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Nicole D. Wagner, André J. Simpson, Myrna J. Simpson

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Running title: Age specific metabolic responses to contaminant exposure

Corresponding author:

Myrna J. Simpson

Environmental NMR Centre and Department of Physical and Environmental Sciences

1265 Military Trail, Toronto

Ontario Canada

M1C 1A4

416-287-7234

Email: myrna.simpson@utoronto.ca

Metabolomic responses to sub-lethal contaminant exposure in neonate and adult *Daphnia magna*

Nicole D. Wagner, André J. Simpson, Myrna J. Simpson *

Environmental NMR Centre and Department of Physical and Environmental Sciences,
University of Toronto Scarborough. Scarborough, ON, Canada.

* Corresponding author Myrna J. Simpson email: myrna.simpson@utoronto.ca

ABSTRACT

The use of consumer products and pharmaceuticals that act as contaminants entering waterways through runoff and wastewater effluents alter aquatic ecosystem health. Traditional toxicological end points may underestimate the toxicity of contaminants as lethal concentrations are often orders of magnitude higher than that found within freshwater ecosystems. While newer techniques examine the metabolic responses of sub-lethal contaminant exposure, there has been no direct comparison with ontogeny in *Daphnia*. We hypothesize that *Daphnia magna* will have distinct metabolic changes to 3 different sub-lethal contaminant exposures caused by differences in the toxic mode of action and ontogeny. To test this hypothesis, we measured the proton nuclear magnetic resonance metabolomic profiles in *D. magna* aged day 0 and 18 after exposure to 28 % of the lethal concentration of 50% of organisms tested (LC50) of atrazine, propranolol and perfluorooctanesulfonic acid (PFOS) for 48 hours. Principal component analysis revealed significant separation of contaminants from the control daphnids in both neonates and adults exposed to propranolol and PFOS. In contrast, atrazine exposure caused separation from the controls in only the adult *D. magna*. Propranolol exposure displayed minimal ontogenetic changes in the targeted metabolites. For both atrazine and PFOS exposures ontogeny exhibited unique changes in the targeted metabolites. These results indicate that depending on the contaminant studied, neonates and adults respond uniquely to sub-lethal contaminant exposure.

Keywords ^1H NMR, PFOS, Propranolol, Atrazine, Environmental metabolomics

INTRODUCTION

Anthropogenic activity is increasingly altering aquatic ecosystem health both directly and indirectly through wastewater effluents and changes in land use [1,2]. Contaminants enter aquatic ecosystems through wastewater effluents containing personal care products and pharmaceuticals that remain in treated wastewater due to inefficient removal methods [3,4]. Land use changes to agriculture, especially to monoculture, leads to increases in run off into nearby riparian zones ultimately increasing turbidity [5], and contaminants (pesticides) [6] entering rivers and lakes. Upon entering aquatic ecosystems the fate of contaminants depends on both abiotic and biotic factors [7,8]; however, the presence of contaminants can potentially disrupt or alter ecosystem dynamics such as changes in biodiversity and nutrient cycling [9,10].

Contaminants elicit lethal and sub-lethal responses to many different aquatic organisms including producers such as chlorophytes [11,12], cyanophytes [11], and consumers such as cladocerans [13–15], benthic invertebrates [16,17], and vertebrates [18,19]. The freshwater consumer, *Daphnia magna*, is an important aquatic species capable of dominating the zooplankton biomass [20] thus controlling both top down and bottom up ecosystem processes [21]. While *Daphnia spp.* are capable of large biomasses within lentic and pelagic zones they are sensitive to many types of environmental stressors including food quality [22], predation [21], and contaminants [23–25] that can change population dynamics [26], and biogeochemical cycles [10,20]. Given the ecological importance and the life history (i.e. short life cycle) of *D. magna* it is often the choice of many toxicity experiments to study and predict the ecological consequences of contaminants in aquatic ecosystems [12,13,23,27,28]. However, traditional toxicity end points such as lethal concentration of 50% of organisms tested (LC50), may

underestimate the stress caused by sub-lethal contaminant exposure as lethal concentrations are often magnitudes higher than what is present within aquatic ecosystems [10].

Recently many toxicity studies have shifted their focus from terminal (e.g. death) to more sub-lethal metrics such as growth [23], reproduction [23], and molecular-level responses [29] to gain a better understanding of how contaminants alter the physiology of organisms. An emerging field well suited to study the molecular responses of organisms exposed to contaminants is metabolomics, which investigates how a subset of metabolites (polar or non-polar) are altered as a result of sub-lethal contaminant exposure [15,30,31]. The advancement of analytical tools such as mass spectrometry [32] and microprobes for nuclear magnetic resonance (NMR) [33] have increased the sensitivity and decreased the mass requirements for metabolomics, thus improving the feasibility to use small organisms such as *Daphnia spp.* Traditional acute toxicity tests such as the LC50 use neonate *D. magna* [12,13,27], whereas metabolomic studies use either adult (greater than 14 days old; [15,34]) or neonate *D. magna* [35,36] to determine the changes in the metabolic profile caused by the contaminant exposure. As the field of toxicology transitions from traditional lethal studies to more modern sub-lethal metrics there will become an increasing need to compare whether animals will respond similarly throughout ontogeny. However, to our knowledge no studies have examined if ontogenetic differences in *D. magna* will alter their metabolic profile to contaminant exposure.

Daphnia spp.'s life history consists of being an r-selected species that emphasizes energy into reproduction [20]. Typically, daphnids have fast growth rates and can reach reproduction at approximately 8 days old under optimal conditions [22]. Once *Daphnia spp.* begin to reproduce, their energetic demands shift from growth to reproduction, and thus growth rates decline. Energetic demands can be thought of as a hieratical system with prioritizing maintenance, then

growth and finally reproduction as described in dynamic energy budget theory [37]. These differences in energetic demand can be amplified especially for acute toxicity tests as reproduction is not a continuous energy demand unlike growth [38]. Given the differences in energetic demand between the two life stages of *Daphnia spp.* in addition to previous research that has shown neonates are more susceptible to toxicity [39,40], a direct comparison between the metabolic profiles of neonate and adult *D. magna* exposed to contaminants is needed to determine if their profiles respond similarly to contaminant stress.

Here we examine the metabolomic responses between neonate and adult *D. magna* exposed to 3 different contaminants for 48 hours. Both neonate and adult *D. magna* were exposed to one concentration of propranolol, atrazine, or perfluorooctanesulfonic acid (PFOS) and their metabolic profiles were measured using ¹H NMR. Propranolol is a pharmaceutical used to treat hypertension by acting as a non-specific β -blocker causing vasodilation. This pharmaceutical enters aquatic ecosystems through inefficient removal during wastewater treatment [23]. Atrazine is a commonly used agricultural herbicide that inhibits the photosystem II in plants, and enters aquatic ecosystems through run off [41]. PFOS is a common consumer product used as a stain repellent on clothing, as well used as a surfactant, and enters aquatic ecosystems through inefficient removal in treating wastewater [12]. Given the difference type of contaminants, we hypothesize *D. magna* will have distinct metabolic profiles caused by differences in the toxic mode of action (MOA) and ontogeny. We expect neonate *D. magna* to respond more severely than adult *D. magna* to contaminant stress because of differences in energy demands for maintenance, growth and reproduction.

MATERIALS AND METHODS

Algae and Daphnia magna Culturing

Raphidocelis subcapitata (Canadian Phycological Culture Centre strain 37) was grown in multiple batch culture jars in Bristol media with a 16 : 8 h light : dark cycle, and a light intensity of approximately 150 $\mu\text{mol/s/m}$ at 20 °C. Each batch culture was concentrated by centrifugation at 4066 *g* for 20 min and fed to brood mother *D. magna* ad libitum.

Daphnia magna (Straus) was purchased from Ward Science Canada in 2013 and has since been raised at 20 °C in municipal de-chlorinated tap (Toronto, Ontario, Canada) water with a hardness of approximately 120 mg CaCO_3/L and pH 8.15. Animals were maintained in a density of 1 daphnid per 30 mL water and fed *R. subcapitata* ad libitum daily with a 16 : 8 h light: dark cycle. Water was changed by replacing 50% of the old water with fresh de-chlorinated tap water to remove excess food and ammonium every other day. In addition to water changes, *D. magna* were supplemented with 1 $\mu\text{g/L}$ of selenium and cobalamin bi-weekly to ensure animals received adequate essential nutrients not provided by the algae [42].

Contaminant exposure

Cultured daphnids were used to study the metabolomic changes in both neonate and adult *D. magna* exposed to sub-lethal concentrations of atrazine, propranolol, and PFOS. We used *D. magna* literature values of acute 48 h LC50 for both atrazine (35.5 mg/L [13,28]) and PFOS (130 mg/L [12]) to determine sub-lethal concentrations for our exposures. However, propranolol literature LC50 value (7.7 mg/L [27]) resulted in 100% mortality, thus 48 h LC50 experiments were conducted using Environment Canada's protocol [42]. Briefly, neonates under 24 h old were removed from brood mother jars and placed in groups of 10 in 200 mL of de-chlorinated

tap water with varying concentrations of propranolol and checked for mortality after 48 h ($n = 5$; Supplemental Data, Figure S1).

For both neonate and adult exposures, we used concentrations consisting of 28 % of the 48 h LC50 as initial trials resulted in no mortality. This represents a nominal concentration of 10 mg/L atrazine (PENSTAL 98.8 % purity Sigma-Aldrich, Oakville, ON), 36 mg/L PFOS (98 % purity, Sigma-Aldrich, Oakville, ON), and 0.67 mg/L propranolol (propranolol hydrochloride, < 99 % purity, Sigma-Aldrich, Oakville, ON). Both PFOS and propranolol were hydrophilic enough to be dissolved directly into the de-chlorinated tap water; however a solvent carrier of 0.15 % acetone was needed to make stock solutions of atrazine. The final concentration of acetone was 0.03 % used in the neonate and adult exposure experiments.

Neonate specific experimental design

Stock concentrations of each contaminant were made in de-chlorinated tap water. An aliquot of the contaminant was added to replicate ($n = 8$ per contaminant exposure) 2 L glass jars and filled with additional de-chlorinated tap water to a final volume of 2 L to obtain the nominal concentration of each contaminant (0.67 mg/L propranolol, 10 mg/L atrazine, 36 mg/L PFOS; $n = 8$ per contaminant exposure). This procedure was repeated for all the treatments (propranolol, atrazine, and PFOS). Additional replicates ($n = 8$) of 2 L glass jars were filled with de-chlorinated tap water as a control treatment for the propranolol and PFOS treatments, whereas the atrazine treatment controls ($n = 8$) contained 0.03 % acetone as a solvent control. *Daphnia magna* neonates (< 24 h old) were removed from the brood mother cultures and pooled into a container then placed into experimental glass jars with the density of 1 neonate per 30 mL (60 neonates in each jar) and fed 6 mg of *R. subcapitata*/L.

To assess *D. magna* growth responses to acute contaminant exposure, we sampled 3 replicates of 20 neonates placed in pre-weighed aluminum cups and dried overnight at 40°C in a drying oven (Fisher Scientific Isotemp Oven) to determine the average initial mass of the daphnids with a microbalance (Sartorius ME 36S, Gorttingen, Germany). After 48 h of growth, differently exposed daphnids were collected from replicate jars ($n = 5$ per treatment) containing 18 *D. magna* placed in pre-weighed aluminum cups and dried overnight at 40°C in a drying oven and reweighed. Mass specific growth rate (MSGR) determined by the equation:

$$MSGR = \frac{[\ln(B2) - \ln(B1)]}{time}$$

Where B2 is the final average mass per *D. magna*, B1 is the average initial neonate mass, and time is number of days of *D. magna* growth. Using mass specific growth rates, instead of mass or length accounts for the small variation in neonate initial size (1 – 2 µg) [43]. MSGR ranges between 0-1, with 1 indicating a doubling of mass per day, and 0 indicating no growth [20,43].

Adult specific experimental design

Fresh stock contaminants in the nominal concentration were prepared (0.67 mg/L propranolol, 10 mg/L atrazine, 36 mg/L PFOS) in de-chlorinated tap water. An aliquot of the contaminant was added to replicate ($n = 10$ per treatment) glass jars which were subsequently filled to 300 mL with a 50 % mixture of old and new de-chlorinated tap water to ensure adult survival. This procedure was repeated for all the treatments (propranolol, atrazine, and PFOS). The control treatments used for the propranolol and PFOS exposures, had replicate ($n = 10$) glass jars filled with 300 mL of a 50 % mixture of old and new de-chlorinated tap water. Atrazine replicate solvent controls ($n = 10$) were prepared with 300 mL of a 50 % mixture of old and new

de-chlorinated tap water containing 0.03 % acetone. We added day 18 old *D. magna* (from day 0 to 18 grown under *D. magna* culturing conditions) to each replicate at the density of 1 adult per 30 mL (10 adults per jar) and they were fed 6 mg of *R. subcapitata*/L at the start of the experiment and after 24 h.

Animal preservation for ¹H NMR metabolomics

After 48 h of sub-lethal contaminant exposure, we transferred replicate jars to de-chlorinated tap water to remove algae and contaminants from their carapace. After rinsing, animals were filtered onto a mesh, transferred to a 1.5 mL microfuge tube and immediately placed in liquid nitrogen. Animals were subsequently lyophilized and stored at -20 °C for 2 weeks before metabolomic analysis was performed.

¹H-NMR Metabolomics extraction

Daphnia magna from replicate jars were weighed on a microbalance (Sartorius ME 36S, Gorttingen, Germany) to approximately 1 mg. The number of *D. magna* required per replicate varied between treatments especially for neonates with 60 – 100 individuals, whereas only 2 adults were needed to obtain 1 mg. For each treatment (control, solvent control, propranolol, atrazine, and PFOS) we extracted 8 replicates from the neonate and 10 replicates from the adult treatments. Extraction procedures follow Nagato et al. [33]. Briefly, daphnids were homogenized into a fine powder with a 5 mm stainless steel spatula. After homogenization 45 µL of 0.2 M phosphate buffered (NaH₂PO₄·2H₂O; 99.3%; Fisher Chemicals) D₂O (99.9% purity, pD 7.4 adjusted with 30 % w/w NaOD 99.5% purity; Cambridge Isotope Laboratories) containing 0.1 w/v sodium azide (99.5% purity; Sigma Aldrich) and 10 mg/L of 2,2-dimethyl-2-silapentane-5-

sulfonate sodium salt (DSS; 97%, Sigma Aldrich) was added as internal calibrant. Samples were then vortexed for 30 s and sonicated for 15 min. After sonication, samples were centrifuged at 14000 RPM (~15000 g) at 4 °C for 20 min. The supernatant was then removed and transferred into a 1.7 mm NMR tube (Norell Inc., NJ, USA).

Data acquisition and processing

The ^1H NMR spectra were acquired with a Bruker BioSpin Avance III 500 MHz NMR equipped with a ^1H - ^{13}C - ^{15}N TXI 1.7 mm microprobe fitted with an actively shielded Z gradient [33]. Briefly, acquisition was performed with 256 scans with a relaxation delay of 3 s, 32k time domain points, and a 90° pulse calibrated on a per sample basis. Water suppression was accomplished through Presaturation Using Relaxation Gradient and Echoes program (PURGE; [44]). All spectra were apodized through multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectra, with a zero filling factor of 2 [30,33]. The resulting NMR spectra were manually phased, baseline corrected and aligned to $\delta = 0.00$ ppm using the trimethyl silyl group of the DSS internal calibrant.

Data analysis

Differences in growth rate caused by contaminant exposure were determined using a t-test (two-tailed, equal variance; Excel 2013 with data analysis add on) between control neonates to the propranolol and PFOS exposed neonates. To determine differences in growth for atrazine exposed animals a t-test (two-tailed, equal variance; Excel 2013 with data analysis add on) between solvent control and atrazine neonates were performed. We set the critical $p \leq 0.05$ adjusted with a Bonferroni correction for multiple comparisons ($p \leq 0.0125$).

The ^1H NMR spectra region from 0.5 to 10 ppm was divided into 0.02 ppm widths resulting in a total of 475 buckets using AMIX software (v3.9.7; Bruker BioSpin Rheinstetten Germany). Residual water peaks residing in the area between 4.70 – 4.85 ppm were excluded to eliminate differences due to water suppression. All spectra were scaled to total intensity. The resulting intensity of each bucket was used to construct a principal component analysis (PCA) plot with corresponding PCA loadings plots. The PCA scores plot was exported from AMIX into Sigma Plot 12.5 to graph the average PCA scores plot. PCA is a multivariate unsupervised technique that enables the visualization of many response variables at one time. This technique is ideal for non-targeted metabolomics that generates 475 buckets (response variables) from 1 spectra. If no separation is observed between the control and exposed treatments it indicates the metabolome was not altered. However if significant separation is observed then individual metabolites can be identified and examined to determine the response between control and exposed treatments.

Metabolites were identified by comparing the resonances in the spectra obtained to the AMIX database as well as published resonances for selected metabolites [15,30,33,34]. The percent change of metabolites was calculated by using the bucket intensities for the exposed groups (atrazine, PFOS, and propranolol) and the control groups (solvent control and control). T-tests (two-tailed, equal variances; Excel 2013 with data analysis add on) were performed for known metabolites between control treatment to the solvent control, PFOS, and propranolol using $p \leq 0.05$ corrected with the false discovery rate [45]. The atrazine treatment was only compared to the solvent control, thus minimizing any metabolomics changes due to the presence of acetone. Additional t-test (two-tail unequal variances; Excel 2013 with data analysis add on)

were performed on the relative percent changes of the targeted metabolite between neonate and adult *D. magna* using $p \leq 0.05$ corrected with the false discovery rate [45].

RESULTS

Daphnia magna growth rates were altered depending on the contaminant exposure. Neonate daphnids in the solvent control (exposed to 0.03% acetone) and PFOS exposure did not decrease their growth rates compared to the control animals, thus maintaining a growth rate of ~50 % per day (doubling their body mass every two days; Figure 1). However, neonates exposed to atrazine ($t(8) = 6.93, p < 0.001$) and propranolol ($t(8) = 13.39, p < 0.001$) caused significant decreases in growth compared to the control neonates (Figure 1). The reduction in growth was more severe in propranolol exposure with neonates only growing ~ 11 % per day, whereas atrazine exposed neonates were able to grow ~ 30 % per day (Figure 1).

The first 2 axes of the average PCA scores plots explained 86% and 85% of the variation for the neonate and adult *D. magna* respectively. Average PCA scores plots of the metabolome identified significant separation in the animals exposed to propranolol and PFOS from the controls in both neonate and adult daphnids (Figure 2). The atrazine treatment caused significant separation from the solvent controls (contains 0.03% acetone) in only the adult daphnids (Figure 2B). There was no significant separation observed in the PCA scores plots between the controls and the solvent controls in both neonate and adult daphnid treatments (Figure 2). Separation along PC1 for the neonate *D. magna* was caused by resonances related to the metabolites arginine ($\delta = 1.91$ ppm), valine ($\delta = 1.03$ ppm), and leucine ($\delta = 0.95$ ppm), and separation along the PC2 axis was caused by alanine ($\delta = 1.47$ ppm; Figure 3A). For adult *D. magna* separation along PC1 was caused by leucine ($\delta = 0.95$ ppm) and overlapping sugars and amino acids

resonances, while separation along PC2 was caused by alkanolic acids ($\delta = 1.27$ ppm and $\delta = 1.29$ ppm; [Figure 3B](#)).

No significant changes were observed in targeted metabolites for the solvent control treatment in both neonates and adult *D. magna* ([Supplemental Data, Figure S2](#)). However the 3 contaminants assessed caused unique changes within the targeted metabolites compared to the controls for both neonate and adult *D. magna* ([Figure 4](#)). Significant differences were observed for most targeted metabolites with the exception of threonine, proline, serine and lactate for all 3 contaminant exposures in both neonates and adults ([Figure 4](#) and [Supplemental Data, Figure S3](#)). Generally, neonates exposed to propranolol significantly increased many of amino acids metabolites (between ~15-70 %) and had decreased glucose and tryptophan compared to the control ([Figure 4](#)). Neonates exposed to atrazine resulted in only significant increases in alanine (increase ~50 %) with all other metabolites remaining the same as the solvent control ([Figure 4](#)). Finally neonates exposed to PFOS decreased some of their amino acids metabolites (leucine, isoleucine, valine, alanine, arginine, phenylalanine, and tyrosine, ~5-20 % change decrease) compared to the controls where other targeted metabolites did not differ ([Figure 4](#)). Adults exposed to propranolol resulted in many amino acids (except glycine) being increased (~20-70 % increase) and decreased glucose compared to the control group. Atrazine exposure in adult *D. magna* caused a similar metabolic profile to propranolol however glycine was significantly decreased (~20%) compared to the solvent controls. Adult exposure to PFOS revealed increases in many amino acid metabolites (except glycine) similar to propranolol, however no decrease in glucose was observed ([Figure 3](#)).

Neonate and adult daphnids exposed to propranolol had similar metabolic profiles, with glucose and lysine being the only targeted metabolites identified as significantly different with

ontogeny (Figure 4). Relative percent metabolite changes for both atrazine and PFOS exposures differed with ontogeny. The distinct metabolic profiles with ontogeny in the atrazine treatment is caused by the neonate profile remaining similar to the control whereas the adult profile was drastically different from the control (Figure 4). Many of the significant metabolic changes produced by ontogeny in animals exposed to PFOS were caused by inverse metabolic responses with neonates decreasing and adults increasing their metabolites (Figure 4)

DISCUSSION

In agreement with our hypothesis, the metabolic profile in *D. magna* likely shifted depending on the contaminant MOA and between neonate and adult acute sub-lethal exposures. Each contaminant tested exhibited unique metabolic responses. In addition to metabolic changes due to contaminant MOA, we also identified differences in the metabolic responses between ontogeny for 2 out of 3 contaminants. While propranolol exposures responded similarly between neonate and adult daphnids, both atrazine and PFOS exposures revealed divergent metabolic profiles with ontogeny. Our results indicate the metabolic profile is altered due to contaminant exposure and these changes are not always consist through ontogeny. Furthermore, these results suggest that the ecological impacts of contaminant exposure are not equal across ontogeny in *D. magna* and could alter ecosystem dynamics due to different stressors on various ages.

When organisms are exposed to a different class of contaminants their biochemistry, and thus metabolic pathways, are altered in a specific way [15,30,34,46]. The use of metabolomics, which investigates the intermediates of metabolic pathways, is becoming increasingly popular in toxicity studies to help elucidate the toxic MOA of contaminants [30,34,46]. However not all metabolic changes are monotonic over a gradient of contaminant exposure [15,34]. Often

metabolic changes can have a threshold where metabolites plateau after a certain concentration of contaminant, or have a unimodal response where the highest concentration of exposure decreases, compared to the intermediate concentrations [15,34]. Given that the metabolite responses can vary considerable with exposure concentration, it is best to determine the MOA over a range of contaminant concentration to gain fine scale information on how metabolites change [15]. Although the precise MOA cannot be determined from our experimental design, our study provides some insight into the possible mechanisms causing the alterations in the metabolic profiles when *D. magna* are exposed to contaminant stress.

In invertebrates, exposure to β -blockers (i.e. propranolol) cause decreases in heart and feeding rates [23]. We found significant decreases in growth with propranolol exposure as well as increases in the relative amount of most amino acids metabolite and decreases in glucose for both the neonate and adult treatments. Taylor et al. [46] identified the toxic MOA of propranolol in *D. magna* to involve impairments of fatty acid synthesis. The extraction of metabolites with a phosphate buffer used in the present study is more polar than the chloroform methanol water extraction used by Taylor et al. [46], thus many fatty acids such as linoleic acid would not be visible in our method. Although insignificant, Taylor et al. [46] did report increases in most amino acid metabolites, the discrepancy between these studies maybe caused by differences in concentration of propranolol with Taylor et al. [46] using 10% of the LC50 compared to 28% of the LC50 in the present study. With the observed decreases in growth rate and glucose, and increases in many amino acid metabolites, we speculate the metabolomic profile of *D. magna* exposed to propranolol may be attributed to protein degradation being used to fuel energetic demands [47]. This general response of increased free amino acids and decreased growth rates is also observed under low food conditions [43], adding evidence that feeding rates may be

decreased when daphnids are exposed to propranolol. Furthermore, increases in aromatic amino acids may indicate impairments to both immune function and nervous system. Tyrosine is a precursor for both melanin formation (L-DOPA) and dopamine synthesis, which is important for immune function [48], and a known neurotransmitters in invertebrates [49] respectively.

The potential toxic MOA for atrazine in invertebrates is acting as an endocrine disruptor and impairing ecdysteroid activity resulting in difficulty molting, impaired embryogenesis [41], and increased male production [50]. We observed contrasting metabolic responses to atrazine exposure in neonate and adult *D. magna*. While the neonate exposures did cause decreases in growth, the only metabolic response significantly different from the solvent controls was an increase in alanine content. Alternatively, the adult exposure to atrazine caused many of the amino acid metabolites to increase, and glycine and glucose decreased relative to the solvent controls. In agreement with atrazine's toxic MOA an increase in free amino acid metabolites may be caused by impaired hormone synthesis leading to difficulty molting and embryogenesis as found in crayfish [51]. Or alternatively, the increase in many amino acid metabolites may be attributed to protein degradation, as seen in the propranolol results. Additionally, decreases in glycine may be related to oxidative stress as glycine is needed for the production of a well-known antioxidant glutathione.

Unlike atrazine and propranolol, the toxic MOA of PFOS in *Daphnia spp.* has yet to be determined at the molecular-level. However the toxic MOA has been reported in other invertebrates such as the earthworm, *Eisenia fetida* [52] and marine mussels [53]. Lankadurai et al. [52] observed a decrease in many metabolites (valine, leucine, arginine, phenylalanine, and lysine, glucose) and concluded the toxic MOA is likely similar to the mammalian MOA that involves fatty acid oxidation and membrane degradation. In the marine green mussel, *Perna*

viridis, an oxidative stress response has been identified by increases in superoxide dismutase, and catalase activity and decreases in glutathione when animals are exposed to PFOS [53]. Unlike the propranolol and atrazine neonate exposure, PFOS did not cause a reduction in growth, but did alter the metabolic profile. Our study observed similar metabolic responses as earthworms within the neonate PFOS exposure treatment, with many amino acid metabolites being decreased relative to the control treatments. If the toxic MOA in daphnids disrupts membranes, or makes membranes more permeable, the metabolic profile of neonates exposed to PFOS may be experiencing osmotic stress, as similar metabolome patterns were identified with *D. magna* exposed to salt stress [43]. Alternatively, many of the amino acids that were found to decrease significantly are essential (e.g. leucine, valine, lysine, and phenylalanine; [54]) this might indicate a slight decrease in feeding rates but not enough to cause growth inhibition. However, to our knowledge no studies have examined the physiological responses of PFOS exposure in *Daphnia spp.* The adult PFOS exposure resulted in many amino acid metabolites (e.g. alanine, isoleucine, leucine, valine, phenylalanine, methionine) increasing relative to the controls. This pattern of increased amino acids may be caused by protein breakdown to generate energy from glucogenic amino acids (e.g. alanine, valine, methionine) [47]. Alternatively, protein breakdown into amino acid metabolites may be caused by oxidative stress [55]; thus adding evidence that PFOS causes oxidative damage in aquatic invertebrates.

Our results indicate the metabolic profiles between neonates and adults differed with the same concentration of contaminant for atrazine and PFOS, while similar responses were observed for propranolol. Previous studies have indicated neonates are more sensitive to contaminant exposure [39,40]; however, our metabolomics study does not support this argument. For example neonates acutely exposed to atrazine had very similar metabolic profiles to the

solvent controls, while the adults exposed to atrazine caused many targeted metabolites to increase compared to the solvent control. We suspect exposing neonates to atrazine caused the decreased in growth as seen with other endocrine disruptors in *D. magna* [56] that allowed for the additional nutrients and energy that would have been required for growth be shuttled into maintenance pools to maintain homeostasis. However when growth is minimal in adult *Daphnia spp.*, exposure to atrazine may alter metabolic pathways involved in reproduction and maintenance leading to changes in the metabolic profile, consistent with our observation. To further explore this explanation for differences between ontogeny we suggest future experiments growing *D. magna* under a range of atrazine at various life stages from neonate, juvenile and young adults where growth is still important life history trait. In addition, to tease apart whether reproduction or maintenance demands are altered in adults, we recommend targeted metabolomics to look at reproduction specific pathways such as vitellogenin formation as well as hormones that could further support the proposed MOA.

Daphnia magna exposed to PFOS had many targeted metabolites decreased in neonates and increased in adults compared to unexposed animals. We suspect these differences in the metabolic profiles of neonate and adult daphnids could indicate a difference in the toxicity to PFOS at different life stages. While our data does not clearly indicate whether neonates are more sensitive to PFOS exposure, it does indicate potential different metabolic pathways are altered between neonate and adult *D. magna*. These differences in metabolic profiles between neonate and adult daphnids may be caused by differences in the energetic demands for maintenance, growth, and reproduction as predicted by dynamic energy budget theory [37]. Alternatively, differences seen daphnids metabolic profiles during ontogeny may be caused by differences in physiology leading to changes in contaminant exposure. While it is unknown if PFOS uptake in

D. magna occurs by dietary or environmental routes [12] it is possible both routes of uptake may result in neonates being exposed to less contaminant. If dietary exposure is possible, adults would experience higher concentrations of PFOS as their overall food intake is higher than neonates even though neonates have a faster feeding rate [57,58]. Additionally, if uptake of PFOS comes directly from the environment it may decrease the mass specific contaminant concentration in neonates caused by fast growth rates resulting in growth dilution of contaminants [59].

CONCLUSIONS

We investigated the sub-lethal responses of acute contaminant exposure in both neonates and adult *D. magna*. Dissimilar metabolic profiles between ages in *D. magna* exposed to contaminants may be caused by differences physiology with neonates prioritizing growth while adults prioritize reproduction. If the differences in the metabolic profiles during ontogeny indicates altered stress responses, it may lead to changes daphnids population structure in natural ecosystems by favoring neonates or adults, thus changing foodweb dynamics. Variation in the molecular-level responses throughout ontogeny may add difficulty for the development of high throughput molecular-based techniques for risk assessments as proposed by Schroeder et al. [60]. The goal of examining molecular-level responses including metabolic profiles is to identify changes at the individual level that will correlate to higher level processes such as population growth rates. However, variation in the metabolic profiles caused by ontogeny will likely make it difficult to predict the outcome of contaminant exposure in daphnids. Future experiments should focus on developing models that will be able to predict how the metabolome responds to

contaminant exposure throughout ontogeny. Our results indicate care should be taken especially for development molecular-level ecological risk assessments, until the metabolic profile between ontogeny and contaminant stress and their interaction can be predicted.

SUPPLEMENTAL DATA

Supplemental Data - The Supplemental Data are available on the Wiley Online Library at DOI:

XXX

Figures S1-S3

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Figure Legends

Figure 1. Average $\pm$ standard error of mass specific growth rate per day (MSGR d^{-1}) of neonate *D. magna* exposed for 48 h of propranolol (Pro; 0.67 mg/L), atrazine (10 mg/L), and PFOS (36 mg/L) and unexposed control and solvent control (0.03% acetone; $n = 5$ per treatment). * indicate $p \leq 0.05$ corrected for multiple comparisons ($p \leq 0.0125$) between control and exposed treatments.

Figure 2. Average principal component analysis (PCA) score plots $\pm$ standard error ($n = 8$ per treatment for neonate; $n = 10$ per treatment for adult) for: (A) neonate and (B) adult *D. magna* grown for 48 h under control, and solvent conditions and exposed to propranolol (0.67 mg/L), atrazine (10 mg/L), and PFOS (36 mg/L). * indicate $p \leq 0.05$ corrected for multiple comparisons ($p \leq 0.0125$) between control and exposed treatments.

Figure 3. Principal component analysis (PCA) loading plots indicating buckets responsible for the separation within PCA. (A) PCA loadings for neonate PCA, (B) PCA loadings for adult PCA.

Figure 4. Average relative percent changes $\pm$ standard error of targeted metabolites from the control (propranolol; pro, 0.67 mg/L, and PFOS, 36 mg/L) or solvent control (atrazine, 10 mg/L) in neonate (grey bars) and adult (white bars) treatments. * indicates significant differences from the control $p \leq 0.05$ corrected for the false discovery rate. + indicates significant differences in the response between neonate and adult treatments relative to their respective controls $p \leq 0.05$ corrected for the false discovery rate. Bolded amino acid names are classified as essential in

641 crustaceans [54]. Nonsignificant metabolite changes are shown in Supplemental Data, Figure
642 S3.