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The synthetic progestogen, Levonorgestrel, but not natural progesterone, affects male mate calling behavior of *Xenopus laevis*

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ABSTRACT

Worldwide, more than 100 million women use hormonal contraceptives, which act through progestogenic modes of action. These man-made hormones can enter the aquatic environment as they are excreted via feces and urine. Xeno-progestins are able to interfere with the endocrine system of female aquatic vertebrates impairing oogenesis and reproduction. However, data on progestogenic effects on reproductive behavior of male aquatic vertebrates are lacking. To evaluate whether progestins affect the mating behavior of male *Xenopus laevis*, we exposed male frogs to three environmentally relevant concentrations (10^{-7} M, 10^{-8} M and 10^{-10} M) of the synthetic progestin Levonorgestrel (LNG) and the corresponding natural steroid progesterone (PRG), respectively. LNG at all exposure concentrations increased the proportions of advertisement calling, indicating a sexually aroused state of the males. Furthermore LNG at 10^{-7} M decreased the relative proportions of rasping, a call type indicating a sexually unaroused state of the male. PRG, on the other hand, did not affect any of those parameters. Temporal and spectral features of the advertisement call itself were not affected by any of the two exposure treatments. Since LNG exhibits slight androgenic activity, the results suggest that LNG effects on male mate calling behavior of *X. laevis* are due to its moderate androgenic but not to its progestogenic activities. However, although males' sexual arousal seems to be enhanced by LNG, the adverse effects of LNG on female reproduction presumably outweigh these enhancing effects and LNG exposure nonetheless might result in reduced reproductive success of these animals.

1. Introduction

Worldwide, more than 100 million women use hormonal contraceptives [26], such as birth control pills, emergency contraceptive pills, hormone implants and injectables. Besides the classical synthetic estrogen ethinylestradiol (EE2), synthetic progestogens, so called progestins, are used in such contraceptives. Levonorgestrel (LNG) is one of these progestins that progestogen-only pills as well as combined oral contraceptives or hormone implants consist of. LNG prevents ovulation and changes the quality of cervical mucus in such a way that spermatozoa cannot penetrate and these effects are mediated via progesterone receptors (PR) [9]. It exhibits high affinity to PR (~300% of total specific binding of the natural ligand progesterone) and moderate affinity to androgen receptors (AR; 58% of total specific binding of the natural ligand testosterone) [1,62]. Alarmingly, LNG and its metabolites are excreted via feces and urine [3,11,69] and enter the environment through wastewater effluents. In the environment, especially in aquatic systems, the highly active LNG can then interfere with the endocrine system of non-target organisms, such as aquatic vertebrates [7], and adversely affect their reproductive biology [36,40,78]. So far, LNG has been detected in effluents at concentrations up to 30 ng/L [70,71] and in surface and ground water samples, it was detected at concentrations of up to 11 ng/L [50,72]. LNG in sewage effluents was moreover shown to bioconcentrate into blood plasma of rainbow trouts (*Oncorhynchus mykiss*) to a high extend [17].

Recently, Zeilinger et al. [79] demonstrated that LNG causes an inhibition of reproduction in fathead minnows at concentrations below 1 ng/L. Higher concentrations (3.3 and 29.6 ng/L) were shown to result in masculinization of females, probably due to the partial androgenic activity of LNG [79]. In the aquatic frog *Xenopus tropicalis*, LNG exposure of tadpoles led to severely impaired oviduct and ovary development and reduced fertility of females at adult age [36]. Exposed females had larger fractions of immature oocytes (meiotic prophase) and only one out of 10 females was able to lay eggs after mating with unexposed males [36]. However, LNG did not affect testicular development, sperm count or male fertility [36]. Sexually mature female *X. tropicalis* exposed to LNG similarly showed interrupted germ cell progression

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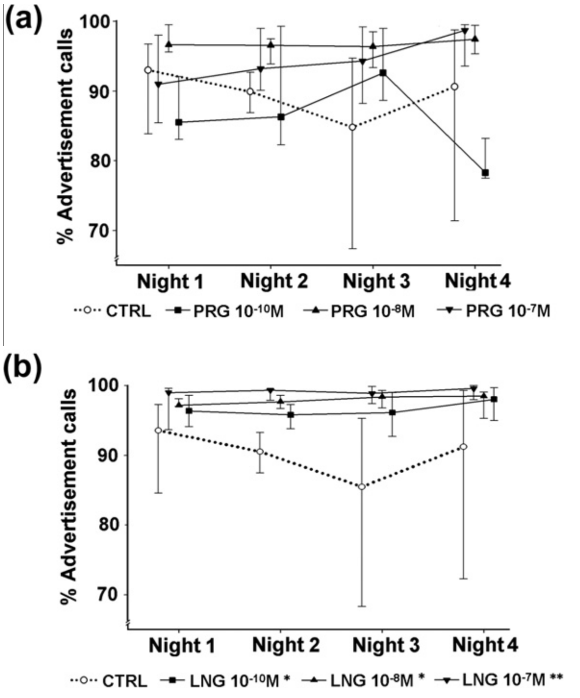

Fig. 1. (a and b) Percentages (median ± interquartile ranges) of advertisement calls of (a) progesterone (PRG) exposed and (b) Levonorgestrel (LNG) exposed male *X. laevis*. Proportions are given for each treatment in each night ($n = 10$ per treatment). Statistical differences were determined using General Linear Mixed models. Significant differences from solvent control (CTRL) are marked by asterisks ($^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$).

into meiosis and inhibited vitellogenesis [57]. In *Xenopus laevis* tadpoles, environmentally relevant concentrations of LNG were shown to affect reproductive biology by interfering with hormones of the thyroid system [39] and the hypothalamic–pituitary–gonad (HPG) axis [40]. Developing *X. laevis* exposed to LNG via the surrounding water displayed repressed mRNA expression of luteinizing hormone (LH) in both genders [40], whereas follicle stimulating hormone (FSH) expression was increased by LNG treatment in males but not females [40]. The authors suggested the disruption of reproduction in adult amphibians as a possible consequence [40].

Natural progesterone (PRG), on the other hand, which is the main natural progestagen in mammals and other vertebrates, is involved in oocyte maturation [37], secretory development of the endometrium to facilitate implantation of fertilized eggs and development of mammary lobules and alveoli [21]. Moreover, PRG and its metabolites were also shown to regulate various social behaviors in vertebrates, like sexual behavior [8,72], addiction [42,54] and aggression [28,60]. In female frogs, for instance, sexual receptivity was increased by PRG treatment [13,31,45,59] and PRG was further shown to affect female mate choice [44]. PRG treatment of male rodents was shown to affect male sexual behavior in opposing ways: supraphysiological plasma PRG levels led to impaired sexual behavior [14,16], while PRG plasma levels in the physiological range could restore sexual behavior in castrated animals [12,73,77]. In frogs, however, PRG treatment failed to evoke sex behavior [43,47].

Male mate calling behavior of *X. laevis* is under control of gonadal steroids, particularly androgens [17,40]. But various non-steroidal hormones, such as gonadotropins [34,76], prostaglandins [75], thyrotropin [65], as well as the neuropeptide arginine vasotocin (AVT) [35,48,53] were shown to act synergistically with gonadal steroids on calling behavior. We recently could show that environmentally relevant concentrations of the antiandrogenic endocrine disrupting compound (EDC) flutamide [2] and vinclozolin (VIN) [23] or the estrogenic contraceptive 17α-ethinyl estradiol (EE2) [24] suppress the androgen-dependent male mate calling behavior of *X. laevis*, whereas the nonaromatizable synthetic androgen 17-methyldihydrotestosterone (MDHT) acts as stimulus [25]. However, to date, nothing is known on how environmentally relevant progestins affect male mate calling behavior of anurans and information is scarce on how potential LNG effects might affect populations.

Thus, the objective of the present study was to investigate whether environmentally relevant concentrations of the synthetic progestin, LNG, affect the mate calling behavior of male *X. laevis*. In parallel, we evaluated the effects of natural progesterone (PRG) on this behavior to identify whether potential LNG effects are due to progestogenic or androgenic modes of action (MOA), respectively.

2. Materials and methods

2.1. Subjects

Seventy adult male *X. laevis* (4-year-old) were obtained from a breeding stock of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Berlin, Germany. The light:dark cycle was 12:12 h and frogs were fed a fish diet (Fisch-Fit; Interquell, Wehringen, Germany) twice a week. After experiments, animals were anesthetized by immersion in buffered MS 222 (tricaine methanesulfonate; 2 g/L for 4 min) [68]. Adequate anesthesia was ensured by the absence of withdrawal and righting reflexes [68]. Frogs were weighed and their snout-to-vent length was measured (weight: 20.8 ± 5.7 g; length: of 6.7 ± 0.6 cm). Afterwards, anesthetized frogs were euthanized by cervical dislocation. The German State Office of Health and Social Affairs (LaGeSo, Berlin, Germany) reviewed and approved all procedures for this study (Reg 0409/08).

2.2. Exposure treatment

Males were placed individually in 60 L glass tanks (50 × 40 × 30 cm) containing 20 L of distilled water supplemented with 5 g marine salt (Tropic Marin Meersalz, Tagis, Dreieich, Germany). D-(−)-Norgestrel (99%; LNG) and PRG ($\geq 99\%$) were obtained from Sigma–Aldrich (Dreieich, Germany). Chemicals were dissolved in dimethyl sulfoxide (DMSO). To mimic natural exposure conditions, animals were exposed by adding the dissolved chemical to the ambient water (equivalent to simultaneous dermal, inhalation and oral exposure [27]) as described previously [23–25]. Test tanks were assigned to the following exposure treatments ($n = 10$): (1) DMSO solvent control (0.001%), (2) LNG exposure at 10^{-10} M (31.24 ng/L), (3) LNG exposure at 10^{-8} M (3.124 μg/L), and (4) LNG exposure at 10^{-7} M (31.24 μg/L), (5) PRG exposure at 10^{-10} M (31.45 ng/L), (6) PRG exposure at 10^{-8} M (3.145 μg/L), and (7) PRG exposure at 10^{-7} M (31.45 μg/L). Exposures lasted 96 h and water temperature was monitored daily (19.5 ± 0.5 °C).

2.3. Acoustic monitoring and call analysis

Calling behavior was recorded for four consecutive nights (18:00–6:00 h) from the day of exposure until the end of the experiment. Acoustic monitoring was performed as described previously [23] using Avisoft Recorder software (Avisoft, Berlin, Germany).

Call analysis was performed using Avisoft SasLab software (Avisoft, Berlin, Germany). In nature as well as in the laboratory, male *X. laevis* produce temporally and spectrally distinct call types [23,66,68,71,74,76]. Within this study, the following five call types were recorded: (1) advertisement calls, which are produced to