

# Impacts of an Anti-Androgen and an Androgen/Anti-Androgen Mixture on the Metabolite Profile of Male Fathead Minnow Urine

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Male and female fathead minnows (*Pimephales promelas*, FHM) were exposed via water to 20 or 200  $\mu\text{g/L}$  of cyproterone acetate (CA), a model androgen receptor (AR) antagonist. FHM were also exposed to 500 ng/L of 17 $\beta$ -trenbolone (TB), a model AR agonist, and to mixtures of TB with both concentrations of CA. The urine metabolite profile (as measured by <sup>1</sup>H NMR spectroscopy) of male FHM exposed to the high concentration of CA was markedly different from that of controls, and this difference was less for males coexposed to the associated TB+CA mixture. The exposure to TB alone had almost no impact on the male urine profile. These results suggest that male FHM urinary metabolite profiling may be useful for directly detecting effects of anti-androgens. In contrast, the urinary profile of male FHM exposed to the lower concentration of CA was not very different from that of controls, but, unexpectedly, this difference was increased when coexposed to the associated TB+CA mixture. This suggests that TB with CA at the lower concentration impacts male FHM through an interactive effect possibly unrelated, or in addition, to AR antagonism. The relative occurrence of male-like nuptial tubercles in female FHM exposed to TB and to the mixtures of TB and CA supported the metabolomics data.

## Introduction

Small fish models, such as the fathead minnow (*Pimephales promelas*, FHM), have proven useful for detecting a chemical's ability to disrupt the normal function of the

hypothalamic-pituitary–gonadal (HPG) axis, which controls reproduction and sexual development in vertebrates (1, 2). Relatively simple and unambiguous end points obtained from fish assays can often help establish an endocrine-disrupting chemical's primary mechanism of action (MOA) within the HPG axis. For example, androgen receptor (AR) agonists can cause the occurrence of male-specific secondary sex characteristics in females, while estrogen receptor (ER) agonists feminize males causing, for example, a marked induction of the egg yolk precursor protein vitellogenin (VTG) (1).

Fish assays have also been used to help identify AR antagonists (3–5). However, measurement of direct responses of common fish models (such as fathead minnows) to anti-androgens are often not as simple, specific, or unambiguous as for ER or AR agonists, making it more difficult to establish whether a chemical possesses this important MOA (1). For example, 21 day exposures of adult FHM to flutamide and vinclozolin (VZ), two model anti-androgens, resulted in changes in HPG-related end points (gonad histopathology, VTG, secondary sex characteristics, etc.) that were often subtle and not particularly MOA-specific (6, 7). For this reason, indirect approaches have been used to more convincingly identify chemicals as AR antagonists in fish. These indirect methods test for a suspected anti-androgen's ability to "block" specific responses induced by a model androgen in coexposure experiments (4, 6, 8). For example, FHM were recently coexposed to VZ and 17 $\beta$ -trenbolone (TB), a potent AR agonist. Exposure to TB alone caused strong expression of male-like nuptial tubercles in female FHM, while tubercles were absent in unexposed females and in females exposed to a mixture of VZ and TB (6). These types of indirect assays provide a useful tool for detecting an anti-androgenic MOA. However, more direct assessments of the impacts of anti-androgens on endocrine function in fish would be desirable relative to predicting adverse effects on reproduction and development. Given increasing concerns over impairments in male sexual development and reproductive function in both humans and wildlife, there remains a need for robust methods that directly assess anti-androgenic behavior of chemicals, particularly at the molecular and biochemical levels where information on specific pathways may be obtained.

We have recently shown that levels of endogenous metabolites in urine from male FHM were altered upon exposure to the anti-androgen VZ (9). However, using similar techniques and exposure scenarios, we subsequently found relatively little or no effect on male FHM urinary metabolites after exposure to several other chemicals that are not anti-androgens, but which could affect HPG function through other mechanisms (specifically, TB and fipronil, a GABA receptor antagonist; unpublished data). These observations led us to consider the possibility that male FHM urine metabolite profiling might be particularly useful for directly characterizing and studying the impacts of exposure to AR antagonists. Indeed, endogenous metabolite profiling, or metabolomics, has proven to be a generally useful tool for studying and elucidating MOAs of environmental toxicants (10).

Here we report on NMR-based metabolite profiling of urine from male FHM that were exposed via the water to 20 or 200  $\mu\text{g/L}$  of cyproterone acetate (CA), which has been shown to be a mammalian AR antagonist (11). We also conducted coexposures using mixtures of CA (at both concentrations) with TB (at 500 ng/L). We hypothesized that, if effects specific to AR antagonism were seen with CA, they

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should be (partially) blocked by the androgen TB. This work contributes to our long-range goal of identifying candidate metabolite-based biomarkers of exposure that are diagnostic and specific for the important MOAs of HPG-active chemicals, including AR antagonists (12). Also, note that our design for this experiment involved both male and female FHM, and that we also conducted an exposure to TB alone. This allowed us to apply the indirect test of AR antagonism to CA using whole-animal assays, such as female FHM tubercle scores, as described above (6). These measurements provided a critical apical “anchor” to support conclusions drawn from the endogenous metabolite measurements.

## Experimental Section

**Experiment Design and Sample Collection.** Sexually mature (ca. 5–6 month old) FHM were from an on-site culture facility at the Duluth EPA lab. Prior to the exposures, the fish were held in a mass culture, which is a setting that is not conducive to reproduction. To initiate the experiment, four males and four females were placed together in a glass test tank holding 10 L of Lake Superior water (4  $\mu$ M filtered, UV sterilized) or test chemical dissolved in Lake Superior water, which was continuously renewed at a rate of about 45 mL/min. There were two such tanks for each treatment condition, yielding an initial  $n = 8$  for each sex and each treatment. (Water quality characteristics are summarized in the Supporting Information (SI).) The animals were held at  $25 \pm 0.5$  °C under a 16:8 L:D photoperiod, and were fed brine shrimp to satiation twice per day.

Solvent-free solutions of TB (Sigma Chemical, St. Louis, MO >98% pure) and CA (Sigma, >98% pure) were prepared as concentrated stocks in Lake Superior water. Exposures consisted of the following six treatments: controls (CON), TB (500 ng/L; nominal), CA low (CAL, 20  $\mu$ g/L; nominal), CA high (CAH, 200  $\mu$ g/L; nominal), TB+CAL (500 ng/L TB + 20  $\mu$ g/L CA) and TB+CAH (500 ng/L TB + 200  $\mu$ g/L CA). Exposures were conducted for 14 days.

Stock solution and tank water concentrations of all test materials were measured at least two times per week. Concentrations of TB were determined using direct injection, high-pressure liquid chromatography (HPLC) with fluorescence detection (13). No TB was detected (detection limit, 50 ng/L) in method blanks, control water, or treatment tanks containing only CA. A modification of the HPLC method with diode array detection described by Christiaens et al. was used for CA analyses (14). No CA was detected (detection limit, 2  $\mu$ g/L) in the method blanks, control, or TB-only tanks. Measured tank water concentrations for the chemical treatments were about 100% and 80% of target for TB and CA, respectively; details concerning chemical exposure characterization appear in the SI.

There were no treatment-related mortalities. At the conclusion of the 14 day exposures, the fish were anaesthetized with buffered tricaine methane sulfonate (MS-222; 100 mg/L buffered with 200 mg/L NaHCO<sub>3</sub>/L; Finquel, Argent, Redmond, WA). Urine was expelled from males by applying gentle pressure on the fish's abdomen and collected using nonheparinized microcapillary tubes. Every effort was made to avoid contaminating the urine sample with sperm (see SI for more details). A sufficient amount of urine was successfully collected from all males ( $n = 8$ ) in all exposure classes, except for one individual belonging to the CAH exposure class (also, unfortunately, one sample from the CAH exposure class was lost in processing, yielding  $n = 6$  for that class). Nuptial tubercles in both sexes were enumerated and scored based on a combination of the count data and a subjective assessment of size/expression using a scale of 1 (smallest) to 3 (6, 15).

**Collecting NMR Spectra of FHM Urine.** Urine samples were lyophilized for 8 h to remove water. Each sample was

then reconstituted in 250  $\mu$ L of 0.1 M sodium phosphate buffered deuterium oxide (pH 7.4) containing 25  $\mu$ M sodium 3-(trimethylsilyl) propionate-2,2,3,3-d<sub>4</sub> (TSP). The samples were then vortexed briefly, and centrifuged at 10 600 rcf for 15 min at 4 °C to remove any insoluble components. The resulting supernatant was then pipetted into a standard 3 mm NMR tube. All <sup>1</sup>H one-dimensional spectra were collected at 20 °C using a standard presaturation pulse sequence on a 600 MHz Varian Inova (599.76 MHz, <sup>1</sup>H) spectrometer equipped with a cryogenic triple-resonance probe. For each spectrum, 1024 transients were collected into 14k points using a spectral width of 7200 Hz and an acquisition time of 2 s. Suppression of the residual water resonance was achieved by a 1-s presaturation delay at a field strength of 40 Hz. All metabolite assignments were made based on previously reported chemical shift values and multiplicities (9).

**NMR Data Processing and Analysis.** Spectra were processed using ACD/1D NMR Manager (Advanced Chemistry Development, Toronto, Canada). Specifically, spectra were zero-filled to 32,768 points, and exponential line broadening of 0.3 Hz was applied prior to Fourier transformation. An automated routine was used to phase and baseline-correct each spectrum, which was then referenced to TSP (at 0.0 ppm). An example of a typical processed spectrum used for the metabolomic analyses is shown in SI as Figure S1.

Next, the spectra (0.50–10.00 ppm) were segmented into 0.005 ppm-wide bins and imported into Microsoft Excel (Microsoft Corporation, Redmond, WA). Within Excel, several spectral regions were omitted (i.e., their Y-values were set to zero). The region 1.20 – 1.42 ppm was omitted because our automated routine was unable to achieve an acceptable baseline in this region, which contained a small but broad and complex resonance. The regions 2.22–2.25 and 3.34–3.37 ppm were omitted to remove resonances from an unexplained contamination of acetone and methanol, respectively. The region 4.70–5.10 ppm was omitted to remove the residual water resonance. Finally, the regions of 3.26–3.28 and 3.90–3.92 were omitted to remove resonances from betaine (trimethylglycine). Betaine was both highly abundant and highly variable in these samples. Preliminary analysis revealed that these resonances dominated multivariate modeling, and that the level of betaine was not exposure dependent. The remaining bins were then normalized within Excel to achieve unit total intensity for each spectrum.

This Excel spreadsheet of binned spectra was then imported into SIMCA-P+ (Umetrics Inc., Umea, Sweden) for multivariate data analysis (which was conducted on mean-centered bins.) First, principal components analysis (PCA) was used to screen for outliers in the data set (none were found using the Hotelling's  $T^2$  test at the 95% confidence interval for a scores plot of the first two components). PCA was also used to help establish and visualize the extent of impact for a given treatment, as well as the relative impacts of different treatments. Additionally, a series of two-class partial least-squares discriminant analysis (PLS-DA) models were constructed. We used the permutation testing routine (16) within SIMCA-P+ for PLS-DA models to assess the validity of assuming that a given treatment had resulted in a discernible effect. This approach was also helpful in comparing the relative impacts of different treatments.

The Excel spreadsheet of binned, edited, and normalized spectra was also used to construct “*t*-test filtered difference spectra,” which aided identification of specific metabolites that were affected by the chemical exposures. In addition, these difference spectra were used to further compare the relative impacts of the different treatments. In order to generate these difference spectra, an “average class spectrum” was first calculated by averaging the binned spectra across all class members (note that class was defined by chemical exposure condition). Next, the difference spectrum for each

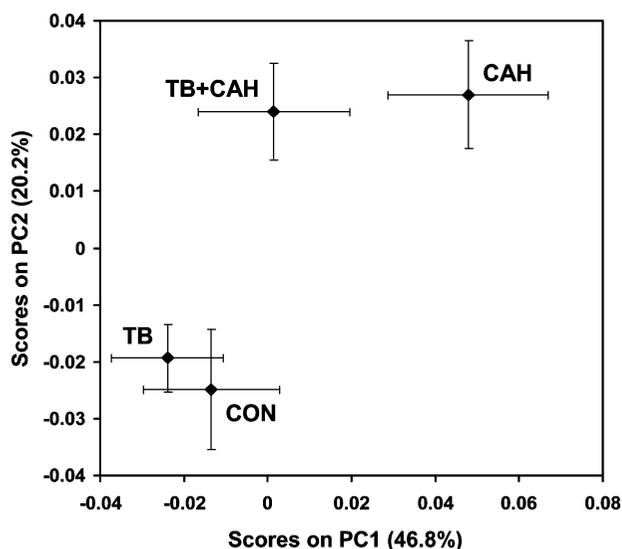
exposed class was generated by subtracting the averaged bins of the control class from those of the exposed class. Then, a *t*-test was conducted on each bin to determine if the average for the exposed class differed significantly from that of the control class, using a *p*-value < 0.05. If not, the bin value for the difference spectrum was replaced with a zero. To greatly reduce the rate of false positives, any single isolated bin that passed the *t*-test (without an adjacent bin also passing) was replaced with a zero (i.e., it was rejected), because legitimate metabolite peaks span more than one bin at this bin size. To further reduce false positives, any occurrences of two (and only two) adjacent bins with opposite arithmetic sign were replaced with zeros, because this outcome is incompatible with NMR peak shapes. The resulting *t*-test filtered difference spectrum for each exposed class displays peaks above the baseline that correspond to metabolites that increase upon exposure, and peaks below the baseline that correspond to metabolites that decrease. (More details on generating these difference spectra, and on limiting their false positives rates, are in the SI.)

It is important to note that the *t*-test filtered difference spectra were used to help identify which metabolites might be treatment dependent. After identification in this manner, treatment dependency was either confirmed or rejected by integrating the area of a given metabolite's most abundant and/or most isolated peak for each exposure- and control-class member. These integrated peak areas were calculated in Excel using the same spreadsheet of binned, edited, and normalized spectra described above. Then, a univariate *t*-test was conducted to assess treatment dependency, using a *p*-value < 0.05. All specific peaks and metabolites discussed herein as changing upon exposure (relative to controls, in the context of difference spectra) passed this univariate test. Integrated peak areas from the Excel spreadsheet of binned, edited, and normalized spectra were also used to compare responses across the various treatments for candidate biomarker peaks.

## Results and Discussion

**Exposures with 200  $\mu\text{g/L}$  CA.** As described in the Introduction, our previous findings led us to consider the possibility that metabolite profiling with male FHM urine might provide the ability to directly classify a chemical as an AR antagonist. While firmly establishing this capability is beyond the scope of any single study, the design of the present experiment allowed us to partially test this hypothesis, and to assess the value of further exploration.

If this hypothesis is correct, from this experiment we would expect that male FHM urine metabolite profiles: (1) would not be significantly impacted upon exposure to TB alone (based on earlier, unpublished findings from our lab), (2) would be significantly impacted by exposure to the anti-androgen CA, (3) would be less impacted by exposure to a mixture of CA and TB (as compared to CA alone), and (4) would change upon exposure to CA in a manner that is similar to our findings with VZ. It is possible to partially evaluate the first three of these expectations with a single scores plot (Figure 1) from a PCA model that was built using spectra from four of the exposure classes: control (CON), exposure to TB, exposure to CA at the high level (CAH), and exposure to a mixture of TB and CA at the high level (TB+CAH). In Figure 1 it is clear that the CON and TB classes are clustered together, indicating little or no impact on the male urine metabolite profile from exposure to TB. Also, the impact due to exposure to CAH is obvious - both PC1 and PC2 score values for the CAH class are quite different from those of the CON class (the *p*-value for PC1 scores from a *t*-test is 0.0153 and for PC2 is 0.00212). Furthermore, this plot suggests that the impact of CAH is partially blocked by TB since the mean score for the TB+CAH class falls in a location along the PC1

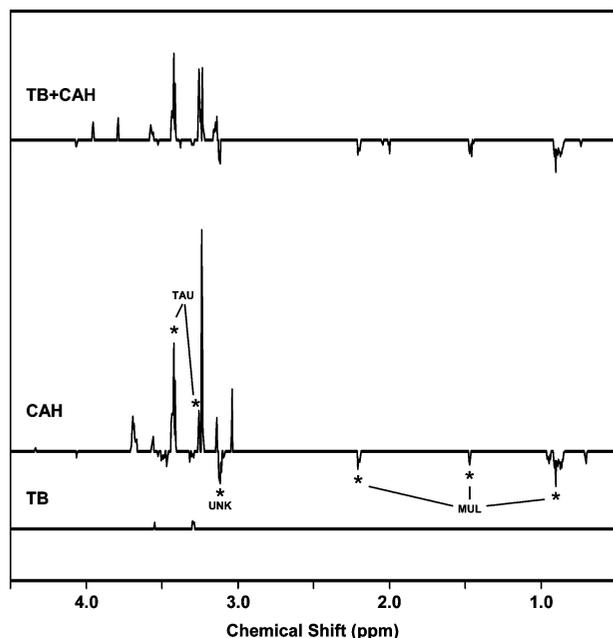


**FIGURE 1.** Two-component scores plot from principal component analysis (PCA) of NMR spectra of urine from unexposed (CON, control) male fathead minnows, and those exposed to: 17 $\beta$ -trenbolone (TB, 500 ng/L), the high treatment of cyproterone acetate (CAH, 200  $\mu\text{g/L}$ ), and a cotreatment of TB and CAH (TB+CAH). Each marker is the mean score value for a given treatment, shown with its associated standard error. The amount of variation in the data set captured by each component is shown parenthetically in the axis titles.

axis that is between those for the CON and CAH classes. In fact, the TB+CAH scores are better separated along PC1 (which captures the largest fraction of variation in the data) from the CAH scores (*p* = 0.0539) than from the CON scores (*p* = 0.275). Although not definitive, these trends suggest that the presence of TB is partially blocking the effects of CA, while the presence of TB alone has no measurable effect on the male urine metabolite profile. (Note that a scores plot of the PCA model from Figure 1 is also shown using individual points, instead of the mean values, as Figure S2 in the SI.)

To test the hypothesis that TB is blocking the effect of CAH, we built several two-class PLS-DA models, where one class was CON and the other was an exposure class. These models were then subjected to permutation testing, which is a common method used to test (or validate) the assertion that classes are measurably different (16). The results from these tests demonstrated (see Figure S3 in the SI) that the model built using the CON and CAH classes was considerably more valid than the model built using the CON and TB+CAH classes. (The model built using the CON and TB classes was clearly invalid.) In addition, a two-class model built using the TB+CAH and CAH classes was also found to be valid (see Figure S4 in the SI), indicating a significant difference between these two exposed classes. This further supports the hypothesis that the FHM urinary metabolite profiling is directly detecting the anti-androgenic effect of CA, which is partially blocked by coexposure with the androgen TB.

Next, we constructed several *t*-test filtered difference spectra, wherein the average spectrum for the CON class was subtracted from that of an average exposed class (as described in the Experimental Section). These difference spectra facilitate the identification of metabolites that are changing in response to exposure. In addition, comparison of multiple difference spectra aids in determining which treatment had the greatest impact. Generally speaking, an exposure that produces a large impact on the urinary metabolite profile will result in a difference spectrum with more numerous and more intense peaks than that of an exposure with lesser impact. Also, the "fingerprints" of difference spectra (i.e., the pattern of peak changes) can aid



**FIGURE 2.** Average NMR urine difference spectra (exposed minus control) generated from male fathead minnows exposed to: 17 $\beta$ -trenbolone (TB, 500 ng/L), the high treatment of cyproterone acetate (CAH, 200  $\mu$ g/L), and a cotreatment of TB and CAH (TB+CAH). Positive-going peaks indicate metabolites that increased (relative to controls) upon exposure, while negative-going peaks indicate those that decreased. Only those peaks for which differences were determined to be significant by *t*-test ( $p < 0.05$ ) and further validation were included. For clarity, only the region of 0.5–4.5 ppm is displayed because peaks outside this region were much less intense. Note that all difference spectra are displayed using the same Y-axis scale. Asterisks denote peaks that are shared (both in peak location and direction of change) with the results from our earlier related exposure study with vinclozolin (9). (TAU = taurine, UNK = unknown metabolite, MUL = a monounsaturated lipid.)

in determining if different chemicals are impacting animals through the same biological effect(s).

The difference spectra for the TB, CAH, and TB+CAH exposures are shown in Figure 2. Again, it is clear that TB had little impact. The fingerprints from the spectra for the CAH and the TB+CAH exposures are similar, suggesting that the two treatments elicited similar biological effects. However, the number of peaks, and the intensity of several peaks, is greater for the CAH exposure compared to that of the TB+CAH exposure. Again, this observation is consistent with the hypothesis that the metabolite changes are related to the anti-androgenic activity of CA, which is partially blocked by TB.

Recall that one of our hypotheses was that metabolite changes caused by CA would be similar to our earlier findings with VZ (9). There are, indeed, some striking similarities, which are illustrated in Figure 2. Specifically, asterisks are used to denote peak changes for the CAH exposure that are shared (both in peak location and direction of change) with the results from our earlier VZ exposure. The denoted peaks account for about 30% of the relatively intense peaks found in the CAH exposure difference spectrum. Interestingly, several of these peaks (at 0.90, 1.47, 2.20, 5.95, and 6.80 ppm) belong to a single metabolite, a monounsaturated lipid (MUL) whose exact identification we have not yet established. MUL was found to be significantly down regulated by the CAH exposure and, earlier, by the VZ exposure. Due to the relatively narrow linewidths observed for these resonances (as well as its presence in solution, it is reasonable to assume despite the lack of a positive identification) that MUL possesses a

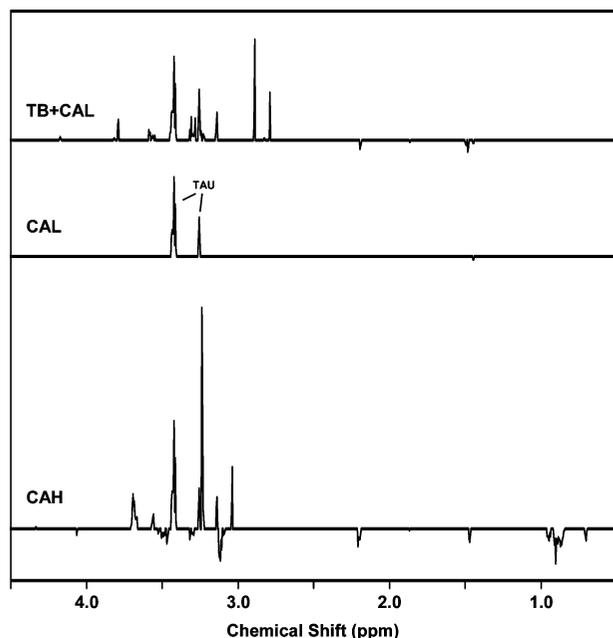
low molecular weight and some degree of polarity, consistent with, for example, a short-chain fatty acid. Other metabolite changes that were also observed in the earlier VZ exposure include taurine, which was up regulated, and an unknown metabolite with a resonance at 3.12 ppm, which was down regulated.

**Exposures with 20  $\mu$ g/L CA.** All of the observations with the TB, CAH, and TB+CAH exposures were consistent with the hypothesis that CA was functioning as an anti-androgen, and that male FHM urine metabolite profiling was useful for directly detecting this. However, this hypothesis was seemingly challenged by data from the CAL and, especially, the TB+CAL treatments. For example, a PCA model was built using spectra from the CON, TB, CAL, and TB+CAL classes. The scores plot from that model is displayed in the SI (Figure S5). In that scores plot, we again saw that the CON and TB classes were clustered together, consistent with little or no impact from exposure to TB. And (in contrast to the results at the higher exposure level), the CAL class was mixed with the CON class as well ( $p$ -values for scores on PC1 = 0.252 and on PC2 = 0.0845). This suggests that the anti-androgenic effects of CA were diminished at the lower exposure concentration, which is certainly reasonable. Unexpectedly, however, members of the TB+CAL class were comparatively well clustered and completely separated along PC2 from the CON class. Indeed, PC2 (but not PC1) score values for the CON and TB+CAL classes were quite different ( $p = 0.000304$ ). This outcome is surprising since neither the separate TB nor the CAL exposures resulted in a significant change from controls. This suggests that the TB+CAL mixture is eliciting an interactive effect on the biology of the fish that is, perhaps, in addition to, or unrelated to, AR antagonism.

To reinforce the assertion that the TB+CAL exposure elicited a significant effect when neither the TB nor the CAL exposure did, we once again built several two-class PLS-DA models, where one class was CON and the other was an exposure class. These models were then subjected to permutation testing. The results from these tests (presented in the SI as Figure S6) demonstrated that the model built using the CON and TB+CAL classes was, indeed, highly valid. Furthermore, both the model built using the CON and TB classes and the model built using the CON and CAL classes were invalid.

The difference spectra for the CAL and TB+CAL exposures are shown in Figure 3, along with the difference spectrum for CAH, which is included for sake of comparison. The only noteworthy features in the CAL difference spectrum are due to an increase in taurine. In comparison, the TB+CAL mixture exposure difference spectrum contains numerous peaks, many with considerable intensity. This is consistent with an interactive effect, since the TB+CAL exposure resulted in many more significant metabolite changes than did either the separate CAL or TB exposures (see Figures 2 and 3). Furthermore, note that, while some peaks are shared, the overall fingerprint of the TB+CAL difference spectrum is quite unlike that of CAH, suggesting different, or additional, biological effects. If the pattern of features we observed in the CAH and TB+CAH difference spectra (particularly those that were in common with our earlier VZ exposure) reflects an anti-androgenic MOA, then these results suggest that the CAL exposure did not result in a measurable AR mediated response. Furthermore, if TB+CAL is eliciting a response mediated through the AR, then it is likely occurring in the presence of additional response(s).

**Female FHM Tubercle Occurrence.** Recall that our experimental design involved the use of both male and female FHM. This allowed us to apply an indirect test of AR antagonism to CA using whole-animal assays, as described in the Introduction. Specifically, in females exposed to the androgen TB, we observed the induction of nuptial tubercles,

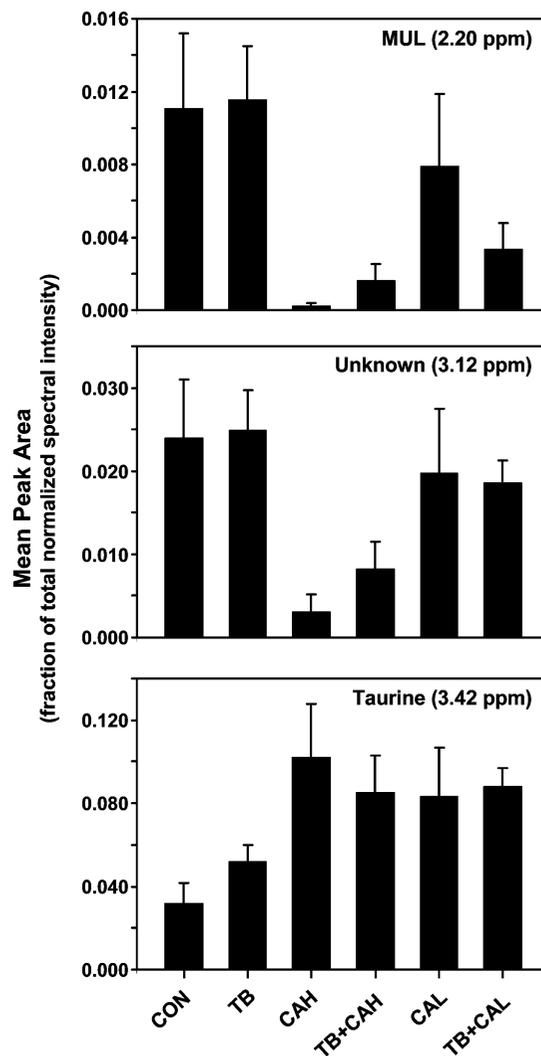


**FIGURE 3.** Average NMR urine difference spectra (exposed minus control) generated from male fathead minnows exposed to: the high treatment of cyproterone acetate (CAH, 200  $\mu\text{g/L}$ ), the low treatment of cyproterone acetate (CAL, 20  $\mu\text{g/L}$ ), and a cotreatment of TB and CAL (TB+CAL). See Figure 2 caption for additional information on the construction of difference spectra. (TAU = taurine.)

which are secondary sex characteristics that naturally occur only in males. We then looked for blockage of this induction by CA when coexposed with TB. CAH functioned as an anti-androgen, effectively blocking TB-induced tubercle formation in females, whereas CAL did not (See Figure S7 in the SI). This is consistent with results from the male urine metabolite profiling presented herein.

**Potential for Exposure Biomarkers.** The weight of evidence discussed above might suggest that some of the peaks denoted with an asterisk in Figure 2 should be considered as candidate urinary biomarkers for exposure of male FHM to anti-androgens. Clearly, much more investigation would be required to establish a reliable biomarker, including confirming the identity of responsive metabolites, and establishing the relationship of their biochemical function to AR activity. Nonetheless, it is interesting to compare the relative intensities of these targeted peaks across treatment classes. For example, in Figure 4, we have plotted the mean areas for peaks from MUL, the Unknown at 3.12 ppm, and taurine. Note that an increase in taurine appears to be a fairly nonspecific response. However, both the MUL and the Unknown peaks follow trends that are generally consistent with expectations for a marker of anti-androgenic behavior by CAH, which is partially blocked by TB, and largely absent in the case of CAL. (Not surprisingly, the situation with TB+CAL is not as easily interpreted.)

**Implications for Exposure and Hazard Assessment.** Problems with sexual development and reproduction in both humans and wildlife are an issue of concern relative to EDCs. Research into the potential links of some of these problems with the environmental occurrence of chemicals that affect the HPG axis via different mechanisms is expanding. For example, there is now a significant body of work on the effects of environmental estrogens on fish (17). However, there is a relative lack of information on the impacts of chemicals that disrupt the normal functioning of ARs, in particular antagonists. In addition, HPG-active chemicals (such as AR antagonists) typically do not occur in isolation in the environment, but are often found in mixtures with other



**FIGURE 4.** Mean areas (shown with the associated standard errors), as a function of treatment class, for some of the NMR peaks from male fathead minnow urine that were found to change significantly upon exposure to 200  $\mu\text{g/L}$  of cyproterone acetate (CAH), and, previously, to vinclozolin (9), both of which are anti-androgens. See Figures 2 and 3 captions for definitions of other treatment class abbreviations.

chemicals that may have similar or opposing MOAs. Therefore, it is important to investigate the ways in which organisms respond to mixtures of these chemicals. The results presented here provide a clear and compelling justification for the further exploration of using male FHM urine metabolite profiling for directly detecting the occurrence of AR antagonists, and for describing the impacts of exposure to simple chemical mixtures.

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## Supporting Information Available

A typical NMR spectrum, additional experimental details, information on limiting false discover rates for *t*-test filtered difference spectra, additional PCA score plots, permutation testing results for PLS-DA models, and data on female FHM tubercle occurrence. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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