



Long-term exposure to environmentally relevant concentrations of progesterone and norgestrel affects sex differentiation in zebrafish (*Danio rerio*)



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ABSTRACT

The aim of this study was to investigate the effects of progestins on the sex differentiation of zebrafish by measuring the sex ratio and transcriptions of genes related to sex differentiation (*Amh*, *Dmrt1*, *Figa*, *Sox9a* and *Sox9b* genes) as well as sex hormone levels and transcriptional expression profiles along the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes in juvenile zebrafish. Exposure of zebrafish to 4, 33, 63 ng L⁻¹ progesterone (P4) or 4, 34, 77 ng L⁻¹ norgestrel (NGT) started at 20 days post fertilization (dpf) and ended at 60 dpf. The results showed that exposure to P4 caused a significant increase in proportion of females as well as significant down-regulation of *Amh* gene and up-regulation of *Figa* at a concentration of 63 ng L⁻¹. However, the shift in the sex ratio toward males was observed following exposure to 34 and 77 ng L⁻¹ NGT, which came along with the significant induction of *Dmrt1* gene and inhibition of *Figa* gene. The sex hormones in exposed fish were measured with estrone being detected only in the fish exposed to the highest P4 concentration; whereas estradiol and androstenedione were detected only in the fish of the control and lowest NGT concentration. Furthermore, the increase in females was associated with the significant up-regulation of several key genes controlling the synthesis of sex hormones (i.e., *Cyp17*, *Cyp19a1a* and *Hsd3b*) following exposure to 63 ng L⁻¹ P4 whereas the significant down-regulation of *Cyp11a1*, *Cyp17*, *Cyp19a1a* and *Hsd3b* genes was observed in the male-biased populations caused by 34 and 77 ng L⁻¹ NGT. The overall results imply that both P4 and NGT could significantly affect sex differentiation in zebrafish, and that changes may be reflected by altered sex hormone levels and transcriptional expression profiles of genes related to synthesis of sex hormones.

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Abbreviations: NGT, norgestrel; P4, progesterone; *Amh*, anti-Mullerian hormone; *Dmrt1*, doublesex and mab-3 related transcription factor 1; *Figa*, factor in the germline alpha; *Sox9a*, sry-related high mobility group box-9a; *Sox9b*, sry-related high mobility group box-9b; *Pgr*, progesterone receptor; *Esr1*, estrogen receptor 1; *Vtg1*, vitellogenin 1; *Ar*, androgen receptor; *Mr*, mineralocorticoid receptor; *Gr*, glucocorticoid receptor; *Star*, steroidogenic acute regulatory protein; *Cyp11a1*, cytochrome P450-mediated side-chain cleavage enzyme; *Cyp17*, 17-alpha-hydroxylase/17,20-lyase; *Cyp19a1a*, ovarian cytochrome P450 aromatase; *Cyp11b*, 11-beta-hydroxylase; *Hsd3b*, hydroxysteroid 3-beta dehydrogenase; *Hsd20b*, hydroxysteroid 20-beta dehydrogenase; *Hsd17b3*, hydroxysteroid 17-beta dehydrogenase type 3; *Hsd11b2*, hydroxysteroid 11-beta dehydrogenase 2; *Gnrh2*, gonadotropin-releasing hormone 2; *Gnrh3*, gonadotropin-releasing hormone 3; *Fshb*, follicle stimulating hormone, beta polypeptide; *Lhb*, luteinizing hormone, beta polypeptide; *Crh*, corticotropin releasing hormone; *Pomc*, proopiomelanocortin.

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1. Introduction

Human pharmaceuticals present in the aquatic environment have drawn widespread concerns as some of them may cause adverse effects on aquatic organisms (Corcoran et al., 2010; Fent et al., 2006; Kloas et al., 2009). Natural and synthetic steroids have been known to be one of the most active pharmaceutical classes, but most studies focus on the adverse effects of estrogens and androgens, for example, feminization of fathead minnow and suppression of sexual behavior in male Japanese medaka by estrogens (Kidd et al., 2007; Oshima et al., 2003), and irreversible masculinization in zebrafish caused by androgens (Morthorst et al., 2010). However, very little attention has been paid to ecotoxicological effects caused by progestins.

Natural progesterone (P4) is mainly derived from the excretion (feces and urine) of humans and animals, and it is an important

regulator of oocyte growth and maturation. P4 is also used in hormone replacement therapy in humans. The synthetic progestins, including norgestrel (NGT), have been used widely in oral contraceptives, contraceptive implants, intrauterine devices, vaginal ring and also as active pharmaceutical ingredients for clinical use in humans (Han et al., 2000; Kejuan et al., 2007; Qureshi and Attaran, 1999). Natural and synthetic progestins occur in the environment due to their incomplete removal in wastewater treatment plants and direct excretion of animals and direct discharge of wastewaters (Liu et al., 2012b). P4 and NGT were found in surface waters at concentrations up to 199 ng L⁻¹ and 22 ng L⁻¹, respectively (Kolpin et al., 2002; Liu et al., 2011). In municipal wastewater treatment plant effluents, P4 and NGT were detected at a concentration of 1 and 11 ng L⁻¹, respectively (Liu et al., 2012b). In addition, P4 and NGT were detected in the receiving surface waters of swine farms with concentrations up to 30.5 and 465 ng L⁻¹, respectively (Liu et al., 2012c). Furthermore, P4 and NGT were also detected in flush waters of swine and dairy cattle farms with relatively high concentrations up to 3470–11900 ng L⁻¹ and 10800–6460 ng L⁻¹, respectively, since these chemicals are often used to control pregnancy time of animals in the livestock industry (Liu et al., 2012a,c). Environmental residues of P4 and NGT may pose potential risks to aquatic organisms in the receiving aquatic environment.

Until recently, some studies have reported the reproductive effects of P4 (Blüthgen et al., 2013a; DeQuattro et al., 2012; Murack et al., 2011) and synthetic progestins (Han et al., 2014; Paulos et al., 2010; Runnalls et al., 2013; Zeilinger et al., 2009) in fish at low concentrations. However, the effects of progestins on sex differentiation in fish remain unclear. In developing fish, the balance between estrogens and androgens plays an important role in the course of sex differentiation. For example, exposure to estrogens or androgens during the early development of zebrafish resulted in a change of the sex ratio toward females or males, respectively (Baumann et al., 2013; Holbech et al., 2006). The synthetic progestin levonorgestrel has been reported to be a potent androgenic chemical in the three-spined stickleback, including induction of the normally male specific glue protein spiggin and suppression of liver vitellogenin in females (Svensson et al., 2013), and disruption of androgen-dependent reproductive cycle in males (Svensson et al., 2014). Yeast-based in vitro assay further demonstrated some synthetic progestins (e.g., levonorgestrel, norethindrone, gestodene, drospirenone, desogestrel and medroxyprogesterone acetate) showed androgenic activity (Runnalls et al., 2013). NGT is a racemic mixture of two optical isomers called enantiomers, i.e., levonorgestrel and dextronorgestrel. Furthermore, levonorgestrel is the only bioactive isomer in the racemic mixture NGT. Therefore, we hypothesize that NGT is also a chemical with androgenic activity, similar to levonorgestrel. P4 also has a weak androgenic activity as it weakly binds to the androgen receptor (Ellestad et al., 2014). In addition, P4 treatment altered transcriptional expression of genes involved in steroid hormone receptor activity, circadian rhythm, cell cycle, cardiac hypertrophy and reproduction (Zucchi et al., 2012, 2013). However, so far, the effects of P4 and NGT on sex differentiation are largely unknown. Therefore, it is necessary to assess and understand adverse effects of P4 and NGT on sex differentiation in fish.

As a protogynic fish, zebrafish is an important vertebrate model for the investigation of chemical effects on sex differentiation. Approximately 10 days post fertilization (dpf) the differentiation of gonads begins and zebrafish develops ovary-like gonads, regardless of chromosomal background. At approximately 20 dpf, around 50% ovaries will be transformed into testes simultaneously with ovarian apoptosis. In the following development period, ovarian development and maturation of fish continue (von Hofsten and Olsson, 2005). Development of gonads is completed at approximately 60 dpf under hormone control. Several studies reported that

exposure of zebrafish to estrogens or androgens in the sensitive phase of gonadal transformation led to the alterations of the sex ratio (Holbech et al., 2006; Orn et al., 2003). Therefore, sex ratio is used as an essential endpoint for the evaluation of sex differentiation following exposure to progestins from 20 to 60 dpf.

Sex differentiation in zebrafish is distinct from mammals, since no sex chromosomes and sex determination genes have been identified in zebrafish until now. However, several genes, such as *Amh*, *Dmrt1*, *Figa*, *Sox9a* and *Sox9b*, have been associated with the process of sex differentiation in zebrafish. In mammals, anti-Mullerian hormone (AMH), produced by Sertoli cells, initiates degeneration of the Mullerian ducts and inhibits the aromatase enzyme that converts androgens to estrogens in the gonads (von Hofsten and Olsson, 2005). Although zebrafish does not have Mullerian ducts, other AMH functions may be important for male differentiation. The sex determining gene *Dmy* (also referred to as *Dmrt1y*) is located on the Y chromosome in Japanese medaka and originates from *Dmrt1* duplication, which suggests that *Dmrt1* gene is also crucial for testis development (Jorgensen et al., 2008). Furthermore, *Amh* and *Dmrt1* are expressed at high levels during testicular differentiation and are expressed at low levels or not expressed or down-regulated during ovarian differentiation in zebrafish (Jorgensen et al., 2008; Schulz et al., 2007; von Hofsten and Olsson, 2005). *Figa* plays an important role in the formation of ovarian follicle and is a stable and efficient gene expressed specifically in the zebrafish female germline, and thus it is useful for the assessment of female development (Jorgensen et al., 2008; Onichtchouk et al., 2003). The *Sox9* (Sry-related high mobility group box-9) gene family encodes a diverse range of developmental regulators involved in sex differentiation, which are characterized by the presence of a DNA-binding high mobility group (HMG) domain with at least 50% similarity to that of Sry, the sex determining factor on the Y chromosome (Jorgensen et al., 2008; Wegner, 1999). In adult zebrafish, *Sox9a* transcript was observed in testis while abundant *Sox9b* expression was seen in the ovary. The different expression patterns of two *Sox9* genes suggest they have unique functions during sex differentiation (Chiang et al., 2001; Jorgensen et al., 2008). Since *Amh*, *Dmrt1*, *Figa*, *Sox9a* and *Sox9b* are involved in sex differentiation, the transcriptional expression levels of these five genes are investigated in the present study.

The objective of this study was to assess the effects of exposure of juvenile zebrafish to environmental levels of P4 or NGT on sex differentiation. The sex ratio and transcriptional expression of genes related to sex differentiation were used as the endpoints for the evaluation of sex differentiation. In addition, the concentrations of sex hormones and transcriptional expression profiles of target genes along the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes in zebrafish were also measured in order to identify potential modes of actions of P4 and NGT on sex differentiation.

2. Materials and methods

2.1. Chemicals

Chemical standard progesterone (P4, CAS 57-83-0, purity 98%) was purchased from Tokyo Chemical Industry (Shanghai, China). Norgestrel (NGT, CAS 6533-00-2, purity 100%) was obtained from US Pharmacopeia (Rockville, MD). Stock solutions (1 mg mL⁻¹) for each compound were dissolved in 100% ethanol and then stored at -20 °C in the dark.

2.2. Test species and experimental design

Juvenile zebrafish (*Danio rerio*) were obtained from spawning adults placed in groups of 6 males and 3 females. Newly fertilized eggs were collected and transferred into petri dishes, and kept at

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