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Review

Endocrine disruption by environmental gestagens in amphibians – A short review supported by new *in vitro* data using gonads of *Xenopus laevis*

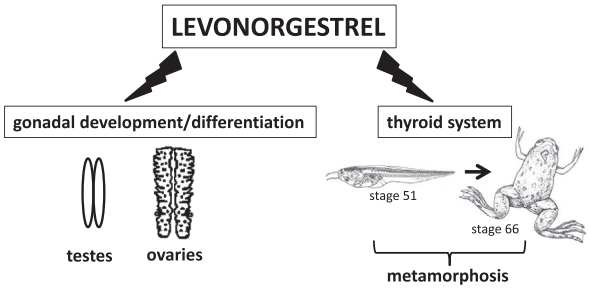
Andrea Ziková [a, *], Claudia Lorenz [a], Frauke Hoffmann [a], Wibke Kleiner [a], Ilka Lutz [a], Matthias Stöck [a], Werner Kloas [a, b]

[a] *Department of Ecophysiology and Aquaculture, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany*
[b] *Department of Endocrinology, Institute of Biology, Humboldt University of Berlin, Berlin, Germany*

HIGHLIGHTS

- Gestagens have impacts on both, reproductive and thyroid system, in amphibians.
- Female gonads are more susceptible to gestagens than male ones.
- Levonorgestrel delayed and disrupted metamorphosis in *Xenopus laevis*.

GRAPHICAL ABSTRACT



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ABSTRACT

Endocrine disruption caused by various anthropogenic compounds is of persisting concern, especially for aquatic wildlife, because surface waters are the main sink of these so-called endocrine disruptors (ED). In the past, research focused on (anti)estrogenic, (anti)androgenic, and (anti)thyroidal substances, affecting primarily reproduction and development in vertebrates; however, other endocrine systems might be also targeted by ED. Environmental gestagens, including natural progestogens (e.g. progesterone (P4)) and synthetic progestins used for contraception, are supposed to affect vertebrate reproduction via progesterone receptors. In the present paper, we review the current knowledge about gestagenic effects in amphibians, focussing on reproduction and the thyroid system. In addition, we support the literature data with results of recent *in vitro* experiments, demonstrating direct impacts of the gestagens levonorgestrel (LNG) and P4 on sexually differentiated gonads of larval *Xenopus laevis*. The results showed a higher susceptibility of female over male gonads to gestagenic ED. Only in female gonads LNG, but not P4, had direct inhibitory effects on gene expression of steroidogenic acute regulatory protein and P_{450} side chain cleavage enzyme, whereas aromatase expression decreased in reaction to both gestagens. Surprisingly, beyond the expected ED effects of gestagens on reproductive physiology in amphibians, LNG drastically disrupted the thyroid system, which resembles direct effects on thyroid glands and pituitary along the pituitary-thyroid axis disturbing metamorphic development. In amphibians, environmental

* Corresponding author. Department of Ecophysiology and Aquaculture, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, 12587 Berlin, Germany.
E-mail address: zikova@igb-berlin.de (A. Ziková).

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gestagens not only affect the reproductive system but at least LNG can impact also development by disruption of the thyroid system.

Contents

1. Introduction

Surface waters are the main sink for environmental pollution by various chemical compounds of anthropogenic origin and therefore there is persisting concern that a number of compounds, including some pharmaceuticals, cause adverse effects on aquatic wildlife by endocrine disruption rather than by direct toxic impacts (Damstra et al., 2002; Kloas et al., 2009; Bergmann et al., 2013; Kumar et al., 2015). Initially, researchers focused on (anti)estrogenic, (anti)androgenic, and (anti)thyroidal substances, the so-called endocrine disruptors (ED), affecting mainly reproduction and development in vertebrates while interacting with their endocrine system (Le Blanc et al., 2011).

Lately environmental gestagens, including the natural progestogens (e.g. progesterone (P4)) and synthetic progestins, have been identified as potential EDs, too (Jenkins et al., 2003; Carson et al., 2008; Besse and Garric, 2009; Liu et al., 2011; Mansell et al., 2011; Fent, 2015; Avar et al., 2016). Gestagens are steroid hormones used for cancer treatment and female contraception. Apart from pharmaceutical products for human use, a variety of industrial chemicals and pesticides displays (anti)gestagenic activities as well (Li et al., 2008, 2010).

Gestagens bind to progesterone receptors (PR) and display important functions in vertebrate reproduction particularly, with regard to gamete maturation, behaviour, and negative feed-back regulation of reproductive cycles (Norris and Carr, 2013; Fent, 2015; Frankel et al., 2016). Progesterone (P4), a derivative of cholesterol and pregnenolone, is the first steroidal hormone giving rise to further gestagens, androgens, estrogens, and corticosteroids. Gestagens are pivotal for final gamete maturation in fish (Tokumoto et al., 2005) and amphibians (Arias Torresa et al., 2016; Ogawa et al., 2011), where they affect the germinal vesicle breakdown (GVBD) (Pickford and Morris, 1999) and sperm motility (Murack et al., 2011). In addition to interactions with nuclear PR, gestagens are able to bind to membrane PR, causing rapid gestagen-mediated biological responses (Thomas, 2000; Thomas and Doughty, 2004). It is known that some progestins can display also androgenic activities by binding to androgen receptors (AR) (Kumar et al., 2015).

However, despite the great importance of gestagens for reproduction in all vertebrates, the close interaction of gestagens, androgens, and estrogens in the regulation of reproductive processes and the crosstalk between these signalling pathways makes clear identification of (anti)gestagenic modes of action inherently difficult. Beyond their endocrine disruptive effects, additional but poorly investigated targets (e.g. photo-transduction cascade and circadian rhythm network) can be also affected (Zhao and Fent, 2016).

Amphibians are suitable and sensitive models to assess endocrine disruption (Kloas, 2002; Kloas and Lutz, 2006). In amphibians, gestagenic effects (Kloas et al., 2009) were found not only on reproductive physiology (Kvarnryd et al., 2011; Lorenz et al., 2011b) and mating behaviour (Hoffman and Kloas, 2012a) but also on the thyroid system (Kloas et al., 2009; Lorenz et al., 2011a, 2016).

Here, we review the current knowledge about impacts of environmental gestagens on amphibians, focussing on the endocrine systems, gonadal development and reproduction as well as the thyroid system. In addition to this brief overview, we provide first empirical *in vitro* data on direct effects of the progestins levonorgestrel (LNG) and progesterone (P4) on immature but sexually differentiated gonads of larval *Xenopus laevis*.

2. Gonadal development

In contrast to fish (Zeilinger et al., 2009; Paulos et al., 2010; Hua et al., 2015; Frankel et al., 2016; Zhao and Fent, 2016), the number of studies investigating impacts of gestagens as ED on reproductive processes in amphibians is rather limited.

2.1. Review of gestagenic impacts on gonads

One potential endpoint for gestagenic effects, the germinal vesicle breakdown (GVBD), was already used as early as 1999 to characterize impacts of ED (Pickford and Morris, 1999). Depending on the examination of intact follicles or denuded oocytes, co-incubation protocols with gonadotropin and/or gestagen and the potential ED of interest are available. Such assays can be performed either directly *in vitro* or after in vivo exposure of the test animals, and the results are evaluated by visual inspection. The natural gestagen P4 stimulated GVBD already at a concentration of 10 nM and the estrogenic pesticide methoxychlor (MCL) was a potent inhibitor of P4 causing inhibition of oocyte maturation with a median concentration of 72 nM, and thus demonstrated clear antigestagenic activity. However, additional experiments, using in parallel the antiestrogen ICI 182,780, revealed that the antigestagenic effect of MCL is not due to suggested estrogenic activity. The authors further showed that MCL did not displace [^{3}H]-P4 from

isolated oocyte plasma membrane, and suggested that MCL may exert its action not directly at the membrane PR. Thus MCL acted rather downstream, during early events in maturational signalling, through interference with P4-regulated processes and by mechanisms other than receptor antagonism (Pickford and Morris, 1999).

Under *in vitro* conditions, pentachlorophenol (PCP) inhibited the ovulatory response for GVBD and ovarian steroidogenesis of *X. laevis* in the nanomolar range (Orton et al., 2009). An additional short-term in vivo exposure of adult females to 0.375 and 3.75 nM PCP over 6 days resulted in minor alterations of plasma steroid hormone levels in vivo and toxic effects on the ovaries, indicated by changes of the *in vitro* steroid production of ovarian follicles, when stimulated by human chorionic gonadotropin (Orton et al., 2009).

Thus the observed antigestagenic endocrine disrupting effects of MCL as well as of PCP might be due to some unknown toxic mechanisms rather than caused by direct specific endocrine interactions affecting the gestagenic pathways.

All other recent studies investigating gestagenic endocrine disrupting effects on amphibians only examined the natural gestagens P4 and/or the progestins levonorgestrel (LNG) and norethindrone (synonymous norethisterone; NET). LNG is a widely distributed synthetic gestagen and ingredient of female contraceptive formulations. It prevents ovulation by exerting negative feedback on pituitary luteinizing hormone (LH) secretion and, furthermore, induces changes in cervical mucus, suppressing penetrability to spermatozoa. The underlying mechanisms are thought to be mediated via the nuclear PR since LNG displays a higher affinity to this receptor, compared to the natural ligand P4 (Africander et al., 2011). In addition, LNG displays also considerable androgenic activity, by binding to the androgen receptor (McRobb et al., 2008). This substance has been detected in surface waters at concentrations up to 30 ng L^{-1} (Kumar et al., 2015). NET as well as P4 also occur in surface waters in the ng L^{-1} range, posing risks for aquatic vertebrates (Runnalls et al., 2010; Creusot et al., 2014; Fent, 2015). Similarly to LNG, NET also poses considerable androgenic activity by androgen receptor binding (McRobb et al., 2008) but exhibits only a comparable binding activity to the nuclear PR as P4 (Kumar et al., 2015).

First evidence that LNG can act as ED in amphibians, demonstrating that larval exposure of *X. laevis* at 5×10^{-7} M did not change sex ratio but disrupted male gonadal development, and delayed metamorphosis, was provided by Kloas et al. (2009). As a follow-up experiment, larval *X. laevis* from developmental stage NF 48 (Nieuwkoop and Faber, 1994) onwards were exposed throughout metamorphosis to LNG ranging from 10^{-11} to 10^{-8} M (Lorenz et al., 2011b). Endpoints measured at stages NF 58 and NF 66 were expressed by the mRNA levels of the hypophyseal gonadotropins, LH, follicle stimulating hormone (FSH), and gonadal steroidogenic factors, namely steroidogenic acute regulatory protein (StAR), P_{450} side-chain cleavage enzyme (P450scc), aromatase (ARO), steroid-5-alpha-reductase type 1 (Srd5a1) and type 2 (Srd5a2) and additionally by histological analysis of the gonads. In both sexes, LH mRNA expression decreased drastically, already at 10^{-9} M LNG at NF 58, and persisted also for 10^{-8} M at NF 66. FSH mRNA was elevated only in males (NF 58) at 10^{-9} and 10^{-8} M, and decreased at NF 66 at 10^{-8} M, whereas in females, only at NF 66 a moderate increase at the concentration of 10^{-9} M LNG occurred. The determination of gonadal steroidogenic factors revealed inhibitory impacts on StAR and P450scc in males at NF 66, while females were not significantly affected. In addition, histological evaluation of gonads also suggested a higher sensitivity of males because testes were only poorly developed, seemingly indicating endocrine disruption (Lorenz et al., 2011b).

2.2. Direct effects of gestagens on gonads of larval Xenopus laevis - in vitro experiment

The existing in vivo studies on impacts of progestins in amphibian gonads have remained insufficient to assess whether effects might directly act on the gonads. To test such effects, we used for the first time an *in vitro* approach to investigate differentiated gonads of male and female *X. laevis* tadpoles. Specifically we focused on the developmental stage NF 58, and applied treatments with LNG being gestagenic and androgenic, in parallel to the natural gestagen P4, resembling pure gestagenic activity, and to the androgen 17 α methyldihydrotestosterone (MDHT), providing pure androgenic activity.

2.2.1. Material and methods

2.2.1.1. Organ culture. Organ culture was performed according to Leal et al. (2009). Briefly: 1,5% agarose was prepared using Ringers solution (147 mM NaCl, 4 mM KCl and 29 mM $CaCl_2$) adjusted to pH 7.4 and autoclaved for 20 min at 121 °C. After cooling down to 60 °C agarose was pour under the sterile conditions into 48 wells plate (ROTH, Karlsruhe, Germany), cooled down over night and afterwards agarose cylinders were transferred into 24 well plates, covered with 22 μm nitrocellulose membrane (ROTH, Karlsruhe, Germany) and each well was filled with 1 ml of basic medium (250 ml Leibovitz L-15 Medium, 10 mM HEPES, 10 nM retinoic acid, 0.5% bovine serum), fungizone (Amphotericine B and Pen-Strep liquid) of pH 7.4.

Tadpoles *of X. laevis* at the developmental stage NF 58 were anesthetized (MS222) and the gonadal-kidney complex was removed for organ culture. Males' and females' gonadal kidney complexes were sorted out on nitrocellulose membrane of an agarose cylinder and incubated at 22 °C for 24 h. The basic medium was changed after 12 h. After 24 h of acclimatization 10 male and 10 female gonadal kidney complexes were exposed to levonorgestrel (LNG), progesterone (P4) and 17 α methyldihydrotestosterone (MDHT) at the concentrations of 10 and 100 nM for 24 h. Experimental chemicals were dissolved in basic medium that was renewed after 12 h. After the exposure, only the gonads were transferred into Eppendorf tubes with 5 μl RNALater stabilization reagent (Qiagen, Hilden, Germany), put into liquid nitrogen and frozen at −80 °C until further analyses.

2.2.1.2. RNA-extraction and reverse transcription. Total RNA was isolated using RNA extraction kits of Qiagen (RNeasy Micro Kit, Hilden, Germany) according to the manufacturer's instructions with carrier RNA and included on-column DNAse ingestion. The amount and purity of RNA were quantified by UV absorbance measurements (260/280 and 230/280 ratio) using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Germany). The analysis of the RNA integrity was performed by Agilent RNA 6000 Nano Kit (Agilent Technologies, Germany) and ranged between 8.4 and 9.6.

Reverse transcription was performed following manufacturer's instruction (AffinityScript QPCR cDNA Synthesis Kit; Agilent Technologies, Berlin, Germany). To test for the potential presence of genomic contaminations, negative control samples were prepared omitting the AffinityScript RT/RNase Block enzyme mixture. Samples were stored at −20 °C until the use for quantitative real time PCR (qPCR).

2.2.1.3. Quantitative real-time PCR. Amplifications of cDNA were run in a Stratagene MX3000 (Agilent, Germany) and PCR reactions were performed with 2 μL cDNA (only in case of mPR was cDNA 1:5 diluted prior to PCR amplification) in 20 μL reaction volume (2.5. or 5 μM primer, PCR buffer, 2 or 3 mM $MgCl_2$, 10 mM dNTPs (Qiagen,

Germany)), 1:200 diluted SYBR-Green solution (Invitrogen, Germany), and 1 U Platinum Taq Polymerase, under the following thermal cycling conditions: initial denaturation at 95 °C for 7 min 40 s, followed by 40 cycles of denaturation at 95 °C for 17 s, primer annealing for 25 s (annealing temperatures in Table 1) and extension at 72 °C for 25 s. Primers were derived from Urbatzka et al. (2009) and Josefsberg Ben-Yehoshua et al. (2007). Reference samples (calibrator) used to determine relative amounts of target transcript were produced by pooling cDNA samples from different treatments. PCR reactions were run in duplicates for all samples.

2.2.1.4. Data analysis and statistics. QPCR data were analyzed using MxPro software (Stratagene) by means of the comparative CT method (ΔΔCT), including the corresponding amplification efficiencies (Pfaffl, 2001). The reference gene elongation factor 1α (EF1a) was used as an internal standard. Expressions of target genes were normalized to the corresponding level of EF1α mRNA. In addition to samples without reverse transcriptase, reactions where the cDNA template was replaced by DNAse/RNAse free water (Invitrogen, Germany) served as additional negative controls to check for the target specificity of the cDNA amplification.

Statistical analyses of mRNA expression were performed using Graphpad Prism 4 software. Data were tested for criteria of normality using Kolmogorov–Smirnov. If data were normally distributed, effects of NPX on gene expression levels were determined by testing for homogeneity of variance across groups, an analysis of variance (one-way ANOVA) and the differences among test groups were assessed with the Tukey's test. For non-normally distributed data Kruskal–Wallis test and Dunn's Multiple Comparison test was used. Significances are indicated by asterisks for $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Gonadal mRNA expression was assessed for the steroidogenic factors StAR, P450scc, ARO, Srd5a1 and Srd5a2 and, in addition, the membrane progesterone receptor (mPR) and deiodinases type 2 (DIO 2) and type 3 (DIO 3), relevant for thyroid hormone metabolism. Gonadal steroidogenic factors are expressed in a sex-specific manner in untreated controls (Fig. 1).

ARO-mRNA was much greater in female gonads, whereas Srd5a2-mRNA was stronger expressed in male gonads, while mPR-mRNA was more abundant in female gonads. All further steroidogenic factors, DIO 2 and DIO 3, did not differ between both sexes.

LNG treatments revealed lowered mRNA expression for StAR and P450scc in the ovaries (Fig. 2). Contrary, P4 seemed to have no impact neither on StAR-mRNA nor P450scc but decreased significantly ARO in females, similarly to LNG.

In male gonads all treatments were without significant differences compared to controls but showed at least similar tendencies (Fig. 2). Thus, because MDHT treatment did not reveal any significant changes, it is obvious that LNG does not only interfere with PR and acts via hypothalamic and hypophyseal mechanisms but exerts some direct effects on the gonads at early stages of steroidogenesis that are neither associated with pure gestagenic effects of P4 nor with androgenic activities of MDHT.

3. Amphibian mating behaviour and reproduction

Exposure of *Xenopus tropicalis* at low LNG concentrations of 0.06 and 0.5 nM throughout the larval development changed neither sex ratio nor obviously affected gonadal development of both sexes at the end of metamorphosis (Kvarnryd et al., 2011). After larval exposure the juveniles were kept further without LNG treatment until maturation and females treated with 0.5 nM LNG completely lacked oviducts, displayed a larger fraction of immature oocytes that were arrested in meiotic prophase and thus being infertile, compared to control females. The males, however, displayed a normal gonadal development and were fertile as controls, suggesting that for long-term consequences of LNG exposure female individuals are more susceptible. LNG exposure of adult female *X. tropicalis* for 28 days, ranging from 1.3 to 1240 ng L^{-1}, reduced the proportions of immature, vitellogenic, mature and atretic oocytes (Säfholm et al., 2012). Even at the lowest concentration of 1.3 ng L^{-1} LNG, the proportions of previtellogenic oocytes increased, while the percentage of vitellogenic oocytes decreased, indicating inhibition of vitellogenesis and interruption of germ cell progression. Larval exposure of *X. tropicalis* to 0.1 nM ethinylestradiol (EE2) separately, or in combination with LNG (0.01, 0.1, 1.0 nM) was used to assess potential impacts of co-exposure of LNG and EE2 on sex ratio and hepatic mRNA expression of vitellogenin, androgen, estrogen, and progesterone receptors (Säfholm et al., 2015). EE2 treatment alone increased a female-biased sex ratio and vitellogenin-mRNA expression as expected. The co-exposure with LNG did not alter the strong estrogenic EE2 effects. LNG alone increased mRNA levels of AR only in females, becoming abolished by co-exposure to EE2. More recently, Säfholm et al. (2016) performed a similar LNG exposure during early life stages of *X. tropicalis* (NF 48 until completion of metamorphosis) as published earlier (Kvarnryd et al., 2011) but, in contrast to the previous experiment, no dramatic effects on oviductal agenesis were revealed. However, sampling in Säfholm et al. (2016) was done only 4 weeks after completion of metamorphosis whereas in the earlier study (Kvarnryd et al., 2011) oviductal agenesis was observed at a later time point in 9 month old mature females. Thus the time required to result in a complete loss of oviducts might be greater than 4 weeks. However, determination of molecular and histological endpoints suggested (Säfholm et al., 2016) that LNG might perturb anti-Müllerian hormone and PR expression and hereby alter oviduct development.

With focus on an important behavioural endpoint for reproduction, namely male mating calls in *X. laevis* during short-term exposure of 4 days, LNG and P4 have been investigated, both at

Table 1
Primer sequences and annealing temperatures: EF 1-a (elongation factor 1-α), ARO (aromatase), Srd5a1 (steroid 5-α-reductase type 1), Srd5a2 (steroid 5-α-reductase type 2), P450scc (P_{450} side-chain cleavage enzyme), StAR (steroidogenic acute regulatory protein), mPR (membrane progesterone receptor), DIO 2 (deiodinase type 2), DIO 3 (deiodinase type 3).

	Primer sequence, forward (5′-3′)	Primer sequence, reverse (5′-3′)	Annealing temp. [°C]
EF 1-a	ACCGCACAGGTTATCATC	CAACAATGGCAGCATCTC	62
ARO	CGGTTCCATATCGTTACTTCC	GCATCTTCCTCTCAATGTCTG	62
Srd5a1	CTGAACCTCTTGGCTATG	GATGCCTAACTCGGATTG	62
Srd5a2	CTTATCCTGCTGCTTATG	AGTCCTGTGGAAATAGTG	62
P450scc	CAGTGTTGGCCAGGATTTTGT	GCGGAAGAGCTCATTGGTCAG	62
StAR	AACCCAAATGTCAAGGAAGTCAAG	ACAAAATCCCGGGCCCCTACAATA	62
mPR	ATAAGCCCTGTTGTCCACCG	TCTGGTGACCGTGCCCTATA	65
DIO 2	GTTGCCGACTTTGTGTTGGTGT	CGTTCTTCTTGGCTTCTGTGTTTC	62
DIO 3	AGGCAACGGGACACAATAAC	GTCGTTTGGTCGCACTTTTT	62

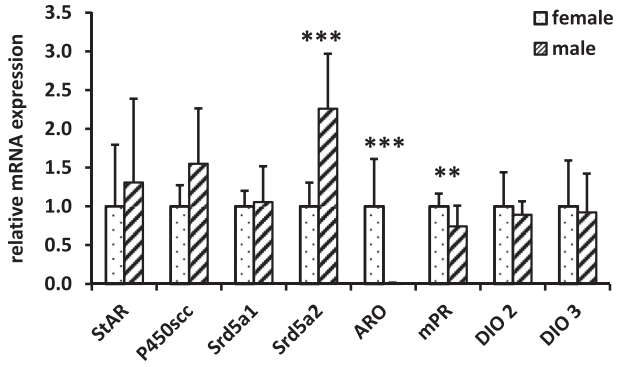

Fig. 1. mRNA-expression of the steroidogenic factors StAR (steroidogenic acute regulatory protein), P450scc (P_{450} side chain cleavage enzyme), ARO (aromatase), Srd5a1 (steroid-5-alpha-reductase type 1) and Srd5a2 (type 2), the membrane progesterone receptor (mPR) and deiodinases type 2 (DIO 2) and type 3 (DIO 3) using testes and ovaries of *X. laevis*, stage 58. Statistical comparisons between male and female gonads (n = 10) of untreated controls are expressed as mean ± SD and analyzed by Mann-Whitney *U* test, significant differences are indicated by asterisks (**$p < 0.01$; ***$p < 0.001$).

concentrations of 10^{-10}, 10^{-8}, and 10^{-7} M (Hoffman and Kloas, 2012a). P4 did not affect any of the male mating call parameters, whereas LNG produced significant effects, already at 10^{-10} M, indicating sexual arousal. However, that finding might be attributed to the considerable androgenic activity of LNG, causing similar impacts on male mating calls as the androgen MDHT (Hoffman and Kloas, 2012b).

In order to investigate the impacts of another progestin, NET, on ovarian gonadogenesis, adult female *X. tropicalis* have been exposed to 1, 10, and 100 ng L^{-1} NET and in parallel to 10 and 100 ng L^{-1} P4 for 28 days by Säfholm et al. (2014). Even at the lowest concentrations, both treatments caused an increased proportion of previtellogenic and a decreased one of vitellogenic oocytes, suggesting general inhibition of vitellogenesis. Thus both progestins, LNG and NET, as well as P4 pose similar gestagenic effects on ovarian gonadogenesis.

4. Thyroid system

The first indication that LNG at higher concentrations might delay and interfere with metamorphosis, thus affecting beside reproductive processes also the thyroid system, was obtained by Kloas et al. (2009) and became later verified by Lorenz et al. (2011a) exposing *X. laevis* tadpoles at stage NF 48 to LNG in a flow-through system at concentrations ranging from 10^{-11} to 10^{-8} M. Tadpoles were sampled just before metamorphic climax at stage NF 58 and after completion of metamorphosis at stage NF 66. Developmental time and organismal responses were assessed and correlated with molecular and histopathological endpoints. The highest concentration of 10^{-8} M LNG arrested metamorphosis at early climax stages or revealed tailed juvenile frogs. Subsampling of tadpoles at stage NF 58 showed no changes in mRNA expression of thyroid stimulating hormone β (TSHβ), thyroid hormone receptor β (TRβ) and DIO 3 in pituitary-brain tissue. However, prolactin (PRL) mRNA, also known as anti-metamorphic hormone, was increased at 10^{-9} M LNG in females and at 10^{-8} M in both sexes. At NF 66, TSHβ mRNA was significantly increased in 10^{-9} and 10^{-8} M LNG treatments, indicating a hypothyroidal state. No further changes in mRNA expression of TRβ, DIO 3, and PRL were observed. In addition, histopathological assessment of the thyroid gland revealed no typical hypothyroidism but rather an inactivated appearance of the thyroid. This indicated a new and unexpected thyroid system

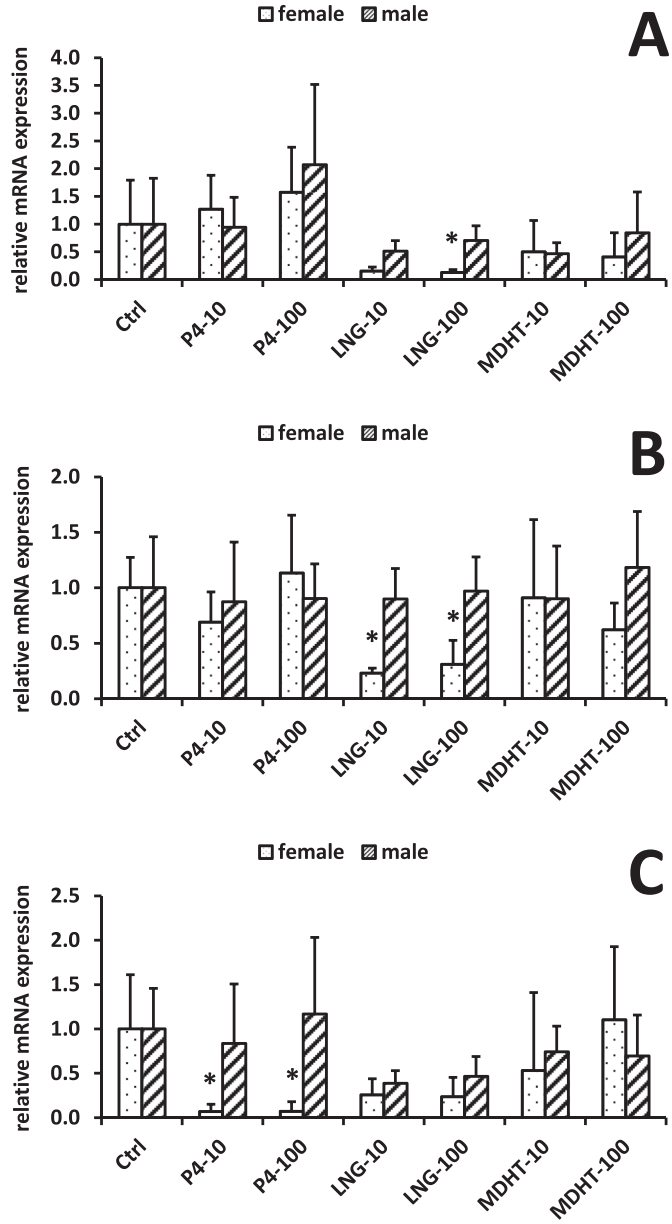

Fig. 2. mRNA-expression of (**A**) steroidogenic acute regulatory protein (StAR), (**B**) P_{450} side chain cleavage enzyme (P450scc), and (**C**) aromatase (ARO) in male and female gonads of larval *Xenopus laevis* (stage NF 58) after the 24 h *in vitro* exposure to 10 and 100 nM of levonorgestrel (LNG), progesterone (P4) and 17 α methyl dihydrotestosterone (MDHT) compared to control. Statistical comparisons among the treatments (n = 5 to 10) using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test is expressed as mean ± SD and significant differences are indicated by asterisks (*$p < 0.05$).

disruption caused by LNG. It is noteworthy that these surprising effects of LNG on the thyroid system occur at a concentration of 10^{-8} M LNG, in the lower range reported for plasma levels of women taking LNG as oral contraceptive (Westhoff et al., 2010).

In order to gain better insights into the mechanisms underlying the LNG-caused effects on the TH system of amphibians, a series of in vivo and *ex vivo* experiments was recently conducted in *X. laevis* by Lorenz et al. (2016). Prometamorphic tadpoles (stage NF 56) were exposed for three days to LNG at concentrations ranging from 0.01 to 10 nM. In brain-pituitary tissue mRNA expression of TSHβ, DIO 2, and DIO 3 was not significantly altered. However, in the thyroid gland mRNA expression of slc5a5 (encoding for sodium

Table 2
Main results of previous studies including new data: DIO 2, 3 (deiodinase type 2, 3), EE2 (ethinylestradiol), FSH, (follicle stimulating hormone), GSI (gonadosomatic index), GVBD (germinal vesicle breakdown), iyd (encoding for iodotyrosine dehalogenase), LH (luteinizing hormone β), LNG (levonorgestrel), MCL (methoxychlor), mRNA (messenger RNA), NET (norethindrone/norethisterone), P4 (progesterone), PCP (pentachlorophenol), PRL (prolactin), slc5a5 (encoding for NIS), StAR (steroidogenic acute regulatory protein), TPO (thyroidperoxidase), TRβ (thyroid hormone receptor β), TSHR (TSH receptor), TSHβ (thyroid stimulating hormone β).

Substance	Concentration	Animal species, developmental stage, sex	Study	Effect	Reference
LNG	5×10^{-7} M	*X. laevis* (NF 50–66)	in vivo	No change of sex ratio, however disruption of male gonadal development, delayed metamorphosis.	Kloas et al. (2009)
LNG	10^{-11},10^{-10},10^{-9}, and 10^{-8} M	*X. laevis* NF 48 (sampling at NF 58 and NF 66)	in vivo flow-through	Exposure to 10^{-8} M caused an inhibition of metamorphosis (developmental arrest: giant tadpoles or tailed frogs), at the stage NF 58 no changes in mRNA expression of TSHβ, TRβ and DIO 3 in pituitary-brain tissue. PRL was increased at 10^{-9} M in females and at 10^{-8} M in both sexes. At NF 66, TSHβ mRNA significantly increased in 10^{-9} and 10^{-8} M (hypothyroidal state). Histopathology: thyroid gland - no typical hypothyroidism but rather an inactivated and disrupted appearance of the thyroid.	Lorenz et al. (2011a)
LNG	10^{-11},10^{-10},10^{-9}, and 10^{-8} M	*X. laevis* NF 48 (sampling at NF 58 and NF 66)	in vivo flow-through	Suppression of LH mRNA in both sexes already at 10^{-9} M at NF 58, and also for 10^{-8} M at NF 66. FSH mRNA sex specific: elevated only in males (NF 58) at 10^{-9} and 10^{-8} M, and decreased at NF 66 at 10-8 M, whereas in females, only at NF 66 a moderate increase at the concentration of 10^{-9} M. StAR and P450scc no change and difference at NF 58 and decrease only in males at NF 66. At 10-^{8} M, Srd5a 1 and 2 increase only in females (NF 58), Srd5a 1 induction in females (NF 66) and reduction of Srd5a 2 in males (NF 66). Histology: poor development of male gonads.	Lorenz et al. (2011b)
LNG	0.06 nM, 0.5 nM	*X. tropicalis* (from NF 47–48 until metamorphosis)		No difference in sex ratio between the treatments and the control group at the end of metamorphosis. After larval exposure the juveniles were kept further without LNG treatment until maturation. Females treated with 0.5 nM LNG completely lacked oviducts, displayed a larger fraction of immature oocytes (arrested in meiotic prophase - being infertile). Males normal gonadal development and were fertile as controls.	Kvarnryd et al., 2011
LNG	10^{-10}, 10^{-8}, and 10^{-7} M	*X. laevis* adult males	in vivo (4 d)	Increased the proportion of advertisement calling at all concentrations due to the androgenic activity of LNG.	Hoffman and Kloas (2012a)
LNG	51 or 307 ng L^{-1} (7 d) 1.3, 18, 160, or 1240 ng L^{-1} (28 d)	*X. tropicalis* adult females	in vivo (7 or 28 d)	Histology: increase of previtellogenic and immature oocytes at 304 ng L^{-1} after 7 d. Decreased GSI and reduced cloacal length occured at 1240 ng L^{-1} after 28 d of exposure. Reduction of oocytes at immature, vitellogenic, mature stages + increase of privitellogenic oocytes (28 d). Even at the lowest concentration of 1.3 ng L^{-1} LNG, the proportions of previtellogenic oocytes increased, while the percentage of vitellogenic oocytes decreased.	Säfholm et al., 2012
LNG + EE2	0.01; 0.1; 1.0 nM LNG + 0.1 nM EE2	*X. tropicalis* larvae		Exposure with LNG at various concentrations and co-exposure of LNG with 0.1 nM EE2 starting at stage 47/48 throughout metamorphosis. EE2 treatment alone increased female-biased sex ratio and vitellogenin-mRNA expression as expected. The co-exposure with LNG did not alter the strong estrogenic EE2 effects. LNG alone increased mRNA levels of AR only in females, becoming abolished by co-exposure to EE2.	Säfholm et al., 2015
LNG	3, 30, 300 ng L^{-1}	*X. tropicalis*, NF 48 until the end of metamorphosis	in vivo	No dramatic effects on oviductal agenesis were revealed in comparison to Kvarnryd et al. (2011). Sampling already at 4 weeks after completion of metamorphosis compared to the former study investigating 9 months old mature females. No dose-response of LNG. LNG is supposed to perturb expression of anti-Müllerian hormone and of PRs. hereby altering oviduct development.	Säfholm et al. (2016)
LNG	0.1–10 nM	*X. laevis*	in vivo (72 h)	Exposure of stage 56 tadpoles for 72 h to LNG from 0.01 to 10 nM. In brain-pituitary tissue mRNA expression of TSHβ, DIO 2, and DIO 3 was unaltered. However, in the thyroid gland expression of slc5a5 and iyd decreased while DIO2 and DIO3 increased.	Lorenz et al. (2016)
LNG	10 nM + 10 nM T3	*X. laevis*	*in vitro* (48 h)	Pituitaries and thyroid glands were excised from tadpoles stage NF 58 and incubated *in vitro*. Hypophyseal expression of TSHβ decreased whereas DIO2 and DIO3 increased. Thyroidal expression of TSHR, TPO, slc5a5, iyd, DIO2, and DIO3 increased.	Lorenz et al. (2016)
LNG	10 nM, 100 nM	*X. laevis* NF 58, male + female gonads	*in vitro* (24 h)	Lower mRNA expression for StAR and P450scc in the ovaries.	recent study
P4	10^{-10}, 10^{-8}, and 10^{-7} M	*X. laevis* adult males	in vivo (4 d)	Did not affect any of the male mating call parameters.	Hoffman and Kloas (2012a)
P4	10, and 100 ng/L	*X. tropicalis* adult female	in vivo (28 d)	Increased proportion of previtellogenic and a decreased one of vitellogenic oocytes even at the lower concentration.	Säfholm et al. (2014)
P4	10 nM, 100 nM		*in vitro* (24 h)		recent study

(*continued on next page*)

Table 2 (*continued*)

Substance	Concentration	Animal species, developmental stage, sex	Study	Effect	Reference
		X. laevis NF 58, male + female gonads		Significant decrease of ARO mRNA expression in females, no impact neither on StAR nor P450scc mRNA expression.	
NET	1, 10, and 100 ng L^{-1}	*X. tropicalis* adult female	in vivo (28 d)	Even at the lowest concentrations an increased proportion of previtellogenic and a decrease of vitellogenic oocytes.	Säfholm et al. (2014)
MCL	4, 40, 400, 4000 nM	*X. laevis*	*in vitro* assay (defolliculated oocytes) 24 h	GVBD induced by 7.5 nM P4 is inhibited by 40 nM MCL with an IC_{50} value of 72 nM MCL, antigestagenic effect, interference with P4-regulated processes	Pickford and Morris (1999)
PCP	6.25, 62.5, 625, 6250, and 62500 nM	*X. laevis* ovarian follicles	*in vitro*	Inhibited the ovulatory response for GVBD already at 625 nM and ovarian steroidogenesis at 6250 nM.	Orton et al. (2009)
PCP	0.375 and 3.75 nM	*X. laevis* adult females	in vivo (6 d)	Alterations of plasma steroid hormone levels in vivo and toxic effects on the ovaries.	Orton et al. (2009)

iodine symporter (NIS)) and iyd (encoding for iodotyrosine dehalogenase) decreased while that of DIO2 and DIO3 increased. For *in vitro* exposure pituitaries and thyroid glands were excised from tadpoles stage NF 58 and incubated for 48 h. Hypophyseal mRNA expression of TSHβ decreased whereas DIO2 and DIO3 increased whereas in the thyroid gland *in vitro* 10 nM LNG elevated mRNA expression of TSHR, TPO, slc5a5, iyd, DIO2, and DIO3. *In vitro* organ culture of thyroid glands treated with bovine TSH and LNG demonstrated that the effects of LNG were directly induced by LNG decreasing TSH stimulated mRNA expression of slc5a5, and increasing mRNA levels of DIO 2 and DIO 3.

In conclusion, the findings on LNG effects in the thyroid system (Kloas et al., 2009; Lorenz et al., 2011a, 2016) indicate that LNG directly alters thyroidal mRNA expression. This is important for the thyroid hormone synthesis and metabolism and furthermore LNG disrupts the negative feedback loop of TSHβ and stimulates antimetamorphic PRL gene expression. This leads to the observed metamorphosis-disrupting effects of chronic LNG treatment. In combination, these effects could account for the absence of histological signs of compensatory TSH overstimulation in hypothyroid tadpoles as observed after chronic LNG treatment (Lorenz et al., 2011a). However, although the anti-thyroidal actions of LNG are certainly verified, further experiments are required to identify intracellular mechanisms of LNG action. Moreover, it is of interest whether the anti-thyroidal actions are unique to LNG, or a common property of all synthetic gestagens.

5. Conclusions and perspectives

The emerging evidence that environmental compounds pose considerable risks of endocrine disruption in surface waters by gestagenic activities has been mostly demonstrated by studies dealing with fish and have demonstrated that these aquatic vertebrates are very sensitive to progestins disrupting reproductive

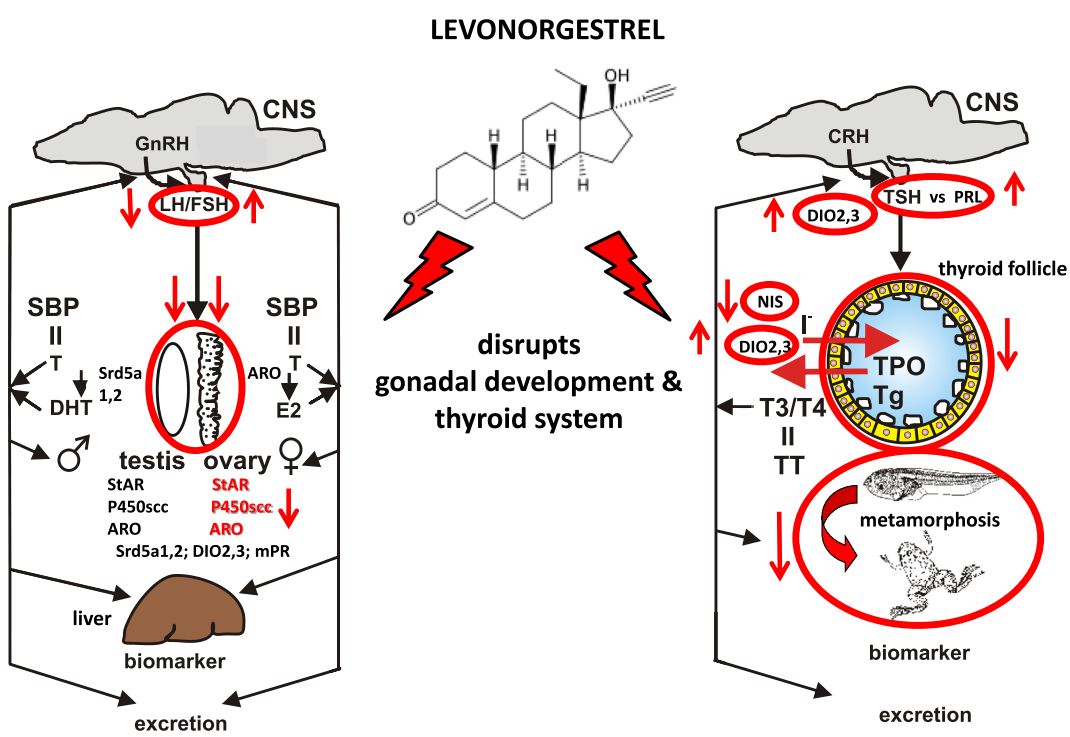

Fig. 3. Schematic summary of in vivo and *in vitro* effects of the most investigated progestin levonorgestrel (LNG) on gonadal and thyroid system. Red arrows indicate stimulatory or inhibitory impacts. ARO (aromatase), CNS (central nervous system), CRH (corticotropin-releasing hormone), DHT (dihydrotestosterone), DIO 2, 3 (deiodinases type 2, type 3), FSH (follicle stimulating hormone), GnRH (gonadotropin releasing hormone), LH (luteinizing hormone), mPR (membrane progesterone receptor), NIS (sodium/iodide symporter), P450scc (P_{450} side chain cleavage enzyme), PRL (prolactin), SBP (sexual steroid binding protein), Srd5a1, 2 (steroid-5-alpha-reductase type 1, type 2), StAR (steroidogenic acute regulatory protein), T3 (triiodothyronine), T4 (tetraiodothyronine/thyroxine), TSH (thyroid stimulating hormone), TT (transthyretin). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

physiology (Kumar et al., 2015). Despite the limited number of studies available for amphibians (see Table 2), similar to fish, they confirm that gestagenic EDs adversely affect the reproductive processes. Importantly, and judging from the few systems which have been examined so far, female amphibians might be more susceptible to progestins than males. Our recent *in vitro* data presented here for the first time show that direct adverse effects of LNG and P4 on the gonads occur in amphibians. The fact that at least the progestin LNG causes a drastic and surprising endocrine disruption of the thyroid system,, specifically by direct and indirect effects on the pituitary and thyroid axis, could only become detected because amphibians are rather sensitive for disruption of the thyroid system (Kloas, 2002; Opitz et al., 2005, 2006a, 2006b, 2009; Opitz and Kloas, 2010). Thus, the combination of in vivo and *in vitro* exposures demonstrated adverse effects for LNG (Lorenz et al., 2011a, 2016) on reproduction and thyroid system. The findings of direct and indirect effects of the most investigated progestin LNG on amphibians affecting reproductive processes and thyroid system are summarized in Fig. 3.

Future research on gestagenic ED effects in amphibians should lead to an extension and intensification, because especially studies focussing on effects of progestins of the second and third generation (Africander et al., 2011; Orlando and Ellestad, 2014; Kumar et al., 2015) are still missing. Furthermore, antigestagenic model compounds, such as mifepristone, should also be included. However, it has to be considered that most progestins also interfere with additional steroid hormone receptors and thus do not solely display gestagenic activities. Consequently and by using in parallel the respective model compounds with single endocrine activity, we here report an experiment, which was specifically designed to assess the complete set of endocrine activities of the compounds of interest. Since the thyroid system is highly conserved among all classes of vertebrates, our finding that the progestin LNG also affects drastically the thyroid system of amphibians, should, in addition to the existing threat for aquatic vertebrates, raise some further concern and stimulate future research to evaluate the potential risks for the thyroid system of women taking progestins for contraception.

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References

Africander, D., Verhoog, N., Hapgood, J.P., 2011. Molecular mechanisms of steroid receptor mediated actions by synthetic progestins used in HRT and contraception. Steroids 76, 636–652. http://dx.doi.org/10.1016/j.steroids.2011.03.001.

Arias Torresa, A.J., Bühlera, M.J., Zelarayána, L.I., 2016. *In vitro* steroid-induced meiosis in *Rhinella arenarum* oocytes: role of pre-MPF activation. Zygote 24, 252–258. http://dx.doi.org/10.1017/S0967199415000143.

Avar, P., Maasz, G., Takács, P., Lovas, S., Zrinyi, Z., Svigruha, R., Takátsy, A., Tóth, L.G., Pirger, Z., 2016. HPLC-MS/MS analysis of steroid hormones in environmental water samples. Drug Test. Anal. 8, 123–127. http://dx.doi.org/10.1002/dta.1829.

Bergmann, A., Heindel, J.J., Jobling, S., Kidd, K.A., Zoeller, R.T., 2013. State of the Science of Endocrine Disrupting Chemicals 2012. Inter-organization Programme for the Sound Management of Chemicals. United Nations Environment Programme and the World Health Organization, Geneva, Switzerland, ISBN 978 92 4 150503 1. http://www.who.int/ceh/publications/endocrine/en/.

Besse, J.P., Garric, J., 2009. Progestagens for human use, exposure and hazard assessment for the aquatic environment. Environ. Pollut. 157, 3485–3494. http://dx.doi.org/10.1016/j.envpol.2009.06.012.

Carson, J.D., Jenkins, R.L., Wilson, E.M., Howell, W.M., Moore, R., 2008. Naturally occurring progesterone in loblolly pine (*Pinus taeda* L.): a major steroid precursor of environmental androgens. Environ. Toxicol. Chem. 27, 1273–1278. http://dx.doi.org/10.1897/07-515.

Creusot, N., Ait-Alissa, S., Tapie, N., Pardon, P., Brion, F., Sanchez, W., Thybaud, E., Porcher, J.M., Budzinski, H., 2014. Identification of synthetis steroids in river water downstream from pharmaceutical manufacture discharges based on a bioanalytical approach and passive sampling. Environ. Sci. Technol. 48, 3649–3657. http://dx.doi.org/10.1021/es405313r.

Damstra, T., Barlow, S., Kavlock, R., Van der Kraak, G.J., 2002. Global Assessment of the State of the Science of Endocrine Disruptors. World Health Organization. http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/.

Fent, K., 2015. Progestins as endocrine disrupters in aquatic ecosystems: concentrations, effects and risk assessment. Environ. Intern. 84, 115–130. http://dx.doi.org/10.1016/j.envint.2015.06.012.

Frankel, T.E., Meyer, M.T., Kolpin, D.W., Gillis, A.B., Alvarez, D.A., Orlando, E.F., 2016. Exposure to the contraceptive progestin, gestodene, alters reproductive behavior, arrests egg deposition, and masculinizes development in the fathead Minnow (*Pimephales promelas*). Environ. Sci. Technol. 50, 5991–5999. http://dx.doi.org/10.1021/acs.est.6b00799.

Hoffman, F., Kloas, W., 2012a. The synthetic progestogen, Levonorgestrel, but not natural progesterone, affects male mate calling behavior of *Xenopus laevis*. Gen. Comp. Endocrinol. 176, 385–390. http://dx.doi.org/10.1016/j.ygcen.2012.02.009.

Hoffman, F., Kloas, W., 2012b. Effects of environmentally relevant concentrations of the xeno-androgen, methyldihydrotestosterone, on male and female mating behavior in *Xenopus laevis*. Chemosphere 87, 1246–1253. http://dx.doi.org/10.1016/j.chemosphere.2012.01.030.

Hua, J., Han, J., Guo, Y., Zhou, B., 2015. The progestin levonorgestrel affects sex differentiation in zebrafish at environmentally relevant concentrations. Aquat. Toxicol. 166, 1–9. http://dx.doi.org/10.1016/j.aquatox.2015.06.013.

Jenkins, R.L., Wilson, E.M., Angus, R.A., Howell, W.M., Kirk, M., 2003. Androstenedione and progesterone in the sediment of a river receiving paper mill effluent. Toxicol. Sci. 73, 53–59.

Josefsberg Ben-Yehoshua, L., Lewellyn, A.L., Thomas, P., Maller, J.L., 2007. The role of Xenopus membrane progesterone receptor β in mediating the effect of progesterone on oocyte maturation. Mol. Endocrinol. 21, 664–673. http://dx.doi.org/10.1210/me.2006-0256.

Kloas, W., 2002. Amphibians as model for the study of endocrine disruptors. Int. Rev. Cytol. 216, 1–57.

Kloas, W., Lutz, I., 2006. Amphibians as model to study endocrine disrupters. J. Chromatogr. A 1130, 16–27.

Kloas, W., Urbatzka, R., Opitz, R., Wuertz, S., Behrends, T., Hermelink, B., Hofmann, F., Jagnytsch, O., Kroupova, H., Lorenz, C., Neumann, N., Pietsch, C., Trubiroha, A., Van Ballegooy, C., Wiedemann, C., Lutz, I., 2009. Endocrine disruption in aquatic vertebrates. Ann. N. Y. Acad. Sci. 1163, 187–200. http://dx.doi.org/10.1111/j.1749-6632.2009.04453.x.

Kumar, V., Johnson, A.C., Tumová, J., Ihara, M., Grabic, R., Kloas, W., Tanaka, H., Kroupová, H.K., 2015. The challenge presented by progestins in ecotoxicological research: a critical review. Environ. Sci. Technol. 49, 2625–2638. http://dx.doi.org/10.1021/es5051343.

Kvarnryd, M., Grabic, R., Brandt, I., Berg, C., 2011. Early life progestin exposure causes arrested oocyte development, oviductal agenesis and sterility in adult *Xenopus tropicalis* frogs. Aquat. Toxicol. 103, 18–24. http://dx.doi.org/10.1016/j.aquatox.2011.02.003.

Leal, M.C., de Waal, P.P., García-López, Á., Chen, S.X., Bogerd, J., Schulz, R.W., 2009. Zebrafish primary testis tissue culture: an approach to study testis function *ex vivo*. Gen. Comp. Endocrinol. 162, 134–138. http://dx.doi.org/10.1016/j.ygcen.2009.03.003.

Le Blanc, G., Norris, D., Kloas, W., Kullmann, S., Baldwin, W., Greally, J., 2011. Draft Detailed Review Paper State of the Science on Novel In Vitro and In Vivo Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors. OECD. http://www.oecd.org/env/ehs/testing/48343294.pdf.

Li, J., Li, N., Ma, M., Giesy, J.P., Wang, Z., 2008. *In vitro* profiling of the endocrine disrupting potency of organochlorine pesticides. Toxicol. Lett. 183, 65–71. http://dx.doi.org/10.1016/j.toxlet.2008.10.002.

Li, J., Ma, M., Wang, Z., 2010. *In vitro* profiling of endocrine disrupting effects of phenols. Toxicol. Vitro 24, 201–207. http://dx.doi.org/10.1016/j.tiv.2009.09.008.

Liu, Z.H., Ogejo, J.A., Pruden, A., Knowlton, K.F., 2011. Occurrence, fate and removal of synthetic oral contraceptives (SOCs) in the natural environment: a review. Sci. Total Environ. 409, 5149–5161. http://dx.doi.org/10.1016/j.scitotenv.2011.08.047.

Lorenz, C., Contardo-Jara, V., Pflugmacher, S., Wiegand, C., Nützmann, G., Lutz, I., Kloas, W., 2011a. The synthetic gestagen levonorgestrel impairs metamorphosis in *Xenopus laevis* by disruption of the thyroid system. Toxicol. Sci. 123, 94–102. http://dx.doi.org/10.1093/toxsci/kfr159.

Lorenz, C., Contardo-Jara, V., Trubiroha, A., Krüger, A., Viehmann, V., Wiegand, C., Pflugmacher, S., Nützmann, G., Lutz, I., Kloas, W., 2011b. The synthetic gestagen Levonorgestrel disrupts sexual development in *Xenopus laevis* by affecting gene expression of pituitary gonadotropins and gonadal steroidogenic enzymes. Toxicol. Sci. 124, 311–319. http://dx.doi.org/10.1093/toxsci/kfr241.

Lorenz, C., Opitz, R., Trubiroha, A., Lutz, I., Zikova, A., Kloas, W., 2016. The synthetic gestagen levonorgestrel is an endocrine disruptor of the amphibian thyroid system: evidence for a direct effect on thyroidal gene expression. Aquat. Toxicol. 177, 63–73. http://dx.doi.org/10.1016/j.aquatox.2016.05.011.

Mansell, D.S., Bryson, R.J., Harter, T., Webster, J.P., Kolodziej, E.P., Sedlak, D.L., 2011. Fate of endogenous steroid hormones in steer feedlots under stimulated rainfall-induced runoff. Environ. Sci. Technol. 45, 8811–8818. http://dx.doi.org/10.1021/es202072f.

McRobb, L., Handelsmann, D.J., Kazlaukas, R., Wilkinson, S., McLeod, M.D., Heather, A.K., 2008. Structure-activity relationship of synthetic progestins in a yeast-based *in vitro* androgen bioassay. J. Steroid Biochem. Mol. Biol. 110, 39–47.

http://dx.doi.org/10.1016/j.jsbmb.2007.10.008.
Murack, P.J., Parrish, J., Barry, T.P., 2011. Effects of progesterone on sperm motility in fathead minnow (*Pimephales promelas*). Aquat. Toxicol. 104, 121–125. http://dx.doi.org/10.1016/j.aquatox.2011.04.006.
Nieuwkoop, P.D., Faber, J., 1994. In Normal Table of *Xenopus laevis* (Daudin). Garland Publishing, Inc., New York, NY.
Norris, D.O., Carr, J.A., 2013. Vertebrate Endocrinology, fifth ed. Academic Press, San Diego, p. 580.
Ogawa, A., Dake, J., Iwashima, Y.K., Tokumoto, T., 2011. Induction of ovulation in Xenopus without hCG injection: the effect of adding steroids into the aquatic environment. Reprod. Biol. Endocrinol. 9, 1–6. http://dx.doi.org/10.1186/1477-7827-9-11.
Opitz, R., Braunbeck, T., Bögi, C., Pickford, D.B., Nentwig, G., Oehlmann, J., Tooi, O., Lutz, I., Kloas, W., 2005. Description and initial evaluation of a Xenopus metamorphosis assay for detection of thyroid-system disrupting activities of environmental compounds. Environ. Toxicol. Chem. 24, 653–664.
Opitz, R., Lutz, I., Nguyen, N.H., Scanlan, T.S., Kloas, W., 2006a. Analysis of thyroid hormone receptor betaA mRNA expression in *Xenopus laevis* tadpoles as a means to detect agonism and antagonism of thyroid hormone action. Toxicol. Appl. Pharmacol. 212, 1–13.
Opitz, R., Trubiroha, A., Lorenz, C., Lutz, I., Hartmann, S., Blank, T., Braunbeck, T., Kloas, W., 2006b. Expression of sodium-iodide symporter mRNA in the thyroid gland of *Xenopus laevis* tadpoles: developmental expression, effects of antithyroidal compounds, and regulation by TSH. J. Endocrinol. 190, 157–170.
Opitz, R., Schmidt, F., Braunbeck, T., Wuertz, S., Kloas, W., 2009. Perchlorate and ethylenethiourea induce different histological and molecular alterations in a non-mammalian vertebrate model of thyroid goitrogenesis. Mol. Cell. Endocrinol. 298, 101–114. http://dx.doi.org/10.1016/j.mce.2008.08.020.
Opitz, R., Kloas, W., 2010. Developmental regulation of gene expression in the thyroid gland of *Xenopus laevis* tadpoles. Gen. Comp. Endocrinol. 168, 199–208. http://dx.doi.org/10.1016/j.ygcen.2010.04.013.
Orlando, E., Ellestad, L., 2014. Sources, concentrations, and exposure effects of environmental gestagens on fish and other aquatic wildlife, with an emphasis on reproduction. Gen. Comp. Endocrinol. 203, 241–249. http://dx.doi.org/10.1016/j.ygcen.2014.03.038.
Orton, F., Lutz, I., Kloas, W., Routledge, E.J., 2009. Endocrine disrupting effects of herbicides and pentachlorophenol: *In vitro* and *in vivo* evidence. Environ. Sci. Technol. 43, 2144–2150.
Paulos, P., Runnalls, T.J., Nallani, G., La, P.T., Scott, A.P., Sumpter, J.P., Huggett, D.B., 2010. Reproductive responses in fathead minnow and Japanese medaka following exposure to a synthetic progestin, Norethindrone. Aquat. Toxicol. 99, 256–262.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.
Pickford, D.B., Morris, I.D., 1999. Effects of endocrine-disrupting contaminants on amphibian oogenesis: methoxychlor inhibits progesterone-induced maturation of *Xenopus laevis* oocytes *in vitro*. Environ. Health Perspect. 107, 285–292.
Runnalls, T.J., Margiotta-Casaluci, L., Kugathas, S., Sumpter, J.P., 2010. Pharmaceuticals in the aquatic environment: steroids and anti-steroids as high priorities for research. J. Hum. Ecol. Risk Assess. 16, 1318–1338.
Säfholm, M., Norder, A., Fick, J., Berg, C., 2012. Disrupted oogenesis in the frog *Xenopus tropicalis* after exposure to environmental progestin concentrations. Biol. Reprod. 86, 1–7. http://dx.doi.org/10.1095/biolreprod.111.097378.
Säfholm, M., Ribbenstedt, A., Fick, J., Berg, C., 2014. Risks of hormonally active pharmaceuticals to amphibians: a growing concern regarding progestagens. Philos. Trans. R. Soc. Lond. B Biol. Sci. 369, 1–7. http://dx.doi.org/10.1098/rstb.2013.0577.
Säfholm, M., Jansson, E., Fick, J., Berg, C., 2015. Mixture effects of levonorgestrel and ethinylestradiol: estrogenic biomarkers and hormone receptor mRNA expression during sexual programming. Aquat. Toxicol. 161, 146–153. http://dx.doi.org/10.1016/j.aquatox.2015.02.004.
Säfholm, M., Jansson, E., Fick, J., Berg, C., 2016. Molecular and histological endpoints for developmental reproductive toxicity in *Xenopus tropicalis*: levonorgestrel perturbs anti-Müllerian hormone and progesterone receptor expression. Comp. Biochem. Phys. C 181–182, 9–18. http://dx.doi.org/10.1016/j.cbpc.2015.12.001.
Thomas, P., 2000. Chemical interference with genomic and nongenomic actions of steroids in fishes: role of receptor binding. Mar. Environ. Res. 50, 127–134.
Thomas, P., Doughty, K., 2004. Disruption of rapid, nongenomic steroid actions by environmental chemicals: interference with progestin stimulation of sperm motility in Atlantic croaker. Environ. Sci. Technol. 38, 6328–6332.
Tokumoto, T.M., Tokumoto, M., Nagahama, Y., 2005. Induction and inhibition of oocyte maturation by EDCs in zebrafish. Reprod. Biol. Endocrinol. 3, 69. http://dx.doi.org/10.1186/1477-7827-3-69.
Urbatzka, R., Watermann, B., Lutz, I., Kloas, W., 2009. Exposure of *Xenopus laevis* tadpoles to finasteride, an inhibitor of 5-alpha reductase activity, impairs spermatogenesis and alter hypophyseal feedback mechanisms. J. Mol. Endocrinol. 43, 209–219. http://dx.doi.org/10.1677/JME-09-0058.
Westhoff, C.L., Torgal, A.H., Mayeda, E.R., Pike, M.C., Stanczyk, F.Z., 2010. Pharmacokinetics of a combined oral contraceptive in obese and normal-weight women. Contraception 81, 474–480. http://dx.doi.org/10.1016/j.contraception.2010.01.016.
Zeilinger, J., Steger-Hartmann, T., Maser, E., Goller, S., Vonk, R., Länge, R., 2009. Effects of synthetic gestagens on fish reproduction. Environ. Toxicol. Chem. 28, 2663–2670. http://dx.doi.org/10.1897/08-485.1.
Zhao, Y., Fent, K., 2016. Progestins alter photo-transduction cascade and circadian rhythm network in eyes of zebra (*Danio rerio*). Sci. Rep. 6, 21559. http://dx.doi.org/10.1038/srep21559.