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Research Paper

Endogenous and exogenous biomarker analysis in terrestrial phase amphibians (*Lithobates sphenocephala*) following dermal exposure to pesticide mixtures

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Environmental context. Metabolomics can be used to provide a snapshot of an organism's physiology as the organism is exposed to varying environmental conditions. In this study, laboratory-reared amphibians were exposed to multiple pesticides, analogous to field exposures, resulting in an impact to both pesticide body concentrations and the amphibians' hepatic metabolome. These data can be used in the environmental and ecological risk assessment of multiple pesticides in non-target species.

Abstract. Pesticide mixtures are frequently co-applied throughout an agricultural growing season to maximise crop yield. Therefore, non-target ecological species (e.g. amphibians) may be exposed to several pesticides at any given time on these agricultural landscapes. The objectives of this study were to quantify body burdens in terrestrial phase amphibians and translate perturbed metabolites to their corresponding biochemical pathways affected by exposure to pesticides as both singlets and in combination. Southern leopard frogs (*Lithobates sphenocephala*) were exposed either at the maximum or 1/10th maximum application rate to single, double or triple pesticide mixtures of bifenthrin (insecticide), metolachlor (herbicide) and triadimefon (fungicide). Tissue concentrations demonstrated both facilitated and competitive uptake of pesticides when in mixtures. Metabolomic profiling of amphibian livers identified metabolites of interest for both application rates; however, the magnitude of changes varied for the two exposure rates. Exposure to lower concentrations demonstrated downregulation in amino acids, potentially owing to their usage for glutathione metabolism and/or increased energy demands. Amphibians exposed to the maximum application rate resulted in upregulation of amino acids and other key metabolites likely owing to depleted energy resources. Coupling endogenous and exogenous biomarkers of pesticide exposure can be used to form vital links in an ecological risk assessment by relating internal dose to pathophysiological outcomes in non-target species.

Additional keywords: application rate, body burden, frog, metabolomics.

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Introduction

Pesticide application practices on agricultural fields change continuously depending on the season, crop, or nuisance pest being targeted (Laetz et al. 2009). Herbicides are usually applied in the springtime on peanuts and cotton while fungicides are applied multiple times throughout the summer in the USA's southern states (Glinski et al. 2018). Owing to the frequent application of pesticides, some pests have developed resistance, which has resulted in higher application rates and greater application frequencies over time (Mann et al. 2009). Pesticides are often applied in combination to help minimise application costs and simultaneously combat multiple pests (Cloyd 2012). As a result, many pesticides can be detected in agricultural settings; moreover, low level residues can persist well after they have been applied. Subsequently, as a result of post-application persistence and the increased use of pesticide mixtures, many non-target species are exposed to multiple pesticides concurrently. Pesticide mixtures have been detected in streams,

sediment, surface water, and rainwater (Gilliom et al. 2006; Smalling et al. 2012; Potter and Coffin 2017).

Amphibians are often considered as integrated indicators of the health of an ecosystem because they are a unique class of vertebrates owing to their biphasic life histories that incorporate both aquatic and terrestrial habitats. As tadpoles, amphibians are continuously exposed to contaminants in the aquatic environment. Many species of terrestrial adults remain near aquatic environments to stay hydrated, thus they can become co-contaminated by terrestrial and aquatic ecosystems. Amphibian populations have been declining globally over the past few decades owing to exposure to multiple stressors such as pesticides, deforestation, pollution, and diseases (Blaustein et al. 1994; Sparling et al. 2001; Christin et al. 2003). Since the 1980s, more than 120 amphibian species have become extinct globally, while over one-third of amphibian species are threatened worldwide (Whitfield et al. 2007).

Multiple studies have investigated the effects of pesticides on aquatic stage amphibians. Boone (2008) found that exposure to three insecticides reduced the tadpole mass at metamorphosis. This was supported by Hayes et al. (2006), where pesticide mixtures resulted in the inhibition of growth and development in *Rana pipiens*. Relyea (2004) noted that combinations of four pesticide formulations reduced survival and growth within exposed amphibian larvae. Similar results were obtained by Relyea (2009) and Hua and Relyea (2014), where mixtures reduced survival more than their corresponding individual pesticides. A synergistic relationship was observed between atrazine and organophosphates effects in midges and in *Xenopus laevis* (Pape-Lindstrom and Lydy 1997; Wacksman et al. 2006). In amphibian larvae that were exposed to a mixture of atrazine and alachlor, the toxicity was observed to be greater than the separate additive toxicities (Howe et al. 1998). Conversely, Boone and Bridges-Britton (2006) observed no effects of multiple chemical stressors on tadpoles; however, this may arise from the fact that the compounds they used had different modes of action. Similar observations of no effects have been found in other studies (Boone and James 2003; Relyea 2009). Overall, there is a lack of information on terrestrial exposure to mixtures because most studies to date have only been in aquatic settings.

Biomarker analysis and metabolite or pathway identification can be used in the assessment of pesticide exposures. Identifying internal biochemical consequences of pesticide exposure can connect exposure to effects and contribute to the assessment of higher level effects within an ecosystem (Venturino et al. 2003). Metabolomics examines the endogenous levels of metabolites, such as amino acids, fatty acids, and sugars, which may be up or downregulated following exposure to pesticides or other xenobiotics. Quantifying differences in fluxes in the metabolome make it possible to identify specific pathways (e.g. citric acid cycle or fatty acid biosynthesis) that are perturbed leading to an adverse outcome. Therefore, metabolomics and biomarker identification can determine impacted key pathway events within the metabolome after a molecular initiating event and before, or simultaneous with, observed changes in measured apical endpoints (i.e. growth, reproduction, and mortality).

The objectives of this study were to measure the body burdens and liver metabolomic profiles in terrestrial phase amphibians (southern leopard frogs, *Lithobates sphenocephala*) after pesticide exposure. Treatments included individual and mixture exposures of three pesticides (metolachlor (Met), triadimefon (Tdn), and bifenthrin (Bif)) at rates representing the maximum and 1/10th maximum labelled application rates. Maximum application rates can be considered as a worst-case, direct exposure scenario, while the 1/10th maximum application rates better represent the post-application residual concentration of a pesticide in agricultural fields. Metabolomic profiles of the amphibian liver were used to identify biochemical perturbations that occurred during the treatments. Understanding and differentiating how single and joint exposures impact amphibian metabolomic profiles is necessary to elucidate synergistic and antagonistic interactions at the biochemical level and can lead to more accurate estimation of ecological risk.

Experimental

Chemicals

Active ingredients (AI) and corresponding metabolites for Met, metolachlor ethane sulfonic acid (MESA), metolachlor oxanilic acid (MOXA), Tdn, triadimenol (Tdl), Bif, permethrin (internal standard), and tetraconazole (internal standard) were obtained from the USA Environmental Protection Agency's National Pesticides Standard Repository (Fort Meade, MD, USA). All pesticides used in the study were ≥96.5 % purity. All solvents used for pesticide extraction and analysis were of highest grade and purchased from Fisher Scientific (Pittsburgh, PA, USA).

Amphibian exposures

Amphibian collection and rearing was previously described by Van Meter et al. (2014) and exposure studies were carried out based on the methods reported by Van Meter et al. (2016). Briefly, soil was processed through a 2-mm sieve and stored in a cold room <4 °C until used; all soil was collected from Newton, GA in July 2015. Soil parameters are available from the literature (Van Meter et al. 2016). Each Pyrex® bowl contained 150 g of sieved soil and a full factorial exposure design with three pesticides (Tdn, Met, and Bif) at two concentrations (maximum or 1/10th maximum application rate) was applied to the soil with 8 individual replicates per treatment. Application rates were scaled down to the size of 8 Pyrex® bowls (1800 cm^2; each bowl 15 cm × 15 cm) to minimise spraying time and variability between replicates. Application rates of Bif, Met and Tdn were 3.45, 30.62 and 2.87 μg cm^{-2}, respectively, for maximum application rate, while for the 1/10th maximum application rate of Bif, Met and Tdn were 0.345, 3.062 and 0.287 μg cm^{-2}, respectively. Active ingredients were combined and dissolved in 75 mL of methanol (MeOH), while 100 % MeOH was used as a control, and applied to the 8 Pyrex® bowls using a Spray Gun® canister attached to a graduated glass jar. After application, all bowls were placed in a fume hood overnight to evaporate the MeOH. The following morning, each bowl was rehydrated with 50 mL of deionised water. Amphibians were dehydrated for 10 h before 8 h exposure to pesticide contaminated soil with one amphibian per bowl, total $n = 8$ per treatment. Leopard frogs used in the study were at least 180 days post-metamorphosis. After the experiment, amphibians were killed and stored at −80 °C while soil sample aliquots (~5 g) were collected and stored at −20 °C until processing and analysis.

Metabolomics and pesticide analysis

Leopard frogs were thawed and a final weight was obtained before livers were excised and placed into pre-labelled 2-mL centrifuge tube and immediately placed on ice. Endogenous hepatic metabolites were extracted and analysed following the bi-phasic procedure described by Viant (2007). Briefly, livers were extracted with a mixture of methanol and chloroform using

2-mm beads in a tissue homogeniser to separate metabolites into a polar and non-polar fraction. Individual fractions were placed into 2-mL vials and evaporated to dryness overnight on a SpeedVac Concentrator. Samples were derivatised with 100 μL of methoxyamine hydrochloride in pyridine (20 mg mL^{-1}; 2.5 h, 60 °C) and then with 100 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 10 % TCMS (1.5 h, 60 °C) for a total of 4 h. After derivatisation, all polar samples were analysed by gas chromatography coupled with mass spectrometry (GC-MS) as described below.

Methods for pesticide and soil extractions for body burden analysis have been described by Van Meter et al. (2014). Briefly, remaining whole body homogenates and soil samples were spiked with 10 μL of 1000 ppm tetraconazole as an internal standard and whole body homogenates were placed on a freeze drier overnight. Soil and amphibian samples were extracted two times with MeOH by sequential sonication and centrifugation. Supernatants from both extractions were combined and blown down to 1 mL under a gentle stream of nitrogen gas. Then, liquid-liquid extraction was completed with methyl *tert*-butyl ether (3 mL MTBE). A 1-mL aliquot was transferred to a 2-mL vial and blown dry under nitrogen, then reconstituted with 1 mL 30 % MeOH (v:v) and analysed on a liquid chromatograph coupled to a mass spectrometer (LC-MS/MS) as described below.

Gas chromatography with time-of-flight mass spectrometry

Metabolomic samples were analysed on a LECO Pegasus® 4D *gas chromatography* with time-of-flight *mass* spectrometer (GC-ToF/MS; St Joseph, MI, USA) with an Agilent 7890B gas chromatograph and all data were processed with *ChromaTOF*® software. The separation column was a DB-5 ms (30 m, 0.25-μm thickness, and 0.25-mm internal diameter; Agilent Technologies, CA, USA). All injections were made in splitless mode (2 μL). The injector temperature was 275 °C, the transfer line temperature was held constant at 280 °C and the ion source temperature was 225 °C. The carrier gas was ultra-high purity grade helium and maintained at a constant flow of 0.8 mL min^{-1}. The oven temperature was held at 60 °C for the first 2 min and then ramped at 8 °C min^{-1} to 300 °C with a hold time of 5 min. Mass spectra were acquired from 50–650 *m*/*z* at an acquisition rate of 20 spectra/second.

LC-MS/MS

Active ingredients as well as their metabolites were analysed on a Varian Prostar HPLC interfaced to a Varian 1200L triple quadrupole mass spectrometer. Compounds were separated with an Eclipse XDB-C18 column (3.5-μm particle size, 3.0 × 150 mm; Agilent Technologies, CA, USA). Initial conditions were held for 2 min at 70 % water with 0.05 % formic acid and 2 mM ammonium acetate (A) and 30 % methanol with 0.05 % formic acid and 2 mM ammonium acetate (B), and then ramped to 95 % B over 22 min and held for 6 min before returning to starting conditions (total runtime 40 min). The flow rate was 400 μL min^{-1} and injection volume was 20 μL. The drying gas was set at 225 °C and the capillary voltage was at 60 V for all compounds analysed. MOXA and MESA were the only analytes analysed in negative mode from 0–17 min; all other pesticides were analysed in positive mode from 17–40 min (Table S1, Supplementary Material).

Statistical methods

Body burden concentrations from each of the treatments were tested for normality with Shapiro-Wilkes with the null hypothesis being that the data came from a normal distribution. *t*-tests with a Bonferroni correction were performed to compare the single pesticide treatment for each of the pesticides with the multiple pesticide treatments for that same pesticide. Six tests (two exposure levels and three multiple treatments per pesticide) were performed for each of the three pesticides for a total of 18 *t*-tests.

All chromatograms related to metabolomics were exported as netCDF files and imported into *MetAlign 041012* for data alignment and processing using recommended parameters for time-of-flight mass spectrometry (Lommen 2009). After alignment, spectra were filtered and duplicate retention times were removed using *Microsoft Excel* as described by Niu et al. (2014). *Excel* was used to generate *t*-test filtered chromatograms and *MetaboAnalyst 3.0* was used for metabolite pathway analysis (Xia and Wishart 2016). Metabolites were identified using the NIST 2014 spectral database.

Results

Tissue concentration for maximum application rate

This study identified body burdens in terrestrial phase amphibians after an 8 h dermal exposure. A total of 18 *t*-tests were performed that compared body burden concentrations from single pesticide treatment to multiple pesticide treatments. Only two of the 18 *t*-tests showed a significant difference between the single and multiple treatment comparisons, which was roughly what would be expected as a false positive rate given a significance test with a *p*-value of 0.05 and 18 tests. Therefore, we did not find any support for the hypothesis that single versus multiple pesticide treatments impacted body burdens within this study. However, we present the descriptive statistics of each of the treatments below to add to the literature since these types of exposure studies are limited for amphibians. Amphibians exposed to Met had a body burden of 7.53 ± 2.20 s.e. μg g^{-1}; however, when exposed as a component of a mixture, the concentrations were lower (3.11 ± 0.51 μg g^{-1}, BifMet; 2.01 ± 0.37 μg g^{-1}, MetTdn), the lowest tissue concentration was 1.99 ± 0.42 μg g^{-1} for the complete mixture (Fig. 1). Amphibian tissue concentrations for Bif were 0.367 ± 0.046 μg g^{-1} for the single treatment and 0.381 ± 0.045 μg g^{-1} for the complete mixture. Observed concentrations for Bif were higher in the presence of Met (0.461 ± 0.089 μg g^{-1}) and Tdn (0.670 ± 0.084 μg g^{-1}). For Tdn, the observed tissue concentrations were 0.326 ± 0.051 μg g^{-1} and 0.311 ± 0.078 μg g^{-1} for the complete mixture. Concentrations of Tdn in the joint presence of Bif (0.413 ± 0.060 μg g^{-1}) and Met (0.313 ± 0.065 μg g^{-1}) were also similar.

Tissue concentration for 1/10th maximum application rate

Metolachlor had a tissue concentration of 0.163 ± 0.032 μg g^{-1} when singly applied and 0.359 ± 0.055 μg g^{-1} for the complete mixtures (Fig. 1). In the presence of Bif, Met decreased to 0.136 ± 0.026 μg g^{-1} (BifMet), while in the presence of Tdn, Met increased to 0.208 ± 0.031 μg g^{-1} (MetTdn). Singlet treatment of Bif resulted in concentrations of 0.088 ± 0.016 μg g^{-1} and 0.103 ± 0.022 μg g^{-1} for the triple mixture. While the Bif body burden was lower in the presence of Met (0.039 ± 0.008 μg g^{-1}), Tdn only slightly lessened the dermal uptake to 0.061 ± 0.009 μg g^{-1} (BifTdn). Independently, Met and Bif did not statistically affect the Tdn total body burden; (0.037 ± 0.005 μg g^{-1}, MetTdn; 0.060 ± 0.009 μg g^{-1}, BifTdn) however, when all three were exposed together, Tdn levels increased from 0.038 ± 0.006 μg g^{-1} (Tdn) to

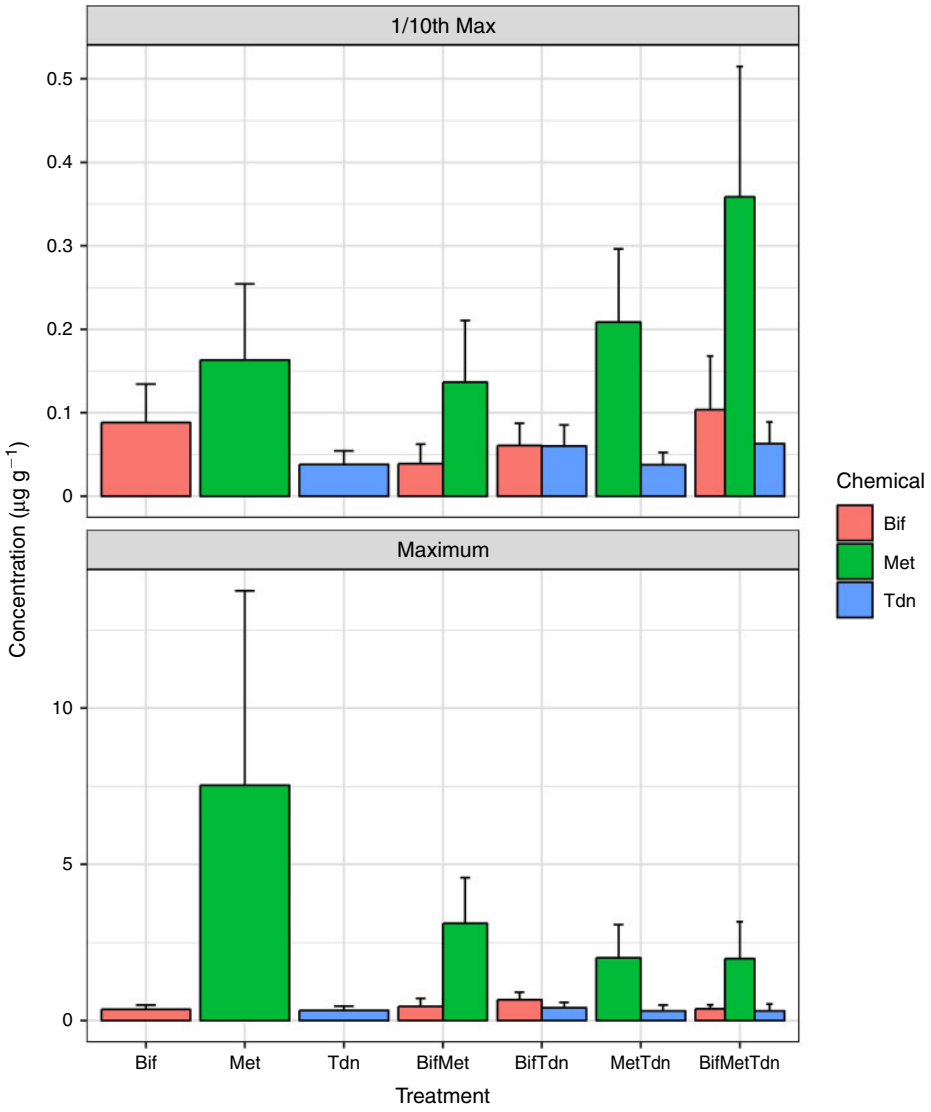

Fig. 1. Tissue concentrations (µg g^{-1}) of pesticide(s) in amphibians exposed at the 1/10th maximum and maximum application rate. Mean ± s.e. of each pesticide that was exposed.

0.063 ± 0.009 µg g^{-1} (BifMetTdn). Interestingly, Met was the only pesticide with statistically increased body burdens when applied in mixtures (Fig. 1).

Metabolomics

To assess the consequence of exposure to pesticide and pesticide mixtures on hepatic biochemical profiles in amphibians, polar metabolites were analysed on a GC-ToF/MS to identify the potential biochemical pathways affected. As a rough aid for visualising the 'biological impact' on the metabolome, the statistically significant retention times (*m*/*z* pairs after *t*-test filtering) were summed for each treatment, which was analogous to how many metabolites were detected that were significantly different (Fig. 2). Surprisingly, the summed biological impact of the maximum application rate was smaller in statistically significant spectral features compared with the 1/10th maximum application rate (Fig. 2). For the 1/10th maximum application exposure, Met had the largest abundance of spectral features changed. However, for the maximum application rate, BifMet exhibited the greatest number of spectral features modified by exposure (Fig. 2).

The chromatograms were further processed to determine the retention times and *m*/*z* values that were statistically different between each pesticide or pesticide mixture and the control to determine similar metabolites affected by exposure (Table 1; Figs S1 and S2, Supplementary Material). Overall, 75 metabolites were identified as statistically up or downregulated for any exposure compared with the controls. Metabolites that were up or downregulated included amino acids (i.e. alanine, serine, leucine, and proline), carbohydrates (i.e. galactose, maltose, and mannose) and nucleic acid content (i.e. adenine, thymine, and hypoxanthine). The maximum exposure to Bif had the fewest number of metabolites ($n = 8$) that were modified by exposure, while BifTdn at the 1/10th maximum application had the highest number of metabolites affected ($n = 44$). On average, the maximum application rates included 22 putatively identified metabolites that changed in concentration compared with 38 metabolites in the 1/10th maximum application rate treatments. Furthermore, pesticides applied as singlets had, on average, fewer metabolites up or downregulated compared with the doublet and triplet mixtures.

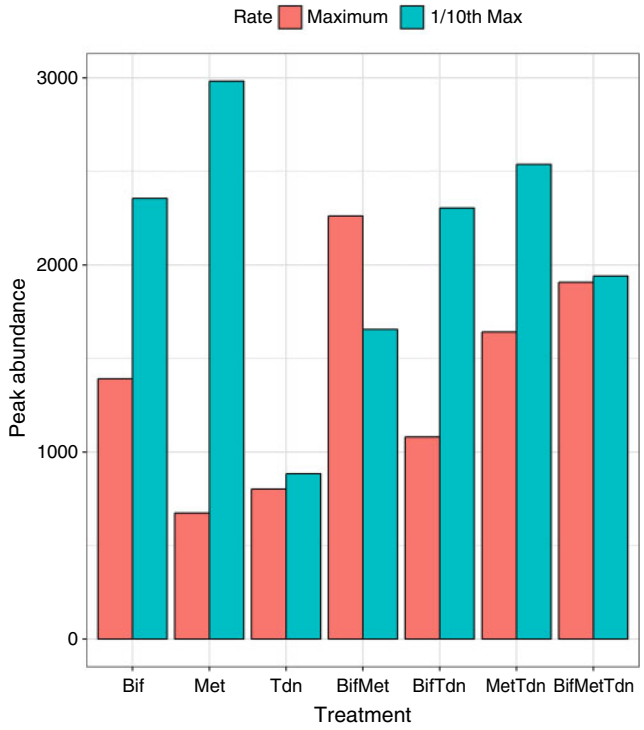

Fig. 2. Summation of spectral features that were affected by pesticide treatment either at the maximum or the 1/10th maximum application rate.

These metabolites were used to identify the biological pathways that were affected by pesticide exposure in amphibian livers using Metaboanalyst's pathway analysis (Table S2, Supplementary Material). Overall, only three pathways were similar among all pesticide treatments and both application rates: 1) aminoacyl-tRNA biosynthesis; 2) alanine, aspartate, and glutamate metabolism; and 3) arginine and proline metabolism. Other pathways that exhibited changes in response to pesticide exposure treatments included valine, leucine and isoleucine biosynthesis; glutathione metabolism; nitrogen metabolism; and butanoate metabolism (Table S2).

Discussion

The objectives of the current study were to identify the effects from pesticides applied as singlets and mixtures in terrestrial phase amphibians by measuring body burdens and examining the metabolic profiles of the liver. The majority of amphibian and pesticide exposure studies in the current literature has focussed on tadpoles (Brühl et al. 2011). To date, limited studies have assessed the effects of pesticide exposure on terrestrial phase amphibians (Brühl et al. 2011; Cusaac et al. 2015; Cusaac et al. 2016; Dinehart et al. 2009; Van Meter et al. 2015; Van Meter et al. 2016; Van Meter et al. 2014). Therefore, there is a paucity of data on exposures to mixtures of pesticides and their impacts on terrestrial phase amphibians. The paper by Van Meter et al. (2018) is the only previously published article that assessed tissue concentrations of multiple active ingredients on terrestrial post-metamorphic amphibians. As in the present study, Van Meter et al. (2018) used a full factorial exposure design by comparing all combinations/pairings of three pesticides.

Few studies have investigated the modification of biochemical profiles in tadpoles exposed to pesticides (Zaya et al. 2011; Dornelles and Oliveira 2014; Güngördü et al. 2016). To date, only two studies have used metabolomics to examine the effects of pesticide exposure on amphibians (Snyder et al. 2017; Van Meter et al. 2018). From these studies, little can be generally concluded about the metabolic impacts of pesticides on amphibians. In the current study, up and downregulations were observed for various metabolite classes, such as amino acids, purine nucleosides, and carbohydrates. Snyder et al. (2017) observed that atrazine exposure can affect purine metabolism, amino acid metabolism, and aminoacyl-tRNA biosynthesis and disrupt amino acid and energy metabolism. In this study, three different biological pathways were affected by all classes of pesticide exposures. This is in contrast to the study by Van Meter et al. (2018), where amphibians were exposed to five pesticides either as a single, double or triple mixture, and either as all herbicides or a mixture of herbicide, insecticide and fungicide. The main metabolites of significance resulted in eight different pathways identified as being affected by a triple herbicide mixture (atrazine, metolachlor, and 2,4-D), while for the mixed pesticide mixture (atrazine, propiconazole, and malathion), twelve biological pathways were affected (Van Meter et al. 2018). However, aminoacyl-tRNA biosynthesis and alanine, aspartate, and glutamate metabolism were the only two pathways that were identified in the current study and by Van Meter et al. (2018).

In the current study, the 1/10th maximum exposures were observed to have a higher abundance of statistically different spectral features compared with maximum application exposures. This could arise from the organism overextending itself to eliminate low levels of xenobiotics or that at high concentrations, overt toxicity results in a 'shutdown' of important homeostatic pathways. The triple pesticide mixture at maximum exposure had the second highest abundance of spectral features modified, which could indicate that the pesticides, all having different modes of action, overwhelmed the system. However, for the 1/10th maximum treatments, the triple pesticide mixture was fifth highest in abundance. In the article by Van Meter et al. (2018), the authors observed that the triple pesticide exposure had the lowest overall biological impact on the metabolome, which they stated was potentially as a result of the different modes of action, also observed in the current study.

Metabolomics

t-tested 'filtered' chromatograms were used to determine the statistical differences between pesticide treated and control samples for all exposures tested. Both up and downregulated metabolites were found in groups of endogenous metabolite classes such as amino acids, carbohydrates, organic acids, and sugar/phosphoric acid derivatives. These endogenous metabolite classes were similar to what was observed by Snyder et al. (2017), which examined the effects of atrazine exposure in tadpoles, and by Van Meter et al. (2018), which explored the effects of mixtures, both one of each class and three different herbicides, on terrestrial phase amphibians. The identification of these metabolites was not unexpected as GC-MS is amenable to derivatised lipids, sugars, aldehydes, ketones esters, amino acids, and fatty acids.

Overall, in the current study, we observed a decrease in the amino acids alanine, glycine, methionine, proline, serine, threonine, and tyrosine in the 1/10th maximum application rate exposure group, while at the same time, there was an upregulation of lactose, maltose, and galactose. This same phenomenon was described by Nagato et al. (2016) in diazinon exposed

Table 1. Metabolites that were statistically ($P \leq 0.05$) up or downregulated following exposure to bifenthrin, metolachlor or triadimefon, compared with control, at the maximum application rate and 1/10th maximum application rate

	Maximum application rate							1/10th maximum application rate						
Pathway/Compound	Bif	Met	Tdn	BifMet	BifTdn	MetTdn	BifMetTdn	Bif	Met	Tdn	BifMet	BifTdn	MetTdn	BifMetTdn
Alanine, aspartate and glutamate metabolism														
Alanine		↑		↑		↑	↑	↓	↓	↓	↓	↓		↓
Aspartic acid			↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓
GABA	↑	↑	↑	↑		↑	↑	↓	↓	↓		↓		↓
Glutamic acid				↑		↑	↑		↓	↓				
Succinic acid	↓		↑	↑	↑	↑	↑				↑	↑		
Amino sugar and nucleotide sugar metabolism														
Fructose											↓		↓	↓
Mannose		↓		↓		↓					↓		↓	
N-acetyl-D-glucosamine					↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
Aminoacyl-tRNA biosynthesis														
Alanine		↑		↑		↑	↑	↓	↓	↓	↓	↓		↓
Aspartic acid			↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓
Glutamic acid				↑		↑	↑		↓	↓				
Glycine				↑		↑	↑	↓	↓	↓				
Isoleucine									↓	↓		↓	↓	↓
Leucine		↓		↓		↑	↑	↓	↓	↓		↓	↓	↑
Lysine		↑		↑		↑	↑	↑	↑		↑	↓	↓	↑
Methionine				↑		↑	↑	↓	↓					
Phenylalanine			↑	↑		↑	↑				↑			↑
Proline	↑	↑	↑	↑		↑	↑	↓	↓	↓	↓	↓		↓
Serine			↑			↑	↑					↓	↓	
Threonine				↑	↑	↑	↑					↓		↓
Valine		↑		↑		↑	↑				↓	↓	↑	↑
Arginine and proline metabolism														
Aspartic acid			↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓
GABA	↑	↑	↑	↑		↑	↑	↓	↓	↓		↓		↓
Glutamic acid				↑		↑	↑		↓	↓				
Ornithine	↑		↑	↑		↑	↑				↓	↓		↓
Proline	↑	↑	↑	↑		↑	↑	↓	↓	↓	↓	↓		↓
Putrescine				↑	↑		↑	↓	↓		↑	↓	↓	↓
Urea								↓	↓	↓			↓	↓
Ascorbate and aldarate metabolism														
Glucuronic acid			↑			↑	↑					↑	↑	
Myo-inositol								↑	↑	↓	↓	↓	↓	↓
Butanoate metabolism														
Butanoic acid											↓	↓		
GABA	↑	↑	↑	↑		↑	↑	↓	↓	↓		↓		↓
Glutamic acid				↑		↑	↑		↓	↓				
Succinic acid	↓		↑	↑	↑	↑	↑				↑	↑		
Cyanoamino acid metabolism														
Glycine				↑		↑	↑	↓	↓	↓				
Serine			↑			↑	↑					↓	↓	

Galactose metabolism																
Glycerol													↓		↓	
Lactose					↓	↓	↓	↓	↓	↓			↑			↑
Mannose		↓		↓		↓					↓				↓	
Myo-inositol								↑	↑	↓	↓		↓		↓	↓
Glutathione metabolism																
Glutamic acid				↑		↑	↑		↓	↓						
Glycine				↑		↑	↑	↓	↓	↓						
Ornithine	↑		↑	↑		↑	↑				↓		↓			↓
Putrescine				↑	↑		↑	↓	↓		↑		↓		↓	↓
Pyroglutamic acid		↓	↓			↓	↓	↓	↓	↓	↓		↓			↓
Glyoxylate and dicarboxylate metabolism																
Glyceric acid									↓	↓			↑		↑	↑
Glycolic acid															↑	↑
Histidine metabolism																
Aspartic acid			↑	↑	↑	↑	↑	↑	↑	↓	↓		↓		↓	↓
Glutamic acid				↑		↑	↑		↓	↓						
Methane metabolism																
Glycine				↑		↑	↑	↓	↓	↓						
Serine			↑			↑	↑						↓		↓	
Nitrogen metabolism																
Glutamic acid				↑		↑	↑		↓	↓						
Glycine				↑		↑	↑	↓	↓	↓						
Phenylalanine metabolism																
Phenylalanine			↑	↑		↑	↑				↑					↑
Tyrosine								↓	↓	↓	↓				↓	
Phenylalanine, tyrosine and tryptophan biosynthesis																
Phenylalanine			↑	↑		↑	↑				↑					↑
Tyrosine								↓	↓	↓	↓				↓	
Pyruvate metabolism																
Acetic acid		↑				↑										
Lactic acid		↑		↑	↑	↑	↑	↑	↑	↓			↑		↑	↑
Selenoamino acid metabolism																
Acetic acid		↑				↑										
Alanine		↑		↑		↑	↑	↓	↓	↓	↓		↓			↓
Starch and sucrose metabolism																
Fructose											↓				↓	↓
Glucuronic acid			↑			↑	↑						↑		↑	
Maltose		↑	↓	↑	↓	↓	↑	↓	↓	↓	↓		↓		↑	↑
Valine, leucine and isoleucine biosynthesis																
Isoleucine									↓	↓			↓		↓	↓
Leucine		↓		↓		↑	↑	↓	↓	↓			↓		↓	↑
Threonine				↑	↑	↑	↑						↓			↓
Valine		↑		↑		↑	↑				↓		↓		↑	↑
Other																
Adenine	↓			↓			↓	↓	↑	↑			↓			↓
Allose											↓					↓

(*Continued*)

Table 1. (Continued)

	Maximum application rate							1/10th maximum application rate						
Pathway/Compound	Bif	Met	Tdn	BifMet	BifTdn	MetTdn	BifMetTdn	Bif	Met	Tdn	BifMet	BifTdn	MetTdn	BifMetTdn
Aminomalonic acid								↓	↓					
Arabinonic acid			↑		↑	↑	↑							
Arabinose								↓	↓	↓				
Arabitol											↑	↑		↑
Azelaic acid											↑		↑	
Cellobiose		↓		↓		↓		↓	↓	↓		↑	↑	↑
Erythronic acid				↑		↑	↑	↑	↑		↑	↓	↑	↑
Galactopyranoside												↑		↑
Galactose	↓	↓	↓	↓	↓	↓	↑	↑	↓	↓	↑	↑	↓	↑
Gluconic acid								↓	↓	↓	↓	↓		↓
Glutaric acid											↑		↑	↑
Glycerol												↓	↓	
Hypoxanthine	↑	↑		↑	↑	↑	↑	↓		↓				
Inosine											↑	↑		
Inositol	↓			↓	↓									
Lactulose				↑	↑		↑	↓	↓		↓	↓	↓	↓
Malic acid		↑		↑		↑	↑						↓	↓
Mannobiose												↓		↓
Methylmalonic acid			↑			↑	↑							
5-Methyluridine								↓	↓	↓				
N-α-acetyllysine												↓		↓
Oxalic acid				↑		↑		↓	↓	↓				
Purine		↑	↑			↑	↑				↓		↓	↓
Putreanine											↓	↓	↓	↓
Ribitol			↑			↑	↑	↓	↓	↓	↓	↑	↓	↓
Ribose								↓	↓	↓	↓	↓		
Threonic acid			↑	↑		↑	↑							
Thymine			↑			↑	↑							
Turanose											↓	↓	↓	↓
Uracil								↓	↓	↓	↑	↑	↑	
Uridine								↓	↓	↓		↓		↓
Xylopyranose								↓		↓				
Xylose								↓	↓	↓				

Daphnia magna, where a decrease in amino acids happened concurrently with an increase in sugar, which suggested that energy resources were being used to combat stress. Moreover, at maximum application rates, aspartic acid, alanine, glutamic acid, lysine, methionine, proline, serine, threonine, and valine were at higher concentrations, while galactose, mannose, maltose, and lactose were downregulated. When *D. magna* were exposed to high concentrations of diazinon (0.135 μg L^{-1}) and malathion (0.47 μg L^{-1}), the authors concluded that the organism was slowing down protein synthesis owing to the depletion of energy which resulted in lowered protein synthesis (Nagato et al. 2016). Together, this suggests that at low pesticide exposure levels, more energy resources are being used to help combat stress, such as aerobic respiration and catabolism of amino acids; however, at higher levels of exposure, all the energy resources of the organism are depleted and it cannot fully compensate (Nagato et al. 2016; Sokolova et al. 2012).

In the current study, 15 amino acids were affected by pesticide exposure, either as a singlet or in combination, and at both application rate exposures. The amino acid arginine is known for being incredibly versatile, since it is a precursor for the synthesis of proteins, other amino acids (glutamate and proline), ornithine, and urea (Wu and Morris 1998). In the groups receiving the maximum application rate, both proline and glutamic acid were upregulated compared with controls owing to the production of these compounds. Conversely, at lower exposures (1/10th maximum application rate) proline and glutamic acid were downregulated compared with the controls. The three branched-chain amino acids (BCAAs), leucine, isoleucine, and valine, are vital for energy production and protein synthesis (Brosnan and Brosnan 2006; Kimball and Jefferson 2006; Ch et al. 2015; Xu et al. 2015). Leucine is the most important amino acid in skeletal muscle protein synthesis, while isoleucine is best known for being incorporated into lymphocytes (Kimball and Jefferson 2006; Calder 2006). In the 1/10th maximum application treatments for Bif exposures, valine, leucine, and isoleucine were all downregulated compared with the controls, though for Met and Tdn, BCAAs were both up and downregulated. Met, at maximum application rate, expressed an upregulation for valine and leucine. Similar to amphibians, Ekman et al. (2006) observed an upregulation in valine when rats were exposed to a high dose of Tdn. Xu et al. (2015) measured a significant increase in these three amino acids when goldfish were exposed to butachlor. Conversely, Ch et al. (2015) showed that cypermethrin treated earthworms expressed a decrease in both valine and isoleucine concentrations, which was hypothesised to occur owing to neurological defects and stress in the muscles. Therefore, in this study, the decreased concentration of BCAAs observed in the 1/10th maximum exposures could similarly arise from the increased synthesis of proteins and not necessarily linked to a pesticide-specific mechanism of action (MOA).

Two of the aromatic amino acids, phenylalanine and tyrosine, are major constituents in producing the biogenic amines dopamine and octopamine, which are used by the nervous system (Miller et al. 1970; McCoole et al. 2012). In the 1/10th maximum application treatments, all pesticides exhibited a downregulation in tyrosine. However, in butachlor-treated goldfish, an increase in abundance for these aromatic amino acids was observed (Xu et al. 2015). In the current study, a downregulation of phenylalanine and tyrosine in the 1/10th maximum application treatments suggest that biogenic amines were being overly produced. Therefore, the catabolism of both BCAAs and aromatic amino acids can increase the production of ketones in the liver which are then used for metabolic energy in these amphibians (Nelson and Cox 2008).

As noted by Liu et al. (2015), glutamate and aspartate are excitatory neurotransmitters, where glutamate can produce GABA through a decarboxylation reaction. GABA is the main inhibitory neurotransmitter in the nervous system, where it regulates psychological and physiological processes along with mediating fast inhibitory synaptic transmissions (Kumar and Goyal 2008). Glutamic acid and GABA were downregulated in the 1/10th maximum application group; however, they were upregulated in the maximum application groups. Madl and Royer (2000) noted that GABA levels can increase as a result of hypoxia inhibiting GABA metabolism and the depletion of ATP inducing glutamate decarboxylase. Both compounds, glutamate and GABA, were significantly increased in goldfish that were exposed to the organophosphate dichlorvos (Liu et al. 2015). Moreover, goldfish exposed to butachlor also expressed an increase in glutamate, which showed that the tissues were hypoxic (Xu et al. 2015). Therefore, this upregulation in glutamic acid and GABA at maximum application would also indicate that amphibian tissues were potentially hypoxic.

Amphibians that are dehydrated can express a decrease in ATP synthesis through aerobic metabolism; therefore, other routes must be used to synthesise ATP (Gatten 1987). One route is through anaerobic metabolism, or glycolysis, which results in the accumulation of lactate (Gatten 1987). Since the amphibians in this study were dehydrated for 10 h before exposure and exposed for 8 h, this could have resulted in a build-up of lactic acid. Therefore, owing to the build-up of lactic acid in the amphibians, anaerobic metabolism was likely used to produce energy, even at a less efficient rate compared with aerobic metabolism owing to the hypoxic conditions. This was also observed by Chang et al. (2006), where the authors noted that lactic acid formed under hypoxic conditions in freshwater prawns as a result of the oxidation of pyruvate.

Myo-inositol is known for generating second messengers by being a precursor of the inositol phospholipids (Downes and Macphee 1990). However, in this study, fluxes in myo-inositol were detected in the treatments at the 1/10th maximum application rate where it was both up and downregulated depending on pesticide exposure. Interestingly, inositol was only detected in Bif for the maximum application rate, where it was downregulated compared with the controls. This could imply that the production of secondary messengers by myo-inositol was increased owing to the downregulation of this compound when the amphibians were under 'low' stress. Ch et al. (2015) reported that cypermethrin treated earthworms expressed an increase in myo-inositol, which demonstrated that cypermethrin disrupted phosphatidyl inositol phosphate metabolism. A similar mechanism is plausible in amphibians in the current study.

Nucleic acids are the main building blocks of life and are known for regulating the synthesis of proteins; however, any change in nucleic acid content can lead to alterations in protein synthesis (Tilak et al. 2009). An upregulation was observed for hypoxanthine and the bases of nucleic acids purine and thymine in amphibians exposed to Bif, Met, and Tdn at maximum application. Additionally, uridine and 5-methyluridine were downregulated in the 1/10th maximum application exposure, while uracil was downregulated in singlet exposures. The downregulation of the nucleic acids would lead to a disruption in nucleic acid and protein synthesis along with other biological processes being hindered. Tilak et al. (2009) showed freshwater

fish that were exposed to alachlor expressed a decrease in nucleic acid content. Moreover, when the freshwater fish *Punctius arenatus* was exposed to the pesticides fenvalerate and monocrotophos, there was a significant decrease in nucleic acid content, which could affect amino acids and proteins (Rathod and Kshirsagar 2010).

In the current study, galactose, lactose, and maltose were upregulated for the 1/10th maximum treatment groups, which indicated that energy metabolism was being impacted. Another biochemical route to obtain energy, when it is in high demand, is through the metabolism of amino acids (Zaya et al. 2011). Additionally, a decrease in amino acids compared with the controls can be attributed to DNA repair mechanisms and defence proteins (Taylor et al. 2009; Spann et al. 2011). However, in the maximum treatment groups, the sugars (i.e. galactose, lactose, and mannose) were downregulated which indicated high levels of stress for the amphibians. This same phenomenon was observed in *D. magna* exposed to diazinon and malathion (Nagato et al. 2016). Ultimately, the changes observed in amphibians suggest biochemical fluxes in energy, which can aid in detoxifying pesticides from organisms (Dornelles and Oliveira 2014).

Pathways

Using the metabolites putatively annotated, the principal biochemical pathways that were perturbed in this study were: aminoacyl-tRNA biosynthesis; alanine, aspartate, and glutamate metabolism; and arginine and proline metabolism.

Aminoacyl-tRNA biosynthesis creates an aminoacyl-tRNA for each amino acid, which is used for protein building and is vital for DNA replication (Ibba and Söll 2000; O'Donoghue and Luthey-Schulten 2003). The function of an aminoacyl-tRNA is to deliver its appropriate amino acid to the ribosomal A site, so the corresponding anticodon and codon can match up in mRNA (Ibba and Söll 2000). The main metabolites that were identified to affect this pathway were the amino acids identified (i.e. proline, tyrosine, and BCAAs), which resulted in the aminoacyl-tRNA biosynthesis pathway being perturbed for each pesticide at both application rates. This same pathway was impacted in studies by Ch et al. (2015), Snyder et al. (2017) and Van Meter et al. (2018). Therefore, pesticide exposure to amphibians can affect the aminoacyl-tRNA biosynthesis pathway and, in turn, impact DNA replication and protein synthesis.

The alanine, aspartate and glutamate metabolism pathway included alanine, aspartic acid, glutamic acid, GABA, and succinic acid as the major metabolites that were affected by exposure. Both of the amino acids aspartic acid and glutamic acid are vital in amino acid metabolism (Wu and Rittenberg 1949). These compounds undergo a transamination reaction, which results in the removal of the amino group in the liver for urea formation (Wu and Rittenberg 1949). This pathway was affected by all exposure treatments. Through the amino acid glutamate, many other metabolism pathways are interconnected, including nitrogen metabolism, glutathione metabolism, butanoate metabolism, glyoxylate and dicarboxylate metabolism, and arginine and proline metabolism. The impact on this pathway would affect the synthesis of other vital amino acids in amphibians. A downregulation in this pathway would result in a smaller supply of amino groups for synthesising amino acids.

The primary metabolites that were annotated in the arginine and proline metabolism pathway were aspartic acid, glutamic acid, proline, urea, ornithine, and putrescine. Additionally, one of the main metabolites of this pathway is nitric oxide, owing to the breakdown of arginine (Wu and Morris 1998; Ichu et al. 2014). Nitric oxide is synthesised in neurons and is an important neurotransmitter and neuromodulator in the central nervous system (Wu and Morris 1998; Ichu et al. 2014). This pathway was impacted in each exposure scenario. Furthermore, alanine, aspartate, and glutamate metabolism and butanoate metabolism pathways are connected to the arginine and proline metabolism pathway through putrescine. After putrescine undergoes two reactions to form first 4-aminobutyraldehyde and, subsequently, GABA, it can then partake in both butanoate metabolism and alanine, aspartate, and glutamate metabolism (Houen 1998; Schneider and Reitzer 2012). Downregulation in this pathway would negatively impact the biosynthesis of proteins and amino acids in amphibians, as described above.

Valine, leucine, and isoleucine biosynthesis pathway regulates brain amino acid uptake, muscle protein synthesis, and insulin secretion (Brosnan and Brosnan 2006). These BCAAs are considered dietary essential amino acids; however, when metabolising BCAAs, the first step is a transamination to a ketoacid, which is reversible (Brosnan and Brosnan 2006; Platell et al. 2000). Dietary restrictions to BCAAs in mice resulted in the immune system being impaired, along with an increase in the susceptibility to other pathogens (Calder 2006). Additionally, the amino acid leucine partakes in the insulin signalling pathway, which can regulate protein synthesis (Layman and Walker 2006). These three amino acids, when upregulated in the brain, can prevent the uptake of the aromatic amino acids (phenylalanine, tyrosine, and tryptophan), which would lead to a decline in the synthesis and release of neurotransmitters (Fernstrom 2005).

Since the BCAAs were both up and downregulated in several treatments, this infers they could also be degrading through catabolism. Valine, leucine, and isoleucine are all catabolised as groups extrahepatically within the skeletal muscle (Platell et al. 2000; Brosnan and Brosnan 2006). The three BCAAs go through a deamination step to produce branched-chain ketoacids that are incorporated into protein or act as an energy substrate in several areas of the body (brain, heart, kidney, and mainly the skeletal muscle) (Abumrad et al. 1982; Platell et al. 2000). Additionally, these amino acids are nitrogen donors that are located in the brain and peripheral tissues (Hutson et al. 2005). The amino group is transported from the muscle to the liver in the form of alanine and glutamine, while in the brain, the amino group is used for the synthesis of glutamate and GABA (Hutson et al. 2005).

Glutathione is involved in Phase II metabolism of xenobiotics and can help control reactive oxygen species that are harmful to the organism (Hansen et al. 2006). Glutathione is made up of three amino acids, glycine, cysteine, and glutamate; the main metabolites that were observed in this pathway were glutamate, glycine, and pyroglutamic acid. The glutathione pathway was observed in the Bif and Met treatments exposed at the 1/10th maximum application rate. Zaya et al. (2011) showed this pathway was over-represented or upregulated, after *Xenopus laevis* tadpoles were exposed to atrazine, which could have assisted in the removal of atrazine and its metabolites. Therefore, the role of the glutathione metabolism pathway in the current study may have been to facilitate the excretion of pesticides from the body.

For nitrogen metabolism, only two metabolites, glutamate and glycine, were putative hits for this pathway. Nitrogen metabolism was impacted in both of the Met treatments and at the 1/10th maximum application for Tdn. Stitt et al. (2002) noted

that glutamate, malic acid, and low sugars can hinder the nitrogen metabolism pathway. Snyder et al. (2017) observed that nitrogen metabolism was altered when *Hyla versicolor* tadpoles were exposed to atrazine.

The metabolites that partake in the butanoate metabolism pathway that were observed in the exposures were succinic acid, malic acid, glutamate, and GABA. This pathway was unique in this study because it was only impacted in Bif exposures at both application rates. Although Ch et al. (2015) only identified one metabolite that affected butanoate metabolism when earthworms were exposed to cypermethrin, Snyder et al. (2017) found this pathway was only impacted in *H. versicolor* tadpoles with three metabolites of interest. Using pathways specific to each pesticide, we noticed that butanoate metabolism was observed only in the Bif exposures and this may help elucidate pesticide specific mechanisms of action.

Conclusions

The effects of pesticides as mixtures on body burden and the metabolome of terrestrial phase amphibians were assessed. Met exposure had the most significant impact on amphibians by eliciting the highest body burden for the maximum application rate as well as by modifying the highest number of spectral features in the metabolome. The metabolomic profile at the 1/10th maximum application rate was more impacted than at the maximum application rate. Pesticide mixtures at both application rates appeared to equally affect the metabolome of amphibians, possibly owing to the same metabolites being required to bring the organism back to a homeostatic state. Exposure at low concentrations exhibited a downregulation in metabolites that was necessary to combat the innate stress of xenobiotic exposure. Prominent amino acids were downregulated potentially as a result of them being used for glutathione metabolism or energy demands such as increased protein formation. However, at higher exposure concentrations, necessary metabolites for energy and synthesis of new compounds demonstrated the opposite trend. Therefore, elucidating the effects of pesticides, both as singlets and mixtures, is vital in assessing the risk these compounds pose to non-target species.

Supplementary material

The list of all the parameters for LC-MS method for the three pesticides and their metabolites (Table S1), biological pathways that were affected by pesticide treatment in amphibians for both application rates (Table S2), along with *t*-test filtered chromatograms for both maximum (Fig. S1) and 1/10th maximum (Fig. S2) application rates are available on the Journal's website.

Data availability

https://github.com/puruckertom/glinski_biomarkers

Conflicts of interest

The authors declare no conflicts of interest.

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