

THE IMPACTS OF 2,4-D HERBICIDE DMA® 4 IVM ON REPRODUCTIVE HEALTH AND
GENE EXPRESSION ALONG THE HYPOTHALAMIC-PITUITARY-GONAD-LIVER
[HPGL] AXIS IN THE FATHEAD MINNOW (PIMEPHALES PROMELAS)

By

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**The impacts of 2,4-D herbicide DMA® 4 IVM on reproductive health and gene expression
along the hypothalamic-pituitary-gonad-liver [HPGL] axis in the fathead minnow
(*Pimephales promelas*)**

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Abstract

Aquatic herbicides are commonly used to control a wide variety of non-native and nuisance plants. One common active ingredient used in commercial herbicide formulations globally is 2,4-dichlorophenoxyacetic acid (2,4-D). Due to the stability of 2,4-D in aquatic environments, many non-target aquatic species experience prolonged exposure throughout critical developmental life stages that could have adverse health consequences. To our knowledge, no studies have investigated cellular, biochemical, and transcriptional effects of 2,4-D exposure on fathead minnows (*Pimephales promelas*) throughout reproductive development (the juvenile life stage). Additionally, no mechanism of action has been described for 2,4-D as an endocrine disrupting chemical in fishes. By investigating changes in biochemistry, histology, and gene expression, we can create a snapshot of the reproductive health of fish. Therefore, we conducted a series of experiments to expand upon previous studies by investigating the chronic effects of 2,4-D exposure on the developing reproductive system in juvenile fathead minnows. Herein, we observed significantly decreased plasma testosterone concentration in male fish exposed to environmentally relevant concentrations (0.50, 2.00, and 4.00 mg/L) of 2,4-D, but no histopathological impacts in the male gonads at the same concentrations. We observed a significant increase in ovarian severity grading and oocyte atresia in female fish exposed to 4.00 mg/L. Of differential expression analysis in fish exposed to 2.00 mg/L 2,4-D in DMA4, we identified significantly decreased expression of the steroid hormone receptors ESR1, ESR2b, and AR in male liver tissue while AR expression was significantly decreased in the female liver. ESR2a expression was significantly increased in male liver suggesting the presence of compensatory receptor expression. We observed significant increases in expression of male steroidogenic genes 3 β HSD in the testis and 11 β HSD2 in the liver of male fish while 17 β HSD4

was significantly decreased in the female liver. Of genes regulating steroid metabolism, testis *SULT1st2* and liver *CBR11* were significantly increased in males while gonadal *SULT2a1* was significantly decreased in both males and females. Altogether, these data suggest that 2,4-D could act as an endocrine disrupting chemical that alters expression of primary genes regulating hormone receptors, steroidogenesis, and steroid metabolism along the hypothalamic-pituitary-gonad-liver (HPGL) axis. The use of 2,4-D herbicides for weed control in aquatic ecosystems could present risks to the reproductive health of non-target aquatic species.

Keywords

Endocrine disruption; Testosterone; Oocyte atresia; Herbicide; 2,4-D; RNA Seq; Differential Expression

1. Introduction

Approximately 2 billion kilograms of pesticides are used each year around the world and nearly half of that global estimate (~1 billion kg/year) are herbicides (Sharma et al., 2019). Many active ingredients in commercial herbicides used on terrestrial crops are water soluble and can enter aquatic ecosystems through terrestrial run-off, leaching, and spray drift (Carter, 2000; Donald et al., 2007; Rice et al., 2001). Commercial herbicides can also be directly applied into aquatic ecosystems to effectively control, combat, and potentially eradicate nuisance and non-native plant species (de Castro Marcato et al., 2017; Nault et al., 2018; Poovey et al., 2007). One common active ingredient in commercial herbicide formulations that is used in both agriculture and aquatic environments is 2,4-Dichlorophenoxyacetic acid (2,4-D) (de Castro Marcato et al., 2017).

The active ingredient 2,4-D mimics the plant growth hormone auxin and induces uncontrollable cell growth that eventually leads to senescence and plant death (Song, 2014). The United States Environmental Protection Agency (USEPA) permits the direct application of 2,4-D in lakes up to 4 mg/L spot-treatment and up to 2 mg/L in whole-lake-treatment with a follow up treatment 21 days after initial application (U.S. EPA, 2005). Moreover, concentrations of 2,4-D measured in runoff frequently exceed water quality guidelines and thus are a source of contamination (Cessna et al., 2013; Mulder & Schmidt, 2011; Waite et al., 2002). In the Great Lakes region of the United States, lakes are commonly treated with commercial 2,4-D herbicide formulations in late spring and/or early summer to increase their efficacy on controlling non-native species and decrease microbial degradation (Nault et al., 2012). Degradation of 2,4-D can vary in aquatic environments with recorded half-lives as short as 4 days and reaching over 75 days (Nault et al., 2014, 2018). Therefore, it is critical to understand how chronic exposure to ecologically relevant

concentrations of 2,4-D impact different life stages of non-target aquatic organisms (e.g., embryo, larval, and juvenile fish).

Recent literature suggests that exposure to ecologically relevant concentrations of 2,4-D can broadly impact early developmental life stages (i.e., embryo and larvae) of freshwater fishes. Specifically, exposure can decrease embryonic and larval survival (Dehnert et al., 2021; DeQuattro & Karasov, 2016; Gaaied et al., 2019; Li et al., 2017), impair locomotion (Gaaied et al., 2019), impair visually guided behaviors such as prey capture (Dehnert et al., 2019), impair ontogenetic development (Dehnert et al., 2018; DeQuattro & Karasov, 2016), alter innate immune function and white blood cell profiles (Anton et al., 2023), and disrupt neural signaling activity in the brain (Anton et al., 2021; da Fonseca et al., 2008; Dehnert et al., 2019). While 2,4-D has been shown to acutely affect early developmental life stages of fish, the impacts of prolonged exposure to 2,4-D on reproductive development and gene expression as an endocrine disrupting chemical (EDC) in fish remains poorly understood.

Many endocrine disrupting chemicals including herbicides with deleterious impacts on reproductive health of fish have been identified (Guerrero-Estévez & López-López, 2016; Solomon et al., 2013). Impairments frequently include delayed gonadal maturation and development, altered concentrations of sex steroids, altered secondary sex characteristics, male feminization, and the presence of testis-ova (Arukwe, 2001; Bautista et al., 2018; Guerrero-Estévez & López-López, 2016; Jin et al., 2018; Solomon et al., 2013; Wolf et al., 2004). In one chronic exposure study, fathead minnows exposed to environmentally relevant concentrations of 17 α -ethinylestradiol (EE2) resulted in feminization and vitellogenin production in males and altered oogenesis in females that led to the elimination of recruitment and near extinction of the species in the experimental lake (Kidd et al., 2007). Endocrine disruption resulting in

physiological changes can occur through a variety of mechanisms. In fish, steroidogenesis is controlled by the hypothalamus-pituitary-gonadal-liver (HPGL) axis and involves a series of feedback loops to maintain homeostasis (Hogan, 2001). Hypothalamic gonadotropin-releasing hormone (GnRH) stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. In turn, these hormones regulate steroidogenesis in the gonads by interacting with cell specific receptors (CYP, StAR, HSD, etc). Levels of circulating steroids then feedback to regulate GnRH production in the hypothalamus. In fish, endocrine hormones can also bind to steroid receptors all over the body to modulate growth and development. Conjugation and excretion of steroid hormones occurs predominantly in the liver and gonad. Thus, at least three primary mechanisms of disruption are possible. First through direct binding and activation or blocking of steroid receptors, classically via the estrogen receptor (ESR) or androgen receptor (AR) and modifying transcription of endocrine-related genes at the nucleus (Arukwe, 2001; Jin et al., 2018; Kidd et al., 2007). A second receptor independent mechanism is through interference with steroid metabolism and excretion (Bautista et al., 2018; Crago & Klaper, 2012). A third mechanism acts via disruption along the pathway of steroidogenesis leading to increases or decreases of various enzymes or hormone products (Hogan et al., 2010).

Though recent research has shown that acute exposure to commercial 2,4-D herbicides can alter neurotransmission, have cardio-toxic effects, and reduce larval survival (Anton et al., 2021; Dehnert et al., 2021; DeQuattro & Karasov, 2016; Gaaied et al., 2019; Li et al., 2017), to our knowledge, no literature has investigated the chronic impacts of 2,4-D exposure throughout the juvenile life stage where endocrine disruption may alter sexual development and growth.

Therefore, we investigated whether chronic 120-day exposure of fathead minnow (*Pimephales*

promelas) to ecologically relevant concentrations of the 2,4-D herbicide formulation DMA®4IVM (DMA4) would impair reproductive health or alter gene expression. Specifically, we evaluated 1) circulating plasma testosterone concentration of males; 2) the presence and severity of histopathology in ovarian and testicular tissue; 3) differential expression of relevant genes isolated from brain, liver, and gonadal tissues involved in sexual development and reproductive health. By assessing levels of circulating hormone, gonad histology, and differential expression of endocrine functioning genes simultaneously we can better discern the impacts of 2,4-D exposure on fathead minnows. Understanding possible mechanisms linking the observed cellular and biochemical changes can shed light on the risks associated with herbicide usage on non-target organisms.

2. Material and methods

2.1. Chemicals and chemical analysis

Commercial 2,4-D amine liquid herbicide DMA®4IVM (DMA4) (46.3% 2,4-D) was purchased from Shore Line Aquatic Solutions (Longmount, CO, USA). Concentrated stock solutions were prepared such that their delivery to the dilution cells would result in target concentrations of 0.00 mg/L (control), 0.50 mg/L, 2.00 mg/L and 4.00 mg/L of 2,4-D at the tank level in our system. The stock solutions for the exposure system were prepared in Pyrex glass by diluting parent herbicide with distilled water. The resulting mixture was aliquoted to 18-L sealed light protected vessels. Stock solutions were prepared every 6 days. A new sealed vessel for each treatment was placed into the exposure system every 6 days. Water samples for 2,4-D concentration analysis were taken from two randomly selected tanks every 6 days to confirm exposure concentrations. Samples were refrigerated and stored at 4°C. Sample concentrations were analytically quantified by high-performance liquid chromatography (HPLC) using a diode-array detector (White et al.,

2022). Measured concentrations of 2,4-D in DMA4 for each nominal concentration in each experiment are recorded in Table 1. Herbicide concentrations are reported here as 2,4-D acid equivalent (a.e.). Target concentrations (mg/L) will be referred to from hereon for simplicity.

2.2. *Fish and husbandry*

Fathead minnows were chosen because they are endemic to North America, abundant in aquatic systems of the Great Lakes region where these formulations are applied, and are accepted as an excellent model fish for studies in toxicology, reproductive endocrinology, and transcriptomics (Gerald T. Ankley & Villeneuve, 2006). Approximately 9-month-old adult fathead minnows maintained in 230-liter tanks (~100 minnows/tank) as large sex-specific groups, in a flow-through system at 25 ± 1 °C under a 16:8-h light:dark photoperiod were bred at the University of Wisconsin–Madison Water Science and Engineering Laboratory. To spawn, adults were separated into 15L tanks each with a 1:3 male:female ratio. Each tank contained two 110×90×70-mm spawning tiles made by cutting a polyvinyl chloride pipe in half lengthwise. Tiles containing adhered eggs were collected the day of deposition and placed into separate tanks and aerated until hatch (5d). Upon hatch, larvae were fed live freshly hatched brine shrimp (Brine Shrimp Direct) ad libitum twice daily until 7 days post hatch (dph). Temperature was measured daily (25 ± 1 °C); dissolved oxygen (8 ± 0.5 mg/L), pH (7.1-7.2), hardness (<250mg/L), and ammonia (non-detectable) were measured weekly. All exposures and laboratory practices using fathead minnows were reviewed and approved by The University of Wisconsin–Madison under RARC protocol A005702.

2.3. *Exposure system*

The present study utilized a flow-through exposure system. Two 4-channel, peristaltic pumps (model 07523-90, Cole Parmer) delivered the control (system water) and three 2,4-D solutions from sealed vessels through tubing (size L/S 13 Masterflex1 Norprene1, Cole Parmer) to individual glass dilution chambers at a rate of 2 mL/min. Heated (25 ± 1 °C), carbon-filtered City of Madison (WI, USA) water was added to the dilution chambers at a flow rate of ~900 mL/min, which diluted the 2,4-D solutions to the target 2,4-D concentrations of 0.00, 0.50, 2.00 and 4.00mg/L of 2,4-D. The water then flowed from each dilution chamber to 6 replicate exposure tanks (15L) per treatment (n = 24 tanks). The flow rate into each tank was ~150 mL/min (~0.6 tank turnover/h). Each dilution chamber was constantly aerated.

2.4. Chronic exposure assay

For experiment 1, unexposed larval fish (7 dph) were randomly allocated to one of four treatment groups 0.00, 0.50, 2.00, and 4.00 mg/L of 2,4-D for 120 days (n = 15 juveniles per tank, 6 tanks per treatment). For experiment 2, unexposed larval fish (7 dph) were randomly allocated to one of two treatment groups 0.00 and 2.00 mg/L of 2,4-D for 120 days (n = 10 juveniles per tank, 12 tanks per treatment). For both experiments 1 and 2, juveniles were fed a mixture of frozen artemia and Bio Olympic dry feed (Bio-Oregon) twice daily and survival per tank was measured weekly by visual counting.

2.5. Endpoints

Following the 120-day exposure period, final survivorship was recorded, and animals were euthanized with buffered concentrations of MS-222. Following euthanasia, total length (± 0.01 cm) and wet mass (± 0.001 g) of animals was determined. Length measurements were made from the tip of the longest jaw or end of the snout to the longest caudal lobe (Kahn et al., 2004). After

blotting animals with Kim-wipes to remove excess fluid, wet mass was determined using an Ohaus analytical balance. Fish were also evaluated for presence of deformities (i.e., folded operculum & spinal conformation) and changes in secondary sex characteristics (i.e., male tubercle count) (Jensen et al., 2001). Fish were dissected and gonad weight and gonadosomatic index ($GSI = \text{gonad mass/body mass}$) were determined (DeQuattro & Karasov, 2016).

At the end of experiment 1, blood from male fish was immediately collected from the caudal vein into a capillary tube for testosterone analysis. Blood was centrifuged at 10,000 rpm for 5 minutes to collect plasma which was then transferred to polypropylene tubes (Eppendorf Safe-Lock tubes) and stored at -20°C . Plasma samples were later thawed and levels of testosterone were measured via 96 well testosterone enzyme immunoassay (EIA) kit Mybiosource (Al-khalaifah et al., 2020). Samples were assayed in duplicate. Absorbance was set to 450 nm and measured with a powerwave plate reader (Bio-Tek Instruments). The testosterone concentrations were calculated as ng/ml blood (Islam et al., 2017). Male and female gonads were dissected over ice and fixed in 10% neutral buffered formalin. Formalin fixed tissues were later prepped, embedded in paraffin, mounted onto slides containing multiple cross sections, and stained with hematoxylin and eosin by the University of Wisconsin – Madison, School of Veterinary Medicine Histology Laboratory. Cells and cell structures of sections were identified and graded subjectively via light microscopy. Each section was evaluated for abnormalities and any potential treatment-related changes. Testis was evaluated for changes in the proportion of spermatogonia, foreign cell types (i.e., testis-ova), testicular degeneration, or interstitial cell hyperplasia/hypertrophy. Ovaries were evaluated for incidence of follicular atresia, parafollicular cell hyperplasia, and changes in gonadal staging and maturation. Macroscopic observations were also recorded (Wolf et al., 2004). The severity of any inflammatory, degenerative, and/or

proliferative change was graded as not remarkable, minimal, mild, moderate, or severe per Wolf (2008).

At the end of experiment 2, brain (including pituitary), liver, and gonadal tissues of both males and females were dissected over ice, placed into microtubes and snap frozen in liquid nitrogen. The dissection area and tools were treated with ThermoFisher RNase AWAY and ethanol between each collection. Tissue samples were stored at -80°C for subsequent analysis. To extract and purify RNA, frozen tissues were thawed, briefly pulse homogenized using a ThermoFisher TH tissue homogenizer, and processed following the method ThermoFisher Purelink RNA minikit (Fu et al., 2021). mRNA samples were tested for initial quality and quantity via Nanodrop spectrophotometer (Thermo Fischer Scientific, Wilmington, DE), gel electrophoresis, and BioAnalyzer (Agilent, Santa Clara, CA). Extracted RNA for RNA sequencing was stored at -80°C until use. Library preparation and sequencing were performed at the University of Wisconsin – Madison Gene Expression Center (Madison, WI). RNA quantity and quality were assessed using Agilent 4200 TapeStation (Agilent Technologies, Santa Clara, California). RNA Integrity Numbers (RINs) were all > 9 . Stranded libraries were prepared using the Illumina Stranded mRNA Prep Kit following manufacturer instructions with Poly(A) enrichment (Illumina, San Diego, CA). Libraries were sequenced using the Illumina NovaSeq 6000 platform using paired end 150 bp reads to a sequencing depth of 30 M reads per sample. To perform bioinformatics, raw fastq files were imported on a local instance of Galaxy and trimmed for primers via Trimmomatic (Bolger et al., 2014; Jalili et al., 2021). A fathead minnow genome assembly file and a transcriptome annotation file were then obtained from the NCBI assembly database (Kitts et al., 2016). This annotation file improves upon previous files and is comparable to that of the zebrafish (*Danio rerio*) GRCz11 reference genome (Martinson et al., 2022).

Trimmed reads were aligned with HISAT2 and reads were counted with featureCounts (Kim et al., 2015; Liao et al., 2014). Differential expression analysis was performed with DESeq2 (Love et al., 2014).

2.6. Data analysis

For genetic analysis, the effect of treatment on tissue expression was determined by comparing them with the control group with Generalized Linear Models (GLM) with the Gaussian family and the identity-link function in the software R (4.3.0, 2023) applying RStudio (2023.06.1+524). The GLM assumption of independence between the treatments was met, and the confidence interval was set at 95% ($p < 0.05$).

Remaining data analysis was performed with GraphPad Prism Software 10.0.2 (GraphPad Software Incorporated, La Jolla, Ca, USA). For survival, GSI, and gonad histology, prior to use of parametric statistics, the assumption of normality was tested using Brown-Forsythe test and Bartlett's test or Anderson-Darling test, Shapiro-Wilk test, and Kolmogorov-Smirnov test. The assumption of normality was tested using Spearman's test for heteroscedasticity on the residuals. When assumptions of normality and/or homoscedasticity were not met, appropriate data transformations were performed (e.g., log transformations). Parametric analyses were performed using a one-way-ANOVA followed by Tukey's multiple comparison analysis (F statistic presented). Data are presented as means \pm standard error of the mean (SEM). Significance was set at $p \leq 0.05$. Sample size of N for each experiment is detailed in the results and/or figure legend.

3 Results

3.1. Growth, survival, GSI & deformity

We did not observe significant impacts to the survival of minnows exposed to 2,4-D in DMA4 at any concentration compared to controls ($F = 2.82$, $p > 0.05$). We did not observe a significant difference in total length in minnows of either sex at any concentration of 2,4-D in DMA4 as compared to control ($F = 0.55$, $p > 0.05$; female $F = 1.49$, $p > 0.05$). We observed a significant increase in total wet weight of male minnows when exposed to 0.50 mg/L as compared to control ($F = 6.84$, $p < 0.05$). We did not observe significant impacts to the total wet weight of female minnows exposed to 2,4-D in DMA4 at any concentration compared to controls ($F = 0.72$, $p > 0.05$). We did not observe significant impacts to gonadosomatic index in minnows of either sex exposed to 2,4-D in DMA4 at any concentration compared to controls (male $F = 0.61$, $p > 0.05$; female $F = 0.57$, $p > 0.05$). We observed an increase in the presence of external deformities in minnows exposed to 2.00 mg/L as compared to control ($F = 1.68$, $p > 0.05$).

3.2. Male Plasma Testosterone

We observed a significant decrease in levels of circulating plasma testosterone in male minnows across all concentrations of 2,4-D (0.50 mg/L, $p = 0.0399$; 2.00 mg/L, $p = 0.0033$; 4.00 mg/L, $p = 0.0228$) in DMA4 exposure as compared to control ($F = 5.27$, $p = 0.0034$, Figure 1).

3.3. Gonad Histology

We observed significantly elevated levels of follicular atresia characterized by perforation of the chorion and parafollicular cell hyperplasia in the gonads of female DMA4 exposed fish at the 4.0 mg/L treatment as compared to control ($F = 2.71$, $p = 0.038$, Figure 2).

We observed no significant change in proportion of spermatogonia, foreign cell types (i.e., testis-ova), testicular degeneration, or interstitial cell hyperplasia/hypertrophy in the gonads of male 2,4-D in DMA4 exposed fish exhibited as compared to control ($F = 0.96$, $p > 0.05$, Figure 3).

3.4 Differential Expression

Exposure to 2,4-D in DMA4 altered expression in all three tissue types in both males and females. Males overall experienced the largest number of changes with the majority being present in the liver as compared to brain and gonad (Figure 4). In male brain tissue, RNA seq differential expression analysis showed 88 significantly upregulated genes and 53 significantly downregulated genes in response to 2.00 mg/L 2,4-D in DMA4. In female brain tissue, 10 genes were significantly upregulated genes and 8 were significantly downregulated. In male liver tissue, 2201 genes were significantly upregulated and 1264 were significantly downregulated. In female liver tissue, 1016 genes were significantly upregulated and 522 were significantly downregulated. In male gonad tissue, 369 genes were significantly upregulated and 180 were significantly downregulated. In female gonad tissue, 14 genes were significantly upregulated and 54 were significantly downregulated (Figure 4).

Exposure to 2,4-D in DMA4 affected expression of 10 of 36 genes that have been previously reported to play a role in the teleost HPGL axis (G. T. Ankley et al., 2002; Crago & Klaper, 2012; Martinović-Weigelt et al., 2011; Villeneuve, Larkin, et al., 2007) (Table 2). In brain tissue of both sexes, we observed no significant change in expression of both pituitary gonadotropins FSHb and LHb or GnRH R1, GnRH R2, and NPY. In male liver tissue, we observed a significant increase in expression of ESR2a, 11 β HSD, and CBR11 and a significant decrease in expression of ESR1, ESR2b, AR, and SULT2a1. In female liver tissue, we observed a significant decrease in expression of AR, 17 β HSD4, and SULT2a1. In male gonad tissue, we observed a significant increase in expression of 3 β HSD and SULT1st2. In female gonad tissue, we observed no significant changes in expression of relevant genes. We observed no change in expression of steroidogenic genes StAR, CYP11a, CYP17, CYP19a, or TSPO in any tissue of either sex. No

significant change in expression was detected among peroxisome proliferation genes PPAR α , ACOX1, EHHADH in any tissue type. No significant change in expression of PXR1 or CYP3A4 was detected in any tissue type (supplemental table 1). Exposure to 2,4-D in DMA4 had no significant impact on expression of 16 suspected marker genes of follicular atresia in teleost fishes from Gonzolaz-kother et al., 2020.

4 Discussion

4.1. Male overview

Chronic exposure to 2,4-D herbicide formulation DMA4 resulted in physiological changes in juvenile male fathead minnows. We found no significant impacts of 2,4-D in DMA4 exposure on survival, presence of deformities, or GSI of male fathead minnows at any of the ecologically relevant concentrations tested. However, we observed significant dose dependent decreases in circulating male testosterone across all ecologically relevant concentrations (0.50, 2.00, and 4.00 mg/L 2,4-D) (Figure 1). EDC exposure in male fish has been previously linked to altered testis morphology (Islam et al., 2017). Thus, we hypothesized that testis architecture could be altered alongside the plasma T however, we observed no change in testis architecture or histopathology at any concentration. Moreover, we identified changes in relative expression of genes associated with hormone receptors, steroidogenesis, and steroid metabolism in male minnows. This is the first time the effects of 2,4-D have been evaluated regarding changes to circulating hormone levels, cell structure, and differential gene expression in fathead minnows. The observed changes in gene expression uncovered in this study suggest that 2,4-D in DMA4 acts as an endocrine disrupting chemical and may interfere with reproductive development during critical periods of growth.

4.2. Male hormone receptors

We observed a significant decrease in hormone receptor expression in male liver tissue (ESR1, ESR2b, and AR). Previous literature has documented that hormone receptor function is critical for the development of gonad, testis organization, spermatogenesis, and fertility in fish (Schulz et al., 2001). In male teleosts, disruption of androgen receptor function has been observed to impact sexual differentiation, testicular development and sex steroid biosynthesis that results in diminished sperm quality (Golshan & Alavi, 2019). In mice (*Mus musculus*), androgen receptor knockouts exhibited reduced serum testosterone (Yeh et al., 2002), thus decreased receptor expression and subsequent reduction in androgen binding may play a role in the observed decline in circulating testosterone of male fathead minnows. Testis of ESR1^{-/-} zebrafish and Nile tilapia (*Oreochromis niloticus*) develops normally such that the gene is considered dispensable as its function can be rescued while ESR2b^{-/-} fish experience normal testis growth with the exception of efferent duct development in tilapia (Lu et al., 2017; Yan et al., 2019). However, testis of both AR^{-/-} zebrafish and ESR2a^{-/-} tilapia is abnormal and disorganized contributing to infertility (Crowder et al., 2018; Lu et al., 2017; Pinto et al., 2021; Tang et al., 2018; Yan et al., 2019; Zhai et al., 2022). In the present study, expression of ESR2a was significantly upregulated in the male liver, this may indicate the presence of a functional redundancy (Lu et al., 2017) and may explain the lack of histopathology or altered testicular architecture in male minnows. While fecundity was not assessed in this study, acute exposure to environmentally relevant levels of 2,4-D in DMA4 in adult male fathead minnows has not previously impacted fecundity or the ability to produce viable offspring (DeQuattro & Karasov, 2016). It is plausible that some biochemical impairments due to decreased AR expression were present yet undetected as we did not evaluate sexual differentiation or sperm quality. We also did not measure plasma concentrations of 11-

ketotestosterone which is the primary androgen involved in stimulation of spermatogonial proliferation in spermatogenesis (Borg, 1994; Miura, 1997). While we did not test circulating 11-KT levels, we hypothesize that levels of 11-KT would decrease with exposure to 2,4-D and future research should evaluate potential anti-androgen mechanisms of 2,4-D exposure.

4.3. Male steroidogenesis

Expression of 2 genes involved in steroidogenesis were significantly altered in males (Figure 5). Expression of 11 β HSD was significantly increased in the male liver. 11 β HSD facilitates the conversion of 11 β -OH testosterone to 11 ketotestosterone, and therefore it is plausible through increased activity could have contributed to the observed decrease in circulating testosterone. In addition, we observed a significant increase in 11 β HSD and CBR11 expression which may provide novel insight into a potential mechanism of action for a previously reported decreased stress response of fathead minnows exposed to 2,4-D (Dehnert et al., Unpublished). Cortisol is inactivated by its conversion to physiologically inert cortisone by 11 β HSD and CBR11, thus increased expression resulting in decreased circulating cortisol would diminish the stress response (Dang, 2014; Sadoul & Geffroy, 2019; Tenugu et al., 2021; Theodoridi et al., 2021). Finally, it is noteworthy that 3 β HSD was significantly upregulated in the male gonad in fathead minnow. 3 β HSD facilitates conversion of pregnenolone to progesterone and the conversion of dehydroepiandrosterone (DHEA) to androstenedione. Previous studies have shown EDC induced inhibition or deficiency in 3 β HSD is linked to reproductive dysfunction in fathead minnows (Villeneuve et al., 2008). However, the increased expression of 3 β HSD observed herein may be indicative of a compensatory mechanism of steroid production (Villeneuve et al., 2007). Future investigations should include multiple time points to monitor for trends in expression as temporal

transcriptional variability in the face of a chemical stressor in fathead minnow (Skolness et al., 2011).

4.4. Male steroid metabolism

Relative expression of 2 genes associated with steroidogenesis were significantly altered (Table 2). In particular, expression of estrogen specific cytosolic sulfotransferase 1st2 (SULT1st2) in the testis was significantly increased. Sulfotransferases conjugate sulfate groups directly to hormones to alter activity and promote excretion (Kauffman, 2004). As such, action of SULT1st2 could be responsible for increased steroid metabolism and the observed decrease in circulating plasma T concentrations in male fathead minnows. Previous research has shown increased transcript abundance of both SULT1st2 and SULT2A1 in the testis to be correlated with declined plasma testosterone in male fathead minnow exposed to the herbicide linuron and a plasticizer (Crago & Klaper, 2012). However, in the present study relative expression of androgen specific SULT2a1 in the liver was significantly decreased. Again, this suggests the activation of compensatory mechanisms to the endocrine impacts of 2,4-D and the complex and dynamic nature of steroid metabolism.

4.5. Female overview

Chronic exposure to 2,4-D in DMA4 herbicide formulation DMA4 resulted in physiological changes in juvenile female fathead minnows. We found no significant impacts of 2,4-D in DMA4 exposure on survival, presence of deformities, or GSI of female fathead minnows at any of the ecologically relevant concentrations tested. We observed significant alteration to ovarian cellular architecture and a significant increase in severity of oocyte atresia at ecologically relevant concentrations of 2,4-D in DMA4 exposure (4.00 mg/L) (Figures 1 & 2). Subsequent

differential expression analysis of potential marker genes of follicular atresia was performed for each female tissue type, but no genes associated with apoptosis, autophagy, or antiapoptotic function exhibited a significant difference in expression. While markers of gross morphological gonadal maturation in fathead minnow have recently been discovered, no putative markers of atresia have yet been identified (González-Kother et al., 2020; Villeneuve et al., 2010). We suspect that any change in expression of atresia modulating genes, if at all present, was masked by the transcriptional activity of the predominant proportion of vitellogenic follicles even in cases of severe atresia. It is noteworthy that differential expression analysis was carried out at the 2.00 mg/L concentration, but ovarian severity grading was not significantly increased until 4.00 mg/L 2,4-D in DMA4. As such, the exposure concentration tested may have been too low to identify significant changes in ovarian expression. For gene expression along the HPGL axis, we observed significant altered expression of three genes (AR, 17 β HSD, SULT2a1) in the liver.

4.6. Female hormone receptors

As in the male liver, expression of AR was significantly decreased in the female liver (table 5). While traditionally associated with male reproductive activity, androgen receptor antagonists have also been shown to enact changes in gene expression associated with reproductive malfunction in female fish (Filby et al., 2007; Martinović-Weigelt et al., 2011). AR antagonists have also been observed to interact with activin signaling which plays a role in oocyte maturation (Wu et al., 2000). Furthermore, the observed decrease in female androgen receptor expression may function in a similar manner and alter ovarian development and function accounting for a portion of the observed ovarian histopathology. ESR1 knockout zebrafish have been found to lose fundamental ovarian maintenance and exhibit increased oocyte atresia (Chen et al., 2018). In the present study, ESR1 expression was significantly decreased in male minnows

but we did not observe significantly decreased expression in female fathead minnows indicating another mechanism is likely responsible for the observed histopathology.

4.7. Female steroidogenesis

Expression of 17 β HSD4 was significantly decreased in the female liver. 17 β HSD4 catalyzes the conversion of androstenedione to testosterone as well as estrone to estradiol. Decreased expression and subsequent depression of estradiol that regulates ovarian development and vitellogenesis may be a cause of female reproductive impairment which could have contributed to the observed ovarian histopathology. A similar mechanism has been described where decreased 17 β -hydroxysteroid dehydrogenase 12a (17 β HSD12a) expression was linked to decreases in both testosterone and estradiol in female fathead minnows exposed to the androgen 17 β -trenbolone (Gerald T. Ankley et al., 2003; Dorts et al., 2009). Future research could evaluate the role of 17 β HSD4 in follicular maturation through loss of function knockout minnows.

4.8. Female steroid metabolism

As in the male liver, the female liver exhibited significantly decreased relative expression of androgen specific SULT2a1. As with the males, decreased SULT2a1 expression may suggest a potential compensatory mechanism by liberating estradiol activity through decreased conjugation and/or excretion. In summary, we are unable to establish a clear mechanism for the observed ovarian histopathology. Future sequencing and single cell proteomics projects should further evaluate markers and regulatory pathways of follicular atresia.

5 Conclusion

Our results demonstrate that chronic exposure to environmentally relevant 2,4-D concentrations in DMA4 has a significant impact on the reproductive health of the fathead minnow. We

establish potential mechanisms of action for the observed interference in circulating male testosterone levels and ovarian tissue development. We demonstrate the impact of exposure on the expression of principal genes in endocrine receptor binding, steroidogenesis, and steroid metabolism in liver and gonads of both male and female minnow. We show that 2,4-D does not alter gene expression in males and females equally. The action of 2,4-D as an endocrine disrupter is complex and merits further study. While the effects of 2,4-D exposure on reproduction in fathead minnows alone may not acutely threaten fish populations, when combined with the impacts on larval survival, larval growth, essential behaviors, and innate immune function in the context of a dynamic multi-stress environment, environmentally relevant 2,4-D may pose a significant threat to fish. It is critical that risk assessment agencies take into account the sub-lethal impacts of herbicide at environmentally relevant levels.

6 Future Directions

The purpose of this study was to investigate the impacts of chronic ecologically relevant exposure to 2,4-D on fathead minnows. Overall this study acts as a first step in uncovering the biochemical mechanisms in which the herbicide interacts with the teleost endocrine system. Future aquatic ecotoxicogenomic and endocrine-related studies can build from these initial findings to progress our knowledge. The differential gene expression information generated in this study will be invaluable in future studies investigating the effects of ecologically relevant 2,4-D on fathead minnows. Future works should evaluate additional circulating hormones (11-KT & E2), time series collection of differential expression data of key genes, additional ecologically relevant concentrations, and proteomics. Finally, it is important that these effects be studied in an integrative multi-stress context as the real world consequence of herbicide exposure is the product of multiple sub-lethal stressors spanning beyond endocrine disruption.

Credit author statement

Brian Anton: Conceptualization, Methodology, Investigation, Data Curation, Formal analysis, Writing - original draft, Project administration. Yushi Oguchi: Methodology, Investigation, Data curation, Writing - review & editing. Amber White: Toxicant analysis, Writing - review & editing. William H. Karasov: Supervision, Funding acquisition, Writing - review & editing, Project administration. Gavin Dehnert: Supervision, Funding acquisition, Writing - review & editing, Project administration.

Declaration of Competing Interest

The authors declare that they have no competing interests: i.e., no financial or personal relationship with other people or organizations that may have inappropriately influenced the present study.

Abbreviations

2,4-D, 2,4-dichlorophenoxyacetic acid; dph, days post hatch; EDC, endocrine disrupting chemical; GSI, gonadosomatic index; HPLC, high pressure liquid chromatography; T, testosterone; HPGL axis, hypothalamic-pituitary-gonad-liver axis; EE2, 17 α -ethinylestradiol; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; ESR1, estrogen receptor 1; ESR2b, estrogen receptor 2b; ESR2a, estrogen receptor 2a; AR, androgen receptor; 3 β HSD, 3 β -hydroxysteroid dehydrogenase; 17 β HSD4, 17 β -hydroxysteroid dehydrogenase 4; 11 β HSD2, 11 β -hydroxysteroid dehydrogenase 2; StAR, steroidogenic acute regulatory protein; TSPO, translocator protein; CYP19a, aromatase; SULT1st2, sulfotransferase 1st2; SULT2a1, sulfotransferase 2a1; CBR11, Carbonyl reductase 1-like (20 β -hydroxysteroid dehydrogenase); NPY, neuropeptide y.

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Data availability — For access to data please contact Brian J. Anton and/or Yushi Oguchi at bjanton@wisc.edu and/or oguchi@wisc.edu.

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Tables and Figures

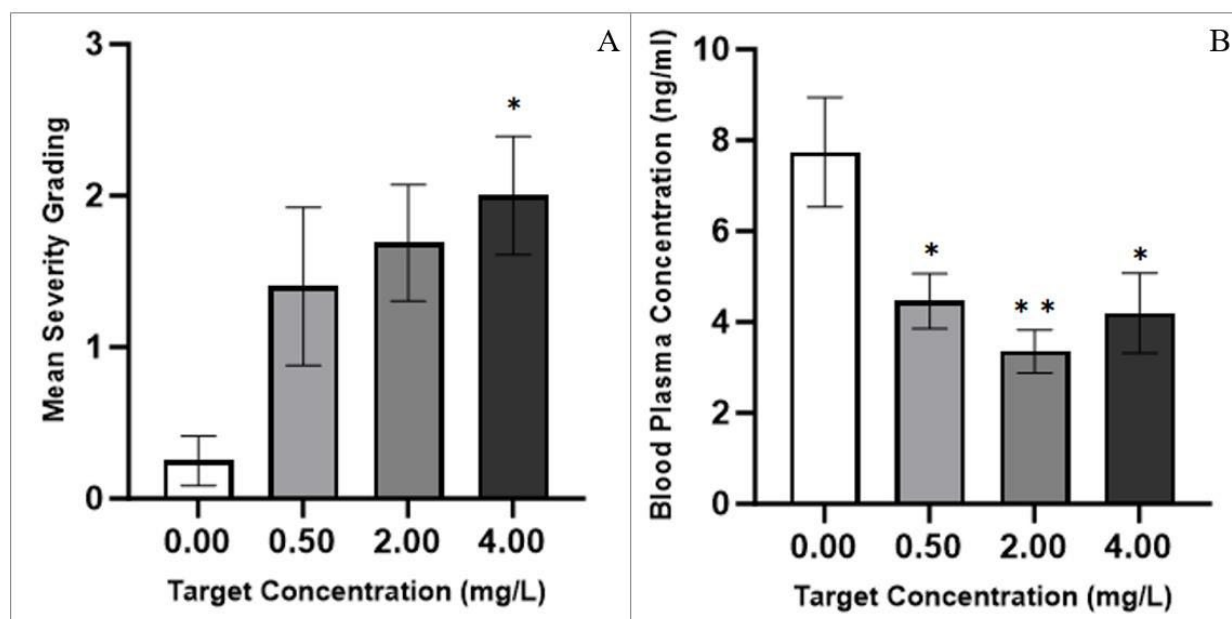


Fig. 1. Exposure to 2,4-D in DMA4 increases severity of oocyte atresia in female minnows ($n = 8-16$ fish per treatment) (a) and decreases circulating plasma testosterone in male minnows ($n = 12$ fish per treatment) (b). $p < 0.05$, $**p < 0.01$, all error bars represent \pm SEM. One-way ANOVA with Tukeys multiple comparison analysis.

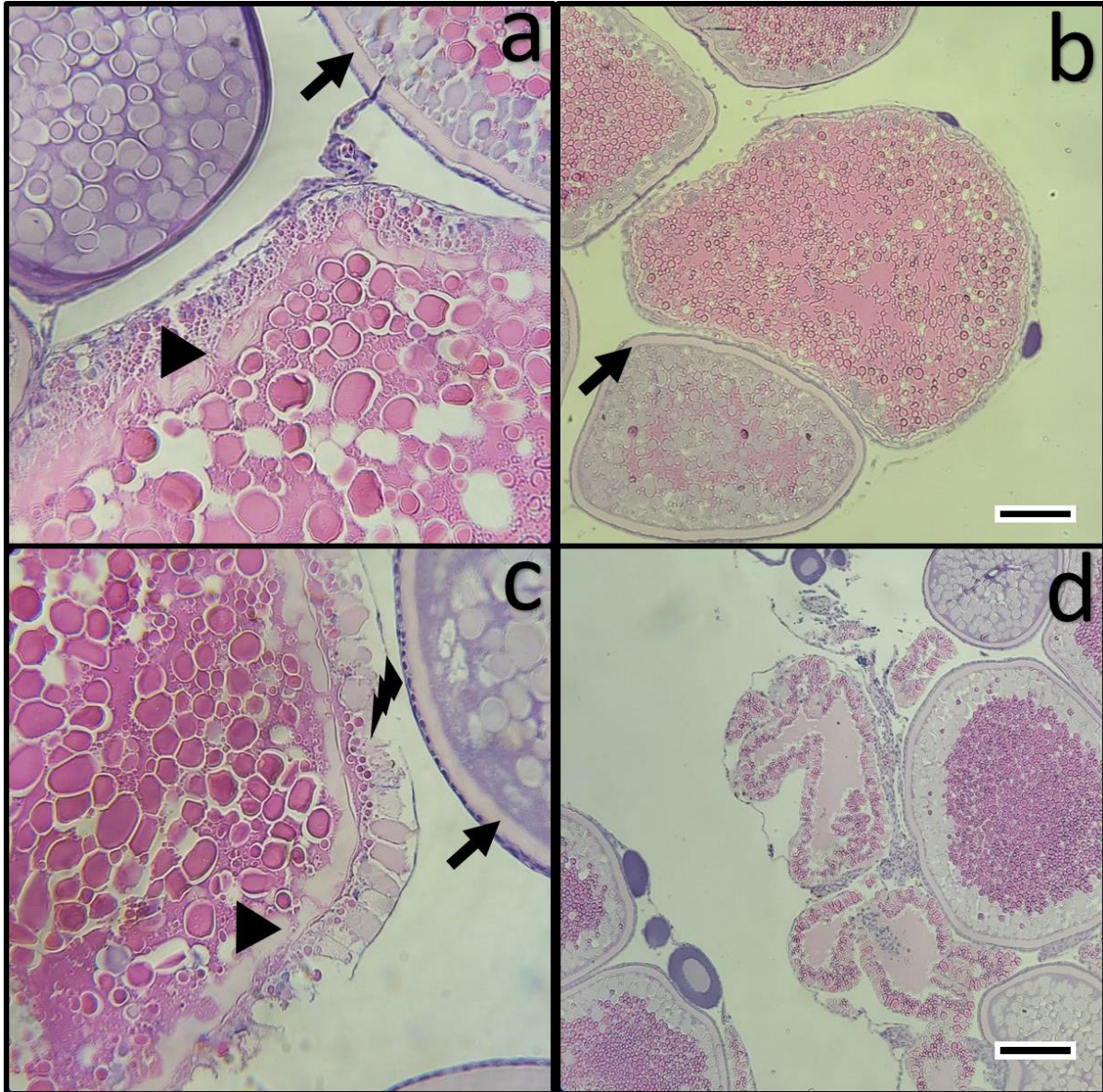


Fig. 2. Photomicrographs of the longitudinal sections of juvenile Fathead minnow ovaries (paraffin, H&E). Arrowheads (▶) denote perforated chorion of atretic oocytes. Arrows (→) denote healthy intact chorion. Bolts (⚡) denote parafollicular cell hyperplasia of atretic oocytes. a) Atretic mature spawning oocyte; 4.00 mg/L, slide severity grading 2 (original mag.: 100x) b) Atretic mature spawning oocyte; 4.00 mg/L, slide severity grading 3 (bar = 100 μm) c) Atretic late vitellogenic oocyte; 0.50 mg/L exposure, slide severity grading 4 (original mag.: 100x) d) Multiple late phase atretic oocytes containing yolk material; 0.50 mg/L exposure, slide severity grading 3. (bar = 100 μm)

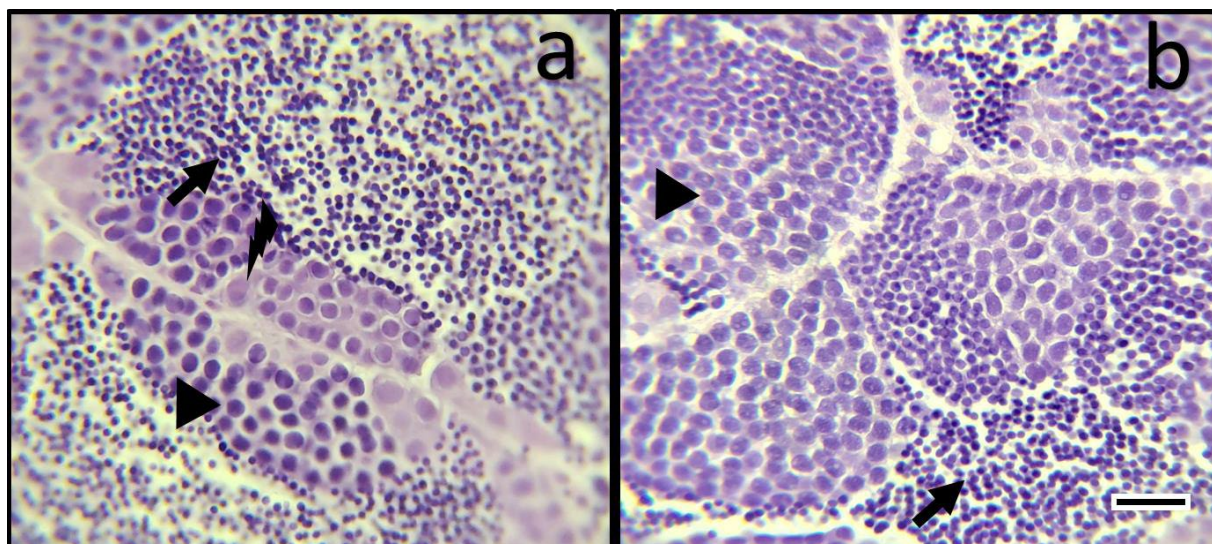


Fig. 3. Photomicrographs of the longitudinal sections of juvenile Fathead minnow testis (paraffin, H&E). Arrowheads (▶) denote primary spermatocytes. Arrows (→) denote mature spermatozoa within the lumen of the seminiferous tubule. Bolts (⚡) denote primary spermatogonium. a) 0.00 mg/L; normal testicular architecture b) 4.00 mg/L; severity grading not remarkable as compared to control (bar = 10 μ m).

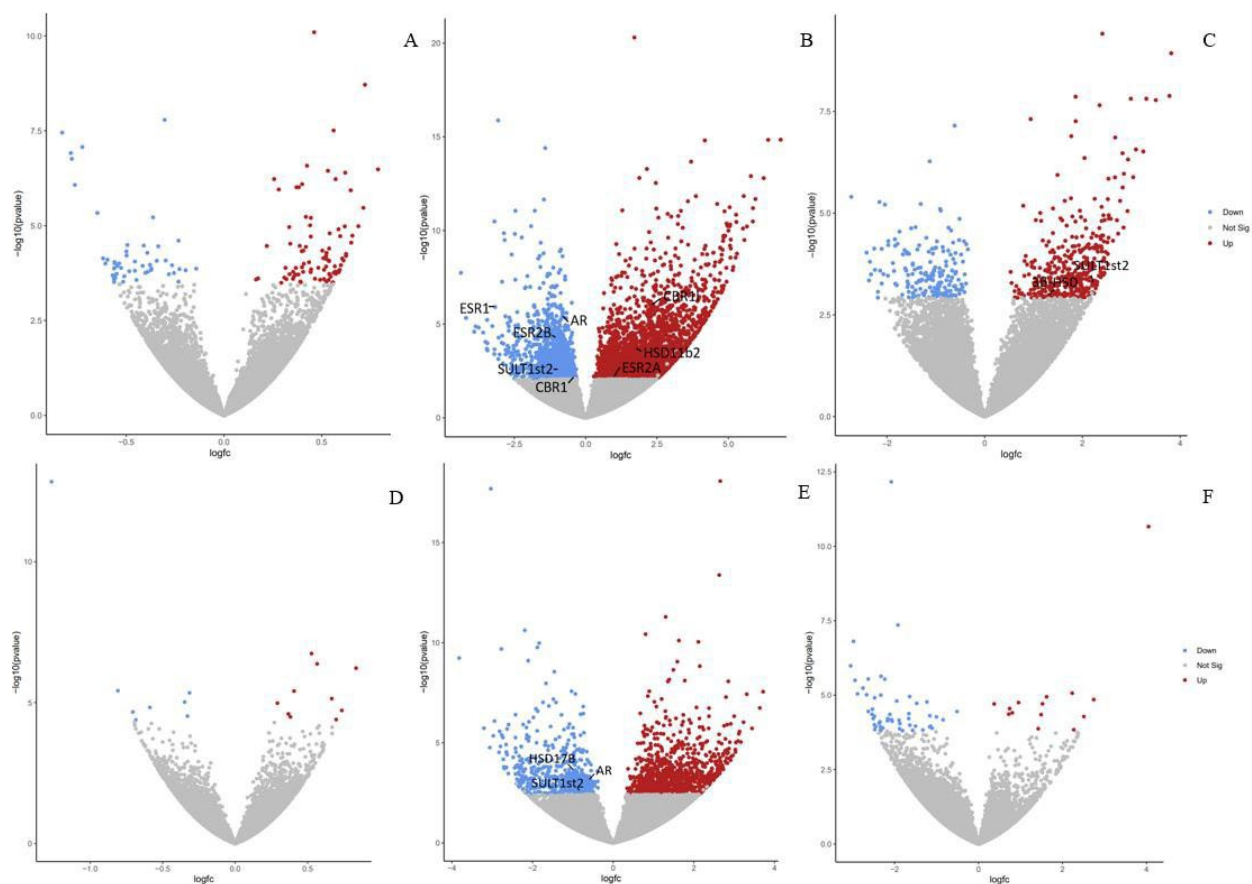


Fig. 4. Analysis of 2,4-D exposure induced gene expression changes ($n = 6$ per treatment per tissue per sex). Volcano plots depicting differential expression results in comparison to control tissues. Each dot represents a gene. Differentially expressed genes with \log_2 (fold change) ≥ 1 were painted in red, and differentially expressed genes with \log_2 (fold change) ≤ -1 were painted in blue. Genes of interest are annotated. a) male brain; b) male liver; c) male gonad; d) female brain; e) female liver; f) female gonad.

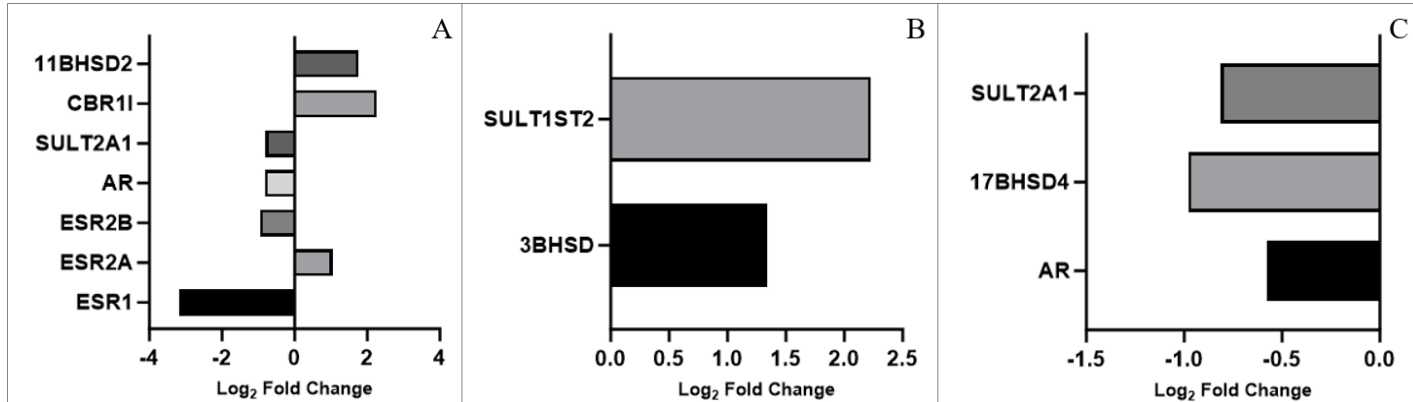


Fig. 5. Differential gene expression analysis, fold changes of significantly expressed genes involved in the HPGL axis (n = 6 per treatment per tissue per sex). Male liver (a); male gonad (b); female liver (c).

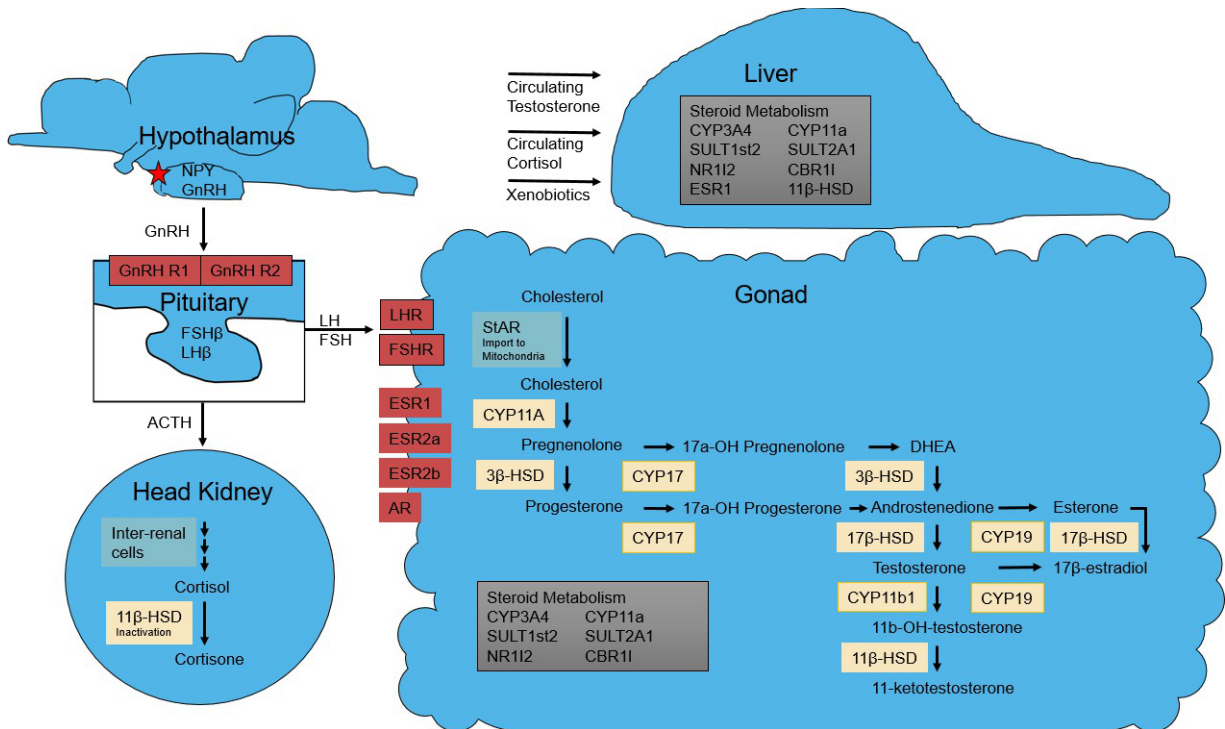


Fig. 6. Diagram of the hypothalamic-pituitary-gonad-liver and hypothalamic-pituitary-interrenal axes depicting tissues and genes involved in steroid biosynthetic and metabolic pathways.

Table 1

Measured 2,4-D concentrations for experiments 1 and 2. Measured values are mean \pm SEM (n = 20 samples for each treatment). ND = nondetectable; 2,4-D = 2,4-dichlorophenoxyacetic acid.

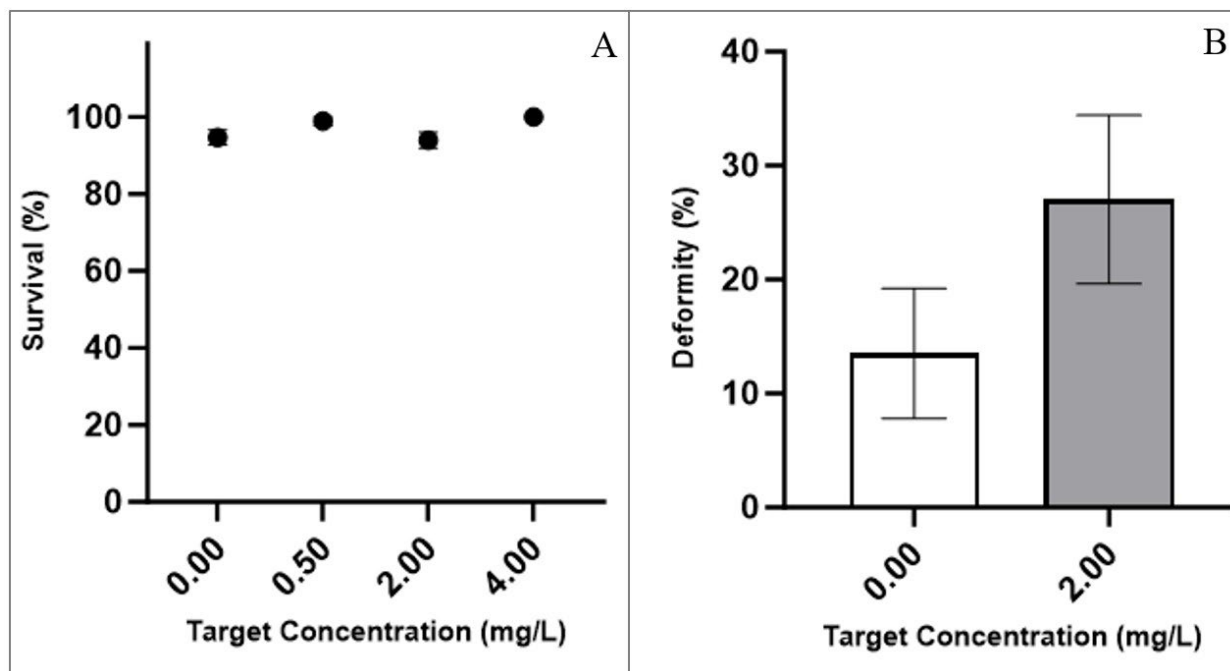
	Experiment 1				Experiment 2	
Target Concentration (mg/L)	0.00	0.50	2.00	4.00	0.00	2.00
Measured Concentration (mg/L)	ND	0.45 \pm 0.014	2.02 \pm 0.027	3.89 \pm 0.056	ND	1.97 \pm 0.031

Table 2

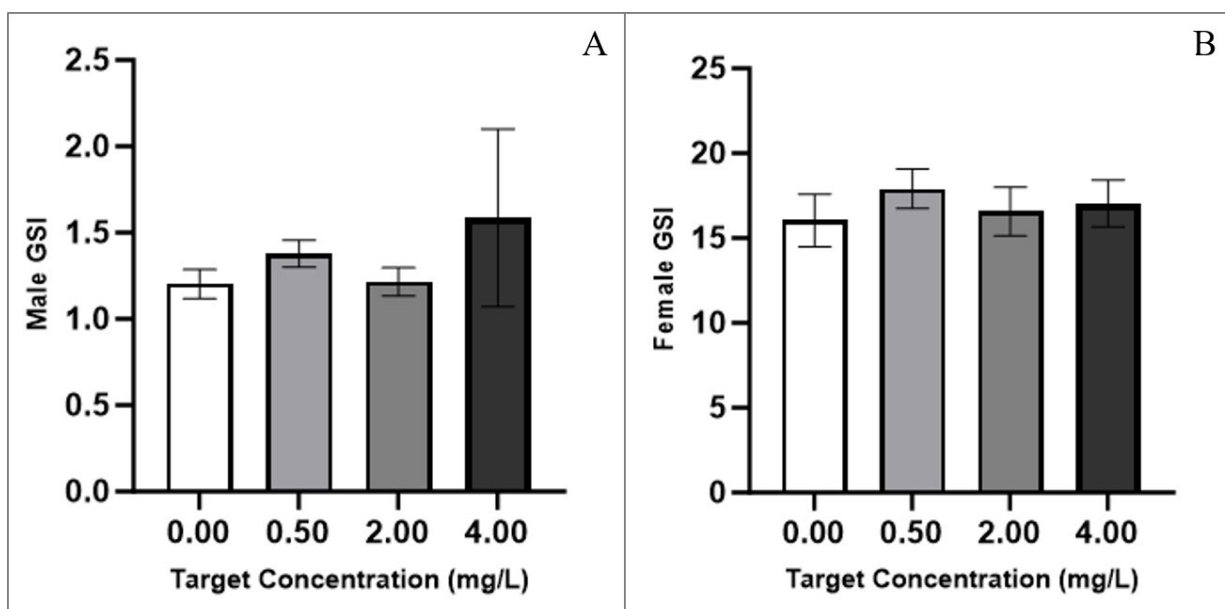
List of differentially-expressed genes ($p < 0.05$) identified that have known roles in the hypothalamic-pituitary-gonad-liver axis.

Sex	Symbol	Tissue	Direction of change	Description	Function
Male	ESR1	Liver	Downregulated	Estrogen receptor 1	Hormone receptor
	ESR2b	Liver	Downregulated	Estrogen receptor 2b	Hormone receptor
	ESR2a	Liver	Upregulated	Estrogen receptor 2a	Hormone receptor
	AR	Liver	Downregulated	Androgen receptor	Hormone receptor
	SULT2A1	Liver	Downregulated	Cytosolic sulfotransferase 2A1	Steroid metabolism
	CBR11	Liver	Upregulated	Carbonyl reductase 1-like (20 β -hydroxysteroid dehydrogenase)	Steroid metabolism
	11 β HSD2	Liver	Upregulated	11 β -Hydroxysteroid dehydrogenase 2	Steroidogenesis
	3 β HSD	Testis	Upregulated	3 β -Hydroxysteroid dehydrogenase	Steroidogenesis
Female	SULT1st2	Testis	Upregulated	Cytosolic sulfotransferase 1st2	Steroid metabolism
	AR	Liver	Downregulated	Androgen receptor	Hormone receptor
	17 β HSD4	Liver	Downregulated	17 β -hydroxysteroid dehydrogenase type IV	Steroidogenesis
	SULT2A1	Liver	Downregulated	Cytosolic sulfotransferase 2A1	Steroid metabolism

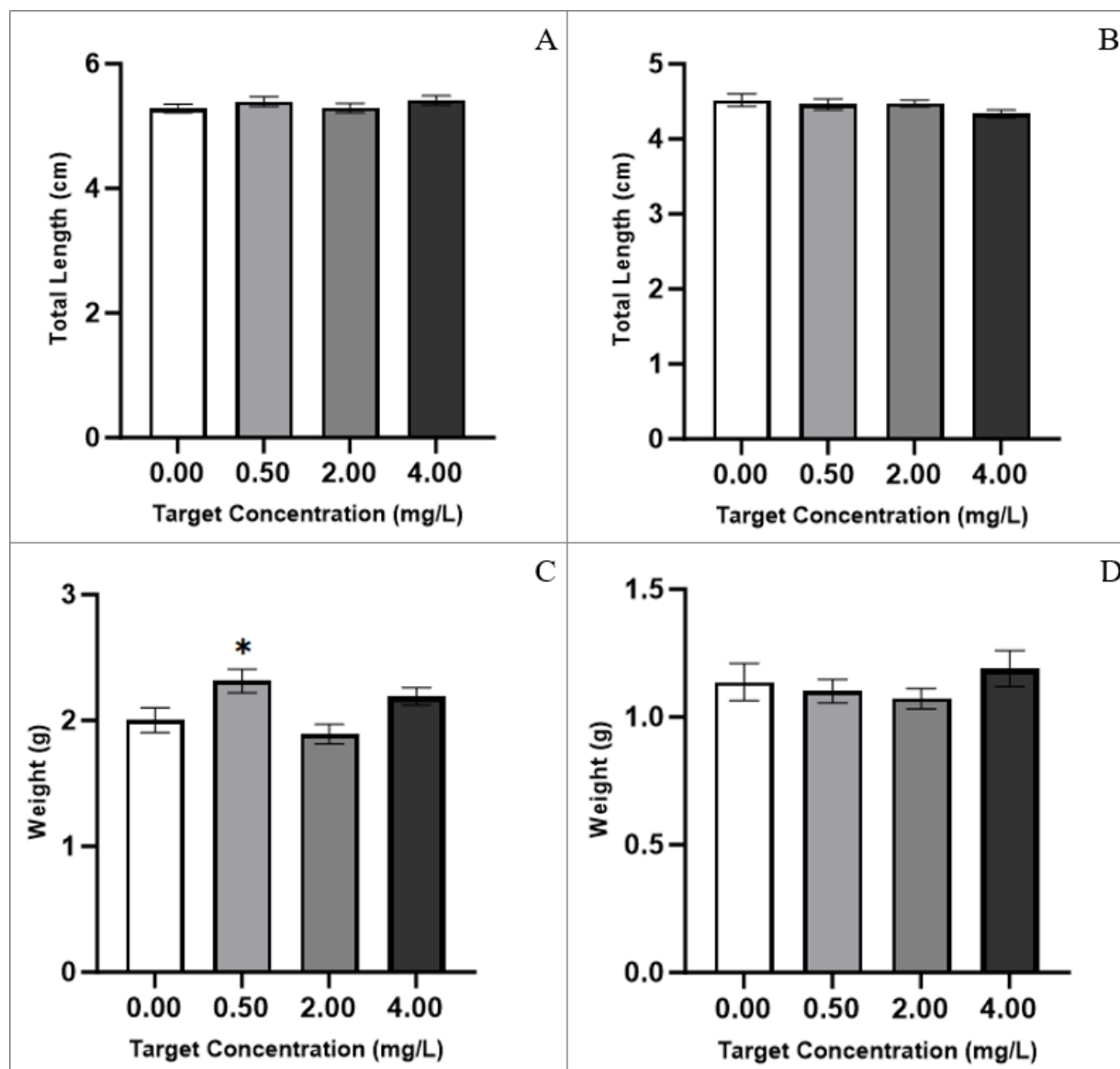
Supplemental Figures:



Sup. Fig. 1. Overall survival (%) of juvenile fathead minnow after chronic 120 d exposure assays (A), Deformity (%) of fathead minnow after chronic 120 d exposure assay II (B). $p < 0.05$, all error bars represent \pm SEM. One-way ANOVA with Tukeys multiple comparison analysis.



Sup. Fig. 2. Gonadosomatic index measures of male (a) and female (b) fathead minnow in experiment I. $p < 0.05$, all error bars represent \pm SEM. One-way ANOVA with Tukeys multiple comparison analysis.



Sup. Fig. 3. Total body length and weight measures of male (a,c) and female (b,d) fathead minnow in experiment I. $p < 0.05$, all error bars represent \pm SEM. One-way ANOVA with Tukeys multiple comparison analysis.

Supplementary Tables

Sup. Table 1

The list of additional genes that have known roles in the hypothalamic-pituitary-gonad-liver axis not affected by exposure to 2,4-D.

Function	Symbol	Description
Steroidogenesis	StAR	Steroidogenic acute regulatory protein
	CYP11a	Cytochrome P450 family 11 subfamily A
	CYP11b	Cytochrome P450 family 11 subfamily B
	CYP17	Steroid 17-alpha-hydroxylase/17,20 lyase
	CYP17a11	Steroid 17-alpha-hydroxylase/17,20 lyase-like
	CYP19a	Aromatase
	HSD17b12a	Hydroxysteroid (17-beta) dehydrogenase 12a
	TSPO	Translocator protein
	SF1	Splicing factor 1
	NPY	Neuropeptide Y
Hormone receptor function	LHb	Gonadotropin subunit beta-2
	LHCGR	Luteinizing hormone/choriogonadotropin receptor
	FSHb	Follicle stimulating hormone subunit beta
	FSHr	Follicle stimulating hormone receptor
	GnRHr1	Gonadotropin releasing hormone receptor 1
	GnRHr2	Gonadotropin releasing hormone receptor 2
Peroxisome proliferation	PPARAA	Peroxisome proliferator-activated receptor alpha a
	ACOX1	Acyl-CoA oxidase 1, palmitoyl
	EHHADH	Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase
Steroid metabolism	NR1I2	Nuclear receptor subfamily 1, group I, member 2
	CYP3A4	cytochrome P450 3A
	SULT2st3	Sulfotransferase family 2, cytosolic sulfotransferase 3
	SRD5a1	Steroid-5-alpha-reductase, alpha polypeptide 1
Regulatory/development	VTG1	Vitellogenin 1
	AMH	Anti-Mullerian hormone