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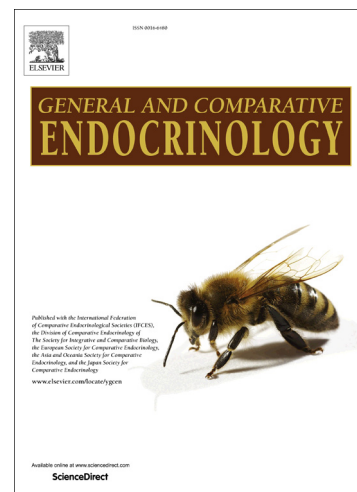
PII: S0016-6480(17)30771-2
DOI: <https://doi.org/10.1016/j.ygcen.2018.05.017>
Reference: YGCEN 12943

To appear in: *General and Comparative Endocrinology*

Received Date: 17 November 2017
Revised Date: 12 April 2018
Accepted Date: 14 May 2018

Please cite this article as: Thomson, P., Langlois, V.S., Developmental profiles of progesterone receptor transcripts and molecular responses to gestagen exposure during *Silurana tropicalis* early development, *General and Comparative Endocrinology* (2018), doi: <https://doi.org/10.1016/j.ygcen.2018.05.017>

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Developmental profiles of progesterone receptor transcripts and molecular responses to gestagen exposure during *Silurana tropicalis* early development

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Abstract

Environmental gestagens are an emerging class of contaminants that have been recently measured in surface water and can interfere with reproduction in aquatic vertebrates. Gestagens include endogenous progestogens, such as progesterone (P4), which bind P4-receptors and have critically important roles in vertebrate physiology and reproduction. Gestagens also include synthetic progestins, which are components of human and veterinary drugs, such as melengestrol acetate (MGA). Endogenous progestogens are essential in the regulation of reproduction in mammalian species, but the role of P4 in amphibian larval development remains unclear. This project aims to understand the roles and the regulatory mechanisms of P4 in amphibians and to assess the consequences of exposures to environmental gestagens on the P4-receptor signaling pathways in frogs. Here, we established the developmental profiles of the P4 receptors: the intracellular progesterone receptor (*ipgr*), the membrane progesterone receptor β (*mpgr β*), and the progesterone receptor membrane component 1 (*pgrmc1*) in Western clawed frog (*Silurana tropicalis*) embryos using real-time qPCR. P4-receptor mRNAs were detected throughout embryogenesis. Transcripts for *ipgr* and *pgrmc1* were detected in embryos at Nieuwkoop and Faber (NF) stage 2 and 7, indicative of maternal transfer of mRNA. We also assessed the effects of P4 and MGA exposure in embryonic and early larval development. Endocrine responses were evaluated through transcript analysis of a suite of gene targets of interest, including: *ipgr*, *mpgr β* , *pgrmc1*, androgen receptor (*ar*), estrogen receptor α (*era*), follicle stimulating hormone β (*fsh β*), prolactin (*prl*), and the steroid 5-alpha reductase family (*srd5 α 1*, 2, and 3). Acute exposure (NF 12 - 46) to P4 caused a 2- to 5-fold change increase of *ipgr*, *mpgr β* , *pgrmc1*, and *ar* mRNA levels at the environmentally relevant concentration of 195 ng/L P4. Acute exposure to MGA induced a 56% decrease of *srd5 α 3* at 1,140 ng/L MGA. We conclude that environmental exposure to P4 and MGA

induced multiple endocrine-related transcript responses; however, data also suggest that the effects of MGA are not mediated through the classical P4 signaling pathway in *S. tropicalis*.

1. Introduction

Gestagens are a class of chemicals that exhibit progestogenic activity, including the endogenous progestogens (e.g., progesterone; P4), and synthetic progestins used in human and veterinary drugs (e.g., melengestrol acetate; MGA) (Fig. 1). Progestogens have critical function in vertebrate physiology and reproduction and P4 is a key metabolic intermediate for the production of other endogenous steroids. In mammals, P4 is crucial to pregnancy with roles in regulating the reproductive cycle, ovulation, preparation of the uterus for implantation, maintaining gestation, and differentiation of the mammary gland (Graham and Clarke, 1997; Rozenbaum, 2001). Progestogens also play an essential role in reproduction and regulation of gamete maturation in lower vertebrates, such as amphibians and fish (Nagahama and Yamashita, 2008). In seasonally breeding amphibians, plasma P4 levels peak during the preovulatory period and gradually decline during breeding (Paolucci and DiFiore, 1994). Progestogens have been shown to induce gamete maturation in frogs (Schuetz and Lessman, 1982; Wasserman et al., 1982) and in several species of teleost fishes (Tubbs et al. 2010; Boni et al. 2007; Kazeto et al., 2005; Zhu et al. 2003a; Nagahama, 1997; Thomas and Trant, 1989).

Physiological functions of gestagens are mediated through binding and activating progesterone receptors (PRs), including intracellular and membrane PRs (iPGR and mPGR, respectively), and P4 membrane receptor (PGRMC) proteins (Thomas, 2008). The intracellular iPGR is a ligand-activated transcription factor that induces transcription of specific P4 response genes (reviewed in Jacobsen and Horwitz, 2012). The biological roles of iPGR in mammalian

species are well documented and include ovulation, sexual behaviour, uterine function, and mammary gland morphology (Conneely et al., 2002; Chappell et al., 1999; Chappell et al., 1997; Lydon et al., 1995). In non-mammalian vertebrates, evidence suggests that iPGR serves critical roles in ovulation (Tang et al., 2016).

Nongenomic progestogenic effects are mediated by membrane bound P4 receptors. mPGRs are G protein-coupled receptors that belong to the progestin and adipoQ receptor (PAQR) family of 7-transmembrane domain proteins (Thomas, 2008; Thomas et al., 2007; Tang et al., 2005; Zhu et al., 2003b). In general, vertebrates express three isoforms of mPGRs (mPGR α , mPGR β , and mPGR γ) (Thomas, 2008; Brinton et al., 2008; Zhu et al., 2003b). The biological functions of mPGRs include final oocyte maturation in female amphibians and fish (Nagahama and Yamashita, 2008) and sperm hypermotility in humans and fish (Thomas, 2008). Similarly, PGRMC1, a membrane bound protein, is capable of mediating rapid progestogenic functions. A diverse range of physiological roles and functions have been demonstrated or suggested for PGRMC1, including: steroid synthesis and metabolism, cholesterol regulation, antiapoptotic action in ovarian follicle cells, and initiation of the acrosome reaction in mammalian sperm cells (Thomas et al., 2014; reviewed in Thomas, 2008; Peluso, 2006; Buddhikot et al., 1999).

Exogenous compounds have been shown to interfere with normal endocrine function in aquatic vertebrates. These endocrine disrupting chemicals (EDCs) pose a threat to wild populations. Recently, gestagens have been identified as an emergent class of EDC in the aquatic environment (reviewed in Orlando and Ellestad, 2014). Gestagens are continuously released the aquatic environment from at least three sources: waste water treatment plant effluent, paper mill plant effluent, and animal agricultural runoff; and are generally detected in the range of 0.1 -30 ng/L (reviewed in Fent, 2015; reviewed in Orlando and Ellestad, 2014). Both P4 and MGA are

administered to beef cattle in animal feeding operations to synchronize estrus and encourage rapid anabolic development (Schiffer et al., 2001). Cattle excrete relatively high concentrations of both endogenous and synthetic steroid hormones in urine and manure (Hanselman et al., 2003) and MGA has been measured at concentrations of 0.3–8.0 ng/g in manure (Schiffer et al., 2001). Elevated levels of both P4 and MGA have been measured in flush water from animal agriculture operations in concentrations up to 11,900 ng/L and 500 ng/L, respectively (reviewed by Fent, 2015; Bartelt-Hunt et al., 2012; Liu et al., 2012).

Despite the presence of gestagens in the aquatic environment, the data set regarding the effects of gestagen exposure in aquatic organisms is limited. Ecotoxicological studies demonstrated that some progestins (e.g., levonorgestrel (LNG), norethindrone (NET), and gestodene (GES)) cause masculinization of females and disruption of testicular development in males in amphibians (reviewed by Ziková et al., 2017; Säfholm et al., 2014 Hoffmann and Kloas, 2012; Säfholm et al., 2012; Kvarnyrd et al., 2011; Lorenz et al., 2011a) and fish (Kroupova et al., 2014; Svensson et al., 2014; Runnalls et al., 2013; Svensson et al., 2013; Zucchi et al., 2012; Murack et al., 2011, Paulos et al., 2010). These effects may be mediated through crosstalk of progestins with receptors other than PRs such as the AR or glucocorticoid receptor (Africander et al., 2011). For example, LNG, NET, and GES are potent androgens with relatively high binding affinity to the AR (45%, 15%, and 85% of metribolone, respectively) (reviewed by Schindler et al., 2003). In contrast, MGA exhibits weak androgenic activity (approximately 1% of T and 0.3% dihydrotestosterone (Bauer et al., 2000)) but glucocorticoid activity comparable with that of hydrocortisone (Elliot et al., 1973, Greig et al., 1970, Duncan et al., 1964). Relative to other progestins, there are few studies to date that examine the ecotoxicity of MGA in aquatic organisms. In African clawed frog (*Xenopus laevis*) tadpoles, MGA exposure induced a

significant reduction in body mass and snout-vent length at 100 ng/L MGA (Finch et al., 2013). While this study suggested that amphibians exposed to MGA in the environment may experience adverse effects in growth and development, further investigation into the molecular mechanism of MGA in frogs remains a nascent topic.

The objectives of the present study were to first establish the ontogenetic expression of *ipgr*, *mpgr β* , and *pgrmc1* during amphibian embryogenesis, and second, to investigate the effects of P4 and MGA exposure on development, survival, and sex steroid-related gene expression in Western clawed frog (*Silurana tropicalis*) larvae. Here, we established for the first time the expression profiles of *ipgr*, *mpgr β* , and *pgrmc1* from the commencement of amphibian embryogenesis at Nieuwkoop and Faber (NF) stage 2 to the beginning of larval development (NF 46) in *S. tropicalis*. We then conducted a series of acute exposures to P4 and MGA from NF 12 – 46 and evaluated the morphological and molecular effects of gestagen treatment.

2. Materials and Methods

2.1. Breeding and maintenance of *S. tropicalis*

S. tropicalis husbandry was conducted as described in Mathieu-Denoncourt et al. (2014) and breeding was performed according to Langlois et al. (2010). Adult frogs were reared in dechlorinated and aerated water from the Queen's University Animal Care Facility. A 12:12 hour light:dark cycle was maintained with the light cycle occurring from 7 am to 7 pm. Water conditions were maintained at 26 ± 1 °C. The care and treatment of animals used in this project are in accordance with the guidelines of the Animal Care Committee of Queen's University (Kingston, ON, CA) and the Canadian Council on Animal Care.

Fertilized eggs were obtained from two pairs of frogs by injecting human chorionic gonadotropin hormone (hCG; standard grade, 3,150 IU/mg potency, Sigma, Oakville, ON, CA) into the dorsal lymph sac of adult *S. tropicalis* to initiate amplexus and ovulation. Both males and females received a priming injection of 40 μ L of hCG (12.5 U) followed by a boosting injection of 160 μ L of hCG (200 U) after 21 h. Pairs were then introduced in the breeding chambers and were kept in the dark.

Fertilized eggs were collected and viable embryos were identified by visual observation with a dissecting microscope. Staging was determined following the NF developmental table (Nieuwkoop and Faber, 1994). Eggs were allowed to develop until NF 8, at which point they were collected and the protective jelly coat surrounding the embryos was removed to facilitate handling and ensure exposure. To do this, the eggs were washed by gentle swirling for 2 min in a 2% w/v L-cysteine solution ($\geq 99\%$, CAS 52-90-4, Sigma, Oakville, ON, CA). Embryos were held in the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX) rearing media (625 mg NaCl, 96 mg NaHCO₃, 75 mg MgSO₄, 60 mg CaSO₄·2H₂O, 30 mg KCl, and 15 mg CaCl₂ per L of deionized water; American Society for Testing and Materials (ASTM), 2004) containing the antibiotic gentamycin sulfate (0.04 mg/L; CAS 1405-41-0; Fisher Scientific, Mississauga, ON, CA).

2.2. Experimental design

For developmental profiles, fertilized embryos were allowed to develop in clean media until sampling occurred. Additionally, two acute toxicity tests were performed as described below following the FETAX protocol (ASTM, 2004). *S. tropicalis* embryos were aqueously exposed to P4 (purity $\geq 99\%$; CAS 57-83-0; Sigma, Oakville, ON, CA) and MGA (purity $\geq 97\%$, CAS 2919-66-6; Enzo Life Sciences, Inc.; Farmingdale, NY, USA) during early development –

from 8 - 96 hpf. Animals were reared in FETAX solution spiked with P4 and MGA. Both chemicals were dissolved in 100% ethanol (EtOH) in order to obtain nominal concentrations of 3.14; 31.4; 314; 400; and 3,140 ng/L P4 (molecular weight = 314.46 g/mol), and 3.97; 39.7; 312; 397; and 3,970 ng/L MGA (molecular weight = 396.519 g/mol), while ensuring that the final EtOH concentration in each treatment was 0.05%. Experimental concentrations were chosen based on measured P4 and MGA concentrations in the environment (reviewed in Fent, 2015; reviewed in Orlando and Ellestad, 2014). Water-only and EtOH-only controls were conducted for each chemical. Each treatment was tested in 5 replicates of 30 mL of FETAX solution containing 40 embryos (NF12) each. Dead embryos were removed and recorded daily. Rearing media was renewed every 24 h to ensure that dissolved oxygen levels remained high. The exposure lasted 96 h, until the developmental stage NF 46 was reached.

2.3. Water and tissue sample collection

For developmental profiling, samples of whole embryos were taken at different NF stages of development: 2, 7, 16, 21, 27, 34, 41, and 46. For gene expression analysis, embryos were pooled at each stage (20 embryos for NF 2-34 and 10 embryos for NF 41 and 46) to ensure sufficient material for RNA isolation. Pools (n = 5-8 per stage) were snap frozen and stored at -80 °C until further analysis.

For acute exposures, whole NF 46 larvae were sampled at exposure completion from each treatment for gene expression analysis (10 individuals per pool; n = 8-10 pools) and stored at -80 °C until gene expression analysis. Additionally, a subset (n = 50 per treatment) of tadpoles was preserved in formalin (10%; CAS 50-00-0; Fisher Scientific; Fair Lawn, New Jersey, USA) and stored in amber vials at room temperature until malformation analysis was conducted.

Rearing media samples ($n = 3$) were collected for all treatments before the introduction of embryos (time 0) and a day later (time 24 h). Media samples were preserved in amber vials at $-20\text{ }^{\circ}\text{C}$ until chemical analyses were performed.

2.4. Media chemistry analysis

The experimental concentrations of P4 and MGA were determined by LC-MS in the Yergeau Laboratory (McGill University, QC, CA). In brief, 7 mL of media sample was transferred to a 15 mL tube (Fisher Scientific; Pittsburgh, PA, USA) and fortified with 30 μL of internal standard solution (10 ng/mL) followed by 3 mL of ethylacetate:hexane (8:2 v/v). Samples were mixed for 1 min on an orbital shaker and centrifuged for 1 min at 1500 $\times g$ and $4\text{ }^{\circ}\text{C}$. The organic layer was transferred to a clean 15 mL tube where 1 mL of 100 mM ammonium formate (pH 9.0) was added and mixed for 1 min on an orbital shaker. Samples were centrifuged and the top organic layer was transferred to a clean 5 mL tube for drying under nitrogen gas. Extracts were reconstituted in 50 μL of 2 mM ammonium formate:methanol at 0.1% formic acid solution and transferred to a vial with an insert for LC-MS analysis.

Separation of P4 and MGA was performed on a Thermo Scientific (Waltham, MA, USA) Accela 600 LC system equipped with refrigerated autosampler set at $4\text{ }^{\circ}\text{C}$, buffer degasser, column oven compartment operated at $60\text{ }^{\circ}\text{C}$ and a quaternary pump. Column configuration consisted of a Thermo in-line filter hardware unit with a 2.1 mm ID and 0.2 μm filter cartridge PN: 22180 (Bellefonte, PA, USA) followed by an Agilent UHPLC guard column Zorbax Eclipse plus C18 2.1 x 5 mm and 1.8 μm PN: 821725-901. Resolution of analytes was carried out on an Agilent analytical column Zorbax Eclipse plus C18 RRHD 2.1 x 50 mm and 1.8 μm PN: 959757-902 (Santa Clara, Cal. USA). When analyzing the media samples, 10 μL of sample or its

dilution were injected at a constant flow rate of 250 $\mu\text{L}/\text{min}$. The initial mobile phase composition was aqueous 2 mM ammonium formate 0.1% formic acid buffer (A) and methanol 0.1% formic acid buffer (B) at 80% A/20% B, with an initial ramp to 65% B from 0 to 3 min, followed by a final ramp to 100% B in 2.5 min. This composition of mobile phases was kept for 2 min and then the column was brought to the initial gradient conditions at 40% mobile phase B in 0.5 min and held for 2 min for equilibration. The total time of the cycle was 10 min.

A Thermo Scientific LTQ XL mass spectrometer equipped with a high resolution Orbitrap detector in tandem with an Accela HPLC system from Thermo Scientific (Waltham, MA USA) was used for chemical determinations using a pneumatic assisted atmospheric pressure equipped with an electrospray ionization source. MS detection was performed by Fourier transform mass spectrometry (FTMS) in positive ion mode. Instrument optimization was performed by infusing standard solutions at 5 $\mu\text{L}/\text{min}$, while determination of optimal source conditions was done by infusion flow analysis. Nitrogen gas was used for all sheath, auxiliary and sweep gasses, while helium gas was used as the collision gas. Data acquisition analysis was done on the precursor-ions for the full scan using FTMS obtained at 30,000 resolution on a mass range from 50-600 m/z on Xcalibur Version 2.1 from Thermo Scientific (San Jose, CA, USA).

2.5. Malformation analysis

Malformation analysis was carried out on fixed tadpoles with a Nikon SMZ18 microscope (Nikon, Chiyoda-Ku, Tokyo) with the software NIS Elements (Nikon, Chiyoda-Ku, Tokyo). Samples were coded to perform blind analysis of the animals. Types of malformations and signs of developmental delay were recorded based on the Atlas of Abnormalities (Bantle et

al., 1998). Briefly, axial, blistering/edema, cranio-facial, eye, gut, and heart abnormalities were assessed.

2.6. Gene expression

Total RNA was obtained from homogenized samples using the e.Z.N.A Total RNA Kit I (Omega Bio-Tek, Norcross, GA, USA). Remaining genomic DNA contamination was removed by DNase treatment with the RQ1 RNase-free DNase kit (Promega, Madison, WI, USA). Nucleic acid quality and concentration were measured with a NanoDrop-2000 spectrophotometer (ThermoFisher, Ottawa, ON, CA). Total cDNA was then generated from 1 µg of total RNA and random primers using reverse transcriptase using the GoScript Reverse Transcription System (Promega, Madison, WI, USA) in a Mastercycler Pro S Thermocycler (Thermo Fisher, Ottawa, ON, CA). The cDNA products were diluted prior to PCR amplification.

Changes in transcript levels were investigated using real-time quantitative polymerase chain reaction (qPCR). The specific forward and reverse primers for the genes of interest are found in Table 1 and Langlois et al. (2010). Primers were designed for a suite of gene targets of interest and optimized to yield an efficiency of $100 \pm 10\%$ and an $R^2 \geq 0.99$. All qPCR assays were performed using a Bio-Rad CFX 96 Real-Time System (Bio-Rad Laboratories Inc., Mississauga, ON, CA) and Promega GoTaq qPCR MasterMix (Madison, WI, USA). The thermocycler program included a hot-start activation step at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and 1 min at a gene-specific annealing temperature of 58 °C, 60 °C, or 62 °C. Denaturation occurred at 95 °C for 1 min, then a dissociation curve was generated to confirm the presence of a single amplicon. The threshold for each gene was assessed automatically by the Bio-Rad CFX Manager Software 3.0. Each qPCR assay was run

with a negative template control and a negative reverse transcriptase control. Standard curves were prepared by serial dilutions (1:4) starting at 50 ng. Samples, controls, and standards were run in duplicate. The relative standard curve method was used to interpolate relative mRNA abundance of target genes within each sample and was automatically calculated by the Bio-Rad CFX Manager Software and corrected manually.

For developmental profiles, gene expression data is presented as fold change relative to stage NF 2 for *ipgr* and *pgrmc1*, and NF 16 for *mpgrβ*. For acute exposures, gene expression data is presented as fold change relative to the EtOH control treatment. Each assay was normalized to the geometric mean of three reference genes: elongation factor 1 alpha (*ef1α*), ornithine decarboxylase (*odc*), and ribosomal protein L8 (*rpl8*). The geometric mean of the reference genes expression was not significantly affected by treatment groups.

2.7. Statistical analysis

Data was analyzed on Prism 6 (GraphPad Software Inc., San Diego, CA, USA). Data and residuals were tested for normality and homoscedasticity using the Shapiro-Wilk and Bartlett's tests, respectively. Statistical outliers were removed using the robust regression and outlier removal method with the maximum false discovery rate set to 1% (a maximum of 2 outliers were identified in any one treatment group for each gene of interest) (Motulsky and Brown, 2006) and the data was transformed if necessary to meet the assumptions of normality. To evaluate fold change in gene expression, treatments were compared to controls by conducting one-way ANOVA's and Dunnett's post hoc analyses. Differences were accepted as significant at an alpha level of $p < 0.05$.

3. Results

3.1. Experimental gestagen water concentrations

Analyses demonstrated that the water and the solvent controls were not contaminated with detectable levels of P4 or MGA (Table 2). At the three highest test concentrations, P4 degraded significantly within 24 h; however, MGA concentrations remained constant throughout the assay. Therefore, experimental concentrations are reported as the average value between 0 h and 24 h. The measured and experimental concentrations for P4 were 3.14; 15.7; 195; 302; and 3,360 ng/L and were similar to the nominal values (3.14; 31.4; 314; 400; and 3,140 ng/L). Likewise, the measured MGA concentrations of 7.93; 59.5; 234; 1,140; and 3,730 ng/L were close to the nominal concentrations (3.97; 39.7; 312; 397; and 3,970 ng/L). All treatments were statistically different from both controls.

3.2. Mortality and malformations

The effects of P4 and MGA on development of *S. tropicalis* larvae are presented in Table 3. Mortality rates in both water and solvent controls were below the recommended 10%, at 6.8 and 6.0%, respectively (ASTM, 2004). The malformation rate in the water control and solvent control was 10 and 12%, respectively, which is at or slightly above the recommended 10%, (ASTM, 2004). There was no observable toxic effect of P4 at the tested concentrations, as mortality and malformation rates did not differ significantly from the solvent control. In the highest MGA treatment (3,140 ng/L MGA), mortality rate was significantly higher than the solvent controls (Table 3). This treatment group also displayed a relatively high frequency of axial and cranio-facial abnormalities compared to the control groups.

3.3. P4-receptor genes are detected in early frog development

We used real-time qPCR to determine the profiles of P4 receptor mRNAs during embryogenesis of *S. tropicalis* (Fig. 2). The expression profiles of the P4 receptor gene transcripts were detected throughout development; however, the genes differ in pattern and magnitude of expression changes throughout development. Detection of transcripts in the earliest two stages studied indicates maternal origin, as transcription in *S. tropicalis* embryos does not commence until NF 12 (Newport and Kirschner, 1982a,b). Levels of *ipgr* and *pgrmc1* were detected at all stages of development and maternally-transferred mRNA was observed at NF 2 and NF 7 for both genes. Transcripts of *mpgr β* however were first detected at NF 16, corresponding to the period of neurulation. Expression of *ipgr* significantly decreased by 70% between NF 2 and NF 7. Likewise, transcript levels of *pgrmc1* decreased 90% between NF 2 and NF 16, and then increased 0.7-fold between NF 34 and NF 41, corresponding to the period of organogenesis. In contrast, *mpgr β* expression remained relatively constant throughout embryonic development after detection at NF 16.

3.4. P4 affects expression of reproduction-related genes

Gene expression analyses following acute gestagen exposure indicated P4 disrupted the expression of genes involved in the reproductive axis and steroidogenesis. The effects of P4 on *ipgr*, *mpgr β* , *pgrmc1*, *ar*, *srd5 α 1*, *srd5 α 2*, and *srd5 α 3* expression in NF 46 larvae are shown in Fig. 3. At 195 ng/L, P4 significantly upregulated the expression of *ipgr*, *mpgr β* , *pgrmc1*, and *ar* by 5.1; 3.5; 2.2; and 2.6-fold, respectively. Changes in expression of the *srd5 α 1*, 2, and 3 were also detected following P4 treatments. In response to P4, *srd5 α 1* mRNA level significantly increased by 2.0, 3.4, and 2.3-fold in the 15.7, 195 and 3,360 ng/L P4 treatment groups,

respectively. Expression of *srd5a2* increased by 3.4-fold following treatment with 195 ng/L P4. Finally, *srd5a3* increased by 2.1-fold in the 15.7 ng/L treatment group. Expression of *era*, *fsh β* , and *prl* were also measured after exposure to P4, but no significant changes were observed (Table S1).

3.5. MGA exposure induced a dissimilar molecular response to P4

The effects of MGA on P4-related gene expression in NF 46 larvae are reported in Fig. 4. In contrast to P4 exposure, treatment with MGA did not induce significant changes in gene expression of *ipgr*, *mpgr β* , *pgrmc1*, *ar*, *srd5a1*, *srd5a2*, *fsh β* , or *prl* in any treatment group (Table S2). However, MGA exposure significantly downregulated *srd5a3* expression by 56% at 1140 ng/L (Fig. 4). The difference in expression patterns between MGA and P4 treatment indicates that these two gestagens act through different molecular cascades in *S. tropicalis*.

4. Discussion

Gestagen signaling is a key regulatory pathway in sexual development and reproduction in vertebrates. However, little is known about the role of P4 signaling during larval development in lower vertebrates. Other endogenous sex steroids (e.g., estradiol and testosterone (T); Bogi et al., 2002) and their receptor transcripts (*er* and *ar*; Duarte-Guterman et al., 2010) have been measured during embryogenesis in frogs. Sex steroids have been shown to have critical roles in survival, growth, and reproductive development during embryogenesis; therefore, P4 may also have crucial functions in developing non-mammalian species.

Few studies have investigated the role of P4 in embryos. In embryonic chicks and fish, *ipgr* transcripts were detected, and increased levels of expression corresponded to the period of

gonadal differentiation (Chen et al., 2010; Camacho-Arroyo et al., 2007; Gasc et al., 1991).

Similarly, expression of *ipgr* and *mpgr β* transcripts were localized in the urogenital complex of developing *S. tropicalis*, and levels increased significantly from larval development through metamorphosis (NF 50 – NF 66 + 4 weeks), suggesting that P4 signaling may be important in gonadal development in amphibians (Jansson et al., 2016). In contrast, Säfholm et al. (2016) found that *ipgr* expression increased from NF 50 on, while *mpgr β* expression level decreased after NF 56 in both male and female *S. tropicalis* gonads. Here, we present complementary profiles of *ipgr*, *mpgr β* , and *pgrmc1* in whole *S. tropicalis* embryos during earlier stages of development, from fertilization through early larval development (NF 2 – NF 46).

From our results, all three *prs* were detected during embryogenesis and before the HPG axis is fully established, suggesting that P4 signaling is requisite in amphibian early development and that the role of P4 extends beyond the reproductive function. Developmentally regulated changes in the levels of all three gene transcripts were also observed. The presence of *ipgr* and *pgrmc1* transcripts in embryos at NF 2 and NF 7 suggests that P4 has a functional role in embryogenesis, as maternally-transferred mRNA may be critical to the earliest stages of embryonic development (Heasman, 2006). Similar maternal transfer data were found for the transcripts of *srd5 α* (important in androgen synthesis) in the *S. tropicalis* fertilized embryo (Langlois et al, 2010). The differences in developmental expression profiles among *prs* in *S. tropicalis* indicate that these receptors serve different functions during these stages of development. Moreover, Säfholm et al. (2016) suggested that *ipgr* and *mpgr β* continue to have disparate functions in post-metamorphic *S. tropicalis*. Taken together, these studies demonstrate that *prs* are present, but differentially expressed from early embryogenesis throughout

metamorphosis, and are therefore, potential targets for endocrine disruption by environmental gestagens.

Exogenous gestagen exposures have been shown to cause reduced fecundity or disrupted gonadal development in amphibians (reviewed by Zikova et al., 2017; Säfholm et al., 2015; Kvarnryd et al., 2011; Lorenz et al., 2011a,b; reviewed in Hayes, 1998). Progestogens being pleiotropic compounds of the HPG axis can modulate gonadotropin levels (Kroupova et al., 2014; Han et al., 2014; Overturf et al., 2014; Svensson et al., 2014; Lorenz et al., 2011b), change circulating sex steroid levels (Kroupova et al., 2014; Runnalls et al., 2013; Paulos et al., 2010), and/or alter the expression of genes encoding for steroid receptors (Kumar et al., 2015; Zucchi et al., 2014). Moreover, P4 is a key intermediate in biosynthetic pathways that lead to the production of all other steroid hormones (Nagahama, 1997). Thus, the presence of P4 in the aquatic environment may pose a particular risk to aquatic species either through direct disruption of P4-signaling, or following metabolic transformation and interaction with other steroid hormone pathways. Taken together with the findings of this study, P4 should be regarded of an EDC of concern and research efforts to investigate the effects of P4 disruption should be intensified.

In this study, treatment with P4 increased the expression of *ipgr* in a concentration-specific manner in *S. tropicalis* whole larvae (NF 46). Several studies in *D. rerio* reported that P4 exposure induced time- and concentration-dependent responses for *ipgr* transcription (Liang et al., 2015a,b; Bluthgen et al., 2013b; Zucchi et al., 2012). P4 exposure induced a significant increase in *ipgr* expression in *D. rerio* embryos (2 – 200 ng/L, 96 hpf; Zucchi et al., 2012) and juveniles (63 ng/L, 20 – 60 dpf; Liang et al., 2015a). Moreover, *ipgr* expression was induced in *D. rerio* at 72 and 144 hpf following treatment with 90 ng/L P4, but decreased at 96 and 120 hpf

following treatment with 6, 45, or 90 ng/L P4 (Liang et al., 2015b). Expression of *ipgr* has been shown to be crucial for normal reproductive tissue development and function in mice (Lydon et al., 1995) and overexpression of *ipgr* in adult *X. laevis* caused an enhanced rate of P4-induced oocyte maturation (Tian et al., 2000). Therefore, disrupted *ipgr* expression during the critical period of organogenesis, as seen in this study, could lead to reproductive toxicity in adults. Due to the high degree of variation in response at different developmental stages in *D. rerio*, time-course transcript profiles of *ipgr* expression following P4 exposure should also be conducted in amphibians to further understand the effects of P4 on *ipgr* expression and disruption of the HPG axis during development and the period of reproductive differentiation.

In this study, the *mpgr β* and *pgrmc1* transcripts were also up-regulated following P4 exposure in a non-monotonic manner. The majority of previous studies on *mpgr* have focused on teleost oocyte maturation and mammalian reproduction, and little is known about the functions and regulations of *mpgr* during development. Similarly, *pgrmc1* is implicated in diverse biological functions, but little is known about its regulation (Cahill, 2007). In ovarian tissue, expression levels of membrane *prs* were positively correlated to circulating progestogen levels in mammals (Kowalik and Kotwica, 2008; Nilsson et al., 2006; Cai and Stocco, 2005) and fish (Zhu et al., 2003a). In mammalian *in vitro* studies, altered *mpgr* and *pgrmc1* expression has been linked to reproductive dysfunction, including breast and ovarian cancers (Cahill, 2007; Schuster et al., 2010; Peluso et al., 2008). Therefore, our finding indicates that P4 can modulate both *mpgr β* and *pgrmc1* transcript levels in a frog species, which suggests that environmental exposure to P4, through cattle manure for example, can further alter the reproductive axis in amphibians.

Exposure to P4 also resulted in an increase in *ar* and *srd5a1*, 2, and 3 expression. While P4 exposure did not cause significantly different effects in gene expression of these transcripts at most of the concentrations tested, the alteration in the expression of genes involved in regulating reproduction and steroidogenesis in some treatments may consequently influence physiological processes. Expression of *ar* is known to be autoregulated by androgens (Cardone et al., 1998), and therefore, P4-induced expression of *ar* suggests the potential for crosstalk between signaling pathways. P4 may act through the AR-mediated signaling pathway either by direct binding and activating the AR (Blüthgen et al., 2013) or by shifting the steroid biosynthesis pathway to increase androgen production (Nagahama, 1997). Several *in vitro* studies in fish exposed to P4 have reported similar upregulations of *ar* transcript levels (*G. affinis*, Hou et al., 2017; Huang et al., 2013; *D. rerio*, Zucchi et al., 2012). Likewise, P4 exposure also altered transcript levels of *srd5as* in fish (*D. rerio* embryos, Liang et al., 2015b; *P. promelas* ovary, Chishti et al., 2014; Garcia-Reyero et al., 2013). The major function of *srd5a1*, 2, and 3 is to convert T to the more potent androgen, 5 α -dihydrotestosterone. Moreover, because P4 is a biological precursor to other steroids, exogenous addition of P4 may cause a shift in the steroidogenic pathway through metabolism to other biologically active metabolites, such as androgens (DeQuattro et al., 2012). *X. laevis* oocytes incubated or injected with radiolabeled P4 rapidly converted P4 to androstenedione within 4 h (Lutz et al., 2001). Moreover, *in vitro* P4 exposure caused significant increased T production in *P. promelas* testes (Chishti et al., 2013). Taken together, these findings suggest that the presence of P4 in the aquatic environment is of ecotoxicological importance and could potentially lead to alterations of several endocrine axes, either directly or following biochemical transformation.

In this study, treatment with P4 altered the expression of *ipgr*, *mpgr β* , *pgrmc1*, and *srd5 α 1-3* in a non-monotonic manner. The non-monotonic concentration response is a common pattern observed in EDC research, which may be explained by several mechanisms (reviewed in Vandenberg et al., 2012). One hypothesis to account for the observed non-monotonic response in transcription is that P4 induces a maximal biological response at a physiologically relevant concentration in *S. tropicalis*. During reproduction, serum P4 levels in adult *X. laevis* and *R. esculenta* are $\leq 9,430$ ng/L (Guerreiro and Ciarcia, 2001; Lutz et al., 2001). While there is no data on circulating P4 levels in frog larvae, it is expected to be significantly lower than this value, as the major organs and networks for P4 synthesis are not yet fully developed. Therefore, the intermediate concentration of P4 tested (195 ng/L) is potentially physiologically and environmentally relevant, and may be an optimal concentration to induce alterations in transcripts of P4-responsive genes.

Noteworthy, our data revealed that MGA exposure did not elicit the same effects on gene expression as P4. While P4 exposure induced transcription of *prs*, MGA did not significantly modulate *ipgr*, *mpgr β* , or *pgrmc1* transcript levels at any of the tested concentrations. Thus, the non-progestogenic effects of MGA observed in this study suggest that MGA does not activate the same molecular pathway as P4 at approximately equimolar concentrations in *S. tropicalis*. However, the possibility that MGA and P4 signal through the same molecular cascades cannot be discounted from this study alone, considering that both compounds did not induce a significant response in many of the concentrations tested. Although MGA is a progestin with approximately 5- to 11-fold higher relative binding affinity for the iPGR compared to P4 (Perry et al., 2005; Bauer et al., 2000), MGA also shows weak androgenic (Bauer et al., 2000) and weak estrogenic (Perry et al., 2005) activity, but relatively high glucocorticoid activity (Lauderdale et

al., 1977; Duncan et al., 1964). Thus, it is possible that the main molecular route for MGA in amphibians is through the stimulation of the glucocorticoid axis.

Glucocorticoids have been shown to affect larval amphibian growth and development (Crespi et al., 2013; Lorenz et al 2009; Glennemeier and Denver, 2002a,b,c). In general, at early life stages (i.e., premetamorphosis), tadpoles exhibit an inverse relationship between glucocorticoid level and growth (Crespi et al., 2013; Glennemeier and Denver 2002b,c). For example, NF 51 *X. laevis* exposed to glucocorticoids for 21 d exhibited reductions in body length and hind limb length and disruption of the progression of metamorphic tissue remodeling (Lorenz et al., 2009). Similarly, *X. laevis* exposed to 100 ng/L MGA for 60 d (NF 33 – NF 50) exhibited a reduction in total body mass, body length, and snout-vent length compared to controls (Finch et al., 2013). Therefore, the MGA-induced increase in mortality observed in this study and the inhibition of growth described by Finch et al. (2013) may be explained by MGA signaling through the glucocorticoid pathway. However, further research is required to address the potential crosstalk between MGA and the hypothalamic-pituitary-adrenal axis in frogs.

In contrast to P4 exposure, MGA inhibited *srd5a3* expression in the 1,140 ng/L treatment group. *Srd5as* are critical during embryogenesis and early larval development in *S. tropicalis* (Bisseger and Langlois, 2016) and inhibition of *srd5a* enzymes has been shown to induce a broad range of reproductive and non-reproductive adverse effects (Traish et al., 2015; Langlois et al., 2011). Although relatively little is known about the biological function of *srd5a3*, it may be implicated in male *S. tropicalis* gonadal steroid synthesis and has been shown to be negatively regulated by T in the *S. tropicalis* ovary (Bisseger and Langlois, 2016). Therefore, it is possible that MGA inhibits *srd5a3* expression following biochemical transformation to an androgenic metabolite (Finch et al., 2013; Lutz et al., 2001), however the structure of the metabolites of

MGA are unknown (Lange et al., 2002) and this hypothesis remains to be addressed. In sum, MGA is a potential EDC of concern, but its effects may be mediated through different molecular pathways than P4. Further research is required to identify the precise mode of action and consequences of MGA exposure in amphibians.

5. Conclusions

Our results show that *prs* are expressed during frog embryogenesis, suggesting that P4-signaling is important in early development. In addition, we showed that larval P4 and MGA exposure alters transcription of genes related to reproduction. Thus, we conclude that PRs may be targets of endocrine disruption by environmental gestagens at sensitive life stages. Future studies should investigate the mixture effects of P4 and MGA, as both occur simultaneously in surface waters receiving run-off from animal agricultural operations.

Acknowledgements

The authors would like to acknowledge Sonja Bissegger and Sarah Wallace (Royal Military College of Canada, ON) and Diana Campbell (Queen's University, ON) for their contributions with animal care, sampling, and molecular analysis, and Dr. Viviane Yargeau and Marco Pineda (McGill University, QC) for P4 and MGA chemical analysis. This research project was funded by the support of a Discovery Grant of the Natural Sciences and Engineering Research Council of Canada [NSERC-DG-418576-2102] and the Canada Research Chair [CRC-950-230442] to VSL.

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Figures captions

Figure 1. Structural formulas of progesterone (P4; A) and melengestrol acetate (MGA; B).

Figure 2. Developmental profiles of the expression of *ipgr* (A), *pgrmc1* (B), and *mpgrβ* (C) during *Silurana tropicalis* embryogenesis. Transcript levels were measured in 5 to 8 replicates of 10 pooled whole embryos and larvae from Nieuwkoop and Faber (NF) 2 (two-cell stage) to NF 46 (beginning of feeding). Data are expressed relative to NF 2 or NF 16, normalized to RNA content. Bars represent the mean + SEM. Data were analyzed using one-way ANOVAs ($n = 5-8$ per treatment; $p < 0.05$). Different letters indicate statistically significant differences between stages. The scales of the y-axes vary between graphs. C: water control, SC: solvent control (0.05% EtOH), *ipgr*: intracellular progesterone receptor, *mpgrβ*: membrane progesterone receptor beta, *pgrmc1*: progesterone receptor membrane component 1.

Figure 3. Effects of progesterone (P4) on *ipgr* (A), *mpgrβ* (B), and *pgrmc1* (C), *srd5a1* (D), *srd5a2* (E), and *srd5a3* (F), *ar* (G) mRNA expression in *Silurana tropicalis* Nieuwkoop and Faber (NF) stage 46 embryos. *S. tropicalis* were exposed to progesterone (P4) from NF 12 to NF 46. The mRNA levels are expressed relative to the solvent control group and are normalized to the mean expression of elongation factor 1 alpha (*ef1α*), ribosomal protein L8 (*rpl8*) and ornithine decarboxylase (*odc*). Bars represent the mean mRNA level + SEM. Data were analyzed using one-way ANOVA ($n = 7-8$ per treatment, $p < 0.05$). Asterisks (*) indicate significant differences from the solvent control group (0.05% EtOH). The scales of the y-axes vary between graphs. C: water control, SC: solvent control (0.05% EtOH), *ipgr*: intracellular progesterone receptor, *mpgrβ*: membrane progesterone receptor beta, *pgrmc1*: progesterone receptor membrane component 1, *ar*: androgen receptor, *era*: estrogen receptor alpha, *srd5a1*: steroid 5 alpha reductase type 1, *srd5a2*: steroid 5 alpha reductase type 2, *srd5a3*: steroid 5 alpha reductase type 3.

Figure 4. Effects of melengestrol acetate (MGA), on *srd5a3* expression in *Silurana tropicalis* embryos. The mRNA levels are expressed relative to the control groups and are normalized to the mean expression of elongation factor 1 alpha (*ef1α*), ribosomal protein L8 (*rpl8*) and ornithine decarboxylase (*odc*). Bars represent the mean mRNA level + SEM. Data were analyzed using one-way ANOVA ($n = 5-7$ per treatment, $p < 0.05$). Asterisks (*) indicate significant differences from the solvent control group (0.05% EtOH). C: water control, SC: solvent control (0.05% EtOH), *srd5a3*: steroid 5 alpha reductase type 3.

Table 1. Genes of interest for acute exposures. For each gene, accession number, primer sequences, annealing temperature (°C), amplicon size (bp) and primer concentrations (nM) are provided. Elongation factor 1 alpha (*ef1a*), ornithine decarboxylase (*odc*), and ribosomal protein L8 (*rpl8*) were used as normalizing genes. Legend: *fshβ*: follicle stimulating hormone beta, *ipgr*: intracellular progesterone receptor, *mpgr*: membrane progesterone receptor, *pgrmc1*: progesterone receptor membrane component 1, *prl*: prolactin, *srd5a1*., *srd5a2*: steroid 5 alpha reductase type 2, *srd5a3*: steroid 5 alpha reductase type 3. Primers for genes not shown (androgen receptor (*ar*), *ef1a*, estrogen receptor alpha (*era*), *odc*, *rpl8*, steroid 5 alpha reductase type 1 (*srd5a1*), steroid 5 alpha reductase type 2 (*srd5a2*), steroid 5 alpha reductase type 3 (*srd5a3*)) were reported by Langlois et al. (2010).

Gene target	Accession No.	Biological function	Direction	Primer sequence (5' - 3')	Annealing temp. (°C)	Amplicon size (bp)	Primer Conc. (nM)
<i>fshβ</i>	XM_018093295.1	Gonadotropin	F R	GTGTACGTGCTCGTCCTGTG GTGTGCGATCCCTCCTTCTC	60	95	400 400
<i>ipgr</i>	XM_002935571.4	Sex steroid	F R	AACTTCAGCACACCACATTCC AGCCACACAACAGAAGTCCA	60	104	400 400
<i>mpgr</i>	NM_001078951	Sex steroid	F R	TGCCTTACTTCACTCCAGAGAGC TGGACACTGATGGATAGCGTG	60	98	250 250
<i>pgrmc1</i>	NM_001006841.1	Sex steroid	F R	ACAGAGGGAGACGTTGAGTG CTGTGTATTCTGTTGGCTCCTC	62	100	250 250
<i>prl</i>	NM_001100229.1	Reproduction and growth	F R	AGAAGACAAGGAGCAAGCAC GCAACCATGTGGAGTAAAGG	60	102	400 400

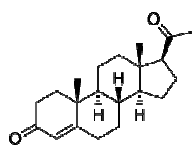
Table 2. Experimental concentrations before and after 24 h of exposure to progesterone (P4) and melengestrol acetate (MGA) in *S. tropicalis* larvae. Data are expressed as mean \pm SD. Two-tailed (paired) t-tests ($p < 0.05$). The limits of detection were 161 and 61 ng/L for P4 and MGA, respectively. Samples were pre-concentrated by a factor of 140x prior to analysis. C: water control, ND: Not detected, SC: solvent control (0.05% EtOH).

Chemical	Nominal concentration (ng/L)	Mean experimental concentration at time 0 h (ng/L \pm SD)	Mean experimental concentration at time 24 h (ng/L \pm SD)	Mean experimental concentration (ng/L \pm SD)
C	0	ND	ND	ND
SC	0	ND	ND	ND
P4	3.14	3.14 \pm 0.31	3.14 \pm 3.14	3.14 \pm 3.14
	31.4	25.2 \pm 6.29	9.43 \pm 6.29	15.7 \pm 91.2
	314	305 \pm 56.6	18.9 \pm 12.6*	195 \pm 167
	400	396 \pm 59.7	9.43 \pm 6.29*	302 \pm 198
	3140	3360 \pm 25.2	18.9 \pm 31.4*	3360 \pm 25.2
MGA	3.97	1.19 \pm 0.39	15.9 \pm 3.97*	7.94 \pm 7.94
	39.7	51.6 \pm 7.94	67.5 \pm 0.39	59.6 \pm 11.9
	317	187 \pm 39.7	288 \pm 35.7	234 \pm 63.5
	397	365 \pm 381	1530 \pm 707	1140 \pm 754
	3970	4000 \pm 615	3260 \pm 3.97	3730 \pm 595

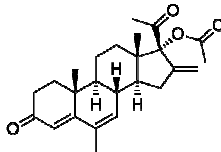
Table 3. Effects of progesterone (P4) and melengestrol acetate (MGA) spiked water on malformation of *S. tropicalis* larvae at exposure completion at Nieuwkoop and Faber (NF) stage 46. Mortality data are expressed as percent mean \pm SD (%) and malformation data are expressed as percentage of malformed animals per treatment. The malformation percentage was calculated from a subset of animals randomly collected in each treatment ($n = 50$). Abnormalities were observed in axial formation (shortening, flexure, and wavy tail), edema (blistering), eyes (reduction in size, asymmetric formation, incomplete separation from the brain, and cyclops), hearts (failure to coil), gut (failure to coil), and cranio-facial (reduction in size and flattened face). Data were analyzed using Fisher's exact tests ($p < 0.05$). Asterisks indicate statistically significant differences between treatments and solvent controls. C: water-only control, SC: solvent control (0.05% EtOH).

Treatments (ng/L)	Mortality (%)	Malformed individuals % (n)	Malformations observed (%)					
			Axial	Edema	Eye	Heart	Gut	Cranio- facial
C	6.8 \pm 2.8	10 (5)	12	8	4	2	4	4
SC	6.0 \pm 2.0	12 (6)	10	8	6	2	12	0
P4 3.14	7.0 \pm 3.7	20 (10)	20	8	20	6	16	0
P4 15.7	8.7 \pm 3.9	16 (8)	6	12	20	4	6	2
P4 195	7.0 \pm 3.0	22 (11)	16	18	12	0	14	4
P4 302	4.3 \pm 2.3	26 (13)	16	4	16	4	16	6
P4 3360	6.3 \pm 3.9	16 (8)	12	6	12	6	12	4
MGA 7.94	4.0 \pm 2.8	16 (8)	10	2	8	2	12	2
MGA 59.6	10.7 \pm 3.3	24 (12)	16	4	10	2	14	4
MGA 234	9.0 \pm 2.4	14 (7)	8	10	12	0	4	0
MGA 1140	9.3 \pm 5.9	24 (12)	14	10	20	4	8	4
MGA 3730	12.7 \pm 6.9 *	28 (14)	26	6	16	4	6	10

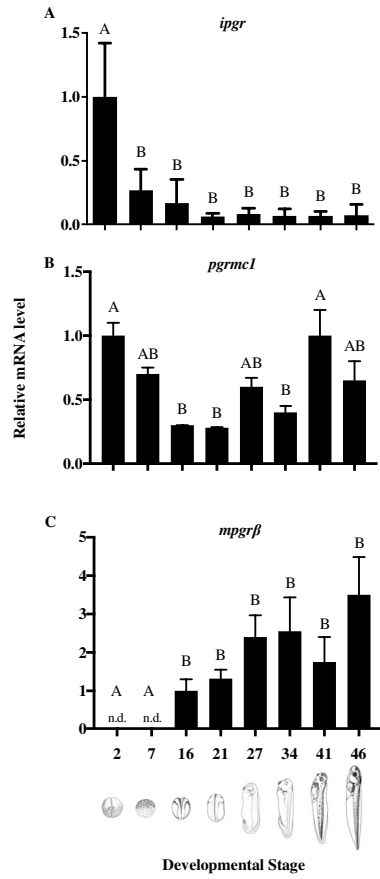
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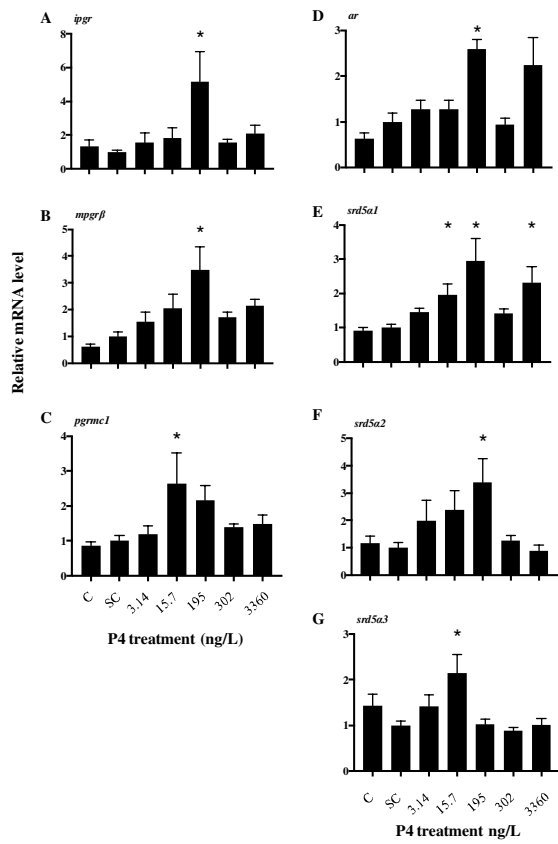


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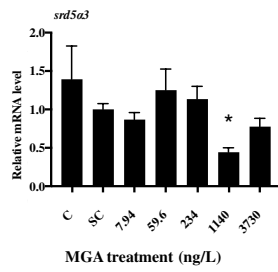
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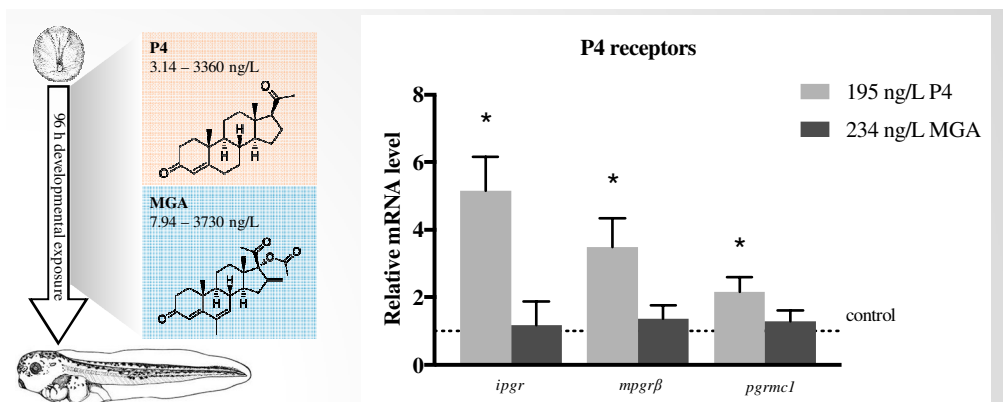
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Highlights

- P4-receptors are expressed in early larval development in *S. tropicalis*
- P4 upregulated gene expression of *ipgr*, *mpgr β* , *pgrmc1*, *ar*, and *srd5 α 1*, 2, and 3
- MGA exposure induced a dissimilar molecular response to P4
- MGA exposure inhibited transcription of *srd5 α 3* in a concentration-dependent manner

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