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

Zita Zrinyi[a], Gabor Maasz[a], Linwen Zhang[b], Akos Vertes[b], Sandor Lovas[a], Tibor Kiss[c], Karoly Elekes[c], Zsolt Pirger[a,*]

[a] MTA-ÖK BLI NAP_B Adaptive Neuroethology, Department of Experimental Zoology, Balaton Limnological Institute, MTA Center for Ecological Research, Tihany, Hungary

[b] Department of Chemistry, W. M. Keck Institute for Proteomics Technology and Applications, The George Washington University, WA, District of Columbia 20052, USA

[c] MTA-ÖK BLI, Department of Experimental Zoology, Balaton Limnological Institute, MTA Center for Ecological Research, Tihany, Hungary

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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.

* Corresponding author at: MTA-ÖK BLI NAP_B Adaptive Neuroethology, MTA Centre for Ecological Research Balaton Limnological Institute, Klebelsberg K. u. 3., H-8237, Tihany, Hungary.

E-mail addresses: pirger.zsolt@okologia.mta.hu, pirgerzs@gmail.com (Z. Pirger).

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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/

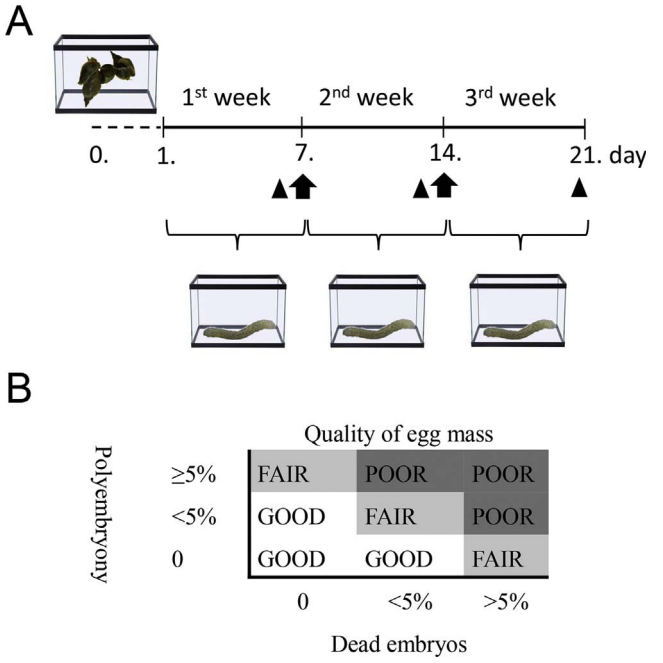


Fig. 1. Management of animal treatment and evaluation of the egg mass quality. (A) Snails acclimatized for 1 day in all tanks with no chemicals (dashed line). From the starting point (1st day) chemicals were applied in the experiment. Recovery measurements were done on the 7th, 14th and 21st days (▲). 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).

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 ≥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 μL) 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 min at 1500 × *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 μL/well). After this, 100 μL 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 μL of chromogen solutions A and B were added to the wells, and incubated for 15 min at 37 °C. Then 50 μL 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 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).

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 ~10,000 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:

$$\text{AEC} = \frac{ATP + 0.5ADP}{ATP + ADP + AMP} \quad \text{and} \quad \text{hexose utilization} = \frac{UDPhexNAc}{UDPhex}$$

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:

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

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. Progestogen-induced alteration in adult snails

VG-like protein content of the hepatopancreas, oocyte production, and the quality of laid egg masses were evaluated after progestogen 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 progestogen 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.

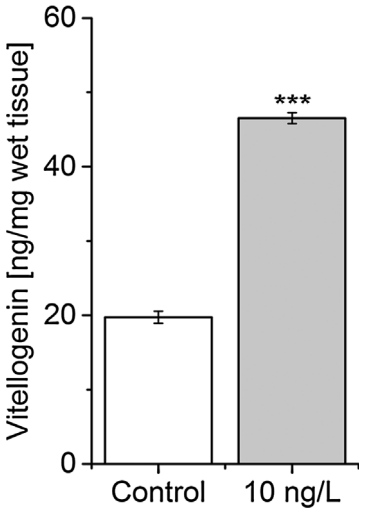


Fig. 2. Alteration of vitellogenin (VG) level during progestogen treatment in adult animals (n = 12). The amount of VG in the two experimental groups (n = 3 animal per group, two independent series) was determined by ELISA at the end of the 3-week long experiment. white column – control group; grey column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

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

Progestogen-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

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

		1st week *			2nd week			3rd week #		
Control	Oocyte number	**73.00±5.36**			**69.33±6.45**			**63.94±3.70**		
	Egg mass quality (%)	GOOD	FAIR	POOR	GOOD	FAIR	POOR	GOOD	FAIR	POOR
		85.19	7.41	7.41	76.00	20.00	4.00	77.80	16.60	5.60
10 ng/L treated	Oocyte number	**37.18±7.20**			**81.60±7.95**			**148.00±5.10**$^{**/***}$		
	Egg mass quality (%)	GOOD	FAIR	POOR	GOOD	FAIR	POOR	GOOD	FAIR	POOR
		50.00	21.43	28.57	68.00	8.00	24.00	72.00	4.00	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 ($P < 0.001$).

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 h after egg laying (single-cell zygote), and after 96 h (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-h old and 96-h 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.

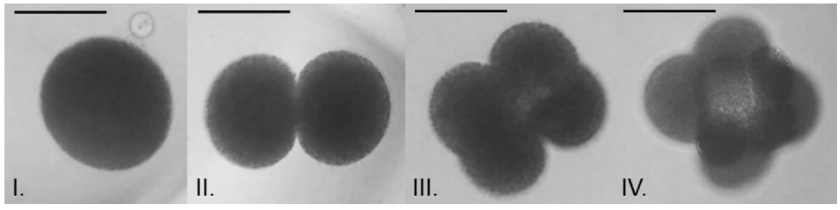
The AEC did not alter in the treated group (0.67 ± 0.03) compared to the control (0.65 ± 0.03) within 1 h after egg laying, but after 96 h the difference was already significant (0.34 ± 0.04 in the control and 0.55 ± 0.05 in the treated group). In other words, the AEC declined during embryogenesis in the control group that contained less ATP and ADP after 96 h compared to the initial 1 h state. However, the AEC was barely reduced [$t_{(1,10)} = -3.691$, $P < 0.01$; n = 11] in the treated group even after 96 h, compared to the 1 h 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-h old [$t_{(1,11)} = -0.52$, P = 0.62; n = 12], or in 96-h 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-h old eggs, while 7.54 ± 1.30 (control) and 6.11 ± 1.30 (treated) in 96-h old eggs. This shift of redox state indicates the increasing reductive capacity of albumen (i.e., more NADH).

4. Discussion

4.1. Progestogen-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* (Miksys and Saleuddin, 1986), *Planorbis* and *Lymnaea* (Bottke, 1986; Bottke et al., 1988). Contradictory reports in the literature stated that the expression

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



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

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

	Molecules detected only in single-cell zygote cytoplasm					
	Accepted Description	Formula	MW [Da]	Calc. Mass [Da]	Meas. Mass [Da]	Δmass [mDa]
1.	Glutamine	$C_5H_{10}N_2O_3$	146.0691	145.0618	145.059	3.2
2.	Glutamic acid	$C_5H_9NO_4$	147.0531	146.0458	146.031	14.4
3.	Glycerol phosphate	$C_3H_9O_6P$	172.0136	171.0063	171.006	0.3
4.	Hexose phosphate	$C_6H_{13}O_9P$	260.0297	259.0224	259.021	1.8
5.	UMP	$C_9H_{13}N_2O_9P$	324.0359	323.0286	323.030	−1.6
6.	CDP-ethanolamine	$C_{11}H_{20}N_4O_{11}P_2$	446.0604	445.0531	445.036	17.0
7.	CTP	$C_9H_{16}N_3O_{14}P_3$	482.9845	481.9772	481.977	0.2
8.	UTP	$C_9H_{15}N_2O_{15}P_3$	483.9685	482.9613	482.954	7.7
9.	UDP-hexose	$C_{15}H_{24}N_2O_{17}P_2$	566.0550	565.0478	565.047	1.0
10.	Oxidized glutathione (GSSG)	$C_{20}H_{32}N_6O_{12}S_2$	612.1520	611.1447	611.118	26.8
	Molecules detected only in egg albumen					
	Accepted Description	Formula	MW [Da]	Calc. Mass [Da]	Meas. Mass [Da]	Δmass [mDa]
1.	IMP	$C_{10}H_{13}N_4O_8P$	348.0471	347.0398	347.052	−12.6
2.	IMP +Na	$C_{10}H_{13}N_4O_8P$	370.0290	369.0218	369.036	−13.9
3.	ADP +Na	$C_{10}H_{15}N_5O_{10}P_2$	449.0118	448.0045	448.009	−4.6
4.	UDP-hexNAc + K	$C_{17}H_{27}N_3O_{17}P_2$	645.0375	644.0302	644.031	−0.4
5.	NADH	$C_{21}H_{29}N_7O_{14}P_2$	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. Mass [Da]	Δmass [mDa]
1.	GSH	$C_{10}H_{17}N_3O_6S$	307.0838	306.0765	306.077	−0.3
2.	CMP	$C_9H_{14}N_3O_8P$	323.0519	322.0446	322.044	0.4
3.	GSH +Na	$C_{10}H_{17}N_3O_6S$	329.0525	328.0452	328.052	7.2
4.	AMP	$C_{10}H_{14}N_5O_7P$	347.0631	346.0558	346.056	0.3
5.	GMP	$C_{10}H_{14}N_5O_8P$	363.0580	362.0507	362.047	3.8
6.	CDP	$C_9H_{15}N_3O_{11}P_2$	403.0182	402.0109	402.011	−0.1
7.	UDP	$C_9H_{14}N_2O_{12}P_2$	404.0022	402.9949	402.995	−0.4
8.	ADP $-H_2O$	$C_{10}H_{15}N_5O_{10}P_2$	409.0189	408.0116	408.014	−1.9
9.	ADP	$C_{10}H_{15}N_5O_{10}P_2$	427.0294	426.0221	426.022	−0.2
10.	GDP	$C_{10}H_{15}N_5O_{11}P_2$	443.0243	442.0171	442.013	4.0
11.	ATP	$C_{10}H_{16}N_5O_{13}P_3$	506.9958	505.9885	505.985	3.8
12.	GTP	$C_{10}H_{16}N_5O_{14}P_3$	522.9907	521.9834	522.002	−18.6
13.	cyclicADP ribose	$C_{15}H_{21}N_5O_{13}P_2$	541.0611	540.0538	540.053	0.6
14.	GDP-hexose	$C_{16}H_{25}N_5O_{16}P_2$	605.0772	604.0699	604.059	10.9
15.	UDP-hexNAc	$C_{17}H_{27}N_3O_{17}P_2$	607.0816	606.0743	606.074	0.6
16.	NAD	$C_{21}H_{27}N_7O_{14}P_2$	663.1091	662.1019	662.102	0.0

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 et al. (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 [μg/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 μg/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

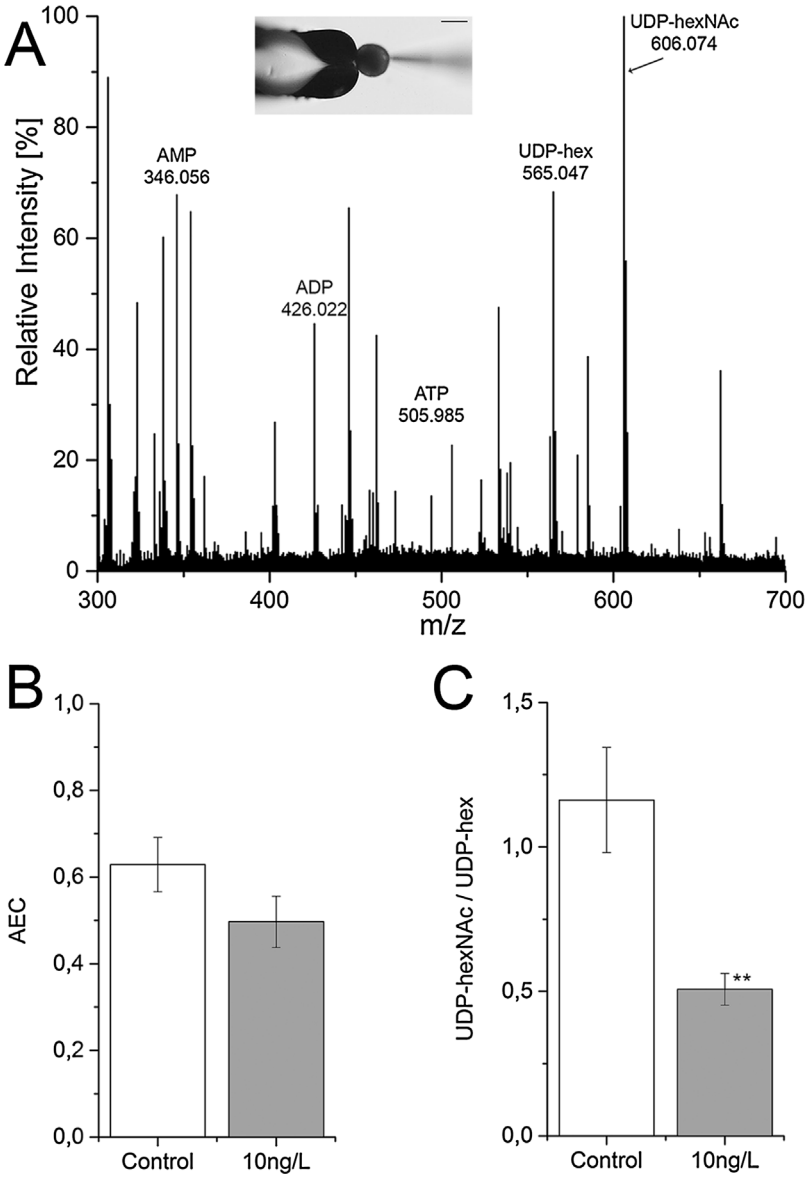


Fig. 3. Metabolomic analysis of a single-cell zygote. (A) A representative mass spectrum of the cytoplasm of a single-cell zygote taken in 300–700 *m/z* detection range and representing the most important molecules detected. The insert shows from left to right the microsampling process of the zygote: the holding pipette, the single-cell zygote (< 1 h) and the sampling capillary. Scale bar: 100 µm. (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; ** – $P < 0.01$.

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 Fig. 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

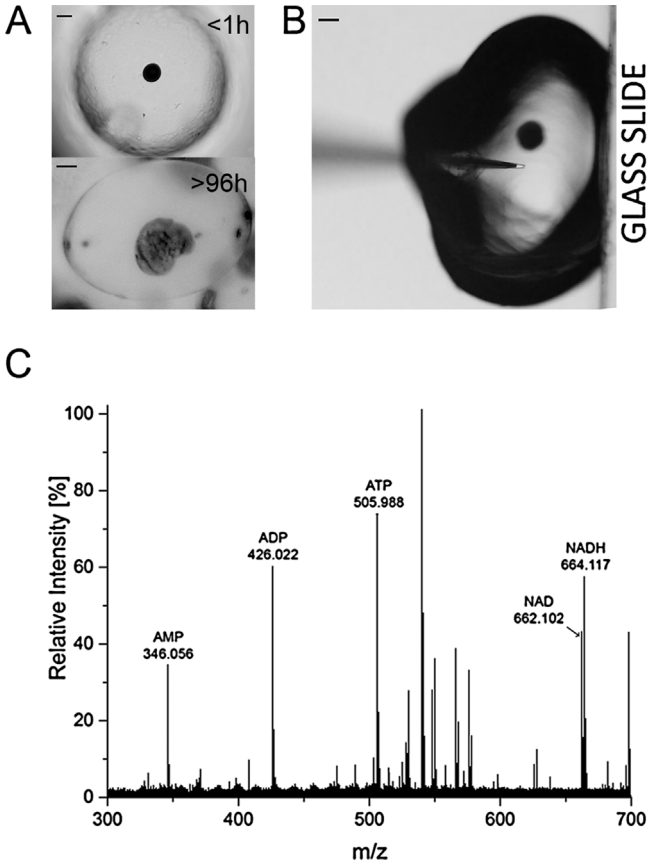


Fig. 4. Microsampling for metabolomic analysis of albumen. (A) A 1-h (upper) and a 96-h (lower) old egg. The foot lobe region is indicated by an arrow. (B) The capillary microsampling of albumen from a 1-h old egg. The microcapillary is surrounded by a dashed line and arrow shows the capillary ending. 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.

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 Fig. 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 Fig. 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 h). 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.

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.

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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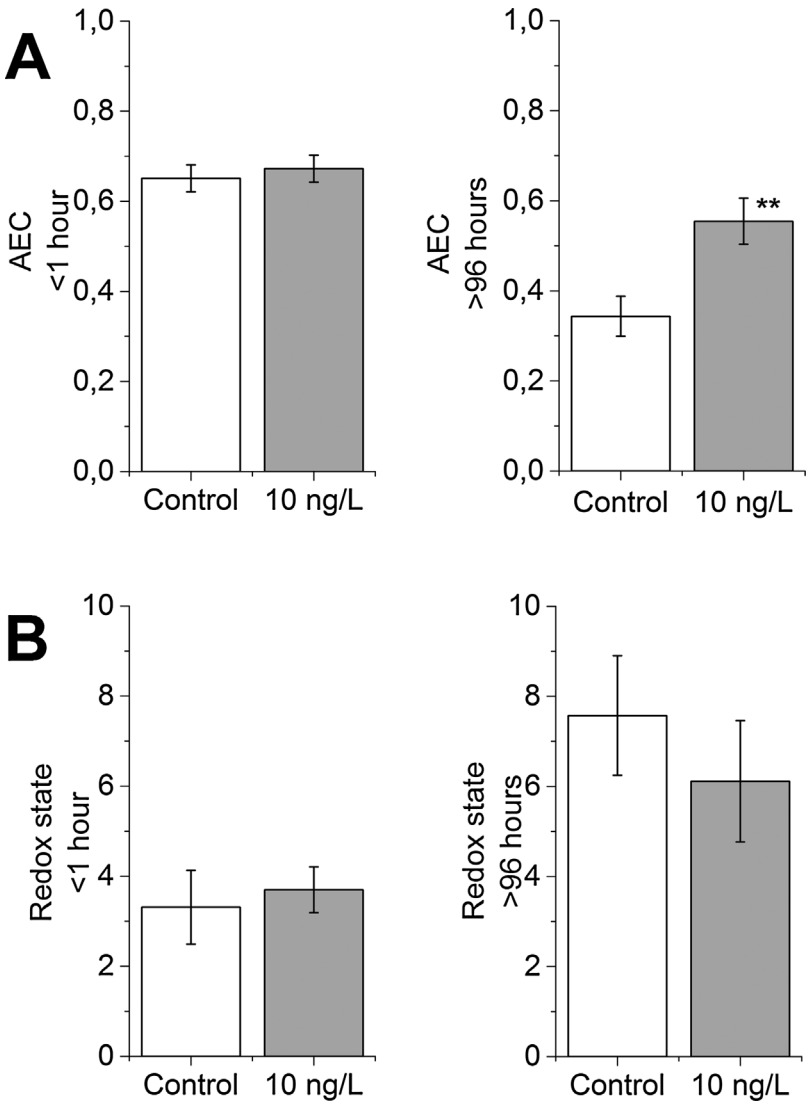

Fig. 5. Metabolomic analysis of the egg albumen. The differences in (A) AEC level, and (B) redox state between control and 10 ng/L treated groups within 1 h (left) and after 96 h (right) are shown. white columns – control; grey columns – 10 ng/L treated group; error bars – standard deviation; ** – $P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

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2017.06.029.

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Supplement

Figure 1. (A) An egg mass of *Lymnaea stagnalis* containing ~100 eggs with ≥96-hour old embryos is presented. Scale bar represents 3 mm. (B) ~24-hour old polyembryonic eggs are shown. The inserted picture represents a polyembryonic egg containing more than 10 embryos after 96 hours. (C) Not-pigmented dead (upper) and alive (lower) embryos after 96 hours are presented. Scale bars represent 500 µm (B, C).

Figure 2. (A) A schematic representation of the signaling molecules and pathways involved in cell-cycle progression and metabolism. GPCR: G-Protein-coupled receptor; AC: adenylate-cyclase; cAMP: cyclic-adenosine monophosphate; PI3K: Phosphoinositide 3-kinase; AKT: RAC-serine/treonine protein kinase or protein-kinase B; PKA: protein-kinase-A. GLI3: zinc finger protein GLI3; ERK5: extracellular-signal-regulated kinase 5. JAK/STAT: Janus kinase (JAK) and two Signal Transducer and Activator of Transcription; MAPK: mitogen activated protein kinase; Hedgehog sign. pw: a signaling pathway consists of hedgehog signaling proteins that transmits information to embryonic cells required for proper development. The different pathways are presented by literature data (Alessi and Cohen, 1998; Han et al., 2016; Nishimoto and Nishida, 2006; Pak and Segal, 2016; Paoli et al., 2013; Shepherd et al., 1998; Syrovatkina et al., 2016). (B) A schematic representation of the usage of hexose in cytoplasm. Hex-6-P: hexose-6-phosphate; Fu-6-P: fructose-6-phosphate; UDP-hexNAc: uridine diphosphate N-acetylhexosamine; UDP-hex: uridine diphosphate hexose; GFAT: glutamine:fructose-6-phosphate amidotransferase enzyme. The hexose utilization is presented by literature data (Baldini et al., 2016; Damerow et al., 2015; Fraga et al., 2013; Pyla et al., 2015; Yi and Huh, 2015).

Detailed method for recovery measurement of progestogens

We used a previously published HPLC-MS method for progestogen measurement (Avar et al., 2016) with slightly modified sample preparation. Tank water was collected in 800 mL glass bottles and basified with sodium-hydroxide (pH=9) to unfold the hydroxypropyl-ß-cyclodextrin – progestogen comlex (Sætern et al., 2004). Samples were filtrated on Whatman glass microfiber filters (0.45 µm) prior to SPE. Oasis HLB (1g/20 mL, 186000117, Waters Kft., Hungary) SPE cartridges were conditioned with 15 mL methanol, equilibrated with 20 mL LC-MS grade water, loaded with 770 mL filtrated sample, washed with 20 mL LC-MS grade water and eluted with 15 mL methanol. After evaporation to dryness extracts were reconstituted in 50 µL methanol. Five µL was injected 3 times from each concentrated sample into the HPLC-MS system (Dionex Ultimate 3000 UHPLC, Q-Exactive HRMS, Thermo Fisher Scientific). Liquid

chromatographic separation was carried out on a Kinetex 2.6 µ C18 100Å HPLC column (100*2.1 mm) maintained at 40 °C. The mobile phase consisted of solvent A (0.01 v/v% formic acid in water) and solvent B (0.01% v/v% formic acid in acetonitrile). The flow rate was 300 µL/min. The mass spectrometer was equipped with a heated electrospray ion source which was operated in positive ion mode. Spray voltage was set to 4.0kV. Capillary temperature was 300 °C. Different HCD (Higher-Energy Collisional Induced Dissociation) cell energies were applied for the fragmentation: 55 % by DRO and 45 % by PRG, LNG and GES. Data analysis was carried out with the software Thermo Xcalibur (version 2.2 SP1.48). For quantitative analysis five point calibration curves were used in the concentration range of 0.5 to 750 ng/L. Correlation coefficients (R2) of the calibration curves were over 0.95. The limit of detection (LOD) calculated from standard mixture was 0.01 (DRO), 0.01 (LNG), 0.003 (PRG) and 0.02 (GES) ng/L while the limit of quantification (LOQ) was 0.11, 0.09, 0.03 and 0.19 ng/L, respectively. Recoveries of 100 µg spiked standards were 72% (DRO), 76% (LNG), 89% (PRG) and 71% (GES). For measured concentrations of progestogens from experimental tanks, the progestogen contents were checked weekly on the seventh day by HPLC-MS method.

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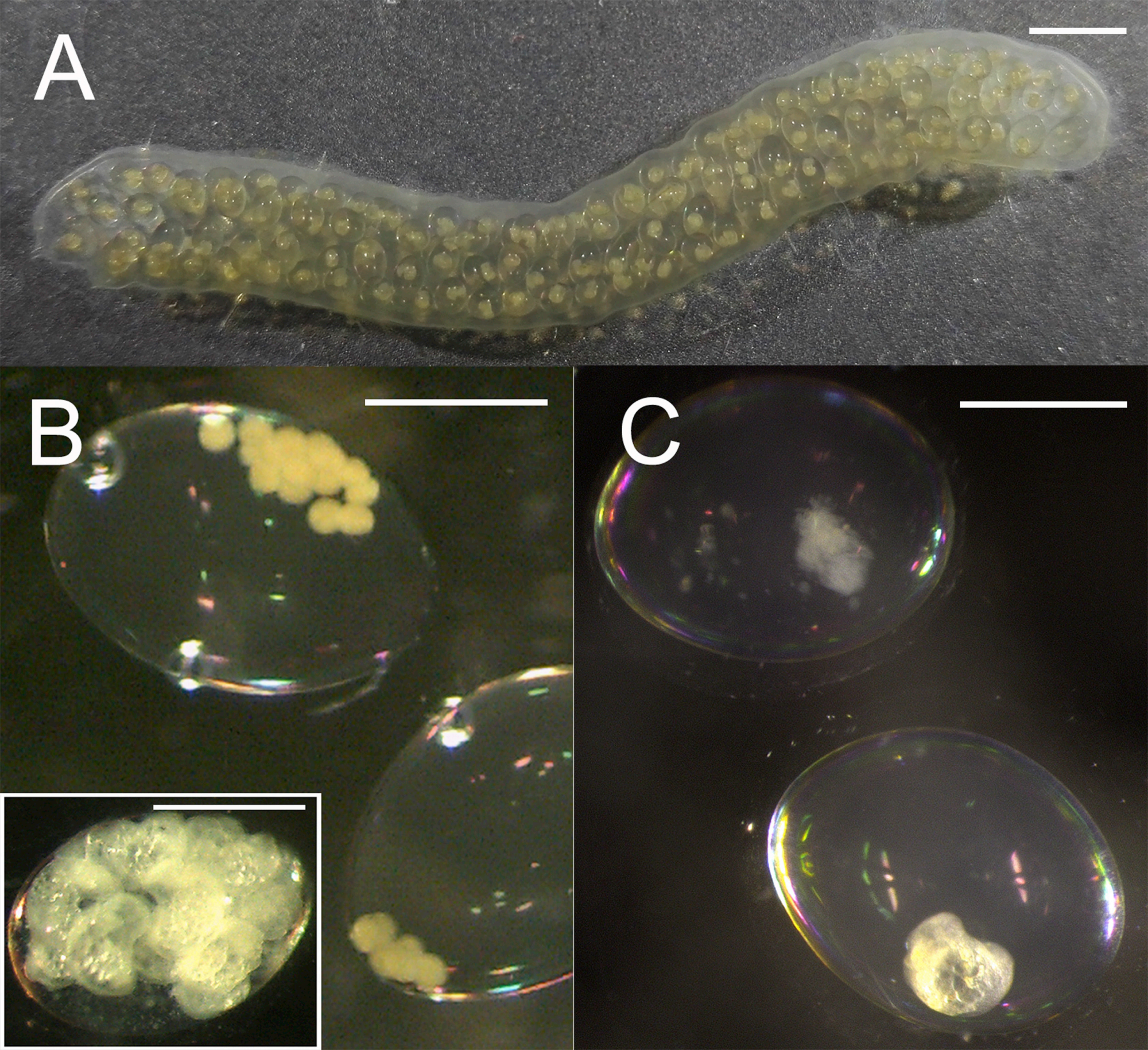
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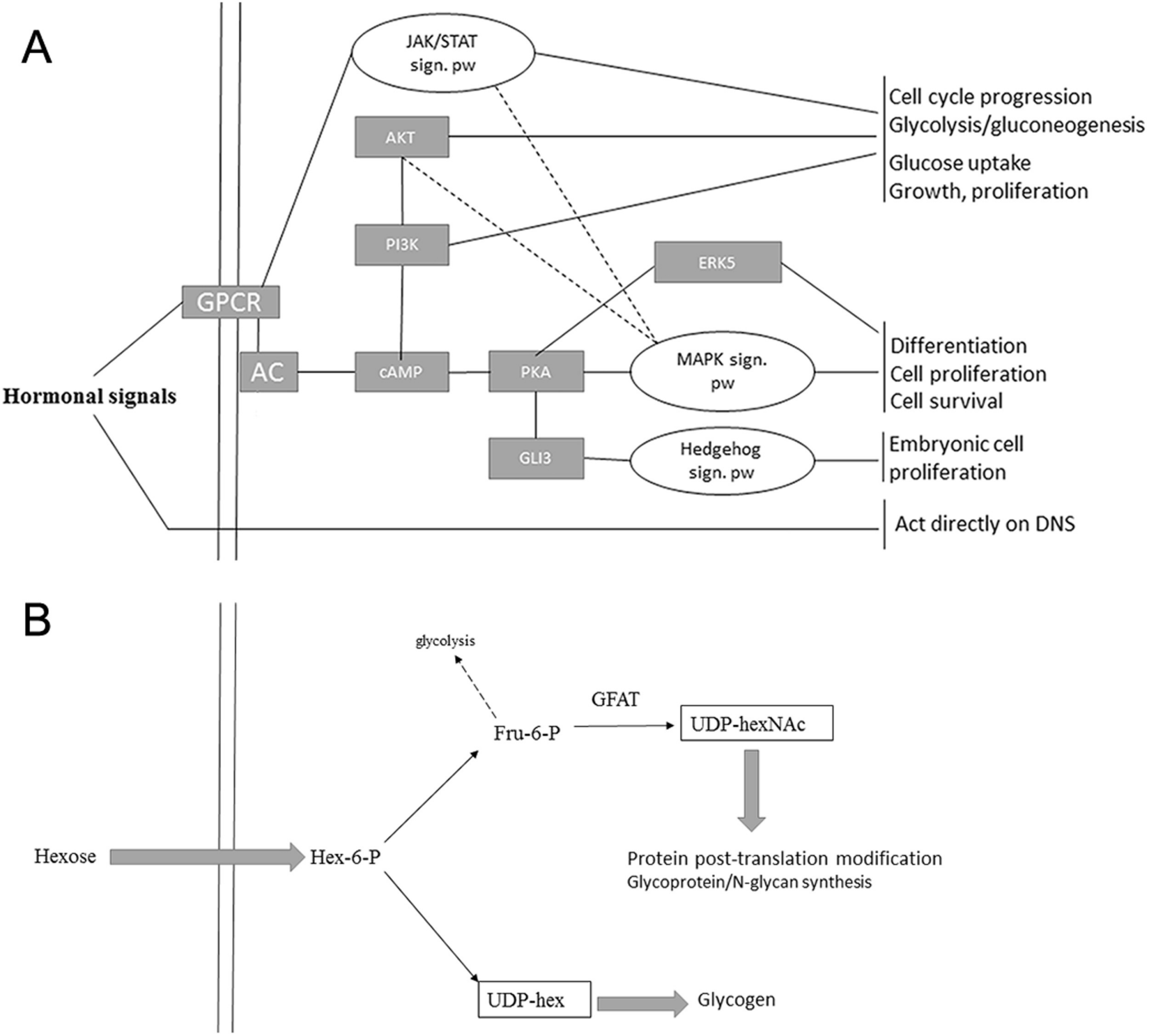
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Glycolysis/gluconeogenesis
Glucose uptake
Growth, proliferation
Differentiation
Cell proliferation
Cell survival
Embryonic cell
proliferation
Act directly on DNS
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glycolysis
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UDP-hexNAc
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UDP-hex
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