Metabolite profiles of striped marsh frog (*Limnodynastes peronii*) larvae exposed to the anti-androgenic fungicides vinclozolin and propiconazole are consistent with altered steroidogenesis and oxidative stress.

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Highlights:

- Pesticides can present a toxicological threat to amphibians
- Our understanding of sub-lethal mechanisms are however quite limited
- *Limnodynastes peronii* larvae were exposed to vinclozolin and propiconazole
- NMR spectroscopy was used to evaluate sub-lethal metabolic responses
- Effects were consistent with altered steroidogenesis and oxidative stress

Abstract

Amphibians use wetlands in urban and agricultural landscapes for breeding, growth and development. Fungicides and other pesticides used in these areas have therefore been identified as potential threats that could contribute towards amphibian population declines. However, relatively little is known about how such chemicals influence sensitive early life-stages or how short episodic exposures influence sub-

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lethal physiological and metabolic pathways. The present study applied untargeted metabolomics to evaluate effects in early post-hatch amphibian larvae exposed to the anti-androgenic fungicides vinclozolin and propiconazole. Recently hatched (Gosner developmental stage 25) striped marsh frog (*Limnodynastes peronii*) larvae were exposed for 96 h to vinclozolin at 17.5, 174.8 and 1748.6 nM and propiconazole at 5.8, 58.4 and 584.4 nM. Nuclear Magnetic Resonance (NMR) spectroscopy was performed on polar metabolites obtained from whole-body extracts. Both fungicides altered metabolite profiles compared to control animals at all concentrations tested, and there were notable differences between the two chemicals. Overall responses were consistent with altered steroidogenesis and/or cholesterol metabolism, with inconsistent responses between the two fungicides likely reflecting minor differences in the mechanisms of action of these chemicals. Broad down-regulation of the tricarboxylic acid (TCA) cycle was also observed and is indicative of oxidative stress. Interestingly, formic acid was significantly increased in larvae exposed to vinclozolin but not propiconazole, suggesting this metabolite may serve as a useful biomarker of exposure to androgen-receptor binding anti-androgenic contaminants. This study demonstrates the power of untargeted metabolomics for distinguishing between similarly acting, but distinct, pollutants and for unraveling non-endocrine responses resulting from exposure to known endocrine active contaminants.

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Introduction

Amphibians commonly use wetlands in urban and agricultural landscapes as breeding grounds and habitat for larval growth and development (Hartel and Wehrden, 2013; Knutson et al., 2004; Mann et al., 2009). Fungicides and other pesticides used in these settings have consequently been identified as factors that may pose a threat to amphibian populations and could contribute to the reported global population declines (Brühl et al., 2011; 2013; Mann et al., 2009; Monastersky, 2014). Early amphibian life-stages are often particularly sensitive to chemical insult (Edginton et al., 2004; Ortiz-Santaliestra et al., 2006; Pauli et al., 1999) due to the permeability of larval skin and their reliance on water for gas exchange and the maintenance of osmotic balances (Berrill and Bertram, 1997; Berrill et al., 1993). Despite this, we know very little about how agricultural pesticides impact physiological parameters and processes in larval amphibians (Broomhall, 2005). This represents an important knowledge gap considering the threatened status of many amphibian populations (Monastersky, 2014) and the continual expansion of agricultural activities necessary to meet global food demands (Tilman et al., 2011).

Untargeted metabolomics offers a powerful approach to broadly explore how pollutants influence physiological and metabolic endpoints in wildlife (Oliveira et al., 2016). Nuclear magnetic resonance (NMR) spectroscopy is a convenient option for metabolite profiling due to relatively simple sample processing and a high level of repeatability, and has proven very useful for applications in ecotoxicology (Emwas et al., 2013; Lankadurai et al., 2013; Viant et al., 2003). The majority of metabolomics studies in aquatic ecotoxicology have focused on fish or shellfish (Cappello et al., 2016; Cappello et al., 2017; Ekman et al., 2007; Viant et al., 2003), with few cases describing effects in amphibians. Those few studies with amphibians have revealed

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correlations between developmental endpoints and metabolite profiles in unexposed amphibian larvae (Ichu et al., 2014), and similarly in those exposed to chemical stressors (Melvin et al., 2017). However, exposure to pesticides is assumed to occur over relatively short timeframes in natural settings since this will intuitively coincide with episodic pulse applications. As such, two reasonable questions are 1) does short-term exposure causes detectable and distinctive effects on the metabolome of early life-stage amphibian larvae, and if so 2) do such effects translate into meaningful higher-level biological outcomes (e.g., altered growth, development or reproduction)? The present study addresses the first of these questions.

Vinclozolin and propiconazole are fungicides that have been used globally for applications ranging from horticulture of fruits and vegetables to the maintenance of turf grass. These chemicals are being phased out in some parts of the world but are still used in many locations (Kidd et al., 2013; Pallavi Srivastava, 2013). Maximal vinclozolin concentrations in natural aquatic matrices have been predicted to be as high as 52 μg/L (Steeger and Garber, 2009), but the limited available monitoring data suggests surface water concentrations closer to 0.5 μg/L (Readman et al., 1997; Tillmann et al., 2001). Expected environmental concentrations for propiconazole have been suggested to be as high as 80 μg/L (DeLorenzo et al., 2001) but monitoring data again reports conazole fungicides at much lower concentrations ($< 0.1 - 7.7$ μg/L) in aquatic environments (Haarstad, 2012; Wightwick et al., 2011). Both fungicides are considered anti-androgenic (Lor et al., 2015; Makynen et al., 2000; Skolness et al., 2013; van Ravenzwaay et al., 2013) and there is evidence that they also elicit oxidative stress (Bruno et al., 2009; Gazo et al., 2013; Radice et al., 1998; Tu et al., 2015). Due to substantial interest in endocrine disruption, studies assessing the toxic impacts of both fungicides have mainly focused on sexual development and related

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reproductive performance, and primarily in rodents and fish (Bayley et al., 2002; Gazo et al., 2013; Hotchkiss et al., 2003; Lor et al., 2015; Makynen et al., 2000; Nesnow et al., 2011). However, as indicated this fails to consider possible subtle biochemical effects that might occur during early developmental life-stages, and overlooks a major class of globally threatened vertebrate species.

We applied ^{1}H NMR spectroscopy to assess changes to global metabolite profiles in striped marsh frog (*Limnodynsastes peronii*) larvae exposed to environmentally relevant concentrations of the fungicides vinclozolin and propiconazole for 96 h during early development.

MATERIALS AND METHODS

Experimental animals

A single fertilized foam nest of the Australian striped marsh frog (*Limnodynastes peronii*) was collected from an ephemeral pond located in Elanora, Queensland, Australia. The nest was transported to the laboratory in water from the collection site and transferred to a 60 L aquaria filled with moderately hard test water (USEPA, 1994) until hatching. The Griffith University Animal Ethics Committee approved the work, according to the principles of the Australian Code for the Care and Use of Animals for Scientific Purposes (Protocol No. ENV/03/16/AEC).

Experimental protocol

Technical-grade vinclozolin (Cat. No. 45705) and propiconazole (Cat. No. 45642) were purchased from Sigma-Aldrich (Castle Hill, Australia). These were dissolved in methanol to create concentrated stock solutions, and administered at less than 0.003% the volume of experimental vessels to achieve target concentrations.

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Tadpoles were allowed to hatch and were subsequently maintained until yolk sac and gills were absorbed. After hatching and yolk sac reabsorption (Gosner developmental stage 25; Gosner 1960), larvae were randomly transferred into individual 50 mL glass beakers filled with moderately hard test water, and each beaker received a single ration of 10 mg of Sera micron® fry food (Sera GmbH). There were nine replicate beakers each of control (water only) and solvent control (0.003% MeOH), and three concentrations of each chemical. Each replicate contained a single tadpole. The vinclozolin treatment was administered at 17.5, 174.8 and 1748.6 nM (equivalent to 5, 50 and 500 μg/L, respectively) and propiconazole at 5.8, 58.4 and 584.4 nM (equivalent to 2, 20 and 200 μg/L, respectively). Following 96 h exposure, larvae were euthanized in buffered 3-aminobenzoic acid ethyl ester (MS-222; Sigma Aldrich, Castle Hill, Australia). Wet weights (mg) were recorded, and animals were snap frozen on liquid nitrogen and stored at -80°C for extraction of global (whole body) metabolites.

Extraction of hepatic metabolites and lipids

Metabolites were extracted and separated from protein and lipids using the modified Bligh-Dyer methanol:chloroform extraction (Bligh and Dyer, 1959), as previously described (Lin et al., 2007; Melvin et al., 2017). Briefly, individual larvae were homogenized in 400 μg/L ice-cold methanol using an Ultra-Turrax® T10 tissue homogenizer (IKA®, Selangor, Malaysi) followed by ultra-sonication using a Q55 probe sonicator (Qsonica, USA), and incubated at -20°C for 1 h. After incubation, 800 μL chloroform and 200 μL ultrapure water were added and the samples were vortexed and centrifuged (10 min, 16,000×g, at 4°C). The polar phase was carefully portioned into glass amber vials, the extraction was repeated with the remaining

protein pellet, and the combined yields were stored at -80°C until further processing.

^{1}H NMR spectroscopy

Extracted metabolites were dried using a Series II centrifugal vacuum concentrator (GeneVac Technologies, England) and subsequently lyophilized. The metabolites were re-suspended in 200 μL phosphate buffer (K_2HPO_4 and NaH_2PO_4; pH 7.4) made with deuterium oxide (D_2O), which contained 0.05% sodium-3-(tri-methylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) as an internal standard. The samples were vortexed and centrifuged (5 min, 2,500×g, at 4 °C) to remove debris, and supernatants were transferred to 3 mm NMR tubes using glass Pasteur pipettes.

NMR spectra were acquired with an 800 MHz Bruker® Avance III HDX spectrometer equipped with a Triple (TCI) Resonance 5 mm Cryoprobe with Z-gradient and automatic tuning and matching, and a SampleJet automatic sample changer. The instrument was controlled using IconNMR™ automation software (Bruker Pty Ltd., Victoria, Australia). Spectra were acquired at 298°K with D_2O used for field locking and TSP (^{1}H δ 0.00) as an internal reference. Proton (^{1}H) spectra were acquired for all samples using zg30 pulse program with 128 scans, 1.0 s relaxation delay, 8.75 μs pulse width and a spectral width of 16 kHz (^{1}H δ -3.75 – 16.28). Edited ^{1}H-^{13}C HSQC spectra were acquired for a pooled sample using the pulse sequence hsqcedetgpsisp2.2 with 16 scans and 128 experiments, 0.8 s relaxation delay, 8.75 μs pulse width and spectral widths of 12.8 kHz (^{1}H δ -3.23 – 12.79) and 33.1 kHz (^{13}C δ -9.40 – 155.2).

Peak assignments and processing of ^{1}H NMR spectra

Post processing was performed with MestReNova v11.0.3 (Mestrelab

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Research S.L., Spain). ^{1}H NMR FIDs were Fourier transformed with a line broadening of 0.3 Hz and HSQC spectra were processed with default Bruker post-processing parameters. All spectra were manually phase corrected, automatically baseline adjusted (ablative) and referenced to TSP ([1]H δ 0.00, [13]C δ 0.0). Spectra were normalized to the TSP peak and stacked and binned into 0.02 ppm buckets. Integrated peak areas of each bucket were exported to an excel file for further processing and multivariate statistical analysis. Metabolites were tentatively assigned to ^{1}H NMR spectra using Chenomx NMR suite 8.2 software (ChenomxInc., Edmonton, Canada). Metabolite identifications were further verified by comparison of HSQC NMR spectra with published reference spectra from the Human Metabolome Database (HMDB).

Univariate and multivariate statistical analysis

Differences in larval weight were assessed by one-way ANOVA using SPSS v22 statistical software for Mac OSX (IBM Inc., Chicago, USA), with α = 0.05. Assumptions of normality and homogeneity of variance were tested with visual inspection of normal probability plots and Shapiro-Wilk's test, respectively. Post-hoc comparisons were performed with Tukey's HSD multiple comparisons test.

Prior to statistical analysis, the exported ^{1}H NMR data was normalized to the weight of each individual. The residual water signal (5.0 ± 0.5 ppm) was excluded, and the spectra were visually inspected to remove regions devoid of chemical signals to ameliorate the quality and validity of the subsequent multivariate analysis (as suggested in Rubingh et al., 2006). Principal Components Analysis was first used to explore differences in metabolite profiles amongst treatment groups using MetaboAnalyst 3.0 (Xia et al., 2015; Xia and Wishart, 2016). Sparse Partial Least-Squares Discriminant Analysis (sPLS-DA) was subsequently used to further

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characterize the separation between the different treatments. One-way ANOVAs with Tukey's HSD post-hoc comparisons were performed to compare the abundances of individual metabolites.

Results and Discussion

Survival and larval mass

There were no mortalities in controls (or solvent controls) and similarly no mortalities in the lowest treatment concentrations for either fungicide after the 96 h exposures. However, four larvae died following exposure to the highest concentration of propiconazole (584.4 nM) and two died in the highest concentration of vinclozolin (1747.6 nM). There was an apparent increase in the mass (mg) of larvae exposed to propiconazole, but this was not significant (p = 0.067; Supplementary Figure S1).

Multivariate analysis of metabolomics data

All surviving larvae were included in the metabolomics analysis. A total of 37 metabolites were identified using a combination of ^{1}H and HSQC spectra (Figure 1) and through comparison with reference spectra in the Human Metabolome Database (Wishart et al., 2012). The pooled HSQC spectrum was also considered to identify optimal peaks for quantification of individual metabolites, and to ensure that metabolites with overlapping ^{1}H resonances were not used since this could produce erroneous conclusions. This resulted in 28 metabolites being deemed suitable for quantification (Figure 1; Supplementary Table S1). Unsupervised PCA analysis identified separation between the various treatment groups (Figure 2a) so metabolite profiles were further analyzed using sPLS-DA to better characterize responses. The more stringent supervised model revealed marked differences between controls (both

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water and solvent) and each of the treatment groups, and also clear differences

between the two fungicides (Figure 2b).

Differences between the fungicides are interesting considering that both are

anti-androgenic (Christen et al., 2014), but are not entirely surprising based on our

knowledge of how these chemicals exert their effects. Specifically, where vinclozolin

is understood to bind with a high affinity to vertebrate androgen receptors (ARs)

(Kavlock and Cummings, 2005), the mechanism for propiconizole is somewhat less

certain but believed to involve inhibition of the enzyme cytochrome p450 aromatase

(CYP p450) necessary for steroidogenesis (Skolness et al., 2013). It should be noted

that there is evidence that neither vinclozolin (Lange et al., 2015; Makynen et al.,

2000) nor propiconazole (Taxvig et al., 2008) behave as (potent) anti-androgens in

fish, and that both fungicides cause oxidative stress (Bruno et al., 2009; Gazo et al.,

2013; Radice et al., 1998; Tu et al., 2015). While there is currently limited

information of this sort specifically for amphibians, changes to several key

metabolites in the present study are indeed suggestive of both altered steroidogenesis

and oxidative stress.

Metabolite alterations consistent with altered steroidogenesis

Formic acid was significantly increased in larvae exposed to vinclozolin at all

concentrations, which is consistent with what has been observed in studies with fish

(Ekman et al., 2007). It has previously been hypothesized that this likely reflects

hepatotoxicity or renal failure (Ekman et al., 2007), but we suggest that this could also

be explained by the anti-androgenic mechanism of action (MoA) of vinclozolin.

Formic acid is a major metabolite by-product of various biochemical processes, most

notably including the steroidogenic conversion of androgens into estrogens (Akhtar et

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al., 1982). Increased formic acid might therefore reflect higher CYP p450 activity, which could be explained by the presence of more unbound androgens available due to competitive displacement by vinclozolin for AR binding sites (Figure 3). We did not measure steroid levels, but increased expression of estrogen receptors (ER) and ER mRNA have previously been reported in fish and frogs exposed to various anti-androgens (Ali et al., 2018; Filby et al., 2007; Langlois et al., 2011), which supports this hypothesis. Formic acid was unchanged in larvae exposed to propiconazole, but this is not surprising considering the MoA involves inhibition of CYP p450 enzymes and would thus impede aromatization (Skolness et al., 2013). We are aware of no studies discussing formic acid as a potential metabolite biomarker of AR-binding anti-androgens, but suggest that this warrants further attention.

Resonances of bile acids were significantly reduced in larvae exposed to vinclozolin (Figure 4a) and marginally so in those exposed to propiconazole (i.e., significantly different from controls but not solvent controls). This is again consistent with studies exploring changes to metabolite profiles in vertebrate wildlife exposed to these fungicides (Ekman et al., 2007; Nesnow et al., 2011). Like formic acid, alterations to bile acids have previously been attributed to hepatotoxicity (Luo et al., 2013), but again we suggest that this could also be symptomatic of broad alterations to steroidogenesis and/or cholesterol metabolism. Bile acids are a primary product of cholesterol degradation, thus reduced levels would be expected as a consequence of lower circulating cholesterol (Chiang, 2009). Anti-androgens have previously been shown to reduce cholesterol levels in humans (Wynn et al., 1986) and to inhibit cholesterol biosynthesis in fish and other vertebrate animals (De Falco et al., 2015; Kumar et al., 2009; Uren Webster et al., 2015). We did not specifically measure cholesterol, but the resonance normally attributed to low-density lipoproteins (LDL)

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was reduced in exposed animals and this likely reflects cholesterol to some extent (Figure 4b). Taken together, this offers supporting evidence of a relationship between observed alterations to metabolite profiles and broad deregulation of various aspects of the steroidogenic pathway. Although we saw only a marginal decrease in bile acids in animals exposed to propiconazole, a study with mice observed marked reductions in bile acids after a similar 96 h exposure (Nesnow et al., 2011). The limited response in this study could relate to the dosage being several orders of magnitude lower than the mice study and/or may suggest lower susceptibility of amphibians to this fungicide.

Metabolite alterations consistent with oxidative stress

Reductions of several key metabolite intermediates of the tricarboxylic acid (TCA) cycle were observed that are characteristic of oxidative stress (Baxter et al., 2006; Boone et al., 2017; Hinder et al., 2013; Tretter and Adam-Vizi, 2000). Specifically, we observed lower levels of citrate, α-ketaglutarate and fumarate (Figure 5). Several amino acids and nucleotides were also decreased in exposed larvae (again, most notably in those exposed to vinclozolin). These observations are reasonable considering that amino acid metabolism and the synthesis of (purine) nucleotide bases are both very closely tied to the TCA cycle (Berg et al., 2002). Reductions to amino acids would thus be expected as a compensatory mechanism when an organism experiences depletions of TCA cycle intermediates (Engelking, 2015). Contrarily, nucleotides are primarily produced by the TCA cycle and depletions are thus a likely outcome of broad inhibition to this pathway.

Niacinamide was also reduced in exposed larvae and is known to play essential roles in nicotinamide adenine dinucleotide redox reactions (i.e., $NAD^+ + H^+$

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+ 2e$^-$ → NADH) of the TCA cycle (Wishart et al., 2012). It is thought that eukaryotic

organisms regulate the TCA cycle as a compensatory mechanism to reduce NADH,

which is a pro-oxidant and has been shown to generate reactive oxygen species (ROS)

that cause oxidative damage (Mailloux et al., 2007). Effects on the TCA cycle and

their relationship to oxidative stress are interesting and should receive further

attention, since we currently know very little about how endocrine active

contaminants elicit responses in wildlife that are not inherently related to the

endocrine system (Mihaich et al., 2017). The fact that many responses in the present

study were apparently highly coordinated highlights the diagnostic strength of

metabolomics for investigating and understanding such effects (Beckonert et al.,

2007). However, the application of metabolomics towards ecological and

toxicological questions is still a rather new approach (Rivas-Ubach et al., 2013; Viant,

2007) and while considerable progress is being made there are still limitations when it

comes to interpreting changes to the metabolome. Greater insights will likely stem

from pairing metabolomics with other omics disciplines (e.g., genomics and

proteomics), and with established techniques in ecotoxicology and endocrinology.

Alterations to other metabolites and interpretive considerations

　　　　Alterations to several metabolites were observed that are not as readily

attributable to known physiological pathways (Table 1), but in some cases are still

consistent with evidence of a broad disruption to energy metabolism. For example,

creatine was significantly decreased in exposed larvae and plays an important role in

replenishing adenosine triphosphate (ATP), which it does by donating a phosphate to

adenosine diphosphate (ADP) (Owen and Sunram-Lea, 2011). Reduced creatine

therefore offers further evidence suggestive of compensatory mechanisms related to

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broad inhibition of the TCA cycle. Another interesting observation was a marked increase in an unknown metabolite with a ^{1}H resonance of 2.82 ppm in larvae exposed to propiconazole. This was initially thought to be methylguanidine since several previous NMR studies have annotated this peak as such (Du et al., 2015; Gupta et al., 2016). However, those studies based metabolite identification upon ^{1}H spectra only, leaving considerable uncertainty. The HSQC spectrum was not consistent with the ^{13}C resonance of 30.08 ppm expected for methylguanidine (Wishart et al., 2012) and we were unable to find a more suitable candidate. We offer this as an example to highlight the importance of acquiring additional 2D NMR data (i.e., beyond simple ^{1}H NMR) when attempting to identify metabolites in complex biological samples.

We would be remiss if we failed to discuss the use of carrier solvents in this and other studies, since it is becoming evident that organic solvents may have an effect on sub-lethal endpoints including aspects of the metabolome (David et al., 2012). In some cases it seems that carrier solvents may even interact with test chemicals (Aliferis and Jabaji, 2011; Melvin et al., 2017; Melvin et al., 2018). There are unfortunately currently limited options for addressing this problem, and the use of solvents is important for pushing solubility limits to facilitate testing dose-response relationships in ecotoxicology (Maes et al., 2012). However, it is important to be aware of potential sources of confounding, and particularly so for sensitive multi-parameter analytical techniques like metabolomics (Simmons et al., 2015). There were few differences between control and solvent control groups for most metabolites in the present study, with the exception of valine and glutamate (Table 1), suggesting the risk of interactive toxicity was limited for these chemicals.

Conclusions

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Metabolomics offers an exciting and powerful approach for exploring the sub-lethal effects of environmental pollutants on wildlife. The provision of broad information about changes to physiologically relevant metabolites makes metabolite profiling applicable to a wide range of pollutants with diverse modes of action. Our results illustrate the potential for using untargeted metabolomics to differentiate between similar pollutants (i.e., anti-androgenic fungicides with distinct MoA), which may be a useful attribute for environmental monitoring. The approach also provided new insights regarding non-endocrine responses to known endocrine disrupting contaminants, thereby contributing to our knowledge of how such pollutants influence biochemical and physiological processes in aquatic wildlife.

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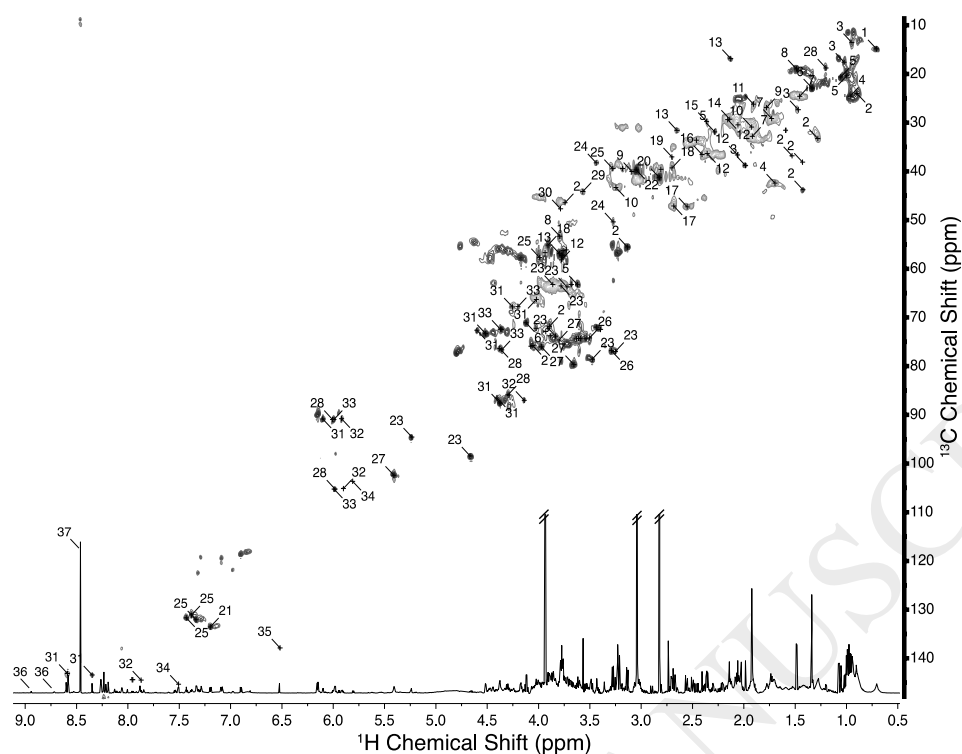
Figures

Figure 1. HSQC spectra with water suppression showing annotated metabolites extracted from pooled sample of larval striped marsh frog (*Limnodynastes peronii*). 1 LDL, 2 Bile acids, 3 Isoleucine, 4 Leucine, 5 Valine, 6 Lactic acid, 7 Lysine, 8 Alanine, 9 Putrescine, 10 Arginine, 11 Acetate, 12 Glutamate, 13 Methionine, 14 Glutamine, 15 Pyruvic acid, 16 Succinic acid, 17 Citric acid, 18 Aspartate, 19 Dimethylamine, 20 Unkown metabolite, 21 Tyrosine, 22 Creatine, 23 Saccharides, 24 Taurine, 25 Phenylalanine, 26 myo-Inositol, 27 Glycogen, 28 UDP Glucose, 29 Glycine, 30 Guanidoacetic acid, 31 Nucleotide bases, 32 Uridine, 33 UDP Galactose, 34 Uracil, 35 Fumarate, 36 Niacinamide, 37 Formic acid.
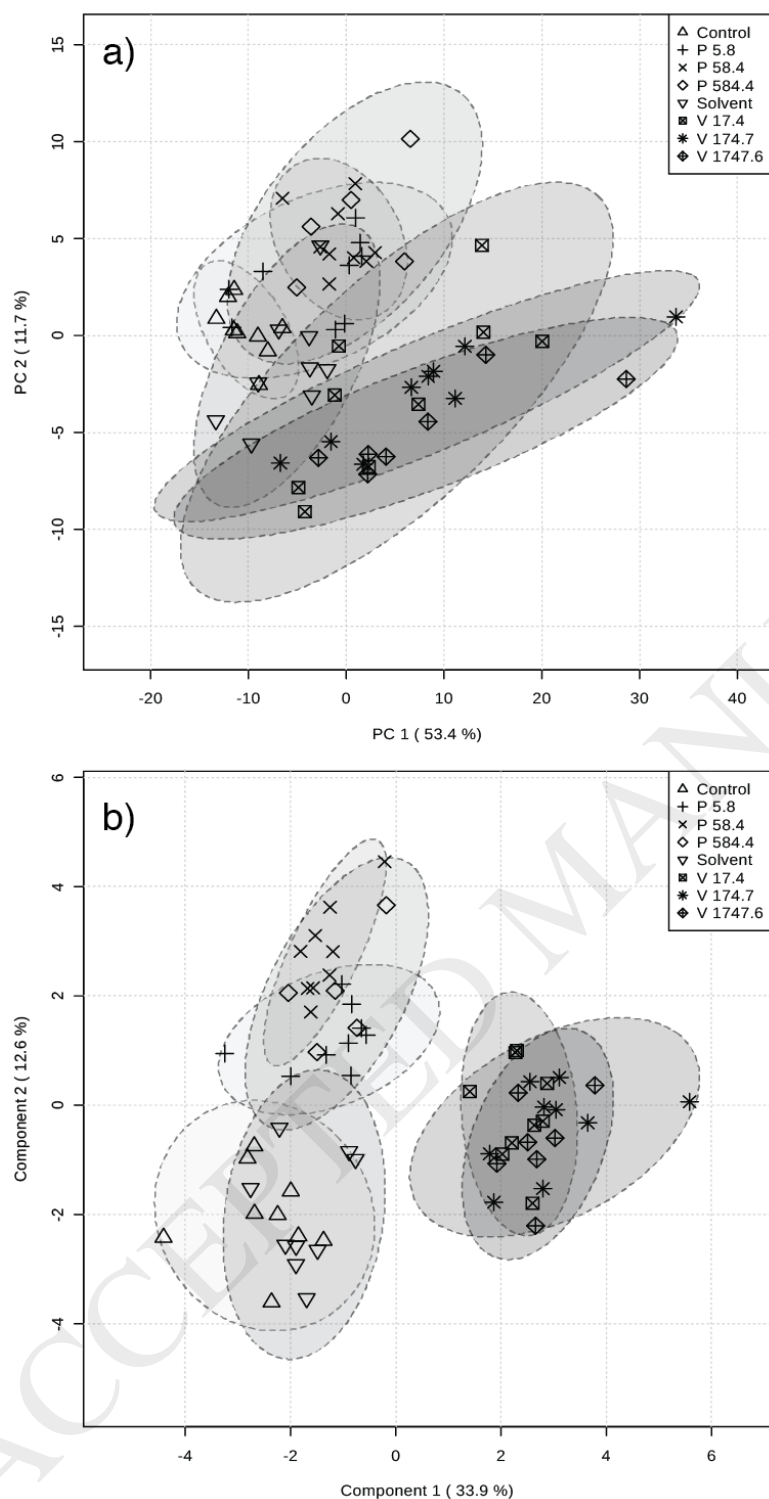
Figure 2. Scores plots for a) unsupervised PCA and b) supervised sPLS-DA showing separation of treatment groups.

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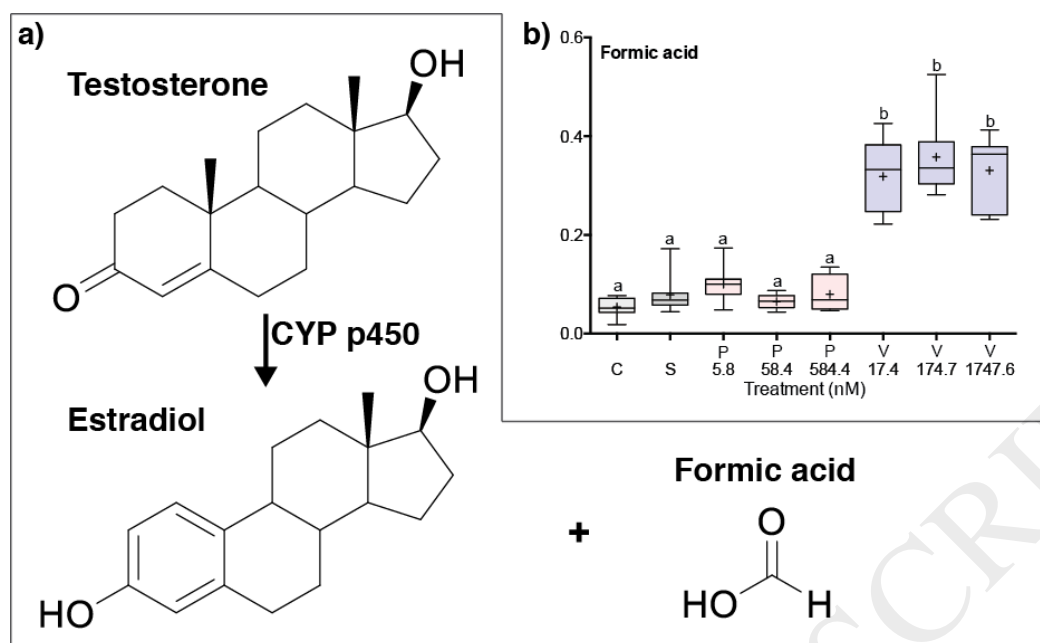
Figure 3. a) the steroidogenic conversion of androgens (e.g., testosterone) into estrogens (e.g., estradiol) is mediated by cytochrome p450 aromatase enzymes and results in the formation of formic acid, b) formic acid was significantly increased in larvae exposed to the AR-binding fungicide vinclozolin but not CYP p450 inhibiting propiconazole. Letters represent groups that are significantly different with $\alpha = 0.05$.

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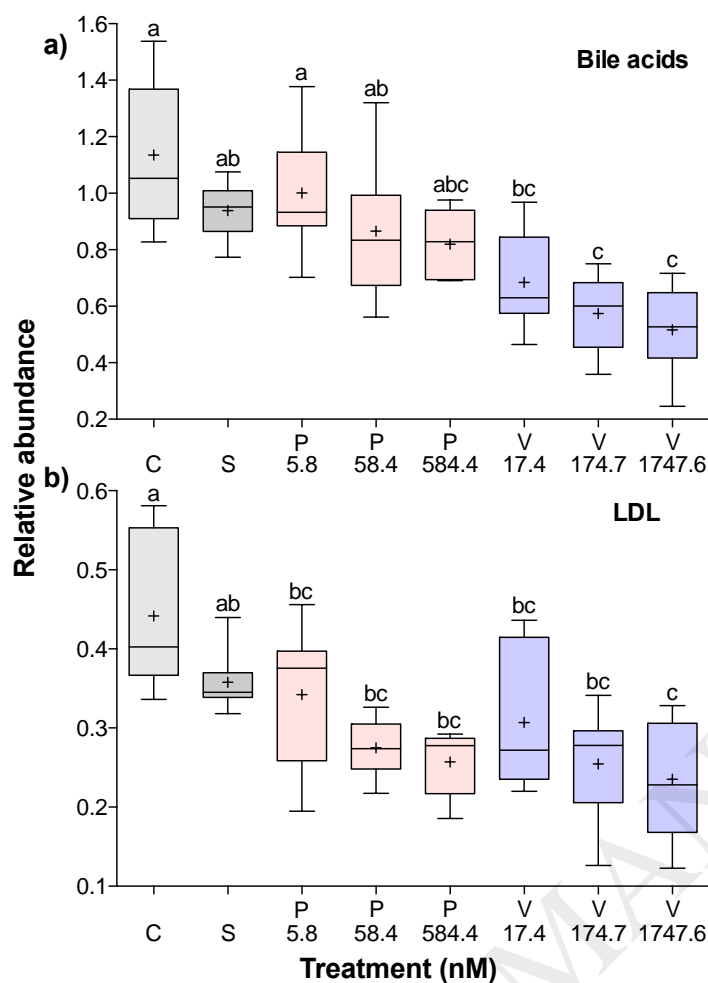
Figure 4. Average relative abundance of a) bile acids and b) low density lipoprotein (LDL) compared to the TSP standard, following exposure of *Limnodynastes peronii* larvae to control (C) and solvent control (S) and a dosing gradient of propiconazole (5.8, 58.4 and 584.4 nM) and vinclozolin (17.4, 174.7 and 1747.7 nM) for 96 hrs. Boxplots represent the interquartile range, median (horizontal line), min and max (whiskers) and average (+) of surviving larvae. Letters represent groups that differ significantly, with α = 0.05.

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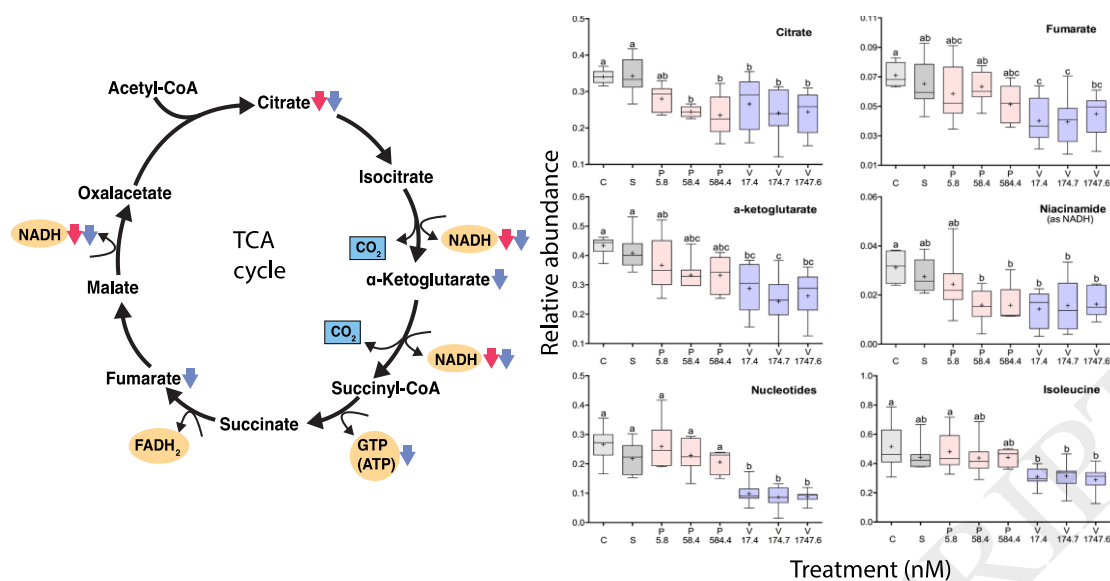
Figure 5. Exposure to vinclozolin and to a lesser extent propiconazole resulted in reduction of key metabolite intermediates of the TCA cycle: citrate, a-ketoglutarate and fumarate. Nucleotide bases, niacinamide and various amino acids (isoleucine shown as an example) are closely tied to this pathway and exhibit similar patterns of reduction. Red arrows represent reductions in propiconazole treatment and blue arrows represent those observed in vinclozolin treatment. Letters in boxplots indicate significant differences with $\alpha = 0.05$.

Metabolite	Control	Solvent	Propiconazole (nM)			Vinclozolin (nM)			p-value
			5.8	58.4	584.4	17.4	174.7	1747.6	
LDL	0.44 ± 0.02^a	0.36 ± 0.02^{ab}	0.35 ± 0.02^{bc}	0.27 ± 0.03^{bc}	0.26 ± 0.03^{bc}	0.31 ± 0.02^{bc}	0.25 ± 0.02^{bc}	0.24 ± 0.03^c	< 0.001
Bile acids	1.14 ± 0.06^a	0.94 ± 0.06^{ab}	0.99 ± 0.08^a	0.89 ± 0.07^{ab}	0.82 ± 0.08^{abc}	0.68 ± 0.06^{bc}	0.57 ± 0.06^c	0.52 ± 0.07^c	< 0.001
Isoleucine	0.52 ± 0.04^a	0.44 ± 0.04^{ab}	0.48 ± 0.03^a	0.44 ± 0.04^{ab}	0.44 ± 0.05^{ab}	0.31 ± 0.04^b	0.32 ± 0.04^b	0.29 ± 0.04^b	< 0.001
Leucine	0.90 ± 0.07^a	0.74 ± 0.07^{ab}	0.82 ± 0.06^a	0.76 ± 0.07^{ab}	0.75 ± 0.09^{abc}	0.47 ± 0.07^{bcd}	0.43 ± 0.07^{cd}	0.37 ± 0.07^d	< 0.001
Valine	0.41 ± 0.02^a	0.27 ± 0.02^b	0.27 ± 0.02^b	0.21 ± 0.03^{bc}	0.23 ± 0.03^{bc}	0.22 ± 0.02^{bc}	0.18 ± 0.02^{bc}	0.14 ± 0.03^c	< 0.001
Lactic acid	1.19 ± 0.28	1.49 ± 0.28	1.30 ± 0.26	1.21 ± 0.29	0.93 ± 0.37	1.42 ± 0.28	1.25 ± 0.28	1.65 ± 0.31	0.857
Alanine	0.23 ± 0.02^a	0.27 ± 0.02^{ab}	0.27 ± 0.02^a	0.28 ± 0.02^a	0.26 ± 0.03^{ab}	0.17 ± 0.02^b	0.18 ± 0.02^b	0.19 ± 0.02^{ab}	< 0.001
Putrescine	0.47 ± 0.04	0.49 ± 0.04	0.46 ± 0.03	0.42 ± 0.04	0.40 ± 0.05	0.36 ± 0.04	0.34 ± 0.04	0.36 ± 0.04	0.02
Acetate	1.39 ± 0.11^{ab}	1.47 ± 0.11^a	1.07 ± 0.10^{ab}	0.95 ± 0.12^b	0.95 ± 0.15^{ab}	0.95 ± 0.11^b	0.97 ± 0.11^b	1.24 ± 0.12^{ab}	0.003
Glutamate	1.00 ± 0.04^a	0.73 ± 0.04^b	0.68 ± 0.04^{bc}	0.62 ± 0.04^{bcd}	0.56 ± 0.05^{bcd}	0.56 ± 0.04^{cd}	0.50 ± 0.04^d	0.49 ± 0.04^d	< 0.001
Methionine	0.52 ± 0.03	0.51 ± 0.03	0.49 ± 0.03	0.45 ± 0.03	0.41 ± 0.04	0.41 ± 0.03	0.42 ± 0.03	0.42 ± 0.03	0.037
Succinate	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.519
Citrate	0.34 ± 0.02^a	0.34 ± 0.02^a	0.28 ± 0.02^{ab}	0.25 ± 0.02^b	0.24 ± 0.02^b	0.27 ± 0.02^b	0.24 ± 0.02^b	0.24 ± 0.02^b	< 0.001
Aspartate	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.039 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.033
Dimethylamine	0.26 ± 0.01^a	0.25 ± 0.01^{ab}	0.21 ± 0.01^{abc}	0.19 ± 0.01^{bc}	0.18 ± 0.02^c	0.19 ± 0.01^{bc}	0.18 ± 0.01^c	0.19 ± 0.02^{bc}	< 0.001
Unidentified	1.47 ± 0.22^c	1.18 ± 0.22^c	2.68 ± 0.21^a	2.76 ± 0.23^a	2.66 ± 0.29^{ab}	1.66 ± 0.22^{bc}	1.37 ± 0.22^c	1.57 ± 0.25^c	< 0.001
Tyrosine	0.03 ± 0.01	0.07 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.12
Creatine	3.39 ± 0.15^a	3.05 ± 0.15^{ab}	2.68 ± 0.15^{bc}	2.52 ± 0.16^{bcd}	2.22 ± 0.21^{cd}	2.17 ± 0.15^{cd}	1.87 ± 0.15^d	2.04 ± 0.17^{cd}	< 0.001
Sugars	0.13 ± 0.03	0.19 ± 0.03	0.20 ± 0.03	0.16 ± 0.03	0.13 ± 0.04	0.19 ± 0.03	0.15 ± 0.03	0.12 ± 0.03	0.386
Taurine	0.06 ± 0.01^a	0.04 ± 0.01^{ab}	0.06 ± 0.01^a	0.03 ± 0.01^b	0.02 ± 0.01^b	0.02 ± 0.01^b	0.02 ± 0.01^b	0.02 ± 0.01^b	< 0.001
Phenylalanine	0.05 ± 0.01^{ab}	0.06 ± 0.01^{ab}	0.05 ± 0.01^{ab}	0.07 ± 0.01^a	0.06 ± 0.01^{ab}	0.03 ± 0.01^b	0.04 ± 0.01^{ab}	0.05 ± 0.01^{ab}	0.029
myo-Inositol	0.41 ± 0.02^a	0.32 ± 0.02^{ab}	0.34 ± 0.02^{ab}	0.28 ± 0.02^{bc}	0.29 ± 0.03^{bc}	0.26 ± 0.02^{bc}	0.22 ± 0.02^c	0.20 ± 0.03^c	< 0.001
Glycine	0.29 ± 0.02^a	0.29 ± 0.02^{ab}	0.24 ± 0.02^{abc}	0.23 ± 0.02^{abc}	0.20 ± 0.02^c	0.25 ± 0.02^{abc}	0.22 ± 0.02^{bc}	0.22 ± 0.02^{abc}	0.003
Nucleotides	0.27 ± 0.02^a	0.22 ± 0.02^a	0.25 ± 0.02^a	0.24 ± 0.02^a	0.21 ± 0.02^a	0.10 ± 0.02^b	0.09 ± 0.02^b	0.09 ± 0.02^b	< 0.001
Uracil	0.025 ± 0.004	0.034 ± 0.004	0.029 ± 0.004	0.029 ± 0.005	0.030 ± 0.006	0.022 ± 0.004	0.035 ± 0.004	0.044 ± 0.005	0.077

Fumarate	0.07 ± 0.01^a	0.07 ± 0.01^{ab}	0.06 ± 0.01^{abc}	0.07 ± 0.01^{ab}	0.05 ± 0.01^{abc}	0.04 ± 0.01^c	0.04 ± 0.01^c	0.05 ± 0.01^{bc}	< 0.001
Niacinamide	0.031 ± 0.003^a	0.026 ± 0.003^{ab}	0.023 ± 0.003^{ab}	0.016 ± 0.00^{3b}	0.016 ± 0.004^b	0.014 ± 0.003^b	0.016 ± 0.003^b	0.016 ± 0.003^b	< 0.001
Formic acid	0.06 ± 0.02^b	0.07 ± 0.02^b	0.09 ± 0.02^b	0.07 ± 0.02^b	0.09 ± 0.02^b	0.32 ± 0.02^a	0.33 ± 0.02^a	0.33 ± 0.02^a	< 0.001

Table 1. Relative abundance (± SEM) of quantifiable metabolites compared to the TSP standard. Different letters represent treatment groups that differ significantly based on one-way ANOVA with Tukey's HSD post-hoc comparisons.