al.* (2002)
	9 (NR)[c]	SWWTP	Thomas *et al.* (2002)
Androstenedione	21 (0.8)[b]	Cattle feedlot	Kolok *et al.* (2007)
	5.3 (0.4)[b]	SWWTP	Kolok *et al.* (2007)
	3.7 (NR)	SWWTP	Borch *et al.* (2009)
	3.5 (2.2)[b]	Cattle feedlot	Kolok *et al.* (2007)
	1.9 (NR)	SWWTP	Borch *et al.* (2009)
	0.2 (NR)	SWWTP	Borch *et al.* (2009)
Androsterone	1.7 (NR)	SWWTP	Borch *et al.* (2009)

[a]Samples with concentrations > limit of quantification.
[b]Units are nanograms collected by a polar organic chemical integrative sampler.
[c]Androgenic activity reported as dihydrotestoterone equivalents.
(—) No standard deviation due to only one sample having concentrations > limit of quantification.
CAFO*, combination of broiler chickens, hens, pigs, and cattle. NR, standard deviation not reported.
CSO, combined sewer overflow.
SWWTP, sewage waste water treatment plan.

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concentrations higher than those of a control site in nearby botanical gardens (i.e. not impacted by cattle). Soto *et al.* (2004) collected samples from a retention pond and two stream locations impacted by cattle feedlots and analyzed them for various androgens and estrogens. Their results showed that natural androgens were responsible for 98.9–99.9% of the androgenic activity in the samples, and that the concentrations decreased with increasing downstream distance from cattle feedlots. Estrogenic activities at the two most upstream sampling sites were found to be high enough to negatively impact target cells in aquatic organisms. Estrone was found to account for 46% of the total estrogenic activity, and the authors hypothesized that most of the remaining estrogenic activity could be attributed to pesticides and other compounds not analyzed in the study, but known to be estrogen-mimicking compounds (Soto *et al.*, 2004).

Many environmental studies that have been conducted to date, such as those described above, examined the contribution of one particular type of manure to the release of hormones into the environment. However, the more realistic scenario involves the presence of different CAFO types (cattle, pig and poultry). Chen *et al.* (2010) sampled eight locations along a stream impacted by a CAFO containing broiler chickens, laying hens, cattle and pigs. They found that, although estrogen concentrations generally decreased with increasing downstream distance from the CAFO, 17β-E_2 equivalents were higher than 10 ng l^{-1} at all sample locations, suggesting that their presence could cause observable effects on aquatic organisms such as fish. These authors also found higher estrogen concentrations during the winter than the spring, probably attributed to lower microbial activity and lower dilution effects.

Gall *et al.* (2011) analyzed approximately 2800 samples collected in 2009 from four tile drain and three agricultural ditch monitoring stations in north central Indiana, approximately 15 km northwest of Lafayette, IN (USA). Details regarding the sampling methodologies are given in Gall *et al.* (2010). The fields received various types of manure applications including swine, poultry, dairy and beef effluent, dairy solids and subsurface injections of swine manure. Hormones were detected in over 80% of the samples, with E_1 being the most frequently detected hormone at all monitoring stations. Synthetic androgens (17β-trenbolone, 17α-trenbolone and trendione) were detected in approximately 10% of samples. Fields treated primarily with poultry effluent experienced the highest tile drain concentrations of E_1 and 17β-E_2, while fields treated primarily with dairy and beef effluent had the highest concentrations of 17α-E_2. In contrast to Chen *et al.* (2010), stream estrogen concentrations were highest in the spring and summer, probably due to the timing of lagoon effluent applications. This research suggests that tile drains play an important role in the export of hormones from tile-drained agricultural fields to nearby surface waters.

Comparison Between Human and Animal Sources

Table 2 provides a summary of the concentrations of hormones observed in surface waters impacted by various sources of hormones. The concentrations range widely, with the highest observed concentrations originating from CAFOs (Chen *et al.*, 2010), and other studies showing similar hormone concentrations originating from CAFOs and SWWTPs (e.g. Baronti *et al.*, 2000; Kolok *et al.*, 2007; Soto *et al.*, 2004). The potent synthetic estrogen EE_2 is associated with human origins, as it is a compound used in human birth control pills. Other synthetic hormones, such as trenbolone (α and β), are associated with livestock origins, as they are metabolites of trenbolone acetate, a synthetic growth-promoting androgen used in cattle implants.

The relative contribution of humans vs livestock to the presence of hormones in surface waters is still largely unknown, although it has been estimated that the application of animal manure contributes over 200 times more estrogens to the environment than human biosolids (Lange *et al.*, 2002; USEPA 1999). However, the contribution from humans is probably more constant than that from livestock, as more factors control the environmental fate and transport of manure-borne hormones than those of human origin. The major factors controlling the input of hormones of human origin into the environment are the occurrence of CSO events and the release of wastewater treatment effluent. The factors controlling the input of manure-borne hormones into the environment are not yet well understood. Laboratory studies have shown that sorption and degradation processes are relatively quick (on the order of hours or days), suggesting that the mobility of hormones in the environment is limited (Lucas and Jones, 2006; Xuan *et al.*, 2008; Ying and Kookana, 2005). However, field studies have shown that hormones can leach from agricultural fields for as long as 11 months after manure is applied (Kjær *et al.*, 2007). Clearly, more field studies are needed that specifically address the environmental factors controlling the fate and transport of manure-borne hormones.

In summary, the input of hormones into the environment from both human and animal sources can occur at concentrations high enough to pose a threat to sensitive aquatic organisms. Environmental concentrations detected have ranged over several orders of magnitude and varied with source type and over time. Many studies have reported large standard deviations, further indicating large ranges in hormone concentration. The timing of human activities, including applying biosolids and manure to agricultural fields and discharging hormones to surface water bodies from both treated and untreated wastewater, probably influences the temporal variability of hormone concentrations. However, seasonal variability of rainfall and temperature also affects hormone concentrations and therefore confounds the ability to discern biogeochemical, hydrologic, and human controls. Understanding the role of these processes in the behavior of hormones in the environment will better allow us to predict both short- and long-term impacts to sensitive aquatic ecosystems.

A BRIEF REVIEW OF SEX DETERMINATION AND SEX DIFFERENTIATION IN FISHES

Sex determination and sex differentiation are related terms but refer to different processes. Sex determination is defined as the genetic or environmental forces that determine whether a fish will become a male or a female, whereas sex differentiation refers to molecular and cellular processes that make a bipotential gonadal primordium develop into a testis or ovary after sex has been determined (Devlin and Nagahama, 2002). In fish, both processes are known to be genetically and environmentally driven. Both of these factors influencing sex determination in fish allow for sex manipulation and sex reversal potential that is

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not possible in mammals and birds. Indeed, sex determination in teleosts is extremely plastic with most of the karyotyped species not having sex chromosomes (Devlin and Nagahama, 2002).Further, with close to 25 000 different fish species, almost all forms of genetic sex determination have been described in this vertebrate group, ranging from dominant monogenic to polygenic or having a sex chromosome control, with heterogametic males in some species (XX/XY) or heterogametic females (ZZ/ZW) in others (for a review see Devlin and Nagahama, 2002). Members of this class of vertebrates also exhibit a wide range of sexuality patterns, from hermaphroditism to gonochorism.

In mammals the Y chromosome gene *sry* (sex-determining region on the Y chromosome) is considered the 'male-inducing master' gene (Sinclair *et al.*, 1990). It is located on the Y chromosome and the absence of this region results in an XX female. This gene belongs to a family of genes that has homology with the High Mobility Group (HMG) box domain genes, or *sox* genes. During testicular development in the mouse, *sry* initiates the differentiation of supporting cell precursors into Sertoli cells (Koopman *et al.*, 1991). In teleosts, the only species known to have a similar sex determination mechanism is the Japanese medaka (*Oryzias latipes*). In medaka, the *dmy* gene is also present on the Y chromosome (a homolog of *dmrt1*, a transcription factor involved in male development in vertebrates; see more about this gene below). This has been identified as the first 'master male sex-determining' gene in a teleost fish (Matsuda *et al.*, 2002).

Temperature, pH and social interactions are known to influence phenotypic sex in several fish species. The first study that discovered sex determination was under the control of both genotype and temperature was published close to 30 years ago in Atlantic silverside (*Menidia minidia*; Conover and Kynard, 1981). Japanese flounder (*Paralichthys olivaceus*) and Nile tilapia (*Oreochromis niloticus*) are additional examples of teleosts that exhibit temperature sex determination (TSD) with genetic females (XX) being sex-reversed to phenotypic males when larvae are reared at high water temperatures (D'Cotta *et al.*, 2001; Kitano *et al.*, 2000). The underlying mechanism of TSD has been related to a suppression of aromatase (*cyp19*) expression at male-promoting temperatures resulting in masculinization, and an increased expression in ovaries at feminizing temperatures (Baroiller and D'Cotta, 2001; Karube *et al.*, 2007). This further supports the importance of this enzyme and its product (estrogen) in ovarian differentiation in teleosts (see more on this gene below).

Socially controlled sex changes can be considered as one of the most extreme examples of environmental sex determination (Godwin, 2009). This can be seen in several families of coral reef fishes in which removal of the large socially dominant males induces sex change in the largest females (Godwin, 2009). The size-advantage hypothesis has been used to understand sex change in teleosts. It predicts that sex change is favored when an individual reproduces most efficiently as one sex when young or small, or as the opposite sex when old or large (Warner, 1975). In addition to the size of an individual relative to that of others within a social group, other factors have also been shown to influence the timing of sex change, including sex ratio of the group and local density (Munday *et al.*, 2006).

Genes Involved in Sex Determination and Sex Differentiation

In mammals, the sex differentiation cascade results from a complex interplay between a large number of cell types and network of genes, and it occurs during a relatively narrow window of time. Although the sex specificity and the timing of expression for some of these genes differ between species, most are well conserved and exhibit a characteristic expression during the period of sex differentiation (Baron *et al.*, 2005). Recent data produced over the last 10 years or so have supported the notion that in teleost fishes several genes that are involved in these processes are also well conserved across families regardless of the sex-determination mechanism involved (Alfaqih *et al.*, 2009, also see Fig. 1). As will be seen in more detail below, these genes belong to several families including transcription factors, germ cell proteins, apoptosis regulators, steroidogenic enzymes, hormones and growth factors as well as their receptors.

Cyp19 (cytochrome P450 19)

Cyp19 encodes for a steroidogenic enzyme, aromatase, which is responsible for catalyzing the aromatization of androgens to estrogens. This is a key gene in ovarian differentiation in all species of teleosts examined (Kitano *et al.*, 1999). Its role in ovarian differentiation has been demonstrated in several studies that have treated fish larvae or fry with *cyp19* inhibitors, resulting in the suppression of estrogen biosynthesis and induction of sex-reversal of genetic females to phenotypic males, which is comparable to what happens when fish in early life stages are treated with androgens (Fenske and Segner 2004; Guiguen *et al.*, 1999; Kitano *et al.*, 2000; Kobayashi *et al.*, 2003; Kwon *et al.*, 2000; Piferrer *et al.*, 1994).

Two isoforms of this gene have been identified in teleosts: *cyp19b*, mostly expressed in the brain, and *cyp19a*, its gonadal counterpart (Blázquez and Piferrer, 2004; Callard and Tchoudakova, 1997; Chang *et al.*, 2005; Chiang *et al.*, 2001a, b; Liu *et al.*, 2007; Tchoudakova and Callard, 1998; Tong *et al.*, 2001; Trant *et al.*, 2001). Of all species examined to date, teleosts are remarkable for their extremely high levels of neural *cyp19b* expression which can reach orders of magnitude higher levels (100- to 1000-fold) compared with mammals (Callard *et al.*, 2001). Even within teleosts, *cyp19b* activity is close to 10 times higher compared with the ovarian isoform. Unlike *cyp19a* mRNA which is predominantly expressed in ovaries, *cyp19b* mRNA is highly expressed in brains of both males and females during early sex differentiation and into adulthood without showing clear sexual dimorphism (Fenske and Segner, 2004; Kwon *et al.*, 2001).

In terms of temporal patterns of expression, *cyp19b* is transcribed much earlier during embryo development compared with *cyp19a*. For instance in zebrafish, *Danio rerio*, *cyp19b* is one of the earliest genes transcribed during development (<7 hpf, hours post-fertilization); *cyp19a* expression is detected later, but still much earlier than the start of ovarian (21 dpf, days post-fertilization) or testicular (25–28 dpf) differentiation (Sawyer *et al.*, 2006; Trant *et al.*, 2001). Similarly, in Nile tilapia, *Oreochromis niloticus*, and rainbow trout, *Onchorynchus mykiss*, aromatase is detected in ovaries many days prior to the first sign of histological sex differentiation (Guiguen *et al.*, 1999; Ijiri *et al.*, 2008; Kobayashi *et al.*, 2003; Kwon *et al.*, 2001). The end result of an early and enhanced *cyp19b* expression and activity is a high neuroestrogen biosynthesis, again supporting the important role of estrogen signaling for normal ovarian development (Callard *et al.*, 2001).

The influence on hormones on sex differentiation of teleost fishes has been studied extensively (for a review see Pandian and Sheela, 1995). It is well known that estrogens are crucial for ovarian differentiation. Furthermore, it is highly possible that *cyp19a* controls sex determination of teleosts by regulating estrogen synthesis (Chang *et al.*, 2005). Indeed, these authors

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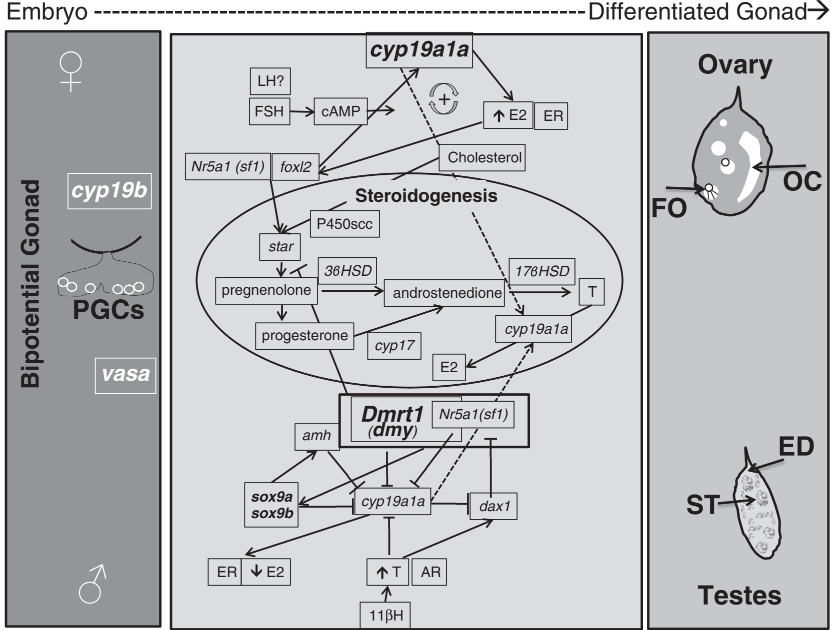

Figure 1. Diagram depicting genes involved in sex differentiation in a “typical” teleost. See text for more information on these genes including complete names. Note that this represents a “typical” gonochorist, but that exceptions exist within this taxonomic group in relation to timing and sexual dimorphism of expression, as well as regulatory mechanisms. D = efferent duct, FO = follicle, OC = ovarian cavity, PGCS = primordial germ cells, ST = seminiferous tubules.

argue that certain factors in teleosts suppress the expression of this gene by binding to its promoter region, causing the bipotential gonad to develop into a testis; in contrast, females are the result of a downregulation of those sex-determining genes which lead to a high production of estrogens thus triggering ovarian development (Chang *et al.*, 2005). This, the authors argue, explains why fish embryos exposed to xenoestrogens respond with an upregulation of *cyp19b* expression leading to similar effects (i.e. feminization) to a physiological increase in *cyp19a* expression in embryos destined to become females. In sum, expression of the extra-gonadal form of *cyp19* (i.e. *cyp19b*) is what probably dictates phenotypic sex differentiation (if not determination) in teleosts. This is further supported by the fact that *cyp19a* lacks estrogen response elements (ERE) and is thus unresponsive to estrogens (Trant *et al.*, 2001). In contrast, the presence of an ERE motif in the *cyp19b* promoter and its high expression in the brain of teleosts very early on during embryo development support the notion that this enzyme is involved in neuroestrogen synthesis in the brain (Chang *et al.*, 2005; Kazeto *et al.*, 2001; Tchoudakova *et al.*, 2001), and this ‘brain’ form is mostly associated with sex determination and differentiation (Trant *et al.*, 2001). Nevertheless, the presence of three putative cAMP response elements in the 5′-flanking region of *cyp19a* offers the potential for transcriptional regulation of this enzyme via gonadotropins (follicle stimulating hormone, FSH), similar to what has been described in mammals (Kazeto *et al.*, 2001).

Several genes have been shown to regulate the transcription of *cyp19a* and thus estrogen synthesis. In the Japanese flounder, a species that exhibits TSD, gonadotropin (FSH) signaling and *foxl2* play important roles in transcriptional regulation of *cyp19a* during gonadal sex differentiation (Yamaguchi *et al.*, 2007). In tilapia, the 5′ flanking region of the *cyp19a* gene has binding sites for *foxl2*, *sf1* (now *Nr5a1*), *wt1-kts*, and *sry*, all of which are key sex-determining factors in mammals (Chang *et al.*, 2005; Kwon *et al.*, 2001; Tong and Chung, 2003). In medaka, *dax1* downregulates *ad4bp/sf1* and *foxl2*-mediated *cyp19a* expression in ovarian follicles (Nakamoto *et al.*, 2007). As with mammals, in zebrafish, the anti-Müllerian hormone (*amh*) has been shown to be an upstream negative regulator of *cyp19a* (Rodríguez-Marí *et al.*, 2005). More information regarding these genes can be found below.

Dmrt1 (double sex and mab-3 related gene 1) and Dmy

Dmrt1 is a member of a gene family of transcription factors that share a highly conserved zinc finger-like DNA-binding domain with a high similarity to proteins that are involved in sex determination in *Drosophila* and *Caenorhabditis elegans*. In mammals, it appears to act at a downstream position in the male sex determination/differentiation cascade, downstream of *sry* (Raymond *et al.*, 2000). *Dmrt1* has been identified exclusively in male gonads of several fish species (Guan *et al.*, 2000; He *et al.*, 2003; Liu *et al.*, 2007; Marchand *et al.*, 2000) and in some species, several isoforms (up to six) have been identified (Guo *et al.*, 2004, 2005; Huang *et al.*, 2002). *Dmrt1* is expressed in Sertoli cells *after* testicular differentiation and thus plays a role in spermatogonial proliferation (Kobayashi *et al.*, 2004). Its temporal pattern of expression, however, has been reported to vary across teleosts. For instance, in rainbow trout and tilapia, *dmrt1* expression is quantifiable in male gonads during sex differentiation and significantly upregulated during the early stages of testicular differentiation (Baron *et al.*, 2005; Ijiri *et al.*, 2008; Marchand *et al.*, 2000). In contrast, zebrafish *dmrt1* expression is also observed in ovarian developing germ cells; thus it appears to also be associated with ovarian differentiation at least in some fish species (Guo *et al.*, 2005).

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In Nile tilapia, *dmrt1* is known to regulate the expression of several genes involved in sex differentiation (Wang *et al.*, 2010). For example, it can suppress *adbp/sf1* and *foxl2*-mediated *cyp19a* transcription. It can also suppress *adbp/sf1*-mediated *StAR* and *cyp17*, but activate *cyp11a* and *cyp11b*. This suggests *dmrt1* can not only induce male phenotypic development via a down-regulation of aromatase, but also via shifting the steroidogenic pathway towards androgen production (Wang *et al.*, 2010).

In Japanese medaka, the male sex-determining gene is a duplicated version of *dmrt1*, known as *dmrt1bY* (or *dmY*) (Matsuda, 2005; Matsuda *et al.*, 2007; Nanda *et al.*, 2002) and thus is considered the nonmammalian vertebrate equivalent of the mammalian sex-determining *sry* gene. In medaka, this gene arose by a duplication event of an autosomal segment containing *dmrt1a*, which was inserted into another chromosome that then became the Y chromosome and the only functional gene that is Y specific. Mutations of this gene result in XY sex-reversed females (Matsuda *et al.*, 2002). In XY embryos medaka, *dmy* is solely expressed in the nuclei of somatic cells surrounding primordial germ (PGCs) and Sertoli cells during early gonadal development and its expression precedes morphological differentiation of the testes (Kobayashi *et al.*, 2004; Matsuda, 2005). Expression of this gene persists into adulthood (Matsuda, 2005). This suggests that *dmy* functions as a transcriptional regulator to direct development of PGCs towards male *before* sexual differentiation has occurred (Kobayashi *et al.*, 2004). This gene, however, is absent from all other fish studied, including other *Oryzias* species, leading to the conclusion that it's duplication occurred recently during the evolution of the genus *Oryzias* and that it has attained the status of the 'male sex-determining' gene only in the medaka lineage (Kondo *et al.*, 2003; Matsuda *et al.*, 2007).

Sox9 (Sry-related HMG box-9)

Sox9 is a member of the *sox* family of transcription factors and contains a 79-amino acid DNA-binding motif known as the HMG domain. In mammals, *sox9* is one of the earliest genes to be upregulated in pre-Sertoli cells of developing male gonads, but it can also be found in female genital ridges until *sry* expression kicks in, at which point its expression is turned off in the ovaries but maintained in Sertoli cells (Morais Da Silva *et al.*, 1996). It interacts with *sf1* (Steroidogenic Factor 1) to upregulate the expression of *amh* (Arango *et al.*, 1999; Rodríguez-Marí *et al.*, 2005; see more about these genes below). Similarly, in teleosts, *sox9* has been predominantly associated with testicular development, with *sox9*-expressing cells being localized directly surrounding the germ cells which then differentiate into Sertoli cells (Nakamura *et al.*, 2008; Qi-Yan *et al.*, 2007; Rodríguez-Marí *et al.*, 2005; Takamatsu *et al.*, 1997; Vizziano *et al.*, 2007). During early gonadal differentiation, an incomplete sexually dimorphic pattern has been described for *sox9* in several fish species, with expression also being reported in females (Ijiri *et al.*, 2008; Nakamoto *et al.*, 2005; Nakamura *et al.*, 2008; Rodríguez-Marí *et al.*, 2005; Vizziano *et al.*, 2007). Upregulation in males occurs at later stages of testicular development. These results suggest that *sox9* does not play a major role in early sex determination and differentiation, but may be involved in the later development of testicular tubules (Nakamoto *et al.*, 2005).

Foxl2 (Fork-head transcription factor 2)

Members of this group of transcription factors are well conserved and usually play key roles regulating cellular determination and differentiation processes. *Foxl2* is a highly conserved winged helix/forkhead transcription factor gene involved in differentiation and proliferation of granulose cells, ovarian development and maintenance of function. Further, it is considered the earliest known sex dimorphic marker of ovarian determination and differentiation in vertebrates including teleosts, with a differential expression (of up to 100-fold) between genders that can be maintained into adulthood (Baron *et al.*, 2004; Cocquet *et al.*, 2003; Ijiri *et al.*, 2008; Liu *et al.*, 2007; Loffler *et al.*, 2003; Nakamoto *et al.*, 2006; Wang *et al.*, 2004). In female tilapia and medaka, expression of *foxl*2 starts in the somatic cells surrounding the germ cells immediately after ovarian differentiation and expression is maintained in the granulosa cells throughout ovarian development and in pre- and vitellogenic follicles of adult females (Nakamoto *et al.*, 2006; Wang *et al.*, 2004). In Nile tilapia, *foxl2* is also expressed very early during gonadal differentiation in the brain and pituitary suggesting that this gene might be regulating steroidogenesis through the brain-pituitary-hypothalamus-gonadal axis (Wang *et al.*, 2004). More recently, it was discovered that *foxl*2 protein can bind to the promoter site of *cyp19a1*during gonadal sex differentiation in the Japanese flounder, a teleost that exhibits TSD (Yamaguchi *et al.*, 2007). This transcriptional activation has also been reported in Nile tilapia (Ijiri *et al.*, 2008; Wang *et al.*, 2007). Similarly, in rainbow trout females treated with aromatase inhibitors, expression of *foxl2* is downregulated, further supporting a direct link between estrogens and *foxl*2 expression in teleost fish (Baron *et al.*, 2004).

Other genes

A series of other genes have also been reported to be differentially expressed between male and female fish and have been suggested as additional players in sex differentiation in this vertebrate group. They include genes involved in steroidogenesis, such as steroidogenic acute regulatory protein (*StAR*), *cyp17a*, *cyp11a*, *3βHSD* and the negative regulator of steroid biosynthesis, the dosage-sensitive sex reversal gene (*dax1*); hormone receptors such as luteinizing hormone receptor (*lhr*); and transcription factors involved in the regulation of steroid enzyme transcription such as *Nr5a1*, anti-Müllerian hormone (*amh*, also referred to as *mis*) and a related gene in eels, eel spermatogenesis related substance (*eSRS21*) (Baron and Guiguen, 2003; Baron *et al.*, 2005; Nakamoto *et al.*, 2007; Vizziano *et al.*, 2007; Von Hofsten *et al.*, 2001; Yoshinaga *et al.*, 2004).

In mammals, *dax1* acts as a negative regulator of steroidogenesis through transcriptional inhibition of *sf1* (Park and Jameson, 2005). Its role in sex differentiation in teleosts is unclear, since in some species, such as rainbow trout, it is dimorphically expressed (higher in developing testes, Baron *et al.*, 2005) whereas in others, such as the medaka, it is not found to be expressed in early periods of sex differentiation (Nakamoto *et al.*, 2007).

Nr5a1 is a member of the nuclear receptors *ftz-f1* (Fushi Tarazu Factor 1) and has been linked with sexual determination in mammals. In teleosts, two functional homologs, *ff1b* and *ff1d*, have been identified (Chai and Chan, 2000; Von Hofsten *et al.*, 2001). They are predominantly expressed in male gonads, but have also been detected in ovaries and hypophysis.

Amh is a glycoprotein member of the transforming growth factor *β* (TGF-*β*) superfamily and it is the first factor secreted by differentiated Sertoli cells in the testis, leading to the regression of Müllerian ducts (Behringer *et al.*, 1990). Despite the absence of Müllerian ducts in teleosts, expression of *amh* has been detected not solely in immature testes, but also in Sertoli cells of

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adult males and in granulosa cells of oocytes from adult females (Rodríguez-Marí *et al.*, 2005). In tilapia, rainbow trout and Japanese flounder, a sexual dimorphic expression has been reported, which suggests an important role of this gene in proliferation and differentiation of spermatogonia (Ijiri *et al.*, 2008; Vizziano *et al.*, 2007; Yoshinaga *et al.*, 2004). At least in zebrafish, it is positively regulated by *sox9* and negatively regulated by *cyp19a* (Rodríguez-Marí *et al.*, 2005).

Transcription factors involved in urogenital development have also been found to be differentially expressed between female and male teleosts. *Wt1* (Wilm's Tumor Supressor-1) encodes for a zinc finger transcription factor critical in the development of the urogenital system. In mammals, mutations of this gene lead to anomalies in kidney and gonad development (Klamt *et al.*, 1998). It is required for *sox9* expression during testis development (Gao *et al.*, 2006) and *in vitro* studies have suggested a role of this gene in sex determination through transcriptional activation of *sry* (Hossain and Saunders, 2001). *Wt1* co-orthologs, *wt1a* and *wt1b*, have been identified in teleosts, with both forms exhibiting co-regulation and probably both involved in PGC development (Bollig *et al.*, 2006; Kluver *et al.*, 2009; Perner *et al.*, 2007).

In rainbow trout *pax2a* is expressed early in testicular development and is absent from ovaries (Baron *et al.*, 2005). More recently, binding sites for this gene have been identified in the promoter sequences of *dmrt1* suggesting members of this gene family are probably involved in sex differentiation of teleosts (Alfaqih *et al.*, 2009).

Ssb4, an homolog of *Gus* (or gustavus) gene which is required for the specification of germ cells, has been identified in zebrafish as playing a role in early development of germ cells (Li *et al.*, 2009). In this fish species, insulin-like growth factor (*igfr*b) has been shown to play an important role in embryonic germline development (Schlueter *et al.*, 2007).

In rainbow trout female, follistatin (*fst*), *ovo11*, *inha*, *gdf9*, *nup62*, *sox23*, *sox24*, *bmp7*, *bcl2lb*, *fancl* and *gcl* are expressed very early during ovarian differentiation and exhibit a clear sexual dimorphic expression (Baron *et al.*, 2005; Vizziano *et al.*, 2007). Some male-specific genes included *cldn11*, *fgf6* and *inha*.

EFFECTS OF STEROID HORMONES ON FISH EARLY LIFE STAGES

Possible Mechanisms of Action of Hormones

The mechanisms of sex determination and sex reversal in fish are complex, vary among different species, and in most cases are not well understood. Exogenous hormones are of concern because they can alter gonad development and sex differentiation. Also in most teleosts, gonads retain bipotentiality even after gonadal differentiation (Devlin and Nagahama, 2002). This means that exogenous hormones have the potential to cause sex reversal and disrupt reproductive processes even after sex differentiation has occurred. As described in more detail below, some mechanisms of action (MOA) of environmental hormones include direct interaction with nuclear receptors, leading to potential changes in the expression of genes involved in sex determination/differentiation and alterations of hypothalamus–pituitary–gonadal (HPG) axis feedback mechanisms.

Steroid hormones are small, hydrophobic hormones that can permeate membranes, therefore can act on receptors in cell nuclei. Sex steroids act through binding to specific nuclear receptors, including estrogen receptors (ER) and androgen receptors (AR) (Sandra and Norma, 2010). These are part of the nuclear receptor superfamily, which are ligand-regulated transcription factors. The general MOA of a steroid hormone is that it can enter a cell, form a complex with a hormone receptor, and enter the nucleus where this complex can bind to DNA and result in the translation of specific mRNA and proteins. These can then lead to specific physiological responses. Exposure to environmental hormones can disrupt normal expression of genes involved in sex differentiation. For example, androgen exposure has been shown to decrease expression of *cyp19a* (Baron *et al.*, 2008), which can lead to masculinizing effects. In contrast, 17β-E_2 can decrease expression of *dmrt1* in males, leading to feminizing effects (Marchand *et al.*, 2000).

Exposure to exogenous sex steroids can also lead to alterations in expression of ERs and ARs. ERs are positively controlled by estrogens to ensure sufficient receptors are present when there are changes in sex hormone levels. In a study by Filby *et al.* (2007), EE_2 was shown to upregulate expression of ERα in liver and downregulate AR expression in gonads and ERα expression in ovary. These nuclear receptors are also found in fish early stages, providing a route for endogenous steroid hormones to act on sex determination and differentiation (Devlin and Nagahama, 2002; Johns *et al.*, 2009b). However, ERs and ARs in ovaries and testes also provide a main route for effects of exogenous hormones on biochemical processes in both males and females. These hormone receptors are also expressed (and co-expressed) in a number of different organs (Matthews and Gustafsson, 2003), leading to potential estrogenic and androgenic effects on many different pathways affected by endocrine processes.

The synthesis and release of steroid hormones is ultimately controlled by feedback mechanisms via the HPG axis, which may also be affected by exposure to natural and synthetic sex hormones. The hypothalamus signals the pituitary to release hormones like gonadotropins. Gonadotropins promote the production of progestins, androgens, and estrogens, which then act to regulate processes in development, reproduction and behavior (Arcand-Hoy and Benson, 1998). Two types of gonadotropins are found in fish, FSH and luteinizing hormone (LH; formerly GTH I and GTH II; Kawauchi *et al.*, 1989; Quérat *et al.*, 2000). FSH levels in the blood increase during early oocyte development, stimulating the synthesis of T, which is then aromatized into E_2 (Cyr and Eales, 1996). E_2 acts on gonads to stimulate oocyte development and acts on gene expression in nongonadal tissues to influence oocyte growth (i.e. vitellogenin, VTG induction). LH alters steroidogenesis toward maturational progestins that induce ovulation (Swanson *et al.*, 1991). Both FSH and LH promote steroidogenesis and spermatogenesis in male fish, FSH being in higher concentrations during development and LH during maturation (Nagahama 1994). Exposure to estrogens and androgens can influence signaling in the HPG axis. For example, metabolites of trenbolone acetate can act indirectly to decrease plasma steroid concentrations by feedback inhibition of the HPG axis (Ankley *et al.*, 2003; Jensen *et al.*, 2006).

Thyroid hormones are also controlled by the HPG axis. In fish the main influence of thyroid hormones is on regulation of growth and development; however, there is evidence of effects on reproduction as well. For instance, triiodothyronine (T_3; metabolized from thyroxine) can act similarly to gonadotropins and stimulate steroidogenesis (Cyr and Eales, 1996). Thyroid receptors are also expressed in fish at early stages, providing a

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mechanism of action on this pathway by exogenous steroid hormones (Johns *et al.*, 2009b).

Effects of Hormones on Fish Early Life Stages

There are many pollutants that are considered endocrine active compounds and exhibit estrogenic or androgenic activity. For the purpose of this review, however, we will only summarize studies that have tested the effects of steroidal hormones of the types released by SWTTPs or CAFOs (see Tables 1 and 2). Tables 3 and 4 summarize the articles reviewed here.

Effects of estrogens

Various common effects of estrogen exposure include increased 17β-E_2 and VTG plasma concentrations; reduced gonad development, spermatogenesis, fecundity and fertility; pathological changes in gonads; decreased male secondary sex characteristics; and female-skewed sex ratios (Filby *et al.*, 2007). Along with classic feminizing effects, estrogen exposure has also been shown to induce responses in expression of genes related to physiological processes other than reproduction (i.e. insulin-like growth factor and growth hormone genes; Filby *et al.*, 2006).

The yolk protein precursor, VTG, is a phospholipoprotein complex induced by E_2 that promotes oocyte growth and is used in larval energy supply (Devlin and Nagahama, 2002). Due to the estrogen control of VTG, levels of this protein have been found to be an effective biomarker for estrogen exposure in fish. Many of the articles reviewed for this paper used VTG induction as a marker of estrogen exposure and response, and many found VTG induction at environmentally relevant concentrations of E_1, E_2, EE_2 and E_3 (Bogers *et al.*, 2006b; Fenske *et al.*, 2005; Holbech *et al.*, 2006; Länge *et al.*, 2001; Liao *et al.*, 2009; Örn *et al.*, 2003; van Aerle *et al.*, 2002). Further, Tyler *et al.* (1999) found induction of this protein to be a more sensitive endpoint than changes on sex ratios or sex reversal in juvenile fathead minnows exposed to 17β-E_2. Similarly, Holbech *et al.* (2006) found that when juvenile zebrafish were exposed to various estrogens, changes in VTG were an equally or more sensitive endpoint than changes in sex ratios.

Although VTG has been well established as a biomarker of exposure to estrogens in fish, variations in responses of this protein are often seen in relation to differences in ages and species tested (see Table 3 for differences in lowest observable effect concentrations, LOECs, across studies). Exposure to 17β-E_2 and E_1 resulted in dose-dependent increases in plasma VTG in adult male fathead minnows (Panter *et al.*, 1998), and similar effects were seen in early life stages (Tyler *et al.*, 1999). However, in a study by Liao *et al.* (2009) adult rare minnows (*Gobiocypris rarus*) were more sensitive than larvae and juveniles when using VTG induction as a marker of estrogen exposure. This was also seen in a similar study with zebrafish (Brion *et al.*, 2004). Liao *et al.* (2009) hypothesized that this may be due to the higher expression of ERs in adults than earlier life stages. This study demonstrates the importance of testing various age classes when evaluating endocrine disrupting effects.

Many studies have demonstrated that the most sensitive time point for exposure to hormones in fish is during sex differentiation (Liao *et al.*, 2009; Maack and Segner, 2004). An example of such an enhanced sensitivity is the irreversible gonadal cellular changes that result from hormonal exposures during this period (Devlin and Nagahama, 2002; Länge *et al.*, 2001). Sensitive exposure time periods are variable among different fish species, so it is important to have knowledge about the developmental biology of the species being studied (Koger *et al.*, 2000). When exposed to hormones during this sensitive period the endpoint of sex ratio or sex reversal may be more sensitive than the typical endpoint of VTG induction, and more objective than gonadal histology such as formation of ovarian-like cavities in developing testes (Hahlbeck *et al.*, 2004a; Liao *et al.*, 2009).

Development of intersex is one endpoint that has often been used to assess feminization of males from exposure to estrogens. Often male fish are 'intersex' when both ovarian and testicular tissue are present (also referred to as ova–testis). This phenomenon has been reported to occur naturally in some teleosts, and can be present in control groups at low percentages (Hahlbeck *et al.*, 2004a, b). Notably, intersex has been shown to occur at a higher prevalence in fish populations exposed to environmentally relevant concentrations of estrogens (i.e. 33.5 ng E_2 l^{-1}) during sex differentiation compared with reference populations (Hirai *et al.*, 2006).

Skewed sex ratios towards females have been reported in several studies after estrogen exposure of fish embryos and larvae. A 40 day exposure to environmentally relevant concentrations of EE_2 (1 ng l^{-1}) caused a significant shift of a zebrafish population toward females, with complete sex reversal (no intersex fish observed) after exposure to 2 ng l^{-1} (Örn *et al.*, 2003). These concentrations are well within those found in the environment (see Table 2), raising concern for fish populations consistently exposed to this synthetic hormone.

Along with the endpoints discussed above, there are also nonreproductive endpoints that can be used to assess estrogen exposure in young fish. Larval growth rate has been shown to be reduced after EE_2 exposure (Johns *et al.*, 2009a; Länge *et al.*, 2001). Disruption of the energy budget during growth towards VTG synthesis is likely to affect survival (van Aerle *et al.*, 2002) since reduced growth rates usually denote reduced fitness.

In summary, there has been an abundance of work published on the endocrine disrupting effects of estrogens in fish. While some of this work has been focused on early life-stage fish, very few studies have employed low level estrogen concentrations (within environmental ranges). This is particularly true for E_2. From these studies it is evident that most effects occur at levels higher that those found in the environment (see Tables 2 and 3). There is also evidence that some of the responses to estrogen exposure (E_2 and EE_2 in particular) are reversible when exposure ceases, including adverse effects on gonadal development, feminization and VTG induction (Fenske *et al.*, 2005; Hahlbeck *et al.*, 2004a, b; Hill and Janz, 2003; Maack and Segner, 2004). However, there are increasing loads of estrogens being released into aquatic ecosystems by SWTTPs and CAFOs, causing pseudopersistent exposure of these steroid hormones to fish and other organisms. Parrott and Beverley (2005) showed that life-cycle exposure of fathead minnows to 0.32 ng l^{-1} EE_2 resulted in demasculinization and reduction in fertilization success. These population-level effects at environmentally relevant estrogen exposures are of concern and further research is needed into the mechanisms by which organisms respond to environmental estrogens.

Effects of androgens

Like estrogens, androgens can also cause alterations in growth and development, occurrence of intersex, as well as alterations in thyroid morphology (Jobling and Tyler, 2003; León *et al.*, 2007). As already discussed, VTG induction has been well

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Table 3. Summary of studies in early life-stage fish exposed to environmental estrogens

Estrogen	Fish species	Fish age (exposure length)	Exposure concentration (route)	Effects	Reference
Estrone	Zebrafish	Juveniles (28 or 40 days)	10, 25, 50, 100, 200 ng l^{-1} (water)	LOEC of sex ratio skewed toward females was 49 ng l^{-1}. LOEC of VTG induction was 14 ng l^{-1}	Holbech *et al.* (2006)
17β-Estradiol	Zebrafish	Embryo, larvae, juvenile (3 weeks)	5, 25, 100 ng l^{-1} (water)	Reversible VTG induction. Sex ratio skewed toward females. Reduced egg production and survival of offspring. LOEC for all endpoints 100 ng l^{-1}	Brion *et al.* (2004)
		Embryo (0, 24, 48, 72, 120 h)	0.1, 1.0 μM (water)	Up-regulated *cyp19b* expression at all lengths of exposure. LOEC 0.1 μM	Sawyer *et al.* (2006)
		Juveniles (28 or 40 days)	5, 10, 25, 50, 100, 250, 500 ng l^{-1} (water)	LOEC of VTG induction and of sex ratios skewed toward females was 54 ng l^{-1}	Holbech *et al.* (2006)
	Three-spined stickleback	Embryo (14, 26–44 or 39–58 days)	0.01, 1.0, 10.0 μg l^{-1} (water)	Feminization occurred at 1.0 and 10.0 μg l^{-1}. Significant numbers of intersex males in most 1.0 μg l^{-1} groups and in all 10.0 μg l^{-1} groups. VTG induction occurred at 10 μg l^{-1}	Hahlbeck *et al.* (2004a, b)
	Japanese medaka	Embryo (12, 14, 20 days)	33.5, 140.6 ng l^{-1} (water)	Formation of ova-testes in males exposed to 33.5 ng l^{-1} for 14 or 20 days. Complete sex reversal towards females occurred at 140.6 ng l^{-1}	Hirai *et al.* (2006)
		Embryo (4 weeks)	0.01, 0.1 μg l^{-1} (water)	LOEC for feminization of genotypic males based on a secondary sex characteristic index was 0.032 μg l^{-1}	Hagino *et al.* (2001)
	Fathead minnows	Juveniles (10, 20 or 30 days)	25, 50, 100 ng l^{-1} (water)	Dose-dependent induction of VTG; significant at 50 ng l^{-1} in longer exposures and at 100 ng l^{-1} in shorter exposures	Tyler *et al.* (1999)
	Rare minnow	Larvae, juveniles (21 days)	5, 25, 100, 1000 ng l^{-1} (water)	LOEC of 25 ng l^{-1} for VTG induction in larvae, and 100 ng l^{-1} for juveniles. Hermaphrodism in juveniles exposed to at least 25 ng l^{-1}	Liao *et al.* (2009)
Estriol	Zebrafish	Juveniles (28 or 40 days)	0.1, 0.3, 1, 3, 10, 30 μg l^{-1} (water)	LOEC of sex ratio skewed towards females was 22 μg l^{-1}. LOEC of VTG induction was 0.6 μg l^{-1}	Holbech *et al.* (2006)

Continues

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Table 3. *(Continued)*

Estrogen	Fish species	Fish age (exposure length)	Exposure concentration (route)	Effects	Reference
17α-Ethinylestradiol	Japanese Medaka	Larvae (4 weeks)	0.001, 0.003, 0.01, 0.032, 0.1 μg l^{-1} (water)	LOEC for feminization of genotypic males based on a secondary sex characteristic index was 0.032 μg l^{-1}	Hagino *et al.* (2001)
	Western Mosquitofish	Juvenile males (150 days)	01., 1, 2.5, 5, 7.5, 10 μg g^{-1} food (diet)	Reduced reproductive conditions with higher exposures; shorter gonopodia and VTG induction. LOEC 1.0 μg g^{-1} food	Angus *et al.* (2005)
	Zebrafish	Embryo (30 days)	1, 3, 10 ng l^{-1} (water)	Could detect estrogen exposure with luciferase reporter gene. LOEC for VTG induction was 3 ng l^{-1}	Bogers *et al.* (2006b)
		Embryo (42 or 118 days)	3 ng l^{-1} (water)	Elevated VTG in females. Arrest of testicular differentiation in males	Fenske *et al.* (2005)
		Juveniles (40 days)	1, 2, 5, 10, 25 ng l^{-1} (water)	LOEC for increase in VTG production was 2 ng l^{-1}. Sex ratios skewed towards females at 1 ng l^{-1}	Örn *et al.* (2003)
		Larvae (60 days)	1, 10, 100 ng l^{-1} (water)	At 60 dph in 10 ng l^{-1} induction of VTG, 100% females, and kidney damage were seen. When reared in clean water 60–160 dph, no significant change in sex ratio or kidney damage	Hill and Janz (2003); Weber *et al.* (2003)
		Juvenile (28 days)	1.6, 3, 10 ng l^{-1} (water)	LOEC for increased ratio of females was 1.6 ng l^{-1}. Significant reductions in fecundity and fertility success when exposed during gonad transition stage (43–71 dpf)	Maack and Segner (2004)
	Fathead minnow	Embryos (0, −4 −5,	10 ng l^{-1} (water)	Slight increase in ratio of females. VTG	van Aerle *et al.* (2002)

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		5–10, 10–15, 15–20 or −4–20 dph)		induction positively correlated with length of exposure. Feminization of testes	
		Embryos (305 days)	0.2, 1.0, 4.0, 16, 64 ng l^{-1} (water)	F_0 larval growth after 28 dph NOEC of ≥1.0 ng l^{-1}, LOEC of 4.0 ng l^{-1} for male sex development. At 56 dph feminization occurred at 4.0 ng l^{-1} and VTG induction at 16.0 ng l^{-1}	Länge *et al.* (2001)
		Juveniles (60 or 150 days)	0.32, 0.96, 3.5, 9.6, 23 ng l^{-1} (water)	Increased female sex characteristics LOEC was 3.5 ng l^{-1} (60 dph). Reduction in fertilization success, increased number of females, and demasculinized males LOEC was 0.32 ng l^{-1} (150 dph)	Parrott and Beverley (2005)
		Embryo (7 days)	2, 10, 50 ng l^{-1} (water)	LOEC for decreased body size was 10 ng l^{-1}. LOEC for induced VTG and upregulation of StAR was 2 ng l^{-1}	Johns *et al.* (2009a)
	Three-spined stickleback	Embryo (40 or 51 days)	0.05 µg l^{-1} (water)	Feminization. Significant portion of intersex males. VTG induction	Hahlbeck *et al.* (2004a, b)
	Mummichog	Embryo, larvae, juvenile (25 or 60 days)	10 to 10,000 ng l^{-1} (water)	Skeletal abnormalities and anal swelling. LOEC 1000 ng l^{-1}	Boudreau *et al.* (2004)
		Embryos (61 weeks)	0.1, 1, 10, 100 ng l^{-1} (water)	Sex ratio skewed toward females. Condition factor index, GSI, and LSI were found to decrease LOEC 10 ng l^{-1}; more consistent responses at 100 ng l^{-1}	Peters *et al.* (2010)

LOEC, lowest observable effect concentration; VTG, vitellogenin; NOEC, no observable effect concentration; dpf, days post-fertilization; dph, days post-hatch; GSI, gonadosomatic index; LSI, liver somatic index.

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Table 4. Summary of studies in early life-stage fish exposed to environmental androgens

Androgens	Fish species	Fish age (exposure length)	Exposure concentration (route)	Effects	Reference
11β-Hydroxyandrostenedione	Rainbow trout	Juveniles (3 months)	10 mg kg^{-1} food (diet)	De-differentiation of granulosa cells (downregulation of genes like *foxl2* and *cyp19a*)	Baron *et al.* (2008)
11-Ketotestosterone	Japanese Medaka (Qurt strain)	Larvae (96 h)	0.01, 0.1, and 1.0 mg l^{-1} (water)	LOEC for germ cell necrosis and increased growth in males was 1.0 mg l^{-1}. LOEC for germ cell necrosis and ovarian atresia in females was 0.01 mg l^{-1}, with decreased growth at 0.1 mg l^{-1}. Thyroid follicle hyperplasia was twice as likely in males as females	León *et al.* (2007)
Methyldihydrotestosterone	Fathead minnows	Embryos (63 or 114 days)	0.1, 0.32, 1.0 μg l^{-1} (water)	Exposure at 1 μg l^{-1} for 63 d showed nuptial tubercles on >80% of fish. LOEC for mix sex or effects on gonad development was 0.32 μg l^{-1}	Bogers *et al.* (2006a)
17α-Methyltestosterone	Three-spined stickleback	Embryo, juvenile (14, 26–44 or 39–58 days)	1.0 μg l^{-1} (water)	Intersex fish in females and males. Testis abnormalities, not fully developed, and abnormal cavity in gonads	Hahlbeck *et al.* (2004a, b)
	Zebrafish	Juveniles (40 days)	26, 50, 100, 260, 500, 1000 ng l^{-1} (water)	Reduced VTG. Complete sex reversal (ovaries to testes) in all concentrations used. High intersex rate at 1000 ng l^{-1}	Örn *et al.* (2003)
	Japanese Medaka	Embryos (4 weeks)	0.01, 0.1 μg l^{-1} (water)	Genetic females converted to functional males at of 0.1 μg l^{-1}	Hagino *et al.* (2001)
17β-Trenbolone	Zebrafish	Juvenile (28 or 39 days)	5, 50, 500, 5000 ng l^{-1} (water)	LOEC for VTG reduction was 193 ng l^{-1}. All male population produced at 9.7 ng l^{-1}	Holbech *et al.* (2006)
Testosterone	Japanese Medaka	Embryos and larvae (1 week)	100 μg l^{-1} (water)	Intersex gonads developed when larvae were exposed for 1 week	Koger *et al.* (2000)

LOEC, lowest observable effect concentration. VTG, vitellogenin.

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established as an effective biomarker of dose-dependent estrogen exposure. It can also be induced by androgens, however the response is much more variable compared with estrogens. For instance, Holbech *et al.* (2006) found induction of VTG after exposure to ≥193 ng l^{-1} 17β-trenbolone, but at ≥9.7 ng l^{-1} an all-male population was observed. There have been other studies that have also shown inconsistent trends of VTG reduction with androgen exposure (Ankley *et al.*, 2003; Bogers *et al.*, 2006a). This discrepancy in the results observed could be due to the aromatization of some androgens and not others as discussed below.

Similar to estrogen assessment, histology of the gonads can be used to evaluate effects of androgen exposure. Some androgens produce intersex fish when applied during early life stages. For example, Koger *et al.* (2000) found that medaka larvae developed intersex gonads after only 1 week of exposure to 100 μg l^{-1} of T. However, changes in gonad histology vary depending on the degree of androgen aromatization making the assessment of responses to androgens potentially more challenging than to estrogens. Since aromatase converts androgenic steroid hormones to estrogens, exposure to aromatizable androgens (i.e. 17α-methyltestosterone, MT) may induce both masculinizing and feminizing effects (i.e. ova-testes, Koger *et al.*, 2000; Seki *et al.*, 2004). A large portion of intersex zebrafish were observed after a 40 day exposure to 1000 ng l^{-1} MT and caused complete sex reversal towards males when exposed to 26–1000 ng l^{-1} MT (Örn *et al.*, 2003). However, studies by Hahlbeck *et al.* (2004a, b) found signs of both masculinization and feminization when analyzing gonad histology after exposure of medaka to the same concentration of MT (1 μg l^{-1}) for a similar amount of time; showing intersex females and underdeveloped male testis with abnormal cavities present. These studies again demonstrate the importance of studying responses of various fish species to sex steroid hormones.

Just like with estrogen exposures, nonreproductive effects have also been seen with androgen exposures in young fish (Länge *et al.*, 2001). Androgen exposure has been shown to increase growth in males and decrease growth in females exposed to 0.01–1.0 mg l^{-1} 11-ketotestosterone for only 96 h (León *et al.*, 2007). The same study found androgen exposure can cause alterations in a wide range of endocrine functions, including thyroid function. Thyroid hypertrophy is probably caused by alterations in T_3 levels, which could have larger effects on organisms due to its role in organ growth and development (León *et al.*, 2007). This is a mechanism of action worth further investigation, as not many studies have focused on thyroid disruption after androgen exposure, particularly in fish early life stages.

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Although evidence from the studies reviewed here point to similar mechanisms of steroid hormone action in early life-stage fish as in adult fish, further research in this area is warranted. We still lack a complete understanding of the finer mechanisms involved in disruption of gonadal development and sex differentiation after estrogen and particularly after androgen exposure, as well as what mechanisms are at play in 'real life' exposure scenarios where mixtures, various species, developmental stages and environmental conditions are factors. Furthermore, there is a lack of studies that have 'replicated' as closely as possible real environmental conditions. In our opinion, the most useful studies are those in which exposure conditions (chemicals in mixture and their concentrations as well as length and timing of exposure) most closely resemble those that could occur in the environment. Perhaps adding more sampling time points from a population of developing teleosts would be more important than increasing the number of replicates and only testing one or two time periods.

Assessments of mixture effects, however, are inherently difficult and are fraught with many complications including, but not limited to, varying degradation rates during testing and little knowledge of the way in which hormones interact when mixed. As a good example of the complexities associated with testing hormone mixtures, Žlábek *et al.* (2009) observed increased VTG induction in early life-stage chubs exposed to a mixture of 17β-E_2 and T. Interestingly, a skewed sex ratio toward females was also seen in this study suggesting that this particular mixture was more 'estrogenic' than 'androgenic.' Nonreproductive effects, such as behavioral changes, after exposure to a mixture of hormones are even harder to predict. A mixture of estrogens (E_1, 17β-E_2 and EE_2) was shown to reduce predator avoidance, but when E_1 and EE_2were tested separately, such effects disappeared (McGee *et al.*, 2009). Also, conventional endpoints such as VTG induction and gonadal histology can be misleading or difficult to interpret when evaluating aromatizable androgens that can cause both feminizing and masculinizing effects (Hahlbeck *et al.*, 2004b). Thus when attempting to relate findings of hormone effects on feral fish, it is important to keep in mind that it is very likely that fish were/are being exposed to a mixture of estrogens and androgens, with only some androgenizing ones in the case of the latter.

When environmental exposure to hormones is in mixtures, which is the case more often than not, effects like inhibited reproduction and growth are also seen (Jobling *et al.*, 2002; Peters *et al.*, 2010). Although many environmental factors (most notably temperature) can contribute to changes in growth, reproductive condition and sex ratios, many of the studies discussed here provide evidence that these may also be seen as signs of hormone exposure. They are endpoints that can be related to overall success of an organism, better relating hormone effects to population level issues.

New assays are needed that allow for a better and more efficient evaluation and interpretation of effects due to hormonal mixtures. Measuring effects on sex ratios should be straightforward and at the same time allow for an assessment of population-level effects. However, genetic markers of sex have not been discovered in many fish species. Exceptions are Chinook salmon (*Oncorhynchus tshawytscha*; Devlin *et al.*, 1991), Japanese medaka (Matsuda *et al.*, 1997), guppy (*Poecilia reticulata*; Khoo *et al.*, 2003), ayu (*Plecoglossus altivelis*; Watanabe *et al.*, 2004) and three-spined stickleback (*Gasterosteus aculeatus*L.; Griffiths *et al.*, 2000). Having a sex marker that can be used as a quick (sometimes just a few hours or days post-hatch) and easy method for determining sex ratios would save significant amounts of time and money. These assays are particularly needed for commonly used toxicological fish models such as the zebrafish and the fathead minnow. A good example of such a model is the Japanese medaka strain (Qurt), in which live genetic males are noninvasively distinguished from genetic females at 2 days post-fertilization by the appearance of leucophores in males and not females (Wada *et al.*, 1998).

In summary, there are obvious variations in response to exogenous steroid hormones depending on factors such as fish

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species, time and duration of exposure, concentration, and types of hormones tested. In the case of estrogens, particularly of EE_2 and 17β-E_2, there is evidence showing feminization, alterations in gonadal development and induction of VTG induction and growth (Table 3). In some species these responses appear to be reversible when fish are transferred to clean water for a period of time. Fewer studies are available on the effects of androgens in fish. From these few studies, there is evidence that male-like hormones cause skewed sex ratios towards males and altered gonadal development (Table 4).

More research is needed on a wider array of teleost species to better determine general involvement of endogenous steroid hormones and effects of exogenous steroid hormones on sex differentiation. Further investigations need to be made into effects of environmental variables on steroidogenesis, steroids in tissues other than gonads, and differences in effects before and after gonadal differentiation. There are still many mechanisms related to steroid responses that we do not understand. Combining what we know about endocrine functions and effects of steroid hormones on receptors and gene expression, predictions can be made about modes of action of environmental hormones. However, it is still difficult to assess what the population level effects of these exposures may be. We have gained a good starting point for this, but more work is needed to develop more streamline methods for assessing effects of hormones in early life-stage fish and detecting potential threats to fish populations.

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