**¹H NMR-based metabolomics reveals interactive effects between the carrier
solvent methanol and a pharmaceutical mixture in an amphibian developmental
bioassay with *Limnodynastes peronii*.**

Steven D. Melvin^{a,*}, Oliver A.H. Jones^b, Anthony R. Carroll^c, Frederic D.L. Leusch^{a,b}

^aAustralian Rivers Institute, Griffith University, Southport QLD 4222, Australia

^b Australian Centre for Research on Separation Science, School of Science, RMIT
University, GPO Box 2476, Melbourne, VIC 3001, Australia

^cGriffith School of Environment, Griffith University, Southport QLD 4222, Australia

*To whom correspondence may be addressed. Email: s.melvin@griffith.edu.au

ABSTRACT

Organic carrier solvents are used in aquatic toxicity testing to improve chemical solubility and facilitate the exploration of dose-response relationships. Both water- and solvent-control groups are normally included in these scenarios to ensure that the solvent itself has no effect on the test organism, but this fails to consider possible interactive effects between carrier solvents and contaminants of interest. We explored this topic by exposing *Limnodynastes peronii* tadpoles to a mixture of common water-soluble pharmaceuticals (diclofenac, metformin and valproic acid) in the presence and absence of the carrier solvent methanol, according to standard developmental bioassay methodology. Nuclear Magnetic Resonance (NMR) spectroscopy was applied as a platform for untargeted metabolomics, to compare broad sub-lethal hepatotoxicity in solvent- and solvent-free exposure scenarios. Considerable interactive effects were identified between the pharmaceutical mixture and a typical dose of methanol (0.003%). Specifically, pronounced differences were observed between the solvent- and solvent-free exposure groups for leucine, acetate, glutamine, citrate, glycogen, tyrosine, arginine, purine nucleotides and an unidentified metabolite at 6.53ppm. Various other metabolites exhibited similar disparity related to the use of carrier solvent, but the interactions were non-significant. These results raise important questions about the use of carrier solvents for chemical exposures in aquatic ecotoxicology, and particularly for studies interested in sub-lethal mechanistic information and/or biomarker discovery.

KEY WORDS

Amphibian; Ecotoxicology; Interactive effect; Metabolomics; NMR spectroscopy

45 GRAPHICAL ABSTRACT

46

47

1. INTRODUCTION

Reliable toxicity data is paramount to the effective identification and management of environmental threats from chemicals (EEA 2010). At the forefront of global research efforts aimed at understanding chemical toxicity are controlled laboratory bioassays, which are extensively used to evaluate sub-lethal effects in non-target aquatic species (Amiard-Triquet et al., 2015). Standard exposure methods have been developed by international research organisations to establish guidelines for achieving robust toxicity information (OECD 2012; ASTM 2014). These and other resources describe situations where carrier solvents are necessary to improve chemical dispersion or increase solubility, for example to facilitate the exploration of dose-response relationships (Maes et al., 2012). In these scenarios, both water- and solvent-control groups are normally included to ensure that the solvent itself does not elicit a toxicological response in the test organism. However, recommended maximal solvent dosing has historically been based on adverse effects for apical endpoints, and the suitability of carrier solvents in the context of robust modern analytical techniques that can detect metabolic changes before major toxicity occurs warrants attention (Turner et al., 2012). Direct solvent toxicity is an important consideration, but another fundamental question exists with major consequence for ecotoxicology – are sub-lethal biochemical responses influenced by interactive effects between solvents and contaminants of interest (Melvin et al., 2017)?

Pharmaceutical contaminants are an emerging environmental concern and offer a particularly appropriate case for exploring this question. The potential for, and occurrence of, interactive effects between pharmaceuticals and alcohol is common knowledge, and humans are frequently advised to avoid mixing alcoholic drinks with therapeutic medicines (NIH 2014; Weathermon and Crabb 1999). The most common

adverse side effect from alcohol-drug interactions is hepatic toxicity (Weathermon and Crabb 1999; Adams 2009). It is hypothesised that this may also hold true when using alcohols as carrier solvents in standardised toxicity bioassays. There has been surprisingly limited research on the topic; perhaps due to several studies indicating that solvent-drug interactions are of limited concern for apical endpoints (Chromcova et al., 2011; Barera and Adams 1983). Surprisingly, methanol has even been deemed one of the least harmful solvents for vertebrate developmental bioassays and is commonly used in aquatic ecotoxicology (Maes et al., 2012), despite evidence that it may influence sub-lethal pathways involved with chemical detoxification (David et al., 2012). Considering the known influence of alcohol on drug metabolism and hepatotoxicity in humans and the widespread use of organic solvents like methanol in toxicity research, it seems prudent to evaluate the potential for interactive effects between solvents and chemical pollutants in sub-lethal ecotoxicology testing.

Metabolomics has been gaining recognition for its ability to provide a comprehensive picture of toxicity in living systems, including quantitative assessment of broad changes to the profiles of numerous small molecules in biological samples (Viant 2007). The ability to simultaneously measure the end products from a wide range of cellular processes yields robust information about sub-lethal toxicological effects in response to environmental stressors (Spurgeon et al., 2010; Viant 2008; Jones et al., 2008; Bouhifd et al., 2013). Somewhat paradoxically, the same sensitivity and comprehensive scope that makes metabolite profiling so attractive may make the approach susceptible to sources of confounding that have gone unnoticed using traditional test methods (Simmons et al., 2015). Understanding such factors is critical for the future successful application of metabolomics and other omics disciplines (e.g., genomics and proteomics) towards ecotoxicology research (Simmons et al.,

2015). More broadly, this may reveal critical insights about the validity of established testing paradigms that could lead to improvements in future experimental strategies aimed at evaluating toxicological risks. The latter is vital for ensuring rigorous science and the provision of meaningful information, for example when attempting to identify biomarkers of exposure or integrate diverse toxicity information into an Adverse Outcome Pathway (Brockmeier et al., 2017; Ankley et al., 2010).

Here, we report alterations to hepatic metabolite profiles of larval amphibians exposed to a mixture of pharmaceutical contaminants in a standard developmental bioassay, using both solvent and solvent-free chemical dosing. Parallel exposures were performed in the presence and absence of methanol, to a concentration gradient of three representative water-soluble chemicals (i.e., diclofenac, metformin and valproic acid). We explored differences in response patterns triggered by solvent-drug interactions to assess the validity of using organic carrier solvents for omics research, including any implications for biomarker discovery or mechanistic research.

2. METHODS

2.1 Chemical standards

The only way to conclusively assess the possibility for common carrier solvents to interact with pharmaceuticals (or other contaminants) is to compare responses to a concentration gradient in the presence and absence of solvent (Green and Wheeler 2013). Importantly, this can only be achieved using water-soluble contaminants. Technical-grade standards (99.9% purity) of metformin, diclofenac and valproic acid were therefore purchased from Sigma-Aldrich (Castle Hill, Australia). These compounds were chosen since they are readily soluble in water, have been detected in sewage and environmental matrices in the low $\mu\text{g/L}$ range, and have been

flagged as potentially interacting with alcohol consumption in humans (Weathermon and Crabb 1999). Concentrated working dilutions were prepared by dissolving each compound individually in Milli-Q water, with fresh stocks made every week throughout the experiment and maintained at -20 °C.

2.2 Experimental animals

A single fertilised foam egg nest of the Australian striped marsh frog (*Limnodynastes peronii*) was collected from an ephemeral pond located in Elanora, Queensland (Australia) and brought to the laboratory in a bucket filled with water from the collection site (QLD Government Collection Permit No. WISP16873916). The egg mass was held in the laboratory in a glass aquaria filled with 50 L moderately hard test water (USEPA 1994) until hatching. Hatched larvae were fed Sera micron® fry food (Sera GmbH) *ad libitum* twice daily until most animals had reached Gosner developmental stage (Gs) 26 (Gosner 1960). Temperature and photoperiod were maintained at 22.0 ± 0.2 °C and 12: 12 light: dark sequence, respectively, through holding and experimentation. This research was approved by the Griffith University Animal Ethics Committee in accordance with the principles in the Australian Code for the Care and Use of Animals for Scientific Purposes (Protocol No. ENV/03/16/AEC).

2.3 Developmental bioassays

Two exposures were performed in parallel, each comprising four replicate aquaria of control (nothing added) and 5, 50 and 500 µg/L of a mixture of metformin, diclofenac and valproic acid. Replicates contained 4 L of the appropriate mixture concentration and housed four *L. peronii* tadpoles (n = 16 per treatment), with gentle aeration throughout the experiment to maintain oxygen levels. After adding the

mixture, every replicate from one of the parallel exposures also received 100 μ L methanol, equivalent to 0.003% of the total exposure volume. Control water used for the experiment was reconstituted moderately hard water made to standard test guidelines (USEPA 1994). Animals were fed Sera micron® powdered larval food twice daily, and excess food waste and faeces were removed daily with a turkey baster. Full water changes and treatment renewals were performed twice weekly throughout the exposure by gently removing animals with a fine mesh dip net, refilling with fresh water and re-spiking the mixture and solvent.

Following 30 days exposure, all tadpoles were euthanized by individual immersion in a solution of buffered 3-aminobenzoic acid ethyl ester (MS-222; Sigma Aldrich, Castle Hill, Australia) dissolved in water. Measurements of snout-vent length (SVL; mm), total length (mm), wet weight (mg) and developmental stage were quickly taken with the animals in weigh boats over ice. Animals were then dissected, and livers were removed and weighed (mg), flash frozen on liquid nitrogen, and stored at -80°C for subsequent extraction of hepatic metabolites. Morphometric measurements were used to calculate Hepatic Somatic Index (HSI; [liver weight/body weight] $\times$ 100) and condition factor (K ; [body weight/SVL³] $\times$ 100).

2.4 Chemical analysis for verification of exposure concentrations

A 2 mL sample was collected from each aquaria immediately following addition of the contaminant(s) on four occasions, and pooled by treatment group for chemical analysis, which was performed using liquid chromatography with mass spectrometry (LC-MS). Analysis was performed using an Agilent 1290 Infinity Series HPLC paired with a 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC-MS system. The HPLC was equipped with a 1.8 μ m 2.1x100 mm Agilent ZORBAX

Eclipse Plus C18 column maintained at 40°C, and an injection volume of 2 µL was used. The system was operated in positive ionisation mode for analysis of diclofenac and metformin, with 95% acetonitrile containing 0.1% formic acid and HPLC grade water containing 0.1% formic acid as mobile phase A and B, respectively. Due to poor detection of valproic acid this compound could not be measured from grab samples. The mixture was therefore spiked into a separate aquaria holding 1L of water and the entire volume was concentrated via solid phase extraction with an Oasis® HPL cartridge (Waters Co., Milford, USA), yielding an approximation of dosing according to an existing method (Gao et al., 2011). Valproic acid was analysed in negative ionisation mode, with 95% acetonitrile containing 0.1% ammonium formate and HPLC grade water containing 0.1% ammonium formate as mobile phase A and B, respectively. For all compounds, a flow rate of 0.35 ml min⁻¹ was used with a 4 min gradient going from 5% mobile phase A for 1 minute to 100% A at 2 minutes, and holding there for 1 minute before dropping back to 5% mobile phase A.

2.5 Extraction of hepatic metabolites and lipids

Liver samples were processed using a modified Bligh-Dyer extraction procedure (Bligh and Dyer 1959), as this has been shown to achieve good extraction and separation of hydrophilic metabolites and hydrophobic lipids (Wu et al., 2008; Lin et al., 2007) and has been successfully applied for prior metabolomics analysis of tadpole liver tissues (Melvin et al., 2017). Briefly, livers were homogenised in 400 µL ice-cold methanol via ultra-sonication using a Q55 probe sonicator (Qsonica, USA), and incubated at -20°C for 24 h. Following incubation, 800 µL chloroform and 300 µL ultrapure water were added and samples were vortexed and centrifuged (10 min,

16,000 $\times$ g, at 4°C). Organic and polar phases were portioned into separate glass vials, the extraction was repeated to increase yield, and samples were stored at -80 °C.

2.6 Quantification of hepatic triglycerides

Lipid fractions were thawed and aliquots were analysed for non-specific triglycerides according to an established protocol (Smolders et al., 2005; Melvin et al., 2017). Briefly, 100 μ L aliquots were transferred to glass test tubes, 500 μ L sulphuric acid (1 M) was added, and samples were charred at 200°C for 30 min. A calibration curve was made using analytical grade triglyceride standard (Sigma Aldrich, Cat No. 17811) for quantification and measurements were standardised by liver weight of each individual animal and averaged by experimental replicates for data analysis. The lipid assay was performed in 96-well plate format and measured colorimetrically using a FLUOstar Omega plate reader (BMG LABTECH Pty, Ltd.), with absorbance measured at 370 nm (Smolders et al., 2005).

2.7 Nuclear Magnetic Resonance Spectroscopy

Extracted polar metabolite fractions were dried using a Series II centrifugal vacuum concentrator (GeneVac Technologies, England) followed by lyophilisation (freeze-drying). Dried samples were resuspended in 200 μ L phosphate buffer (K_2HPO_4 and NaH_2PO_4 ; pH 7.4) made with D_2O containing 0.05% sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) as an internal standard. Samples were vortexed, centrifuged (5 min, 2500 $\times$ g, at 4 °C), and supernatants were transferred to 3 mm NMR tubes using glass pipettes. A blank containing 100 μ L Milli-Q water was put through the full extraction and analytical workflow to ensure no contamination resulting from sample processing or NMR spectroscopy.

NMR spectra were acquired with an 800 MHz Bruker[®] Avance III HDX spectrometer. The system was equipped with a Triple (TCI) Resonance 5 mm Cryoprobe with Z-gradient and automatic tuning and matching, and a SampleJet automatic sample changer controlled via the software IconNMR™ (Bruker Pty Ltd., Victoria, Australia). Spectra were acquired at 298°K, using D₂O for field locking, and TSP (¹H δ 0.00) as an internal reference. ¹H spectra were acquired by using the zg30 pulse program with 128 scans, 0.8 s relaxation delay, 8.75 μs pulse width and 16 kHz spectral width (¹H δ -3.75 – 16.28). Edited ¹H-¹³C HSQC spectra were acquired for a pooled sample using the pulse sequence hsqcedetgpsisp2.2 with 32 scans and 128 experiments, 0.8 s relaxation delay, 8.75 μs pulse width and spectral widths of 12.8 kHz (¹H δ -3.23 – 12.79) and 33.1 kHz (¹³C δ -9.40 – 155.2).

2.8 Peak assignments and processing of ¹H NMR spectra

MestReNova v8.1.4 (Mestrelab Research S.L., Spain) was used for post processing of NMR spectra. ¹H NMR FIDs were Fourier transformed with 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 and normalised to TSP (¹H δ 0.00, ¹³C δ 0.0). Once processed, spectra were stacked and binned into 0.01 ppm bins (integrated spectral regions) between δ 0.00-9.50. Integrated peak areas of each bin were exported for further processing and multivariate statistical analysis.

2.9 Univariate and multivariate statistical analysis

Data were analysed for differences in growth, morphometric indices, and hepatic triglycerides using SPSS v22 statistical software (IBM Inc., Chicago, USA),

for Mac OSX (Apple, Cupertino, USA) with $\alpha = 0.05$. Assumptions of normality and homogeneity of variance were tested with visual inspection of normal probability plots and Shapiro-Wilk's test, respectively. Differences in Gosner stage, SVL, *K*, *HSI*, and triglycerides amongst treatment groups were compared using two-way ANOVAs. Post-hoc comparisons were performed with Tukey's multiple comparisons test.

The exported ^1H NMR data for each animal was normalised by liver weight and Log_{10} transformed prior to statistical analysis. The spectral region between 0.00 to 9.00 ppm was used for analysis, with the residual water signal (5.0 ± 0.5 ppm), TSP ($^1\text{H} \delta$ 0.00) and any regions devoid of chemical signals excluded to improve analysis and subsequent model validation (Rubingh et al., 2006). Initial data exploration was performed using the Principal Component Analysis (PCA) Toolbox plugin for MATLAB. Partial Least-Squares Discriminant Analysis (PLS-DA) with auto-scaling was then performed using the Classification Toolbox plugin for MATLAB (Milano Chemometrics and QSAR Research Group, University of Milano, Italy), to explore separation between different treatments. Venetian blinds cross-validations were used to optimise the number of latent variables for the analysis and assess the quality of the PLS-DA model (Rubingh et al., 2006). Heat map analysis was applied to further visualise differences in metabolite abundance between treatment and dosing groups, using the metaboanalyst online platform (Xia et al., 2016).

Metabolites were tentatively identified using Chenomx NMR suite 8.2 software (ChenomxInc., Edmonton, Canada). Where possible, further validation of metabolite identification was achieved by comparison of HSQC NMR spectra with published reference spectra available through the Human Metabolome Database (Wishart et al., 2007, 2013). Two-way ANOVAs with Bonferroni post-hoc comparisons (McDonald 2014) were performed to compare the abundance of each

identified metabolite relative to TSP. One-way ANOVAs with Bonferroni post-hoc comparisons were also performed to illustrate the hypothetical outcomes that would have originated had these been two individual experiments (i.e., situations where solvent-drug interactions are not of interest).

3. RESULTS AND DISCUSSION

3.1 Water quality and tadpole survival, growth and morphometric indices

Mortality was less than 5% in all treatment groups throughout the experiment. There were no significant differences in basic water quality parameters (i.e., temperature, dissolved oxygen, pH, conductivity) between treatment groups (Supplementary Table 1) and chemical analysis revealed exposure concentrations as being similar to nominal concentrations. A clear dosing gradient was achieved for the pharmaceutical mixture with negligible differences in chemical concentrations apparent between solvent- and solvent-free groups (Supplementary Table 2).

There was a significant effect of the pharmaceutical mixture ($F_{(3,24)} = 14.87, p < 0.001$) and methanol ($F_{(1,24)} = 20.48, p < 0.001$) on developmental (Gosner) stage but no interaction between the two (Figure 1a; $F_{(3,24)} = 0.45, p = 0.718$). Snout-vent length (SVL) was similarly influenced by both the mixture ($F_{(3,24)} = 12.40, p < 0.001$) and methanol ($F_{(1,24)} = 57.92, p < 0.001$), and again there was no interaction (Figure 1b; $F_{(3,24)} = 0.18, p = 0.907$). There was no main effect of the mixture ($F_{(3,24)} = 1.73, p = 0.187$) or methanol ($F_{(1,24)} = 3.79, p = 0.063$) on K , and no interaction (Figure 1c; $F_{(3,24)} = 2.29, p = 0.104$). Methanol significantly influenced HSI ($F_{(1,24)} = 31.70, p = 0.001$) and an interaction was observed between methanol and the mixture (Figure 1d; $F_{(3,24)} = 5.09, p = 0.007$). However, despite an apparent dose-dependent decrease in HSI in the water-only group and apparent increase in the methanol group, the overall

treatment effect was not significant (Figure 1d; $F_{(3,24)} = 3.21$, $p = 0.536$). Hepatic triglycerides were unchanged by the treatment ($F_{(3,24)} = 1.04$, $p = 0.393$) but were increased by methanol ($F_{(1,24)} = 7.24$, $p = 0.013$), and there was no significant interaction (Supplementary Figure 1; $F_{(3,24)} = 2.25$, $p = 0.109$).

3.2 NMR-based metabolomics assessing solvent-drug interactions

Results of the PCA analysis revealed separation between groups and thus supported proceeding with supervised PLS-DA analysis (Supplementary Figure 2). Six latent variables were included in the PLS-DA based on the outcomes of the venetian blinds cross validations. Analysis of individual tadpoles showed clear separation between exposure concentrations for both the solvent-free and methanol groups, but with considerable variability (Figures 2a,b). To improve this and address potential issues of pseudo-replication we also analysed tank averages, which resulted in PLS-DA models that explained at least 80% of the variance in the data and had an acceptably low 0.13 error rate (Figures 2c,d). Heat map analysis further illustrated the differences amongst treatment groups, and exposed a greater overall effect of the pharmaceutical mixture in the absence of methanol (Supplementary Figure 3). A total of 31 metabolite features were tentatively identified based on the ^1H spectra and confirmed based on the HSQC spectra (Figure 3). Formate was identified in the ^1H spectra but the ^{13}C region fell outside of the viewable range of the HSQC spectra. One notable unidentified feature with a ^1H chemical shift of 6.53 ppm was also included in the analysis. Of the identified metabolites, 27 were determined to be quantifiable based on visual inspection of the HSQC spectra (i.e., non-overlapping regions).

Analysis of the identified features revealed significant interactive effects between methanol and the pharmaceutical mixture for 8 metabolites (Figure 4), and

apparent but non-significant interactions for several others (Figure 5). There were also a number of metabolites that exhibited no interactive effects but were significantly influenced by the pharmaceutical mixture, the carrier solvent, or both (Figure 6). Due to the large number of statistical outcomes and to achieve improved clarity in the figures, results from the statistical analysis of individual metabolites are summarised in Table 1. HSQC chemical shift data (^1H and ^{13}C ; ppm) and numerical annotations relevant for Figure 3 are provided in Supplementary Table 3. The findings are perhaps not surprising since alcohol-drug interactions are well documented in humans (Weathermon and Crabb 1999), but could have far-reaching implications for environmental science since organic solvents are widely used in contemporary ecotoxicology research. Carrier solvents including methanol are generally accepted as being necessary to improve chemical solubility and dispersion for certain substances. On the one hand, using carrier solvents to achieve dose-response relationships can lend confidence by confirming subtle effects observed at lower concentrations. However, the use of carrier solvents can make it difficult to interpret data and identify true ecological risks (Turner et al., 2012). In the past the inclusion of solvent-control groups largely satisfied such concerns, and several researchers have asserted that there is little evidence to suggest that solvents interact with chemical test items (Barera and Adams 1983; Green and Wheeler 2013). Contrarily, our results suggest that the inclusion of solvent-control groups fails to address the potential for interactive toxicity with the chemicals being tested (Melvin et al., 2017). The relevance of testing chemicals above their solubility limits is itself arguably questionable (Rufli 1998) but is often unavoidable, for example with highly lipophilic contaminants. Nevertheless, the present study suggests that the benefits of using solvents to push solubility boundaries needs to be weighted against the risks of forming erroneous conclusions.

Specifically, despite limited difference between the water-controls and solvent-controls for most metabolites (Figures 4, 5 and 6), interactive effects were observed in many cases with increasing concentrations of the pharmaceutical mixture. Independent analysis of solvent- and solvent-free exposure groups (using One-Way ANOVAs) resulted in fundamentally different response patterns (Table 1). This raises concerns about the validity of research aimed at biomarker discovery or the exploration of mechanistic pathways when chemical dosing is achieved using organic carrier solvents such as methanol.

3.3 Implications for mechanistic information and biomarker discovery

One of the foremost approaches used for higher-level interpretation of metabolomics data is enrichment analysis, a statistical technique that considers significant changes to the relative abundance of identified metabolites to discern alterations to functional metabolic pathways (Booth et al., 2013). This has some recognised limitations, including the inability to identify all metabolites in a sample and databases that are specific to only a limited number of organisms (Booth et al., 2013; Chagoyen and Pazos 2013). Understanding these limitations, enrichment analysis was performed separately with metabolites identified as being significantly altered in the solvent- and solvent-free exposure groups (Table 1). Despite the narrow list of annotated metabolites and specificity for mammalian species, this revealed differences in key pathways identified as being influenced by the mixture in the presence or absence of methanol. The primary pathways identified as being affected by the pharmaceutical mixture in the absence of methanol were ammonia recycling and the urea cycle (Supplementary Figure 4). This is consistent with previous studies implicating both valproic acid (Aires et al., 2011; Machado and da Silva 2014) and

metformin (Rotroff et al., 2016) with adverse effects on these specific pathways. Contrarily, the foremost pathway influenced by the mixture in the presence of methanol was protein biosynthesis (Supplementary Figure 5). This is more consistent with the established effect of chronic alcohol consumption on protein biosynthesis in humans (Lang et al., 2000; Karinch et al., 2008), which relates to inhibition of aminoacyl-tRNA synthetase activity (David et al., 1983; Ibba and Soll 2000; Fleming et al., 1975). When therapeutic drugs and solvents are combined, interactive toxicity may be influenced by enhanced gastric solubility, altered metabolism and excretion of the chemical and various other physical and biochemical factors (Linnoila et al., 1979), the net sum of which can obscure specific drug-induced mechanisms of action. We reiterate that there are known limitations to this approach, and suggest that these results should only be considered as anecdotal evidence to illustrate how overall outcomes and interpretations may differ between studies with and without solvents.

Animal exposure bioassays hold a prominent place in ecotoxicology research, and yield critical information about higher-level effects in complex organisms. Sophisticated analytical techniques such as metabolomics stand to significantly advance whole-animal testing by facilitating comprehensive evaluation of broad sub-lethal toxicity pathways. Technologies have advanced to the point where technical variability is minimal (Bencic 2015), but issues related to experimental variability have yet to be thoroughly addressed. The present study reveals that using methanol as a carrier solvent can represent a source of confounding (Simmons et al., 2015), but other solvents and experimental factors should face similar scrutiny. Differences in food quality, temperature, dissolved oxygen and other variables must be adequately assessed to understand their influence on sub-lethal physiology and metabolic endpoints in toxicity testing (Connor et al., 2004; Hines et al., 2007). This type of

information is needed to move techniques like metabolomics beyond proof-of-concept applications (Fent and Sumpter 2011), and may ultimately yield better information about sub-lethal toxicity (Langley et al., 2015).

4. CONCLUSIONS

As analytical technologies advance we become better equipped to evaluate and understand the various ways that anthropogenic stressors influence the environment. However, it is crucial that the appeal of novel technologies does not distract from the thorough appraisal of their meaningful application in research. Beyond understanding physiological upsets and unravelling mechanistic pathways related to toxicity, advanced analytical techniques should also be viewed as an opportunity to critically assess and improve upon fundamental approaches used in environmental research. From this perspective, the present study applied untargeted NMR-based metabolomics to investigate whether methanol, a common carrier solvent used in standard aquatic toxicity bioassays, influences sub-lethal physiological and metabolic endpoints in larval amphibians. Results demonstrate the potential for interactive effects between what would typically be considered a low dose of methanol (0.003%) and a sub-lethal pharmaceutical mixture. Use of carrier solvents is unavoidable for some contaminants and it is not our intention to discredit studies where they have (or will) been used, but merely to highlight that caution may be necessary when interpreting biochemical data since solvents may influence sub-lethal responses. The identification of solvents that do not interact with chemical test items and the development of novel solvent-free approaches for chemical administration warrant attention, since this could result in improved reliability of sub-lethal ecotoxicology testing.

422 ACKNOWLEDGEMENTS

423 Funding was provided through a 2016 Griffith University Postdoctoral Fellowship
424 (No. 219059) and New Researcher Grant (No. ARI2551) awarded to S.D.M. Thanks
425 to Marie Petit for assistance in the laboratory and Wendy Lao-Kum-Cheung for
426 technical support with NMR spectroscopy.

FIGURE CAPTIONS

Figure 1. Average a) Gosner developmental stage, b) snout-vent length, c) condition factor (K) and d) Hepatic-Somatic Index (HSI) of larval striped marsh frog (*Limnodynastes peronii*) reared in control water and the pharmaceutical mixture at 5, 50 and 500 $\mu\text{g/L}$. Clear boxes represent water-only dosing and shaded boxes represent dosing with carrier solvent (0.003% MeOH). Boxplots show the interquartile range, median (horizontal line), min and max (whiskers), and average (+) of four replicates (N) per treatment. Lower-case letters represent differences related to the pharmaceutical mixture and upper-case letters represent significant differences related to the solvent, based on Two-Way ANOVA with $\alpha = 0.05$.

Figure 2. Scores plots from PLS-DA analysis showing separation of a) water-only dosing group based on analysis of all individual tadpoles, b) solvent dosing group (MeOH) based on analysis of all individual tadpoles, c) water-only dosing group based on analysis of tank averages, and d) solvent dosing group (MeOH) based on analysis of tank averages. Solid lines represent water-only groups and dashed lines represent dosing with MeOH as a carrier solvent.

Figure 3. Superimposed typical 800 MHz ^1H and HSQC NMR spectra showing labelled metabolites: 1 L-Isoleucine, 2 L-Leucine, 3 L-Valine, 4 β -Hydroxybutyric acid, 5 L-Lactic acid, 6 L-Lysine, 7 L-Alanine, 8 Putrescine, 9 L-Ornithine, 10 Acetate, 11 L-Arginine, 12 L-Glutamate, 13 L-Glutamine, 14 Oxalacetic acid, 15 Pyruvic acid, 16 Citrate, 17 L-Aspartate, 18 Creatine, 19 L-Tyrosine, 20 O-Phosphocholine, 21 D-Glucose, 22 D-Maltose, 23 Taurine, 24 Myo-inositol, 25 Glycogen, 26 UDP Glucose, 27 UDP Galactose, 28 Glycine, 29 L-Serine, 30 Nucleotides (AMP, ATP), 31 NAD^+ .

Figure 4. Relative abundances of hepatic metabolites (relative to TSP; ^1H δ 0.00) identified as exhibiting significant interactive responses with methanol. Clear boxes represent water-only dosing and shaded boxes represent dosing with carrier solvent (0.003% MeOH). Boxplots represent the interquartile range, median (horizontal line), min and max (whiskers), and average (+) of four replicates (N) per treatment group. A summary of the specific outcomes of statistical analysis is provided in Table 1.

Figure 5. Relative abundances of hepatic metabolites (relative to TSP; ^1H δ 0.00) identified as exhibiting no significant treatment, solvent or interactive effects with methanol. Clear boxes represent water-only dosing and shaded boxes represent dosing with carrier solvent (0.003% MeOH). Boxplots represent the interquartile range, median (horizontal line), min and max (whiskers), and average (+) of four replicates (N) per treatment group. A summary of the specific outcomes of statistical analysis is provided in Table 1.

Figure 6. Relative abundances of select metabolites (relative to TSP; ^1H δ 0.00) exhibiting significant effects of treatment, solvent or both, but no interaction with methanol. Clear boxes represent water-only dosing and shaded boxes represent dosing with carrier solvent (0.003% MeOH). Boxplots represent the interquartile range, median (horizontal line), min and max (whiskers), and average (+) of four replicates (N) per treatment group. A summary of the specific outcomes of statistical analysis is provided in Table 1

476

477

478 Figure 1.

Figure 2.

483

Figure 3.

Figure 4.

Figure 5.

Figure 6.

REFERENCES

- EEA., 2010. Pharmaceuticals in the environment - Result of an EEA workshop, European Environment Agency.
- Amiard-Triquet, C., Amiard, J.C., Mouneyrac, C., 2015. Aquatic ecotoxicology: advancing tools for dealing with emerging risks, Academic Press.
- OECD., 2012. Fish Toxicity Testing Framework: Series on Testing and Assessment No. 171. 1–174. Environment Directorate, Organization for Economic Co-operation and Development, Paris.
- ASTM., 2014. Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians, ASTM International.
- Maes, J., Verlooy, L., Buenafe, O.E., de Witte, P.A.M., Esguerra, C.V., Crawford, A., 2012. Evaluation of 14 Organic Solvents and Carriers for Screening Applications in Zebrafish Embryos and Larvae. PLoS ONE. 7, e43850–9.
- Turner, C., Sawle, A., Fenske, M., Cossins, A., 2012 Implications of the solvent vehicles dimethylformamide and dimethylsulfoxide for establishing transcriptomic endpoints in the zebrafish embryo toxicity test. Environ. Toxicol. Chem. 31, 593–604.
- Melvin, S.D., Habener, L.J., Leusch, F.D.L., Carroll, A.R., 2017 ¹H NMR-based metabolomics reveals sub-lethal toxicity of a mixture of diabetic and lipid-regulating pharmaceuticals on amphibian larvae. Aquat. Toxicol. 184, 123–132.
- NIH., 2014. Harmful Interactions: mixing alcohol with medicines. Department of Health and Human Services, National Institute on Alcohol Abuse and Alcoholism, USA, 1–11.
- Weathermon, R., Crabb, D.W., 1999. Alcohol and medication interactions. Alcohol Res. Health. 23, 40–54.
- Adams, W.L., 2009. Interactions between Alcohol and Other Drugs. Int. J. Addict. 30, 1903–1923.
- Chromcova, L., Stepanova, S., Plhalova, L., 2011. Effect of four selected carrier solvents on embryonal stages of *Danio rerio*. Neuroendocrinol. Lett. 33, 60–65.
- Barera, Y., Adams, W.J., 1983. Resolving some practical questions about *Daphnia* acute toxicity tests, in: Bishop, W. E., Cardwell, R.D., Heidolph, B.B., (Eds.), Aquatic Toxicology and Hazard Assessment, Sixth Symposium, ASTM International. pp. 509–518.
- David, R.M., Jones, H.S., Panter, G.H., Winter, M.J., Hutchinson, T.H., Chipman, J.K., 2012. Interference with xenobiotic metabolic activity by the commonly used vehicle solvents dimethylsulfoxide and methanol in zebrafish (*Danio rerio*) larvae but not *Daphnia magna*. Chemosphere. 88, 912–917.

- Viant, M.R., 2007. Metabolomics of aquatic organisms: the new 'omics' on the block. *Mar. Ecol-Prog Ser.* 332, 301–306.
- Spurgeon, D.J., Jones, O.A.H., Dorne, J-L.C.M., Svendsen, C., Swain, S., Stürzenbaum, S.R., 2010. Systems toxicology approaches for understanding the joint effects of environmental chemical mixtures. *Sci. Total Environ.* 408, 3725–3734.
- Viant, M.R., 2008. Recent developments in environmental metabolomics. *Mol. Biosyst.* 4, 980–986.
- Jones, O., Dondero, F., Viarengo, A., Griffin, J.L., 2008. Metabolic profiling of *Mytilus galloprovincialis* and its potential applications for pollution assessment. *Mar. Ecol-Prog Ser.* 369, 169–179.
- Bouhifd, M., Hartung, T., Hogberg, H.T., Kleensang, A., Zhao, L., 2013. Review: Toxicometabolomics. *J. Appl. Toxicol.* 33, 1365–1383.
- Simmons, D.B.D., Benskin, J.P., Cosgrove, J.R., Dunker, B.P., Ekman, D.R., Martyniuk, C.J., Sherry, J.P., 2015. Omics for aquatic ecotoxicology: Control of extraneous variability to enhance the analysis of environmental effects. *Environ. Toxicol. Chem.* 34, 1693–1704.
- Brockmeier, E.K., Hodges, G., Hutchinson, et al., 2017. The role of omics in the application of Adverse Outcome Pathways for chemical risk assessment. *Toxicol. Sci.* 158, 252–262.
- Ankley, G.T., Bennett, R.S., Erickson, R.J. et al., 2010. Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environ. Toxicol. Chem.* 29, 730–741.
- USEPA., 1994. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. United States Environmental Protection Agency.
- Gosner, K.L., 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica.* 16, 183–190.
- Green, J., Wheeler, J.R., 2013. The use of carrier solvents in regulatory aquatic toxicology testing: Practical, statistical and regulatory considerations. *Aquat. Toxicol.* 144–145, 242–249.
- Gao, S., Miao, H., Tao, X., Jiang, B., Xiao, Y., Cai, F., Yun, Y., Li, J., Chen, W., 2011. LC-MS/MS method for simultaneous determination of valproic acid and major metabolites in human plasma. *J. Chromatogr. B.* 879, 1939–1944.
- Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917.
- Wu, H., Southam, A.D., Hines, A., Viant, M.R., 2008. High-throughput tissue extraction protocol for NMR- and MS-based metabolomics. *Anal. Biochem.* 372,

- 204–212.
- Lin, C.Y., Wu, H., Tjeerdema, R.S., Viant, M.R., 2007. Evaluation of metabolite extraction strategies from tissue samples using NMR metabolomics. *Metabolomics*. 3, 55–67.
- Smolders, R., De Coen, W., Blust, R., 2005. Integrative measures of toxicant exposure in zebra fish (*Danio rerio*) at different levels of biological organization, in: Ostrander, G.K., (Ed.), *Techniques in Aquatic Toxicology* 2, 766., CRC Press, USA.
- Rubingh, C.M., Bijlsma, S., Derks, E.P.P.A., Bobeldijk, I., Verheij, E.R., Kochhar, S., Smilde, A.K., 2006. Assessing the performance of statistical validation tools for megavariate metabolomics data. *Metabolomics*. 2, 53–61.
- Xia, J., Wishart, D.S., 2016. Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis. *Curr. Protoc. Bioinformatics*. 55, 14.10.1 – 14.10.91 (doi:10.1002/cpbi.11)
- Wishart, D.S., Tzur, D., Knox, C. et al., 2007. HMDB: the Human Metabolome Database. *Nucleic Acids Res.* 35, D521–D526.
- Wishart, D.S., Jewison, T., Guo, A.C. et al., 2013. HMDB 3.0 – The Human Metabolome Database in 2013. *Nucleic Acids Res.* 41, D801–D807.
- McDonald, J.H., 2014. *Handbook of Biological Statistics*, third ed. Sparky House Publishing, Baltimore, Maryland, USA.
- Fent, K., Sumpter, J.P., 2011. Progress and promises in toxicogenomics in aquatic toxicology: Is technical innovation driving scientific innovation? *Aquat. Toxicol.* 105, 25–39.
- Rufli, H.E.A., 1998. Aquatic toxicity testing of sparingly soluble, volatile, and unstable substances and interpretation and use of data. *Ecotoxicol. Environ. Saf.* 39, 72–77.
- Booth, S.C., Weljie, A.M., Turner, R.J., 2013. Computational tools for the secondary analysis of metabolomics experiments. *Comput. Struct. Biotechnol. J.* 4, e201301003–13.
- Chagoyen, M., Pazos, F., 2013. Tools for the functional interpretation of metabolomic experiments. *Brief. Bioinform.* 14, 737–744.
- Aires, C.T.C.P., van Cruchten, A., Ijlst, L., de Almeida, I.T., Duran, M., Wanders, R.J.A., Silva, M.F.B., 2011. New insights on the mechanisms of valproate-induced hyperammonemia: Inhibition of hepatic N-acetylglutamate synthase activity by valproyl-CoA. *J. Hepatol.* 55, 426–434.
- Machado, M.C., Pinheiro da Silva, F., 2014. Hyperammonemia due to urea cycle disorders: a potentially fatal condition in the intensive care setting. *J Intensive Care*. 2, 22–5.

- Rotroff, D.M., Oki, N.O., Liang, X., Yee, S.W., Stocker, S.L., Corum, D.G., Meisner, M., Fiehn, O., Motsinger-Reif, A.A., Giacomini, K.M., Kaddurah-Daouk, R., 2016. Pharmacometabolomic Assessment of Metformin in Non-diabetic, African Americans. *Front. Pharmacol.* 7, 3941–12.
- Lang, C.H., Frost, R.A., Kumar, V., Wu, D.Q., Vary, T.C., 2000. Impaired protein synthesis induced by acute alcohol intoxication is associated with changes in eIF4E in muscle and eIF2B in liver. *Alcohol. Clin. Exp. Res.* 24, 322–331.
- Karinch, A.M., Martin, J.H., Vary, T.C., 2008. Acute and chronic ethanol consumption differentially impact pathways limiting hepatic protein synthesis. *Am. J. Physiol. Endoc. M.* 295, E3–E9.
- David, E.T., Fischer, I., Moldave, K., 1983. Studies on the Effect of Ethanol on Eukaryotic Protein Synthesis. *J. Biol. Chem.* 258, 7702–7706.
- Ibba, M., Soll, D., 2000. Aminoacyl-tRNA synthesis. *Ann. Rev. Biochem.* 69, 617–650.
- Fleming, E.W., Tewari, S., Noble, E.P., 1975. Effects of chronic ethanol ingestion on brain aminoacyl-tRNA synthetases and tRNA. *J. Neurochem.* 24, 553–560.
- Linnoila, M., Mattila, M.J., Kitchell, B.S., 1979. Drug interactions with alcohol. *Drugs.* 18, 299.
- Bencic, D.C., 2015. The Challenge: Real-world application of ‘omics endpoints. *Environ. Toxicol. Chem.* 34, 700–700.
- Connor, S.C., Wu, W., Sweatman, B.C., Manini, J., Haselden, J.N., Crowther, D.J., Waterfield, C.J., 2004. Effects of feeding and body weight loss on the ¹H-NMR based urine metabolic profiles of male Wistar Han rats: implications for biomarker discovery. *Biomarkers.* 9, 156–179.
- Hines, A., Oladiran, G.S., Bignell, J.P., Stentiford, G.D., Viant, M.R., 2007. Direct Sampling of Organisms from the Field and Knowledge of their Phenotype: Key Recommendations for Environmental Metabolomics. *Environ. Sci. Technol.* 41, 3375–3381.
- Langley, G., Austin, C.P., Balapure, A.K. et al., 2015. Lessons from Toxicology: Developing a 21st Century Paradigm for Medical Research. *Environ. Health Perspect.* 123, A268–A272.

Table 1. Statistical comparisons of 27 quantifiable hepatic metabolites from larval *Limnodynastes peronii* following exposure to a pharmaceutical mixture with solvent-free and carrier-solvent (methanol) dosing through the developmental period.

Metabolite [#]	Treatment			Solvent			Interaction			One-Way ANOVAs	
	SS	F Ratio	p-value	SS	F Ratio	p-value	SS	F Ratio	p-value	Water	MeOH
L-Isoleucine	0.015	0.320	0.811	0.012	0.756	0.393	0.080	1.699	0.194	0.735	0.133
L-Leucine	0.109	3.383	0.035*	0.000	0.037	0.849	0.112	3.486	0.031*	0.965	0.002*
L-Valine	0.026	0.603	0.619	0.005	0.331	0.570	0.102	2.409	0.092	0.859	0.050*
B-Hydroxybutyric acid	0.227	5.681	0.004*	0.513	38.500	<0.001*	0.033	0.822	0.495	0.007*	0.46
L-Lactic acid	0.400	2.668	0.070	0.005	0.985	0.331	0.016	1.054	0.387	0.098	0.827
L-Lysine	0.013	1.374	0.275	0.003	0.911	0.349	0.026	2.778	0.063	0.054*	0.817
L-Alanine	0.017	0.457	0.715	0.004	0.317	0.579	0.075	1.999	0.141	0.876	0.064
Putrescine	0.025	0.699	0.562	0.135	11.540	0.002*	0.039	1.108	0.365	0.307	0.903
Acetate	0.078	5.174	0.007*	0.000	0.050	0.825	0.101	6.766	0.002*	0.603	<0.001*
L-Arginine	0.007	0.553	0.651	0.037	8.810	0.007*	0.098	7.845	<0.001*	0.069	0.015*
L-Glutamate	0.040	4.245	0.015*	0.014	4.616	0.042*	0.005	0.517	0.675	0.054*	0.351
L-Glutamine	0.044	4.530	0.012*	0.008	2.364	0.137	0.076	7.851	<0.001*	0.3239	<0.001*
Citrate	0.006	0.866	0.472	0.003	1.390	0.250	0.064	9.327	<0.001*	<0.001*	0.155
L-Aspartate	0.056	2.774	0.063	0.057	8.459	0.008*	0.049	2.449	0.088	0.014*	0.936
Creatine	0.081	1.976	0.145	0.099	7.182	0.013*	0.098	2.403	0.092	0.121	0.168
O-Phosphocholine	0.006	0.278	0.841	0.146	17.600	<0.001*	0.041	1.653	0.204	0.767	0.271
Glucose/Maltose	0.008	0.339	0.797	0.015	1.841	0.188	0.054	2.131	0.123	0.109	0.777
Myo-inositol	0.034	1.107	0.366	0.126	12.140	0.002*	0.046	1.494	0.241	0.436	0.202
Glycogen	0.012	0.519	0.673	0.002	0.319	0.578	0.082	3.686	0.026*	0.168	0.138
UDP Glucose	0.044	2.794	0.062	0.115	22.070	<0.001*	0.014	0.867	0.472	0.016*	0.490
UDP Galactose	0.168	2.976	0.052*	0.392	20.840	<0.001*	0.004	0.063	0.979	0.046*	0.479
Glycine	0.094	6.567	0.002*	0.039	8.174	0.009*	0.037	2.603	0.075	<0.001*	0.6006
Nucleotides	0.009	0.889	0.461	0.029	8.850	0.007*	0.046	4.633	0.011*	0.017*	0.3461
NAD+	0.127	0.522	0.671	0.072	8.860	0.007*	0.009	0.389	0.762	0.594	0.967
Formate	0.022	0.766	0.524	0.065	6.735	0.016*	0.022	0.761	0.527	0.920	0.204
L-Tyrosine	0.005	0.265	0.850	0.027	4.082	0.055	0.124	6.330	0.003*	0.149	0.018*
Unknown at 6.53	0.456	7.855	<0.001*	0.046	3.189	0.087	0.069	12.020	<0.001*	<0.001*	0.286

NMR spectra consistent with the metabolites L-Ornithine, Taurine, L-Serine, Oxalacetic acid, AND Pyruvic acid were identified but could not be quantified with confidence due to overlapping ¹³C chemical shifts in the HSQC spectra. * Asterisks and bold indicates statistical significance.

Melvin et al., CHEM50400 _ Highlights

- Carrier solvents are commonly used for chemical exposures in ecotoxicology.
- Test protocols overlook possible interactions between solvents and pollutants.
- We exposed tadpoles to a mixture of pharmaceuticals throughout development.
- The exposure was performed with and without the carrier solvent methanol.
- Metabolomics revealed considerable interactive toxicity caused by the solvent.