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Investigation of somatic and molecular changes induced by oral contraceptive residues in invertebrate and vertebrate aquatic model species

PhD thesis

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1 Introduction

Estrogens and progestogens in combination are widely used as oral contraceptives and in hormone replacement therapy. Progestins or their metabolites are eliminated from the body mainly through the renal system and a remarkable amount is excreted unchanged or in the form of active metabolites. These agents enter waste-water treatment plants (WWTP) or watercourses as the active pharmaceutical, metabolites, or other transformation products. 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 in 1985. Since then, the development of analytical techniques have decreased the limit of detection, resulting in an increasing number of sex-steroids detected. 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). The most extensively studied steroid EDCs are various estrogens (e.g., 17α-ethinylestradiol, 17β-estradiol, and estrone) that exhibit an impairing effect on reproduction. Furthermore, these molecules also have impairing effects on morphological (e.g. liver, spawn, kidney), as well as, on molecular (e.g. vitellogenin - VTG) level in fish. However, limited data are available on the adverse effects of other steroid EDCs, for example the progestogens, on reproduction, especially at environmentally relevant (e.g., ∼10 ng/L) concentrations. These molecules can accumulate in invertebrate (e.g. zebra mussels) and vertebrate (e.g. rainbow trout) species. 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. The concentrations of individual progestogens are generally low in the environment, but the simultaneous presence of several of these chemicals might already be enough even in low concentrations to cause endocrine disruption in aquatic ecosystems. Therefore, studying the effect of exposure to a mixture of these chemicals may provide a more realistic environmental risk assessment. In the recent years, only a few studies have applied hormone treatment in a realistic mixture to understand how non-target aquatic animals respond to these contaminants, rather than individual chemicals. The presented results contribute to this knowledge by applying an invertebrate and a vertebrate model animal, and by applying a treatment with an equi-concentration mixture of four progestogens in environmentally relevant, relatively low concentrations.

2 Aims

Our aim was to investigate the physiological effect of progestogen contaminants in an invertebrate (*Lymnaea stagnalis*) and in a vertebrate (*Rutilus rutilus*) aquatic model species. On the one hand, we applied a relevant concentration from an environmental point of view (10 ng/L), on the other hand, we investigate the effects of a mixture, which allow us to interpret the environmental situation. We also applied higher, 50 and 500 ng/L concentrations in some of the experiments.

Our purposes were the followings:

1. Investigate the application and suitability of VTG, which is a common biomarker in estrogen contamination, in case of progestogen contamination.

2. Test the DJ-1 protein, as a possible biomarker for prediction of progestogen contamination.
3. Explore the effect of progestogen treatment on reproductive system of *L. stagnalis*, a hermaphrodite species via mapping the presence of GnRH-like peptides in the CNS, quantify the gametes on peripheral level, and investigation of quality of laid egg masses.
4. Investigation of snail offspring viability (time-window of cell proliferation during early embryonic development and metabolomic composition of egg albumen, as well as, single-cell zygote) in case of parents progestogen treatment.
5. Describe the effect of progestogen mixture on somatic (brain, liver, gonad and kidney indices) and molecular (lipid metabolism and stress proteins) level in roach.

3 Materials and methods

3.1 Animals and treatment

Adult (3–6 months old) specimens of the pond snail, *Lymnaea stagnalis*, originating from laboratory-bred stocks were used in the experiments. Roaches were caught in the Sárvíz stream situated in the drainage system of Lake Balaton in March 2014 and were brought to the MTA-ÖK Balaton Limnological Institute, Tihany. Mixture of HPLC grade PRG (P0130), LNG (L0551000), GES (L0551000) and DRO (SML0147) were used for the treatments as progestogen agents (Sigma Aldrich). Progestogens were dissolved in 0.5 M cyclodextrine (WXBB5296, 2-hydroxypropyl-ß-cyclodextrin, Sigma Aldrich). Concentrations of all progestogens were measured weekly in the experimental tank before refreshing the water via an HPLC–MS method.

Snails were acclimatized for 1 day in all tanks with no chemicals. After 1 day 10 ng/L progestogen mixture (PRG, LNG, GES, DRO) were applied in one of the two groups, the other group was exposed to the solvent (5 μL, 0.5 M cyclodextrin in 3 L water) as a vehicle control (21-day long experiment in 5 independent series, 55 animals in total per group). Hepatopancreas (midgut gland), which is an organ of the digestive tract of molluscs, was removed and homogenized (10 mg/100 μL) in ice cold PBS (0.02 mol/L, and pH 7.4) for ELISA measurements (VTG and DJ-1 protein). CNS was also removed and homogenized for DJ-1 measurement or fixed in 4% paraformaldehyde for GnRH immunohistochemistry (IHC) experiments. The seminal vesicle (signed by arrow) running on the proximal surface of the hepatopancreas was prepared and the sperm number was counted by flow cytometry (Partec CyFlow ML II) after nuclear staining (Hoechst 33342, Life Technologies). Following egg-laying, each egg mass of the control and the 10 ng/L treated adult snails was removed daily and left to develop in untreated water.

After one month acclimatization period, the healthy mixed gender fish were divided into 4 groups (n=20 fish/group in 100 L tank). Animals in the control experiments were exposed to the solvent (100 μL, 0.5 M cyclodextrine/60 L water) as a vehicle control. Fish of the other three groups were exposed for 42 days to 10, 50, 500 ng/L mixture of progestogen (PRG, LNG, DRO) treatment. After this exposure period, fish were anaesthetized and decapitated. Their brain, liver, gonads and kidney were removed on ice and weighted. Somatic indices (brain-somatic index: BSI, liver-somatic index: LSI, gonadosomatic index: GSI, kidneysomatic index: KSI) were determined (calculated as: [tissue weight/body weight]×100).

Livers from control and 10, 50, 500 ng/L treated animals (each n=3) were homogenized in ice cold PBS (10 mg wet tissue/100 μL) for VTG and DJ-1 ELISA measurements. On the other hand, livers and brains were fixed (in 4% PFA) for DJ-1 IHC experiment. One mL blood was collected from the anaesthetized fish from control and 500 ng/L treated animals using a closed blood collection system. Free cholesterol, HDL-, LDL-cholesterol and triglyceride were measured by automatized enzymatic and colorimetric methods from serum using Roche Integra

800 System (La-Roche Ltd.). Serum was also used for cell stress array studies (Human Cell Stress Array Kit, R & D Systems) in control and 500 ng/L treated animals.

3.2 Evaluation of egg mass quality

Egg masses of the control and the treated adult snails were classified individually according to their quality using our own evaluation system. This simple evaluation system is based on the commonly used 5% decision level in statistics and contains two parameters: polyembryonic eggs (more than one embryos per egg) and dead embryos (unmoving, not pigmented) relative to the total number of eggs in the mass. Polyembryony and total number of eggs were counted on the first day, dead embryos were identified on the fourth day. The parameters were classified as absent, occurrence under 5%, equal, or over 5%. The quality of the egg mass was described by a three-part grading scheme: I – good, II – fair, and III – poor.

3.3 Embryonic development

Time lapse observation of Lymnaea embryogenesis was performed using a Leica M205c stereomicroscope equipped with time lapse software (LAS V4.5). At the end of the 3-week long treatment period of the adult snails, the freshly laid zygotes were observed from the single-cell to the eight-cell stage. Pictures were taken every 10 min, starting with the 1st cell proliferation (beginning of the two-cell stage), and following the changes until the 3rd proliferation (beginning of the eight-cell stage).

3.4 Capillary microsampling

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.

Samples for metabolomic analysis were collected from a good quality egg mass on the third week. Snail eggs with an ellipsoidal shape, and containing ~0.5 μL viscous albumen were used. The eggs were taken out from the egg mass and the gelatinous material was removed. During microsampling, a single egg was propped with a sampling capillary and a glass slide in a Petri dish in order to keep the egg immobilized. Approximately 0.3 μL albumen was collected by a sharp sampling capillary (TW100-F-3, WPI) from the egg of control and treated parents (n=12 per group). The single-cell zygote was spherical with ~100 – 120 μm diameter. The estimated volume of the sampled cells was ~0.5 nL (n=10 per group). During the zygote sampling procedure, a holding capillary was used to immobilize the cell. Holding capillaries were made from non-filamented borosilicate glass (B100-75-10, Sutter Instrument), and sampling capillaries were prepared from filamented borosilicate glass (BF100-50-10, Sutter Instrument). After extraction of the albumen or cell cytoplasm, metabolite composition was examined using ion mobility separator (IMS) eqipped with time-of-flight (TOF) analizator mass spectrometer (Synapt G2-S, Waters Co., Milford, MA), as well as, a Bruker AmaZon SL ion trap MS equipped with an off-line NanoElectrospray source (73084, Bruker Daltonics, Bremen, Germany).

3.5 Statistical analysis

Statistical analysis was performed using the OriginPro® 2015 (OriginLab Corp., Northampton, Massachusetts, USA) and IBM SPSS Statistics Version 20 (IBM Magyarország Kft.) softwares. Normality of the dataset was investigated using the Kolmogorov-Smirnov test,

homogeneity of variances between groups investigated using Levene's statistic. Based on the results of these statistics, the following tests were used: independent sample t-test, repeated measures ANOVA and one-way ANOVA (with Welch correction, Scheffe or Tamhane post hoc test) comparing VG-like proteins, DJ-1 protein, oocyte production, time-window of early embryonic development, AEC, hexose utilization, and redox states, cholesterol, LDL-, HDL-cholesterol and triglyceride. Comparison of egg mass quality in *L. stagnalis* and differences between starting and final body lengths and weights such as in the case of somatic indexes of different treatments in *R. rutilus* were performed using nonparametric Kruskal-Wallis test. Differences were considered statistically significant at $P < 0.05$.

4 Results and discussion

4.1 Vitellogenin and vitellogenin-like proteins

Invertebrates and vertebrates were also found sensitive for progestogen treatment during our experiments. The VTG content of hepatopancreas in *L. stagnalis* was significantly increased in the 10 ng/L treated group. In contrast, in liver of female roach was slightly increased in 10 and 50 ng/L treated groups, but significantly increased in 500 ng/L treated groups. VTG synthesis depends on available amount of hexose and amino acids, in accordance with the fact that reproduction in adults is induced upon positive change in systemic nutritional status. VTG or VTG-like protein level is high in sexually mature females and increases gradually during vitellogenesis. Vitellogenesis in vertebrates generally occurs outside the ovary, mainly in liver. The synthesized VTG secreted into the blood and taken up by oocytes. In invertebrates, where vitellogenesis has also been described, different tissues (e.g. hepatopancreas orglandula albuginea) are the source of circulating VTG-like proteins in hemolymph. In most oviparous species, the VTG-like proteins and the VTG are precursors of typical eggyolk proteins, e.g., vitellins in oocytes that serve as energy reserves during embryogenesis. In gastropods, vitellins, such as yolk ferritin, have also been described in the oocytes of *Helix*, *Helisoma*, *Planorbis* and *Lymnaea*. The VTG-like protein content of hepatopancreas was significantly higher in the experimental group treated with the 10 ng/L mixture, compared to the control group at the end of the 3rd week. This observation could also explain the elevated oocyte number. Giusti (2013) described that yolk ferritin expression in the reproductive organ of *L. stganalis* did not change significantly after being exposed to 2 – 50 µg/L progestogen cyproterone acetate (CPA) for 21 days. Results of Giusti's experiments contradict our observations, which show that exposure to progestogen mixture significantly changes the VTG-like protein content in *L. stagnalis*. The discrepancy might be because of the concentration used in the present study was lower (10 ng/L) than the one used in Giusti's experiments (1.1 and 28.7 µg/L), furthermore, we applied a mixture of four progestogens instead of the CPA. To date, other progestogen effects on VTG-like protein content were not investigated in freshwater snails. Contradictory data of VTG content measured in vertebrates can be found in the literature. According to DeQuattro (2012) the VTG mRNA content decreased in fathead minnow after 21-day long 10 ng/L PRG treatment. Zucchi (2014) reported that 50 ng/L DRO was capable of significantly decreasing the hepatic VG mRNA level in zebrafish, but in combination with 4 ng/L PRG it had no effect after 14-day long treatment. However, 500 ng/L DRO alone or in combination with 40 ng/L PRG significantly reduced the hepatic VG mRNA. In parallel, the plasma VTG content was unchanged during the experiments. According to other studies 0.5 and 5 ng/L LNG had no effect but 25 ng/L did already cause a significant increase, while 100 ng/L concentration significantly decreased the plasma VTG level in fathead minnow. Based on the above, we suggested that due to variable effects of progestogens the VTG cannot be used as indicator to reveal progestogens contamination in lower vertebrates, moreover, only females can be used.

4.2 DJ-1 protein

Due to the above mentioned results, the testing of DJ-1 protein, as a possible biomarker for prediction of progestogen contamination have been arised. DJ-1 protein is a gender-independent molecule, which shows sensitivity for environmental changes, and has several common molecular target (e.g. NRF2, PSF, LDL-receptor) with progestogens. In addition, DJ-1 plays an important role in cell survival, proliferation, gene transcription and in protection against oxidative stress. Based on these, the elevation of DJ-1 level was hypothesized in progestogen treatment. Even so, it was significantly decreased at the end of 1st week, but showed no change at the end of the 3rd week of the treatment in *L. stagnalis*. Thus, this protein was capable to predict the early progestogen exposure at low concentration, but not the chronic exposure. It suggests that DJ-1 protein and progestogens do not have common target in this species, and the direction of changes might mean that the snail implements the protection against stress on another, energy-consuming ways, however, the DJ-1 protein level returns to the physiological level relatively soon. In the vertebrate model animal DJ-1 protein was a potential marker molecule of chronic progestogen treatment, because the DJ-1 level was significantly increased at the low concentration of progestogen, in contrast to VTG. The elevation was also visualized by IHC experiments. In summary, DJ-1 protein seems to be more suitable biomarker to check progestogen contamination, than VTG. However, the usability of DJ-1, as a potent biomarker for progestogen contamination of aquatic ecosystems, needs to validate by experiments on other aquatic species.

4.3 Progestogen-induced alteration in adult *L. stagnalis*

The damage of reproductive system of *L. stagnalis* was observed at both central and peripherial level in average environmental concentration of progestogen mixture. However, the chronic treatment of this hermaphrodite species caused different effect in regulation of male and female reproductive system. We localized the GnRH-like immunpositive neurons in *L. stagnalis* CNS. The neuropeptide GnRH is responsible for regulation of male as well as female reproduction, but to date the exact role is unclear. GnRH-like peptide expressing small (<50 μm), median (50 - 100 μm) and large (100 - 150 μm) neurons were found in cerebral, pedal, right parietal and visceral ganglia as well as the varicose fibers of neuropil also displayed immunoreactivity.

Immunopositive fibers running out through the anal and intestinal nerv of VG could be observed, similar to earlier described in *Helisoma trivolvis* and *Haliotis asinina*. Besides, similar to Young (1999) described in *L. stagnalis*, the lateral lobe of L/RCG was the most intense stained region, and L/RBG or LPG did not contain immunopositive neurons or fibers. In contrast to earlier studies, L/RPlG was not, but L/RPeG stained in our experiments. Five immunopositive neuron groups stained symmetrically in L/RPeG. The staining of RCG was different between control and treated snails. Neurons and fibers also stained in the control group, but the intensity of GnRH signal decreased in 10 ng/L group. This fading could be observed in anterior lobe, which contains neurons responsible for regulation of male reproduction. GnRH immunopositivity was not changed after progestogen treatment in regions responsible for regulation of female reproduction (RPG, CDC cells of L/RCG, anal and intestinal nerv of VG).

At the peripherial level, gamete production followed the changes of GnRH staining: the sperm number was significantly decreased, but oocyte production was increased in the treated group at the end of the 3-week long period. Earlier studies described decreased sperm motility in fathead minnow (300 ng/L PRG treatment for 1 week), decreased sperm number in stickleback (65 ng/L LNG treatment for 45 days), or decreased motility and fusion ability with oocyte in treatment of human sperm (1, 10, 100 ng/mL LNG). The oocyte number of treated

snails was significantly decreased during the 1st week, but increased in the 3rd week compared to control group. Similar to our results, Ducrot (2010) described the same in *L. stagnalis* treated with PRG agonist vinclozoline (25 ng/L for 21 days). Further experiments on determination of progestogen receptor localisation in the CNS or female reproductive organs of *L. stagnalis* could ascertain the cause of oocyte elevation

4.4 Progestogen-induced alteration in offsprings of *L. stangalis*

Our results obtained at the physiological level suggested that the progestogen treatment of parents influenced the hormonal signals and molecular composition of the eggs. Hormonal signals are able to impact the cell cycle directly or indirectly (differentiation, proliferation and survival), and/or the hexose metabolism by affecting the G proteincoupled receptor and the associated signaling pathways: (GPCR)-ACcAMP-PKA-MAPK, GPCR-AC-cAMP-PI3K-AKT, GPCR-JAK/STAT, and GPCR-AC-cAMP-PKA-GLI3-Hedgehog. The cellular (prolipheration) and molecular (adenylate energy carriers – AEC, hexose utilization and redox state) endpoints described above have not been used for the assessment of progestogen exposure. A remarkable observation was that cell proliferation was accelerated by mixed progestogen hormones in the early stage embryos, however, the hatching time was not changed significantly. Egg formation and content depend on the parents and determine the viability of offspring. The general molecular pattern of egg albumen, as well as, single-cell cytoplasm, including 31 identified metabolites were investigated. Qualitative differences in molecular pattern were not observed between the control and the treated groups. Considering the relative ratios of certain metabolites differences could be found between offsprings of control and treated parents in early stage. These changes are related to carbohydrate metabolism. The cytoplasmic AEC level of single-cell zygotes, which represents the amount of metabolically available energy stored in adenine nucleotides, did not show any difference between the control and progestogen treated groups. In contrast, the UDP-hex level was elevated, and the UDP-hexNAc/UDP-hex ratio was significantly decreased in zygotes obtained from treated parents. This ratio depends on the utilization of hexose molecules (glucose, galactose) in the cytoplasm. UDP-hex is important in glycogen synthesis, which provides nutrition for the growing embryo. Thus, the UDP-hex level depends on the energy status of the cell and the hexose excess. Our data suggests that zygotes obtained from treated parents seemingly produce more glycogen because of the elevated UDP-hex level. Although glycogen synthesis requires energy, the AEC ratio was found to be the same in treated single-cell zygote as in the control. Considering that hormonal signals act through the GPCR-AC-cAMP-PI3K-AKT cascade and block the glycogen-synthase kinase 3 (GSK3) enzyme, the glycogen synthesis stops and UDPhex is accumulated in zygotes obtained from treated parents. In parallel, the GPCR-AC-cAMP-PI3K-PIP3-PDK1 system intensifies the hexose uptake, thus hexose metabolism is not disrupted. According to Wells, the UDP-hexNAc pool directly depends upon hexose concentration. UDPhexNAc is generated through the hexosamine biosynthesis pathway and it is utilized for post translational modifications of proteins and/or for the synthesis of glycoproteins and N-glycans. The latter also provides nutrition for the growing embryos, thus the nutrient supply of embryos is not disrupted by the hormone treatment. It would be worth examining the later effects of parental progestogen treatment in a multi-generation experiment to monitor tolerance/adaptation.

4.5 Progestogen-induced alterations in *R. Rutilus*

Progestogen treatment caused changes at somatic, as well as, molecular level. Adult roaches were exposed to 10, 50 and 500 ng/L of mixture of progestogen for 42 days and some

of their somatic indices (liver and kidney hypertrophy, gonad hypotrophy) were significantly different between the groups.

Similarly to our results, kidney hypertrophy were also observed due to ≥40 ng/L LNG treatment on female sticklebacks in a previous study. Zucchi (2014) observed siginificant decrease in GSI value due to 5000 ng/L DRO combined with 400 ng/L PRG treatment in zebrafish, but DeQuattro (2012) described no changes dur to 1000 ng/L PRG exposure in fathead minnows. Previous studies of breams in Lake Balaton described seasonal changes in GSI value, which is lower in March, then growing steeply from April, decrease during the summer and slowly increase til November. Our experiments was performed after the summer rest period, when gonads started to increase. This phenomena could cause the high standasd deviation in the control group, however, significant difference between control and 500 ng/L treated group can still be described. At molecular level, cholesterol and LDL-cholesterol of 500 ng/L progestogen treatment were decreased, as well as, several other proteins with various biological functions (e.g. PON3, Phospho-p38a, SIRT2, SOD2, HIF-1 alpha, HIF-2alpha, p27 and NFKB1, FABP-1).

Our results show that mixture of progestogens was a stress factor in this model organism, and animals tried to adapt to changed metabolism. Our results also strengthen the fact that progestogen mixture in low concentration is a relevant biological risk factor in the catchment area of the largest shallow lake of Central Europe today and in the future. Several endpoints among the examined parameters in invertebrate, as well as, vertebrate model species could be used for a further additional toxicological interaction (concentration addition) experiment to specify the the effect of each progestogen on each other. Further impacts of changes in environmental pollution (e.g. change in contraceptive use trends, transformation of sewage treatment processes) on aquatic non-target species could be explored and extimated in this way.

5 Novel findings

1. The investigation of VTG in progestogen exposure resulted in elevated levels in both model animal and all treated group. However, the treatment with environmentally relevant concentration caused significant elevation only in *L. stagnalis*. Thus, VTG has positive predictive value for progestogen exposure only in invertebrate species.

2. Alternatively, a novel biomarker DJ-1 protein was investigated, which has not been used so far in progestogen exposure. This molecule is able to predict the chronic presence of low concentration of progestogen exposure in *R. rutilus*. In *L. stagnalis* is able to predict only acute progestogen contamination.

3. The chronic presence of low contentration of progestogen affect male and female reproductive system in *L. stagnalis*. Gamete production resulted in contrary changes, which showed correlation with the related molecular changes (VTG and GnRH). The elevated VTG concentration of hepatopancreas reflected the elevated oocyte production, and decreased GnRH immunostaining of CNS manifested in decreased sperm number in the treated group.

4. An egg mass evaluation system has been worked out, which enabled to compare egg mass of control and treated snails.

5. According to our results viability and development of offsprings from treated adults has been affected by progestogens at environmentally relevant concentration. Although, the molecular composition of the single-cell zygote cytoplasm and the albumen was not changed, differences could be found in metabolic rates and the speed of cell prolipheration. Progestogen treated adults provided more hexose in eggs compared to the control.

6. Some of the somatic indices showed significant changes in roach treated with progestogen at environmentally relevant concentration. At molecular level, cholesterol and LDL-cholesterol were decreased, as well as, several other proteins with various biological functions (e.g. PON3, SIRT2, HIF-1 alpha).

List of publications

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Publications related to the present Ph.D. thesis

Zrinyi Z, Maasz G, Zhang L, Vertes A, Lovas S, Kiss T, Elekes K, Pirger Z (2017) Effect of progesterone and its synthetic analogs on reproduction and embryonic development of a freshwater invertebrate model. *Aquat Toxicol 190:94-103.* ($IF_{2012-2016}$: 4,425)

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