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

Steven D. Melvin [a,*], Chantal M. Lanctôt [a], Paul M. Craig [a], Thomas W. Moon [a], Kerry M. Peru [b], John V. Headley [b], Vance L. Trudeau [a]

[a] Centre for Advanced Research in Environmental Genomics, Department of Biology, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada
[b] Aquatic Ecosystem Protection Research Division, Water Science and Technology Directorate, Environment Canada, 11 Innovation Boulevard, Saskatoon, Saskatchewan S7N 3H5, Canada

ARTICLE INFO

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

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 ($26 \pm 1\ ^{\circ}C$) 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 mm² 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/SVL³] × 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 $-80\ ^{\circ}C$ 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 $\pm$ 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 ($\pm$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

by sex with ANCOVA, using JMP version 9.0.1 (SAS Institute, Inc.). All other response variables (i.e. glycogen, glucose, triglycerides, and cholesterol) were analyzed with two-way ANOVA to test for differences amongst males and females using SPSS version 17. Post-hoc comparisons were performed with Tukey's multiple comparisons test. Sex ratios were analyzed using a chi-square test.

3. Results

There were no significant differences in survival amongst treatment groups. There was a significant overall difference in sex ratios amongst treatment groups ($p = 0.018$). This reflects slightly male-skewed populations in the NA-exposed groups and female-skewed populations in EE2-exposed groups (Fig. 1). The greatest deviation from a 50:50 (M:F) ratio was in the EE2 treatment group with 61% females.

Repeated-measures ANOVA for SVL (Fig. 2a; $F(3, 36) = 74.35$, $p < 0.001$) and full body length (Fig. 2b; $F(3, 36) = 60.17$, $p < 0.001$) revealed a reduction in NA-exposed tadpoles throughout the first two weeks of the exposure. No differences were observed between controls and the EE2 group for these endpoints at this time. At the termination of the experiment (day 75), SVL was reduced in the 1 mg/L group compared to controls (Fig. 3a; $F(3, 8) = 11.67$, $p = 0.003$) but there was no difference between controls and the 2 mg/L NA group. The full body length of controls was not significantly different from either NA treatment at day 75, but individuals exposed to EE2 were significantly larger than those exposed to 1 mg/L NA (Fig. 3b; $F(3, 8) = 4.802$, $p = 0.034$). Development was significantly delayed by two Gosner stages in tadpoles at both NA concentrations (Fig. 3c; $F(3, 8) = 9.429$, $p = 0.005$), but there was no difference in development between controls or NA groups and the EE2 group.

There was no interaction between tadpole weight and any of the endpoints tested with ANCOVA ($p > 0.05$ for each), so for all cases the interaction term was removed and the analysis repeated. There was no effect of treatment on the relative size of male livers (LSI; $F(3, 7) = 3.897$, $p = 0.063$) or gonads (GSI; $F(3, 7) = 4.020$, $p = 0.059$) amongst treatment groups, but there was a significant increase in condition of male tadpoles in NA-exposed groups compared to controls ($F(3, 7) = 12.829$, $p = 0.003$). In females, there was no difference in relative gonad size (GSI; $F(3, 7) = 1.062$, $p = 0.424$) or condition ($F(3, 7) = 3.353$, $p = 0.085$), but livers were significantly larger in females exposed to 2 mg/L NA throughout development ($F(3, 7) = 5.008$, $p = 0.037$).

No differences were found between males and females in levels of hepatic glucose ($F(1, 24) = 0.26$, $p = 0.613$) or glycogen ($F(1, 24) = 0.78$, $p = 0.385$). Glucose levels were similar across all treatments (Fig. 4; $F(3, 24) = 0.32$, $p = 0.811$) but glycogen levels
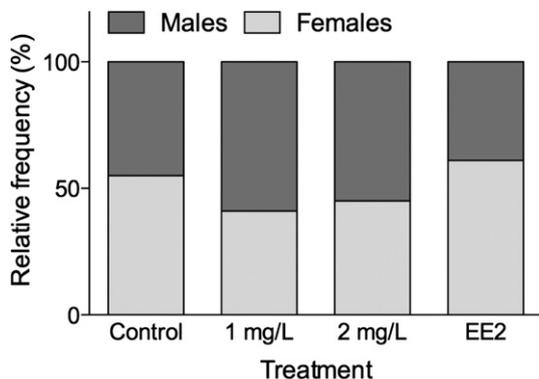

Fig. 1. Relative sex ratios (M:F) of *Lithobates pipiens* tadpoles exposed for 75 days to control (solvent), 1 mg/L NA, 2 mg/L NA, and 10 nM EE2. Chi-square analysis identified an overall difference, but this was not related to differences between any treatment group and controls.
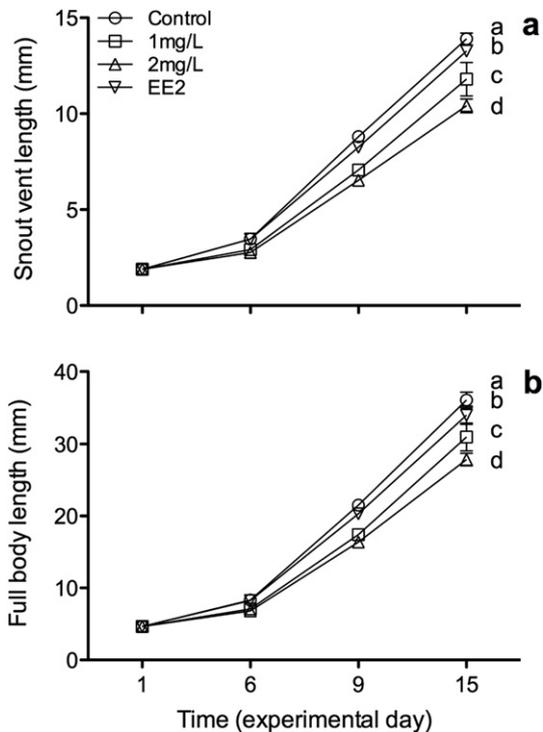

Fig. 2. Snout-vent length (a) and full body length (b) of *Lithobates pipiens* tadpoles throughout the first two weeks of exposure to control (solvent), 1 mg/L NA, 2 mg/L NA, and 10 nM EE2. The exposure started with animals at Gosner stage 26. Bars represent ±1 SE. Different letters represent groups that are significantly different from each other.

were significantly reduced by 32% and 43% in the 1 mg/L and 2 mg/L NA treatment groups (Fig. 4; $F(3, 24) = 5.11$, $p = 0.007$), respectively, compared to the controls. There was no difference in triglyceride concentrations amongst males and females ($F(1, 24) = 0.22$, $p = 0.646$), and tadpoles exposed to 2 mg/L NA and EE2 had significantly higher triglyceride content than controls (Fig. 5; $F(3, 24) = 3.46$, $p = 0.032$). Females had higher liver cholesterol concentrations than males ($F(1, 24) = 4.50$, $p = 0.044$), but there was no overall difference in cholesterol concentrations amongst treatment groups (Supplementary Fig. 4; $F(3, 24) = 0.10$, $p = 0.961$).

4. Discussion

Our results demonstrate delayed growth and development, and disruption of normal metabolic function in *L. pipiens* tadpoles exposed to sub-lethal concentrations of a commercial NA preparation. Decreased growth was observed early in the developmental period at both NA concentrations, with the greatest decrease occurring at the highest concentration. Differences were less pronounced following 75 days of exposure, which may indicate compensatory growth (Räsänen et al., 2002; Squires et al., 2010) with continued low-dose exposure. Reduced growth and delayed development is consistent with our previous observations of embryonic exposures of tadpoles (Melvin and Trudeau, 2012b), and reveals a very marked response of amphibian larvae exposed to a commercial preparation of NA at early developmental stages. That these represent conceivable real world concentrations (Clemente and Fedorak, 2005) is of concern because it indicates that even short-term exposure to NA can influence the ability of tadpoles to compete or to escape predation at critical early life stages. However, it is difficult to predict population level responses from controlled laboratory experimentation and so further research is needed to determine the actual persistence of low levels of NA in natural
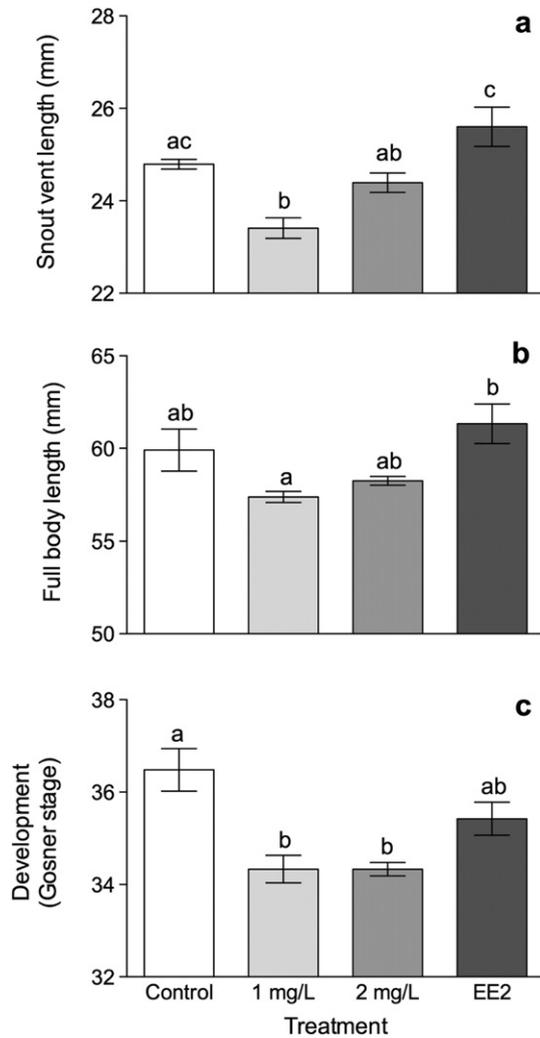
Fig. 3. Snout-vent length (a), full body length (b), and Gosner developmental stage (c) of *Lithobates pipiens* tadpoles exposed for 75 days to control (solvent), 1 mg/L NA, 2 mg/L NA, and 10 nM EE2 starting at Gosner stage 26. Bars represent ±1 SE. Different letters represent groups that are significantly different from each other.

Fig. 4. Glycogen (μmol/mg tissue) and glucose (μmol/mg tissue) levels in livers of *Lithobates pipiens* tadpoles exposed for 75 days to control (solvent), 1 mg/L NA, 2 mg/L NA, and 10 nM EE2 starting at Gosner stage 26. Individuals analyzed for these endpoints were chosen because they were at similar stages of development (Gosner stages 34–36). Bars represent ±1 SE. Different letters represent groups that are significantly different from each other.

systems, and the likelihood that natural tadpole populations would experience similar exposure scenarios in the real world. Furthermore, it is important to note that commercial preparations of NA may have different properties and behave somewhat differently than those from OSPW, thus we encourage further investigation of similar responses with other sources of NA.

At the end of the experiment, tadpoles exposed to 1 mg/L NA were significantly smaller than controls but there was no difference in the size of tadpoles exposed to 2 mg/L NA at this time, which is consistent with our observations in an earlier study (Melvin and Trudeau, 2012a). Contrary to this, and our current observation of decreased growth, we observed increased growth in tadpoles at 4 mg/L in that previous study. However, we now believe this to reflect either (i) a release from a density-induced decrease in body size, or (ii) an indication of selective mortality of smaller individuals (66% mortality of *Lithobates sylvaticus* at 4 mg/L), and not a true treatment-induced increase in size in our previous work (Melvin and Trudeau, 2012a). Neither of these explanations for increased growth would be surprising, since laboratory studies demonstrate extreme decreases in growth of tadpoles at high densities and smaller individuals are sometimes more sensitive to chemical exposure than larger individuals (Hogan et al., 2006; Mann and Bidwell, 1999; Jones et al., 2011). Moreover, in this experiment
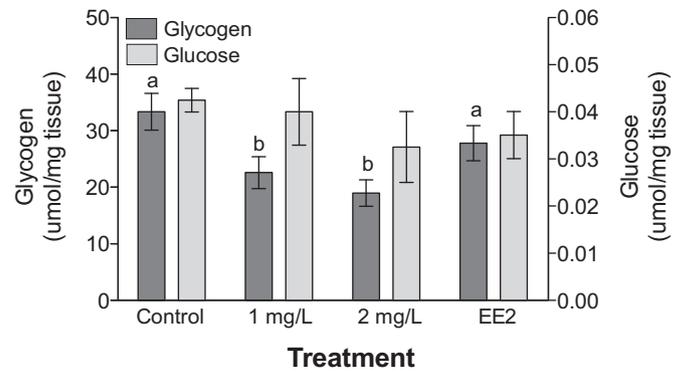
and our previous exposure of frog embryos to NA (Melvin and Trudeau, 2012b), the results indicate reduced growth and development. This seems to be the clearest response pattern in vertebrates exposed to NA (Peters et al., 2007), and to NA-containing OSPW (Pollet and Bendell-Young, 2000; Hersikorn and Smits, 2011).

Exposure to both concentrations of NA disrupted liver metabolic processes in leopard frog larvae. We observed decreased glycogen in NA-exposed individuals, but no difference amongst treatment groups in glucose content. Decreased hepatic glycogen content has been described in fish exposed to 5 mg/L NA, but lower concentrations (0.5 and 1 mg/L) resulted in increased glycogen in the same study (Dokholyan and Magomedov, 1983). In another study, Rogers et al. (2002) observed increased glycogen stores in 90-day-old rats exposed orally to prolonged high doses of NA. While it is currently difficult to meaningfully compare responses across species, concentrations, and routes of exposure, it is nevertheless clear from our results and these previous studies that relatively low concentrations of NA disrupt energy storage in the form of glycogen in livers of exposed vertebrates. For amphibian species such as *L. pipiens*, this could have deleterious implications for overwintering survival since *Ranidae* species metabolize mainly glycogen while dormant (Loumbourdis and Kyriakopoulou-Sklavounou, 1991).
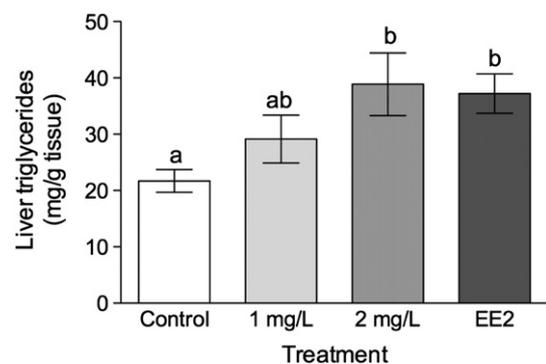

Fig. 5. Levels of triglycerides (mg/g tissue) in the livers of *Lithobates pipiens* tadpoles exposed for 75 days to control (solvent), 1 mg/L NA, 2 mg/L NA, and 10 nM EE2 starting at Gosner stage 26. Individuals analyzed for these endpoints were chosen because they were at similar stages of development (Gosner stages 32–34). Bars represent ±1 SE. Different letters represent groups that are significantly different from each other.

In addition to reduced glycogen stores in livers of NA exposed groups, we also observed increased levels of triglycerides in the NA and EE2 groups. Hepatic lipids are important primary energy sources for fueling anuran metamorphosis (reviewed in Sheridan and Kao, 1998), so increased levels of triglycerides in NA-exposed individuals may help to explain the observation of decreased development in these groups. If exposed individuals were not able to metabolize lipid stores to satisfy metamorphosis-related energy demands, then delayed metamorphosis would be expected. In addition, like glycogen, triglycerides are important fuel sources used by amphibians during estivation (dormancy). However, triglycerides are generally metabolized initially, and once depleted a dormant amphibian then switches to other energy reserves, such as glycogen and protein (Feder and Burggren, 1992). As such, smaller animals are less able to survive extreme durations of dormancy (Feder and Burggren, 1992). Since tadpoles exposed to NA were significantly smaller than controls, increased triglycerides might not present sufficient energy stores to counter-balance reduced glycogen stores, especially under extreme overwintering conditions. These scenarios ensuing from the observed metabolic disruption are speculative and require further investigation, but it is clear that exposure to NA throughout growth and development disrupts normal liver function in vertebrates (see also Dokholyan and Magomedov, 1983; Rogers et al., 2002), and therefore may pose a significant concern for individual and population fitness. Correspondingly, recent computer modeling predicted some form of liver enzyme disruption resulting from each of 54 NA forms (Scarlett et al., 2012), indicating a broader significance of these results.

One additional possibility is that NA might have endocrine disrupting activities. Exposure of adult goldfish to OSPW suppressed sex steroids and increased cortisol levels in blood plasma (Lister et al., 2008), but there is little in vivo evidence directly related to the effects of NA. There are, however, structural similarities between NA and steroidal compounds (Rowland et al., 2011), and some petrogenic NA are weak agonists of the human estrogen receptor and antagonists of the human androgen receptor in vitro (Thomas et al., 2009). In the present study, we observed little congruity between EE2 exposed groups and those exposed to NA, which suggests that the commercial mixture of NA used contains little estrogenicity. However, NA species in oil sands are comprised of a much more diverse range of components than the commercial blend tested here, including those found to have estrogenic properties (Rowland et al., 2011). On the other hand, Hersikorn and Smits (2011) reported that metamorphosis was delayed or never occurred in tadpoles raised in young reclaimed oil sands wetlands. They suggested that thyroid hormone (TH) disruption likely played a role in the metamorphic delay because the ratio of triiodothyronine (active TH) to thyroxine was lowest in tadpoles raised in young, tailings-affected wetlands. Delayed development, as indicated by the lower Gosner stages in tadpoles exposed to both levels of NA, is also suggestive of thyroid disruption. Given the diversity of NA chemical structures, it is therefore conceivable that a few may interfere with some aspects of thyroid physiology (e.g. synthesis or transport of TH, or interaction with TH receptor subtypes).

To conclude, the current study demonstrates that exposure of tadpoles to NA negatively influences their growth, development, and metabolic function. We have portrayed scenarios where these responses could translate to effects on population fitness, but further research is encouraged to investigate the potential for NA exposure to affect amphibian populations in natural systems. Regardless, developing amphibians represent informative model organisms and the responses observed in this study raise the possibility of similar metabolic and developmental upsets as a consequence of exposure to NA in other vertebrates. Considering the abundance of NA species in OSPW, their widespread use in commercial and industrial capacities, and the observed effects on developmental and metabolic processes in this study, we strongly encourage further research investigating this group of compounds.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2013.02.003.

References

Al-Ansari, A.M., Saleem, A., Kimpe, L.E., Trudeau, V.L., Blais, J.M., 2011. The development of an optimized sample preparation for trace level detection of 17α-ethinylestradiol and estrone in whole fish tissue. Journal of Chromatography B. Analytical Technologies in the Biomedical and Life Sciences 879, 3649–3652.

Atkinson, S.K., Marlatt, V.L., Kimpe, L.E., Lean, D.R., Trudeau, V.L., Blais, J.M., 2011. Environmental factors affecting ultraviolet photodegradation rates and estrogenicity of estrone and ethinylestradiol in natural waters. Archives of Environmental Contamination and Toxicology 60, 1–7.

Bergmeyer, H.U., Bergmeyer, J., Grassl, M., 1983. Methods of Enzymatic Analysis, third ed. Academic Press, New York.

Brient, J.A., Wessner, P.J., Doyle, M.N., 1995. Naphthenic acids. In: Kroschwitz, J.I. (Ed.), Kirk-Othmer Encyclopedia of Chemical Technology. John Wiley, New York, pp. 1017–1029.

Clemente, J.S., Fedorak, P.M., 2005. A review of the occurrence, analyses, toxicity, and biodegradation of Naphthenic acids. Chemosphere 60, 585–600.

Dokholyan, B.K., Magomedov, A.K., 1983. Effects of sodium naphthenate on survival and some physiological–biochemical parameters of some fishes. Journal of Ichthyology 23, 125–132.

Dorn, P.B., 1992. Case histories – the petroleum industry. In: Ford, D.L. (Ed.), Toxicity Reduction: Evaluation and Control. Water Quality Management Library, vol. 3. Technomic Publishing Company, Lancaster, PA, pp. 183–223.

Duarte-Guterman, P., Trudeau, V.L., 2011. Transcript profiles and triiodothyronine regulation of sex steroid- and thyroid hormone-related genes in the gonad-mesonephros complex of Silurana tropicalis. Molecular and Cellular Endocrinology 331, 143–149.

Environment Canada, 2010. Proposal by Environmental Defence to Add Naphthenic Acids to the NPRI (accessed 06.02.12.). http://www.ec.gc.ca/inrp-npri/default. asp?lang=en&n=AC708134-1.

Farrar, E.S., Frye, B.E., 1973. Comparison of blood glucose and liver glycogen of larval and adult frogs (Rana pipiens). General and Comparative Endocrinology 21, 513–516.

Feder, M.E., Burggren, W.W., 1992. Environmental Physiology of the Amphibia. University of Chicago Press, Chicago.

Filby, A.L., Neuparth, T., Thorpe, K.L., Owen, R., Galloway, T.S., Tyler, C.R., 2007. Health impacts of estrogens in the environment, considering complex mixture effects. Environmental Health Perspectives 115, 1704–1710.

Frank, R.A., Fischer, K., Kavanagh, R., Burnison, B.K., Arsenault, G., Headley, J.V., Peru, K.M., Van Der Kraak, G., Solomon, K.R., 2009. Effect of carboxylic acid content on the acute toxicity of oil sands naphthenic acids. Environmental Science and Technology 43, 266–271.

Gentes, M.-L., Waldner, C., Papp, Z., Smits, J.E.G., 2007. Effects of exposure to naphthenic acids in tree swallows (Tachycineta bicolor) on the Athabasca oil sands, Alberta Canada. Journal of Toxicology and Environmental Health, Part A 70, 1182–1190.

Gosner, K.L., 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16, 183–190.

Hassid, W.Z., Abraham, S., 1957. Chemical procedures for analysis of polysaccharides. In: Colowick, S.P., Kaplan, N.O. (Eds.), Methods Enzymology. Academic Press, New York, pp. 34–37.

Headley, J.V., McMartin, D.W., 2004. A review of the occurrence and fate of naphthenic acids in aquatic environments. Journal of Environmental Science and Health, Part A 39, 1989–2010.

Headley, J.V., Peru, K.M., 2007. Characterization of naphthenic acids from Athabasca oil sands using electrospray ionization: the significant influence of solvents. Analytical Chemistry 79, 6222–6229.

Hersikorn, B.D., Smits, J.E.G., 2011. Compromised metamorphosis and thyroid hormone changes in wood frogs (Lithobates sylvaticus) raised on reclaimed wetlands on the Athabasca oil sands. Environmental Pollution 159, 596–601.

Hogan, N.S., Lean, D.R.S., Trudeau, V.L., 2006. Exposures to estradiol, ethinylestradiol and octylphenol affect survival and growth of Rana pipiens and Rana sylvatica tadpoles. Journal of Toxicology and Environmental Health, Part A 69, 1555–1569.

Hogan, N.S., Duarte, P., Wade, M.G., Lean, D.R., Trudeau, V.L., 2008. Estrogenic exposure affects metamorphosis and alters sex ratios in the northern leopard frog (Rana pipiens): identifying critically vulnerable periods of development. General and Comparative Endocrinology 156, 515–523.

Jones, D., Scarlett, A.G., West, C.E., Rowland, S.J., 2011. Toxicity of individual naphthenic acids to Vibrio fischeri. Environmental Science and Technology 45, 9776–9782.

Korsgaard, B., 2005. Metabolic changes associated with 17alpha-ethinylestradiol-exposure in the pregnant teleost Zoarces viviparous (L). Electronic Journal of Ichthyology 1, 10–20.

Lister, A., Nero, V., Farwell, A., Dixon, D.G., Van Der Kraak, G., 2008. Reproductive and stress hormone levels in goldfish (Carassius auratus) exposed to oil sands process-affected water. Aquatic Toxicology 87, 170–177.

Loumbourdis, N.S., Kyriakopoulou-Sklavounou, P., 1991. Reproductive and lipid cycles in the male frog Rana ridibunda in Northern Greece. Comparative Biochemistry and Physiology, Part A 99, 577–583.

Madill, R.E.A., Orzechowski, M., Chen, G., Brownlee, B.G., Bunce, N.J., 2001. Preliminary risk assessment of the wet landscape option for reclamation of oil sands mine tailings: bioassays with mature fine tailings pore water. Environmental Toxicology 16, 197–208.

Mann, R.M., Bidwell, J.R., 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Archives of Environmental Contamination and Toxicology 36, 193–199.

Maruo, K., Suda, M., Yokoyama, S., Oshima, Y., Nakamura, M., 2008. Steroidogenic gene expression during sex determination in the frog Rana rugosa. General and Comparative Endocrinology 158, 87–94.

Melvin, S.D., Trudeau, V.L., 2012a. Toxicity of naphthenic acids to wood frog tadpoles (Lithobates sylvaticus). Journal of Toxicology and Environmental Health, Part A 75, 1–4.

Melvin, S.D., Trudeau, V.L., 2012b. Growth, development and incidence of deformities in amphibian larvae exposed as embryos to naphthenic acid concentrations detected in the Canadian oil sands region. Environmental Pollution 167, 178–183.

Peters, L.E., MacKinnon, M., Van Meer, T., van den Heuvel, M.R., Dixon, D.G., 2007. Effects of oil sands process-affected waters and naphthenic acids on yellow perch (Perca flavescens) and Japanese medaka (Orizias latipes) embryonic development. Chemosphere 67, 2177–2183.

Pollet, I., Bendell-Young, L.I., 2000. Amphibians as indicators of wetland quality in wetlands formed from oil sands effluent. Environmental Toxicology and Chemistry 19, 2589–2597.

Räsänen, K., Laurila, A., Merilä, J., 2002. Carry-over effects of embryonic acid conditions on development and growth of Rana temporaria tadpoles. Freshwater Biology 47, 19–30.

Rogers, V.V., Wickstrom, M., Liber, K., MacKinnon, M.D., 2002. Acute and subchronic Mammalian toxicity of naphthenic acids from Oil Sands tailings. Toxicological Sciences 66, 347–355.

Rowland, S.J., West, C.E., Jones, D., Scarlett, A.G., Frank, R.A., Hewitt, L.M., 2011. Steroidal aromatic 'naphthenic acids' in oil sands process-affected water: structural comparisons with environmental estrogens. Environmental Science and Technology 45, 9806–9815.

Scarlett, A.G., West, C.E., Jones, D., Galloway, T.S., Rowland, S.J., 2012. Predicted toxicity of naphthenic acids present in oil sands process-affected waters to a range of environmental and human endpoints. Science of the Total Environment 425, 119–127.

Sheridan, M.A., Kao, Y.-H., 1998. Regulation of metamorphosis-associated changes in the lipid metabolism of selected vertebrates. American Zoologist 38, 350–368.

Squires, Z.E., Bailey, P.C.E., Reina, R.D., Wong, B.B.B.M., 2010. Compensatory growth in tadpoles after transient salinity stress. Marine and Freshwater Research 61, 219–222.

Tollefsen, K.E., Petersen, K., Rowland, S.J., 2012. Toxicity of synthetic naphthenic acid and mixtures of these to fish liver cells. Environmental Science and Technology 46, 5143–5150.

Thomas, K.V., Langford, K., Petersen, K., Smith, A.J., Tollefsen, K.E., 2009. Effect-directed identification of naphthenic acids as important in vitro xeno-estrogens and anti-androgens in North Sea offshore produced water discharges. Environmental Science and Technology 43, 8066–8071.

Trudeau, V.L., Somoza, G.M., Natale, G.S., Pauli, B., Wignall, J., Jackman, P., Doe, K., Schueler, F.W., 2010. Hormonal induction of spawning in 4 species of frogs by coinjection with a gonadotropin-releasing hormone agonist and a dopamine antagonist. Reproductive Biology and Endocrinology 8, 36.