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Effect of progesterone and its synthetic analogs on reproduction and embryonic development of a freshwater invertebrate model

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ABSTRACT

The presence of a mixture of progestogens at ng/L concentration levels in surface waters is a worldwide problem. Only a few studies explore the effect of progestogen treatment in a mixture as opposed to individual chemicals to shed light on how non-target species respond to these contaminants. In the present study, we used an invertebrate model species, *Lymnaea stagnalis*, exposed to a mixture of four progestogens (progesterone, levonorgestrel, drospirenone, and gestodene) in 10 ng/L concentration for 3 weeks. Data at both physiological and cellular/molecular level were analyzed using the ELISA technique, stereomicroscopy combined with time lapse software, and capillary microsampling combined with mass spectrometry.

The treatment of adult *Lymnaeas* caused reduced egg production, and low quality egg mass on the first week, compared to the control. Starting from the second week, the egg production, and the quality of egg mass were similar in both groups. At the end of the third week, the egg production and the vitellogenin-like protein content of the hepatopancreas were significantly elevated in the treated group. At the cellular level, accelerated cell proliferation was observed during early embryogenesis in the treated group. The investigation of metabolomic changes resulted significantly elevated hexose utilization in the single-cell zygote cytoplasm, and elevated adenylate energy charge in the egg albumen. These changes suggested that treated snails provided more hexose in the eggs in order to improve offspring viability. Our study contributes to the knowledge of physiological effect of equi-concentration progestogen mixture at environmentally relevant dose on non-target aquatic species.

1. Introduction

Estrogens and progestogens in combination are widely used as oral contraceptives and in hormone replacement therapy. In recent years, steroidal estrogen and progestogen compounds have become part of the most studied pharmaceutical pollutants in freshwater ecosystems worldwide. The first review, which described the presence of estrogen and progestogen hormones in original form as endocrine disrupting chemicals (EDCs) occurring at ng/L concentration range in natural water samples was published by Richardson and Bowron (Richardson and Bowron, 1985). Since then, the development of analytical techniques have decreased the limit of detection, resulting in an increasing number of sex-steroids detected (Aris et al., 2014; Runnalls et al., 2015). In wastewater and surface water, which are relevant from an ecotoxicological point of view, their presence is reported in the concentration range from a few ng/L to often tens or hundreds of ng/L (estrogens: 0.20–180 ng/L, and progestogens: 0.07–22.2 ng/L) (Aris et al., 2014; Fent, 2015; Santos et al., 2010).

The most extensively studied steroid EDCs are various estrogens (e.g., 17α-ethinylestradiol, 17β-estradiol, and estrone) that exhibit an impairing effect on reproduction (Aris et al., 2014; Caldwell et al., 2008; Giusti and Joaquim-Justo, 2013; Ketata et al., 2008). However, limited data are available on the adverse effects of other steroid EDCs, for example the progestogens, on reproduction (Giusti et al., 2014; Tillmann et al., 2001), especially at environmentally relevant (e.g., ~10 ng/L) concentrations. The concentrations of individual progestogens are generally low in the environment, but the simultaneous presence of several of these chemicals (Avar et al., 2016; Chang et al., 2011; Vulliet et al., 2008) might already be enough even in low concentrations to cause endocrine disruption in aquatic ecosystems.

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Therefore, studying the effect of exposure to a mixture of these chemicals may provide a more realistic environmental risk assessment. Bioaccumulation of progestogens in both vertebrate and invertebrate freshwater species is well-known (Contardo-Jara et al., 2011; Liu et al., 2011). In these non-target species, progestogens can influence the circulating hormone levels and interfere with the endocrine system, negatively affecting reproduction, development, gamete maturation, and eliciting changes in mating behavior or secondary sex-characteristics (Orlando and Ellestad, 2014; Runnalls et al., 2013).

For the investigation of progestogen mixture effects on an invertebrate model animal, the pond snail (*Lymnaea stagnalis*) was used (Ducrot et al., 2014). The reproductive biology of *Lymnaea* has been well-studied (Koene, 2010; Mescheriakov, 1990; Morill, 1982; Nakadera et al., 2015). It is a hermaphrodite species, but during mating behavior one individual acts as male and the other as female. During oviposition, masses containing 50–100 eggs embedded in a gelatinous mass are deposited on the substrate, from which juvenile snails of adult form emerge following about 10 days of intracapsular embryogenic development, without any free-living larval stages (Mescheriakov, 1990; Morill, 1982). The catchment area of the largest shallow lake of Central Europe, Lake Balaton, is a habitat of *Lymnaea,* where the simultaneous presence of progestogens (0.23–13.67 ng/L) was published in our previous paper (Avar et al., 2016). Therefore, we analyzed the effect of a progestogen hormone mixture (progesterone [PRG], levonorgestrel [LNG], gestodene [GES], drospirenone [DRO]) at a 10 ng/L nominal concentration on this snail focusing on female reproduction. According to literature data, vertebrate-type steroids are also present in mollusks, but contradictory evidence is present regarding their endogenous biosynthesis or endocrine roles (Scott, 2012). However, cholesterol which is the direct precursor of pregnenolone (P5), has been detected in *Lymnaea* neurons (Altelaar et al., 2005). P5 is a key molecule in the biosynthetic pathway of main vertebrate steroids (e.g., PRG, testosterone, and 17ß-estradiol) that have been proposed as functional hormones in mollusks (Scott, 2012). In fact, *Lymnaea* can transform PRG from P5 (Jong-Brink et al., 1981).

A variety of endpoints were assessed in adult animals, including oocyte production, the quality of egg masses (clutches), and vitellogenin (VG)-like protein content. Following egg-laying, the time window of cell division in the offspring, the metabolite content of single-cell zygotes, as well as the metabolite content of egg albumen were investigated before and after the treatment of parents.

Measurements of unstable metabolites during progestogen-induced changes requires a rapid, sensitive and reliable method with fast response time. Capillary microsampling combined with mass spectrometry (MS) is an excellent tool for rapid non-targeted, and qualitative biomolecular analysis (e.g., metabolites) of single cells due to its high sensitivity and specificity (Svatos, 2011; Tomos and Sharrock, 2001; Zhang et al., 2014; Zhang and Vertes, 2015). Enabled by this novel technique, another aim was to observe the progestogen mixture induced molecular changes at the level of metabolites, focusing on nicotinamide adenine dinucleotide in its two forms (NAD^+ and NADH), uridine diphosphate hexose (UDP-hex), to uridine diphosphate N-acetylhexosamine (UDP-hexNAc) ratios, as well as on the possible shift in composition of adenylate energy carrier molecules, such as adenosine-monophosphate (AMP), adenosine-diphosphate (ADP) and adenosine-triphosphate (ATP) in the single-cell zygote and albumen.

2. Materials and methods

2.1. Chemicals and instrumentation

Mixtures of PRG (P0130-25G, HPLC grade), LNG (L0551000, HPLC grade), GES (L0551000, HPLC grade) and DRO (SML0147-10MG, HPLC grade) were used for the treatments as progestogen agents (Sigma-Aldrich, Hungary). Progestogens were dissolved in 0.5 M cyclodextrin (H-107, 2-hydroxypropyl-ß-cyclodextrin, Sigma Aldrich) and added to the water in the experimental tank to reach 10 ng/L final concentrations. Cyclodextrins (cyclic oligosaccharides used as non-toxic solubilizers) have been applied in pharmaceutical fields, as well. Distilled water of LC–MS grade was obtained from VWR International (Debrecen, Hungary). HPLC grade methanol, ATP (A26209) and phosphate buffer saline (PBS – P5368) were also purchased from Sigma-Aldrich (Hungary). AmaZon SL iontrap (Bruker Daltonics Gmbh., Germany) and Synapt G2-S (Waters Co., Milford, MA) mass spectrometers were used for metabolomic analysis. A pipette puller, (P-1000, Sutter Instrument, Novato, CA) was used to produce capillaries for single-cell analysis, and a Leica M205c stereomicroscope helped the monitoring of embryo development.

2.2. Experimental animals and treatment

Adult (3–6 months old) specimens of the pond snail, *Lymnaea stagnalis*, originating from laboratory-bred stocks (MTA ÖK BLI, Tihany, Hungary, and The George Washington University, Washington, DC, USA) were used in the experiments. There are no differences between the two strains (age, shell-size, and maintenance). Groups of animals were maintained until use in large tanks containing low-copper water at 20 °C on a 12:12 h light-dark regime. Animals were fed on lettuce three times a week. The experiments consisted of control and treated groups (n = 55 total number of animals in each) and data were obtained from 5 independent treatment series (n = 11 snails/group/tank in 3 L water). Animals in the control experiments were exposed to the solvent (5 µL, 0.5 M cyclodextrin in 3 L water) as a vehicle control. The cyclodextrin did not evoke any changes in the investigated parameters of controls. Animals in the treated group were exposed to 10 ng/L mixture of PRG, LNG, GES and DRO for 3 weeks. Water was refreshed weekly and recovery measurements were performed before refreshing (Fig. 1A). All procedures on snails were performed according to the protocols approved by the Scientific Committee of Animal Experimentation of the Balaton Limnological Institute (VE-I-001/01890-10/

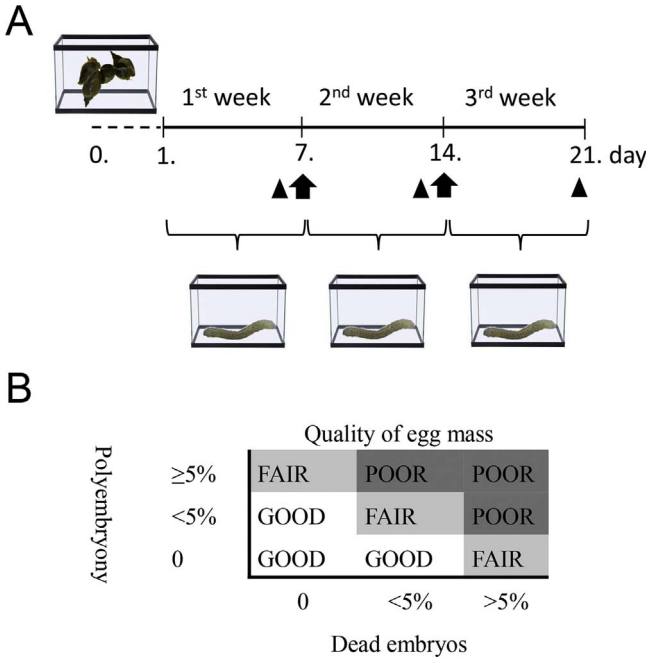

Fig. 1. Management of animal treatment and evaluation of the egg mass quality. (A) Snails acclimatized for 1 day in all tanks with no chemicals (dashed line). From the starting point (1st day) chemicals were applied in the experiment. Recovery measurements were done on the 7th, 14th and 21st days (▲). Total water renewal was performed in all tanks on the 7th and 14th days (⮝). Egg masses were collected and left to develop in clean water week by week. (B) Quality of the egg mass was described by a three-part grading scheme in a quality-map (see chapter 2.5): I – good (white), II – fair (light gray) and III – poor (dark gray).