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Effects of naphthenic acid exposure on development and liver metabolic processes in anuran tadpoles

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ABSTRACT

Naphthenic acids (NA) are used in a variety of commercial and industrial applications, and are primary toxic components of oil sands wastewater. We investigated developmental and metabolic responses of tadpoles exposed to sub-lethal concentrations of a commercial NA blend throughout development. We exposed *Lithobates pipiens* tadpoles to 1 and 2 mg/L NA for 75 days and monitored growth and development, condition factor, gonad and liver sizes, and levels of liver glucose, glycogen, lipids and cholesterol following exposure. NA decreased growth and development, significantly reduced glycogen stores and increased triglycerides, indicating disruption to processes associated with energy metabolism and hepatic glycolysis. Effects on liver function may explain reduced growth and delayed development observed in this and previous studies. Our data highlight the need for greater understanding of the mechanisms leading to hepatotoxicity in NA-exposed organisms, and indicate that strict guidelines may be needed for the release of NA into aquatic environments.

1. Introduction

Naphthenic acids (NA) comprise a diverse group of naturally occurring petrogenic carboxylic acids, and are widely used in a variety of commercial and industrial applications such as tire fabrication, and as surfactants and wood preservatives (Brient et al., 1995; Clemente and Fedorak, 2005). Several lines of evidence point to NA as the primary toxic component of Oil Sands Process-affected Water (OSPW) (Frank et al., 2009; Dorn, 1992; Madill et al., 2001). Tailings ponds contain concentrations as high as 120 mg/L (reviewed in Clemente and Fedorak, 2005), but environmental concentrations of NA downstream of oil sands mining activities are commonly not higher than approximately 1–2 mg/L (Environment Canada, 2010; Headley and McMartin, 2004). Commercial NA may be structurally and symptomatically dissimilar from those occurring in OSPW, and there is very little information pertaining to environmental concentrations of these and other forms of NA. Nonetheless, commercial NA has been observed to cause acute lethality and sub-lethal effects in aquatic vertebrates at low exposure concentrations (1–4 mg/L) (Peters et al., 2007; Melvin and Trudeau, 2012a,b). With the widespread use of NA in numerous industrial capacities, there is a need to investigate potential mechanisms of adverse effects of these contaminants on wildlife.

Overt NA toxicity has been investigated in bacteria (Jones et al., 2011), aquatic invertebrates (Frank et al., 2009), fish (Peters et al., 2007), mammals (Rogers et al., 2002), and birds (Gentes et al., 2007), but until recently there has been little information regarding NA exposure in amphibians (Melvin and Trudeau, 2012a,b). Studies with anuran larvae demonstrate that they suffer acute toxicity (i.e. mortality) with exposure to low concentrations of a commercial NA (Melvin and Trudeau, 2012a), and experience significantly delayed growth and development at sub-lethal concentrations (Melvin and Trudeau, 2012b). Amphibian larvae are extremely vulnerable and sensitive to contaminants introduced into aquatic environments, and so increased effort should be directed at understanding how potentially widespread environmental contaminants like NA affect developmental, metabolic, and physiological processes in exposed tadpoles.

Computer modeling was recently used by Scarlett et al. (2012) to show that each of 54 previously identified NA structures would be predicted to affect at least one type of human liver enzyme. Similarly, a commercial NA mixture was observed to cause metabolic inhibition *in vitro* using fish liver cells (Tollefsen et al., 2012). There is some *in vivo* evidence of metabolic disruption resulting from NA

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exposure in fish and mammals (Dokholyan and Magomedov, 1983; Rogers et al., 2002), but no information of this kind pertaining to amphibian larvae despite the fact that they experience complex, energy-intensive periods of morphological change as they grow and develop. Levels of hepatic glycogen and phospholipids may be particularly relevant biomarkers of exposure since these are important energy reserves utilized by amphibian tadpoles for the completion of metamorphosis (Farrar and Frye, 1973; Sheridan and Kao, 1998). As such, changes to these compounds may signify potential mechanisms responsible for the observation that NA-exposed tadpoles suffered significantly reduced size and decreased developmental rates in a previous study (Melvin and Trudeau, 2012b).

We describe the results of a study exposing *Lithobates pipiens* (formerly *Rana pipiens*) tadpoles throughout the developmental period to sub-lethal concentrations (1–2 mg/L) of a commercial mixture of NA. We compared the effects of NA to ethinyl-estradiol (EE2) because it is a well-studied endocrine disruptor with known effects on development in this species (Hogan et al., 2006, 2008) and because of the capacity for it to also disrupt some aspects of metabolic function (Korsgaard, 2005; Filby et al., 2007). We monitored survival, growth and development, gonad and liver sizes, and levels of hepatic glucose, glycogen and lipids following exposure, to investigate the potential for NA to influence developmental and metabolic processes at concentrations that are known to occur in natural Canadian waterways.

2. Materials and methods

2.1. Experimental animals

Eggs were obtained by artificially induced spawning of adult leopard frogs in the laboratory, according to the AMPHIPLEX method described by Trudeau et al. (2010). Fertilized eggs were reared in a 20 L aquarium until their yolk sacs were reabsorbed and they commenced free swimming. These experiments were performed at the University of Ottawa Aquatic Care Facility in accordance with protocols approved by the Ottawa Protocol Review Committee and adhere to the guidelines of the Canadian Council of Animal Care (CCAC) for research on animals.

2.2. Chemicals for waterborne exposures

Technical grade NA (Cat No. 70340, Lot No. BCBC9959V) was purchased from Sigma–Aldrich and prepared as previously reported (Melvin and Trudeau, 2012a,b). We characterized the NA by electrospray ionization paired with a high resolution Orbitrap mass spectrometer according to validated methods (Headley and Peru, 2007). As expected, mass spectral analysis confirmed that this commercial NA product is composed primarily of $C_nH_{2n+Z}O_2$ species (see Supplementary Figs. 1–3). Ethinyl-estradiol was also obtained from Sigma–Aldrich, and prepared for addition to water according to previously published procedures (Al-Ansari et al., 2011; Atkinson et al., 2011).

2.3. Experimental procedure

Tadpoles were added to experimental aquaria once all individuals had commenced free swimming and feeding, at approximately Gosner stage (Gs) 26 (Gosner, 1960). The exposure started on 2 December 2011 and continued for 75 days at ambient temperature (26 ± 1 °C) and pH (7.00 ± 0.25), at which time some of the most developmentally advanced individuals were nearing metamorphic climax (Gs 42). There were four (4) replicates each of solvent control (EtOH; final concentration 0.003%), 1 mg/L NA, 2 mg/L NA, and a 2.96×10^{-3} mg/L (10 nM) EE2 treatment group until experimental day 60, at which point one replicate from each treatment group was randomly selected and sampled to assess the level of gonadal differentiation. These nominal exposure concentrations were selected because they fall below acutely toxic doses in previous studies (Melvin and Trudeau, 2012a,b), and represent the range of NA concentrations occurring in natural Canadian surface waters (Clemente and Fedorak, 2005; Environment Canada, 2010; Headley and McMartin, 2004). Based on preliminary sampling at day 60, it was decided to continue the experiment for two additional weeks with the remaining three (3) replicates so that median developmental stages were advanced enough that individuals could be sexed visually by inspection of gonadal morphology. Replicates consisted of 20 L aquaria containing 15 tadpoles in 15 L of aerated de-chlorinated water. Tadpoles in all treatments were fed an equal mixture of rabbit chow and Wards tadpole food each day, *ad libitum*, and feces and food waste was removed twice weekly prior to performing 90% water changes and treatment renewals.

Tadpoles were measured periodically throughout the first two weeks of the experiment by removing them from their aquaria with a fine mesh dip-net, placing them onto a 1 mm^2 grid and photographing them for measurement with ImageJ image analysis software (U.S. National Institute of Health; http://rsbweb.nih.gov/ij). At the end of the exposure (day 75), tadpoles were euthanized in buffered 3-aminobenzoic acid ethyl ester (MS-222; Sigma) and measurements of snout-vent length (SVL; mm), full body length (mm), body mass (g), and developmental stage (Gosner, 1960) were taken. Following external measurements, livers and gonads were weighed (g); because of the small size and fragility of tadpole gonadal tissue in early development, the gonad–mesonephros complex (GMC) was dissected out under a dissecting microscope rather than individual ovaries and testes (e.g. Maruo et al., 2008; Duarte-Guterman and Trudeau, 2011). Average liver somatic index (LSI; [liver weight/body weight] × 100), GMC index (GMCI; [GMC/body weight] × 100), and condition factor (K; [body weight/SVL^3] × 100) were calculated for all replicates (Table 1). Animals were sexed based on visual inspection of gross gonadal morphology. After dissection and weighing, liver samples were flash frozen and stored at −80 °C for subsequent analyses.

2.4. Quantification of liver glucose and glycogen

All chemicals were purchased from Sigma–Aldrich (St Louis, MO, USA), and reaction buffers were prepared fresh daily. Livers from individuals at approximately the same developmental stage (Gs 34–36) were selected from each treatment group for analysis; three males and three females were analyzed individually from each replicate, and statistical analysis (described below) was performed on tank averages. Liver tissues were sonicated for 10 s each in 20× volumes of ice-cold $HClO_4$ (6%) to liberate glucose and glycogen. Aliquots used for glycogen analysis were neutralized with 1M $NaHCO_3$, enzymatically digested according to Hassid and Abraham (1957) using amyloglucosidase, and all neutralized samples were assayed for glucose spectrophotometrically following the protocol of Bergmeyer et al. (1983).

2.5. Quantification of liver triglyceride and cholesterol

Livers from individuals at the same developmental stage (Gs 32–34) were selected from each treatment group for analysis; three males and three females were analyzed as above. Lipids were extracted from liver tissue using a 2:1 chloroform:methanol (Folch) extraction protocol. Briefly, liver tissue was homogenized in 5 mL Folch reagent using a polytron® style homogenizer (Kinematica, Switzerland) and incubated for 20 min at room temperature; 2.5 mL 2 M KCl buffered with 5 mM EDTA was added, and the solution was vortex and allowed to incubate for an additional 10 min prior to centrifugation at 2000 g for 3 min. The lower phase was placed in a clean glass tube and the solution was evaporated under nitrogen until only the lipid residue remained. The extracted lipids were reconstituted in 0.25 mL ethylene glycol monomethyl ether (EGEM). Triglycerides and cholesterol were quantified from the tissue extracts using diagnostic kits following the manufacturer's protocol (TECO Diagnostic, Anaheim, CA).

2.6. Statistical analysis

Data were analyzed for statistical significance with SPSS version 17 statistical software (IBM Inc., Chicago, USA), with α set as 0.05. All data are reported as mean ± standard error of the mean (SEM). Prior to analysis, data were tested for assumptions of normality and homogeneity of variance, the results of which indicated that parametric analysis was appropriate. Growth throughout the first two weeks was analyzed with repeated measures ANOVA, with treatment as a fixed factor and time as a random factor. SVL, full body length, and developmental stage (Gs) at the time of sampling (day 75) were analyzed with one-way ANOVA. Differences in liver size, gonad size, and the length of animals relative to their weight (represented graphically as LSI, GMCI, and K, respectively) were analyzed separately

Table 1
Mean (±SE) condition factor (K), liver somatic index (LSI), and gonad mesonephros complex-index (GMCI) of *Lithobates pipiens* tadpoles exposed to solvent control (EtOH), 1 mg/L NA, 2 mg/L NA, and 10 nM EE2 for 75 days starting at Gosner stage 26. Different letters indicate groups that are significantly different.

Sex	Treatment	n	K	LSI (%)	GMCI (%)
Males	Control	3	1.06 ± 0.04^{ab}	2.28 ± 0.07	0.67 ± 0.02
	1 mg/L NA	3	1.19 ± 0.02^{c}	2.55 ± 0.06	0.84 ± 0.02
	2 mg/L NA	3	1.12 ± 0.01^{b}	2.56 ± 0.30	0.78 ± 0.03
	EE2 (10 nM)	3	1.05 ± 0.03^{a}	2.02 ± 0.25	1.29 ± 0.47
Females	Control	3	1.12 ± 0.06	2.01 ± 0.06^{a}	0.73 ± 0.04
	1 mg/L NA	3	1.21 ± 0.08	2.09 ± 0.15^{a}	0.78 ± 0.05
	2 mg/L NA	3	1.08 ± 0.05	2.36 ± 0.13^{b}	0.72 ± 0.03
	EE2 (10 nM)	3	1.04 ± 0.01	2.07 ± 0.06^{a}	0.84 ± 0.03