Aquatic Toxicology 154 (2014) 154–162

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

The progestin levonorgestrel disrupts gonadotropin expression and sex steroid levels in pubertal roach (*Rutilus rutilus*)

H.K. Kroupova[a,*], A. Trubiroha[b], C. Lorenz[b], V. Contardo-Jara[b,c], I. Lutz[b], R. Grabic[a], M. Kocour[a], W. Kloas[b,d]

[a] Research Institute of Fish Culture and Hydrobiology, South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, Zatisi 728/II, CZ-38925 Vodnany, Czech Republic
[b] Department of Ecophysiology and Aquaculture, Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB), Mueggelseedamm 310, D-12587 Berlin, Germany
[c] Department Ecological Impact Research and Ecotoxicology, Technische Universität Berlin, Ernst Reuter Platz 1, 10587 Berlin, Germany
[d] Department of Endocrinology, Humboldt University Berlin, Invalidenstrasse 42, D-10099 Berlin, Germany

ARTICLE INFO

Article history:
Received 11 January 2014
Received in revised form 7 May 2014
Accepted 10 May 2014
Available online 17 May 2014

Keywords:
Fish
Oestrogen
Androgen
Steroid receptors
Vitellogenin
Gonads

ABSTRACT

The aim of the present study was to investigate the effects of the synthetic progestin levonorgestrel (LNG) on the reproductive endocrine system of a teleost fish, the roach (*Rutilus rutilus*). Pubertal roach were exposed for 28 days in a flow-through system to four concentrations of LNG (3, 31, 312, and 3124 ng/l). Both males and females treated with 3124 ng/l LNG exhibited the upregulated levels of vitellogenin and oestrogen receptor 1 mRNA in the liver. At the same concentration, LNG caused a significant upregulation of the mRNA expression of the gene encoding luteinising hormone β-subunit (*lhβ*) and the suppression of the mRNA expression of the gene encoding follicle-stimulating hormone β-subunit (*fshβ*) in the pituitary of both male and female roach. A lower LNG concentration (312 ng/l) suppressed mRNA expression of *fshβ* in males only. Females treated with 3124 ng/l LNG exhibited significantly lower plasma 11-ketotestosterone (11-KT) and oestradiol (E2) concentrations, whereas their testosterone (T) level was higher compared with the control. Females exposed to 312 ng/l LNG presented significantly lower plasma E2 concentrations. Males exposed to ≥31 ng/l LNG exhibited significantly reduced 11-KT levels. As determined through a histological analysis, the ovaries of females were not affected by LNG exposure, whereas the testes of males exposed to 31 and 312 ng/l LNG exhibited a significantly higher percentage of spermatogonia B compared with the control. The results of the present study demonstrate that LNG disrupts the reproductive system of pubertal roach by affecting the pituitary gonadotropin expression and the sex steroid levels. This disruption was determined to occur in males after exposure to an environmentally relevant concentration (31 ng/l). Moreover, the highest tested concentration of LNG (3124 ng/l) exerted an oestrogenic effect on fish of both sexes.

1. Introduction

Pharmaceutical products and metabolites present in the aquatic environment have become an increasingly important issue in environmental protection (Fent et al., 2006; Corcoran et al., 2010). In particular, considerable attention has been paid to endocrine disruption associated with the oral contraceptive ingredient 17β-ethinyloestradiol (EE2; Desbrow et al., 1998; Jobling et al., 2002). Recently, however, studies have also investigated synthetic progestins (Zeilinger et al., 2009; Paulos et al., 2010), which are ingredients of oral contraceptives and other hormonal medicines (Africander et al., 2011). Synthetic progestins (also called gestagens, gestogens, progestogens, and progestagens) to some extent mimic endogenous progesterone but also exhibit a wide range of biological activities that differ from those of progesterone (Stanczyk, 2003; Besse and Garric, 2009; Paulos et al., 2010; Africander et al., 2011). These compounds are known to interact not only with progesterone receptors (PR) but also with androgen (AR), oestrogen (ESR), glucocorticoid (GR), and mineralocorticoid (MR) receptors (Africander et al., 2011). In the past, when data on the environmental concentrations of progestins were scarce, it was thought that synthetic progestins are found in sub-nanogram per litre concentrations or in the very low ng/l range in surface waters (Zeilinger et al., 2009; Scott et al., 2010). However, since then, more analytical

* Corresponding author. Tel.: +420 387 774 621; fax: +420 387 774 634.
E-mail address: kroupova@frov.jcu.cz (H.K. Kroupova).

http://dx.doi.org/10.1016/j.aquatox.2014.05.008
0166-445X/

surveys have been conducted, and these have reported concentrations ranging from several ng/l (Vulliet and Cren-Olive, 2011) to tens of ng/l (Al-Odaini et al., 2010; Liu et al., 2011). These findings suggest that progestins in surface water may pose a substantial risk to aquatic organisms. Indeed, recent studies have reported the negative effects of progestins at the low ng/l range on the reproductive functions of fish (Zeilinger et al., 2009; Paulos et al., 2010; Runnalls et al., 2013) and amphibians (Säfholm et al., 2012).

One of the commonly used pharmaceutical progestins is levonorgestrel (LNG), a synthetic steroid hormone structurally related to testosterone that has not only progestagenic but also androgenic activity (Besse and Garric, 2009). In addition, metabolites of LNG exhibit oestrogenic activity (García-Becerra et al., 2002). It was recently demonstrated that fish exposed to very low concentrations of LNG strongly bio-concentrate this substance in their blood plasma (Fick et al., 2010). Because it has been suggested that LNG is a potentially harmful substance (Fick et al., 2010; Christen et al., 2010), an increasing number of studies describing its effects on different aquatic organisms has been published recently (Zeilinger et al., 2009; Contardo-Jara et al., 2011; Kvarnryd et al., 2011; Lorenz et al., 2011a,b; Säfholm et al., 2012; Hoffmann and Kloas, 2012; Runnalls et al., 2013; Svensson et al., 2013). Given its use as a contraceptive in humans, it is not surprising that its main effects in aquatic vertebrates were exerted on reproduction, and LNG was also found to be a very potent endocrine disruptor in fish. For example, Zeilinger et al. (2009) showed that a water concentration of LNG as low as 0.8 ng/l, which is its lowest tested concentration, caused reduced fertility in the exposed adult fathead minnows (*Pimephales promelas*). Svensson et al. (2013) found androgenic effects at higher LNG concentrations (at ≥40 ng/l) in three-spined stickleback (*Gasterosteus aculeatus*). However, few data are available regarding the target sites of LNG in fish or the modes of action underlying its endocrine disrupting activity.

Therefore, the aim of the present study was to describe in detail the effects of LNG on the hypothalamus-pituitary-gonad (HPG) axis in a teleost fish, the roach (*Rutilus rutilus*). This widespread cyprinid fish lives in fresh and brackish waters of Europe and is a well-established sentinel for assessing endocrine disruption under both laboratory and field conditions (Tyler et al., 2007). Because progestins are not only important for final reproductive events but also known to be involved in the regulation of other steps during gametogenesis (Schulz et al., 2010; Lubzens et al., 2010), the present study focused on the effects of LNG on pubertal roach. Sub-chronic LNG-exposure was achieved using a flow-through system. The endpoints included gonad histology, plasma levels of sex steroids, and the expression of key genes (here and later the phrase gene expression is used as a synonym for gene transcription, although it is acknowledged that gene expression can also be regulated, e.g., at translation and protein stability level) involved in the regulation of reproduction. The mRNA expression levels of genes encoding gonadotropin β-subunits (*fshβ* and *lhβ*) in the pituitary and vitellogenin (*vtg*), sex steroid receptors (*ar, esr1, ers2a*, and *esr2b*), and steroid-hormone binding globulin (*shbg*) in the liver were studied. Furthermore, because steroid hormones, including LNG, have been demonstrated not to affect only reproductive physiology (Filby et al., 2006; Lorenz et al., 2011b), we included genes encoding the pituitary thyroid-stimulating hormone β-subunit (*tshβ*) and growth hormone (*gh*) in our gene expression analysis.

2. Materials and methods

2.1. Chemicals and dilution water

D(−)-norgestrel (=levonorgestrel, LNG; CAS number: 797-63-7, purity: 99%) obtained from Sigma–Aldrich (Steinheim, Germany) was used as the test substance and for the preparation of analytical standards for LC–MS/MS. To achieve the desired test concentrations, dimethylsulfoxide (DMSO, ≥99.8%, Roth, Karlsruhe, Germany) was used as a solvent. Milli-Q-grade water was used for the preparation of stock solutions. As a dilution water, the artificial tank water (ATW) was used. ATW was continuously filtered (0.45-mm filter), UV-sterilised, and temperature-conditioned Milli-Q-grade water containing 100 mg/l of Instant Ocean sea salt, 200 mg/l $CaCl_2$, and 103 mg/l $NaHCO_3$.

2.2. Experimental animals

Roach of one year of age (*R. rutilus*; mass: 9.3 ± 1.0 g) were obtained from a local fish farm, cultured in a pond (Vodnany, Czech Republic), and maintained for three months (over winter) in an indoor tank (300 l). Three weeks before the start of the experiment, the fish were transferred to the experimental flow-through system and acclimatised to this system until the start of the exposure. During acclimation period, the temperature was gradually increased to 21.0 ± 0.2 °C, the photoperiod was gradually changed from 12:12 h (light–dark) to 14:10 h, and the flow rate was 250 l of ATW per tank per day. At the beginning of the acclimation period there were several cases of fish death (up to 4 fish per tank) before the fish adapted to the experimental conditions. A subset of fish was subjected to a histological analysis of the gonads before the experiment to determine their maturational stage. Female and male roach displayed signs of puberty, as indicated by the presence of oocytes in the cortical alveolus stage in the ovaries and spermatogonia B in the testes, respectively (Levavi-Sivan et al., 2010; Schulz et al., 2010; Taranger et al., 2010).

2.3. Experimental design

The LNG exposure of roach lasted 28 days. A flow-through system with two parallel tanks (40 l) for each treatment was used to maintain constant conditions throughout the experiment. Fish at a density of 25 fish per tank (6 g/l) were exposed to the following nominal LNG concentrations: 3, 31, 312, and 3124 ng/l. Moreover, two control groups were included: (1) control group containing dilution water only (water control) and (2) solvent control (SC) containing 0.0005% DMSO (the same concentration used in the LNG groups to facilitate LNG dissolving). The flow-through system was operated at a flow rate of 250 l of exposure medium per tank per day, which is equivalent to a water exchange rate of approximately six tank volumes per day. One mixing chamber per tank received ATW and the corresponding concentrated stock solution. The exposure medium containing 0.0005% DMSO was supplied to the corresponding tanks. The actual test concentrations in each tank were verified weekly (five times in total) through LC–MS/MS. All of the exposure tanks were permanently aerated (oxygen saturation ≥80%). The temperature was set to 21.0 ± 0.2 °C, and the water pH was 8.1 ± 0.1. The photoperiod was maintained at 14:10 h. The light intensity at the tank level was 335 ± 58 lux. The fish were fed a commercial diet (Aller Futura; 64% protein, 12% fat) twice a day at an amount equal to 2% of the total body mass. The fish that died were removed from the tanks, and the mortality was recorded. The experiment was conducted in compliance with the local laws on animal welfare.

2.4. Sampling

At the end of the experiment, every experimental group was sampled randomly. The fish were anaesthetised with ethyl 3-aminobenzoate methansulfonate (MS222, Sigma). The standard length and body mass of each individual fish were measured to the nearest 1 mm and 100 mg, respectively. Blood was collected from