**Metabolomics for informing adverse outcome pathways: androgen receptor activation and
the pharmaceutical spironolactone**

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Abstract:

One objective in developing adverse outcome pathways (AOPs) is to connect biological changes that are relevant to risk assessors (i.e., fecundity) to molecular and cellular-level alterations that might be detectable at earlier stages of a chemical exposure. Here, we examined biochemical responses of fathead minnows (*Pimephales promelas*) to inform an AOP relevant to spironolactone's activation of the androgen receptor, as well as explore other biological impacts possibly unrelated to this receptor. Liquid chromatography with high resolution mass spectrometry (LC-MS) was used to measure changes in endogenous polar metabolites in livers of male and female fish that were exposed to five water concentrations of spironolactone (0, 0.05, 0.5, 5, or 50 $\mu\text{g L}^{-1}$) for 21 days. Metabolite profiles were affected at the two highest concentrations (5 and 50 $\mu\text{g L}^{-1}$), but not in the lower-level exposures, which agreed with earlier reported results of reduced female fecundity and plasma vitellogenin (VTG) levels. We then applied partial least squares regression to assess whether metabolite alterations covaried with changes in fecundity, VTG gene expression and protein concentrations, and plasma 17β -estradiol and testosterone concentrations. Metabolite profiles significantly covaried with all measured endpoints in females, but only with plasma testosterone in males. Fecundity reductions occurred in parallel with changes in metabolites important in osmoregulation (e.g., betaine), membrane transport (e.g., L-carnitine), and biosynthesis of carnitine (e.g., methionine) and VTG (e.g., glutamate). Based on a network analysis program (i.e., *mummichog*), spironolactone also affected amino acid, tryptophan, and fatty acid metabolism. Thus, by identifying possible key events related to changes in biochemical pathways, this approach built upon an established AOP describing spironolactone's androgenic properties and highlighted broader implications potentially unrelated to androgen receptor activation, which could form the basis for development of an AOP network.

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24 **Keywords:** metabolomics, fathead minnow, mineralocorticoid, reproduction

1. Introduction:

To improve understanding and prediction of contaminant toxicity, the adverse outcome pathway (AOP) concept was developed to identify a series of key events that relate a molecular initiating event (e.g., receptor activation) elicited by a chemical exposure to adverse outcomes relevant to risk assessors (Ankley et al., 2010; Villeneuve et al., 2014). This can improve the efficiency of risk assessment programs, for example, by predicting adverse outcomes of a contaminant based on its ability to trigger molecular and biochemical changes linked to a given molecular initiating event in an established AOP. Although this approach can be effective for predicting toxic outcomes in many cases, some limitations exist. For instance, AOPs are inherently pathway specific and cannot predict toxic effects due to molecular perturbations that are outside the key events that define it (Villeneuve et al., 2014). Thus, when a contaminant acts via several toxicological pathways, consideration needs to be given to the potential involvement of multiple AOPs that may be integrated into larger AOP networks and help to better understand interactions among the various pathway perturbations (Knapen et al. 2015). However, the full list of applicable AOPs may not be known *a priori*. Further analyses (e.g., data-mining and case-studies) may be required to identify those molecular initiating events and AOPs that might co-occur with exposure to a particular chemical (Villeneuve et al., 2014).

Metabolomics represents an effective approach for not only identifying the presence of additional AOPs that may be occurring in parallel with an already assigned AOP, but also for providing physiological support and expanding knowledge of those AOPs relevant to a particular exposure. For example, decreased vitellogenin (VTG) synthesis is a biomarker of endocrine disruption and a key event in several AOPs, including those relating reproductive dysfunction to androgen or estrogen receptor interactions (AOP:23 and 30, respectively; Adverse Outcome Pathway Wiki, 2016a, b). Compounds that affect VTG synthesis have also been shown, via

metabolomics, to elicit changes in endogenous metabolites important to synthesis of this lipoprotein (e.g., glutamate; Ekman et al., 2011). Thus, metabolomics has the potential to inform and provide additional physiological support for AOPs that incorporate this particular key event, including the possible identification of previously undefined key events. Indeed, metabolomics can measure changes in endogenous metabolites involved in a wide variety of biochemical pathways, and associate those changes with exposure to specific chemicals (e.g., Bundy et al., 2007; Davis et al., 2016) and also to adverse biological outcomes (e.g., reduced earthworm fecundity; Åslund et al., 2013; Guo et al., 2009). Thus, examination of those pathways perturbed by a chemical exposure may be useful for identifying a range of impacts and any potential receptor interactions. This may subsequently point toward additional molecular initiating events and previously unsuspected AOPs that may be relevant to a particular chemical and may help forecast toxicity of contaminants acting via numerous pathways.

To demonstrate how metabolomics can enhance understanding of a contaminant's toxicological effects and potentially inform AOP development for those contaminants that might perturb multiple toxicological pathways, we measured endogenous metabolite responses to spironolactone exposure. Spironolactone is a widely prescribed pharmaceutical that interacts with several nuclear steroidal receptors (Creusot et al., 2014; Macikova et al., 2014) and has been detected downstream of wastewater treatment plants, particularly those receiving influent from hospitals and pharmaceutical manufacturers (Macikova et al., 2014). Spironolactone is a known mineralocorticoid receptor antagonist in humans and is a frequently prescribed diuretic (Doggrell and Brown, 2001). Other evidence indicates that it may have implications for aquatic biota, as it can interact with mineralocorticoid receptors in fish (Sturm et al., 2005). In addition, spironolactone is an antagonist of androgen receptors in humans (Doggrell and Brown, 2001); however, results from fish-based studies have shown that spironolactone can act as an androgen

receptor agonist in fish and result in anal fin elongation and decreased hepatic VTG mRNA in females (Howell et al., 1994; Raut et al., 2011). Spironolactone exposure also leads to modifications of progesterone and glucocorticoid receptor signaling in human cell lines (Creusot et al., 2014), which may be due to its rapid conversion to secondary metabolites (i.e., 7 α -thiomethylspironolactone and canrenone) that interact with multiple receptors (Los et al., 1994).

The broad array of evidence demonstrating spironolactone's potential to interact with multiple receptors suggests that several AOPs may be needed to better understand its impacts on fish. In fact, those impacts have begun to be elucidated by the recent application of the androgen receptor activation AOP (Adverse Outcome Pathway Wiki, 2016a). Specifically, LaLone et al. (2013) showed that female fathead minnows (*Pimephales promelas*) exposed for 21-days exhibited declines in fecundity, and plasma VTG and steroid concentrations consistent with what would be expected for a chemical operating through this particular AOP. Furthermore, biochemical and fecundity results for spironolactone were similar to those observed for the model androgen receptor agonist 17 β -trenbolone (i.e., Ankley et al., 2003). Although LaLone et al. (2013) provided convincing evidence of spironolactone's androgenic properties, as a result of their focus on key events associated with the androgen receptor activation AOP, possible impacts on other pathways and potential need for additional AOPs were not assessed. Metabolomics offers an approach for identifying these other impacts of spironolactone, and, more generally, may help to identify AOPs relevant for predicting biological effects of other contaminants that act on various receptors (e.g., progesterone, mineralocorticoid, and glucocorticoid receptors) and can reach biologically-relevant concentrations in aquatic ecosystems (e.g., Creusot et al., 2014; Runnalls et al., 2010).

Here, we applied a metabolomics-based approach to identify changes in endogenous metabolites in fathead minnows from the previous study by LaLone et al. (2013), helping to

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4 97 further develop and assess the androgen receptor AOP. Building on results of that analysis, we
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6 98 first confirmed that spironolactone exposure altered profiles of endogenous metabolites in
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8 99 fathead minnow livers. Then, we related those alterations to changes in biological endpoints (i.e.,
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11 100 fecundity, VTG mRNA in liver tissue, and concentrations of VTG, 17 β -estradiol [E2], and
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13 101 testosterone in plasma) that were sensitive to spironolactone exposure and linked to an AOP
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15 102 based on androgen receptor activation. Finally, we employed a metabolite network-analysis
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17 103 approach based on untargeted metabolite changes to investigate additional altered biochemical
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19 104 pathways that may reflect the involvement of additional AOPs initiated by spironolactone
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21 105 exposure, but potentially unrelated to androgen receptor activity.
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26 106 **2. Materials and Methods:**

27 28 107 **2.1 Experimental design:**

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33 109 changes to reproduction related endpoints (e.g., fecundity and VTG production), we conducted a
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35 110 21-day study that followed established protocols (Ankley et al., 2001). As reported by LaLone et
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37 111 al. (2013), two pairs of sexually-mature male and female fathead minnows were placed in a 20-L
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41 113 continuously renewed in a flow-through system. Aquaria were divided by a porous, plastic
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43 114 screen, with each side containing a single breeding pair and spawning substrate. After a 14-day
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45 115 acclimation period, fish that had successfully reproduced were allocated to one of five treatment
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47 116 groups, four exposed to different concentrations of spironolactone (0.05, 0.5, 5, or 50 $\mu\text{g L}^{-1}$) and
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49 117 one to control water (Lake Superior). Five aquaria were used for each concentration, yielding 10
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51 118 pairs of fish per treatment. Concentrations of spironolactone in test aquaria were verified using
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53 119 liquid chromatography mass spectrometry (LC-MS; LaLone et al., 2013). Throughout the study,
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55 120 fish were maintained at 25 ± 1 °C and under a 16:8 hour light:dark photoperiod, and fed twice
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4 121 daily with frozen brine shrimp. Fecundity of each breeding pair (i.e., numbers of spawned eggs
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6 122 and fertile eggs produced) was monitored daily and summed for the entire 21-day exposure.
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11 124 concentrations in aquaria water, and experimental design were reported by LaLone et al. (2013).
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14 125 At the end of the 21-day exposure, fish were anesthetized in tricaine methane-sulfonate (MS-
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16 126 222; 100 mg L⁻¹ buffered with 200 mg L⁻¹ NaHCO₃), weighed, and dissected. Blood (~20-50 µL)
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18 127 was collected from the caudal vasculature, centrifuged, and stored at -80°C until analyzed for
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21 128 testosterone, E2, and VTG concentrations using established methods (see Jensen et al., 2001;
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23 129 LaLone et al., 2013 for details). After blood collection, the liver was dissected and a sample from
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26 130 females, but not males, was allocated for RNA extraction. LaLone et al. (2013) used quantitative
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28 131 real-time polymerase chain reaction to quantify relative abundances of mRNA transcripts for
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31 132 VTG protein in livers. For analysis of endogenous metabolites, a liver sample was collected from
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33 133 both males and females, transferred to a 2 mL microcentrifuge tube containing a stainless steel
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36 134 ball bearing (3 mm), flash frozen in liquid nitrogen, and stored at -80 °C until extracted.
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38 135 **2.2 Processing of metabolomics samples:**

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43 137 extracted in a 96-well plate format using established protocols for a dual phase extraction (see
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45 138 Davis et al., 2013 for additional details). A 440 µL aliquot of the polar extract was subsequently
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48 139 collected from each sample. An additional aliquot (ca. 75-100 µL) was then collected from the
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51 140 remaining polar extract for each sample, pooled, and split into multiple quality control (QC)
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53 141 samples for high resolution LC-MS/MS analysis. The QC samples and polar extracts were
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55 142 vacuum dried overnight and stored at -80°C. Prior to analysis, polar extracts and QC samples
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58 143 were reconstituted in 100 µL of methanol:water (50:50), after which the samples were allowed to
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60 144 sit for 10 min and then vortexed at 500 RPM for 10 min. Samples were allowed to sit again for
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10 min and then vortexed a final time for 10 min (500 RPM). To remove particulates, the reconstituted samples were transferred to a prewetted 96-well PVDF filter plate (0.45 μ m pore size) and centrifuged at 1000 $\times$ g for 5 min (20 $^{\circ}$ C). The filtrate was transferred to a 96-well plate with 300 μ L glass inserts and briefly centrifuged to remove air bubbles (1 min at 1000 $\times$ g). Samples were stored in a refrigerated tray (4 $^{\circ}$ C) during the LC-MS/MS analysis. Quality control samples were analyzed at the beginning, end, and after every tenth sample of the analytical run.

2.3 Acquisition parameters for mass spectrometry:

Relative intensities of endogenous metabolite features were first assessed using high-resolution LC-MS (full-scan mode), with a follow-up tandem LC-MS/MS fragmentation analysis to aid in metabolite annotation. These analyses were performed using an Accela 1250 UHPLC system with an Open Accela Autosampler (Thermo Fisher Scientific), coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific) via a heated electrospray ionization source (HESI-II) operating in positive (+ESI) and negative (-ESI) modes. A Synchronis aQ C18 column (100 mm $\times$ 2.1 mm $\times$ 1.7 μ m; Thermo Fisher) was maintained at 50 $^{\circ}$ C and operated at a flow rate of 500 μ L min⁻¹. A 3 μ L sample injection was used with an elution gradient consisting of ultrapure water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid. The gradient started at 100% solvent A (0 min), and declined to 80% solvent A by 5 min, 40% solvent A by 6.5 min, and 0% solvent A by 9.5 min. Prior to the injection of the next sample, the column was rinsed for 4 min at 100% solvent B and then re-equilibrated for 5 min at 100% solvent A. The following parameters were used during the mass spectrometry analyses: probe heater temperature, 350 $^{\circ}$ C; sheath gas, 50; auxiliary gas, 10; sweep gas, 2.38; capillary temperature, 320 $^{\circ}$ C; S-lens RF level, 35; and spray voltage, 3.5 kV. The m/z scan range was set to 60 to 850 and the resolution was 70,000. The automatic gain control target was set at 1 $\times$ 10⁶ and maximum injection time was 120 ms. For the data-dependent MS-MS (Top 5), the resolution

was set at 17,500 and the HCD normalized collision energy was set at 40% with a stepped normalized collision energy of 50%. The automatic gain control target was set at 1×10^5 and maximum injection time was 60 ms.

2.4 Data processing:

All raw data were processed using SIEVE software (v2.1, Thermo Fisher Scientific). The small molecule component extraction signal detection algorithm was used for peak detection and integration. For a detected peak to be included in the final dataset, it had to exhibit a percent coefficient of variation (CV) of $<30\%$ in the QC samples and had to be present in $\geq 80\%$ of samples in at least one exposure group. Prior to multivariate and univariate analyses, we identified and removed duplicate records in datasets collected using the positive and negative electrospray ionization modes. All remaining metabolite features were normalized to unit total integrated intensity for each ion mode individually. Then, the data from the positive and negative ionization modes were combined and divided by 2 to renormalize to unit total integrated intensity.

Detected peaks of interest were putatively annotated (level 2 metabolite identification according to the Metabolomics Standards Initiative (Sumner et al., 2007)) by searching the Human Metabolome Database (www.hmdb.ca) and LipidMaps (www.lipidmaps.org) using accurate mass of the peak with a mass error tolerance of 3 ppm. When possible, metabolite identifications were further assigned by matching MS/MS spectra with those recorded in the public database Metlin (www.metlin.scripps.edu) and MZCloud (www.mzcloud.org), or by matching MS/MS spectra to *in silico* MS/MS spectra that were generated for possible candidates.

2.5 Statistical analyses:

After data processing, 1177 peaks that met QC criteria were detected in livers from male and female fish. We expect these peaks represented endogenous metabolites because they were

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4 193 detected in all treatment groups, including controls. To determine whether any change in
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16 198 subsequent PCA models excluded QC samples and assessed whether exposure altered overall
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21 200 data were unit-variance scaled and mean-centered, and statistical outliers were identified based
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23 201 on preliminary PCA comparisons and a Hotelling's T^2 test (95% confidence limit). To assess
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28 203 ANOVA with posthoc Tukey's test (R v3.2.2, R Core Team) were run for first and second
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33 205 To evaluate how changes in endogenous metabolite profiles covaried with endpoints related
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35 206 to reproductive function in these fish, we used partial least squares (PLS) regression (SIMCA v
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38 207 13.0, Umetrics Inc.). This framework can simultaneously compare multiple X and multiple Y
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40 208 variables where the number of variables exceeds the number of replicates or when the variables
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43 209 possess collinearity (Wold et al., 2001). Specifically, PLS-regression was used to evaluate
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45 210 whether changes in female metabolites significantly covaried with changes in fecundity, VTG
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48 211 mRNA in liver tissue, or concentrations of VTG, E2, and testosterone in plasma. For males, we
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50 212 only compared changes to plasma VTG, E2, and testosterone. Metabolite data were unit
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53 213 variance-scaled and mean-centered, and reproduction related endpoints were unit variance-
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55 214 scaled, mean-centered, and $\log_{10}(x+1)$ transformed. All PLS models were validated using cross-
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58 215 validation, whereby the full dataset was divided into seven randomly distributed subsets and one
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60 216 of the seven data subsets was left out to build a PLS model (i.e., leave-one-out cross validation).

To evaluate whether the model predicted those variables better than random chance, we then statistically compared the respective residuals of the measured values of that subset to those values predicted based on the PLS model (CV-ANOVA < 0.05; Eriksson et al., 2008). The best-fitting model was then identified by repeating this cross-validation in an iterative fashion based on subsequently generated data subsets (1000 permutations). Their predictive ability was assessed using cumulative Q^2 (Q^2_{cum} ; Eriksson et al., 2006), which assesses how well the model predicts bootstrapped data while also controlling for model overfit. To further assess the fit of the validated PLS models, we then used the best-fitting model to predict biological endpoint values for each fish used in the cross-validation, which were statistically compared to the measured values using simple linear regression (r^2_{adj} : adjusted r^2).

In addition to assessing how overall metabolite profiles were affected, we determined which specific metabolites significantly changed among treatment groups using two separate analyses. First, to assess whether the relative abundance of a detected feature differed across exposure groups, we applied a one-way ANOVA and post-hoc pairwise t-tests (R v3.2.2) to each metabolite feature. A false-discovery rate (FDR) correction (i.e., q-value) was also applied (Strimmer, 2008). Second, we identified metabolites that significantly covaried with fecundity based on the PLS-regression model. We focused on fecundity because it is an apical measurement relevant to risk assessment that integrates changes in other measured endpoints. For this, we utilized variable importance in the projection (VIP) values, which indicates the relative importance of an X-variable (i.e., metabolite) for the Y-variable (i.e., fecundity) in a PLS-regression model (Eriksson et al., 2006). Features with VIP values ≥ 1.0 are considered important and explain a substantial proportion of the variability in fecundity. Because fecundity significantly declined at higher concentrations of spironolactone, we indirectly assessed the direction and magnitude of the relationship between fecundity and biologically-relevant

metabolite features (i.e., $VIP \geq 1.0$) by examining the one-way ANOVA results and percent change calculations for each metabolite feature.

In order to provide a broader understanding of spironolactone effects, we also explored how the exposures may have altered biochemical pathways other than those directly related to reproductive function by using a network analysis approach (i.e., *mummichog*, v1.0.5; Li et al., 2013). This software can help elucidate those pathways significantly impacted in a treatment group relative to a control group (Hoffman et al., 2014; Li et al., 2013). Significantly impacted biochemical pathways are those exhibiting an adjusted p-value < 0.05 . For this comparison, we focused on endogenous metabolite features that significantly changed (pairwise t-test q-value < 0.05) in females exposed to $50 \mu\text{g L}^{-1}$ of spironolactone, as it would represent the greatest potential impact of the chemical exposure. To meet assumptions of the network approach, this comparison was conducted separately for positive and negative ionization modes. Detailed descriptions of the overall network approach can be found elsewhere (Li et al., 2013).

3. Results:

3.1 Summary of previously reported biological responses:

Results of the current study, which focused on the endogenous metabolite responses to spironolactone exposure, build upon previously published work that assessed the response of endpoints related to reproduction measured in these same fish (LaLone et al., 2013). As reported by LaLone et al. (2013), females exposed to the two highest concentrations (5 and $50 \mu\text{g L}^{-1}$) exhibited significant declines in plasma VTG concentrations and VTG mRNA expression in liver tissue compared to controls. These endpoints were not significantly different from controls in the two lowest exposure concentrations (0.05 and $0.5 \mu\text{g L}^{-1}$). Similarly, fecundity was significantly lower in females exposed to 5 and $50 \mu\text{g L}^{-1}$ of spironolactone, but no change was detected for the other exposure groups. Levels of plasma E2 and testosterone in females significantly

declined at a spironolactone concentration of 0.5 and 5 $\mu\text{g L}^{-1}$, and were lowest in females exposed to 50 $\mu\text{g L}^{-1}$ of spironolactone. In males, plasma E2 and VTG concentrations were not significantly affected by spironolactone exposure at any concentration; however, plasma testosterone declined at the highest test concentration (50 $\mu\text{g L}^{-1}$). Thus, females exposed to spironolactone exhibited reductions in several measures of reproductive endocrine function, largely at the two highest concentrations; however, exposure of males reduced only plasma testosterone levels, and only in the highest treatment group (50 $\mu\text{g L}^{-1}$) (LaLone et al., 2013).

3.2 Comparing metabolite profiles across exposure groups:

To assess whether overall metabolite profiles of the fathead minnows were significantly impacted by exposure to the five concentrations of spironolactone (0, 0.05, 0.5, 5, and 50 $\mu\text{g L}^{-1}$), responses were initially assessed with PCA and subsequent one-way ANOVA. Preliminary PCA comparisons and a Hotelling's T^2 test (95% confidence limit) detected a single statistical outlier (1 male from the control group), which was removed from all subsequent analyses. Furthermore, technical variation was found to be nominal as results from a separate preliminary PCA showed that variability in the pooled QC samples was substantially lower than variability measured in either female or male fish (Fig. S1A and S1B). Based on the final PCA comparisons that excluded pooled QC samples, metabolite profiles of females significantly differed along the second (ANOVA: $F_{4,49} = 50.41$, p-value < 0.001), but not the first component (ANOVA: $F_{4,49} = 0.886$, p-value = 0.48) of the PCA model (Fig. 1A). Pairwise comparisons indicated that differences along the second component were largely due to metabolite profiles of females from the 5 and 50 $\mu\text{g L}^{-1}$ exposure groups being statistically different from each other, and from the other groups (0, 0.05, and 0.5 $\mu\text{g L}^{-1}$; Tukey's test, p < 0.05). For males, metabolite profiles were also significantly different along the second component of the PCA model (ANOVA: $F_{4,48} = 17.88$, p-value < 0.001), such that males from the 5 and 50 $\mu\text{g L}^{-1}$ exposure groups were

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4 289 statistically different from each other and from other exposure groups (Tukey's test, $p < 0.05$;
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6 290 Fig. 1B). Males did not significantly differ along the first component (ANOVA: $F_{4,48} = 0.523$, p -
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9 291 value = 0.719). Thus, spironolactone exposure altered overall metabolite profiles of males and
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11 292 females, but only for the two highest concentrations, as metabolite profiles measured in
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16 294 Based on the one-way ANOVA and associated post-hoc pairwise tests that compared relative
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21 296 significantly affected by spironolactone exposure in females than in males and this number was
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23 297 greater at higher concentrations (Table S1). For instance, exposure to spironolactone
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26 298 significantly altered relative abundances of 289 features from females (ANOVA q -value < 0.05).
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36 302 different from the controls (Table S1). In males only 164 features significantly differed in
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38 303 relative abundance across exposure classes (ANOVA q -value < 0.05). However, pairwise
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43 305 145 features in males exposed to 50 $\mu\text{g L}^{-1}$ of spironolactone were significantly different from
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45 306 controls and only 5 features from the 5 $\mu\text{g L}^{-1}$ exposure were significantly different from controls
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48 307 (pairwise t -test, $q < 0.05$; Table S1). Thus, spironolactone exposure elicited significant changes
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50 308 in a number of metabolite features; however, those impacts were only at the two highest
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53 309 concentrations of the chemical and were more prevalent in females than males.

54 55 310 **3.3 Impacted biochemical pathways in females:**

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57 311 In addition to identifying changes in the relative intensities of individual metabolites, we
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60 312 applied a network analysis approach (i.e., *mummichog*) to identify biochemical pathways in
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females that were significantly altered by the 50 $\mu\text{g L}^{-1}$ exposure. Based on permutation testing that mapped significantly changing metabolite features in females (pairwise t-test q-value < 0.05) onto known biochemical pathways, we found that 33 pathways were significantly impacted by spironolactone exposure when comparing features detected using the positive ionization mode. Forty-one pathways were impacted when comparing features from the negative ionization mode (Table 1, *mummichog* adjusted p-value < 0.05). However, there was considerable overlap in these lists, as 25 pathways were identified as significantly impacted in both comparisons. For those metabolites detected in positive ionization mode, the five biochemical pathways with the lowest adjusted p-value were tryptophan metabolism (*mummichog* adjusted p-value = 0.0007), glycine, serine, alanine, and threonine metabolism (0.0007), xenobiotic metabolism (0.0007), arginine and proline metabolism (0.0008), and the urea cycle (0.0008). When comparing features detected in negative ionization mode, several pathways had equally low adjusted p-values. These eight pathways were glycosphingolipid metabolism (adjusted p-value = 0.0003), purine metabolism (0.0003), sialic acid metabolism (0.0004), glycosphingolipid biosynthesis (0.0004), urea cycle (0.0004), methionine and cysteine metabolism (0.0004), glycerophospholipid metabolism (0.0004), and nitrogen metabolism (0.0004; Table 1). Thus, the application of this network approach helped to elucidate a variety of biochemical pathways in females that were significantly altered by exposure to 50 $\mu\text{g L}^{-1}$ of spironolactone.

3.4 Comparing metabolite profiles to endpoints related to reproductive function:

Based on the PLS regression, changes in metabolite profiles significantly covaried with changes in several reproductive endpoints (plasma E2, testosterone, VTG, VTG mRNA, and fecundity). The strength of those relationships varied between sexes and across endpoints. For instance, metabolite changes in males significantly covaried with changes in plasma testosterone ($Q^2_{\text{cum}} = 0.277$; CV-ANOVA < 0.05), but only exhibited a weak linear relationship between

measured levels and those predicted based on the cross-validated PLS-regression model (p-value < 0.001, $r_{adj}^2 = 0.284$; Table S2; Fig. 2). The PLS-regression models examining covariance of plasma VTG or E2 in males with changes in their metabolite profile did not validate (Table S2). In contrast to males, changes in all five reproductive endpoints in females were significantly related to metabolite profile changes (CV-ANOVA < 0.001; Table S2), with the strength of those relationships, as measured by Q^2_{cum} , ranging from 0.374 to 0.726 (Figs. 3A-D and 4; Table S2). The model relating metabolites and plasma VTG in females exhibited the strongest relationship ($Q^2_{cum} = 0.726$; Table S2), and showed significant agreement between predicted and measured values of plasma VTG (p-value < 0.001; $r_{adj}^2 = 0.477$, Fig. 3B). The PLS-regression relating female fecundity and metabolite changes also exhibited a relatively strong relationship ($Q^2_{cum} = 0.547$; Table S2) with a significant linear relationship between predicted and measured values of fecundity (p-value < 0.001; $r_{adj}^2 = 0.471$; Fig. 4). Although the model relating metabolites and VTG mRNA exhibited comparable strength as models describing fecundity and plasma VTG ($Q^2_{cum} = 0.654$), it poorly predicted VTG mRNA levels (p-value < 0.001; $r_{adj}^2 = 0.272$), partly due to overestimation in the control group (Fig. 3A). Lastly, models describing plasma E2 and testosterone exhibited a weaker relationship with metabolite changes ($Q^2_{cum} = 0.495$ and 0.374 , respectively; Table S2), but their ability to predict steroid levels was comparable to other models (p-value < 0.001; $r_{adj}^2 = 0.468$ and 0.353 , respectively; Fig. 3C-D). The combination of such evidence suggests that changes in metabolite profiles were significantly covarying with changes in a variety of reproductive endpoints comprising the androgen receptor activation AOP in females, including fecundity and plasma VTG (Figs. 3-4). The strength of those relationships varied and was generally weaker in males (Table S2).

3.5 Individual metabolites that covaried with fecundity:

We also used results of the PLS-regression analyses to discern those hepatic metabolites that significantly covaried with fecundity changes at the individual fish level. Specifically, we selected the 420 metabolite features that had VIP values ≥ 1.0 , but were only able to putatively annotate 41 of these features, partly due to limitations of publicly available fragmentation libraries (Table 2). Nonetheless, examination of this list of putatively annotated metabolites points to several important biochemical pathways as being related to decreased fecundity.

Based on VIP results from the PLS-regression model and results of the ANOVA that compared changes in relative abundances across exposure groups, we evaluated how individual metabolites changed across the five groups and with respect to fecundity. For instance, several metabolites associated with carnitine biosynthesis and fatty acid oxidation were significantly related to fecundity changes (L-carnitine VIP: 1.73, acetylcarnitine: 1.63, propionylcarnitine: 1.37, (+/-) hexanoylcarnitine: 1.27, 2-methylbutyrylcarnitine: 1.10, and methionine: 1.04; Table 2). This relationship was accompanied by concomitant changes in the abundances of these metabolites at the highest concentrations of spironolactone, although the overall pattern of those alterations differed among metabolites (Table 2 and Figs. 5A-F). Relative to controls, L-carnitine and propionylcarnitine significantly decreased at both the 5 and 50 $\mu\text{g L}^{-1}$ concentrations (pairwise t-test, $q < 0.05$; Figs. 5A and 5D). Although (+/-) hexanoylcarnitine was marginally lower at the 5 $\mu\text{g L}^{-1}$ level (pairwise t-test, $q < 0.10$), it was significantly lower only at the 50 $\mu\text{g L}^{-1}$ concentration ($q < 0.05$; Fig. 5B). In contrast, acetylcarnitine significantly decreased at 5 $\mu\text{g L}^{-1}$ (pairwise t-test, $q < 0.05$), but was only marginally lower at the 50 $\mu\text{g L}^{-1}$ concentration ($q < 0.10$; Fig. 5C). The abundance of 2-methylbutyrylcarnitine was marginally lower at the highest concentration (pairwise t-test, $q < 0.10$; Fig. 5E), whereas methionine increased with exposure concentration ($q < 0.05$; Fig. 5F). Despite some variability in the overall responses, results indicate that relative intensities of these particular metabolites were significantly covarying with

female fecundity at the individual fish level, and were generally lower in the groups of females exhibiting decreased fecundity at higher levels of spironolactone exposure.

In addition to changes in metabolites associated with carnitine biosynthesis, relative intensities of other selected metabolites covaried with fecundity and also were significantly different among exposure concentrations. For example, lactic acid significantly covaried with fecundity (VIP = 1.32) and was significantly lower at the highest exposure concentrations (5 and 50 $\mu\text{g L}^{-1}$) relative to the controls (pairwise t-test q-value < 0.05; Fig. S2A). Similarly, glutamate and gamma-aminobutyric acid (GABA) were important for the fecundity PLS-regression model (VIP = 1.50 and 1.71, respectively), and their relative abundances were significantly lower at the 5 and 50 $\mu\text{g L}^{-1}$ exposure levels (pairwise t-test q-value < 0.05; Fig. S2B-C). In contrast, betaine significantly covaried with fecundity (VIP = 1.43) and increased at the highest dose of spironolactone exposure (pairwise t-test, q < 0.05; Fig. S2D).

4. Discussion:

4.1 Androgen receptor AOP informed by linking metabolite and reproductive endpoints

In this study, we identified effects of a 21-day spironolactone exposure on polar metabolite profiles in fathead minnow livers and linked those changes to declines in fecundity and other reproductive-related endpoints that were previously reported in a companion study (i.e., LaLone et al., 2013). This builds upon earlier evidence of the androgenic properties of spironolactone in fish (i.e., Howell et al., 1994; Raut et al., 2011), as metabolite changes in this study correlated with reproductive endpoints measured in females (VTG mRNA expression, plasma VTG and steroids, and fecundity), whereas changes in endogenous metabolites only weakly correlated with changes in plasma testosterone levels in males. This stronger relationship in females is possibly related to the direct functional role of the liver in the biosynthesis of VTG (e.g., Parks et al., 1999), which is important in female reproduction. This direct relationship is less apparent in

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4 408 males, which may attenuate the strength of the relationship between hepatic metabolite changes
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6 409 and alterations of other reproduction-related endpoints measured in this study. Also, in
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9 410 agreement with earlier measurements of reproductive endpoints (i.e., LaLone et al., 2013),
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11 411 metabolite profiles (as analyzed by PCA) and relative intensities of individual metabolites (as
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14 412 analyzed by ANOVA) were significantly altered at 5 and 50 $\mu\text{g L}^{-1}$ of exposure, but not at the
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16 413 lowest concentrations. However, metabolite and reproductive endpoint responses did diverge for
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19 414 males exposed to 5 $\mu\text{g L}^{-1}$ of spironolactone, whereby metabolite profiles were affected despite
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21 415 the lack of a similar response in male reproductive endpoints measured by LaLone et al. (2013).
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23 416 This inconsistency suggests that changes in endogenous metabolites could be indicative of
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33 420 In addition to strengthening the evidence of spironolactone's androgenic properties, we
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36 421 provided insight into the mechanistic basis of relevant key events and linkages within the
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38 422 androgen receptor AOP by relating metabolite changes to indicators of reproductive function.
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41 423 For example, reproductive endpoints that significantly covaried with metabolite changes
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43 424 comprise several key events in the androgen receptor AOP described for female fish (AOP 23;
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45 425 Adverse Outcome Pathway Wiki, 2016a); thus, our results provide further confidence in the
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48 426 scientific robustness of this AOP. In particular, the relative abundance of glutamate in livers (the
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51 427 primary site of VTG synthesis; Parks et al., 1999) significantly decreased with exposure and
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53 428 covaried with fecundity declines. Glutamate is, in fact, an important constituent of VTG in
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55 429 fathead minnows (Parks et al., 1999). Thus, the significant relationship between hepatic
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58 430 glutamate and fecundity are likely related to concomitant changes in plasma VTG, and are
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60 431 consistent with a spironolactone-induced decline in VTG production (LaLone et al., 2013).
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Indeed, VIP results from the PLS-regression comparing plasma VTG and liver metabolite changes found that hepatic glutamate significantly covaried with plasma VTG levels (VIP = 1.72; data not shown). Because this particular endogenous metabolite is an important constituent of VTG, there is a very direct biological link between these two measurements. On the other hand, there are a greater number of intermediate steps that occur between changes in VTG mRNA levels and endogenous metabolites, which may attenuate the strength of their relationship. This may be a contributing factor in the stronger relationship between changes in endogenous metabolites and plasma VTG versus changes in VTG mRNA levels.

Our approach also identified other endogenous metabolites of potential importance; for example, L-carnitine and related metabolites significantly covaried with fathead minnow fecundity. This agrees with earlier studies showing that reductions in L-carnitine can be associated with hyperandrogenism in human females (Fenkci et al., 2008) and reduced fecundity in other fish species (Schreiber et al., 1997, but see Dzikowski et al. 2001). Furthermore, targeted *in vitro* inhibition of L-carnitine biosynthesis significantly reduced oocyte maturation in bovine ovaries, likely due to changes in fatty acid oxidation (Sanchez-Lazo et al., 2014), while *in vitro* L-carnitine supplementation improved rates of mouse oocyte maturation, fertilization, and blastocyst development (Dunning et al., 2011). Thus, considerable biological evidence provides indirect support that our observed decline in hepatic L-carnitine levels may be a contributing factor in decreased female fecundity, especially as L-carnitine is primarily biosynthesized in the liver (Bøhmer, 1974; Vaz and Wanders, 2002). Such results are largely correlative and any conclusion based upon them should be viewed accordingly. Results from other studies have suggested that changes in certain metabolites measured in this current study (e.g., glutamate and L-carnitine) may be related to biological pathways unrelated to androgen receptor signaling (i.e., Karst et al. 2005; Grube et al. 2006). Nonetheless, the strong relationships observed among

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4 456 endogenous metabolites and biological endpoints helped elucidate specific metabolites that could
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6 457 be targeted by future studies. Those studies could more fully evaluate metabolite relationships
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9 458 described here and explore whether they should be considered for inclusion as key events in the
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11 459 androgen receptor activation AOP, or whether they are related to other biological pathway
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16 461 This study provides further evidence for the potential utility of metabolomics to identify
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21 463 refine the development of this particular AOP. Despite this possibility, the proportion of detected
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23 464 peaks that were putatively annotated in this study, while comparable to other LC/MS-based
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26 465 metabolomics studies, also demonstrates that metabolite annotation continues to represent a
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28 466 significant challenge for LC-MS-based metabolomics studies (Zhou et al., 2012). In particular,
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31 467 there is a considerable need to expand publically available MS/MS spectral libraries, as there are
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33 468 a large number of endogenous metabolites and few of them have been reliably identified in
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36 469 currently available libraries. Such limitations are largely due to the scarce resources available to
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38 470 develop these libraries. Nonetheless, focusing those resources on metabolite features that have
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43 472 exposures may represent a cost-effective means of fully realizing the potential strength of
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45 473 metabolomics for informing AOP discovery and ecotoxicology in general.

48 474 **4.2 Potential key events supported by similar responses to other androgen exposures**

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53 476 largely agree with earlier studies that have assessed fish responses to other androgens. For
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55 477 example, similar to spironolactone, a 21-day exposure of fathead minnows to 17 β -trenbolone
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58 478 significantly decreased plasma VTG (Ankley et al., 2003) and correspondingly decreased hepatic
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60 479 glutamate (Ekman et al., 2011). Consistent with the present study, exposure to 17 β -trenbolone
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also reduced hepatic lactate and glutamine. In addition to changes in metabolite profiles, 17 β -trenbolone altered gene expression in fathead minnows (Dorts et al., 2009), with many of the genes exhibiting functional overlap with changes in biochemical pathways that were observed in this present study with spironolactone. For example, a 4-d 17 β -trenbolone exposure of fathead minnows down-regulated genes important for fatty acid and lipid metabolism, and also sphingolipid metabolism in female gonad tissue (Dorts et al., 2009). Alteration of gene expression related to lipid metabolism and transport have also been reported for fish exposed to other types of androgens (11-ketotestosterone and methyl-testosterone; Martyniuk and Denslow, 2012). Such substantial agreement of transcriptomic and metabolomic changes in multiple studies using known androgens provide additional evidence that some of the metabolite alterations observed in the present study may be generalizable responses that could eventually serve as additional key events for diagnosing exposure to and effects of this class of chemicals.

4.3 Metabolite alteration indicates additional potential impacts and AOPs

When utilizing an AOP to characterize or predict possible effects of a given chemical, an important question is whether additional effects might be exerted through other AOPs. Such potential is supported by the results of the present study, as spironolactone exposure reduced the ability of fathead minnows to biosynthesize L-carnitine. Specifically, we measured significant decreases in several forms of endogenous metabolites involved with fatty acid oxidation (i.e., L-carnitine, (+/-) hexanoylcarnitine, acetylcarnitine, propionylcarnitine, and 2-methylbutyrylcarnitine). In addition, we detected significant increases in methionine, a crucial building block in L-carnitine biosynthesis (Bremer, 1983), suggesting that the reduction in L-carnitine biosynthesis may occur during later stages of biosynthesis. Due to the essential role of L-carnitine in many processes, including fatty acid, lipid, and amino acid metabolism (Bremer, 1983), such declines may not only contribute to the reduced fecundity discussed above, but may

encompass biological effects that extend beyond those measured by LaLone et al. (2013). For example, spironolactone exposure may have implications for energy production because L-carnitine deficiencies can increase the reliance of fish on protein metabolism to maintain metabolic rates (Harpaz, 2005). In the context of other research, results from this current study suggest that spironolactone exposure could potentially interact with other environmental changes, as L-carnitine can ameliorate the negative impacts of environmental stressors on fish, including thermal stress, ammonia toxicity, and oxidative stress. Specifically, cichlids (*Pelvicachromis pulcher*) fed diets supplemented with L-carnitine exhibited reduced mortality in response to cold shock (Harpaz et al., 1999). Because of its functional role as an osmolyte, L-carnitine also improved resistance to ammonia toxicity in Chinook salmon (*Oncorhynchus tshawytscha*) injected with ammonium acetate, whereby 33% of fish receiving L-carnitine supplemented diets exhibited ammonia toxicity and only 4% died, in contrast to 98% and 69% of reference fish, respectively (Tremblay and Bradley, 1992). Due to its role in membrane transport, L-carnitine declines may also affect the susceptibility of exposed fish to other xenobiotics when spironolactone is present in complex chemical mixtures. Under thermal stress, L-carnitine supplementation of guppies (*Poecilia reticulata*) reduced the transport of an anionic xenobiotic (i.e., fluorescein) across membranes of epithelial and gill cells, subsequently reducing its accumulation in liver and kidney tissue (Schreiber et al., 1997). Furthermore, oxidative stress declined in fathead minnow cells that were pretreated with L-carnitine and subsequently exposed to hydrogen peroxide (Wang et al., *In press*). Given the variety of physiological functions associated with L-carnitine, this highlights the potential for additional adverse physiological outcomes as a result of these pathway alterations.

The potential for spironolactone exposure to elicit biological impacts beyond those associated with androgen receptor activation is also supported by other biochemical alterations

reported here and their overall agreement with pharmacological effects. For example, results from previous studies have shown that spironolactone can bind to the glucocorticoid and progesterone receptors (Garthwaite and McMahon, 2004; Fagart et al., 2010). Perturbations of mineralocorticoid receptor signaling may also represent a molecular initiating event through which spironolactone is acting, as it has been shown to be an antagonist of mineralocorticoid receptors in humans (Doggrell and Brown, 2001). In fact, it can display the highest affinity for the mineralocorticoid receptor (Rogerson et al. 2004) and alter signaling of this nuclear receptor in mammals at a lower concentration ($MR\ IC_{50} < AR\ IC_{50} < PR\ EC_{50} < GR\ IC_{50}$; Garthwaite and McMahon, 2004; Fagart et al., 2010). This affinity for the mineralocorticoid receptor has led to its use as a diuretic and to treat hypertension in humans due to the functional importance of the mineralocorticoid receptor for ion transfer and osmoregulation (Doggrell and Brown, 2001). There is also some evidence that spironolactone is an agonist of this receptor in teleost fish (Pippal et al., 2011; Sturm et al., 2005). This points to its activation as a possible molecular initiating event related to spironolactone exposure, with other evidence suggesting possible physiological effects. Several endogenous metabolites (i.e., GABA, L-carnitine, and betaine) that were affected by spironolactone exposure are involved with fish osmoregulation (Peluso et al., 2000) and may be partly related to agonism of this steroidal receptor. This agrees with another study showing that dosing of killifish (*Fundulus heteroclitus*) with spironolactone inhibited the expression of genes important for ion transfer across gill membranes, and affected ion concentrations in plasma (Scott et al., 2005). In addition, other studies indicate spironolactone can reduce the prevalence of fatty livers in mice fed a diet high in fat and fructose and down-regulate genes involved with lipidogenesis and β -oxidation (Wada et al., 2010). In agreement with such findings, the network analysis from the present study indicated overall changes in fatty acid oxidation and β -oxidation in exposed fathead minnows. It also showed that tryptophan

metabolism was significantly altered by spironolactone exposure, which aligns with evidence indicating that spironolactone may affect depression in mammals, possibly via changes in mineralocorticoid receptor activation and serotonin levels, a neurotransmitter derived from tryptophan (Wu et al., 2013; Young et al., 2003).

4.3 Predicting effects via alteration of multiple receptors requires an AOP network approach

Results of our metabolomics-based approach and previous pharmacological studies contribute to the mounting evidence that the development of AOP networks may be required to effectively forecast net impacts of compounds that alter multiple molecular pathways. For instance, an AOP network approach (*sensu* Knapen et al. 2015) helped to predict overall changes in fathead minnow gluconeogenesis, immune system function, and androgen receptor activation due to several molecular initiating events related to beclomethasone dipropionate exposure (Margiotta-Casaluci, et al. 2016). As evidence suggests that spironolactone similarly interacts with a range of nuclear receptors, an AOP network that integrates impacts via a number of molecular changes may similarly improve understanding of how aquatic biota respond to similar types of compounds. Such a possibility is supported by the range of endogenous metabolite changes reported here and the functional overlap of some of the receptors impacted by spironolactone, including the potential importance of mineralocorticoid and glucocorticoid receptors in gill permeability and osmoregulation (Kelly and Chasiotis 2011). Although spironolactone exposure may elicit interactive effects that may require an AOP network approach, the relative importance of these various pathway changes may be largely concentration-dependent. For instance, mineralocorticoid and androgen receptor signaling in mammals can be altered at a substantially lower concentration of spironolactone (as measured by IC₅₀ or EC₅₀) than changes in progesterone and glucocorticoid signaling (e.g., Garthwaite and

McMahon, 2004; Fagart et al., 2010). Thus, exposure to lower concentrations of spironolactone may elicit effects primarily via alteration of mineralocorticoid and androgen receptors, with the effects related to progesterone and glucocorticoid receptors increasing in importance at higher exposure levels. These dose-dependent interactions suggest the potential complexity and need for AOP networks to integrate dynamic relationships when forecasting the overall effects of compounds that alter multiple molecular pathways.

Overall results from this current study highlight how the application of metabolomics within an AOP framework may help to elucidate other pathway alterations and associated effects that may be occurring in parallel with androgen receptor activation and could be explored in the future. Consideration of these additional alterations may provide a more complete understanding of the overall effects associated with contaminants that act upon multiple receptors, and could be targeted for inclusion in a broader AOP network focused on these nuclear receptors. The relative importance of the various molecular changes within such a network could be assessed by future studies that utilize the more recently developed compounds (e.g., eplerenone) that exhibit greater selectivity for the mineralocorticoid receptor and reduced side effects associated with androgen receptor binding (Garthwaite and McMahon 2004; Fagart et al. 2010). These and other types of studies (e.g., time-course experiments) may help to refine the AOPs focused on these particular nuclear receptors, partition the relative importance of these molecular pathways in a broader AOP network, and evaluate the utility of applying changes in those endogenous metabolites measured in this current study as indicators of exposure to similar classes of compounds. Understanding those relationships and how they interact with other factors may be particularly important in complex multi-stressor environments where co-occurring environmental stressors related to energy regulation, ammonia toxicity, and osmotic gradients could potentially exacerbate responses directly attributable to contaminant exposure.

5. Conclusions:

Results from this study provide an example of how high-resolution mass spectrometry and metabolomics can complement existing knowledge of an established AOP and help identify potential molecular indicators of a biological perturbation. For example, parallel analysis of reproductive endpoint measurements and endogenous metabolites in fathead minnow livers identified several metabolites (i.e., glutamate and L-carnitine) that significantly covaried with fecundity and other endpoints that have been used as biomarkers of exposure for a variety of endocrine disrupting compounds (i.e., plasma VTG levels; Ankley et al., 2003; Parks et al., 1999). This comparison also helped substantiate aspects of the physiological basis of the AOP centered on androgen receptor activation, as these endpoints have been previously recognized as key events that link this AOP's molecular initiating event (i.e., activation of androgen receptor) to an adverse outcome (i.e., decreased fecundity). Furthermore, we identified alterations in other endogenous metabolites (i.e., glutamine and lactate) that have been similarly affected by known androgenic compounds (e.g., Ekman et al., 2011), which supports the potential for applying pathway-based metabolomics to identify generalizable molecular changes that may help signal exposure to a range of androgenic compounds.

In addition to highlighting potential molecular changes that could be tied to the androgen receptor AOP, examination of biochemical pathways that were significantly affected by spironolactone helped generate testable hypotheses for additional biological impacts. Future studies or AOP development could target such effects to help determine if they could be more generally applied to other compounds similar to spironolactone, and be linked together under a broader AOP network-based approach. In particular, metabolite analysis identified changes in several endogenous metabolites important for osmoregulation and membrane transport, indicating that spironolactone may increase the susceptibility of exposed fish to changes in

osmotic gradients and nitrogen concentrations, two environmental variables known to vary at outflows of wastewater treatment plants. In addition to identifying this impact that points toward possible involvement of an AOP based on mineralocorticoid receptor signaling, other evidence points to impacts that could not be tied any specific receptor activation, but may have comparable implications for aquatic biota. Results from the metabolite analysis suggest the potential for spironolactone to exacerbate exposure effects for other contaminants, as we observed declines in an endogenous metabolite (i.e., L-carnitine) important for maintaining membrane integrity, reducing xenobiotic transport across cell membranes, and protecting against oxidative stress. This suggests that spironolactone could have interactive effects when found in combination with other contaminants. Similar integrative studies, which utilize multivariate analysis of pathway-based metabolomic and reproductive endpoint data, could help build on such results by evaluating the robustness of relationships described here and contribute to mounting evidence of metabolomics' utility for AOP development and risk management (e.g., Åslund et al., 2013; Davis et al., 2016; Guo et al., 2009).

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Table 1: Biochemical pathways that were altered by spironolactone exposure. Significantly changing biochemical pathways (adjusted p-value < 0.05) based on *mummichog* network analyses of significantly changing metabolites in females (50 µg L⁻¹ exposure vs. controls). Separate comparisons were run for positive and negative ionization modes. A dash indicates that a biochemical pathway was not significantly impacted based on endogenous metabolites detected in that particular ionization mode. The list is sorted based on adjusted p-values for pathways detected in positive ionization mode.

Biochemical pathway	Positive ionization mode		Negative ionization mode	
	No. of significant putatively annotated metabolites	Adjusted p-value	No. of significant putatively annotated metabolites	Adjusted p-value
Tryptophan metabolism	24	0.0007	13	0.0035
Glycine, serine, alanine and threonine metabolism	20	0.0007	13	0.0059
Xenobiotics metabolism	13	0.0007	-	-
Arginine and proline metabolism	16	0.0008	10	0.0049
Urea cycle/amino group metabolism	20	0.0008	14	0.0004
Alanine and aspartate metabolism	9	0.0009	10	0.0006
Glutamate metabolism	8	0.0009	6	0.0008
Histidine metabolism	9	0.0011	8	0.0007
Lysine metabolism	12	0.0011	5	0.0122
Beta-Alanine metabolism	7	0.0012	6	0.0008
Glutathione metabolism	6	0.0013	-	-
Caffeine metabolism	5	0.0014	-	-
Methionine and cysteine metabolism	12	0.0018	15	0.0004
Vitamin B3 metabolism	8	0.0022	5	0.0012
N-Glycan degradation	4	0.0028	4	0.0007
Aspartate and asparagine metabolism	23	0.0028	14	0.0230
Fatty acid metabolism	3	0.0035	-	-
Keratan sulfate degradation	3	0.0035	3	0.0083
Nitrogen metabolism	3	0.0035	4	0.0004
Vitamin B9 (folate) metabolism	5	0.0040	5	0.0008
Glycosphingolipid metabolism	6	0.0052	12	0.0003
Aminosugars metabolism	8	0.0074	8	0.0016
Butanoate metabolism	8	0.0074	7	0.0296
Valine, leucine and isoleucine degradation	8	0.0074	6	0.0095
Tyrosine metabolism	21	0.0076	16	0.0111
Carnitine shuttle	3	0.0089	-	-
Glycosphingolipid biosynthesis - globoseries	3	0.0089	5	0.0004
Fatty acid oxidation, peroxisome	2	0.0197	-	-
Saturated fatty acids beta-oxidation	2	0.0197	-	-
Glycosphingolipid biosynthesis - ganglioseries	3	0.0201	5	0.0008
Linoleate metabolism	4	0.0211	-	-
Sialic acid metabolism	9	0.0383	11	0.0004
Pyruvate metabolism	3	0.0400	6	0.0095
Alkaloid biosynthesis II	-	-	2	0.0146
Biopterin metabolism	-	-	4	0.0103
Blood group biosynthesis	-	-	3	0.0020
Drug metabolism - cytochrome P450	-	-	5	0.0184
Glycerophospholipid metabolism	-	-	11	0.0004
Glycosphingolipid biosynthesis - lactoseries	-	-	3	0.0020
Glycosphingolipid biosynthesis - neolactoseries	-	-	3	0.0020
Keratan sulfate biosynthesis	-	-	2	0.0146
N-Glycan biosynthesis	-	-	6	0.0008
Propanoate metabolism	-	-	7	0.0017
Purine metabolism	-	-	17	0.0003
Pyrimidine metabolism	-	-	15	0.0015
Selenoamino acid metabolism	-	-	6	0.0005
Ubiquinone biosynthesis	-	-	2	0.0305
Vitamin B1 (thiamin) metabolism	-	-	3	0.0083
Vitamin B2 (riboflavin) metabolism	-	-	2	0.0055

Table 2: Endogenous metabolites that covaried with female fecundity. List of 41 putatively annotated endogenous metabolites that were significantly related to female fecundity based on the partial least squares (PLS) regression, as measured with variable importance in the projection (VIP; > 1.0). Direction of change indicates whether the relative intensity of a metabolite in the 50 µg L⁻¹ exposure group increased or decreased relative to those measured in the control group, regardless of whether that change was statistically significant. Asterisks indicate that the metabolite was significantly changing among exposure groups based on an ANOVA analysis of individual metabolite features (q-value < 0.05). ‘Pos’ indicates that the metabolite was detected in positive ionization mode and ‘Neg’ indicates that it was detected in negative ionization mode.

Putative metabolite annotation	Detection mode	VIP	Direction of change for females	Direction of change for males
(+/-) Hexanoylcarnitine	Pos	1.27	Decreased*	Increased
1-O-palmitoyl-2-O-acetyl-sn-glycero-3-phosphorylcholine	Pos	1.13	Increased	Decreased
2,2'-(3-methylcyclohexane-1,1-diyl)diacetic acid	Neg	1.22	Decreased	Decreased
2-Methylbutyrylcarnitine	Pos	1.10	Decreased*	Decreased
2-Phenylacetamide	Neg	1.07	Increased	Increased
3-Aminopropanesulphonic acid	Neg	1.23	Increased*	Increased*
4-Oxoproline	Neg	1.45	Decreased*	Decreased
5β-cholanic acid-3α, 12α-diol N-(2-sulphoethyl)-amide	Pos	1.25	Increased*	Increased
7α,12α-dihydroxy-4-cholestene-3-one	Pos	1.60	Decreased	Decreased
Acetylcarnitine	Pos	1.63	Decreased*	Decreased*
Acetylcholine	Pos	1.09	Increased*	Increased*
Adenosine diphosphate (ADP)	Neg	1.07	Decreased	Decreased
Adenosine triphosphate (ATP)	Neg	1.03	Decreased	Decreased*
Aspartic acid	Neg	1.05	Decreased	Decreased
Betaine	Pos	1.43	Increased*	Increased*
Citrulline	Pos	1.79	Increased	Decreased*
Creatine	Pos	1.06	Decreased*	Decreased*
Gamma-aminobutyric acid (GABA)	Pos	1.71	Decreased*	Decreased
Glutamate	Pos	1.50	Decreased*	Decreased
Guanosine diphosphate (GDP)	Neg	1.21	Decreased	Decreased
Guanosine triphosphate (GTP)	Neg	1.12	Decreased	Decreased
Hypoxanthine	Pos	1.57	Increased*	Increased*
Lactic acid	Neg	1.32	Decreased*	Increased*
L-α-Lysophosphatidylserine	Neg	1.37	Increased	Decreased
L-Carnitine	Pos	1.73	Decreased*	Decreased*
L-Glutamine	Neg	1.50	Decreased*	Decreased
L-Histidine	Neg	1.34	Decreased*	Increased*
Malic acid	Neg	1.16	Decreased*	Decreased
Methionine	Pos	1.04	Increased*	Decreased
N-Acetylneuraminic acid	Neg	1.23	Decreased*	Increased
N-Carboxyethyl-γ-aminobutyric acid (spermidic acid)	Pos	1.21	Increased*	Decreased*
Niacinamide	Pos	1.15	Increased	Decreased
Proline	Pos	1.40	Decreased*	Increased
Propionylcarnitine	Pos	1.37	Decreased*	Decreased*
Pyrophosphate	Neg	1.02	Decreased*	Decreased
Sarcosine	Neg	1.19	Decreased	Decreased*
Succinic acid	Neg	1.48	Decreased*	Increased
Taurochenodeoxycholic acid	Neg	1.41	Increased*	Increased
Taurocholic acid	Pos	1.14	Increased	Increased
Threonine	Neg	1.19	Decreased*	Decreased
Uridine monophosphate (UMP)	Neg	1.09	Decreased	Increased

Fig. Legends:

Figure 1: Average scores plot (mean $\pm$ SE) for the principal component analysis (PCA) that compared profiles of endogenous metabolites from either (A) female or (B) male fathead minnows exposed to five concentrations of spironolactone (0, 0.05, 0.5, 5, and 50 $\mu\text{g L}^{-1}$). The amount of variation captured by each component is indicated on its respective axes.

Figure 2: Measured versus predicted testosterone levels in plasma from male fathead minnows exposed to five concentrations of spironolactone (0, 0.05, 0.5, 5, and 50 $\mu\text{g L}^{-1}$). Predicted values were calculated based on the best-fitting partial least squares (PLS) regression that compared changes in endogenous metabolites from livers and testosterone in plasma from male fathead minnows. See Table S2 for model-fitting metrics. The dashed line is the 1:1 line. The solid line and associated statistical results represents the fit of the simple linear regression that compared predicted and measured values.

Figure 3: Measured versus predicted (A) VTG mRNA from livers, (B) plasma VTG, (C) plasma testosterone, and (D) plasma E2 from female fathead minnows exposed to 5 concentrations of spironolactone (0, 0.05, 0.5, 5, and 50 $\mu\text{g L}^{-1}$). Predicted values were calculated based on the best-fitting partial least squares (PLS) regression that compared changes in endogenous metabolites from livers and biological endpoints in female fathead minnows. See Table S2 for model-fitting metrics. The dashed line is the 1:1 line. The solid line and associated statistical results represents the fit of the simple linear regression that compared predicted and measured values.

Figure 4: Measured versus predicted fecundity from female fathead minnows exposed to 5 concentrations of spironolactone (0, 0.05, 0.5, 5, and 50 $\mu\text{g L}^{-1}$). Predicted values were calculated based on the best-fitting partial least squares (PLS) regression that compared changes in endogenous metabolites from livers and fecundity in female fathead minnows. See Table S2 for model-fitting metrics. The dashed line is the 1:1 line. The solid line and associated statistical results represents the fit of the simple linear regression that compared predicted and measured values.

Figure 5: Percent change (mean $\pm$ SE) values for relative abundances of (A) L-carnitine, (B) hexanoylcarnitine, (C) acetylcarnitine, (D) propionylcarnitine, (E) 2-methylbutyrylcarnitine, and (F) methionine in female fathead minnows. A positive percent change value indicates that the relative intensity of a given metabolite increased with spironolactone exposure and a negative value indicates that it's intensity decreased in response to spironolactone exposure (relative to the control group). Symbols indicate a significant difference between a particular exposure group of females and control females based on ANOVA and post-hoc tests (* = q-value < 0.05 ; # = q-value < 0.10).

Figure 1:

Figure 2:

Figure 3:

Figure 4:

Figure 5: