



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

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Highlights

- Physiological and cellular/molecular effects of mixture of progestogen were tested
- 10 ng/L hormone concentration was applied on *Lymnaea* in the 21 days experiment
- Evaluation system of egg masses quality was created based on our observations
- VG level and oocyte production was significantly increased after treatment
- Cell proliferation was accelerated, hexose utilization and AEC were decreased

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 progestogens mixture at environmentally relevant dose on non-target aquatic species.

Keywords: Adenylate Energy Charge; Capillary microsampling; Egg mass quality; *Lymnaea*; Metabolomic analysis; Progestogen exposure

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 Brown (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. Therefore, studying the effect of exposure to a mixture of these chemicals may provide a more realistic environmental risk assessment. Bio-accumulation 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 17 β -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- β -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 12:12h 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-001/01890-10/2013). Efforts were made to minimize both the suffering and the number of animals used in the experiments.

2.3 Recovery measurement of progestogens

Concentrations of all progestogens were measured weekly in the experimental tank via an HPLC-MS method according to Avar (Avar et al., 2016) complemented with GES measurements (see the Supplementary Material). Our recovery results indicate that the actual concentrations in the experiment were always $\geq 80\%$ of the nominal concentration (10 ng/L) for DRO (8.94 ± 0.29 ng/L), LNG (8.76 ± 0.79 ng/L), PRG (9.2 ± 0.35 ng/L), and GES (8.58 ± 0.16 ng/L), and no progestogens were detected in the control water samples. Based on these recovery data, the water in all experimental tanks was changed and treated with the progestogen mixture every week (Fig. 1A).

2.4 Vitellogenin ELISA

At the end of the 3-week long treatment period, the hepatopancreas (midgut gland), which is an organ of the digestive tract of molluscs, was removed ($n=6$ in each group) and homogenized (10 mg/100 μ l) in ice cold PBS (0.02 mol/L, and pH 7.4) with a glass homogenizer. The homogenates were ultra-sonicated on ice and centrifuged for 15 minutes at $1500\times g$. VG-like protein content in the supernatant was measured by a fish VG ELISA Kit (MBS010726, MyBioSource; sensitivity 5.0 ng/mL; detection range: 31.2 – 1000 ng/mL). The VG standards were added to each standard well (at 31.2, 62.5, 125, 250, 500, and 1000 ng/ml) and the hepatopancreas samples were deposited in the sample wells (50 μ l/well). After this, 100 μ l horseradish peroxidase conjugate was added to each well and the wells were incubated for 1 h at 37 °C. The plates were washed four times and 50-50 μ l of chromogen solutions A and B were added to the wells, and incubated for 15 minutes at 37 °C. Then 50 μ l stop solution was added to each sample and the optical density was measured by a microplate reader (Victor3 1420 multilabel counter, PerkinElmer) at 450 nm. Finally, the VG-like protein concentrations of the hepatopancreatic tissues for the different groups were calculated in ng/mg wet tissue.

2.5 Evaluation of egg mass quality

Following egg-laying, each egg mass (Supplementary Fig. 1A) of the control and the treated adult snails was removed and left to develop in untreated water (Fig. 1A). They were classified individually according to their quality using our own evaluation system (Fig. 1B). 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, see Supplementary Fig. 1B) and dead embryos (unmoving, not pigmented, see Supplementary Fig. 1C) 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 (Fig. 1B). In addition, the number of eggs compared to the size of the egg mass was taken into consideration. There are some parameters, such as too much gelatinous material, oocytes out of the eggs, or the presence of empty eggs, which could be interpreted as a negative indication regarding damaged reproduction of the snails, but we did not see any of these phenomena in our tests. Simultaneous occurrence of these phenomena and dead embryos or polyembryony could be an exclusion criterion in this evaluation system.

2.6 Embryonic development

Time lapse observation of *Lymnaea* embryogenesis was performed using a Leica M205c stereomicroscope equipped with a DFC3000G (Leica) digital camera and 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 minutes, 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).

2.7 Single-cell and albumen sampling

A single-cell sampling technique was used for sample preparation according to Zhang et al. (Zhang et al., 2014). 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 using wet precision wipes. 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 (see Fig. 4B). Approximately 0.3 μL albumen was collected by a sharp sampling capillary (TW100-F-3, WPI) from the egg. The single-cell zygote was spherical with ~ 100 μm diameter. The estimated volume of the sampled cells was ~ 0.5 nL. During the oocyte 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).

2.8 Metabolomic analysis of single-cell zygote and egg albumen by MS methods

2.8.1 Synapt G2-S-TOF MS

After extraction of the albumen or cell cytoplasm, the sample capillary was backfilled with 1 μL electrospray solution using a microloader pipette tip (930001007, Eppendorf). The capillary was gently flicked to remove air bubbles before being attached to electrode holder custom modified to have an insulating stem (H-12-S, Narishige, Tokyo, Japan). A platinum wire of ~ 1.5 cm in length was inserted into the capillary from the back end until it contacted the electrospray solution. The tip of the capillary was placed ~ 5 mm away from the orifice of a high-resolution MS (tuned to ~ 10000 FWHM resolution) equipped with a traveling wave ion mobility separation (IMS) system (Synapt G2-S). Two-hundred volts were applied to the wire by a power supply, and an electrospray containing analyte droplets was produced. The generated ions were retarded by the collisions with the drift gas in the IMS system, separated

according to their collision cross sections, and transferred to the time of flight MS for mass analysis. Nitrogen gas was used as the drift gas. It was supplied at a flow rate of 90 mL/min and a pressure of 3.25 mbar in the IMS system. The voltage wave velocity and amplitude in the IMS cell were optimized to 650 m/s and 40 V, respectively. The ion abundances as a function of drift time and mass-to-charge ratio (m/z) were recorded. Single-cell spectra were averaged over 3 scans (1 scan/second). Structure identification was facilitated by tandem MS based on collision induced dissociation in Ar background gas at 20–30 eV energy.

2.8.2 Off-line NanoElectrospray-Ion Trap MS

A Bruker AmaZon SL ion trap MS equipped with an off-line NanoElectrospray source (73084, Bruker Daltonics, Bremen, Germany) was used for mass detection. The loaded sample capillary was inserted into the holder. The holder with capillary tip first was inserted into the mounting tool and then it was fixed with the mounting tool into the off-line NanoElectrospray source. The mounting tool from the holder was pulled off and the ionization source was closed. The sample capillary position was adjusted in the x-, y-, z-direction, and the capillary tip was observed through the monitors. The ionization source was operated with an On Volt potential of 950 V in the negative ion mode. The following electrospray parameters were kept constant during the analysis: drying gas (N_2) flow rate of 4 L/min, drying gas temperature of 120 °C, and nebulizer gas pressure of 3 psi. 20 ms Pre-time was applied in order to ensure stable spray conditions during the accumulation period. The trap drive was set to 55 V. The ionization source in pulsing mode was applied for the molecule identification. Rest Volt Offset was set to a level of - 250 V. With an On Volt of 950 V the absolute Rest Voltage is set to 700 V. By using the pulsing mode, the spray voltage was set to a lower level outside the accumulation period. During accumulation, the spray voltage was set to the On Voltage, outside the accumulation period the spray voltage is set to the Rest Voltage (On Volt + Rest Volt Offset). The pulsing mode has two important advantages: it saves the sample during signal analysis, and some

compounds in the spray remain more stable, but there is also one disadvantage: the actual spray current cannot be observed. In the fragmentation, automatic top 5 precursor ion selection was used. MS/MS fragmentation amplitude was set to 2 V. Data acquisition and spectrum evaluations were performed by means of the Bruker DataAnalysis 4.1 software (Build 359).

2.9 Data analysis

Mass Lynx V4.1 (Waters Inc.) and Compass Data Analysis V4.1 (Bruker Daltonics) software were used for data analysis. Metabolomic ratios were calculated according to literature data (de Graef et al., 1999; Obrosova and Stevens, 1999; Zhang and Vertes, 2015). Of the identified molecules, we focused on AMP (346.05 *m/z*), ADP (426.02 *m/z*), ATP (505.98 *m/z*), UDP-hexNAc (606.07 *m/z*), and UDP-hex (565.04 *m/z*) in single-cell zygote cytoplasm. Their ratios indicate the energy state of the cell expressed by the adenylate energy charge (AEC), and hexose utilization:

$$AEC = \frac{ATP + 0.5 ADP}{ATP + ADP + AMP} \quad \text{and} \quad \text{hexose utilization} = \frac{UDP\text{hexNAc}}{UDP\text{hex}}$$

AEC was also investigated in egg albumen, as well as redox state, the ratio of NADH (664.11 *m/z*) to NAD⁺ (662.10 *m/z*) intensities:

$$\text{redox state} = \frac{NADH}{NAD^+}$$

2.10 Statistical analysis

Statistical analysis was performed using the OriginPro® 2015 software (OriginLab Corp., Northampton, Massachusetts, USA). Differences in the amount of the VG-like proteins, oocyte production, AEC, hexose utilization, and redox states were analyzed using the independent-samples *t*-test, the one-way ANOVA with Welch correction, and the Tamhane post hoc test. The time-window of early embryonic development was tested using repeated measures ANOVA. Normality of the dataset was investigated using the Kolmogorov-Smirnov test, homogeneity of variances between groups investigated using Levene's statistic. Comparison of

egg mass quality was performed using nonparametric Kruskal-Wallis test. Differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 Progesterone-induced alteration in adult snails

VG-like protein content of the hepatopancreas, oocyte production, and the quality of laid egg masses were evaluated after progesterone treatments and compared with controls of untreated adult snails. In adult snails, an increased level of VG-like protein was detected following the application of progesterone mixture. The VG-like protein content was significantly higher [$t_{(8)} = -54.79$, $P < 0.001$] in the experimental group treated with the 10 ng/L mixture (46.51 ± 0.35 ng/mg wet tissue), compared to the control group (19.74 ± 0.40 ng/mg wet tissue) (Fig. 2). This observation could also explain the elevated oocyte number. Table 1 summarizes data about the average oocyte number of individuals. No significant differences can be observed between control and treated groups on the first [$F_{(1,39)} = 0.11$; $P = 0.91$; $n = 40$] and second [$F_{(1,48)} = 0.91$; $P = 0.38$; $n = 49$] weeks. In contrast, the average oocyte production significantly changed in the treated group at the third [$F_{(1,41)} = 8.84$; $P < 0.01$; $n = 42$] week compared to the control, and compared to the first and second weeks within the treated group [$F_{(2,61)} = 8.27$; $P < 0.001$; $n = 63$]. No significant difference was observed in weekly average oocyte number in the control group [$F_{(2,67)} = 2.45$; $P = 0.094$; $n = 69$]. Investigation of the egg mass revealed significantly lower quality in the treated group in the first ($\chi^2 = 6.31$; $P < 0.05$; $n = 41$) week, but not during the second ($\chi^2 = 3.65$; $P = 0.56$; $n = 50$) and the third ($\chi^2 = 0.15$; $P = 0.70$; $n = 43$) weeks. No significant difference was observed in the egg mass quality between weeks within the control or the treated groups.

3.2 Progesterone-induced changes in the time-window of early embryonic development

Progesterone-induced differences were also investigated at the cellular level in zygotes using a time-lapse software. Table 2 represents the time-window of embryogenesis following from single-cell zygotes (stage I). Timing started with the first cleavage of the zygote (stage II, two

cells) and continued through the second cleavage (stage III, four cells) until the third cleavage (stage IV, eight cells). The time-window of cell proliferation was significantly different [$F_{(1,12)}=6.747$; $P<0.05$; $n=13$] in zygotes obtained from treated animals compared to controls.

3.3 Metabolomic analysis of single-cell zygote after progestogen treatment

The previously described accelerated cell proliferation in the progestogen treated group can be explained by a need of surplus substances, or by changes in metabolism due to the hormonal effect. Therefore, the metabolomic pattern of the single-cell zygote cytoplasm was investigated. An overview of the 26 identified metabolites is given in Table 3. Based on our MS data (Fig. 3A), differences in the molecular composition of single-cell zygote cytoplasm were not observed between eggs obtained from control and treated animals. There was no significant difference [$t_{(1,9)}=-1.77$, $P=0.110$; $n=10$] in the AEC between the groups, i.e., the AEC ratios were 0.63 ± 0.11 for the control and 0.49 ± 0.12 for the treated cells (Fig. 3B). The hexose utilization was significantly reduced [$t_{(1,9)}=3.83$, $P<0.01$; $n=10$] in the treated group (0.50 ± 0.11) compared to the control (1.16 ± 0.36) (Fig. 3C).

3.4 Metabolomic analysis of albumen after progestogen treatment

The metabolite content of albumen was also examined. Table 3 summarizes the general molecular pattern of egg albumen, including 21 identified metabolites. Two developmental stages were investigated. Albumen sampling was performed within 1 hour after egg laying (single-cell zygote), and after 96 hours (at the beginning of the metamorphosis, which is the halftime of the average hatching time) (Fig. 4A and B). Differences in molecular pattern were not observed between 1-hour old and 96-hour old egg albumen, or between the control and the treated groups. Fig. 4C shows a representative mass spectrum (300–700 m/z) of the molecules detected in the albumen.

The AEC did not alter in the treated group (0.67 ± 0.03) compared to the control (0.65 ± 0.03) within 1 hour after egg laying, but after 96 hours the difference was already significant

(0.34 ± 0.04 in the control and 0.55 ± 0.05 in the treated group). In other words, the AEC declined during embryogenesis in the control group that contained less ATP and ADP after 96 hours compared to the initial 1 hour state. However, the AEC was barely reduced [$t_{(1,10)} = -3.691$, $P < 0.01$; $n = 11$] in the treated group even after 96 hours, compared to the 1 hour state [$t_{(1,11)} = -0.516$, $P = 0.61$; $n = 12$] (Fig. 5A). The redox state did not change in the treated group compared to the control either in 1-hour old [$t_{(1,11)} = -0.52$, $P = 0.62$; $n = 12$], or in 96-hour old [$t_{(1,9)} = 0.90$, $P = 0.39$; $n = 10$] egg albumen (Fig. 5B). However, its value showed an increasing tendency during embryogenesis: 3.31 ± 0.92 (control) and 3.69 ± 0.51 (treated) in 1-hour old eggs, while 7.54 ± 1.30 (control) and 6.11 ± 1.30 (treated) in 96-hour old eggs. This shift of redox state indicates the increasing reductive capacity of albumen (i.e., more NADH).

4 Discussion

4.1 Progesterone-induced changes at physiological level

VG or VG-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 VG secreted into the blood and taken up by oocytes. In invertebrates, where vitellogenesis has also been described, different tissues are the source of circulating VG-like proteins in hemolymph. Remarkably altered levels of the VG-like proteins and VG content are widely accepted as a biomarker for monitoring of xenoestrogen/estrogen contaminations both in amphipod, mollusc and fish species (Gagnaire et al., 2009; Jubeaux et al., 2012; Kokokiris et al., 2006; Lee et al., 2008; Matozzo and Marin, 2008; Miracle et al., 2006; Soverchia et al., 2005). In most oviparous species, the VG-like proteins and the VG are precursors of typical egg-yolk proteins, e.g., vitellins in oocytes that serve as energy reserves during embryogenesis. Vitellins are a nonpolar molecular carrier and storage proteins that transfer lipids, peptides and vitamins to the oocyte. In gastropods, vitellins, such as yolk ferritin, have also been described in the oocytes of *Helix* (Barre et al., 1991), *Helisoma* (Miksysis and Saleuddin, 1986), *Planorbis*

and *Lymnaea* (Bottke, 1986; Bottke et al., 1988). Contradictory reports in the literature stated that the expression of yolk ferritin declined after 21 days of xenoestrogen (tributyltin) exposure in *Lymnaea* (Giusti et al., 2013), whereas its precursor, the VG-like proteins, decreased after 14 days and increased after 28 days of tributyltin treatment in other freshwater gastropods (Gagnaire et al., 2009). Additionally, the up- or downregulation of VG-like protein levels depends on the applied concentration of other xenoestrogens (bisphenol A, and octylphenol), and the exposure period in snail species (Gagnaire et al., 2009). Similarly, our results also show that the VG-like protein content of the hepatopancreas of *Lymnaea* was significantly increased at the end of the 21-day long treatment with the progestogens, which is consistent with the elevated oocyte production observed during the third week. Giusti (Giusti et al., 2013) described that yolk ferritin expression in the reproductive organ of *Lymnaea* did not change significantly after being exposed to progestogen cyproterone acetate for 21 days. Results from Giusti's experiments contradict our observations, which show that exposure to progestogen mixture significantly changes the VG-like protein content in *Lymnaea*. The discrepancy might be because 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 cyproterone acetate. To date, other progestogen effects on VG-like protein content were not investigated in freshwater snails. Based on literature data, VG measurements after exposure to a progestogen mixture were only performed in fish. 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. However, 500 ng/L DRO alone or in combination with 40 ng/L PRG significantly reduced the hepatic VG mRNA.

The next physiological endpoint considered in the present study was oocyte production, which was reduced by the end of the first week, but showed a two-fold elevation in the treated

group at the third week. Our observations contradict other data where oviposition was not significantly altered after progestogen treatment. Giusti (Giusti et al., 2014) published that the total number of egg masses and eggs per *Lymnaea* did not change significantly, however, the mean number of eggs per mass decreased after 21-day long progestogen (cyproterone acetate [$\mu\text{g/L}$]) or non-steroid progestogen (vinclozolin [ng/L]) exposure. Other observation showed that the mean cumulative number of eggs per *Lymnaea* was significantly reduced by a 21-day exposure to vinclozolin. The most pronounced reductions were observed during the first week in a wide concentration range of exposure (from ng/L to $\mu\text{g/L}$). After the first week, vinclozolin in low (25 ng/L) concentration stimulated egg production in the exposed snails (Ducrot et al., 2010). The changes in oocyte production in this experiment were similar to our observations despite the differences in exposure and the selection of progestogens. The effect of the progestogen mixture used in our study has not been investigated in mollusks but similar hormones have been utilized mainly in fish experiments. In fathead minnows, individual application in a 21-day exposure to 10 ng/L GES, 10 ng/L PRG, or 100 ng/L DRO had no effect, but at the 100 ng/L level GES significantly reduced egg production (DeQuattro et al., 2012; Runnalls et al., 2013). Several studies on fathead minnows demonstrated that LNG reduced the egg production in a dose-dependent manner from 0.8 ng/L to 100 ng/L (Runnalls et al., 2013; Zeilinger et al., 2009). The results of our experiments suggest that the tested progestogens significantly disturb the VG-like protein levels or oocyte production of adult *Lymnaea*. Our observations show that invertebrate aquatic species could be sensitive to the simultaneous presence of these hormones in the environment. It would be interesting to know whether the same physiological changes have been observed if *Lymnaea* was exposed to some of the compounds alone at similarly low levels.

4.2 Progestogen-induced changes at cellular and molecular levels

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 protein-coupled receptor and the associated signaling pathways: (GPCR)-AC-cAMP-PKA-MAPK (Nishimoto and Nishida, 2006; Paoli et al., 2013), GPCR-AC-cAMP-PI3K-AKT (Alessi and Cohen, 1998; Shepherd et al., 1998), GPCR-JAK/STAT (Nishimoto and Nishida, 2006; Syrovatkina et al., 2016), and GPCR-AC-cAMP-PKA-GLI3-Hedgehog (Pak and Segal, 2016; Syrovatkina et al., 2016) (see Supplementary Figure 2A). The cellular and molecular endpoints described above have not been used for the assessment of progestogen exposure. Egg formation and content depend on the parents and determine the viability of offspring (Tills et al., 2013). A remarkable observation was that cell proliferation was accelerated by mixed progestogen hormones in the early stage embryos. 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 in the cytoplasm (Baldini et al., 2016; Damerow et al., 2015; Fraga et al., 2013; Pyla et al., 2015; Yi and Huh, 2015) (Supplementary Figure 2B).

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 (Dehennaut et al., 2009; Dehennaut et al., 2007). This observation means that if the cell is in a good energy state the regulatory enzymes of anabolic sequences increase their activity and glycogen synthesis starts. 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 (Han et al., 2016; Wei et al., 2016; Zhang et al., 2016), the glycogen synthesis stops and UDP-hex is accumulated in zygotes obtained from treated parents. In parallel, the GPCR-AC-cAMP-PI3K-PIP3-PDK1 system (Paoli et al., 2013) intensifies the hexose uptake (Supplementary Figure 2A), thus hexose metabolism is not disrupted. According to Wells (Wells et al., 2003), the UDP-hexNAc pool directly depends upon hexose concentration. UDP-hexNAc 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 (Park et al., 2008), thus the nutrient supply of embryos is not disrupted by the hormone treatment.

The egg albumen participates in embryonic growth by providing essential nutrients as well as securing the proper environment in each developmental stage. According to our results, this albumen environment initially was similar from a metabolic point of view (redox state and AEC) in the control and treated animals. The redox state is determined in part by glycolysis which sets the steady-state of the NADH/NAD⁺ ratio. This ratio increased in both groups during embryogenesis, but no significant difference could be observed between them. Therefore, it is concluded that the progestogen treatment of parents has no effect on the enzymes of glycolysis. Furthermore, the most important redox buffer is the glutathione (GSH) – oxidized glutathione (GSSG) pair, and the redox buffer effect of GSH dominates that of NADH/NAD⁺ (Obrosova and Stevens, 1999; Zhang and Vertes, 2015). In case of albumen MS measurements, despite of the stable GSH signal GSSG cannot be detected, thus we discussed only the NADH/NAD⁺ ratio. In contrast, AEC was significantly higher in the treated group at the half-time of embryogenesis (96 hours). The elevated AEC suggests that the treated parents provided more

hexose in eggs compared to the control. The surplus hexose in albumen may serve on the one hand to provide more energy for the upcoming metamorphosis, and, on the other, to improve the viability of the offspring through strengthening tolerance/adaptation to the steroid contaminated environment.

5 Conclusion

Contamination of rivers and lakes with various steroidal pharmaceuticals is a general problem worldwide. 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 model animal, and by applying a treatment with an equi-concentration mixture of four progestogens in environmentally relevant, relatively low concentrations. The results show that invertebrate aquatic species could be sensitive to the simultaneous presence of hormones in the environment.

6 Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

7 Authors contribution

The study was design by ZZ and PZ, and the experimental work was performed by ZZ, MG, LZ and AV. Statistical analyses was made by ZZ, with additional interpretation by MG and LS. The manuscript was written by ZZ and PZ with feedback from LS, KT, EK and AV.

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10 References

- Alessi, D.R., Cohen, P., 1998. Mechanism of activation and function of protein kinase B. *Curr Opin Genet Dev* 8, 55-62.
- Altelaar, A.F., van Minnen, J., Jimenez, C.R., Heeren, R.M., Piersma, S.R., 2005. Direct molecular imaging of *Lymnaea stagnalis* nervous tissue at subcellular spatial resolution by mass spectrometry. *Anal Chem* 77, 735-741.
- Aris, A.Z., Shamsuddin, A.S., Praveena, S.M., 2014. Occurrence of 17alpha-ethynylestradiol (EE2) in the environment and effect on exposed biota: a review. *Environ Int* 69, 104-119.
- Avar, P., Maasz, G., Takacs, P., Lovas, S., Zrinyi, Z., Svigruha, R., Takatsy, A., Toth, L.G., Pirger, Z., 2016. HPLC-MS/MS analysis of steroid hormones in environmental water samples. *Drug Test Anal* 8, 124-128.
- Baldini, S.F., Steenackers, A., Olivier-Van Stichelen, S., Mir, A.M., Mortuaire, M., Lefebvre, T., Guinez, C., 2016. Glucokinase expression is regulated by glucose through O-GlcNAc glycosylation. *Biochem Biophys Res Commun* 478, 942-948.
- Barre, P., Bride, M., Beliard, R., Petracca, B., 1991. Localization of yolk proteins and their possible precursors using polyclonal and monoclonal antibodies, in *Helix aspersa*. *Cell Mol Biol* 37, 639-650.
- Bottke, W., 1986. Immunolocalization of ferritin polypeptides in oocytes and somatic tissue of the freshwater snails *Lymnaea stagnalis* and *Planorbarius corneus*. *Cell Tissue Res* 243, 397-404.
- Bottke, W., Burschkyk, M., Volmer, J., 1988. On the origin of the yolk protein ferritin in snails. *Roux's Arch Dev Biol* 197, 377-382.
- Caldwell, D.J., Mastrocco, F., Hutchinson, T.H., Lange, R., Heijerick, D., Janssen, C., Anderson, P.D., Sumpter, J.P., 2008. Derivation of an aquatic predicted no-effect concentration for the synthetic hormone, 17 alpha-ethinyl estradiol. *Environ Sci Technol* 42, 7046-7054.
- Chang, H., Wan, Y., Wu, S., Fan, Z., Hu, J., 2011. Occurrence of androgens and progestogens in wastewater treatment plants and receiving river waters: comparison to estrogens. *Water Res* 45, 732-740.
- Christen, V., Crettaz, P., Oberli-Schrammli, A., Fent, K., 2012. Antiandrogenic activity of phthalate mixtures: validity of concentration addition. *Toxicol Appl Pharmacol* 259, 169-176.
- Contardo-Jara, V., Lorenz, C., Pflugmacher, S., Nutzmann, G., Kloas, W., Wiegand, C., 2011. Molecular effects and bioaccumulation of levonorgestrel in the non-target organism *Dreissena polymorpha*. *Environ Pollut* 159, 38-44.
- Damerow, S., Hoppe, C., Bandini, G., Zarnovican, P., Buettner, F.F., Luder, C.G., Ferguson, M.A., Routier, F.H., 2015. Depletion of UDP-Glucose and UDP-Galactose using a degran system leads to growth cessation of *Leishmania major*. *PLoS Negl Trop Dis* 9, e0004205.

- de Graef, M.R., Alexeeva, S., Snoep, J.L., Teixeira de Mattos, M.J., 1999. The steady-state internal redox state (NADH/NAD) reflects the external redox state and is correlated with catabolic adaptation in *Escherichia coli*. *J Bacteriol* 181, 2351-2357.
- Dehennaut, V., Lefebvre, T., Leroy, Y., Vilain, J.P., Michalski, J.C., Bodart, J.F., 2009. Survey of O-GlcNAc level variations in *Xenopus laevis* from oogenesis to early development. *Glycoconj J* 26, 301-311.
- Dehennaut, V., Lefebvre, T., Sellier, C., Leroy, Y., Gross, B., Walker, S., Cacan, R., Michalski, J.C., Vilain, J.P., Bodart, J.F., 2007. O-linked N-acetylglucosaminyltransferase inhibition prevents G2/M transition in *Xenopus laevis* oocytes. *J Biol Chem* 282, 12527-12536.
- DeQuattro, Z.A., Peissig, E.J., Antkiewicz, D.S., Lundgren, E.J., Hedman, C.J., Hemming, J.D., Barry, T.P., 2012. Effects of progesterone on reproduction and embryonic development in the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 31, 851-856.
- Ducrot, V., Askem, C., Azam, D., Brettschneider, D., Brown, R., Charles, S., Coke, M., Collinet, M., Delignette-Muller, M.L., Forfait-Dubuc, C., Holbech, H., Hutchinson, T., Jach, A., Kinnberg, K.L., Lacoste, C., Le Page, G., Matthiessen, P., Oehlmann, J., Rice, L., Roberts, E., Ruppert, K., Davis, J.E., Veauvy, C., Weltje, L., Wortham, R., Lagadic, L., 2014. Development and validation of an OECD reproductive toxicity test guideline with the pond snail *Lymnaea stagnalis* (Mollusca, Gastropoda). *Regul Toxicol Pharm* 70, 605-614.
- Ducrot, V., Teixeira-Alves, M., Lopes, C., Delignette-Muller, M.L., Charles, S., Lagadic, L., 2010. Development of partial life-cycle experiments to assess the effects of endocrine disruptors on the freshwater gastropod *Lymnaea stagnalis*: a case-study with vinclozolin. *Ecotoxicology* 19, 1312-1321.
- Fent, K., 2015. Progestins as endocrine disruptors in aquatic ecosystems: Concentrations, effects and risk assessment. *Environ Int* 84, 115-130.
- Fraga, A., Ribeiro, L., Lobato, M., Santos, V., Silva, J.R., Gomes, H., da Cunha Moraes, J.L., de Souza Menezes, J., de Oliveira, C.J., Campos, E., da Fonseca, R.N., 2013. Glycogen and glucose metabolism are essential for early embryonic development of the red flour beetle *Tribolium castaneum*. *PLoS One* 8, e65125.
- Gagnaire, B., Gagne, F., Andre, C., Blaise, C., Abbaci, K., Budzinski, H., Devier, M.H., Garric, J., 2009. Development of biomarkers of stress related to endocrine disruption in gastropods: alkali-labile phosphates, protein-bound lipids and vitellogenin-like proteins. *Aquat Toxicol* 92, 155-167.
- Giusti, A., Joaquim-Justo, C., 2013. Esterification of vertebrate like steroids in molluscs: a target of endocrine disruptors? *Comp Biochem Physiol C Toxicol Pharmacol* 158, 187-198.
- Giusti, A., Lagadic, L., Barsi, A., Thome, J.P., Joaquim-Justo, C., Ducrot, V., 2014. Investigating apical adverse effects of four endocrine active substances in the freshwater gastropod *Lymnaea stagnalis*. *Sci Total Environ* 493, 147-155.
- Giusti, A., Leprince, P., Mazzucchelli, G., Thome, J.P., Lagadic, L., Ducrot, V., Joaquim-Justo, C., 2013. Proteomic analysis of the reproductive organs of the hermaphroditic gastropod *Lymnaea stagnalis* exposed to different endocrine disrupting chemicals. *PloS one* 8, e81086.
- Han, C.C., Ma, Y., Li, Y., Wang, Y., Wei, W., 2016. Regulatory effects of GRK2 on GPCRs and non-GPCRs and possible use as a drug target (Review). *Int J Mol Med* 38, 987-994.
- Jong-Brink, M.d., Schot, L.P.C., Schoenmakers, H.J.N., Bergamin-Sassen, M.J.M., 1981. A biochemical and quantitative electron microscope study on steroidogenesis in ovotestis and digestive gland of the pulmonate snail *Lymnaea stagnalis*. *Gen Comp Endocrinol* 45, 30-38.
- Jubeaux, G., Simon, R., Salvador, A., Queau, H., Chaumot, A., Geffard, O., 2012. Vitellogenin-like proteins in the freshwater amphipod *Gammarus fossarum* (Koch, 1835): functional characterization throughout reproductive process, potential for use as an indicator of oocyte quality and endocrine disruption biomarker in males. *Aquat Toxicol* 112-113, 72-82.

- Ketata, I., Denier, X., Hamza-Chaffai, A., Minier, C., 2008. Endocrine-related reproductive effects in molluscs. *Comp Biochem Phys C* 147, 261-270.
- Koene, J.M., 2010. Neuro-endocrine control of reproduction in hermaphroditic freshwater snails: mechanisms and evolution. *Front Behav Neurosci* 4, 167.
- Kokokiris, L., Fostier, A., Athanassopoulou, F., Petridis, D., Kentouri, M., 2006. Gonadal changes and blood sex steroids levels during natural sex inversion in the protogynous Mediterranean red porgy, *Pagrus pagrus* (Teleostei: Sparidae). *Gen Comp Endocrinol* 149, 42-48.
- Kunz, P.Y., Fent, K., 2009. Estrogenic activity of ternary UV filter mixtures in fish (*Pimephales promelas*) - an analysis with nonlinear isobolograms. *Toxicol Appl Pharmacol* 234, 77-88.
- Lee, K.W., Hwang, D.S., Rhee, J.S., Ki, J.S., Park, H.G., Ryu, J.C., Raisuddin, S., Lee, J.S., 2008. Molecular cloning, phylogenetic analysis and developmental expression of a vitellogenin (Vg) gene from the intertidal copepod *Tigriopus japonicus*. *Comp Biochem Phys B* 150, 395-402.
- 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. *Science Total Environ* 409, 5149-5161.
- Matozzo, V., Marin, M.G., 2008. Can 17-beta estradiol induce vitellogenin-like proteins in the clam *Tapes philippinarum*? *Environ Toxicol Phar* 26, 38-44.
- Mescheriakov, V.N., 1990. Animal species for developmental studies
in: Detlaff, D.A., Vassetzky, S.C.J. (Eds.), *The common pond snail, Lymnaea stagnalis* Plenum Press, New York-London, pp. 69-132.
- Miksysis, S., Saleuddin, A.S.M., 1986. Ferritin as an exogenously derived yolk protein in *Helisoma duryi* (Mollusca: Pulmonata). *Can J Zool* 64, 2678-2682.
- Miracle, A., Ankley, G., Lattier, D., 2006. Expression of two vitellogenin genes (vg1 and vg3) in fathead minnow (*Pimephales promelas*) liver in response to exposure to steroidal estrogens and androgens. *Ecotoxicol Environ Saf* 63, 337-342.
- Morill, J.B., 1982. Developmental Biology of the Freshwater Invertebrates in: Harrison, F.W., Cowden, R.R. (Eds.), *Development of the pulmonate gastropod, Lymnaea.*, New York, pp. 399-483.
- Nakadera, Y., Swart, E.M., Maas, J.P., Montagne-Wajer, K., Ter Maat, A., Koene, J.M., 2015. Effects of age, size, and mating history on sex role decision of a simultaneous hermaphrodite. *Behav Ecol* 26, 232-241.
- Nishimoto, S., Nishida, E., 2006. MAPK signalling: ERK5 versus ERK1/2. *EMBO Rep* 7, 782-786.
- Obrosova, I.G., Stevens, M.J., 1999. Effect of dietary taurine supplementation on GSH and NAD(P)-redox status, lipid peroxidation, and energy metabolism in diabetic precataractous lens. *Invest Ophthalmol Vis Sci* 40, 680-688.
- Orlando, E.F., Ellestad, L.E., 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.
- Pak, E., Segal, R.A., 2016. Hedgehog signal transduction: key players, oncogenic drivers, and cancer therapy. *Dev Cell* 38, 333-344.
- Paoli, P., Giannoni, E., Chiarugi, P., 2013. Anokis molecular pathways and its role in cancer progression. *Biochim Biophys Acta* 1833, 3481-3498.
- Park, Y., Zhang, Z., Laremore, T.N., Li, B., Sim, J.S., Im, A.R., Ahn, M.Y., Kim, Y.S., Linhardt, R.J., 2008. Variation of acharan sulfate and monosaccharide composition and analysis of neutral N-glycans in African giant snail (*Achatina fulica*). *Glycoconj J* 25, 863-877.
- Pyla, R., Pichavaram, P., Fairaq, A., Park, M.A., Kozak, M., Kamath, V., Patel, V.S., Segar, L., 2015. Altered energy state reversibly controls smooth muscle contractile function in human

- saphenous vein during acute hypoxia-reoxygenation: Role of glycogen, AMP-activated protein kinase, and insulin-independent glucose uptake. *Biochem Pharmacol* 97, 77-88.
- Richardson, M.L., Bowron, J.M., 1985. The fate of pharmaceutical chemicals in the aquatic environment. *J Pharm Pharmacol* 37, 1-12.
- Runnalls, T.J., Beresford, N., Kugathas, S., Margiotta-Casaluci, L., Scholze, M., Scott, A.P., Sumpter, J.P., 2015. From single chemicals to mixtures - Reproductive effects of levonorgestrel and ethinylestradiol on the fathead minnow. *Aquat Toxicol* 169, 152-167.
- Runnalls, T.J., Beresford, N., Losty, E., Scott, A.P., Sumpter, J.P., 2013. Several synthetic progestins with different potencies adversely affect reproduction of fish. *Environ Sci Technol* 47, 2077-2084.
- Santos, L.H., Araujo, A.N., Fachini, A., Pena, A., Delerue-Matos, C., Montenegro, M.C., 2010. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. *J Hazard Mater* 175, 45-95.
- Scott, A.P., 2012. Do mollusks use vertebrate sex steroids as reproductive hormones? Part I: Critical appraisal of the evidence for the presence, biosynthesis and uptake of steroids. *Steroids* 77, 1450-1468.
- Shepherd, P.R., Withers, D.J., Siddle, K., 1998. Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. *Biochem J* 333, 471-490.
- Soverchia, L., Ruggeri, B., Palermo, F., Mosconi, G., Cardinaletti, G., Scortichini, G., Gatti, G., Polzonetti-Magni, A.M., 2005. Modulation of vitellogenin synthesis through estrogen receptor beta-1 in goldfish (*Carassius auratus*) juveniles exposed to 17-beta estradiol and nonylphenol. *Toxicol Appl Pharmacol* 209, 236-243.
- Svatos, A., 2011. Single-cell metabolomics comes of age: new developments in mass spectrometry profiling and imaging. *Anal Chem* 83, 5037-5044.
- Syrovatkina, V., Alegre, K.O., Dey, R., Huang, X.Y., 2016. Regulation, signaling, and physiological functions of G-proteins. *J Mol Biol.* 428, 3850-68.
- Tillmann, M., Schulte-Oehlmann, U., Duft, M., Markert, B., Oehlmann, J., 2001. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part III: Cyproterone acetate and vinclozolin as antiandrogens. *Ecotoxicology* 10, 373-388.
- Tills, O., Rundle, S.D., Spicer, J.I., 2013. Parent-offspring similarity in the timing of developmental events: an origin of heterochrony? *Proc Biol Sci* 280, 20131479.
- Tomos, A.D., Sharrock, R.A., 2001. Cell sampling and analysis (SiCSA): metabolites measured at single cell resolution. *J Exp Bot* 52, 623-630.
- Vulliet, E., Wiest, L., Baudot, R., Grenier-Loustalot, M.F., 2008. Multi-residue analysis of steroids at sub-ng/L levels in surface and ground-waters using liquid chromatography coupled to tandem mass spectrometry. *J Chromatogr A* 1210, 84-91.
- Wei, A., Fan, B., Zhao, Y., Zhang, H., Wang, L., Yu, X., Yuan, Q., Yang, D., Wang, S., 2016. ST6Gal-I overexpression facilitates prostate cancer progression via the PI3K/Akt/GSK-3beta/beta-catenin signaling pathway. *Oncotarget* 7, 65374-65388.
- Wells, L., Vosseller, K., Hart, G.W., 2003. A role for N-acetylglucosamine as a nutrient sensor and mediator of insulin resistance. *Cell Mol Life Sci* 60, 222-228.
- Yi, D.G., Huh, W.K., 2015. PKA, PHO and stress response pathways regulate the expression of UDP-glucose pyrophosphorylase through Msn2/4 in budding yeast. *FEBS Lett* 589, 2409-2416.
- Zeilinger, J., Steger-Hartmann, T., Maser, E., Goller, S., Vonk, R., Lange, R., 2009. Effects of synthetic gestagens on fish reproduction. *Environ Toxicol Chem* 28, 2663-2670.
- Zhang, B., Lai, G., Wu, J., Sun, R., Xu, R., Yang, X., Qi, Y., Zhao, Y., 2016. 20-HETE attenuates the response of glucose-stimulated insulin secretion through the AKT/GSK-3beta/Glut2 pathway. *Endocrine* 54, 371-382.

Zhang, L., Foreman, D.P., Grant, P.A., Shrestha, B., Moody, S.A., Villiers, F., Kwak, J.M., Vertes, A., 2014. In situ metabolic analysis of single plant cells by capillary microsampling and electrospray ionization mass spectrometry with ion mobility separation. *Analyst* 139, 5079-5085.

Zhang, L., Vertes, A., 2015. Energy charge, redox state, and metabolite turnover in single human hepatocytes revealed by capillary microsampling mass spectrometry. *Anal Chem* 87, 10397-10405.

Zucchi, S., Mirbahai, L., Castiglioni, S., Fent, K., 2014. Transcriptional and physiological responses induced by binary mixtures of drospirenone and progesterone in zebrafish (*Danio rerio*). *Environ Sci Technol* 48, 3523-3531.

Figure legends

Figure 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).

Figure 2. Alteration of vitellogenin (VG) level during progestogen treatment in adult animals (n=12). The amount of VG in the two experimental groups (n=3 animal per group, two independent series) was determined by ELISA at the end of the 3-week long experiment. white column - control group; grey column - 10 ng/L treated group; error bars - standard deviation; *** - P<0.001

Figure 3. Metabolomic analysis of a single-cell zygote. (A) A representative mass spectrum of the cytoplasm of a single-cell zygote taken in 300–700 *m/z* detection range and representing the most important molecules detected. The insert shows from left to right the microsampling process of the zygote: the holding pipette, the single-cell zygote (<1 h) and the sampling capillary. Scale bar: 100 μ m. (B) The adenylate energy charge (AEC), and (C) the UDP-hexNAc/UDP-hex ratio between control and 10 ng/L treated groups are shown. white columns

– control group; grey columns - 10 ng/L treated group; error bars - standard deviation; ** - $P < 0.01$

Figure 4. Microsampling for metabolomic analysis of albumen. (A) A 1-hour (upper) and a 96-hour (lower) old egg. The foot lobe region is indicated by an arrow. (B) The capillary microsampling of albumen from a 1-hour old egg. The microcapillary is surrounded by a dashed line and arrow shows the capillary ending. Scale bars represent 100 μm . (C) A representative mass spectrum of the albumen taken in 300–700 m/z detection range, presenting the highlighted molecules of AEC and redox state.

Figure 5. Metabolomic analysis of the egg albumen. The differences in (A) AEC level, and (B) redox state between control and 10 ng/L treated groups within 1 hour (left) and after 96 hours (right) are shown. white columns – control; grey columns - 10 ng/L treated group; error bars - standard deviation; ** - $P < 0.01$

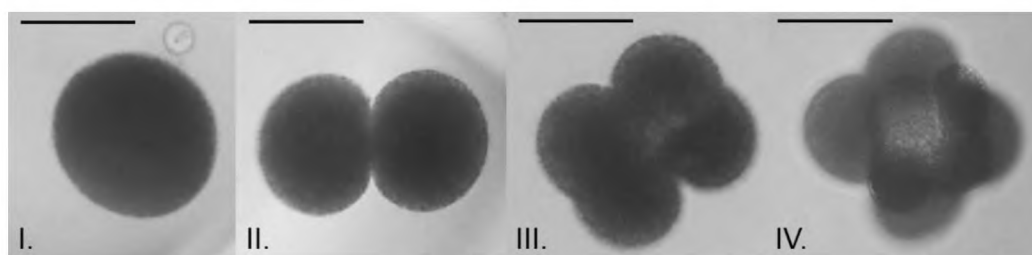
Table 1. Oocyte production refers to individual animals and the egg mass quality during progestogen treatment.

		1 st week *			2 nd week			3 rd week #		
Control	Oocyte number	73.00±5.36			69.33±6.45			63.94±3.70		
	Egg mass quality (%)	GOOD 85.19	FAIR 7.41	POOR 7.41	GOOD 76.00	FAIR 20.00	POOR 4.00	GOOD 77.80	FAIR 16.60	POOR 5.60
10 ng/L treated	Oocyte number	37.18±7.20			81.60±7.95			148.00±5.10^{**/**}		
	Egg mass quality (%)	GOOD 50.00	FAIR 21.43	POOR 28.57	GOOD 68.00	FAIR 8.00	POOR 24.00	GOOD 72.00	FAIR 4.00	POOR 24.00

* The egg mass quality was significantly different ($P<0.05$) between the control and the treated group at the 1st week.

In the 10 ng/L treated group a statistically significant difference was observed in oocyte number at the 3rd week compared to the control ($P<0.01$), as well as compared to the 1st and 2nd weeks within the treated group ($P<0.001$).

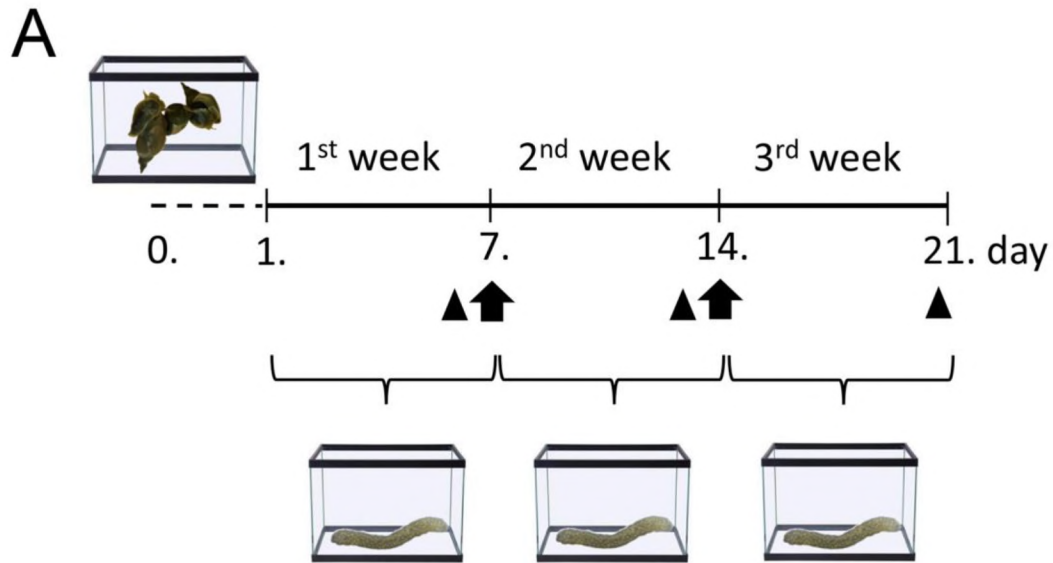
Table 2. Time-window of cell proliferation during early snail embryogenesis. Scale bars represents 100 μm .



Stage development	of Control	10 ng/L treatment	Cell size (μm)
I. Single-cell zygote			~120
II. 2 cells (1 st cleavage)	Start point	Start point	~100
III. 4 cells (2 nd cleavage)	131 \pm 12 min	115 \pm 7 min	~100
IV. 8 cells (3 rd cleavage)	110 \pm 4 min	106 \pm 5 min	Different cell size
Total:	241 \pm 14 min	222 \pm 4 min	
Hatching:	13 \pm 0.7 Days	14 \pm 0.8 Days	

Table 3. Identified molecules detected in the single-cell zygote (within 1 hour after egg laying) and egg albumen (1 and 96 hours after egg laying).

Molecules detected only in single-cell zygote cytoplasm						
	Accepted Description	Formula	MW [Da]	Calc. Mass [Da]	Meas. [Da]	Mass Δ mass [mDa]
1.	Glutamine	C ₅ H ₁₀ N ₂ O ₃	146.0691	145.0618	145.059	3.2
2.	Glutamic acid	C ₅ H ₉ NO ₄	147.0531	146.0458	146.031	14.4
3.	Glycerol phosphate	C ₃ H ₉ O ₆ P	172.0136	171.0063	171.006	0.3
4.	Hexose phosphate	C ₆ H ₁₃ O ₉ P	260.0297	259.0224	259.021	1.8
5.	UMP	C ₉ H ₁₃ N ₂ O ₉ P	324.0359	323.0286	323.030	-1.6
6.	CDP-ethanolamine	C ₁₁ H ₂₀ N ₄ O ₁₁ P ₂	446.0604	445.0531	445.036	17.0
7.	CTP	C ₉ H ₁₆ N ₃ O ₁₄ P ₃	482.9845	481.9772	481.977	0.2
8.	UTP	C ₉ H ₁₅ N ₂ O ₁₅ P ₃	483.9685	482.9613	482.954	7.7
9.	UDP-hexose	C ₁₅ H ₂₄ N ₂ O ₁₇ P ₂	566.0550	565.0478	565.047	1.0
10.	Oxidized glutathione (GSSG)	C ₂₀ H ₃₂ N ₆ O ₁₂ S ₂	612.1520	611.1447	611.118	26.8
Molecules detected only in egg albumen						
	Accepted Description	Formula	MW [Da]	Calc. Mass [Da]	Meas. [Da]	Mass Δ mass [mDa]
1.	IMP	C ₁₀ H ₁₃ N ₄ O ₈ P	348.0471	347.0398	347.052	-12.6
2.	IMP +Na	C ₁₀ H ₁₃ N ₄ O ₈ P	370.0290	369.0218	369.036	-13.9
3.	ADP +Na	C ₁₀ H ₁₅ N ₅ O ₁₀ P ₂	449.0118	448.0045	448.009	-4.6
4.	UDP-hexNAc + K	C ₁₇ H ₂₇ N ₃ O ₁₇ P ₂	645.0375	644.0302	644.031	-0.4
5.	NADH	C ₂₁ H ₂₉ N ₇ O ₁₄ P ₂	665.1248	664.1175	664.115	2.4
Molecules detected both in single-cell zygote cytoplasm and egg albumen						
	Accepted Description	Formula	MW [Da]	Calc. Mass [Da]	Meas. [Da]	Mass Δ mass [mDa]
1.	GSH	C ₁₀ H ₁₇ N ₃ O ₆ S	307.0838	306.0765	306.077	-0.3
2.	CMP	C ₉ H ₁₄ N ₃ O ₈ P	323.0519	322.0446	322.044	0.4
3.	GSH +Na	C ₁₀ H ₁₇ N ₃ O ₆ S	329.0525	328.0452	328.052	7.2
4.	AMP	C ₁₀ H ₁₄ N ₅ O ₇ P	347.0631	346.0558	346.056	0.3
5.	GMP	C ₁₀ H ₁₄ N ₅ O ₈ P	363.0580	362.0507	362.047	3.8
6.	CDP	C ₉ H ₁₅ N ₃ O ₁₁ P ₂	403.0182	402.0109	402.011	-0.1
7.	UDP	C ₉ H ₁₄ N ₂ O ₁₂ P ₂	404.0022	402.9949	402.995	-0.4
8.	ADP -H ₂ O	C ₁₀ H ₁₅ N ₅ O ₁₀ P ₂	409.0189	408.0116	408.014	-1.9
9.	ADP	C ₁₀ H ₁₅ N ₅ O ₁₀ P ₂	427.0294	426.0221	426.022	-0.2
10.	GDP	C ₁₀ H ₁₅ N ₅ O ₁₁ P ₂	443.0243	442.0171	442.013	4.0
11.	ATP	C ₁₀ H ₁₆ N ₅ O ₁₃ P ₃	506.9958	505.9885	505.985	3.8
12.	GTP	C ₁₀ H ₁₆ N ₅ O ₁₄ P ₃	522.9907	521.9834	522.002	-18.6
13.	cyclicADP ribose	C ₁₅ H ₂₁ N ₅ O ₁₃ P ₂	541.0611	540.0538	540.053	0.6
14.	GDP-hexose	C ₁₆ H ₂₅ N ₅ O ₁₆ P ₂	605.0772	604.0699	604.059	10.9
15.	UDP-hexNAc	C ₁₇ H ₂₇ N ₃ O ₁₇ P ₂	607.0816	606.0743	606.074	0.6
16.	NAD	C ₂₁ H ₂₇ N ₇ O ₁₄ P ₂	663.1091	662.1019	662.102	0.0



B

		Quality of egg mass		
Polyembryony	$\geq 5\%$	FAIR	POOR	POOR
	$< 5\%$	GOOD	FAIR	POOR
	0	GOOD	GOOD	FAIR
		0	$< 5\%$	$> 5\%$
		Dead embryos		

