The Synthetic Gestagen Levonorgestrel Disrupts Sexual Development in *Xenopus laevis* by Affecting Gene Expression of Pituitary Gonadotropins and Gonadal Steroidogenic Enzymes

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In the present study, *Xenopus laevis* tadpoles were chronically exposed to four concentrations of the synthetic gestagen Levonorgestrel (LNG; 10⁻¹¹, 10⁻¹⁰, 10⁻⁹, and 10⁻⁸M) starting at Nieuwkoop and Faber (NF) stage 48 until completion of metamorphosis. At NF 58 and 66, brain-pituitary and gonad samples were taken for gene expression analyses of gonadotropins and gonadal steroidogenic enzymes. Exposure to 10⁻⁹ and 10⁻⁸M LNG until NF 58 repressed messenger RNA (mRNA) expression of luteinizing hormone (LH) β in both genders. This decrease was persistent after further treatment until NF 66 in the 10⁻⁸M LNG treatment. Expression of follicle-stimulating hormone (FSH) β was affected sex-specifically. No effect was present in NF 58 females, whereas LNG at 10⁻⁹ and 10⁻⁸M significantly increased FSHβ mRNA levels in males. In NF 66 females, 10⁻⁸M LNG treatment increased FSHβ gene expression, whereas a decrease was observed in NF 66 males exposed to 10⁻⁹M LNG. In gonads, expression of steroid-5-alpha-reductase was affected sex-specifically with increased mRNA levels in females but repressed levels in males. Gene expression of further gonadal steroidogenic factors was decreased by 10⁻⁸M LNG in both genders at NF 66. Assessment of gonad gross morphology and histology revealed poorly developed testes in the 10⁻⁷M LNG treatment. Our results suggest that LNG-exposed females (Zeilinger et al., 2009). Synthetic sex steroids, namely estrogen derivatives and progestins, are widely used components of oral contraceptives and hormone replacement therapy. In contrast to estrogens that have been subject of numerous ecotoxicological studies (Caldwell et al., 2008; Gyllenhammar et al., 2009), there has been paid only little attention on potential impacts of progestins on aquatic nontarget organisms. There are several progestins in use that may vary in their molecular core structure and thus in their affinity to steroid receptors other than the progesterone receptor. Levonorgestrel (LNG) is a second-generation progestin derived from 19-nortestosterone and known to exhibit significant binding affinity to the androgen receptor in mammals (Africander et al., 2011). Environmental concentrations of LNG are mostly reported to be in the low ng/l range (1–10 ng/l; Fick et al., 2010; Liu et al., 2011; Petrovic et al., 2002); however, also higher concentrations of 17 and 30 ng/l LNG were measured in the effluents of French and Canadian sewage treatment plants (STP) (Viglino et al., 2008; Vulliet et al., 2007).

In teleost fish, an androgenic activity of LNG was clearly demonstrated by the occurrence of significant masculinization characteristics in LNG-exposed females (Zeilinger et al., 2009).

Of particular concern is the high potential of LNG in decreasing reproductive success of fish already at concentrations below 1 ng/l (Zeilinger et al., 2009). Correspondingly and based on a mode of action concept, LNG was assessed as a biologically active compound with a considerable risk to affect nontarget organisms (Christen et al., 2010). Due to their mainly aquatic habitat, also amphibians have to be considered as potentially endangered nontarget species. In particular, during the very sensitive period of metamorphosis, when
sexual development occurs, larvae are chronically exposed to xenobiotics, including LNG. Since the key players of reproductive biology are well conserved within vertebrates and based on the enormous potency in disrupting fish reproduction, an LNG impairment of amphibian fertility seems to be likely. In fact, larval exposure to LNG was reported to affect sexual development and fertility in Xenopus tropicalis (Kvarnryd et al., 2011). However, gonad development appeared unremarkable at completion of metamorphosis, and adverse effects of LNG treatment were detected not until adult age.

Recently, we reported that larval exposure to LNG impairs thyroid hormone–dependent development (Lorenz et al., 2011) of Xenopus laevis. The present paper focuses on potential LNG impacts on sexual development occurring in the course of metamorphosis.

The reproductive system is regulated by the hypothalamus-pituitary-gonad (HPG) axis. The gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), synthesized, and released from the pituitary are primarily known to trigger gonadal steroidogenesis and gametogenesis (Kloas and Lutz, 2006). However, the presence of both hormones and their receptors already in premetamorphic tadpole stages (Urbatzka et al., 2010) suggest a relevance of gonadotropins for the endocrine maturation of the reproductive system as well as for gonad differentiation. Synthesis of sex steroids requires a number of proteins, which are directly or indirectly gonadotropin dependent (Urbatzka et al., 2009; Weltzien et al., 2004). Thus, endocrine disruption of the reproductive system can be mediated via affecting pituitary gonadotropin synthesis/release or gonadal steroidogenesis. The progestin LNG is designed to exhibit progestogenic actions. That is, in mammals with respect to gonadotropin regulation, the repression of pituitary responsiveness to gonadotropin–releasing hormone and consequently the inhibition of gonadotropin synthesis and release (Nett et al., 2002). Additionally, LNG exhibits androgenic activity. In X. laevis, exposure to testosterone or methyl-dihydrotestosterone was demonstrated to significantly decrease LHβ messenger RNA (mRNA) expression (Urbatzka et al., 2006, 2009). In addition to indirect actions on steroidogenesis via regulatory effects on gonadotropins, androgens may directly regulate gonadal steroidogenic enzymes as well (Baron et al., 2005). Thus, the present study was based on the hypothesis, that chronic LNG exposure over the period of larval development alters endocrine maturation of the reproductive system and accordingly gonadal steroidogenesis and gametogenesis in X. laevis. To this end, a long-term exposure of X. laevis tadpoles was performed in a flow-through system, starting at the premetamorphic Nieuwkoop and Faber (NF) stage 48 (Nieuwkoop and Faber, 1994). At NF 58 and after completion of metamorphosis (NF 66), brain–pituitary and gonadal tissue were dissected to analyze mRNA expression of pituitary gonadotropins (LHβ; FSHβ) and gonadal steroidogenic enzymes (steroid-5-alpha-reductase type 1 and 2, steroidogenic acute regulatory protein, P450 side-chain cleavage enzyme, and aromatase). Furthermore, gonadal development was evaluated gross morphologically and histologically to complement the insight into LNG induced processes affecting sexual development.

MATERIALS AND METHODS

The experimental design of the respective exposure experiment was recently described in detail (Lorenz et al., 2011). In the following, a brief description is given.

Chemicals. D(−)-Norgestrel (99%; LNG) obtained from Sigma-Aldrich (Steinheim, Germany) was used for preparation of LNG stock solutions and high-performance liquid chromatography-mass spectrometry/mass spectrometry (HPLC-MS/MS) standards. To achieve the desired test concentrations, ethanol (≥ 99.5%, Roth, Karlsruhe, Germany) was used as solvent. Milli-Q grade water was used for preparation of stock solutions. E2OH concentration in the stock solutions was 0.1%.

Animals and husbandry. Adult X. laevis were taken from the breeding stock of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries. Spawning was induced by injection of human chorionic gonadotropin (Sigma-Aldrich) into the dorsal lymph sac. Fertilized eggs and tadpoles were reared in 50-l tanks. Tanks were aerated, temperature was adjusted to 22°C ± 1°C, and photoperiod to 12:12 h (light:dark). Seven days post fertilization (dpf; NF 45–46) tadpoles were transferred into 7-l glass aquaria into a flow-through culture system and were acclimatized until exposure started in water according to standards of the American Society for Testing and Materials (Lutz et al., 2008).

Experimental conditions. The LNG exposure test was performed starting with premetamorphic tadpoles at NF stage 48 (dpf 11) and carried out until two sampling points: the first, when tadpoles had reached prometamorphic stage 58 (sampling 1) and the second, when tadpoles had metamorphosed completed (NF stage 66; sampling 2). A flow-through culture system with three parallel tanks per treatment was used to maintain constant conditions over the period of the experiment. Tadpoles at a density of 25 tadpoles per 7-l tank were exposed to the following nominal LNG concentrations: 10⁻¹¹, 10⁻¹⁰, 10⁻⁹, and 10⁻⁸M that correspond to 3.124, 31.24, 312.4, and 3,124 ng/l, respectively, and a solvent control (SC) containing 0.0005% E2OH. Tadpoles that died were removed from the tanks, and mortality was recorded. The experimental conditions are described in detail in Lorenz et al. (2011).

The experiment was conducted in compliance with the local animal protection committee (LAGESO G 0078/08).

Sampling. Two sampling points were defined. Sampling 1 included only one of three parallel treatment tanks (n = 25 tadpoles per treatment). Each tadpole that had reached NF stage 58 was removed from the test tank and euthanized by immersion in MS 222 (tricaine methanesulfonate). Prior to tissue sampling, gonads were photographed and sex was determined by assessment of gonad gross morphology using a binocular microscope. For gene expression analyses, brain–pituitary and gonad tissue were dissected from six males and six females. Samples were immediately frozen in liquid nitrogen and stored at −80°C until further processing.

Sampling 2 included tadpoles from the two remaining replicate tanks (n = 50), which were continuously treated with LNG. Each tadpole that had completed metamorphosis (NF stage 66) was removed from the test tank. Exposure was conducted for 80 days, and all animals that had failed to achieve metamorphosis within that period were sampled at study day 80. Prior to tissue sampling, tadpoles were euthanized as described above. Gonads were photographed, and sex was recorded.

For gene expression analyses, brain–pituitary tissue and additionally one part of the paired gonad were dissected from eight males and eight females per replicate tank that had completed metamorphosis. Because 10⁻⁸M LNG treatment caused developmental arrest in the majority of tadpoles (Lorenz et al.,
sections of 5 μm thickness were mounted on glass slides and stained with hematoxylin and eosin.

**Statistics.** All statistical analyses were performed using the software GraphPad Prism4 (GraphPad Software, Inc.). Data sets were first analyzed for normal distribution and homogeneity of variances. Data that met these criteria were analyzed by ANOVA following Dunnett’s multiple comparison test to compare control data with all other treatment groups. All other data were analyzed using the nonparametric Dunn’s multiple comparison test to determine whether significant differences existed between control and the other treatment groups. Differences were regarded as being significant at \( p < 0.05 \).

**RESULTS**

**Organismal Responses**

Organismal responses including mortality, developmental progress, morphometric measurements, and sex ratio are given in Lorenz et al. (2011).

The key finding being of relevance for the discussion of the present data was that LNG at \( 10^{-8} \)M affected metamorphic progress resulting in developmental arrest in 80% of tadpoles from this treatment group (Lorenz et al., 2011).

For simplification, all tadpoles included in sampling 2 are termed as NF 66 tadpoles in the “Results and Discussion” sections, although metamorphosis was not completed in the majority of \( 10^{-8} \)M LNG-exposed animals, and thus, NF 66 was not achieved. Sex ratio was not changed by any treatment.

**Gene Expression**

**Sampling 1 (NF stage 58).** Gene expression of gonadotropins and steroidogenic enzymes was analyzed in brain-pituitary and gonad samples using quantitative PCR.

At NF 58, LHβ mRNA levels were decreased significantly in brain-pituitary tissue of males and females exposed to \( 10^{-9} \) and \( 10^{-8} \)M LNG \(( p < 0.01 \); Fig. 1A). FSHβ mRNA levels were increased in males treated with \( 10^{-9} \) and \( 10^{-8} \)M LNG \(( p < 0.01 \); Fig. 1A) but were not affected in females.
In gonad tissue, gene expression of a subset of steroidogenic factors was examined. Gene expression of steroidogenic acute regulatory protein (StAR) and P450 side-chain cleavage enzyme (p450scc) was not changed by any treatment, neither in males nor in females (Fig. 2A). However, mRNA levels of steroid-5-alpha-reductase type 1 and 2 (Srd5a 1 and 2) were significantly increased in female tadpoles treated with 10^{-8}M LNG (p < 0.05; Fig. 3A) but not affected in males (Fig. 3A). Transcript levels of aromatase (ARO) were unchanged in females (Fig. 3A). In males, ARO mRNA was detectable, but expression was too low for quantification.

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Sampling 2 (NF stage 66). Gene expression analyses of brain-pituitary tissue revealed significantly decreased LHβ mRNA levels in 10^{-8}M LNG-treated males and females (p < 0.01 and p < 0.05, respectively; Fig. 1B). FSHβ mRNA levels were affected sex-specifically. Expression levels were significantly decreased in males treated with 10^{-8}M LNG (p < 0.05), whereas an increase of FSHβ mRNA was detected in 10^{-8}M LNG-treated females (p < 0.05; Fig. 1B).

In gonads, a significant decrease of StAR and p450scc mRNA levels was detected in males (p < 0.01; Fig. 2B) but not in females of the 10^{-8}M LNG treatment group (Fig. 2B). Gene

**FIG. 2.** Relative mRNA expression of steroidogenic acute regulatory protein (StAR) and P450 side-chain cleavage enzyme (P450scc) in gonad tissue of (A) NF 58 females and males and (B) NF 66 females and males after exposure to 10^{-11}, 10^{-10}, 10^{-9}, and 10^{-8}M LNG and SC (0.0005% ethanol). Data are shown as means ± SEM (n = 6 for each sex per treatment at NF 58; n = 8 for each sex per treatment at NF 66) and significant differences compared with SC are marked by asterisks (**p < 0.01, Dunn’s multiple comparison test).**

**FIG. 3.** Relative mRNA expression of aromatase (ARO), steroid-5-alpha-reductase (Srd5a) type 1 and 2 in gonad tissue of (A) NF 58 females and males and (B) NF 66 females and males after exposure to 10^{-11}, 10^{-10}, 10^{-9}, and 10^{-8}M LNG and SC (0.0005% ethanol). In males, ARO mRNA levels were detectable but due to low expression not quantifiable. Data are shown as means ± SEM (n = 6 for each sex per treatment at NF 58; n = 8 for each sex per treatment at NF 66) and significant differences compared with SC are marked by asterisks (*p < 0.05, Dunn’s multiple comparison test).
expression of ARO was significantly repressed by $10^{-8}$M LNG exposure in females ($p < 0.05$; Fig. 3B). In males, ARO mRNA was detectable, but expression was too low for quantification.

Treatment with $10^{-8}$M LNG induced Srd5a 1 mRNA expression in females ($p < 0.05$; Fig. 3B) and reduced Srd5a 2 mRNA expression in males ($p < 0.05$; Fig. 3B).

**Gonad Gross Morphology**

At NF 66, prior to tissue dissection, gonads were photographed to evaluate possible changes in gross morphology. Testes of males from the SC group were characterized by compact and smooth tissue (Fig. 4A). Treatment with the three lowest LNG concentrations did not impact testes gross morphology. However, males exposed to $10^{-8}$M LNG exhibited poorly developed testes composed of translucent tissue appearing less compact compared with SC (Fig. 4B). In an attempt to quantify LNG effects on testes development, testes length was measured revealing significantly shorter testes in the $10^{-8}$M LNG treatment group ($p < 0.005$; Fig. 5).

In females from the SC group, ovaries revealed segmented lobular structure and clearly visible internal melanocytes (Fig. 6A). Compared with testes, ovarian tissue appeared less dense. Remarkably, a high variability of gonadal development was observed depending on age at completion of metamorphosis, when tissue sampling occurred. The higher the age of the tadpoles, the more enhanced was gonad development.

![FIG. 4.](image1)

![FIG. 5.](image2)
Treatment with LNG did not disrupt ovarian development and structure as observed in male testes. Instead, females exposed to $10^{-8}$M LNG were characterized by advanced ovary development indicated by a more pronounced lobular structure compared with SC ovaries (Fig. 6B). Histological evaluation revealed a high variability of germ cell maturation stages within all treatment groups correlated with age at tissue sampling. The higher the age, the more advanced was ovarian development. Gonad sections of SC group revealed immature testes containing varying quantities of primary spermatogonia (Fig. 4C). Treatment with LNG did not alter gonad maturation of male animals. However, although germ cell maturation was not affected, the majority of $10^{-8}$M LNG-exposed males exhibited testicular tissue characterized by an increased occurrence of dilated tubules indicating qualitative abnormalities of those testes (Fig. 4D).

In females, LNG exposure did not cause the disruption of ovarian tissue. However, as observed macroscopically, a high variability of gonadal development was assessed within females of all treatment groups. The higher the age at tissue sampling, the more advanced was ovarian development and germ cell maturation comprising different proportions of oogonia and primary oocytes (Figs. 6C and 6E).

**Gonad Histopathology**

Histopathological evaluation of gonad sections of males from the SC group revealed immature testes containing varying quantities of primary spermatogonia (Fig. 4C). Treatment with LNG did not alter gonad maturation of male animals. However,
In the $10^{-8}$M LNG treatment group, according to their higher age at sampling 2 (see Lorenz et al., 2011), females possessed mainly ovaries of advanced development compared with the SC group. As observed in all other treatment groups, composition of the different germ cell stages varied according to the tadpoles’ age at tissue sampling. Females that completed metamorphosis within the experimental period of 80 days exhibited ovaries comprising a substantial amount of early primary oocytes. However, all $10^{-8}$M LNG-treated female tadpoles that did not complete metamorphosis and were therefore sampled not until the finalization of the experiment (study day 80) possessed ovaries of clearly advanced maturation stages with a high fraction of primary oocytes (Figs. 6D and F).

**DISCUSSION**

Because metamorphosis of most amphibians occurs in the aquatic environment, sexual development may be affected by exposure to xenobiotics, including progestins. Previously, a strong inhibitory effect of progestin exposure on fish (Paulos et al., 2010; Zeilinger et al., 2009) and amphibian (Kvarnryd et al., 2011) reproduction has been reported. However, the data collected in these studies do not elucidate the underlying molecular mechanisms.

Recently, we reported a metamorphosis disrupting effect of LNG treatment over the period of larval development (Lorenz et al., 2011). The present paper deals with impacts of larval exposure to LNG on sexual development of *X. laevis*, thereby focusing on molecular and histological endpoints. The data obtained strongly suggest an endocrine disruption of reproductive function by LNG presumably by the impairment of pituitary gonadotropin and gonadal steroidogenic gene expression during the period of larval development.

LNG exposure was conducted from metamorphic tadpole stages until the end of metamorphosis including sensitive stages for sex reversal (Villalpando and Merchant-Larios, 1990). LNG is known to possess androgenic activity (Sitruk-Ware, 2008), and LNG treatment of adult fish induced strong masculinization of secondary sex characteristics (Zeilinger et al., 2009). In contrast, LNG exposure over the period of larval development of *X. tropicalis* was reported not to affect sex ratio (Kvarnryd et al., 2011). Consistently, no effects on sex ratio were observed in the present study by gross morphological and histological evaluation of the gonads (Lorenz et al., 2011). Analyses of gene expression patterns and gonad histopathology showed some sex-specific responses to LNG treatment. Common to both sexes was a strong repression of LHβ mRNA at NF 58 after exposure to $10^{-9}$ and $10^{-6}$M LNG being persistent until NF 66 in the $10^{-8}$M LNG treatment. In contrast to LHβ, LNG effects on FSHβ gene expression were less consistent regarding time course and sex-specificity. Accounting for progestogenic as well as androgenic properties of LNG, the changes of gonadotropin mRNAs are likely to be a direct effect at the hypothalamus-pituitary level.

In amphibians, androgens have been shown to feedback on gonadotropin gene expression and plasma levels, thereby having sex-specific and different regulatory effects on LH and FSH (Tsai and Jones, 2005; Tsai et al., 2005; Urbatzka et al., 2006, 2009). Progestogenic effects on gonadotropin mRNA levels are not yet investigated in amphibians. However, in mammals, progesterone affects gonadotropin synthesis and release by repression of pituitary responsiveness to hypothalamic gonadotropin-releasing hormone (Nett et al., 2002).

LH is known to mainly act on steroidogenesis by promoting expression of steroidogenic enzymes (Laughlin et al., 2010; Urbatzka et al., 2009; Weltzien et al., 2004). Furthermore, a role for sexual differentiation during the larval phase is likely since LH receptor mRNA expression is already present at premetamorphic tadpole stages rising with developmental progress (Urbatzka et al., 2010). Based on this, the persistently decreased LHβ mRNA levels detected in the $10^{-8}$M LNG treatment group are suggested to affect gonadal development and function. In males, exposure to $10^{-8}$M LNG resulted in a considerable disruption of testicular structure and function, as evidenced by gene expression data as well as gross morphological and histological evaluation. Treatment until prometamorphic tadpole stage NF 58 did not affect gonad mRNA expression of our candidate genes, suggesting that the HPG feedback was not functional yet. In agreement, no gross morphological abnormalities of the gonads were observed at this time. However, after further treatment until completion of metamorphosis or alternatively finalization of the experiment, gene expression of StAR, P450sc, and Srd5a 2 was significantly decreased. Given the essential and rate-limiting role of these enzymes for steroidogenesis, a reduction of androgen synthesis is assumed. Based on what is known from sex steroid exposure in general, it is likely that there are at least two modes of action leading to altered mRNA levels in testes: (1) a direct LNG action on testicular gene expression particularly related to steroid synthesis (Baron et al., 2005) and (2) an indirect LNG effect via reduced pituitary LH expression and secretion (Zohar et al., 2010).

In females, the strong LHβ mRNA repression in the $10^{-8}$M treatment group was not mirrored by decreased StAR and P450sc gene expression in ovaries after long-term treatment until completion of metamorphosis. Instead, a significant increase of gonadal Srd5a 1 and 2 mRNAs was detected in ovaries from prometamorphic NF 58 females treated with $10^{-8}$M LNG. Remarkably, $10^{-8}$M LNG did not change ovarian Srd5a expression levels, although LHβ mRNA was as strong reduced as in the $10^{-8}$M LNG treatment. Thus, the impact on gonadal Srd5a gene expression might be a direct effect of LNG on the gonads and not mediated via altered gonadotropin levels. In mammals, progestogenic as well as androgenic transcriptional regulation of the Srd5a 1– and 2–encoding genes has been reported (Matsui et al., 2002; Torres
and Ortega, 2003) supporting a direct action of LNG on gonadal gene expression.

*Srd5a* catalyzes the 5α reduction of testosterone (T) to dihydrotestosterone (DHT), thereby competing with the estradiol producing ARO for their common substrate T. Considering that ARO gene expression was significantly decreased in NF 66 females concomitant with upregulated *Srd5a* 1 mRNA, enhanced androgen levels at the expense of estrogen synthesis might be the consequence of 10⁻⁸M LNG treatment. However, due to methodological limitations, no sex steroid plasma levels were determined leaving conclusions concerning circulating hormone hypothetical.

In addition to gene expression analyses, the impairment of gonadal function was further assessed by gross morphological as well as histological evaluation. According to sex-specific gene expression responses, the effects of LNG on gonad morphology differed between males and females. In males, gross morphological evaluation clearly evidenced a negative impact of LNG long-term exposure on gonadogenesis. Testes from 10⁻⁸M-treated males were significantly growth retarded and displayed a translucent appearance being clearly different from the compact testicular structure observed in control males. Qualitative abnormalities of testicular tissue in 10⁻⁸M LNG-treated males were histologically verified by an increased occurrence of dilated tubules.

With reference to the biomolecular data, our results suggest that the disruption of testicular development is associated with changed pituitary gonadotropin and gonadal steroidogenic gene expression. Generally, there is no detailed information about hormonal regulation of early gonad development in amphibians. In a previous study, using *X. laevis* (Urbatzka et al., 2010), ontogenetic expression profiles of pituitary gonadotropins and their gonadal receptors were reported, and it was hypothesized that LH contributes to an accurate testis development.

In *X. laevis*, similar histopathological effects on testis development were previously reported after exposure to finasteride (FIN), an inhibitor of *Srd5a* 2 (Urbatzka et al., 2009). Contrary to the present study, LHβ gene expression was upregulated by FIN probably via negative feedback due to decreased DHT levels. However, a crucial similarity of both studies is the assumed repression of gonadal androgen synthesis. Hence, combining data of both studies suggest that the disruption of testicular development by LNG as well as by FIN is associated with decreased androgen synthesis.

Interestingly, Urbatzka et al. (2009) reported sex-specific differences of pituitary and gonadal gene expression responses to FIN treatment, suggesting differing feedback mechanisms in males and females. Moreover, FIN effects on gonad development varied sex-specifically. Although testis development was severely affected by FIN, no impact was observed in ovaries. According to this, ovarian growth and development was not disrupted by LNG treatment. However, ovaries from females exposed to 10⁻⁸M LNG exhibited advanced development compared with SC animals. In most of these animals, ovaries contained a substantial amount of primary oocytes, whereas the majority of SC animals displayed ovaries containing a high amount of primary and secondary oogonia and clearly less oocytes. Importantly, it has to be considered that exposure to 10⁻⁸M LNG severely affected metamorphosis (Lorenz et al., 2011). Hence, at the day of tissue sampling, tadpoles from this treatment group were considerably older compared with animals from the other groups. A previous study demonstrated that ovarian maturation depends rather on age than on developmental state (Wolf et al., 2010). Based on this, the advanced ovarian maturation stage in 10⁻⁸M LNG-treated females is suggested to be related more to the higher age rather than to accelerated gonad development by direct LNG effects. Nevertheless, in a recent study (Kvarnryd et al., 2011), severe LNG effects on ovary development and fertility were reported using *X. tropicalis*. Notably, the LNG impact was not evident until adult age. LNG was highly effective at a measured concentration of 0.5 × 10⁻⁹M in this study. Our gene expression data confirm LNG effectiveness in this concentration range since 10⁻⁹M exposure significantly decreased LHβ mRNA levels after treatment until NF 58. However, in contrast to our study, Kvarnryd et al. (2011) did not observe any LNG effects on testicular development and male fertility. Presumably, this discrepancy is due to lower LNG concentrations tested in the study of Kvarnryd et al. (2011). The gonadotropin gene expression responses to 10⁻⁹M LNG in NF 58 male tadpoles indicate that LNG affects male sex development at concentrations of approximately 300 ng/l and above.

In summary, the findings presented here clearly demonstrate that LNG affects the HPG axis of *X. laevis* during sexual development at concentrations being approximately 100-fold higher than those usually detected in the environment (STP effluents). The endocrine disruption by LNG is mediated by feedback on pituitary gonadotropins and the modulation of gonadal steroidogenesis. Amphibians are generally established as model system to study endocrine disruption in lower vertebrates, and our results confirm recent studies demonstrating adverse effects of progestin exposure on amphibian and fish reproduction. Hence, contraceptive progestins have necessarily to be taken into account as potent EDs of the reproductive system of aquatic vertebrates.

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