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Effects of multiple life stage exposure to the fungicide prochloraz in *Xenopus laevis*: manifestations of antiandrogenic and other modes of toxicity

Jonathan T. Haselman^{a,*}, Patricia A. Kosian^a, Joseph J. Korte^a, Allen W. Olmstead^{a,b}, and Sigmund J. Degitz^a

^aUS EPA Mid-Continent Ecology Division, 6201 Congdon Blvd, Duluth, MN, 55804, USA.

^bPresent address: Environmental Toxicology and Risk Assessment, Development North America, Bayer CropScience LP, Research Triangle Park, NC 27709, USA.

Abstract

The Larval Amphibian Growth and Development Assay (LAGDA) is an internationally harmonized testing guideline for evaluating effects of chronic chemical exposure in amphibians. In order to evaluate the effects of chronic exposure to an antiandrogenic chemical in an amphibian model, prochloraz was tested using a variation of the LAGDA design. Exposure was initiated with < 1d post-fertilization embryos at nominal concentrations of 0, 6.7, 20, 60 and 180 µg/L (0, 18, 53, 159, 478 nM) and continued in flow-through conditions until two months following the median time that controls completed metamorphosis. Growth, developmental rate, circulating thyroid hormone and thyroid gland histopathology were evaluated in a subsample at completion of metamorphosis. There were no effects on growth or development at this stage, but circulating thyroid hormone was elevated in the 20, 60 and 180 µg/L treatments and minimal to mild thyroid follicular cell hypertrophy was observed histologically in the 180 µg/L treatment. Growth, overt toxicity, and reproductive development were evaluated at test termination. There were no effects on growth in either gender, but livers and kidneys exhibited treatment-related pathologies consistent with organ toxicity related to metabolism and presumably impaired excretion of prochloraz metabolites. Histological assessments of female ovaries resulted in minimal pathologies only in the 180 µg/L treatment while male testes exhibited numerous treatment-related pathologies that are consistent with previously reported antiandrogenic effects of prochloraz in other species. The most severe testis pathologies occurred in the 180 µg/L treatment; however, incidences of treatment-related pathologies occurred in all prochloraz treatments. Müllerian duct involution in males was inhibited by prochloraz exposure while Müllerian duct maturation in females was accelerated, characteristic of a feminizing effect. Gene expression levels of potential biomarkers of testis function were also measured. Relative abundance of *cyp17a1* transcripts was generally unaffected by prochloraz exposure whereas the *Ins13* orthologue, *rflcii*, was elevated by 3 and >5-fold in the 60 and 180 µg/L treatments, respectively indicating an adaptive response to increase Leydig cell differentiation and ultimately increase testosterone production. Overall, prochloraz exposure caused effects characteristic of an antiandrogenic mode of action, which is

*Corresponding author Jonathan T. Haselman, haselman.jon@epa.gov, T: 1-218-529-5244, F: 1-218-529-5003.

consistent with previously reported results in other species and supports the utility of the LAGDA design for chemical testing.

Keywords

Xenopus laevis; prochloraz; LAGDA; endocrine disruption; *Ins13*; *Rflcii*; *Cyp17*

1. Introduction

The Larval Amphibian Growth and Development Assay (LAGDA) is a second tier, multiple life stage, chemical testing guideline that was developed and globally harmonized under the Endocrine Disruptor Screening Program of the U.S. Environmental Protection Agency (<https://www.epa.gov/endocrine-disruption>) in collaboration with members of the Organization for Economic Cooperation and Development (OECD) to support ecological risk assessment (OECD, 2015; US EPA, 2015). The LAGDA is intended to be a definitive test that identifies apical effects following chronic chemical exposure in the model amphibian species *Xenopus laevis* and establishes a quantitative relationship between exposure concentrations and any adverse effects. During the development of the assay guideline, it was necessary to validate the assay design using chemicals with known modes of action within the endocrine system. Previous reports of LAGDA validation studies include exposures to 4-*tert*-octylphenol and 17 β -trenbolone, a weak estrogen receptor agonist and potent androgen receptor agonist, respectively (Haselman et al., 2016a) and exposure to benzophenone-2, a chemical having estrogenic and thyroid disrupting properties (Haselman et al., 2016b). The objectives of the current study were to test a model chemical with antiandrogenic properties in order to further validate the diagnostic capabilities of the LAGDA design and to develop gene expression markers that could provide mechanistic insight into the antiandrogenic mode of action.

Prochloraz is an imidazole fungicide that disrupts ergosterol synthesis by inhibiting cytochrome P450 (CYP) 14 α -demethylase (CYP51) through an interaction with iron in the heme cofactor, effectively weakening fungal cell membranes (Henry and Sisler, 1984; Bossche, 1985). Prochloraz has been implicated as an endocrine disrupting compound because it is an antagonist of the androgen receptor (Andersen et al., 2002; Ankley et al., 2005; Birkhøj et al., 2004; Kojima et al., 2004; Noriega et al., 2005; Vinggaard et al., 2002, 2006) and also inhibits steroidogenesis enzymes CYP19 aromatase (Anderson et al., 2002; Ankley et al., 2005; Sanderson et al., 2002; Vinggaard et al., 2000, 2006) and CYP 17 α -hydroxylase/17,20-lyase (CYP17) (Blystone et al., 2007a, 2007b; Laier et al., 2006). The timing and life-stage of *in vivo* prochloraz exposure likely influences the predominant toxic mode of action as evidenced by a series of studies in rodents and fish. Exposure to prochloraz *in utero* and in immature male rats caused reproductive malformations in androgen-dependent tissues, whereas effects on female reproductive development were minimal (Noriega et al., 2005; Laier et al., 2006; Vinggaard et al., 2002, 2005). Exposure to prochloraz in reproductively active fathead minnows caused decreased circulating estradiol in females with a corresponding decrease in circulating vitellogenin (estrogen-regulated egg yolk protein) in addition to oocyte atresia, while males exhibited decreased circulating

testosterone and increased spermatogonia (Ankley et al., 2005). These studies support a consistent series of mechanisms by which prochloraz elicits antiandrogenic effects; however, the timing of exposure in relation to the ontogeny of the endocrine targets during reproductive development and maturation appears to have a substantial influence on the gender-specific phenotypes following exposure. The LAGDA prescribes chemical exposure throughout embryonic, larval and juvenile development, so it was expected that developmental effects in the current study would occur more consistent with the rodent studies that resulted in malformations of the male reproductive system. Following this hypothesis, a parallel investigation was conducted to evaluate potential gene expression biomarkers of chemical-induced testis dysfunction in this amphibian model.

Insulin-like 3 protein (INSL3) is a Leydig cell-specific peptide hormone necessary for testis descent and gubernacular development in mammals and plays a role in follicle selection in the adult ovary (Bathgate et al., 2013). Recently, INSL3 has been increasingly employed as a biomarker of Leydig cell function in mammals (Roth et al., 2013; Ivell et al., 2013). Relaxin family locus C type II (Rflcii) in *Xenopus sp.* is orthologous to the mammalian INSL3 protein. Global gene expression analysis in *Xenopus tropicalis* gonads during differentiation indicated that *rflcii* was the transcript showing the highest normalized expression level overall and was the most differentially expressed transcript between ovary and testis tissues (testis-enriched by ~300-fold) (Haselman et al., 2015). This profound difference in expression between tissues provided evidence of this gene's importance in *Xenopus* testis development and is consistent with mammalian testis *Insl3* expression exclusively in Leydig cells (Adham et al., 1993). However, the mechanistic function of INSL3 orthologues has not been well-characterized across species, nor has its response to chemical perturbation been investigated in amphibians. The results presented herein provide precedent for *rflcii* being a testis-specific biomarker for impaired androgen production and/or signaling in *Xenopus laevis*.

Another key protein closely associated with the androgen axis and male reproductive development is CYP17. This enzyme is active within the steroidogenesis pathway and is necessary for synthesis of testosterone and consequently affects downstream estradiol synthesis due to the required aromatization of testosterone to produce estradiol. A growing body of evidence supports that prochloraz inhibits CYP17 (Ankley et al., 2009; Blystone et al., 2007a, 2007b; Hecker et al., 2006; Laier et al., 2006; Villeneuve et al., 2007) and other CYPs due to the imidazole interaction with iron in the heme cofactor required by all CYPs, which could be a major contribution to the antiandrogenic mode of action of prochloraz. *Cyp17* gene expression has been shown to be upregulated in the testis of fathead minnows (Ankley et al., 2009) and medaka (Zhang et al., 2008) exposed to prochloraz, while rodents exposed to prochloraz upregulate CYP17 protein production in the testis without upregulating gene transcription (Blystone et al., 2007b; Laier et al., 2006). These CYP17-related responses to prochloraz exposure across species appear to be adaptive responses to compensate for impaired androgen signaling. Altogether, the current study provided a sensible means to evaluate gene expression of *rflcii* as a potential biomarker of testis dysfunction and *cyp17* as a bioindicator of compensation due to chemical stress in an amphibian model.

2. Materials and methods

2.1 Experimental design

The experimental design of this study was similar to the previously described studies contributing to the development of the LAGDA (Haselman et al., 2016a) and the final published guidelines (OECD, 2015; US EPA, 2015). However, due to slight differences in execution of this study during guideline development, much of the methods will be described. *Xenopus laevis* embryos (<1d post-fertilization) were exposed to four different chemical concentrations and control water until two months after the median time to Nieuwkoop and Faber ([NF], 1994) stage 66 (completion of metamorphosis) in the control. There were five replicate tanks in each test concentration with eight replicate tanks for the control. Generalized toxicity was evaluated by mortality rates, abnormal behavior and growth (weight and snout-vent length) while toxicity affecting metabolism and excretion was evaluated via histopathology of the juvenile liver and kidney at test termination. Thyroid-specific toxicity was evaluated by rates of larval development (time to NF stage 66) and NF stage 66 thyroid histopathology assessments. Histopathology assessments of the juvenile gonads and reproductive ducts at test termination were also performed to evaluate effects on reproductive development. Additionally, gene expression of *rflcii* and *cyp17a1* in testis tissues was evaluated by randomly choosing either the left or right testis from each male for gene expression while the contralateral testis was left *in situ* for histopathology assessments. Sex ratios and possible sex reversal were also determined by comparing individual genotypes to phenotypes.

2.2 Test concentrations

The high test concentration was set based on range finding experiments (data not shown). In those studies, conducted with *Xenopus tropicalis*, concentrations in excess of 200 µg/L resulted in mortality in early larval organisms. Therefore, the high test concentration was set at 180 µg/L with a 0.33 dilution factor resulting in nominal test concentrations of 180, 60, 20 and 6.7 µg/L (478, 159, 53, 18 nM).

2.3 Environmental conditions

The source water used for the study was from Lake Superior (LSW), which was initially filtered through sand, UV sterilized and filtered again to 5 µm before entering the in-house culture or exposure aquaria. The incoming culture water is tested on a yearly basis for contaminants including copper, chlorine, chloramine, estrogenic or androgenic chemicals, fluoride, perchlorate and chlorate as measurable levels of any of these compounds could confound results of the bioassay. Flow-through conditions were maintained by delivering toxicant solutions and control LSW to 6.5 L glass exposure tanks at a constant rate of 44 ml/min, achieving approximately 9.5 tank replacements per day. The pump-driven dilution system was the same system as was used for the 4-*tert*-octylphenol study described in Haselman et al. (2016a). Exposure tanks were held at 21°C throughout the duration of the exposure by controlling the temperature of the delivery/dilution water and also by a water bath in which the exposure tanks were partially submerged. Water quality measurements including dissolved oxygen, pH and conductivity were taken in all exposure tanks once per week throughout the duration of the test (Supplemental table 1). Alkalinity and hardness

were measured once per month in one replicate tank per treatment. There were no treatment-related effects on water quality. A 12-hour light and 12-hour dark cycle was used with fluorescent lighting at intensities between 600 and 2,000 lux (lumens/m²) at the water surface. Exposure tanks were randomly assigned to positions within the exposure system to reduce potential effects from systematic environmental differences in temperature or light.

2.4 Chemical stock preparation

18 mg/L prochloraz stock solutions were prepared by dissolving the neat material (Sigma-Aldrich, St. Louis, MO, USA; CAS 67747-09-5, purity: 99.1%) in 20 mL of high purity acetone and quantitatively transferring to a 19 L carboy. The side walls of the carboy were rinsed with acetone and it was carefully tipped onto its side and placed on a roller. Filtered and desiccated compressed air was gently blown into the bottle as it was rolled to evaporate the acetone and shell-coat the carboy with prochloraz. Once the acetone was completely evaporated, the carboy was filled with reverse osmosis water and stirred overnight to dissolve the prochloraz.

2.5 Test initiation and feeding

All animal care and use procedures for these experiments were reviewed and cleared by the Mid-Continent Ecology Division's Animal Care and Use Committee. Procedures for test initiation and feeding are the same as described by Haselman et al. (2016a) except that embryos were transferred to the test chambers at approximately NF stage 20 for initiation of exposure.

2.6 Sub-sampling at completion of metamorphosis (NF stage 66)

Sub-sampling was performed by dividing the exposure tank and separating individuals that completed metamorphosis (NF stage 66) from actively metamorphosing larvae and recording the date on which each individual reached NF stage 66. One out of each group of four consecutive individuals completing metamorphosis in any given tank was randomly sampled for growth metrics including weight and snout-vent length, serum for circulating thyroid hormone and thyroid gland histopathology. At any given time during metamorphosis, developmental stages of organisms within an experimental unit are generally normally distributed. This larval sub-sampling method was intended to reduce bias toward sampling individuals on the leading or trailing edges of development in a particular tank and ensure all individuals in a tank had equal likelihood of being sampled upon reaching NF stage 66. Sub-sampled individuals were euthanized by submersion in LSW with 200 mg/L tricaine methanesulfonate ([MS-222], Argent Chemical Laboratories, Redmond, WA, USA) buffered with 400 mg/L sodium bicarbonate. Following growth measurements, blood was collected directly from the aorta using a 25 µl capillary tube and then a transverse cut was made at the posterior margin of the arms for decapitation; the head was fixed in Davidson's solution for thyroid histopathology. Individuals not sampled were left in the divided tank until all individuals had reached NF stage 66, at which time the tank dividers were removed.

2.7 Cull and test termination

Two to three weeks following the time at which all individuals completed metamorphosis (NF stage 66), gender genotyping was conducted and a cull was performed to reduce tank density to five males and five females in each tank until test termination. The cull and associated genotyping were performed according to the procedures previously described by Haselman et al. (2016a). Briefly, non-invasive unique toe clips were taken from each individual in each tank resulting in both an identification scheme and DNA samples that were analyzed by PCR for the presence/absence of a W chromosome-linked gene (DM-W) that effectively identified genotypic females, a method previously described by Yoshimoto et al. (2008). Juvenile sampling at test termination was also performed according to procedures previously described by Haselman et al. (2016a). Briefly, following euthanasia by injection of buffered MS-222, frogs were laid in dorsal recumbency, chest cavities were opened and blood was collected by severing the aorta. Next, livers were removed, weighed, transferred to individual cassettes and fixed in Davidson's solution for 48 hours. Finally, internal viscera were removed leaving the gonads, kidneys and Müllerian ducts *in situ* except that one of the juvenile testes per male was randomly chosen to be processed for total RNA purification and subsequent quantitative PCR analyses. Removed testes were homogenized in lysis buffer from an RNeasy Plus kit (Qiagen Inc., Valencia, CA, USA), and immediately frozen on dry ice until samples could be moved to storage at -80°C . Abdomens containing the kidneys, Müllerian ducts and gonad(s) were trimmed at the anterior and posterior margins of the kidneys, transferred to individual cassettes and fixed in Davidson's fixative for 48 hours. Tissue from the lower leg was kept for subsequent DNA purification and genotypic sex determination.

2.8 Histological procedures

All tissues preserved for histological analyses were fixed, processed and evaluated according to the procedures previously described by Haselman et al. (2016a). Following fixation in Davidson's fluid for 48 hours and subsequent storage in 10% neutral buffered formalin, liver, kidney, gonad and reproductive duct tissues were shipped to Integrated Laboratory Systems, Inc. ([ILS[®]], Research Triangle Park, NC, USA) where they were processed, embedded, sectioned, mounted and stained with H&E using typical histological procedures. Histopathologic findings were scored for severity by an ILS[®] pathologist according to the grading system previously described by Haselman et al. (2016a) for all tissues except for thyroid. Thyroid tissues were shipped to Experimental Pathology Laboratories, Inc. ([EPL], Sterling, VA, USA) where they were processed as described above and histologic findings scored for severity by a pathologist using the grading system for thyroid histopathology developed and described by Grim et al. (2009).

2.9 Serum thyroid hormone

Following blood collection from NF stage 66 organisms, the capillary tubes were placed on ice and allowed to clot over a period of 4 hours. They were subsequently spun at $13,700 \times g$ for 2 min in a serological centrifuge and serum was transferred to vials and stored at -80°C until they were pooled, diluted and analyzed. To quantify serum thyroxine (T4) and triiodothyronine (T3) levels, serum samples were initially pooled by combining equal

volumes across individuals within a replicate tank ($n = 4$ or $5/\text{pool}$), they were then diluted 1:8 in mobile phase A (1% methanol with 25 mM formic acid) and analyzed using high performance liquid chromatography (HPLC) interfaced with an inductively coupled plasma mass spectrometer (ICP-MS) as described previously by Tietge et al. (2010, 2013).

2.10 Plasma vitellogenin

Plasma vitellogenin concentrations were measured by ELISA using samples taken from frogs at test termination. Blood samples were processed and evaluated for vitellogenin concentrations according to methods previously described by Haselman et al. (2016a).

2.11 Testis RNA processing

Total RNA was isolated from testis homogenates using RNeasy Plus mini kits according to kit instructions. RNA concentrations were measured in three samples randomly chosen per tank using a fluorescence-based RNA quantitation assay (Quant-iTTM Ribo-green[®], Life Technologies, Grand Island, NY, USA). One tank from the 20 $\mu\text{g/L}$ prochloraz treatment was excluded from subsequent analyses due to extremely low yields from all testis samples. RNA quality was checked on all chosen samples using a 2100 Bioanalyzer with an RNA 6000 pico assay (Agilent, Santa Clara, CA, USA). If sample integrity was compromised based on the electropherogram generated by the Bioanalyzer, a new sample was randomly chosen from that tank and its concentration and integrity were measured prior to pooling. The three samples per tank were then diluted to between 0.5 and 1 $\text{ng}/\mu\text{l}$ (depending on the lowest concentration of the three samples to be pooled) and combined in equal amounts to create one pool per tank ($n = 3$ per pool; 1 pool/tank). A new total RNA concentration was determined for each pool using the same fluorescence method previously described and then divided into multiple aliquots to avoid freeze/thaw cycles. All subsequent gene expression levels were normalized to the total RNA concentration in each respective pool (Control: $n = 8$; 6.7 $\mu\text{g/L}$: $n = 5$; 20 $\mu\text{g/L}$: $n = 4$; 60 $\mu\text{g/L}$: $n = 5$; 180 $\mu\text{g/L}$: $n = 5$).

2.12 Quantitative PCR

Xenopus tropicalis accession numbers for *rflcii* and *cyp17a1* were aligned with the National Center for Biotechnology Information (NCBI) Expressed Sequence Tag (EST) database for *Xenopus laevis* using the nucleotide Basic Local Alignment Search Tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The highest scoring sequences were used to design primers and probes for a quantitative real-time PCR (qPCR) assay using a TaqMan[®] EZ RT-PCR assay (Applied Biosystems[®], Foster City, CA, USA) (Table 1). Primers and probes were designed using Primer Express version 3.0 (Applied Biosystems[®], Foster City, CA, USA). Standard RNA templates were generated by first cloning the cDNA target sequence using a total RNA sample extracted as described in section 2.11 above and specifically designed “outer” primers with a one-step reverse-transcription PCR kit (Sigma-Aldrich, St. Louis, MO, USA; Enhanced Avian HS RT-PCR kit). Next, target cDNA was amplified using separate “middle” primers that flanked the “inner” qPCR primer sequences and contained the T7 promoter region for subsequent *in vitro* transcription using a MEGAScript kit (Ambion[®] | Thermo Fisher Scientific, USA) according to kit instructions. The resulting transcribed RNA template was purified using an RNeasy Plus mini kit according to kit instructions. Purified RNA standard template concentrations were determined using the

mean of three measurements from a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., <http://www.nanodrop.com>); the mean concentration and calculated molecular mass was used to derive estimated copy numbers. Standard RNA templates were log₁₀ serially diluted and each concentration was run in duplicate with each sample set to serve as a standard curve. Additionally, no template controls (NTC) were run in duplicate on each plate; no amplification occurred in any NTC well. Pooled samples were also run in duplicate in 96-well plates according to TaqMan[®] kit instructions on an Agilent Stratagene Mx3005P qPCR instrument. Standard curves and data were processed using MxPro–Mx3005P9[®] software version 4.10 Build 389, Schema 85 (Stratagene, San Diego, CA, USA). Amplification efficiency was 99% for *rflcii* (standard curve slope = –3.341; R² = 0.998) and 97% for *cyp17a1* (standard curve slope = –3.385; R² = 1).

2.13 Exposure verification

Water samples (1 ml) were collected weekly from the prochloraz exposure tanks (0, 6.7, 20, 60 and 180 µg/L) and placed into amber sample vials. The water samples were amended with 0.1 ml methanol, vortex mixed and immediately analyzed using an Agilent 1100 series HPLC with diode array detection (λ = 205 nm). An aliquot (150 µl) of sample was injected directly into a Zorbax SB-C18, 2.1 × 75 mm column (Agilent, Wilmington, DE, USA) that was maintained at 25°C. The auto sampler temperature was maintained at 5°C. Prochloraz eluted from the column under isocratic conditions with 22% Burdick & Jackson (B&J) HPLC grade water and 78% methanol at 0.3 ml/min. Prochloraz concentrations were determined by the external standard method of quantification using linear regression. Prochloraz standards range from 5 to 200 µg/L and were prepared in 90% B&J water and 10% methanol. Quality assurance samples were conducted with each sample set and included LSW blanks, duplicate samples and matrix spiked samples at each of the prochloraz treatment levels. No prochloraz was detected in the LSW blanks (n = 15). Mean ± SD (n = 15) recoveries of spiked samples were 102.2 ± 3%, 99.5 ± 2.5%, 99.3 ± 2.5% and 100.7 ± 2.2% at the 6.5, 20, 60 and 180 µg/L spiked levels, respectively. Mean ± SD (n = 15) agreement among duplicate samples were 99.8 ± 1.9%, 99.3 ± 3.3%, 99.4 ± 1.5% and 100.3 ± 1.5% for the 6.7, 20, 60 and 180 µg/L treatments, respectively. The detection limit was 2.5 µg/L. Mean ± SD (n = 75) measured concentrations of the 6.7, 20, 60 and 180 µg/L treatment tanks were 6.10 ± 0.69, 18.11 ± 2.14, 52.83 ± 5.90 and 169.76 ± 14 µg/L, respectively, over the duration of the experiment. Among-tank variability was 12% with the coefficient of variation (averaged across the duration of the experiment) of 11.4%, 11.8%, 11.2% and 8.2 %, respectively for the 6.7, 20, 60 and 180 µg/L treatments. No prochloraz was detected in the control water treatments.

2.14 Statistical analyses

Statistical analyses were performed according to methods previously described by Haselman et al. (2016a). All data from the NF stage 66 subsample were analyzed independent of genetic sex while all data from juveniles were analyzed separately according to genetic sex. Continuous data (e.g., weight, length, liver-somatic index [LSI], mortality, serum T4, gene expression) were checked for monotonicity by rank transforming the data, fitting to an ANOVA model and comparing linear and quadratic contrasts. All data was monotonic with the exception of female weight at the final sample. A step-down Jonckheere-Terpstra trend

test was performed on replicate medians for endpoints meeting the monotonicity assumptions whereas female weight was analyzed using an ANOVA followed by Dunnett's multiple comparison procedure. Mortality data were analyzed for the time period encompassing the full test and was expressed as percent that died in any particular tank. Tadpoles that did not complete metamorphosis in the given time frame, those tadpoles that were randomly selected for the larval sub-sample or that were culled, and any animal that died due to experimenter error were censored within the mortality analysis. Prior to any statistical analyses, mortality ratios were arcsine-square root transformed and analyzed as described above. Time to NF stage 66 data were analyzed using a mixed effects Cox proportional hazard model (Therneau, 2015). Gene expression data were expressed and analyzed as copies mRNA per ng of total RNA. Histopathology data (both incidence and severity scores) were analyzed using the Rao-Scott Cochran-Armitage by slices (RSCABS) test (Green et al., 2014) implemented in R (R Core Team, 2017) using the StatCharms package (Swintek, 2017).

3. Results

3.1 Generalized toxicity

Prochloraz treatment did not affect survival (data not shown) or growth in the larval phase (Supplemental table 2) nor in either gender in the juvenile phase (Table 2). Liver-somatic indices were significantly lower in the 60 and 180 µg/L treatments in both genders at two months post-metamorphosis ($p < 0.05$, Jonckheere-Terpstra; Table 2). Histopathological evaluations identified a concentration-dependent increase in incidence and severity of hepatocellular degeneration (Supplemental table 3; Figure 1) and is consistent with the concentration-dependent decrease in liver-somatic indices (Table 2). In kidneys of 30 (60%) animals from the 180 µg/L treatment, large numbers of renal tubules contained ~12 µm, irregularly oval, amphophilic intracytoplasmic bodies (inclusions) (Supplemental table 4; Figure 2C). Minimal (infrequent) accumulations of protein within the Bowman's capsule were observed in all study groups with a slightly higher percentage of animals affected in the 180 µg/L treatment (Supplemental table 4; Figure 2D). The prevalence of liver and kidney pathologies was generally not gender-specific.

3.2 Endocrine toxicity

3.2.1 Thyroid—The purpose of the NF stage 66 subsample was to evaluate thyroid-related effects. Prochloraz exposure had no effect on thyroid-mediated metamorphic rates as measured by days to NF stage 66 (Supplemental table 5). Thyroid histopathology evaluations showed a significant increase in incidences and severity of follicular cell hypertrophy in the 180 µg/L treatment ($p < 0.05$, RSCABS), but there were no other corresponding treatment-related pathologies observed (Supplemental table 5). Circulating T4 increased with increasing exposure concentrations of prochloraz and was significantly different than control in the 20, 60 and 180 µg/L treatments ($p < 0.05$, Jonckheere-Terpstra; Figure 3A). Circulating T3 was unaffected by prochloraz treatment (Figure 3B).

3.2.2 Gonad and Müllerian duct histopathology—Evaluations of gonad and Müllerian duct development were performed on the final sampling at two months post-

metamorphosis. Prochloraz did not cause any incidences of sex reversal or gonadal intersex, as assessed by comparing the individual histological gonad phenotype to the genotype, determined by the presence/absence of the sex-specific gene DMW on the W chromosome. Histopathological observations of female ovaries included increased incidences of mononuclear cellular infiltrate, granulomatous inflammation and ovarian hypoplasia, all of minimal severity in only the 180 µg/L treatment (Supplemental table 6). Testis histopathology evaluations revealed a series of effects from prochloraz exposure mainly in the 180 µg/L treatment (Table 3). Gonadal degeneration that presented as germinal epithelial thinning/loss without apoptosis or necrosis was differentiated by the pathologist from gonadal degeneration with evidence of apoptosis and necrosis. Although incidences of these diagnoses occurred across all treatments and control, a higher percentage of individuals were affected in the higher treatments (Table 3; Figure 4). During normal testis development, all germ cells associated with a single spermatocyst mature at the same rate and the spermatocyst generally maintains a circular or ovoid shape. Prochloraz treatment caused asynchronous development of germ cells within a spermatocyst and caused some spermatocysts to become irregular in shape (Table 3; Figure 5); this occurred in a concentration-dependent manner. The 180 µg/L treatment experienced increased incidences of interstitial fibrosis, interstitial cell hypertrophy and hyperplasia, and mononuclear cellular infiltrate indicating inflammatory and compensatory responses from prochloraz insult in the testis.

By two months post-metamorphosis in *Xenopus laevis*, Müllerian ducts are typically resorbed to a fibrous tag in males and are actively maturing to become oviducts in females. Both male and female Müllerian duct development were affected by prochloraz exposure. Male Müllerian duct regression was generally inhibited in a concentration-dependent manner, and in some cases, the ducts partially matured to be characteristic of early female oviducts (Table 4). Female Müllerian ducts exhibited accelerated development with prochloraz exposure. The majority (88-100% among treatments) of prochloraz-exposed females had partially matured oviducts while only 56% of control females had oviducts at this same stage of development.

3.2.3 Circulating vitellogenin—Overall, constitutive vitellogenin levels in the blood of *Xenopus sp.* are very low to absent at two months post-metamorphosis (test termination) and are not significantly elevated unless exposed to potent estrogens (Haselman et al., 2016a, 2016b; Olmstead et al., 2009). In the current study, there was a slight, but significant, concentration-dependent increase in circulating vitellogenin in females exposed to 20, 60, and 180 µg/L prochloraz with a maximum increase of approximately seven-fold above the control ($p < 0.05$, Jonckheere-Terpstra; Table 2). Circulating vitellogenin was also significantly increased in males exposed to 60 and 180 µg/L prochloraz with a maximum increase of approximately three-fold above the control ($p < 0.05$, Jonckheere-Terpstra; Table 2). Although the maximum increases were approximately seven- and three-fold above the control for females and males respectively, these vitellogenin concentrations are sub-ng/ml whereas mature female *Xenopus sp.* have circulating vitellogenin levels six to seven orders of magnitude higher in the mg/ml range (Olmstead et al., 2009), providing context for the lack of biological significance of these increases due to prochloraz exposure.

3.2.4 Testis gene expression—Gene expression was measured in testis tissue of frogs at test termination. *Rflcii* gene expression in the testis exhibited a significant increase in the 60 and 180 µg/L treatments ($p < 0.05$, Jonckheere-Terpstra) and showed three and greater than five-fold increases respectively (Figure 6A). *Cyp17a1* was constitutively expressed at fairly high levels in all treatments and control in the testis at this stage of development (Figure 6B); however, the 180 µg/L treatment was slightly increased as compared to the control ($p < 0.05$, Jonckheere-Terpstra). Additionally, two control ovary samples were analyzed for each *rflcii* and *cyp17a1* expression levels in separate analyses. In these ovaries, the mean normalized *rflcii* transcript level was less than one and the mean *cyp17a1* transcript level was approximately 54% of the mean control testis level (Figure 6A & 6B).

4. Discussion

Prochloraz exposure throughout *Xenopus laevis* embryonic, larval and juvenile development resulted in a range of toxicological effects characteristic of multiple modes of toxicity. The observed hepatocellular degeneration, together with measured decreases in liver-somatic indices and intracytoplasmic inclusions in renal tubular cells are all indicative of overt toxicity in the 60 and 180 µg/L treatments (159 and 478 nM, respectively). The histologic appearance of the hepatic inclusions is suggestive of excess protein and ribonucleic acid aggregations, presumably related to metabolism of prochloraz and/or impaired clearance of endogenous metabolites. A study by Needham and Challis (1991) reported that one of the major prochloraz metabolites in rats was a glucuronide conjugate and high concentrations of prochloraz metabolites were found in liver tissue. In the same study, high concentrations of prochloraz metabolites were also found in kidneys. The similar appearance of hepatic and renal tubular cell inclusions in the current study is suggestive of metabolism, aggregation and excretion of prochloraz metabolites consistent with observations in rats (Needham and Challis, 1991). Despite the treatment-related pathologies in these organs, there were no measurable treatment-related effects on growth or developmental rates.

Rates of metamorphic development through the larval phase can be affected by thyroid disrupting chemicals. In the current study, thyroid-related effects from prochloraz exposure included thyroid follicular cell hypertrophy and increased circulating T4 levels at NF stage 66 (completion of metamorphosis). These effects occurred without concurrent differences in metamorphic rates or circulating T3 across treatments. One explanation for the concentration-dependent increase in circulating T4 levels is that following NF stage 62 (metamorphic climax and peak circulating TH levels), there is a rapid decrease in circulating TH levels (Sternberg et al., 2011) that is mediated by enhanced metabolism and excretion of TH. Given the compromised state of the liver and kidneys due to prochloraz exposure, T4 clearance could have also been compromised resulting in retention of higher blood concentrations at NF stage 66. This impaired clearance could also explain the slight elevation in plasma vitellogenin protein levels seen in juveniles of primarily the 60 and 180 µg/L treatments (159 and 478 nM, respectively). The difference between T4 and T3 profiles seen here is likely due to T3 having a much shorter half-life than T4 (hours vs. days) and being tightly regulated at a fraction of the circulating T4 concentrations. Another possibility for the increased T4 levels could be that prochloraz caused TH resistance by impacting one or more processes necessary to affirm negative feedback in the hypothalamus-pituitary (e.g.,

TH membrane transport, deiodination and/or transcription). Taken together, these data suggest that the compensatory capacity of the thyroid axis in *Xenopus laevis* is sufficiently robust to have supported apparently normal metamorphic development during prochloraz exposure.

Assessments of reproductive development took place at two months post-metamorphosis when the gonads are fully differentiated and actively maturing. At this stage of development, Müllerian duct regression in males is actively occurring and is mediated by testosterone and anti-Müllerian hormone (Kelley, 1996; Piprek et al., 2014). In females, Müllerian ducts are actively maturing to become oviducts, which is estrogen-mediated (Kelley, 1996). Prochloraz exposure caused concentration-dependent effects in all of these tissues with the most remarkable effects occurring in male reproductive organs. Histopathological evaluations of testes revealed degeneration of the germinal epithelium from the high test concentrations of prochloraz. Although the severities of the degenerative effects were quite variable within the high treatments, one individual exhibited such severe thinning of the germinal epithelium that the tissue presented with many large voids throughout the sections. Germinal epithelial thinning was generally accompanied by several other sub-acute testicular lesions (e.g., interstitial cell hyperplasia, interstitial fibrosis, asynchronous spermatocyst development) in the higher treatments. This was in contrast to the lower treatments that, more often, presented with one or very few different lesions of minimal to mild severity. These chronic and sub-acute antiandrogenic effects of prochloraz are consistent with what has been reported in fathead minnows (Ankley et al., 2005) and rats (Blystone et al., 2007a, 2007b; Noriega et al., 2005). Interestingly, the appearance of the testis sections suggest that normal morphogenesis was not initially inhibited by prochloraz, as the macrostructure was generally completely formed. More specifically, overall sizes of testes in the high prochloraz treatment were similar to control. Also, testis cords and spermatocysts were formed and appeared to be progressing through spermatogenesis, albeit atypically. The degenerative process caused by prochloraz exposure appears to occur either concurrently with unabated testicular growth and structural formation, or is manifested following the completion of testis morphogenesis. The most plausible cause for the appearance of these testis pathologies is antagonism of the androgen-mediated phase of spermatogenesis that is initiated in the later stages of testis morphogenesis.

Our previous work indicates that the transcriptional activity in the testis around completion of metamorphosis is primarily associated with morphogenesis including organization of the seminiferous tubules, differentiation of interstitial cells and production of connective tissue (Haselman et al., 2015). Once testis cords are formed, pre-spermatogenesis takes place during late larval development and the early juvenile period. In two species of *Rana*, pre-spermatogenesis has been shown to occur at different points in the mating season independent of hormonal activity in interstitial tissue (reviewed by Ogielska, 2009). Androgens become essential for the second meiotic division of germ cells, which convert secondary spermatocytes to spermatids. Control testes in this study exhibited spermatocysts often containing spermatids and in some cases containing spermatozoa, indicating androgen-mediated “active” spermatogenesis is taking place at two months post-metamorphosis. Therefore, critical levels of androgens are produced by steroidogenesis enzymes in the Leydig cells beginning at some point between the completion of metamorphosis and two

months post-metamorphosis, presumably mediated by gonadotropins released by the pituitary. This late metamorphic climax period has been described as the onset of the functional hypothalamus-pituitary-gonadal (HPG) axis, before which larval gonad development occurs independent of gonadotropins from the pituitary (reviewed by Ogielska, 2009). We postulate that the point at which the testis becomes sensitive to the antiandrogenic action of prochloraz is when androgens become necessary to support spermatogenesis. In effect, prochloraz arrests meiosis and results in degeneration of the already formed germinal epithelium.

Cyp17a1 gene expression levels in testes also support the notion that steroidogenesis is active and prochloraz treatment may cause weak compensatory induction of the enzyme's transcript. This was not a strong response and suggests that *cyp17a1* gene expression may not be a primary mode of compensation in this particular system when androgen production and/or signaling is impaired. There appears to be reasonably high constitutive levels of the *cyp17a1* transcript already present in all treatments and control, which may act as an available reserve for compensatory activation of translation and protein production. Reports of *cyp17* gene regulation following prochloraz exposure have been varied. In a study by Ankley et al. (2009), male fathead minnows exposed to 300 µg/L (796 nM) for eight days exhibited significantly reduced circulating testosterone levels with a corresponding significant increase in testis *cyp17* mRNA. Laier et al. (2006) exposed fetal rats perinatally to 150 mg/kg/day prochloraz and reported upregulation of CYP17 protein by immunohistostaining whereas *Cyp17* gene expression was unchanged in gestational day 21 fetal testes. This provides precedent that compensatory action to increase testosterone levels in the developing testis via CYP17 could be occurring more at the translational level. Although testosterone levels in the testis are not known in this study, the weak *cyp17a1* expression increase seen in the 180 µg/L (478 nM) treatment could be an indication of a compensatory response caused by decreased testosterone levels and/or impaired androgen receptor signaling, but generally lacks usefulness as a biomarker in this particular system.

Transcript expression levels of *rflcii* in the testis showed a marked increase in the 60 and 180 µg/L treatments (159 and 478 nM, respectively) where the majority of treatment-related pathologies were observed in testis tissues. *Xenopus rflcii* is orthologous to mammalian *INSL3* Leydig cell-specific growth factor peptide hormone. INSL3 has been well-characterized in mammalian reproductive development; however, this peptide has only recently been considered in teleosts and is essentially uncharacterized in amphibians (Bathgate et al., 2013; Good-Avila et al., 2009). The relaxin family of genes within and between species shares very little sequence similarity, but the resulting peptides share structural similarity with insulin and confer similar functionality (Wilkinson et al., 2005). INSL3 during early mammalian reproductive development is highly expressed in fetal Leydig cells and is responsible for testis descent by acting on the gubernaculum (Zimmermann et al., 1999; Nef and Parada, 1999). Prevention of germ cell apoptosis by paracrine signaling has also been a suggested role of INSL3 in the adult rat testis (Anand-Ivell et al., 2006; Kawamura et al., 2004). Unlike acute testosterone regulation by luteinizing hormone (LH), INSL3 is not acutely regulated (Ivell et al., 2013). Instead, its constitutive expression is correlated with Leydig cell number, function and differentiation status, which is regulated by long-term stimulation by LH from the pituitary. Previous studies in rat dams

exposed to prochloraz for several days have shown that *InsI3* expression is unchanged in fetal testes, supporting the lack of acute regulation (Wilson et al., 2004). If the relationship between LH and *InsI3* regulation is conserved, chronic exposure to antiandrogen prochloraz in the current study may have caused prolonged LH stimulation as evidenced by *rflcii* expression levels. This would be indicative of a lack of either Leydig cell competence or mature differentiation status, perhaps due to reduced testosterone levels and/or impaired androgen signaling, either of which would reduce negative feedback on the hypothalamus-pituitary causing sustained LH release (from late metamorphic climax to two months post-metamorphosis). Although we did not measure circulating LH, sustained elevation of LH could be causing the elevated expression of *rflcii* in the prochloraz-exposed testes, whereas control *rflcii* expression levels are more representative of the mature status of Leydig cells and fully functional status of the HPG axis. With regard to *rflcii* expression in the testis of *Xenopus laevis*, it appears to be an exceptional biomarker of impaired testis development when evaluated in this chronic exposure paradigm. However, further investigations are necessary to understand the predictive utility of this biomarker in relation to apical reproductive success.

Another notable antiandrogenic effect of prochloraz exposure occurred in both male and female Müllerian ducts. Müllerian duct regression was attenuated in prochloraz-treated males and Müllerian duct maturation was accelerated in prochloraz-treated females. The effects on the Müllerian ducts in both genders with prochloraz treatment is indicative of a feminizing effect similar to the feminization of secondary sex characteristics of male rats during perinatal prochloraz exposure (Noriega et al., 2005; Vinggaard et al., 2005). In the testes of fetal male rats, testosterone has been shown to increase post-translational processing of anti-Müllerian hormone that results in cleavage of the full length protein to N- and C-terminus peptides, of which the C-terminus peptide is bioactive toward Müllerian duct regression (Kuroda et al., 1991; Lee and Donahoe, 1993). If these processes are conserved across rodents and anurans, the results from the current study suggest that testosterone-mediated post-translational bioactivation of anti-Müllerian hormone was impaired by prochloraz treatment via reduced testosterone availability and/or inhibition of androgen receptor signaling. The acceleration of oviduct development in females could be viewed as an estrogenic response resulting from prochloraz exposure. However, anti-Müllerian hormone is present at some level in both testes and ovaries during *X. laevis* development (Piprek et al., 2014), suggesting that there may be competing biochemical pathways in both sexes that eventually results in a developmental trajectory of the Müllerian duct that is characteristic of the predominant hormone action. In this case, we suggest that females exposed to an antiandrogen experience no influence of the constitutive anti-Müllerian hormone due to the lack of testosterone signaling and undergo unabated growth of the oviduct. This study, in addition to our previous studies (Haselman et al., 2016a, 2016b; Porter et al., 2011), emphasize that Müllerian duct fate is highly sensitive to xenobiotics that affect androgen and estrogen signaling.

5. Conclusion

Chronic prochloraz exposure throughout multiple life stages in model amphibian species *Xenopus laevis* caused developmental and apical effects characteristic of multiple modes of

toxicity. Liver and kidney pathologies suggested rate-limited metabolism and clearance of prochloraz which may have impacted proper thyroid hormone homeostasis during the late stages of metamorphosis. Reproductive development was also impacted by prochloraz exposure as evidenced by testicular lesions including abnormal germ cell patterning and degeneration with corresponding effects on *rflcii* and *cyp17a1* gene expression suggestive of reduced testosterone production and/or androgen signaling. The feminization of the Müllerian ducts in both juvenile males and females is consistent with previous reports of feminized reproductive development in rodents following perinatal exposure to prochloraz. Overall, the effects on reproductive development caused by prochloraz exposure are consistent with an antiandrogenic mode of action and are in agreement with a number of studies implicating prochloraz as an antiandrogenic endocrine disruptor. This study further supports the diagnostic utility of the LAGDA for chemical testing in a chronic exposure paradigm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Histological sections of control and prochloraz-exposed liver tissue at two months post-metamorphosis. Normal control liver (A). Hepatic cords are two cells thick (arrow) separated by bile canaliculus; hepatocyte cytoplasm is wispy and eosinophilic (arrowhead). Higher magnification of normal control liver (B). Note wispy eosinophilic cytoplasm (arrowhead) and condensed (basophilic) cytoplasm adjacent to bile canaliculus (arrow). 180 µg/L treated liver exhibiting moderate hepatocellular degeneration (C). Cytoplasmic inclusions are present within many hepatocytes (arrows); inclusion location is often pericanalicular (arrowheads). Panel D is higher magnification of panel C. Degenerative hepatocytes are smaller and contain pericanalicular inclusions (arrows); note prominent bile canaliculus (arrowhead). Another example of a 180 µg/L treated liver exhibiting moderate hepatocellular degeneration (E). Degenerative hepatocytes containing cytoplasmic inclusions may be swollen (arrows); hepatic cords appear jumbled. Panel F is higher magnification of panel E. Inclusions often consist of punctate granular material (arrow); note smudgy basophilic material at periphery of inclusion (arrowhead). Scale bars represent 100 µm in panels A, C and E; 25 µm for panels B, D and F.

Figure 2.

Histology sections of control and prochloraz-treated kidneys at two months post-metamorphosis. Control kidney (A) and higher magnification control kidney (B). Profiles of the thick limb of the loop of Henle (a) fill the cortex; glomeruli (b) and collecting tubules (c) are largely confined to the cortex. Small clusters of myeloid cells are present (d) and large numbers of red blood cells (e) fill the interstitium. Intracytoplasmic amphophilic inclusions in the 180 µg/L study group (C). Renal tubular cells contain large, irregular, intracytoplasmic, amphophilic inclusions (arrows). 60 µg/L study group (D). The Bowman's capsule of several glomeruli contains small accumulations of pale, eosinophilic material (arrowheads) diagnosed as grade 1 glomerular protein. Scale bars represent 200, 100, 50 and 50 µm for panels A, B, C and D, respectively.

Figure 3.

Serum T4 (A) and T3 (B) levels from control and prochloraz-exposed NF stage 66 *X. laevis* (n = 8, 5, 5, 5, 5 for the 0.0, 6.7, 20, 60, 180 µg/L treatments, respectively). Horizontal lines represent medians, boxes represent the 25th-75th percentile and whiskers represent minimum and maximum values. Asterisks (*) indicate treatments that were statistically different than control (p < 0.05; Jonckheere-Terpstra).

Figure 4.

Histology sections of control and prochloraz-exposed testis tissues at two months post-metamorphosis. Control group male provided for reference (A). The dashed boxes in panels A, C, and E are represented in higher magnification in corresponding panels B, D and F. 180 µg/L treatment group male showing mild gonadal degeneration thinning/loss (C and D). The germinal epithelium lining of numerous spermatid tubules is thin to absent (black arrows) with occasional exposure of the underlying basement membrane (arrowhead). 180 µg/L treatment group male exhibiting severe gonadal degeneration thinning/loss (E and F). Diffusely, the germinal epithelium is missing or thin with frequent exposure of the underlying basement membranes (arrows). Scale bars represent 200 µm in panels A, C and E; 50 µm in panels B, D and F.

Figure 5. Histological section of asynchronous cyst development in testis tissue of prochloraz-exposed male in the 180μg/L study group. Asynchronous development within cysts, Grade 3. The normal architecture is distorted by large, irregular spermatocysts (outlined in black dashed lines) which contain cells of asynchronous maturity. Spermatocytes are indicated by arrows, and spermatids are shown by arrowheads. Scale bar represents 50 μm.

Figure 6.

Gene expression levels in testis tissue of two months post-metamorphosis control and prochloraz-exposed *X. laevis* (levels from control ovaries are included for comparison; n = 2). Means $\pm$ SD (error bars) are expressed as copies mRNA per ng total RNA (n = 8, 5, 4, 5, 5 for the 0.0, 6.7, 20, 60, 180 µg/L treatments, respectively). Asterisks (*) indicate

treatments statistically different than control ($p < 0.05$, Jonckheere-Terpstra). Ovary values were not included in statistical analyses.

Table 1.

Accession numbers, primer and probe sequences used for qPCR of selected gene transcripts.

Gene symbol	<i>X. tropicalis</i> accession	<i>X. laevis</i> accession	Primers (5' -> 3')	Probe (5' -> 3')
cyp17a1	NM_001127045.1	NM_001097071.2	For: CCACGTGCTGTGACAACAGATA Rev: GCACTACCTTGCGGTGAAACT	ACATTGCTTTCGCCAACTACAGCCCA
rflcii	NM_001126835.1	CV081081.1	For: TGACACCATGGAACAGTTGCA Rev: CCGCACTCCTCCTCATCCT	TAGGTCAAGAACCACGGAGCACCAGC

Table 2.

Mean $\pm$ standard deviation for growth, liver-somatic index and plasma vitellogenin concentrations in control and prochloraz-exposed *Xenopus laevis* at two months post-metamorphosis. Asterisks (*) represent measurements that are statistically different than control ($p < 0.05$; Jonckheere-Terpstra).

Male	Control (n = 8)	6.7 $\mu\text{g/L}$ (n = 5)	20 $\mu\text{g/L}$ (n = 5)	60 $\mu\text{g/L}$ (n = 5)	180 $\mu\text{g/L}$ (n = 5)
Weight (g)	13.2 $\pm$ 1.0	12.5 $\pm$ 1.4	11.8 $\pm$ 0.6	12.5 $\pm$ 0.7	12.5 $\pm$ 0.8
Snout-to-vent length (mm)	47.4 $\pm$ 1.5	46.4 $\pm$ 1.9	45.1 $\pm$ 0.9	46.7 $\pm$ 1.0	46.4 $\pm$ 1.6
Liver-somatic index (%)	5.9 $\pm$ 0.7	5.6 $\pm$ 0.5	5.6 $\pm$ 0.5	5.1 $\pm$ 0.4*	5.0 $\pm$ 0.2*
Vitellogenin (ng ml ⁻¹)	0.03 $\pm$ 0.01	0.05 $\pm$ 0.03	0.05 $\pm$ 0.02	0.11 $\pm$ 0.06*	0.10 $\pm$ 0.05*
Female	(n = 8)	(n = 4)	(n = 5)	(n = 5)	(n = 5)
Weight (g)	13.0 $\pm$ 0.6	13.6 $\pm$ 1.3	14.1 $\pm$ 0.6	13.9 $\pm$ 0.4	13.3 $\pm$ 1.4
Snout-to-vent length (mm)	47.3 $\pm$ 0.7	47.9 $\pm$ 1.8	47.9 $\pm$ 0.8	47.9 $\pm$ 0.9	48.1 $\pm$ 1.4
Liver-somatic index (%)	6.1 $\pm$ 0.3	5.6 $\pm$ 1.0	6.0 $\pm$ 0.4	5.2 $\pm$ 0.2*	5.2 $\pm$ 0.2*
Vitellogenin (ng ml ⁻¹)	0.03 $\pm$ 0.02	0.04 $\pm$ 0.01	0.08 $\pm$ 0.06*	0.13 $\pm$ 0.03*	0.25 $\pm$ 0.17*

Table 3.

Prevalence (%) and severity of juvenile male gonad histopathological observations showing significant treatment-related effects. Values in parentheses are significantly different than control based on RSCABS analysis ($p < 0.05$). NR = Not Remarkable.

Nominal PZ concentration ($\mu\text{g l}^{-1}$)	0	6.7	20	60	180
Total n	36	25	24	24	25
Replicate tanks	8	5	5	5	5
Testicular interstitial cell hyperplasia/hypertrophy					
NR	83	68	96	50	16
Minimal	17	32	4	(46)	(52)
Mild	0	0	0	4	(32)
Moderate	0	0	0	0	0
Increased spermatogonia					
NR	94	96	92	88	76
Minimal	6	4	4	8	(12)
Mild	0	0	0	0	(8)
Moderate	0	0	4	4	4
Germinal epithelium, thinning					
NR	92	84	92	54	12
Minimal	0	8	4	(38)	(40)
Mild	6	4	4	8	(16)
Moderate	3	4	0	0	(28)
Severe	0	0	0	0	4
Gonadal degeneration (necrosis / apoptosis)					
NR	92	64	96	83	32
Minimal	8	32	4	17	(64)
Mild	0	4	0	0	4
Moderate	0	0	0	0	0
Mononuclear cellular infiltrate					
NR	92	92	79	88	56
Minimal	8	8	21	12	40
Mild	0	0	0	0	4
Moderate	0	0	0	0	0
Asynchronous development within cysts					
NR	100	80	75	67	40
Minimal	0	(16)	(17)	(25)	(44)
Mild	0	(4)	(8)	(8)	(12)
Moderate	0	0	0	0	4
Interstitial fibrosis					
NR	75	52	71	75	12
Minimal	19	40	29	21	(36)
Mild	6	8	0	0	(52)

Moderate	0	0	0	4	0
Intratubular eosoniphilic droplets					
NR	94	96	100	96	76
Minimal	3	4	0	4	(24)
Mild	3	0	0	0	0
Moderate	0	0	0	0	0

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Table 4.

Müllerian duct staging at two months post-metamorphosis following exposure to prochloraz. Values are expressed as percentages of total *n*. Values in parentheses are significantly different than control based on RSCABS analysis ($p < 0.05$).

Nominal PZ concentration ($\mu\text{g l}^{-1}$)	0	6.7	20	60	180
Replicate tanks	8	5	5	5	5
Male Müllerian duct stage					
Total <i>n</i>	37	25	24	26	25
Fibrous tag	84	88	50	31	4
Rudimentary	16	8	(42)	(58)	(92)
Partial oviduct	0	4	8	12	4
Female Müllerian duct stage					
Total <i>n</i>	43	26	22	24	25
Fibrous tag	0	0	0	0	0
Rudimentary	44	0	9	4	12
Partial oviduct	56	100	91	96	88