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4 **Chronic exposure to two gestagens differentially alters morphology and**
5 **gene expression in *Silurana tropicalis***
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54 **Funding**
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Conflicts of interest/Competing interests

The authors declare no conflict of interest.

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4 **Abstract**
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6 Gestagens are active ingredients in human and veterinary drugs with progestogenic activity. Two
7 gestagens, progesterone (P4), and the synthetic P4 analogue, melengestrol acetate (MGA), are
8 approved for use in beef cattle agriculture in North America. Both P4 and MGA have been
9 measured in surface water receiving runoff from animal agricultural operations. This project
10 aimed to assess the morphometric and molecular consequences of chronic exposures to P4,
11 MGA, and their mixture during Western clawed frog metamorphosis. Chronic exposure (from
12 embryo to metamorphosis) to MGA (1.7 µg/L) or P4 + MGA (0.22 µg/L P4 + 1.5 µg/L MGA)
13 caused a considerable dysregulation of metamorphic timing, as evidenced by an inhibition of
14 growth, narrower head, and lack of forelimb emergence in all animals. Molecular analysis
15 revealed that chronic exposure to the mixture induced an additive upregulation of neurosteroid-
16 related (GABA_A receptor subunit α6 (*gabra6*) and steroid 5-alpha reductase 1 (*srd5a1*) gene
17 expression in brain tissue. Chronic P4 exposure (0.26 µg/L P4) induced a significant
18 upregulation of the expression hypothalamic-pituitary-gonadal (HPG)-related genes (*ipgr*, *era*)
19 in the gonadal mesonephros complex (GMC). Our data suggest that exposure to P4, MGA, and
20 their mixture induces multiple endocrine responses and adverse effects in larval Western clawed
21 frogs. This study helps to better our understanding of the consequences of chronic gestagen
22 exposure and suggests that the implications and risk of high gestagen use in beef cattle feeding
23 operations may extend to the aquatic environment.
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53 **Keywords:** progesterone; melengestrol acetate; endocrine disrupting chemical; amphibian
54 metamorphosis; mixture effects
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Introduction

Gestagens are compounds that bind and activate progesterone receptors (PRs), including endogenous progestogens (e.g., progesterone; P4) and synthetic progestins used in human and veterinary drugs (e.g., melengestrol acetate; MGA). 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 approximately 279 tons of gestagens are excreted by farm animals in the US annually (Lange et al., 2002). Gestagens have been detected in the aquatic environment at levels generally in the range of 0.1 -30 ng/L (reviewed by Fent, 2015; Kumar et al., 2015; Orlando and Ellestad, 2014). However, elevated levels of both P4 and MGA have been measured in flush water from animal agriculture operations in concentrations up to 11.9 µg/L and 0.5 µg/L, respectively (reviewed by Fent, 2015; Liu et al., 2015a,b; Ray et al., 2013; Bartelt-Hunt et al., 2012; Liu et al.; 2012; Mansell et al., 2011; Shore and Pruden, 2009; Lange et al., 2002). The environmental fate of these compounds in the aquatic environment is not well understood. P4 has been shown to rapidly degrade in aqueous environments, with a reported half-life of 4.3– 40 h (Ojogoro et al., 2017; Peng et al. 2014; Liu et al., 2013). MGA is known to be persistent in soil and manure matrices (Challis et al., 2021; Qu et al., 2014; Schiffer et al., 2001) but undergoes direct photolysis, with a half-life of approximately 45 min (Qu et al. 2012). However, the aquatic fate of MGA has not yet been evaluated.

Despite their co-occurrence in the aquatic environment, the ecotoxicological impact of these gestagens and their combination is poorly understood. Agricultural ponds are known to be attractive habitats to amphibians as they lack fish predators and provide static aquatic habitat in

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4 an otherwise fragmented landscape (Swartz and Miller, 2019; Knutson et al., 2004). Therefore, it
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6 is especially pertinent to evaluate the effect of exposure to agricultural contaminants during
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8 larval amphibian development as amphibians may breed in these ponds.
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11 Gestagens have recently been identified as an emergent class of endocrine disrupting
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13 compounds (EDCs) with the ability to interfere with the hypothalamic-pituitary-gonadal (HPG)
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15 axis at ng/L levels in aquatic vertebrates (Säfholm et al., 2012; Kvarnryd et al., 2011; reviewed
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17 by: Zikova et al., 2017; Orlando and Ellestad, 2014; Säfholm et al., 2014). Several gestagens,
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19 including P4, have been shown to bind with high affinity to amphibian PRs (Liu and Patiño,
20
21 1993). Therefore, endocrine disruption by gestagen action may be mediated through binding and
22
23 activating of the intracellular progesterone receptor (iPGR), the membrane progesterone
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25 receptors (mPGR α , mPGR β , and mPGR γ), and the progesterone receptor membrane components
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27 (PGRMC1 and PGRMC2). P4 signaling mediated through mPGR β has been shown to serve a
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29 critical role of P4 in amphibian oocyte maturation and ovulation (Liu et al., 2005; Liu and
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31 Patiño, 1993). Additionally, transcripts for *ipgr* and *mpgr β* have been detected in *S. tropicalis*
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33 throughout embryonic development (Nieuwkoop and Faber stage (NF) 12-46) (Thomson and
34
35 Langlois, 2018), larval development, during-, and post- metamorphosis (NF 51 – 66 + 4 weeks,
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37 Jansson et al., 2016; NF 50 – 66 + 4 weeks, Säfholm et al., 2014), representing potential
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39 molecular targets of endocrine disruption by gestagens (Nieuwkoop and Faber, 1994). Moreover,
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41 we previously observed that acute P4- (15.7 – 195 ng/L), but not MGA- (7.94 – 3730 ng/L)
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43 exposure induced an upregulation of the genes encoding the PRs (*ipgr*, *mpgr β* , and *pgrmc1*) in
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45 *Silurana tropicalis* larvae (Thomson and Langlois, 2018).
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55 In addition to acting on the HPG axis, it has recently been reported that some progestins
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57 have the ability to interfere with the hypothalamic-pituitary-thyroidal (HPT) axis (Lorenz et al.,
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4 2018; reviewed by Ziková, 2017). In amphibians, exposure to levonorgestrel (LNG; 312 – 3124
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6 ng/L) and norethindrone (2984 ng/L) caused asynchronous abnormal development and
7
8 inactivation of the thyroid gland in developing tadpoles (Lorenz et al., 2018; Lorenz et al.,
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10 2011a). One study demonstrated that *Xenopus laevis* tadpoles, exposed to 100 ng/L MGA
11
12 exhibited a reduction in body mass and snout-vent length (Finch et al., 2013). However, there are
13
14 limited data regarding the ecotoxicological effects of both P4 (Säfholm et al., 2014) and MGA
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16 (Finch et al., 2013) in amphibians, and the chronic effects of developmental exposure to
17
18 gestagens has been scarcely studied (Kvarnryd et al., 2011). Moreover, in our previous study, P4
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20 and MGA induced dissimilar molecular responses (Thomson and Langlois, 2018), emphasizing
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22 the need to further examine the mechanisms mediating the biological response to gestagens in
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24 frogs.
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31 Despite the co-occurrence of multiple progestogens in the environment (reviewed by:
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33 Bartelt-Hunt et al., 2012; Fent, 2015; Orlando and Ellestad, 2014; Liu et al., 2012a,b; Mansell et
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35 al., 2011; Besse and Garric, 2009; Lange et al., 2002), most studies to date have focused on
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37 single-compound toxicity. Thus, there is limited research concerning joint toxicity of mixtures
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39 progestogens. The objectives of the present study were to examine the effects of chronic
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41 exposure to P4, MGA, and their mixture on development, morphology, and gene expression
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43 during amphibian metamorphosis.
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50 **Materials and methods**

51 **Chemistry**

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53 Progesterone (P4; purity \geq 99%; CAS 57-83-0) was obtained from Sigma (Oakville, ON, CA),
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55 whereas melengestrol acetate (MGA; purity \geq 97%; CAS 2919-66-6) was purchased from Enzo
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4 Life Sciences, Inc. (Farmingdale, NY, USA). Stock solutions for each treatment group were
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6 made by dissolving the powdered hormone in Ethanol (EtOH) and then stored at – 20 °C in the
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8 dark.
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11 To determine gestagen concentrations in experimental treatment groups over the course of
12
13 the 72 h static treatment period, water samples were collected in triplicate from one aquarium for
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15 each treatment at 0 h and 72 h, during the fourth week of the exposure. The experimental
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17 concentrations of P4 and MGA were determined by Liquid Chromatography-High Resolution
18
19 Mass Spectrometry (LC-HRMS) in the Yargeau Laboratory (McGill University, QC, CA). Water
20
21 samples (12 mL) were collected in amber glass vials and stored at -20 °C until analysis. Briefly,
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23 7 mL of the sample was transferred to a 15 mL tube (Fisher Scientific; Pittsburgh, PA, USA) and
24
25 fortified with 30 µL of internal standard solution (10 ng/mL; P4-d9 Cat: P755902 and MGA-d3
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27 Cat: M215352 obtained from Toronto Research Chemicals (North York, ON,CA) followed by 3
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29 mL of ethylacetate:hexane (8:2 v/v).
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36 Samples were mixed for 1 min on an orbital shaker and centrifuged for 1 min at 1500g and
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38 4 °C. The organic layer was transferred to a clean 15 mL tube where 1 mL of 100 mM
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40 ammonium formate (pH 9.0) was added and mixed for 1 min on an orbital shaker. Samples were
41
42 centrifuged and the top organic layer was transferred to a clean 5 mL tube for drying under
43
44 nitrogen gas. Extracts were reconstituted in 50 µL of 2 mM ammonium formate:methanol at
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46 0.1% formic acid solution and transferred to an HPLC vial with an insert.
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51 Separation of P4 and MGA was performed on a Thermo Scientific (San Jose, CA, USA)
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53 Accela 600 LC system equipped with refrigerated autosampler set at 4 °C, buffer degasser,
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55 column oven compartment operated at 60 °C, and a quaternary pump. Column configuration
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57 consisted of a Thermo in-line filter hardware unit with a 2.1 mm ID and 0.2 µm filter cartridge
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4 PN: 22180 (Bellefonte, PA, USA) followed by an Agilent UHPLC guard column Zorbax Eclipse
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6 plus C18 2.1 x 5 mm and 1.8 μm PN: 821725-901. Resolution of analytes was carried out on an
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8 Agilent analytical column Zorbax Eclipse plus C18 RRHD 2.1 x 50 mm and 1.8 μm PN:
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10 959757-902 (Santa Clara, Cal. USA). Ten μL of sample or its dilution were injected at a constant
11
12 flow rate of 250 $\mu\text{L}/\text{min}$. The initial mobile phase composition was aqueous 2 mM ammonium
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14 formate 0.1% formic acid buffer (A) and methanol 0.1% formic acid buffer (B) at 80% A/20%
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16 B, with an initial ramp to 65% B from 0 to 3 min, followed by a final ramp to 100% B in 2.5
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18 min. This composition of mobile phases was kept for 2 min and then the column was brought to
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20 the initial gradient conditions at 40% mobile phase B in 0.5 min and held for 2 min for
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22 equilibration. The total time of the cycle was 10 min.
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29 A Thermo Scientific LTQ Orbitrap XL mass spectrometer (San Jose, CA, USA) equipped
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31 with an electrospray ionization source was used for chemical determinations. MS detection was
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33 performed by Fourier transform mass spectrometry (FTMS) in positive ion mode. Instrument
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35 optimization was performed by infusing standard solutions at 5 $\mu\text{L}/\text{min}$, while determination of
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37 optimal source conditions was done by infusion flow analysis. Nitrogen gas was used for all
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39 sheath, auxiliary and sweep gasses, while helium gas was used as the collision gas. Data
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41 acquisition analysis was done on the precursor-ions for the full scan using FTMS obtained at
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43 30000 resolution on a mass range from 50-600 m/z on Xcalibur Version 2.1 from Thermo
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45 Scientific (San Jose, CA, USA).
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53 **Maintenance and breeding of *S. tropicalis***

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55 Adult Western clawed frog (*S. tropicalis*) husbandry was performed in the Queen's University
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57 Animal Care Facility (Kingston, ON, Canada) in accordance with the guidelines of the Animal
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4 Care Committee of Queen's University and the Canadian Council on Animal Care as described
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6 by Mathieu-Denoncourt et al. (2014). Sexually mature male and female frogs were reared in
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8 tanks containing dechlorinated and aerated water on a 12:12 h light: dark regime, with the light
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10 cycle commencing at 7:00 am. Fertilized eggs were obtained from two pairs of adult frogs
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12 according to an established method (Langlois et al. 2010). Briefly, human chorionic
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14 gonadotropin hormone (hCG; standard grade, 3,150 IU/mg potency, Sigma, Oakville, ON, CA)
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16 was injected into the dorsal lymph sac of each adult frog to initiate amplexus and ovulation. Both
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18 males and females received a priming injection of 40 μ L of hCG (12.5 U) and the animals were
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20 kept in isolation in glass aquaria with the pH adjusted to 6.0-6.1 with HCl. At 21 h post-
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22 injection, the pH of the aquaria was re-adjusted to 6.0-6.1 and a boosting injection of 160 μ L of
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24 hCG (200 U) was administered to each frog. Pairs were then introduced in the breeding
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26 chambers and were kept in the dark.
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34 Amplexus began approximately 3 h after the second injection; eggs were present between
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36 4-6 h post-injection from two pairs. Eggs were collected and pooled together to minimize
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38 parental effects. Embryos were held in the Frog Embryo Teratogenesis Assay: *Xenopus*
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40 (FETAX) rearing media (625 mg NaCl, 96 mg NaHCO₃, 30 mg KCl, 15 mg CaCl₂, 60 mg
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42 CaSO₄ 2H₂O, and 75 mg MgSO₄ per L of dechlorinated water; American Society for Testing
43
44 and Materials, 1998) containing gentamycin sulfate (0.04 mg/L; CAS 1405-41-0, Fisher
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46 Scientific, Mississauga, ON, Canada). Viable embryos were identified by visual observation with
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48 a dissecting microscope. Developmental stages were determined following the Nieuwkoop and
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50 Faber (NF) developmental staging system (Nieuwkoop and Faber, 1994). Eggs were allowed to
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52 develop to NF stage 8, at which point the coating surrounding the embryos was removed by
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54 gentle swirling in a 2% (w/v) L-cysteine Solution (>99%, CAS 52-90-4, Acros Organics, Fair
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4 Lawn, New Jersey, USA) adjusted to pH pH 8.1 with NaOH for 2 min (Williams et al., 2015;
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6 Brausch et al., 2010; Pickford et al., 2003). The eggs were then washed three times with FETAX
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8 solution (ASTM, 1998). Viable embryos were identified by visual inspection with a dissecting
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10 microscope.
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13 14 15 16 **Experimental design**

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19 *S. tropicalis* embryos were aqueously exposed to one of seven exposure regimes beginning at the
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21 gastrulae stage, NF 12 (13 hpf) in replicates of n = 200 until metamorphic climax (NF 60). P4
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23 (molecular weight = 314.46 g/mol) and MGA (molecular weight = 396.519 g/mol) were
24
25 dissolved in EtOH in stock solutions, which were pipetted into aquaria to obtain the target
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27 concentrations while maintaining a final concentration of 0.01% v/v EtOH. Treatment groups
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29 included: 0.01% v/v EtOH (solvent control; SC), measured concentrations of 0.16 µg/L P4
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31 (named P4-L), 0.27 µg/L P4 (P4-M), or 1.4 µg/L P4 (P4-H), a mixture treatment of 0.22 µg/L P4
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33 and 1.5 µg/L MGA (P4-M + MGA), and a singular MGA treatment of 1.7 µg/L MGA. The range
34
35 of exposure concentrations represents both environmentally realistic levels of P4 (P4-L and P4-
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37 M), and elevated concentrations (P4-H, P4-M+MGA, and MGA). The latter may be
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39 representative exceptional levels found in highly intensified agricultural areas (e.g., concentrated
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41 animal feeding operations) (Liu et al., 2015a,b; Orlando and Ellestad, 2014).
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48 At NF 12, healthy embryos were placed into 125 mL glass jars containing either FETAX
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50 solution or one of six test solutions in a density of 40 embryos per jar and 5 jars per treatment.
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52 The antibiotic gentamycin sulfate (0.04 mg/L; Sandoz Canada, Inc. Boucherville, QC, Canada)
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54 was also administered to each jar. FETAX solution and gentamycin sulfate were replaced every
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57 24 h until embryos reached NF 46 to maintain adequate dissolved oxygen levels and remove
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4 waste. Once the free-feeding stage (NF 46) was reached, tadpoles were assigned to one of two
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6 replicate 8-L glass tanks per treatment group containing dechlorinated, aerated water. Tanks
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8 were not evaluated as individual replicates, as tadpoles of the same treatment were sorted by size
9
10 and developmental stage weekly to avoid any metamorphic effects associated with housing, and
11
12 to maintain a target density between 0.8 – 1 g/L (Mathieu-Denoncourt et al., 2015). For the
13
14 remainder of the experiment, rearing media and chemicals were completely replaced every 72 h.
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19 Tadpoles were fed twice daily with equal amounts of a high-protein commercially available
20
21 food (Sera Micron®; AniDis, St. Laurent, QC, Canada) from NF46 onward. Dead animals were
22
23 removed and recorded daily. Experimental conditions were maintained throughout the duration
24
25 of the exposure, including: water temperature ($24 \pm 1^\circ\text{C}$), pH 7.3 – 7.8, constant aeration by air
26
27 stones, photoperiod (12:12 h; light commencing at 0700 h local time), and animal density (0.8 - 1
28
29 g/L). Ammonia levels were measured at weeks 4 and 8 of the experiment and were below the
30
31 safety value of 0.03 mg/L. The experiment was terminated once all SC animals reached NF 60.
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35 At NF 60, animals were anesthetized by immersion in 0.01% ethyl 3-aminobenzoate
36
37 methanesulfonate (MS-222; 98%, CAS 886-86-2, Sigma Canada Ltd., Oakville, ON, Canada),
38
39 and subsequently sacrificed by decapitation and tissue samples of gonadal mesonephros complex
40
41 (GMC) and brain were dissected from each animal and mass was recorded. Tissue samples were
42
43 snap frozen on dry ice and then stored at -80°C until gene expression analysis was conducted
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45 and samples were analyzed individually.
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53 **Morphological analysis**

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55 Morphological measurements were taken throughout the experiment and at exposure completion.
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58 Each week, a subset ($n = 10/\text{treatment}$) of surviving animals were photographed with a scale bar
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4 and whole-body length (snout-to-tail) was calculated using ImageJ image analysis software
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6 (Rasband, 1997). The developmental stage of each animal was recorded weekly. At NF 60, after
7
8 anesthesia but before sacrificing, animals were blotted dry with a Kimwipe®, body mass (BM)
9
10 was measured (wet weight; Pioneer scale; ± 0.1 mg; Ohaus, Pine Brook, New Jersey, USA), and
11
12 dorsal and ventral photographs were taken. Snout-vent length (SVL), interocular distance
13
14 (IOD), and hind limb length (HLL) measurements were digitally calculated to the nearest
15
16 millimeter using ImageJ. Subsequently, hepatosomatic index (HSI) and renosomatic index (RSI)
17
18 were calculated from wet weight of tissue samples. GMC tissue was used in RSI calculations, as
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20 the kidney of NF 60 animals contributes the majority of the mass of the GMC.
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28 **RNA isolation and cDNA synthesis**

29 NF 60 GMC and brain samples were used for gene expression analyses and each treatment was
30
31 tested in 13-21 replicates.
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35 GMC samples were homogenized using a Retsch Mixer Mill MM400 (Fisher Scientific,
36
37 Mississauga, ON, CA). Total RNA was isolated from individual samples using TRIzol reagent
38
39 extraction (Life Technologies, Burlington, ON, CA) according to the manufacturer's protocol
40
41 and was then purified using TURBO DNase treatment (Ambion; ThermoFisher Scientific,
42
43 Ottawa, ON, CA). Isolated RNA was re-suspended in Nuclease-free water and nucleic acid
44
45 concentration and purity were measured using a NanoDrop-2000 spectrophotometer
46
47 (Thermofisher, Ottawa, ON, CA). After isolation, RNA was stored at -80 °C. Complementary
48
49 DNA (cDNA) was synthesized from 0.8 µg of total RNA template and random hexamer primers
50
51 following the QuantiTect Reverse Transcription Kit protocol (Qiagen Inc., Mississauga, ON,
52
53 CA) on the PCR Vapo.protect Mastercycler pro S (Eppendorf, Hamburg, Germany).
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4 Brain samples were homogenized by sonication using the Ultrasonic Dismembrator-150T
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6 (Thermo Fisher, Ottawa, ON, Canada). Total RNA was isolated and purified from individual
7
8 samples using the RNeasy Micro Kit with RNase-free DNase I (Qiagen Inc., Mississauga, ON,
9
10 Canada). Isolated RNA was re-suspended in nuclease-free water and RNA concentration was
11
12 determined on a Qubit 4 Fluorometer (Thermofisher, Ottawa, ON, Canada). After isolation,
13
14 RNA was stored at -80 °C. cDNA was synthesized from 0.5 μ g total RNA template and random
15
16 hexamere primers following the QuantiTect Reverse Transcription Kit protocol (Qiagen Inc.,
17
18 Mississauga, ON, CA) on the PCR Vapo.protect Mastercycler pro S (Eppendorf, Hamburg,
19
20 Germany).

21 22 23 24 25 26 27 28 **Real-time RT-PCR**

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30 Changes in transcript abundance were investigated using real-time quantitative polymerase
31
32 change reaction (qPCR). The cDNA products were diluted either 40-, or 80-fold according to the
33
34 transcript abundance of each gene prior to qPCR analysis. All qPCR assays were performed
35
36 using a Bio-Rad CFX 96 Real-Time System (Bio-Rad Laboratories Inc., Mississauga, ON) and
37
38 Promega GoTaq qPCR MasterMix (Madison, WI, USA), which includes the BRYT Green and
39
40 carboxy-X-Rhodamine dyes. Each reaction consisted of a 20 μ L amplification reaction
41
42 containing 4 μ L cDNA, 0.1 – 0.65 μ g forward and reverse primers, 10 μ L GoTaq Master Mix
43
44 (Promega, Madison, WI, USA) for a final concentration of 1x, and Nuclease-free H₂O. For all
45
46 real-time qPCR assays, primer concentrations were optimized, and concentrations and annealing
47
48 temperatures varied according to the primer set. Gene-specific primer sequences for estrogen
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50 receptor (*era*), androgen receptor (*ar*), aromatase (*cyp19*), 5 α -reductase type 1, 2, and 3 (*srd5a1*,
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52 *srd5a2*,*srd5a3*), and the reference genes ornithine decarboxylase (*odc*), ribosomal protein 8
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4 (*rpl8*), and elongation factor-1 alpha (*ef1a*) were previously designed and validated by Langlois
5
6 et al. (2010). We previously described primers for additional genes of interest including PRs
7
8 (*ipgr*, *mpgrβ*, and *pgrmc1*) (Thomson and Langlois; 2018). Finally, the forward and reverse
9
10 primer sequences for glucocorticoid receptor (*gcr*) and GABA_A receptor subunit α6 (*gabra6*)
11
12 were designed an optimized for the present study (Table S1).
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16 The thermocycler program used included an enzyme activation step at 95 °C for 2 min,
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18 followed by 40 or 50 cycles at 95 °C for 15 s and 1 min at a gene-specific annealing temperature
19
20 of 58, 60, or 62 °C, followed by a denaturation step of 1 min at 95 °C. After the thermocycles
21
22 were complete, a melt curve analysis was conducted by performing 40 cycles starting at 55 °C
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24 and increasing 1 °C every 30 s. The relative standard curve method was applied to interpolate
25
26 relative mRNA abundance of target genes. Standard curves were prepared by serial dilution (1:4)
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28 of cDNA input pooled from each treatment. For quality assurance, two cDNA controls were
29
30 performed on each qPCR run, including products from the cDNA synthesis step that were
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32 generated without the addition of reverse-transcriptase (NoRT) and no template control (NTC)
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34 reactions without the addition of cDNA template. All qPCR reactions including samples, cDNA
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36 controls, and standard curves were performed in duplicate. Reaction efficiencies were 100 ± 10%
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38 with an $R^2 \geq 0.98$. The threshold for each gene was assessed automatically by the Bio-Rad CFX
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40 Manager Software 3.0. Gene expression data is presented as mean fold change relative to the
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42 mean SC treatment. The genes *odc*, *rpl8*, and *ef1a* were selected as reference genes based on
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44 previous evidence of their stable expression in *S. tropicalis*. Fold change data for GMC were
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46 normalized to the geometric-mean of these three reference genes, which was not significantly
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48 affected across treatment groups (one-way ANOVA; $p > 0.64$). Fold change data for brain
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4 samples were normalized to the mean fold change of the reference gene *odc*, which was not
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6 significantly different across treatment groups (one-way ANOVA; $p > 0.55$).
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10 11 **Statistical analysis**

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14 Statistical analysis was performed using GraphPad Prism version 8.2.1 (GraphPad
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16 Software Inc., San Diego, CA, USA). The proportion of animals achieving specific
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18 developmental stages each week was analyzed using Fisher's exact test. Growth and
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20 development over the course of the exposure were analyzed using two-way analysis of variance
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22 (ANOVA) followed by Dunnett's test for multiple comparisons. Morphometric data (body mass,
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24 SVL, HLL, IOD, RSI, and HSI) were analyzed using one-way ANOVA relative to the SC
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26 treatment followed by Dunnett's post hoc test. The mean relative mRNA levels of treatment
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28 groups were compared to the mean of each other treatment group using one-way ANOVAs and a
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30 Tukey post hoc test. Data and residuals were assessed for normality and homogeneity of
31
32 variances using the Shapiro-Wilk and Bartlett's tests, respectively. Statistical outliers were
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34 removed using the robust regression and outlier removal method with the maximum false
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36 discovery rate set to 1% (Motulsky and Brown, 2006). Outliers were identified evenly
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38 throughout the dataset and the maximum number of outliers removed from a treatment was 2.
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40 When necessary, the data was subsequently square root, \log_{10} , or natural log transformed to
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42 achieve normality and homoscedasticity. For all comparisons, significance level was set at $p <$
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55 **Results**

56 57 **Experimental gestagen concentrations**

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4 Measured concentrations at both t = 0 h and t = 72 h timepoints are presented in Table 1.
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6 The solvent control treatment was not contaminated with detectable levels of either P4 or MGA.
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8 After 72 h incubation time, P4 significantly degraded by approximately 95-98% in all P4
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10 treatment groups. In contrast, MGA concentrations increased by approximately 2.8-3.5x. As
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12 concentrations changed with time, the average concentrations (between 0 h and 72 h) were
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14 calculated and are presented in Table 1. These averaged concentrations were then used to report
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16 data in figures.
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23 **Organismal responses to gestagen treatments**

24 *Growth and rate of development*

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26 To evaluate the effects of chronic exposure to waterborne gestagens throughout tadpole
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28 development, developmental rate was evaluated by the proportion of tadpoles achieving specific
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30 developmental stages (Fig. 1, Fig. S1a). Chronic exposure to the highest concentration of P4
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32 accelerated *S. tropicalis* development, which was significant at weeks 6, and 8-15. All animals
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34 from the P4-H group developed hind limbs (NF 52) at week 10, while only 76% of the SC
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36 animals were at NF60 at that time (Fischer's exact test; $p < 0.0001$). In contrast, the MGA
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38 treatment group exhibited a significantly lower proportion of NF 52 animals at weeks 3 – 8.
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40 Initially, the mixture treatment inhibited development at weeks 3 – 9, however, this pattern was
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42 reversed later in the exposure and the mixture treatment hastened development at weeks 14 and
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44 15. Exposure to P4-L and P4-M, mixture, or MGA treatments did not significantly alter the
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46 percent occurrence of NF 60 tadpoles compared to the SC treatment. However, P4-H caused a
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48 developmental acceleration to NF 60 (Fig. 1b), and a significantly higher occurrence was
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50 observed from week 9 and this pattern persisted for the remainder of the experiment.
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Morphology

Tadpole morphology was significantly affected by gestagen exposure (Fig. 2). Within the experimental period, all surviving tadpoles treated with MGA or the mixture treatment displayed abnormal phenotypes and developed asynchronously according to the NF normal table of development. All animals in these treatments exhibited uncharacteristic narrowing of the head, buildup and darkening of tadpole skin, and failure to undergo forelimb emergence (FLE), which characterizes the transition from NF57 to NF58. However, forelimbs were visibly developing beneath the opercular skin and hind limb development often corresponded to stages >58 in these animals. Thus, the determination of developmental stages using the normal staging table was not possible. Instead, NF stage from stages 56 onward was determined based on hindlimb development for these treatment groups.

At NF 60, tadpoles in MGA and mixture treatments exhibited morphological inhibitions (Fig. 2a-f, Fig. S1b), indicated by decreased: SVL by 49% and 42%, wet weight by 50% and 42%, HLL by 37.4% and 32.6%, and IOD by 33.9% and 27.2% in the mixture treatment and MGA treatments, respectively ($p < 0.0001$). Finally, RSI was significantly increased in both the mixture and MGA treatments by 70% ($p = 0.0017$) and 74% ($p = 0.0005$), respectively. HSI was not significantly affected by MGA or mixture treatments. In contrast, P4 treatment did not induce significant changes on SVL, IOD, HLL, BL, or RSI compared with the control group. However, HSI was significantly increased by 58, 83, and 62% ($p < 0.05$) in P4-L, P4-M, and P4-H, respectively.

Gene expression

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4 GMC and brain gene expression data are presented as standardized means \pm SEM relative to
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6 mean reference gene(s) level. In the GMC, expression of *mpgr β* , *ar*, *cyp19*, *srd5 α 2*, and *srd5 α 3*
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8 was analyzed and differences between treatments were not statistically significant (Table S2).
9
10 The expression of several genes of interest was significantly altered by chronic gestagen
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12 exposure in the GMC of tadpoles, when compared to the SC treatment (Fig. 3a). Expression
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14 levels of *srd5 α 1* mRNA were significantly downregulated (0.5-fold, adjusted $p = 0.0495$) and
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16 (0.5-fold, adjusted $p = 0.0474$) in the GMC of the P4-H and mixture exposure groups,
17
18 respectively. A statistically significant increase (1.9-fold, adjusted $p = 0.0347$) of in the
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20 expression of *ipgr* was observed in GMC of P4-L-treated tadpoles. Finally, *era* expression was
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22 induced (1.8-fold, adjusted $p = 0.0197$) and (1.8-fold, adjusted $p = 0.0110$) in the P4-L and P4-H
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24 groups, respectively.
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31 In brain tissue, gestagen exposure did not significantly alter the expression of *pgrmc1*,
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33 *ipgr*, *ar*, *srd5 α 2*, or *gcr* (Table S3). Expression of several genes related to neuroendocrine
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35 signaling was significantly altered in the brain of tadpoles treated with the mixture of P4-M +
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37 MGA (Fig. 3b). Relative mRNA expression level of *srd5 α 1* was significantly upregulated (1.7-
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39 fold, adjusted $p = 0.0290$) in the tadpoles from the mixture treatment relative to the control
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41 group. Expression of *srd5 α 3* was significantly higher (adjusted $p = 0.0466$) in the mixture
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43 treatment, compared to the P4-M group. Expression of *era* was upregulated in the mixture
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45 treatment group, compared to the solvent control (1.6-fold, adjusted $p = 0.458$). Finally, *gabra6*
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47 expression was induced (2.0-fold, adjusted $p = 0.0458$) in the brain tissue of tadpoles in the
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49 mixture treatment compared to the control group. Of note, comparison between the mixture
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51 treatment group and the relevant single-chemical treatment groups (P4-M and MGA), indicated a
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53 statistically significant additive effect in each of these genes (Fig. 3b).
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Discussion

The objectives of this study were to evaluate the single and mixture effects of chronic gestagen exposure during tadpole metamorphosis. We employed a static renewal exposure design, mimicking environmental processes, such as precipitation events that transport environmental gestagens in a pulsatile nature (Biswas et al., 2017; Schiffer et al., 2004). We demonstrated that P4 concentrations significantly decreased in the 72 h renewal period. This finding is supported by the literature that suggest P4 is not a highly persistent pollutant. Ojogoro and colleagues (2017) demonstrated that P4 concentration in surface water dropped from of 1000 µg/L to < 0.7 µg/L after 72 h. Moreover, P4 actively and in humans, has a biological half-life, ranging from 3 – 90 min (Aufrère and Benson, 1976). In contrast, the observed increase in MGA concentration over time may be explained by incomplete dissolution or homogenization at t = 0 h. MGA is predicted to have very low water solubility (0.55 mg/L at 25 °C (US EPA, 2012)). Alternatively, it is possible that there was an incomplete removal of MGA during water changes (e.g., adsorption by aquaria or animals and later release into the media). The aqueous fate and behaviour of MGA is not well understood, however, it the biological half-life of MGA in humans is 3 – 5 d (Cooper et al., 1967). While there is no literature on the ability of MGA to bioconcentrate in aquatic organisms, this property has been demonstrated for other progestins (LNG, norgestrel, and medroxyprogesterone acetate) in mollusks (Contardo-Jara et al., 2011) and fish (Liu et al., 2015c; Steele et al., 2013; Fick et al., 2010).

A major finding of the present study is the disparate effects induced by these two gestagens. This is surprising due to the conserved mechanisms of action in other vertebrate species. In laboratory mammals, MGA is reportedly a highly potent gestagen compared to

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4 endogenous P4 and other synthetic gestagens (Bauer et al., 2001; Kazensky et al., 1998;
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6 Lauderdale et al, 1977; Duncan et al., 1964). Moreover, in bovine uterine cells, the relative
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8 binding affinity of MGA was reportedly 526% that of P4 (Bauer et al., 2001). However, the
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10 current findings suggest that this pattern may not be consistent in larval amphibians and that
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12 MGA may not act through classical P4-signalling pathways. These differences may be explained
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14 by differential molecular mechanisms of action, binding affinity, or activity in amphibians. Our
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16 previous work also found that P4 and MGA induced dissimilar effects at approximately
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18 equimolar concentrations in larval *S. tropicalis* (Thomson and Langlois, 2018). Nonetheless, the
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20 affinity and potency of MGA on amphibian PRs remains to be evaluated on a molecular level.
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26 Chronic exposure to a high concentration of P4 significantly accelerated frog
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28 metamorphic timing. As metamorphosis is primarily driven by thyroid hormones (THs), these
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30 effects may be explained by crosstalk between the gestagen signalling pathway and the HPT axis
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32 in non-mammalian species. While Liang et al. (2015) concluded that the influence of P4 on TH-
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34 related gene expression is possible through the upregulation of the sodium iodide symporter, it
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36 remains minimal in larval zebrafish (*Danio rerio*). Because anuran metamorphosis is a TH-
37
38 dependent process, larval amphibians may be more sensitive to P4 exposure than fish. Indeed,
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40 several synthetic progestins have been shown to affect the HPT axis in fish (Zucchi et al., 2013)
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42 and frogs (Lorenz et al., 2011a,b). Alternatively, high P4 level may have perturbed other
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44 morphogens involved in metamorphosis, such as antagonizing the anti-metamorphic hormone
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46 prolactin (PRL) or hypothalamic pituitary adrenal axis (HPA).
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53 In contrast, exposure to a high concentration of MGA or a mixture of P4 + MGA did not
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55 accelerate, but instead, retarded pre-metamorphic development, caused inhibited growth, and
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57 asynchronous tissue remodeling. Tadpoles exposed to MGA and mixture treatments were
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4 significantly smaller throughout development (beginning at week 3; Fig. S1) and were slower to
5 reach NF 52 (development of hindlimbs). Similarly, in the only other study that investigated the
6 effects of chronic exposure to MGA to date, Finch et al. (2013) demonstrated that *X. laevis*
7 tadpoles exposed to 100 ng/L MGA for 60 d exhibited reductions in body mass and snout-vent
8 length by 34 % and 20%, respectively. In addition to hormone mediated actions, this inhibition
9 of growth could be related to changes in energy balance, feeding behaviour, and/or swimming
10 performance. The developmental delay to reach NF 52 in mixture and MGA-only treatments is
11 unlikely to be due to disruption of the HPT-axis because premetamorphic development is
12 considered to be independent of the HPT-axis, as the thyroid gland is not yet functional (Dodd
13 and Dodd, 1976). Instead, future investigations should aim to elucidate the molecular pathways
14 involved in premetamorphic developmental delay.
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31 Results from the present study indicate that chronic exposure to P4 increased HSI while
32 exposure to MGA or a mixture of P4 + MGA caused a pronounced inhibitory effect on
33 morphology and induced an asynchronous pattern of metamorphic tissue remodeling. At all
34 tested concentrations, chronic P4 exposure was hepatotropic, as indicated by the 58-83%
35 increase in HSI. While there is a lack of data in aquatic vertebrates, hepatomegaly and liver
36 damage has been documented in humans following chronic use of progestogenic contraceptives
37 (Suarez et al., 2001; Jhingran et al., 1977; Fisher, 1975; Sherlock, 1975). Interestingly, the liver
38 undergoes little or no cell division during metamorphosis (Kaywin, 1936); therefore, the
39 observed effect is not likely to be hepatic hyperplasia or an artifact from the observed
40 acceleration of metamorphosis induced by P4. The increase in HSI may be due to disruptions of
41 the liver's roles in glycogen storage or enzymatic metabolism of xenobiotics and endogenous
42 compounds, such as steroid hormones (Waxman et al., 1988; Parkinson, 1996). Some evidence
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4 suggests that HSI is a potential indicator of glycogen storage in the liver and is correlated to
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6 growth in fish (Chellappa et al., 1995; Cui and Wootton, 1988; Adams and McLean, 1985;
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8 Holdway and Beamish, 1984). Other sex steroids have been shown to induce similar hepatotoxic
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10 effects. For example, androgen exposure significantly increased HSI in rainbow trout
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12 (*Oncorhynchus mykiss*) (Hirose and Hibiya, 1968) and channel catfish (*Ictalurus punctatus*)
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14 (Simone, 1990; Bulkley and Swihart, 1973). Moreover, P4 is a key intermediate in the steroid
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16 biosynthetic pathway and the biochemical precursor to other steroids. P4 has shown to increase
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18 the availability of cholesterol in rats (Nervi et al., 1983) through inhibition of cholesterol
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20 esterification (Erickson et al., 1980; Lichtenstein et al., 1980). Future studies could study hepatic
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22 cholesterol levels, expression and activities of steroidogenic enzymes (e.g., StAR, P450scc and
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24 3 β -HSD), and histopathology to elucidate the mechanism and determine the ecotoxicological
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26 implications of this effect.
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34 In contrast to the effects of P4 treatment, the mixture and MGA treatments induced
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36 conspicuous developmental abnormalities and inhibited all morphometrics other than RSI. The
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38 abnormal external morphology induced by both mixture and MGA treatments was characterized
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40 by a narrower head, smaller hindlimbs, skin abnormalities, and complete lack of FLE. These
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42 developmental malformations are indicative of an asynchronous pattern of metamorphic tissue
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44 remodeling that was never observed in any control or P4-treated tadpoles, suggesting that these
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46 effects were driven by MGA exposure in the mixture treatment. During prometamorphosis,
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48 complex biochemical and morphological changes are TH-dependent (reviewed in Brown and
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50 Cai, 2007; Degitz et al., 2005) and include perforation of the opercular membrane, permitting
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52 FLE (Hayes, 1995a), reconstruction of the head (Berry et al., 1998; Hanken and Summers,
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54 1988), and keratinization of the skin (Schreiber and Brown, 2003; Nishikawa et al., 1989).
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4 Metamorphic delay or arrest has been demonstrated in amphibians exposed to TH-antagonizing
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6 chemicals (reviewed by Kloas, 2002), and treatment of tadpoles with anti-thyroidal substances
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8 causes a delay or lack of FLE by down regulating thyroid receptor β (*th β*) expression and thus
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10 arresting metamorphosis (Opitz et al., 2006). Several progestins have been shown to exhibit
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12 thyroid disrupting properties in *X. laevis* (Lorenz et al., 2018; 2011a, Kloas et al., 2009).
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15 However, our observations do correspond with those metamorphic alterations induced by anti-
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17 thyroidal EDCs. While metamorphosis was disrupted in our study, some metamorphic events
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19 (e.g., forelimb development) were not affected, while others were strongly inhibited (e.g.,
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21 forelimb emergence).
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26 Interestingly, similar morphological abnormalities to those documented here (complete
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28 lack of FLE, extensive narrowing of the head) have been reported in tadpoles treated with
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30 relatively high concentrations of exogenous corticosteroids (Lorenz et al., 2009a, b) or reared at
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32 crowded densities, which subsequently results in elevated circulating CORT levels (Hayes,
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34 1997). Therefore, MGA may mimic the actions of CORT on the GCR. Glucocorticoids (GCs)
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36 have been shown to affect tadpole growth and development (Crespi et al., 2013; Lorenz et al
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38 2009a,b; Glennemeier and Denver, 2002a,b,c; reviewed in Hayes, 1997) and treatment with
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40 exogenous corticosteroids during premetamorphic stages has been shown to inhibit
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42 metamorphosis in tadpoles (Hayes, 1995b; Wright et al., 1994; Hayes et al., 1993). *X. laevis*
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44 chronically treated with 35 or 173 $\mu\text{g/L}$ CORT failed to undergo FLE and exhibited upregulated
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46 *prl* gene expression in brain (Lorenz et al., 2009b). Similarly, corticosteroids have a stimulatory
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48 effect on *prl* gene expression in mammalian and chicken pituitary cells (Fu and Porter, 2004;
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50 Camper et al., 1985). Thus, indicating that disruptors of the HPA axis may also regulate HPT-
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52 and PRL-signaling at the transcriptional level. Therefore, we suggest that the metamorphic
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4 dysregulation by MGA and mixture treatments did not act through classical disruption of the
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6 HPT-axis, but instead may be mediated by complex crosstalk with PRL, HPT, and/or HPA-axes.
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9 While the observed morphological abnormalities are consistent with those induced by
10 corticosteroids, we report that MGA blocked FLE at a concentration approximately 25-times
11
12 lower than the effects threshold of CORT for this endpoint. This may be explained by the cross-
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14 reactivity that MGA exhibits for other steroid hormone receptors. MGA was shown to have GC
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16 activity comparable with that of hydrocortisone (Elliott et al., 1973, Greig et al., 1970, Duncan et
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18 al., 1964). However, there is a need to further characterize the affinity and action of MGA on the
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20 GCR, specifically in frogs. In general, the patterns of expression of genes of interest were
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22 differentially affected by P4, MGA, and the mixture treatment, indicating that the dominant
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24 molecular mechanisms of action are different for these two gestagens. We previously reported
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26 that acute exposure to P4 or MGA does not activate the same molecular pathways (Thomson and
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28 Langlois, 2018). Nonetheless, we report that P4 and MGA acted additively on a transcriptional
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30 level in brain tissue. In GMC, our results indicated that P4 acts on the HPG-axis in frogs by
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32 altering gene expression of sex steroid-related transcripts. Treatment with 156.1 ng/L P4 induced
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34 an upregulation of *ipgr* and *era*. Similarly, we previously demonstrated that acute exposure to
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36 195 ng/L induced a 5-fold upregulation of *ipgr* in larval *S. tropicalis* (Thomson and Langlois,
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38 2018). Normal expression of *ipgr* is essential for reproductive function in mice (Lydon et al.,
39
40 1995) and frogs (Tian et al., 2000). And P4-signaling through the iPGR is essential to ovulation
41
42 in *D. rerio* (Tang et al., 2016). Therefore, disrupted *ipgr* expression, especially during the period
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44 of reproductive development, could lead to reproductive toxicity. Indeed, exogenous gestagen
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46 exposures have been shown to cause reduced fecundity or disrupted gonadal development in
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48 amphibians (reviewed by Ziková et al., 2017; Säfholm et al., 2015; Kvarnryd et al., 2011; Lorenz
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4 et al., 2011a,b; reviewed in Hayes, 1998). Interestingly, P4 exposure did not cause an alteration
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6 of expression of genes encoding the membrane *prs*, suggesting that their regulation differs from
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8 that of the *ipgr*.
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11 Expression of *era* was also upregulated by a low concentration of P4 in GMC. P4 is
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13 known to have anti-estrogenic activity (reviewed by Schindler et al., 2003)) and downregulate *er*
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15 expression (Stelmanska et al., 2012; Satyaswaroop et al., 1992; reviewed by Mauvais-Jarvis et
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17 al., 1986). However, in contrast, ER activation tends to upregulate both *era* and *ipgr* expression
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19 in humans (Jacobsen and Horwitz, 2012), rodents (Chappell and Levine, 2000), and reptiles
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21 (Custodia-Lora et al., 2004). Estrogen response elements have been identified in the *ipgr* of
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23 humans (Petz et al., 2004) and rat (Kraus and Montano, 1994). Therefore, it is possible that the
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25 inductions of *ipgr* and *era* in the lowest P4 treatment was due to signaling through the ER
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27 pathway. We also observed that chronic exposure to 1.4 µg/L P4 inhibited *srd5a1* gene
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29 expression. Inhibition of *srd5a* enzymes has been shown to induce a broad range of reproductive
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31 and non-reproductive adverse effects (Traish et al., 2015; Langlois et al., 2011). Interestingly,
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33 while P4 inhibited *srd5a1* in the GMC, we observed the opposite pattern in brain tissue.
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41 Because gestagens readily cross the blood-brain barrier, the brain represents a potential
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43 site of endocrine disruption by environmental gestagens. Here we demonstrate that joint
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45 exposure to P4 and MGA induced additive effects on the level of transcripts encoding proteins
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47 involved in neuroendocrine signaling. In the mixture treatment, both *srd5a1* and *gabra6* were
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49 significantly upregulated. SRD5a1 is responsible for neurotrophic activities during amphibian
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51 larval development (Bruzzone et al., 2010). Therefore, altered expression of *srd5a1* as a result of
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53 chronic, larval gestagen exposure may lead to neurotoxic effects during sensitive stages of
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55 development. Moreover, the main role of SRD5a1 in the brain is the production of neurosteroids
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4 from the biosynthetic conversion of P4 to 5 α -dihydroprogesterone and 3 α , 5 α -
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6 tetrahydroprogesterone (reviewed by Robitaille and Langlois, *submitted*; Bruzzone et al., 2010).
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8 These P4-metabolites act as potent allosteric modulator of the GABA_A receptor by increasing
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10 chloride conductance (Lambert et al., 1995; Callachan et al., 1987; Majewska et al., 1986). In the
11
12 rat brain, P4 has been shown to regulate the number of GABA_A receptors (Maggi and Perez,
13
14 1984). Moreover, in amphibians, GABA plays a role in controlling the biosynthesis of
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16 neurosteroids (Do-Rego et al., 2000), suggesting that the observed upregulation of *srd5a1* and
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18 *gabra6* may be a result of feedback loops from GABAergic actions of gestagens or their
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20 metabolites. Endogenous gestagens have roles in behaviour (reviewed by Baulieu and
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22 Schumacher, 2000), and 4 d exposure of up to 341 ng/L P4 altered reproductive behaviour in
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24 male *X. laevis* (Hoffmann and Kloas, 2012). Together, these finding suggest that the effects of
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26 chronic gestagen exposure includes neuromodulatory alterations on a transcriptional level in the
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28 tadpole brain and may extend to behaviour.
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36 Here we report that chronic exposure to two gestagens and their mixture had significant,
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38 but opposite effects on tadpole development, morphology, and gene expression. We demonstrate
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40 evidence that MGA and mixture treatments caused severe effects on metamorphic tissue
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42 remodeling similar to those induced by high concentrations of GCs. Because GCs induce
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44 opposing effects depending on if exposure begins during the premetamorphic or prometamorphic
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46 phases (reviewed by Denver, 2009), and MGA is a pollutant that is periodically flushed from
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48 agricultural operations, it is pertinent to evaluate the consequences of pulsatile MGA exposure in
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50 amphibians. Our evidence suggests that disruptions of tadpole embryogenesis and
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52 metamorphosis may occur in environments where elevated levels and complex mixtures of
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54 agricultural contaminants occur.
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Table 1 Measured concentrations of progesterone (P4) and melengestrol acetate (MGA) in exposure groups. Concentrations are expressed as mean \pm standard deviation (n= 3 technical replicates/treatment/sampling time). The limits of detection (LOD) were 0.161 and 0.061 $\mu\text{g/L}$ for P4 and MGA, respectively. The limits of quantification (LOQ) were 0.487 and 0.183 $\mu\text{g/L}$, respectively. Samples were pre-concentrated by a factor of 140x prior to analysis. ND: not detected SC: solvent control (0.01% v/v EtOH).

Treatment group	Mean experimental concentration ($\mu\text{g/L}$)		
	0 h	72 h	Average
SC	ND	ND	ND
P4-L	0.31 ± 0.02	$0.01^a \pm 0.00$	0.16 ± 0.01
P4-M	0.45 ± 0.02	$0.01^a \pm 0.00$	0.27 ± 0.01
P4-H	2.65 ± 0.06	$0.13^a \pm 0.07$	1.4 ± 0.07
P4-M + MGA	0.43 ± 0.02 $+ 0.75 \pm 0.05$	$0.01^a \pm 0.01$ $+ 2.16 \pm 0.13$	0.22 ± 0.01 $+ 1.5 \pm 0.09$
MGA	0.70 ± 0.03	2.64 ± 0.19	1.7 ± 0.11

^a < LOQ

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4 **Figure Captions**

5 **Fig. 1** Effects of gestagens on Western clawed frog metamorphosis, characterized by the
6 proportion of tadpoles reaching stage NF 52 (a), and NF 60 (b). Due to the asynchronous
7 development of tadpoles in the mixture and MGA treatments, NF 60 was determined based on
8 morphology corresponding to the illustrations shown. Data are expressed as absolute percentage
9 of animals per treatment that have reached each developmental stage, ranging from 28 – 139
10 animals. Boxes indicate statistically significant differences between treatments and the control
11 group. The illustration of stage NF 52 is from Nieuwkoop and Faber (1994). Legend: P4-L: 0.16
12 $\mu\text{g/L}$ progesterone, P4-M: 0.27 $\mu\text{g/L}$ progesterone, P4-H: 1.4 $\mu\text{g/L}$ progesterone, P4-M + MGA:
13 0.22 $\mu\text{g/L}$ progesterone and 1.5 $\mu\text{g/L}$ ng/L melengestrol acetate, MGA: 1.7 $\mu\text{g/L}$ melengestrol
14 acetate, and SC: solvent control (0.01% v/v EtOH)
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19 **Fig. 2** Morphology of *S. tropicalis* tadpoles chronically treated with exogenous gestagens. NF 60
20 morphometric measurements are presented as means +/- SEM for: snout-vent length (a), hind
21 limb length (HLL) (b), interocular distance (IOD) (c), wet body weight (d), renosomatic index
22 (RSI) (e), and hepatosomatic index (HSI) (f). Asterisks indicate significant differences from SC
23 group. ($n = 21 - 42$ per treatment). Legend: P4-L: 0.16 $\mu\text{g/L}$ progesterone, P4-M: 0.27 $\mu\text{g/L}$
24 progesterone, P4-H: 1.4 $\mu\text{g/L}$ progesterone, P4-M + MGA: 0.22 $\mu\text{g/L}$ progesterone and 1.5 $\mu\text{g/L}$
25 ng/L melengestrol acetate, MGA: 1.7 $\mu\text{g/L}$ melengestrol acetate, and SC: solvent control (0.01%
26 v/v EtOH)
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30 **Fig. 3** Significant changes in of genes of interest in GMC (a) and brain (b) after chronic gestagen
31 exposure. Normalized fold change data are presented as mean \pm SEM ($n = 13 - 17$ per
32 treatment). Letters indicate significant differences between treatment groups. Legend: *srd5 α 1*:
33 steroid 5-alpha reductase 1, *ipgr*: intracellular progesterone receptor, *pgrmc1*: progesterone
34 receptor membrane component 1, *era*: estrogen receptor alpha, *srd5 α 3*: steroid 5-alpha reductase
35 3, *gabra6*: GABA_A receptor, P4-L: 0.16 $\mu\text{g/L}$ progesterone, P4-M: 0.27 $\mu\text{g/L}$ progesterone, P4-
36 H: 1.4 $\mu\text{g/L}$ progesterone, P4-M + MGA: 0.22 $\mu\text{g/L}$ progesterone and 1.5 $\mu\text{g/L}$ ng/L
37 melengestrol acetate, MGA: 1.7 $\mu\text{g/L}$ melengestrol acetate, and SC: solvent control (0.01% v/v
38 EtOH)
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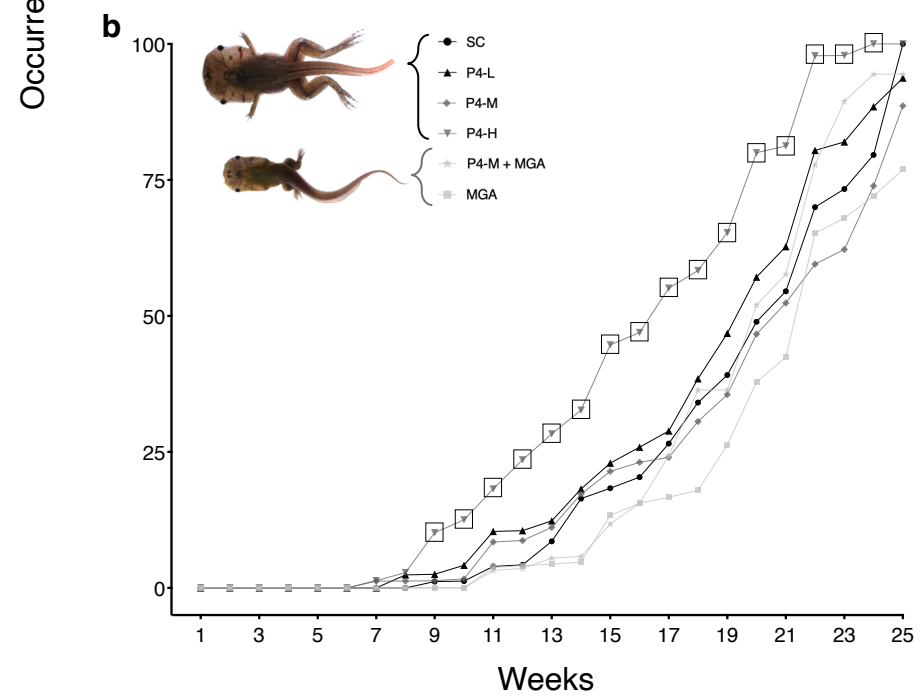
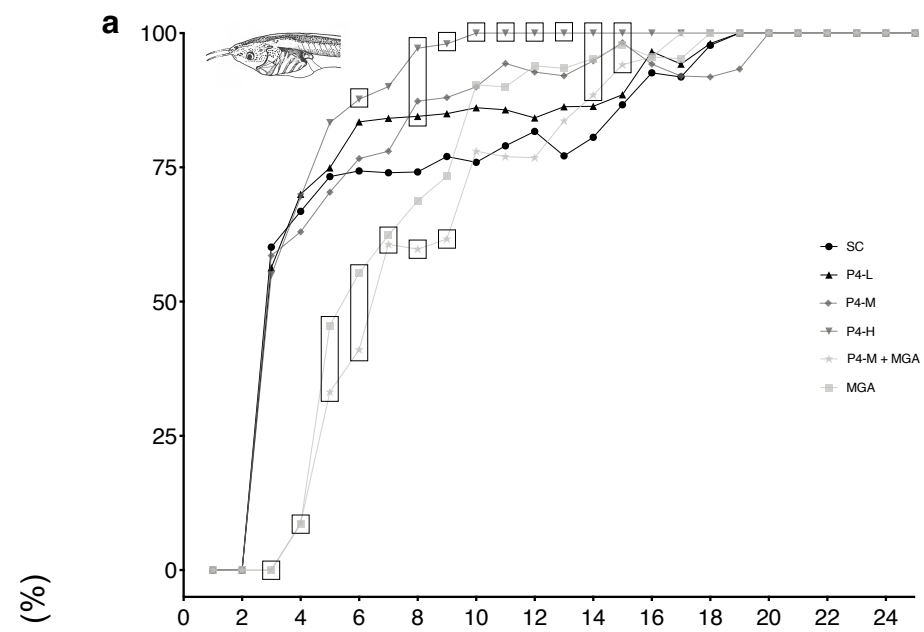
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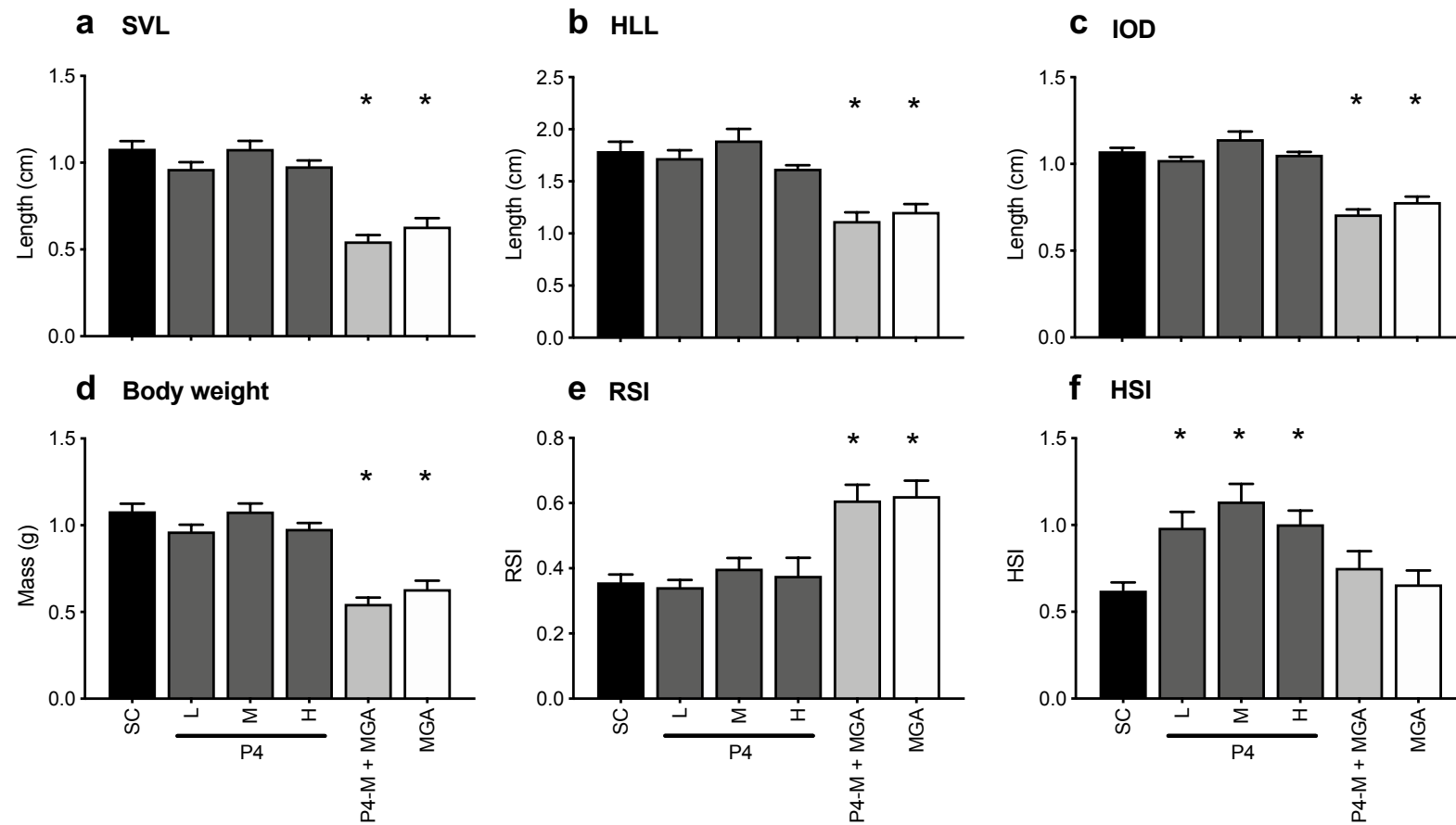
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Figure 1

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