Metabolite profiles of repeatedly sampled urine from male fathead minnows (*Pimephales promelas*) contain unique lipid signatures following exposure to anti-androgens

Timothy W. Collette^{a*}, David M. Skelton^{a1}, John M. Davis^a, Jenna E. Cavallin^b, Kathleen M. Jensen^b, Michael D. Kahl^b, Daniel L. Villeneuve^b, Gerald T. Ankley^b, Dalma Martinović-Weigelt^c, and Drew R. Ekman^a

^a*U.S. EPA, National Exposure Research Laboratory, Athens, GA USA 30605*

^b*U.S. EPA, National Health and Environmental Effects Research Laboratory, Duluth, MN USA 55804*

^c*University of St. Thomas, 2115 Summit Ave. Saint Paul, MN USA 55105*

¹ Current Address: U.S. FDA, Cincinnati OH USA 45237

Running title: Urinary metabolite profiles from fathead minnows exposed to anti-androgens

* Corresponding author. Timothy W. Collette, U.S. EPA, National Exposure Research Laboratory, 960 College Station Rd., Athens, GA USA 30605, Tel.: +1 706 355 8211 Fax +1 706 355 8302, collette.tim@epa.gov

This paper is a contribution to the special issue on Environmental Omics and Toxicology

Abstract

The purpose of this study was twofold. First, we sought to identify candidate markers of exposure to anti-androgens by analyzing endogenous metabolite profiles in the urine of male fathead minnows (mFHM, *Pimephales promelas*). Based on earlier work, we hypothesized that unidentified lipids in the urine of mFHM were selectively responsive to exposure to androgen receptor antagonists, which is otherwise difficult to confirm using established fish toxicity assays. A second goal was to evaluate the feasibility of non-lethally and repeatedly sampling urine from individual mFHMs over the time course of response to a chemical exposure. Accordingly, we exposed mFHM to the model anti-androgens vinclozolin or flutamide. Urine was collected from each fish at 48 hour intervals over the course of a 14 day exposure. Parallel experiments were conducted with mFHM exposed to bisphenol A or control water. The frequent handling/sampling regime did not cause apparent adverse effects on the fish. Endogenous metabolite profiling was conducted with gas chromatography-mass spectrometry (GC-MS), which exhibited lower variation for the urinary metabolome than was found in earlier work with nuclear magnetic resonance (NMR) spectroscopy. Specifically, for inter- and intra-individual variation, the median spectrum-wide relative standard deviation (RSD) was 32.6% and 33.3%, respectively, for GC-MS analysis of urine from unexposed mFHM. These results compared favorably with similar measurements of urine from other model species, including the Sprague Dawley rat. In addition, GC-MS allowed us to identify several lipids (e.g., certain saturated fatty acids) in mFHM urine as candidate markers of exposure to androgen receptor antagonists.

Keywords: anti-androgens, fathead minnows, metabolite profiling, metabolomics, toxicogenomics

Abbreviations: GC-MS, gas chromatography-mass spectrometry; mFHM, male fathead minnows; NMR, nuclear magnetic resonance spectroscopy

1. Introduction

Endogenous metabolite profiling, or metabolomics, has emerged as a useful tool for toxicological and disease-state studies targeted at both humans (Bouhifd et al., 2013; Emwas et al., 2013) and ecologically-relevant organisms (Bundy et al., 2009). Using nuclear magnetic resonance (NMR) spectroscopy- or mass spectrometry (MS)-based platforms, metabolomic studies are now carried out on a wide variety of biological tissues and fluids. For application to human health, urine has proven to be a very useful matrix (Ryan et al., 2011), in part because urine is indicative of an organism's integrated metabolic function. As a result, it has shown great utility for investigating multiple organ responses to toxicity or disease. When using humans or rodents as test subjects, urine is also an advantageous matrix from a practical analytical standpoint – it contains numerous abundant endogenous metabolites and requires minimal sample preparation. Another very important advantage is that urine can be collected non-invasively and, hence, repeatedly from the same test subject. This allows assessment of intra-individual biological variation in unstressed animals, which can help contextualize changes associated with disease or toxicant exposure. Furthermore, temporal responses to chemical-induced toxicity can be tracked by obtaining multiple urine samples from the same individual before, during, and after exposure to a stressor. Studies of this type have yielded a wealth of information, with application to human toxicology (Azmi et al., 2002).

Environmental (or ecological) metabolomics is generally concerned with metabolic responses of organisms other than humans to stressors that they might encounter in the environment (Viant, 2007). A wide variety of species have been studied (Bundy et al., 2009), with a considerable focus on fish (Ekman et al., 2009; Ekman et al., 2011; Samuelsson and Larsson, 2008). Our group has advanced the notion that urine from male fathead minnows (mFHM) may offer many of the advantages for environmental metabolomics that have been demonstrated in studies with rodents and humans. However, endogenous metabolites are far less concentrated in urine from freshwater fish as a result of living in a hypotonic environment. Also, with comparatively small fish such as the FHM, urine collection is non-trivial, and somewhat limited quantities ($\sim 30 \mu\text{L}$) can be obtained, and only from males. But, in spite of these limitations, we have demonstrated the feasibility, and also some important advantages, of mFHM urine as a biofluid for NMR-based metabolomic studies, using optimized sample collection, processing and analysis approaches (Collette et al., 2010; Ekman et al., 2012; Ekman et al., 2007; Martinović-Weigelt et al., 2012). One of our important findings is that urine may be particularly useful for directly detecting and characterizing the impacts of chemicals that function as anti-androgens (specifically, androgen receptor (AR) antagonists) in mFHM (Collette et al., 2010; Ekman et al., 2012; Ekman et al., 2007).

At present, there are several relatively simple and unambiguous whole-animal assays that can be conducted with FHM to establish some endocrine-active chemicals' primary mechanism-of-action (MOA). This includes a test for AR agonists, which induce male-specific secondary sex characteristics in females, and a test for estrogen receptor (ER) agonists, which cause induction of the egg yolk precursor protein vitellogenin (VTG) in males (Ankley and Jensen, 2014). However, direct measurement of responses to AR antagonists using whole-animal assays

with FHM has not been as simple, specific, or unambiguous (Ankley et al., 2010). This has made it more difficult within the aquatic toxicology community to firmly establish that a chemical exhibits this important MOA. Thus, the capability to directly detect AR antagonistic behavior at the molecular level with metabolomics is quite promising.

Our ability to assign the anti-androgenic MOA using NMR-based metabolomics has largely involved observing systematic changes in lipids in urine from mFHM exposed to model anti-androgens. But, unfortunately, NMR is not well-suited for specific identification of individual lipids, due, in large part, to spectral crowding. Furthermore, lipids in FHM urine have relatively low abundance, and are observed only slightly above the limit of detection in our NMR analyses. Therefore, while we have been able to associate patterns of changes in lipids with exposure to known anti-androgens; we have not been able to uniquely identify individual lipids that might serve as markers for this important MOA, due, in part, to the limitations of NMR with regard to sensitivity and spectral overlap.

On the other hand, gas chromatography-mass spectrometry (GC-MS) is well suited for analysis of lipids below approximately 500 Da (Sandra and Sandra, 2013). The MS platform is inherently more sensitive than NMR, and the chromatographic separation step in GC-MS can also provide better selectivity. Also, carbon chain length, which is difficult to discern with NMR-based mixture studies, can usually be determined with certainty with GC-MS. These advantages, coupled with the availability of excellent gas-phase MS spectral libraries, often enable confidence of identification with GC-MS that is not typically obtainable with NMR-based metabolomics. This is particularly true for lipids. For these reasons, in the present study, we have analyzed urine from mFHM exposed to the model anti-androgens vinclozolin (VIN, at two different concentrations) and flutamide (FLU), using GC-MS-based metabolomics. We have also

conducted exposures using bisphenol A (BPA), which in addition to being estrogenic, also antagonizes AR function by competitive inhibition of androgen binding and inhibits the nuclear translocation of AR (Rubin, 2011; Teng et al., 2013). In our earlier metabolomics studies, BPA was found to behave as an anti-androgen in female FHM at the level of 100 µg/L, but not at 10 µg/L (Ekman et al., 2012). For the present study, we exposed mFHM to these two different concentrations of BPA, two concentrations of VIN, or FLU in an effort to identify specific lipids in urine that may be indicative of the anti-androgenic MOA.

Another important goal of this study was to assess our ability to sample urine non-lethally, and repeatedly, from mFHM. In our past work, urine was collected after fish were anesthetized, prior to dissection (for collecting other fluids and tissues) (Collette et al., 2010; Ekman et al., 2007). For the present study, we collected urine eight times from each individual fish (at 48 hr intervals) over the course of the chemical exposures. Individuals were removed from test aquaria and lightly anesthetized, urine was expressed, and the fish were returned to the aquaria. In addition, a control group of fish was likewise sampled. This allowed us to determine whether this sampling regime might adversely affect the fish, assess the variability associated with repeat sampling of mFHM urine, and observe the trajectory of metabolic responses to chemical exposures over time.

2. Materials and methods

2.1. Experimental design and mFHM urine sample collection

Sexually-mature (ca. 5-6 month old) FHM were from an on-site culture facility at the Duluth, MN EPA laboratory. Prior to the study, fish were held in a mass culture, which is a

setting that is not conducive to reproduction. To initiate the experiments, two males and two females were placed together in glass test tanks holding 10 L of Lake Superior water (4 μ M filtered, UV sterilized), which was continuously renewed at a rate of ca 45 mL/min for the duration of the study (complete tank renewal was achieved every 3.5 h). Each tank contained a mesh divider, with one male, one female and a breeding tile in each side of the tank. (Females were included in the tank not for analysis, but to help ensure normal male reproductive behavior and endocrinology.) Fish were acclimated to the tanks for one week prior to initiating the study. For each “treatment condition” there were five such tanks, yielding an N of 10 per treatment per sex. There were a total of seven treatment groups. One treatment group was a “non-repeat-sampled” control that experienced no test chemical exposure (i.e., held in the control Lake Superior water) and was not sampled until termination of the experiment. For the remaining six groups, urine was collected from each male every 48 h following initiation of the chemical exposures, which lasted for 14 d. These six groups included a repeat-sampled control (CON) that experienced no test chemical exposure, but was sampled in the same fashion as the chemically exposed groups. The five remaining groups included individuals that were exposed to: bisphenol A at a target concentration of 10 μ g/L (BPA-LO), bisphenol A at a target concentration of 100 μ g/L (BPA-HI), flutamide at a target concentration of 500 μ g/L (FLU), vinclozolin at a target concentration of 200 μ g/L (VIN-LO), and vinclozolin at a target concentration of 700 μ g/L (VIN-HI). These test concentrations were based on previous work focused on anti-androgenic properties of the three chemicals in the FHM (Ankley et al., 2004; Ankley et al., 2010; Martinović et al., 2008). For all of the test chemical exposures, solvent-free solutions were prepared as concentrated stocks in Lake Superior water. These stock solutions were then introduced into the continually flowing lines that supplied Lake Superior water to the appropriate

exposure tanks in order to achieve the targeted test chemical exposure concentrations. Both BPA and FLU were 99% purity and supplied by Sigma-Aldrich, St. Louis, MO, USA. VIN was 99% purity and supplied by Chem Service, West Chester, PA, USA.

Stock solution and tank water concentrations of the test chemicals were measured on days 1, 2, 6, 8, 12, and 14 of the exposures using methods and quality assurance procedures described in detail elsewhere (Ankley et al., 2004; Ankley et al., 2010; Martinović-Weigelt et al., 2011; Martinović et al., 2008). Briefly, all analyses were done on an Agilent LC-MSD using both atmospheric pressure photoionization (at 227 nm for BPA and 316 nm for VIN) and diode-array (at 310 nm for FLU) detectors using a gradient program with methanol and water. An external method of quantification was used. A Lake Superior water blank, chemical-spiked water sample, and a duplicate from one of the exposure tanks were analyzed with each set of samples. The target concentrations for BPA and FLU were well achieved and quite stable over the entire course of the exposures. However, the VIN concentrations were about a factor of two below the target levels during the early stages of the test, and further diminished at the later time points. A summary of these measured tank water concentrations of the test chemicals is shown in Table SII in Supplemental Information.

After all tanks were populated, the fish were allowed to acclimate for 5 d prior to initiation of the test chemical exposures. Urine was sampled for all of the males on the fourth day of the acclimation period, then once just before initiation of the exposures (d 0), and then six more times from each individual male fish (at 48 h intervals, and at approximately the same time of day) over the course of the chemical exposures. Individuals were removed from test aquaria and lightly anesthetized with buffered tricaine methane sulfonate (MS-222; 100 mg/L buffered with 200 mg/L NaHCO₃; Finquel, Argent, Redmond, WA, USA). Urine was expelled by

applying gentle pressure on the fish's abdomen and collected using non-heparinized micro-capillary tubes (Ekman et al., 2007; Martinović-Weigelt et al., 2012). The urine was immediately transferred to a 2 mL Safe-Lock tube (Eppendorf North America, Hauppauge, NY, USA), placed on dry ice, and stored at -80°C. Every effort was made to avoid contaminating the urine with sperm, which can enter samples during the later phase of the collection process (Martinović-Weigelt et al., 2012). Finally, the fish were returned to the aquaria and the test chemical exposure continued. Recall that, in addition to the fish exposed to the chemicals, a repeat-sampled control group of fish was likewise sampled. The exposures were terminated after 14 days when fish were anaesthetized with MS-222 and not allowed to recover. Also, at this time, urine from the non-repeat sampled males was collected for the first time.

2.2. *mFHM urine sample preparation*

Male FHM urine samples of 5 – 10 µL were centrifuged at 15400 x g for 10 min at 4°C to remove particulates and then transferred to 2 mL amber glass HPLC-vials with fused 300 µL conical inserts and dried using a vacuum concentrator (Thermo Scientific, Waltham, MA, USA). A pool of control male FHM urine was uniformly aliquoted and used to generate quality control samples.

We were not able to collect a sufficient amount of urine to prepare an acceptable sample for GC-MS analysis from all fish at all sampling times. In addition, a few samples were lost due to sampling or processing errors. However, for the control fish and for all of the chemical exposures, the number of samples included in our analysis (N) ranged from 7 to 10 per sampling event, except for the VIN-HI exposure. For the case of VIN-HI, our ability to collect a sufficient

amount of urine decreased at the later time points, yielding an N of 10, 5, 5, 3, 3, 4, 4 and 5 for exposures days 0, 2, 4, 6, 8, 10, 12 and 14, respectively.

2.3. *Gas chromatography – mass spectrometry*

The dried urine samples were reconstituted in 10 μ L N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS). The samples were vortexed for 30 sec and incubated at 70°C for 30 min. Derivatized samples were analyzed in random order on an Agilent 6890N gas chromatograph connected to an Agilent 5973 mass spectrometer using a DB-5MS stationary phase column (30m length, 0.25 mm i.d., 0.25 μ m film thickness, and 10 m Duraguard; Agilent J&W Scientific, Folsom, CA, USA). A volume of 1 μ L was injected in pulsed splitless mode at 35 psi for 1.50 min using an empty hot needle technique (Grob and Rennhard, 1980). Helium was used as the carrier gas at a constant flow of 1 mL/min. The injector, transfer line, and ion source were all maintained at 250°C. The oven was maintained at 60°C for 2 min and then increased at 10°C/min to 325°C where it was maintained for 1 min. The solvent delay was 8 min. The MS was operated in electron ionization mode at 70 eV and data acquisition was performed in full scan mode from m/z 85-500 at 4 scans s^{-1} .

The GC-MS chromatograms were subjected to baseline correction, background and noise reduction, peak smoothing and picking, and retention time alignment using MetAlign software (www.metalalign.wur.nl) (Lommen and Kools, 2012), and ion groups assembled into metabolites using the MSClust software (www.metalalign.wur.nl) (Tikunov et al., 2012). Peaks with signal-to-noise (S/N) ratios less than three were rejected. Only metabolites consistently detected in at least 80% of a class were retained (Bijlsma et al., 2006). Metabolites with relative standard deviations (RSD) of greater than 30% in the quality control samples were excluded from the final dataset

(Dunn et al., 2011). Putative annotation of metabolites was conducted by comparing the retention index (Δ of ± 10) and EI mass spectrum (similarity $\geq 70\%$) to those recorded in the NIST02, Wiley 7, and Golm Metabolome Database mass spectral libraries (Kopka et al., 2005). Ions unique for each metabolite were used for relative quantification. Artifact peaks arising from plasticizers, column bleed, and MSTFA reagent, as well as peaks observed in our method blanks were excluded. Following these steps, the final dataset contained a total of 99 unique metabolites, 30 of which were putatively annotated. Normalization to a constant sum of all metabolites in each sample was performed using Microsoft[®] Excel (Microsoft Corporation, Redmond, WA, USA) prior to statistical analyses.

2.4. *GC-MS data analysis*

GC-MS data were imported from Excel into SIMCA-13.0.2 (Umetrics Inc., Umea, Sweden) for multivariate data analysis, which was conducted on mean-centered and unit-variance-scaled variables. Scores plots from principal component analysis (PCA) models were used to visualize both variation of metabolite profiles within given classes, and also the trajectory of responses over time due to the chemical exposures. Note that we collected urine every 48 hours over the 14 day exposure for all of the exposure classes, and also for the repeat-sampled control class. However, due to limitations in personnel schedules, we were not able to have a single analyst conduct all of the urine collections. Specifically, our most experienced analyst collected all of the urine on days 0, 2, 4, 6, and 10, while a second analyst conducted all collections on days 8 and 12. A third analyst conducted collections on day 14 when the exposures were terminated. From the initial PCA model for the repeat-sampled control (unexposed) class, we observed a strong influence of the analyst who collected urine on the

metabolite profile. These same trends were observable for all treatments. For this reason, we restricted further data analysis to only those time points where urine was collected by the most experienced analyst. Another benefit of limiting our analysis to these time points is elimination of concern over the decline in tank water concentrations of VIN observed at days 12 and 14.

To identify potential biomarkers of anti-androgen exposure, we used univariate analysis of relative metabolite abundances (using Excel) within each exposure class (including repeat-sampled controls). For these analyses, a Student's t-test was conducted for each metabolite, comparing metabolite levels from day 0 (before exposure) to those from day 2 of a given exposure. Day 2 was chosen in part because, as discussed later, direct biological effects of endocrine-disrupting chemicals (EDCs) have typically been found to occur within 48 hours or less (Ankley and Villeneuve, 2015). To correct for multiple hypothesis testing, we then used the 'fdrtool' package (R v. 3.1.2) to calculate FDR-corrected p-values (i.e., q-values) based on those generated by the Student's t-test (Strimmer, 2008). A list of metabolites that exhibited FDR-corrected p-values < 0.05 was assembled for each exposure class. Since any metabolites that were observed to change in the repeat-sampled controls would not be due to anti-androgenic impacts, they were omitted from further consideration in this part of the study.

For investigating the inter-individual variation in metabolite profiles among a given group of unexposed fish, and for tracking intra-individual variation in unexposed individuals over time, we used the method of spectrum-wide relative standard deviation (RSD). This approach, which was developed for benchmarking metabolomic datasets, has been described in detail elsewhere (Parsons et al., 2009). Briefly, for inter-individual variation among N members of a given class, the RSD (expressed as a percentage) is calculated for each metabolite across all N members. The median value for all of the metabolite-specific RSDs (99 of them, in this case),

is the statistic that is used as a metric for comparison. For intra-individual variation across N sampling events over some elapsed time, the RSD is calculated for each metabolite across all N sampling events for a given individual. Again, the median value of the metabolite-specific RSDs is used as the relevant metric. For the present study, there were seven individuals in the control class for which urine was collected for all five of the sampling events included in the final dataset. In order to maximize consistency with a NMR / rat urine data set, which, as described below, was used as a benchmark of comparison, we limited the analysis of RSD in the GC-MS / mFHM urine dataset to five individuals because only five rats were used in the experiment that yielded the NMR / rat urine dataset. To determine which five of the seven mFHMs to include, we first calculated intra-individual variation for all seven individuals in the control class for which urine was collected for all five of the sampling events. The individual with the lowest median RSD and the individual with the highest median RSD were excluded from the final RSD calculations.

2.5. *NMR analysis of rat urine*

NMR analysis of male Sprague Dawley rat urine, which is a widely used and well-established approach in metabolomics, served as a useful benchmark through which to compare variability of GC-MS analysis of mFHM urine. To make this comparison, we used NMR spectra that were collected for, and reported in, an earlier study from our laboratory; details on experimental conditions, sample collection, data acquisition, and pre-processing can be found there (Cherney et al., 2007). Briefly, for the present study, we used spectra that were previously collected for the five individuals that constituted the control (unexposed) group in our earlier toxicology study (Cherney et al., 2007). Urine was collected daily for six consecutive days.

Bacterial growth was controlled throughout the collection times by maintaining urine at 0°C and by the addition of 1mL of a 1% sodium azide solution. Preparation of rat urine samples was as follows: 250 µL of 0.1M sodium phosphate buffered deuterium oxide (pH 7.4) containing 3mM sodium azide and 1mM sodium 3-(trimethylsilyl) propionate-2,2,3,3-d₄ (TSP) was added to 400 µL of urine for each animal. Each sample was then centrifuged at 10,600 rcf for 10 min at 4°C to remove solids and the resulting supernatant was pipetted into a standard 5mm NMR tube.

1D ¹H-NMR spectra for rat urine samples were acquired at 25°C on a Varian Inova 800 NMR spectrometer (799.73 MHz, ¹H) using a cryogenic triple resonance probe. All spectra were acquired using a standard 1D pulse sequence with pre-saturation of the water resonance. Acquisition parameters for these spectra include a spectral width of 10,000 Hz and an acquisition time of 2 sec. Sixty-four transients were collected for each spectrum. Processing included zero filling to 64K points and application of 0.5 Hz line broadening. NMR spectra were phased, baseline corrected, and referenced to TSP. Then, resonances from the carrier vehicle, urea, and water were excluded during binning. The remaining spectral data from 0.5 to 9.5 ppm were reduced by segmenting into bins of 0.005 ppm in width, and then normalized to unit total intensity. All spectral processing and binning were done using the ACD/SpecManager software package (Advanced Chemistry Development, Toronto, Canada). The reduced dataset was then imported into Excel.

The NMR rat urine dataset was exported from Excel into SIMCA-13.0.2 (Umetrics Inc., Umea, Sweden) for PCA analysis, which proceeded as described above for the GC-MS mFHM urine dataset, except that the NMR data were Pareto-scaled. In addition, RSDs were determined for the NMR rat urine dataset in the same fashion as described for the GC-MS mFHM dataset, except that bins containing only noise were removed from the NMR data, leaving only signal-

containing bins. The process for identifying noise-only bins is described in the original report of the spectrum-wide RSD method for benchmarking metabolomic data sets (Parsons et al., 2009).

3. Results and Discussion

3.1 Urine metabolite profile variability of repeat-sampled controls

Assessing our ability to repeatedly sample urine from individual live fish over a substantial course of time (e.g., 1-2 weeks) was an important objective for this study. For example, we wanted to evaluate the extent of inter- and intra-individual variation that would be encountered in control mFHM due to repeated episodes of anesthetizing the fish, expressing the urine, and then returning the fish to the exposure tank. As with all metabolomics experiments, this type of biological variation (along with the technical variation, which is usually much smaller) will set the limit at which one is able to determine meaningful metabolite differences that result from, for example, a chemical exposure.

We (and others) have advocated the use of spectrum-wide RSD (also commonly called coefficient of variation, CV), as a way to quantify unwanted variation in metabolomics, and as a benchmark in efforts to minimize it (Parsons et al., 2009). Metabolomic studies involve the measure of a large number of metabolites. Each metabolite will exhibit some amount of inter-individual variation in relative abundance among individuals of a given class (e.g., the control class). In addition, if repeatedly sampling a single individual over time, intra-individual variation in an unexposed animal will also be observed. For NMR-based metabolomics, where spectra are binned (or bucketed), this variation will be manifested as differences in the relative intensities of individual bins. For MS-based metabolomics utilizing chromatographic separation,

the variation will be observed as differences in relative intensities of individual metabolite peaks. The benchmark that is useful for evaluating these data is the median RSD (expressed as a percentage) when considering all detected MS peaks, or all signal-containing NMR bins.

When previously developing median RSD as a benchmark for metabolomics, we compared 10 different datasets, spanning a wide range of sample types (e.g., urine, plasma, and tissue extracts), including those from fish, invertebrates, mammals, and a cell line (Parsons et al., 2009). One of the datasets in that study was from NMR analysis of mFHM urine from this laboratory. That previous dataset exhibited a median RSD of 52.9% for inter-individual variation, which was among the highest observed for all individual species/sample-type datasets we evaluated. The finding was consistent with a general trend of higher variation observed with biological fluids as compared to tissue extracts, presumably because tissues are under greater homeostatic control. In addition, we also found predictable differences among different species (Parsons et al., 2009). For example, variation was considerably lower for NMR analysis of urine from male Sprague Dawley (S-D) rats than for that of mFHM. This is likely due, at least in part, to the more highly in-bred conditions of the rats, as well as the more controlled conditions under which they are reared and housed. In addition, urine must be manually expressed from mFHM while they are anesthetized, which is not the case with rats. Furthermore, one factor contributing to the higher variation with mFHM urine is likely related to the dramatically lower level of metabolites, as compared to rats. This situation was exacerbated in our earlier work by the relatively low sensitivity of NMR. It was our hope that lower variation would be observed in the present study due to the higher sensitivity of GC-MS.

For control mFHM, we found a median RSD of 32.6% for inter-individual variation using GC-MS (Table 1), which is considerably lower than the value of 52.9% that we observed

in a previous study using NMR. While a number of factors could contribute to this difference (e.g., fewer “signal bins” and fewer individuals for GC-MS), we believe an important factor is, indeed, the higher sensitivity of GC-MS relative to NMR, given the relatively dilute fish urine samples. Other important comparisons in Table 1 are those of inter- and intra-individual variations for GC-MS analysis of mFHM urine relative to NMR analysis of male Sprague Dawley rat urine, a useful benchmark for urine-based metabolomic studies. Since all of these values were determined in the present study, we were able to match – to the extent possible – complicating factors that might otherwise influence measures of variation (e.g., number of individuals, duration of time, and number of samples for the intra-individual variation, etc.) For both inter- and intra-individual variation, the median RSD for GC-MS analysis of mFHM urine (32.6% and 33.3%, respectively) was found to be only slightly higher than that for NMR analysis of male rat urine (26.4% and 30.9%, respectively). In addition, it is interesting to note that, for males from a given species (rat or mFHM), inter- and intra-individual variation is quite similar, particularly in the case of mFHM. This suggests that – at least over this time frame – it is neither more nor less advantageous, from a standpoint of variability, to track temporal responses in a given individual, as opposed to aggregating responses among cohorts at a given time.

Table 1 also contains measures of variation found in the literature for NMR analysis of human and guinea pig urine. It is more difficult to make direct comparisons of our present results with these values however, because the studies differ in important ways. For example, with humans, diet is not controlled, and time elapsed for the intra-individual component of the human study was significantly longer than for the present study. Nonetheless, it is interesting to note that the median RSDs for GC-MS analysis of mFHM is considerably lower than those reported for both humans and guinea pig.

Another useful way to compare variation over time for the GC-MS analysis of control mFHM urine with the NMR analysis of control male rat urine is via principal component analysis (PCA). While less quantitative than RSDs, PCA is useful for detecting variation in a dataset, which can then be easily displayed in a visually informative manner using scores plots. Note that there are some clear differences in the trends from the scores plots from these two datasets (Figure 1). For example, more of the variation in the dataset is captured in the first principle component (PC1) for the rat urine analysis. But, importantly, the extent of change over time, relative to the variation among cohorts at a given time (as depicted by standard error of the mean (SEM) error bars), is comparable for the two sets of analyses.

3.2 *Metabolite profile trajectories from chemical exposures*

PCA analysis of the GC-MS data yielded trends over time (i.e., metabolic trajectories) that exhibited some interesting differences relative to the chemical exposures. Scores plots for PCA analysis of the four most impactful exposures – BPA-HI, FLU, VIN-LO, and VIN-HI – are shown in Figure 2. (The BPA-LO exposure, which did not yield changes over time in the PCA analysis that were more extensive than for the CON fish, is shown in Figure SI1 of Supplemental Information.) It is important to recall that, for a given scores plot (Figure 2), all markers arise from the same group of fish, which were sampled five separate times, before (day 0) and during (days 2-10) the chemical exposure.

For the trajectory of BPA-HI (Figure 2A), there was a discernable impact from the exposure at day 2, based on the separation of the day 2 data from that of day 0. This impact remained relatively constant for days 4 and 6, but increased notably (and in the same “direction” in PCA space) at day 10. For the FLU exposure (Figure 2B), the impact was large at day 2 and

remained relatively constant for days 4 and 6. However, by day 10, the large initial impact is clearly diminished as the metabolite profile of the fish on day 10 was more similar to that of day 0 than that of day 2. For the VIN-LO exposure (Figure 2C), we see a discernable response at day 2, which increases slightly (and in the same direction) throughout the remaining sampling periods. With VIN-HI (Figure 2D) we see a large impact at day 2, which persists throughout the exposure.

3.3 *Trends in metabolite changes at day 2 of the exposures*

A key goal of this study was to identify putative endogenous metabolite-based markers that might be indicative of exposure to anti-androgens. Based on our earlier work, we suspected that some types of lipids (that we were previously unable to uniquely identify) might be potential candidates (Collette et al., 2010; Ekman et al., 2012; Ekman et al., 2007). After an initial analysis of the wealth of information collected on temporal changes in metabolite profiles in the present study, we decided to focus on a comparison of day 2 with day 0 to investigate biomarkers. As seen in the metabolite profile trajectories (Figure 2), some of the exposures appeared to exhibit recovery at later time points, while others exhibited an enhancement of effect. We believed that the most meaningful indicator of the chemical's primary MOA would likely be obtained during the initial phase of the impact, prior to the onset of any of these secondary mechanisms. This is consistent with the findings of Ankley and Villeneuve (2015) who recently published a meta-analysis of dose-response and time-course data on exposure of FHM to eight model EDCs. They found that direct biological effects of EDCs typically occurred within 24 to 48 h (or less), and that system recovery was generally observed at later time points. In addition, there are obvious practical advantages to a short time-course exposure when

developing a potential method for detecting and characterizing a specific MOA. For example, a shorter time course provides for higher-throughput and greater ease of implementation, and minimizes the chance that ontogeny (i.e., developmental changes) will confound the results.

To determine if a metabolite was changing due to chemical exposure, we first calculated an FDR-corrected p-value for that metabolite at day 2 versus day 0 for a given exposure. We then calculated the fold changes using the average relative intensities for all significantly changing metabolites. This process was conducted independently for all exposure classes. Results of these analyses for FLU, VIN-LO, and VIN-HI are displayed as a set of three stacked “bar graphs” – one for each of these three exposures – in Figure 3. For a given graph, a bar above the zero line represents a metabolite that significantly increased upon exposure, while a bar below the zero line represents a metabolite that significantly decreased. Note that if a given metabolite was not found to change with statistical significance for at least one exposure, it is not displayed in Figure 3. While the FLU exposure clearly had the largest impact on the metabolome (42 significant metabolite changes; Figure 3), the VIN-HI exposure also had considerable impact (31 significant changes). The VIN-LO exposure produced numerous (13) significant metabolite changes; however, the number of metabolites impacted was substantially less than observed for FLU or VIN-HI. Conversely, both BPA-LO and BPA-HI exposures produced very little response at day 2 – one and zero significant metabolite changes, respectively – and, thus, are not displayed in Figure 3. The one significant metabolite change observed for BPA-LO was indole-3-acetic acid, exhibiting a fold change of -2.28.

Examination of individual metabolite changes reveals considerable similarity between the FLU and the two VIN exposures (Figure 3), likely reflecting the shared MOA for these model anti-androgens. First, it is interesting to note that when a metabolite change is observed for more

than one of these exposures, the change is always in the same direction. In other words, there is no case where a metabolite is observed to increase for one exposure and decrease for another exposure. Further, it is interesting to note that 24 of the 31 metabolite changes observed for VIN-HI also change (and in the same direction) for the FLU exposure. Likewise, nine of the 13 metabolite changes observed for VIN-LO are also altered (and in the same direction) for both the VIN-HI and the FLU exposures. These results are consistent with anti-androgenicity being the primary MOA for both FLU and VIN. On the other hand, there are a sizable fraction of the FLU metabolite changes (18 of the 42) that are not observed for any of the other exposures, suggesting that FLU is affecting other pathways as well.

There is a paucity of comprehensive mechanistic studies evaluating relative potency and/or comparative mechanisms of toxicity of FLU and VIN in fish. Nonetheless, our findings on trends in metabolite changes comport well with responses reported by one such study (Martinović-Weigelt et al., 2011) on the transcriptome of male zebrafish (*Danio rerio*; ZF) exposed to FLU or VIN for a similar duration, although at different chemical concentrations (632 µg/L for VIN and 1920 µg/L for FLU). Specifically, Martinović-Weigelt et al. (2011) observed considerable similarity in the types of changes in the ZF testis transcriptome for exposure to FLU and VIN, with expression profiles for the two chemicals being difficult to differentiate using hierarchical clustering analysis. Furthermore, the authors reported an overall greater impact from the FLU exposure as measured by the numbers of genes exhibiting altered expression levels. Note that for the present study, we used a higher concentration of VIN (700 µg/L) than of FLU (500 µg/L), yet FLU still produced a larger overall biological response as measured by the number of significant metabolite changes at day 2. Martinović-Weigelt et al. (2011) reported that the greater impact on the transcriptome from exposure to FLU (as compared to VIN) was

largely due to genes that were associated with a generalized response to toxicity and stress. These results, in addition to the metabolomic responses reported here, are consistent with both FLU and VIN functioning as anti-androgens, with FLU likely exhibiting additional MOA(s). In addition to these *in vivo* responses, high throughput *in vitro* toxicity testing from the US EPA's iCSS Dashboard report that FLU and VIN target the AR via common mechanisms, and that FLU was more biologically active (in 17.9% of the assays, many of which were indicative of general toxicity responses) than VIN (active in only 4.3% of the assays) (EPA, 2015). Collectively, these findings suggest that the metabolomic responses shared by FLU and VIN likely reflect anti-androgen activity, and thus support the utility of the specific urinary metabolite changes as suggestive of this perturbation of the AR.

3.4 *Specific metabolite changes for the anti-androgen exposures*

A comparison of metabolite changes observed for the FLU and VIN-HI exposures shows a concerted increase in select fatty acids and a fatty alcohol, namely: myristic acid (C14), palmitic acid (C16), stearic acid (C18), and stearyl alcohol (C18) (see Figure 3). These and other fatty acids/alcohols have been associated with anti-androgenic activity in rodents through inhibition of 5 α -reductase (*srd5a*), the enzyme catalyzing the reduction of testosterone (T) to dihydrotestosterone (DHT), the potent and physiologically important mammalian androgen (Liu et al., 2009). While 11-ketotestosterone (11KT) has long been accepted as the physiologically relevant androgen in teleosts, recognition of the relevance of *srd5a* and DHT in fish has increased considerably in recent years through the reporting of various isoforms of *srd5a* (and the mRNAs that code for them) in multiple tissues (i.e., brain, skin, liver, and gonad) (Martyniuk et al., 2013). In a recent study, DHT was detected at physiologically relevant concentrations in

FHM plasma and at relatively high levels in FHM testis (Margiotta-Casaluci et al., 2013). Moreover, in a separate study, Margiotta-Casaluci and Sumpter (2011) found that DHT had androgenic potency on par with 11KT in male FHMs, and greater potency than 11KT in females. What role the increases in the fatty acids/alcohol in mFHM urine might play in relation to the activity of *srd5a* and DHT is uncertain. However, one possibility is that the presence of increased fatty acids/alcohol in the urine reflects an adaptive mechanism in mFHM in response to anti-androgen exposure. Given the potential androgenic relevance of DHT, the presence of fatty acids that inhibit its synthesis might be particularly detrimental in conjunction with exposure to chemical anti-androgens. Therefore, their increased excretion into the urine might reflect an attempt to reduce endogenous inhibition of *srd5a* (and in turn decrease inhibition of DHT synthesis). In addition, it has been reported that FLU directly inhibits *srd5a* activity at higher concentrations (low to mid μM range) in mammalian systems (Graef et al., 1988; Lo et al., 2007). Again, any endogenous inhibition would potentially exacerbate the effects of FLU, potentially triggering the excretion of these fatty acids/alcohol. Clearly, further research is needed to test these hypotheses, and in the larger context, to determine what role DHT (and *srd5a*) plays in FHM endocrinology.

The increased levels of these fatty acids may also reflect a broader response to anti-androgen exposure, particularly when viewed alongside the concomitant decrease in glucose across all of the anti-androgen exposures (Figure 3). In light of recent reports in which ZF or FHM were exposed to these same anti-androgens, this effect may be due at least in part to changes in the effective activity of the enzyme 11 β -hydroxysteroid dehydrogenase (11 β -HSD) (Filby et al., 2007; Martinović-Weigelt et al., 2011; Martinović et al., 2008). In humans, 11 β -HSD is recognized for regulating glucose and lipid metabolism through regulation of the

cortisol/cortisone ratio (Groegebakan et al., 2012). Martinović-Weigelt et al. (2011), reported decreases in the expression of the gene for the 11 β -HSD2 isoform in the testis of male ZF exposed to either FLU or VIN for 14 days. Conversely, Martinović et al. (2008) and Filby et al. (2007) separately reported an increase in the expression of the 11 β -HSD2 transcript in the testis of male FHM exposed for 21 days to VIN or FLU, respectively. Despite this discrepancy in responses (which could be due to any number of differences in the studies: species, exposure duration, exposure concentration, etc.), altered 11 β -HSD2 expression appears to be a primary response to exposure to these anti-androgens in FHM and ZF (and perhaps teleosts in general). Given the importance of this enzyme in the regulation of glucocorticoid receptor activity (via binding to either cortisone (synthesized by the 11 β -HSD1 isoform) or cortisol (synthesized by the 11 β -HSD2 isoform)), altered transcript expression would likely produce impacts on the levels of lipids and glucose.

Phenylethanolamine (PEOH), which was upregulated in the FLU, VIN-LO and VIN-HI treatments, may also be of interest as a potential biomarker of anti-androgen exposure. PEOH can be synthesized by the action of dopamine β -hydroxylase (DBH) on phenylethylamine (PEA) (Boulton et al., 1985). Both, PEA and PEOH are endogenous trace amines (Shannon and Degregorio, 1982). The relative composition/quantity of trace amines in urine has been shown to be influenced by dominance status in FHM (Martinović-Weigelt et al., 2012), and is sexually dimorphic in rodents (Liberles and Buck, 2006), and may thus be associated with endogenous androgens. Furthermore, the activities of enzymes that are involved in the synthesis of PEA and PEOH (DBH, aromatic L-amino acid decarboxylase), and metabolism of PEA (monoamine oxidases) have been shown to be modulated by androgens (Bethea et al., 2015; Bustamante et al., 1989; Lopez-Contreras et al., 2008). Exposure to the anti-androgens di-(2 ethylhexyl) phthalate,

FLU, and VIN has been shown to alter the activity of trace amine-associated receptors and/or their signaling pathways (Skinner et al., 2013; Vo et al., 2009). Our finding of upregulation of PEOH in the FLU, VIN-LO, and VIN-HI treatments may provide further support that anti-androgens interact with trace amine metabolic and signaling pathways.

4. Conclusions

An important goal of this study was to evaluate the feasibility of repeatedly sampling urine from individual mFHM over the course of a chemical exposure study. Results were largely encouraging. For example, we found that the fish tolerated the repeat sampling regime in a seemingly robust manner; no adverse effects were apparent. Furthermore, variation in metabolite profiles (from unexposed fish) compared favorably with that of other model species. While repeat collection from individual fish over time did not substantially reduce (nor increase) the overall variation in the metabolite profile (compared to aggregating responses among individuals at a given time), this approach offers greater potential for detailed time-course characterization, using more-manageable study designs with smaller numbers of animals.

Another important goal of this study was to identify potential markers of exposure to anti-androgens among the urinary metabolites of mFHM. In our earlier work with NMR, we had hypothesized that certain unidentified lipids might be candidates. The advantages of GC-MS for analysis of lipids in urine allowed us to identify specific fatty acids/alcohols, whose increased abundance in urine was associated with exposure to known anti-androgens. While there are some biologically plausible explanations for these findings, at this point these are untested hypotheses and further investigation is needed to support or reject them. However, regardless of the mechanistic rationale, given the paucity of effective biomarkers for anti-androgen exposure,

further investigation of the overall sensitivity, specificity, and reproducibility of these responses would be worthwhile. For example, if these responses prove robust, it might be feasible to incorporate specific measures of these common metabolites into the fish short-term reproduction assay currently used for EPA's Endocrine Disruptor Screening Program.

Acknowledgements. J.M.D. was supported by the Great Lakes National Program Office and an appointment to the Postdoctoral Research Program at the National Exposure Research Laboratory, administered by Oak Ridge Institute for Science and Education (ORISE) through interagency agreement (DW8992298301) between U.S. DOE and U.S. EPA. JEC was also supported by an ORISE Fellowship. The views expressed in this article are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. Mention of trade names, products, or services does not convey, and should not be interpreted as conveying, official EPA approval, endorsement or recommendation.

Supplemental information for this article can be found online.

References

- Ankley, G.T., Defoe, D.L., Kahl, M.D., Jensen, K.M., Miracle, A., Hartig, P., Gray, L.E., Cardon, M., Wilson, V., 2004. Evaluation of the model anti-androgen flutamide for assessing the mechanistic basis of responses to an androgen in the fathead minnow (*Pimephales promelas*). Environ. Sci. Technol. 38, 6322-6327.
- Ankley, G.T., Jensen, K.M., 2014. A novel framework for interpretation of data from the fish short-term reproduction assay (FSTRA) for the detection of endocrine-disrupting chemicals. Environ. Toxicol. Chem. 33, 2529-2540.

- Ankley, G.T., Jensen, K.M., Kahl, M.D., Durhan, E.J., Makynen, E.A., Cavallin, J.E., Martinovic, D., Wehmas, L.C., Mueller, N.D., Villeneuve, D.L., 2010. Use of chemical mixtures to differentiate mechanisms of endocrine action in a small fish model. *Aquat. Toxicol.* 99, 389-396.
- Ankley, G.T., Villeneuve, D.L., 2015. Temporal changes in biological responses and uncertainty in assessing risks of endocrine-disrupting chemicals: Insights from intensive time-course studies with fish. *Toxicol. Sci.* 144, 259-275.
- Azmi, J., Griffin, J.L., Antti, H., Shore, R.F., Johansson, E., Nicholson, J.K., Holmes, E., 2002. Metabolic trajectory characterisation of xenobiotic-induced hepatotoxic lesions using statistical batch processing of NMR data. *Analyst* 127, 271-276.
- Bethea, C.L., Phu, K., Kim, A., Reddy, A.P., 2015. Androgen metabolites impact CSF amines and axonal serotonin via MAO-A and -B in male macaques. *Neuroscience* 301, 576-589.
- Bijlsma, S., Bobeldijk, L., Verheij, E.R., Ramaker, R., Kochhar, S., Macdonald, I.A., van Ommen, B., Smilde, A.K., 2006. Large-scale human metabolomics studies: A strategy for data (pre-) processing and validation. *Anal. Chem.* 78, 567-574.
- Bouhifd, M., Hartung, T., Hogberg, H.T., Kleensang, A., Zhao, L., 2013. Review: Toxicometabolomics. *J. Appl. Toxicol.* 33, 1365-1383.
- Boulton, A.A., Maitre, L., Bieck, P.R., Riederer, P., 1985. Neuropsychopharmacology of the trace amines. Experimental and clinical aspects. Humana Press, Clifton, New Jersey.
- Bundy, J.G., Davey, M.P., Viant, M.R., 2009. Environmental metabolomics: a critical review and future perspectives. *Metabolomics* 5, 3-21.
- Bustamante, D., Lara, H., Belmar, J., 1989. Changes of norepinephrine levels, tyrosine-hydroxylase and dopamine-beta-hydroxylase activities after castration and testosterone treatment in vas-deferens of adult-rats. *Biol. Reprod.* 40, 541-548.
- Cherney, D.P., Ekman, D.R., Dix, D.J., Collette, T.W., 2007. Raman spectroscopy-based metabolomics for differentiating exposures to triazole fungicides using rat urine. *Anal. Chem.* 79, 7324-7332.
- Collette, T.W., Teng, Q., Jensen, K.M., Kahl, M.D., Makynen, E.A., Durhan, E.J., Villeneuve, D.L., Martinovic-Weigelt, D., Ankley, G.T., Ekman, D.R., 2010. Impacts of an anti-androgen and an androgen/anti-androgen mixture on the metabolite profile of male fathead minnow urine. *Environ. Sci. Technol.* 44, 6881-6886.
- Dunn, W.B., Broadhurst, D., Begley, P., Zelena, E., Francis-McIntyre, S., Anderson, N., Brown, M., Knowles, J.D., Halsall, A., Haselden, J.N., Nicholls, A.W., Wilson, I.D., Kell, D.B., Goodacre, R., Human Serum Metabolome, H.C., 2011. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nat. Protoc.* 6, 1060-1083.
- Ekman, D.R., Hartig, P.C., Cardon, M., Skelton, D.M., Teng, Q., Durhan, E.J., Jensen, K.M., Kahl, M.D., Villeneuve, D.L., Gray, L.E., Collette, T.W., Ankley, G.T., 2012. Metabolite profiling and a transcriptional activation assay provide direct evidence of androgen receptor antagonism by Bisphenol A in fish. *Environ. Sci. Technol.* 46, 9673-9680.

- Ekman, D.R., Teng, Q., Jensen, K.M., Martinovic, D., Villeneuve, D.L., Ankley, G.T., Collette, T.W., 2007. NMR analysis of male fathead minnow urinary metabolites: A potential approach for studying impacts of chemical exposures. *Aquat. Toxicol.* 85, 104-112.
- Ekman, D.R., Teng, Q.N., Villeneuve, D.L., Kahl, M.D., Jensen, K.M., Durhan, E.J., Ankley, G.T., Collette, T.W., 2009. Profiling lipid metabolites yields unique information on sex- and time-dependent responses of fathead minnows (*Pimephales promelas*) exposed to 17 alpha-ethynylestradiol. *Metabolomics* 5, 22-32.
- Ekman, D.R., Villeneuve, D.L., Teng, Q., Ralston-Hooper, K.J., Martinovic-Weigelt, D., Kahl, M.D., Jensen, K.M., Durhan, E.J., Makynen, E.A., Ankley, G.T., Collette, T.W., 2011. Use of gene expression, biochemical and metabolite profiles to enhance exposure and effects assessment of the model androgen 17 beta-trenbolone in fish. *Environ. Toxicol. Chem.* 30, 319-329.
- Emwas, A.H.M., Salek, R.M., Griffin, J.L., Merzaban, J., 2013. NMR-based metabolomics in human disease diagnosis: applications, limitations, and recommendations. *Metabolomics* 9, 1048-1072.
- EPA, U.S., 2015. iCSS Dashboard, <http://actor.epa.gov/dashboard/>, accessed on 9/16/2015.
- Filby, A.L., Thorpe, K.L., Maack, G., Tyler, C.R., 2007. Gene expression profiles revealing the mechanisms of anti-androgen-and estrogen-induced feminization in fish. *Aquat. Toxicol.* 81, 219-231.
- Graef, V., Golf, S.W., Tyrell, C., Fehler, M., 1988. Effect of flutamide on 5-alpha reductases 5-beta reductases and 3 hydroxysteroid dehydrogenases in rat liver. *Steroids* 49, 553-560.
- Grob, K., Rennhard, S., 1980. Evaluation of syringe handling techniques for injections into vaporizing GC injectors. *Journal of High Resolution Chromatography* 3, 627-633.
- Groegebakan, O., Andres, J., Biedasek, K., Mai, K., Kuehnen, P., Krude, H., Isken, F., Rudovich, N., Osterhoff, M.A., Kintscher, U., Nauck, M., Pfeiffer, A.F.H., Spranger, J., 2012. Glucose-dependent insulinotropic polypeptide reduces fat-specific expression and activity of 11 beta-hydroxysteroid dehydrogenase type 1 and inhibits release of free fatty acids. *Diabetes* 61, 292-300.
- Kopka, J., Schauer, N., Krueger, S., Birkemeyer, C., Usadel, B., Bergmuller, E., Dormann, P., Weckwerth, W., Gibon, Y., Stitt, M., Willmitzer, L., Fernie, A.R., Steinhauser, D., 2005. GMD@CSB.DB: the Golm Metabolome Database. *Bioinformatics* 21, 1635-1638.
- Liberles, S.D., Buck, L.B., 2006. A second class of chemosensory receptors in the olfactory epithelium. *Nature* 442, 645-650.
- Liu, J., Shimizu, K., Kondo, R., 2009. Anti-androgenic activity of fatty acids. *Chem. Biodivers.* 6, 503-512.
- Lo, S., King, I., Allera, A., Klingmuller, D., 2007. Effects of various pesticides on human 5 alpha-reductase activity in prostate and LNCaP cells. *Toxicol. In Vitro* 21, 502-508.
- Lommen, A., Kools, H.J., 2012. MetAlign 3.0: performance enhancement by efficient use of advances in computer hardware. *Metabolomics* 8, 719-726.

- Lopez-Contreras, A.J., Galindo, J.D., Lopez-Garcia, C., Castells, M.T., Cremades, A., Penafiel, R., 2008. Opposite sexual dimorphism of 3,4-dihydroxyphenylalanine decarboxylase in the kidney and small intestine of mice. *J. Endocrinol.* 196, 615-624.
- Margiotta-Casaluci, L., Courant, F., Antignac, J.-P., Le Bizec, B., Sumpter, J.P., 2013. Identification and quantification of 5 alpha-dihydrotestosterone in the teleost fathead minnow (*Pimephales promelas*) by gas chromatography-tandem mass spectrometry. *Gen. Comp. Endocrinol.* 191, 202-209.
- Margiotta-Casaluci, L., Sumpter, J.P., 2011. 5 alpha-Dihydrotestosterone is a potent androgen in the fathead minnow (*Pimephales promelas*). *Gen. Comp. Endocrinol.* 171, 309-318.
- Martinović-Weigelt, D., Ekman, D.R., Villeneuve, D.L., James, C.M., Teng, Q., Collette, T.W., Ankley, G.T., 2012. Fishy aroma of social status: Urinary chemo-signalling of territoriality in male fathead minnows (*Pimephales promelas*). *Plos One* 7, e46579.
- Martinović-Weigelt, D., Wang, R.L., Villeneuve, D.L., Bencic, D.C., Lazorchak, J., Ankley, G.T., 2011. Gene expression profiling of the androgen receptor antagonists flutamide and vinclozolin in zebrafish (*Danio rerio*) gonads. *Aquat. Toxicol.* 101, 447-458.
- Martinović, D., Blake, L.S., Durhan, E.J., Greene, K.J., Kahl, M.D., Jensen, K.M., Makynen, E.A., Villeneuve, D.L., Ankley, G.T., 2008. Reproductive toxicity of vinclozolin in the fathead minnow: Confirming an anti-androgenic mode of action. *Environ. Toxicol. Chem.* 27, 478-488.
- Martyniuk, C.J., Bissegger, S., Langlois, V.S., 2013. Current perspectives on the androgen 5 alpha-dihydrotestosterone (DHT) and 5 alpha-reductases in teleost fishes and amphibians. *Gen. Comp. Endocrinol.* 194, 264-274.
- Parsons, H.M., Ekman, D.R., Collette, T.W., Viant, M.R., 2009. Spectral relative standard deviation: a practical benchmark in metabolomics. *Analyst* 134, 478-485.
- Rubin, B.S., 2011. Bisphenol A: An endocrine disruptor with widespread exposure and multiple effects. *J. Steroid Biochem. Mol. Biol.* 127, 27-34.
- Ryan, D., Robards, K., Prenzler, P.D., Kendall, M., 2011. Recent and potential developments in the analysis of urine: A review. *Anal. Chim. Acta* 684, 17-29.
- Samuelsson, L.M., Larsson, D.G.J., 2008. Contributions from metabolomics to fish research. *Mol. Biosyst.* 4, 974-979.
- Sandra, K., Sandra, P., 2013. Lipidomics from an analytical perspective. *Curr. Opin. Chem. Biol.* 17, 847-853.
- Saude, E.J., Adamko, D., Rowe, B.H., Marrie, T., Sykes, B.D., 2007. Variation of metabolites in normal human urine. *Metabolomics* 3, 439-451.
- Shannon, H.E., Degregorio, C.M., 1982. Self-administration of the endogenous trace amines beta-phenylethylamine, n-methyl phenylethylamine and phenylethanolamine in dogs. *J. Pharmacol. Exp. Ther.* 222, 52-60.
- Skinner, M.K., Haque, C., Nilsson, E., Bhandari, R., McCarrey, J.R., 2013. Environmentally induced transgenerational epigenetic reprogramming of primordial germ cells and the subsequent germ line. *Plos One* 8, e66318.

- Strimmer, K., 2008. A unified approach to false discovery rate estimation. *BMC Bioinformatics* 9, 14.
- Teng, C., Goodwin, B., Shockley, K., Xia, M.H., Huang, R.L., Norris, J., Merrick, B.A., Jetten, A.M., Austin, C.P., Tice, R.R., 2013. Bisphenol A affects androgen receptor function via multiple mechanisms. *Chem.-Biol. Interact.* 203, 556-564.
- Tikunov, Y.M., Laptinok, S., Hall, R.D., Bovy, A., de Vos, R.C.H., 2012. MSClust: a tool for unsupervised mass spectra extraction of chromatography-mass spectrometry ion-wise aligned data. *Metabolomics* 8, 714-718.
- Viant, M.R., 2007. Metabolomics of aquatic organisms: the new 'omics' on the block. *Mar. Ecol. Prog. Ser.* 332, 301-306.
- Vo, T.T.B., Jung, E.M., Dang, V.H., Yoo, Y.M., Choi, K.C., Yu, F.H., Jeung, E.B., 2009. Di-(2 ethylhexyl) phthalate and flutamide alter gene expression in the testis of immature male rats. *Reprod. Biol. Endocrinol.* 7, 104.

Table 1: Summary of inter- and intra-individual urinary metabolite profile variation (as measured by median %RSD) for the current datasets, along with others from the published literature.

Animal (Sex)	Class	Analytical technique	# of “signal bins”	# of individuals Inter / Intra	Inter-individual variation		Intra-individual variation			Reference
					Median %RSD	%RSD Range	Median %RSD ^a	%RSD Range ^a	#samples/ elapsed time(D)	
FHM (M)	Control	GC-MS	99 ^b	5 / 5	32.6 ^c	134 ^c	33.3 (2.0)	144 (24)	5 / 11	This study
Rat (M)	Control	¹ H NMR	1091 ^d	5 / 5	26.4 ^c	185 ^c	30.9 (3.2)	182 (19)	6 / 6	This study
FHM (M)	Control	¹ H NMR	273	7 / N.A.	52.9	146	-----	-----	-----	(Parsons et al., 2009)
Human (M,F)	Healthy	¹ H NMR	23(inter) 6(intra) ^e	57 (M,F) / 6 (F)	72.2	234	42.6 (9.3)	73 (28)	30 / 30	(Saude et al., 2007)
Guinea pig (F)	Control	¹ H NMR	22 ^f	25 / N.A.	94.1	259	-----	-----	-----	(Saude et al., 2007)

a) Arithmetic means (STDs) of those that were calculated separately for each individual

b) “signal bins” are normalized relative abundances of 99 metabolites, of which 30 were putatively annotated

c) Values are for the Day 0 sampling. The means (STDs) of those calculated individual for all sampling days was:

for FHM: median %RSD = 31.7 (1.5) and %RSD range = 135 (31), and for Rat: median %RSD = 27.8 (5.1) and %RSD range = 186 (7)

d) Value is for the Day 0 inter-individual variation class. For intra-individual variation, this ranges from 990 to 1065 for the various individuals

e) “signal bins” are concentrations (normalized to creatinine) of 23 identified metabolites for the inter-individual analysis, a randomly selected 6 of these 23 were used for the intra-individual analysis

f) “signal bins” are concentrations of 22 identified metabolites

Abbreviations: M = male, F = female, N.A. = not applicable

Figure Captions

Figure 1: Scores plots (mean $\pm$ SEM) from principal component analyses (PCA) of: A) ^1H -NMR spectra of urine collected daily for six consecutive days (D0-D5) from five control (unexposed) male Sprague Dawley rats, and B) GC-MS spectra of urine collected every 48 hours for seven consecutive days (D0-D6), and then once again 96 hours later (D10) from five control (unexposed) male fathead minnows. The percentage of variation captured by each principal component is indicated in its axis label.

Figure 2: Scores plots (mean $\pm$ SEM) from principal component analyses (PCA) of GC-MS spectra of urine collected every 48 hours for seven consecutive days (D0-D6), and then once again 96 hours later (D10), from male fathead minnows exposed to target concentrations of: A) 100 $\mu\text{g/L}$ of bisphenol A (BPA-HI), B) 500 $\mu\text{g/L}$ of flutamide (FLU), C) 200 $\mu\text{g/L}$ of vinclozolin (VIN-LO), and D) 700 $\mu\text{g/L}$ of vinclozolin (VIN-HI). In each case, urine was collected on day zero (D0) prior to initiating the exposures. The percentage of variation captured by each principal component is indicated in its axis label.

Figure 3: Aligned bar graphs depicting the 51 metabolites that significantly changed (with FDR-corrected p-values < 0.05) from day 2 compared to day 0 for exposure to target concentrations of: A) 500 $\mu\text{g/L}$ of flutamide (FLU), B) 200 $\mu\text{g/L}$ of vinclozolin (VIN-LO), and C) 700 $\mu\text{g/L}$ of vinclozolin (VIN-HI). A positive fold change represents a metabolite that significantly increased upon exposure, while a negative fold change represents a metabolite that significantly decreased. Note that 51 of the 99 detected metabolites changed with statistical

significance for at least one exposure. Of these, the 14 putatively annotated metabolites are labeled along the X-axis and grouped to the left of the graphs. The remaining 37 metabolites were not identified.

Figure 1

Figure 2

Figure 3