2015-05-01

Toxicology of Model Naphthenic Acids in the Great Pond Snail Lymnaea stagnalis

Johnston, Christina

UNIVERSITY OF CALGARY

Toxicology of Model Naphthenic Acids in the Great Pond Snail *Lymnaea stagnalis*

by

Christina Ursula Johnston

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOLOGICAL SCIENCES

CALGARY, ALBERTA

APRIL, 2015

Abstract

Naphthenic acids (NAs), carboxylic acids with large aliphatic tail groups, are an important class of oil sands tailings pond toxins. In this study, the effects of a mixture of model NAs on the pond snail *Lymnaea stagnalis* were examined during various life stages. Hatching rate was progressively affected over a range of 20-75 mg/L NAs, while embryo somatic growth was progressively affected over a range of 30-75 mg/L NAs. My data also provides evidence that NA molecular geometry is a factor in selective permeability of NAs into *Lymnaea*'s gelatinous egg masses. Adult survival, growth, feeding, fecundity and behavior were progressively affected over a range of 10-50 mg/L NAs, but not at 0.1-4 mg/L NAs. Taken together, my data support the conclusion that low molecular weight NAs have a negative impact on various physiological and behavioral aspects of this freshwater gastropod that escalates over a range of environmentally realistic doses.

Acknowledgements

I would like to thank my supervisor, Dr. Wic Wildering, and my co-supervisor, Dr. Petra Hermann, for introducing me to the field of environmental toxicology, and for their guidance and support throughout this thesis project.

I am also grateful to my supervisory committee, Dr. Gordon Chua and Dr. Jeff Goldberg, for their insight and advice as my thesis project progressed, and to Dr. Judit Smits and Dr. Ray Turner for their participation in my thesis exam.

I would like to thank the members of the Wildering lab and the Biological Sciences department for their assistance. I appreciate the assistance of the Gieg lab for gas chromatography-mass spectrometry analysis, and of the Chua lab for obtaining and extracting tailings-derived naphthenic acids.

I gratefully acknowledge the funding support I received from the Natural Sciences and Engineering Research Council, Alberta Innovates Technology Futures, and the University of Calgary, as well as material support from Canada's Oil Sands Innovation Alliance (COSIA, formerly CONRAD).

Finally, I would like to thank my family for instilling in me a love of science and education, and for providing support of all kinds during my academic pursuits.

Table of Contents

List of Tables

List of Figures and Illustrations

List of Symbols, Abbreviations and Nomenclature

4-TBCA	4-*tert*-butylcyclohexane Carboxylic Acid
APW	Artificial Pond Water
CDCH	Caudodorsal Cell Hormone
CHBA	Cyclohexanebutyric Acid
CHSA	Cyclohexylsuccinic Acid
CNA	Commercial Naphthenic Acids
DMSO	Dimethyl Sulphoxide
ESRD	Alberta Environment and Sustainable Resources Development
GABA	γ-Aminobutyric Acid
GC-MS	Gas Chromatography-Mass Sectrometry
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
IC50	Inhibitory Concentration 50%
IUPAC	International Union of Pure and Applied Chemistry
LC50	Lethal Concentration 50%
MNA	Model Naphthenic Acid
NA	Naphthenic Acid
NAPW	Naphthenic Acid Pond Water
OSPW	Oil Sands Process-Affected Water
PAH	Polycyclic Aromatic Hydrocarbon
TNA	Tailings-derived Naphthenic Acids
UNEP	United Nations Environment Programme
USEPA	United States Environmental Protection Agency
USNRC	United States National Research Council

Chapter One: **Introduction**

1.1 Anthropogenic Alterations of Aquatic Ecosystems

Water resources are most often examined and understood from the perspective of the needs of human populations. Historical studies of water are almost exclusively focused on water supplies for cities and agriculture, and how these supplies were managed (e.g., Winiwarter *et al*., 2013; Bohman, 2012; Wilson, 2012). Increasingly, however, aquatic ecosystems have been entering public discourse on water resources, placing human water needs in the context of the environment from which that water comes (e.g., UNEP, 2012). Much less attention has been devoted to the effects of human activities on the health of aquatic ecosystems when they do not impact human consumption of that water.

Despite this relative lack of attention in public discourse, there is evidence that human activities have been altering aquatic environments since ancient times. For example, the land surrounding the Rio Tinto in south-western Spain has been mined for a number of metals, including iron, copper, lead, gold, and silver, since about 3000 B.C.E. (Davis *et al*., 2000). The water in this river system is highly acidified, and sediments contain high concentrations of heavy metals dating to Roman times, and likely earlier (Davis *et al*., 2000). Although the only organisms inhabiting the river today are fungi, bacteria, and algae, sediment cores contain many shells of clams and oysters at depths also containing high copper and zinc concentrations, suggesting that these mollusks were able to survive and reproduce in this highly polluted water (Davis *et al*., 2000). We may not know what the ecosystem looked like in this river before mining activity started, or why the mollusk population disappeared after surviving for a time

despite the metal contamination. But by studying aquatic organisms in contaminated locations, we can gain insight into how best to preserve aquatic ecosystems in a changing environment.

Aquatic ecosystems often bear the brunt of contamination on land due to surface water runoff. Indeed, the U.S. National Research Council has identified surface runoff as a major, and growing, source of water pollution affecting both limnic ecosystems and human drinking water (USNRC, 2008). One such phenomenon, which is occurring worldwide, is the advent of coastal dead zones due in large part to fertilizer runoff from agriculture (Vitousek *et al*., 1997). These dead zones have appeared in the Gulf of Mexico, as well as the Baltic, Black, and East China seas (Diaz & Rosenberg, 2008). As the human population increases, contamination of surface runoff from both known and as-yet unknown sources is likely to increase as well (USNRC, 2008). Thus, effective and efficient ways of monitoring the health of aquatic ecosystems will become ever more important as well.

This thesis on the toxicology of naphthenic acids, a natural component of oil deposits and a common by-product of the petrochemical resources industry, in the freshwater snail *Lymnaea stagnalis*, describes my efforts to advance the science in this field.

1.2 Ecosystems in Contaminated Sites

Organisms living in contaminated sites must be able to withstand exposure to their local toxicants throughout their life cycles, through multiple generations. Determining conditions in which this can occur is a challenge due to the time and resource investment that such studies require. In the following section, I will discuss the ways in which aquatic toxicity studies are used to establish water quality guidelines, especially with respect to species diversity, exposure durations, measured endpoints, and the selective use of available data.

Water quality regulations usually take sub-lethal effects such as growth and reproductive deficits into account (e.g., ESRD, 2014; USEPA, 2013). However, toxicity studies considered for United States Environmental Protection Agency (USEPA) guidelines must meet very specific criteria. For example, acute exposure studies considered for ammonia had exposure durations ranging from 6 hours to 4 days (USEPA, 2013). However, other acute lethality studies, even several with the same exposure durations that were included in the guidelines, were excluded from the analysis for having durations considered non-standard (e.g., 24 hours, 48 hours, 96 hours, 7 days) (USEPA, 2013).

Although acute exposure studies nominally include sub-lethal endpoints, in practice, studies with acute exposure durations measuring sub-lethal endpoints are considered chronic exposure (e.g., a 4-day exposure of juvenile brook trout measuring swimming success) (USEPA, 2013). Exposures as short as 7 days are considered chronic, even for animals that live several years (USEPA, 2013). Most of the chronic exposure studies used for the ammonia guidelines had exposure durations of about 30 days, while one particularly long exposure study followed rainbow trout for 5 years (USEPA, 2013). Chronic exposure studies are required to cover specific life stages, and can be disregarded if, for example, juvenile exposure does not begin immediately after hatching (USEPA, 2013).

Although the USEPA guidelines only apply to the United States, they have a much wider influence. For example, when Alberta first developed water quality guidelines in the 1990s, they were modeled exclusively on USEPA methods (ESRD, 2014). Subsequently, Canadian environmental regulators, including the Canadian Council of Ministers of the Environment and Alberta Environment & Sustainable Resources Development, have updated their water guidelines following USEPA updates (ESRD, 2014).

While the exclusion of relevant toxicity data is concerning, the types of studies used by the USEPA can provide a reasonable picture of full life cycle toxicity. Life cycle studies conducted for water quality assessment usually start with newly hatched animals, and exposure lasts through their maturation until they produce offspring, then the early-stage health of their offspring is also assessed (Munley *et al*., 2013).

Multi-generation studies such as these provide insight into the health of populations over time that single-generation studies cannot. For example, in three *Daphnia* species exposed to nanosilver over five generations, sensitivity to the toxicant, as measured by survival and offspring production, increased over successive generations (Volker *et al*., 2013). After three generations of exposure, two successive generations raised in fresh water did not recover from these reproductive deficits (Volker *et al*., 2013). Similarly, the pond snail *Pseudosuccinea columella* exposed to the herbicide glyphosate showed no detrimental effects on survival, growth, or reproduction for two successive generations (Tate *et al*., 1997). The third generation, however, had reduced growth, increased mortality, and reproductive impairments (Tate *et al*., 1997).

It is also possible for populations to become less sensitive to toxicants during multi-generation exposures. For example, two species of midges were exposed to various heavy metals at a concentration of 30% of their initial 96-hour LC50 in that metal for six generations (Vedamanikam & Shazilli, 2008). LC50 concentrations increased over the successive generations (Vedamanikam & Shazilli, 2008). After the sixth generation, midges were raised in fresh water for two more generations (Vedamanikam & Shazilli, 2008). The 9th generation had lost some, but not all, of the tolerance built up by their ancestors (Vedamanikam & Shazilli, 2008). Although these multi-generation studies can be resource-intensive, and are often not

practical, as with species that take several years to reach sexual maturity, they provide valuable information that is not otherwise available.

When considering the health of ecosystems in contaminated environments, it is important to consider all trophic levels present in the ecosystem. Water quality guidelines tend to focus on animals, and often fail to consider ecological interactions between species. For example, USEPA water quality assessments for particular toxicants require acute exposure tests on eight animal species: two fish species, one vertebrate species which could also be a fish, two crustacean species, one insect species, one invertebrate that is not an arthropod, and one other invertebrate (USEPA, 2013). This range of animals is fairly extensive and would likely cover all relevant trophic levels from primary consumers to tertiary consumers, although only three animal species (one fish, one invertebrate, and one acutely sensitive species) are required for chronic toxicity testing (USEPA, 2013). These same guidelines, however, only require testing of one primary producer, which may be either an alga or a vascular plant (USEPA, 2013). A second plant species must be tested if plants are particularly sensitive to the toxicant in question (USEPA, 2013). These guidelines clearly indicate an effort to assess whole ecosystems. However, considering the geographic and ecological diversity of the areas governed by these guidelines, the relatively small number of species tested, especially primary producers, means that there may be ecosystems whose inhabitants are not included in assessments of relevant toxicants.

The discrepancy in USEPA species testing requirements between acute and chronic exposure raises the issue of appropriate exposure durations. Organisms can survive toxicant exposure for durations considered chronic without apparent detrimental effects, only to show those detrimental effects when longer exposure periods are observed. For example, adult *Lymnaea stagnalis* exposed to cadmium showed no lethal effects after 14 days of exposure, long

enough to be considered chronic exposure under USEPA guidelines (Gomot, 1998). However, during the third to seventh week of exposure, mortality increased considerably (Gomot, 1998).

Although it can be appealing to use relatively short exposure durations when testing chronic toxicity, the organism's lifespan and life cycle should be taken into account. It is possible to reduce the resource input necessary for such life cycle toxicity assessments by identifying life stages and endpoints that approximate whole-life toxicity. In the cadmium experiment examining *Lymnaea*, for example, the author was able to identify embryonic development as the most sensitive life stage (Gomot, 1998). Similarly, Munley *et al*. (2013) identified growth in the first 28 days after hatching as a reasonable approximation of whole-life toxicity of lead in *Lymnaea*. Similar early life-stage tests have been shown to represent whole-life toxicity in many fish species (McKim, 1977). For example, growth during the first 3 months after hatching was found to be a reasonable representation of whole-life toxicity of copper to brook trout (*Salvelinus fontinalis*) (McKim & Benoit, 1974). Thus, even though these assessments require a whole life toxicity test to be conducted at least once, assessments of equivalent sensitivity can be conducted much faster once the most sensitive endpoints and life stages have been identified.

1.3 Mollusks as Environmental Indicators

I performed my thesis research using the gastropod mollusk *Lymnaea stagnalis* as the model system. Mollusks are a very diverse phylum, including cephalopods, bivalves, and gastropods. Most of these animals live in marine environments, but freshwater and terrestrial species are also common. Mollusks engage in many different feeding strategies, including herbivory, carnivory, detritivory, and parasitism (Graham, 1955; Doi *et al*., 2010). Mollusks,

therefore, can be primary, secondary, or tertiary consumers. Mollusks are generally not apex predators. Even large marine cephalopods like the colossal squid are prey for whales and other marine animals (Evans & Hindell, 2004). Thus, in ecosystems that include mollusks, they play a central yet diverse role in energy transfer from primary producers to other consumers.

Snails can make up 20 to 60% of total macroinvertebrates in some freshwater ecosystems (Habdija *et al*., 1995). They are able to regulate populations of primary producers such as filamentous green algae (Hunter, 1980). Freshwater mollusks are preyed upon by invertebrates including leeches (Kelly & Cory, 1987), crayfish (Webber & Lodge, 1990; Vaentine-Darby *et al*., 2015), and flatworms (Tripet & Perrin, 1994), as well as vertibrates including fish (Graber *et al*., 1981; Vaentine-Darby *et al*., 2015), reptiles (Vaentine-Darby *et al*., 2015) and birds (Hamilton *et al*., 1994; de Szalay *et al*., 2003). They can be hosts to a number of parasites, including trematodes that are capable of infecting humans (Cort *et al*., 1945; Hofkin *et al*., 1991; Adema & Loker, 2015). Given these complex interactions, freshwater snails play a very important role in regulation and energy transfer within limnic ecosystems, as well as presenting a potential risk to human health.

Mollusks are particularly sensitive to human activities, representing 42% of all documented animal extinctions since the year 1500 (Lydeard *et al*., 2004). Gastropods and bivalves are among the most sensitive species to a number of toxicants, including ammonia (USEPA, 2013), lead (Munley *et al*., 2013), copper (USEPA, 2007), and tributyltin (USEPA, 2003). Adult bivalves are particularly well suited to surveys of the geographical distribution of toxicants due to their sessile lifestyles (Rittschof & McClellan-Green, 2005). Indeed, the US National Oceanic and Atmospheric Administration established a monitoring program in 1986 called Mussel Watch to monitor anthropogenic alterations of coastal habitats (O'Connor, 2002).

Mollusks have received a fair amount of attention in toxicology studies, and are sometimes specifically included in water quality guidelines, especially in the European Union (e.g. Segner *et al*., 2003; Janer & Porte, 2007). USEPA water quality assessment endpoints can include a mollusk species among the animals assessed for acute toxicity, but this spot can be filled by any non-arthropod invertebrate (USEPA, 2013).

1.4 Model Organism: *Lymnaea stagnalis*

In this project, I have studied *Lymnaea stagnalis*, a pond snail native to vegetated bodies of fresh water in much of the northern hemisphere. *Lymnaea* are among the most common freshwater mollusks in Europe (Czech *et al*., 2001), and the largest freshwater gastropod native to Alberta (Prescott & Curteanu, 2004; Clarke, 1981). Photographs of an adult and a mature embryo of *Lymnaea stagnalis* can be seen in Figure 1.1.

These snails are both scavengers and primary consumers, making them an important link for energy transfer through freshwater ecosystems. *Lymnaea* consume a much wider variety of foods than other pond snail species, with the *Lymnaea* diet including plants, algae, detritus, insects and amphibian eggs (Doi *et al*., 2010).

Although authoritative information on *Lymnaea*'s life span can be difficult to find, they have been observed to live 2 to 2.5 years both in the wild and in captivity (Doi *et al*., 2010; Watson *et al*., 2014). They can reach sexual maturity at 3 to 4 months (Nowland & Carriker, 1946). They are simultaneous hermaphrodites (Baker, 1905), and can reproduce as male or female for most of their adult lives (Nowland & Carriker, 1946). They are also able to self-fertilize (Cain, 1956). They have a single gonad that produces both eggs and sperm, called the ovotestis (Baker, 1905). Adults produce egg masses, containing 10 to over 100 embryos, every 3

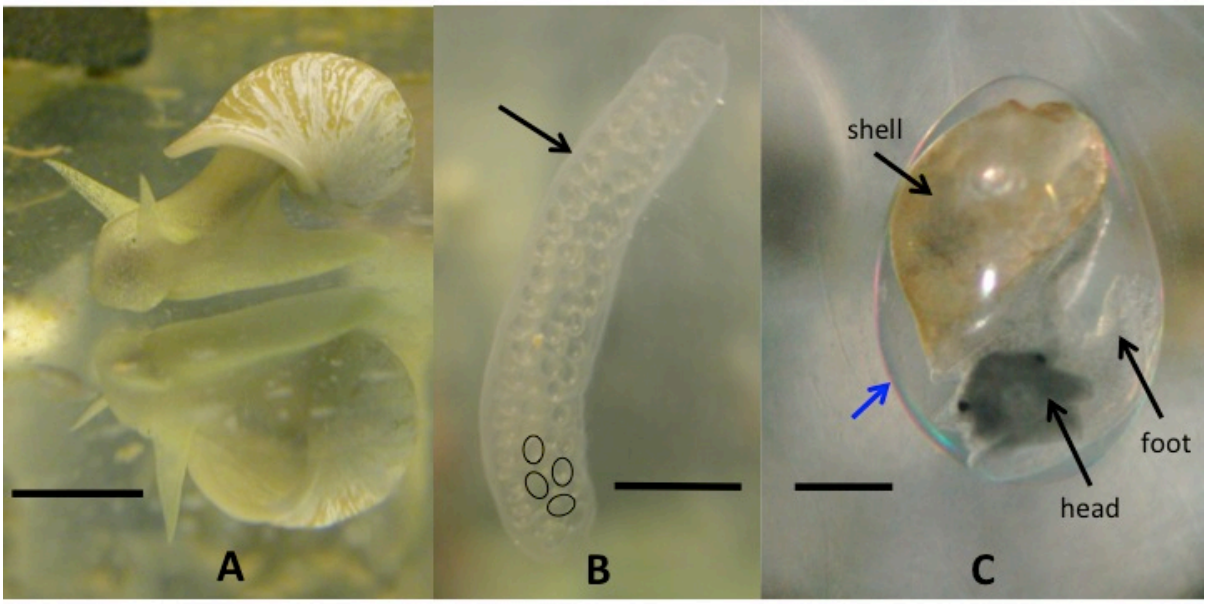

Figure 1.1 ***Lymnaea stagnalis***

A: Adult. Scale bar indicates 1 cm. B: Egg mass on the glass wall of an aquarium. It contains 85 eggs, which are in an early stage of development (1-3 days). Black ovals represent the size of eggs in the egg mass. The black arrow indicates the outer edge of the egg mass casing. Scale bar indicates 1 cm. C: Mature embryo in its egg. The embryo is 20 days old. The blue arrow indicates the outer edge of the egg. The shell, head, and foot of the embryo are marked. Scale bar represents 0.5 mm.

to 10 days (van Duivenboden *et al*., 1985; Gomot, 1998; van der Schalie & Berry, 1973; Nowland & Carriker, 1946). Eggs hatch approximately 2 weeks after laying, although the maturation rate of embryos is strongly temperature-dependent (Gomot, 1998; Morrill, 1982.).

As pulmonates, *Lymnaea* can perform gas exchange across their skin as well as with their lung (Nowland & Carriker, 1946; Jones, 1961). Because of their ability to breathe air with their lung, they are able to survive outside the water for short periods of time. This allows researchers to perform activities that need the snails' shells to be dry, such as labeling and weighing. Wild *Lymnaea* populations in France (Coutellec & Lagadic, 2006), Russia (Karanova & Gakhova, 2007), the Netherlands (Orr *et al*., 2009), the United Kingdom (Dalesman *et al*., 2006) and Canada (Orr *et al*., 2009) have been used directly in experiments, or to start laboratory-reared populations. *Lymnaea* have been used in toxicology studies since at least 1931, when embryonic development and hatching was examined during thiol exposure (Gaunt, 1931).

The control of many physiological systems is highly conserved between mollusks and other orders of animals (Rittschof & McClellan-Green, 2005). For example, γ-aminobutyric acid (GABA) receptors in invertebrates have very similar structures to those in vertebrates, differing only slightly in their pharmacological properties (Varro *et al*., 2009). The nuclear estrogen receptors found in mollusks and vertebrates are believed to be homologous, but the effects of this hormone are not necessarily conserved between the two groups of animals (Janer & Porte, 2007). *Lymnaea* also produce molluscan insulin-like peptides, part of the insulin superfamily, although their function and regulation is still being elucidated (Smit *et al*., 1988; Murakami *et al*., 2013).

Lymnaea stagnalis has been used as a model species for many different disciplines, including neuroscience (e.g., Rosza & Nagy, 1967; Benjamin & Rose, 1980; Staras *et al*., 2000; Vesprini *et al*., 2015), parasitology (e.g., Duerr, 1967; Dejongbrink *et al*., 1991; Hofkin *et al*.,

1991; Adema & Loker, 2015), embryology (e.g., Beadle, 1969; Elbers, 1969; Voronezhskaya *et al*., 1999; Kuroda, 2014), biochemistry and endocrinology (e.g., Dogterom & Robles, 1980; Geraerts *et al*., 1985; Koene, 2010). Because of this long history of attention from a wide range of perspectives, there is a wealth of information available on their anatomy, physiology, behavior and life cycle. The extent of information available on *Lymnaea*'s physiology and biochemistry could assist in determining mechanisms of toxicity.

1.5 Naphthenic Acids

This thesis concerns itself with naphthenic acids (NAs), a class of carboxylic acids with aliphatic tails. NAs are generally recognized as the main toxic component of oil sands tailings ponds (Scott *et al*., 2008; Clemente *et al*., 2004; Madill *et al*., 2001). They are classified by their carbon number and their hydrogen deficiency, the latter being a result of ring formation (Yen *et al*., 2004). Examples of generic NA structures are shown in Figure 1.2.

NAs are produced by incomplete catagenesis (the process by which organic matter becomes petroleum), or biodegradation of mature petroleum (Tissot & Welte, 1978). The NAs that occur in the Athabasca oil sands deposit were generated by the latter process (Tissot & Welte, 1978). Every petroleum deposit has a unique set of NAs, which can be represented in a 3-dimentional plot of carbon number (n) vs. hydrogen deficiency (z) vs. abundance (Yen *et al*., 2004). Each combination of n and z (ie. each column in a composition plot) also has a number of possible isomers (Yen *et al*., 2004). Thus, naturally-occurring NAs are always found in complex mixtures (Yen *et al*., 2004).

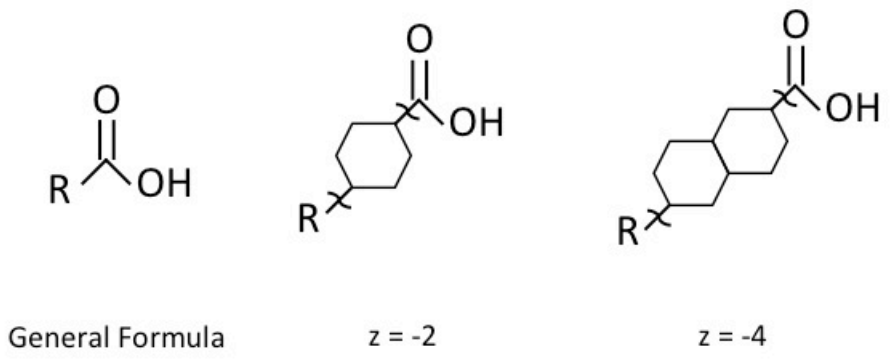

Figure 1.2 Naphthenic Acid Structures

Naphthenic acids (NAs) are carboxylic acids with aliphatic tail groups. Their general chemical formula is $C_nH_{2n+z}O_2$. They are characterized by their carbon number (n) and their hydrogen deficiency (z). The hydrogen deficiency is associated with ring formation, where an acyclic NA molecule would have a z of 0, an NA molecule with one ring would have a z of -2, a bicyclic NA molecule would have a z of -4, and so on.

1.5.1 Naphthenic Acid Sources and Uses

NAs are found in almost all crude oils at concentrations up to 3% w/w (Brient *et al*., 2000). NAs in petroleum cause corrosion in processing equipment, thus they must be removed before upgrading or refining (Zeinalov *et al*., 2009; Brient *et al*., 2000). Naturally-occurring NAs removed from crude petroleum are not commercially useful, since they are mixed with asphalts and pitch (Brient *et al*., 2000). Manufacture of commercial NAs is performed by reacting petroleum distillates with sodium hydroxide in water at 200 to 370°C (Brient *et al*., 2000). In 2000, nearly 13 000 metric tons of NAs were produced annually in the United States (Brient *et al*., 2000). Most commercial NAs are converted into metal naphthenates, mostly copper naphthenate (Brient *et al*., 2000). About 40% of commercial NAs are used for wood preservation, while other uses include paint driers, lubricants, and additives in concrete, plastics, and cosmetics (Nora *et al*., 2001; Brient *et al*., 2000).

1.5.2 Environmental Occurrence of Naphthenic Acids

Due to their extensive commercial uses, NAs are likely entering the environment by many routes. As wood preservatives, NAs are considered environmentally friendly replacements for creosote, pentachlorophenol, and chromated copper–arsenic (Brient *et al*., 2000). Since preserved wood is often exposed to rain, it is reasonable to expect that some of these NAs would be washed away by runoff into surface waters (USNRC, 2008). Nearly all of the attention on the environmental occurrence of NAs, however, has been focused on the Athabasca oil sands deposit in north-eastern Alberta, and to a much lesser extent on undersea oil production (Thomas *et al*., 2009). NAs occur in bitumen deposits, and are able to enter surface waters by erosion, as well as by human industrial activities (Kelly *et al*., 2009). The Athabasca River contains 0.1 to 0.9 mg/L

NAs (Schramm *et al*., 2000), while ground waters in the region have been found to contain 0.4 to 51 mg/L NAs (Clemente & Fedorak, 2005). They are also extracted from bitumen during processing and stored in tailings ponds (Giesy *et al*., 2010; Schramm *et al*., 2000). Tailings pond NA concentrations are highly variable, ranging from 20 to 120 mg/L (Holowenko *et al*., 2002; Holowenko *et al*., 2000; Schramm *et al*., 2000).

1.5.3 Naphthenic Acid Toxicity Mechanisms

NAs generally receive the most attention for remediation efforts among the toxic components of oil sands process-affected water (Giesy *et al*., 2010; Clemente & Fedorak, 2005; Madill *et al*., 2001). The mechanisms of NA toxicity are not known for certain, but there are two main possibilities. The first possibility, which applies to all NAs, is membrane disruption (Frank *et al*., 2009). NAs are amphipathic, having both a hydrophilic region (their carboxyl group(s)) and a hydrophobic region (their aliphatic tail). In this way, their structure resembles the phospholipids that make up biological membranes. Phospholipids have polar, hydrophilic head regions that are located adjacent to aqueous solutions on the inside and outside of the cell. They also have hydrophobic tails, the lipids that face the inside of the membrane bilayer. Although NAs are water-soluble, incorporation into a biological membrane would allow their polar region to interact with the polar region of the bilayer, while their hydrophobic tail could interact with the hydrophobic lipids (Frank *et al*., 2009). A diagram of an NA interacting with a biological membrane is shown in Figure 1.3.

Since NA tail regions are usually bulky with rings and branches, their incorporation among straight or kinked lipids would cause phospholipids to move out of their normal position in the membrane, perhaps disrupting the local shape of the membrane's surface (Frank *et al*.,

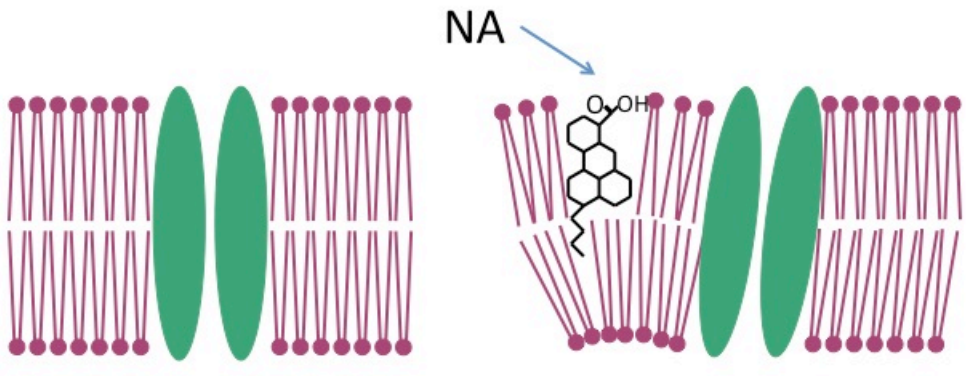

Figure 1.3 Membrane Disruption Hypothesis

Naphthenic acids (NAs) are believed to act via membrane disruption. NAs will preferentially enter biological membranes from aqueous solutions due to their polar carboxyl group and hydrophobic aliphatic tail structure. Incorporation of these bulky molecules into biological membranes could change local membrane architecture, membrane fluidity, and localization of membrane proteins.

2009). This could also change the position and orientation of non-lipid components of the membrane, such as trans-membrane proteins (Frank *et al*., 2009). The incorporation of NAs into biological membranes could also alter interactions between adjacent phospholipids, or between phospholipids and membrane proteins (Frank *et al*., 2009). Due to the ubiquity of biological membranes and membrane-associated functions, this mechanism could affect any system in an organism.

The second toxicity mechanism, which applies to a subset of NAs, is endocrine disruption. The acid-extractible organic fraction typically referred to as 'naphthenic acids' contains aromatic carboxylic acids, some of which have similar structures to steroid hormones (Rowland *et al*., 2011b). These substances can act as estrogen receptor agonists and androgen receptor antagonists (Thomas *et al*., 2009), although another study has observed non-receptor-mediated androgenic effects (Knag *et al*., 2013). Endocrine disruptive effects have been observed in several fish species exposed to NAs and oil sands process-affected water (Kavanagh *et al*., 2012; Lister *et al*., 2008), and this could help to explain observations of feminization in male North Sea fish exposed to process affected water from offshore oil drilling (Thomas *et al*., 2009). These endocrine disrupting compounds do not fit the classical definition of NAs due to their aromatic structure, but they are part of typical mixtures nominally considered naphthenic acids (Rowland *et al*., 2011b).

1.5.4 Toxic Effects of Naphthenic Acids

NAs have been shown to have toxic effects on plants (Leishman *et al*., 2013), trees (Kamaluddin & Zwiazek, 2002), fish (Dokholyan & Magomedov, 1983; Nero *et al*., 2005; Hagen *et al*., 2012), frogs (Melvin & Trudeau, 2012), mammals (Rogers *et al*., 2002b; Garcia-

Garcia *et al*., 2011), and bacteria (Frank *et al*., 2008). These studies cover a wide range of trophic levels, however invertebrate animals are conspicuously under-represented. Despite their importance to limnic ecology, very little is known about the effects of NAs on mollusks. Dokholyan and Magomedov (1983) reported the concentrations considered harmless for several species of clams (*Abra ovate, Didaona trigonoides, Cerastoderma lamarcki*), although they did not specify the criteria used to assess harm. Cairns and Scheier (1962) found that the sensitivity of the European Physa pond snail (*Physella acuta*) to NAs was dependent on both temperature and water hardness.

Although the specific effects of NA exposure vary between species, a few patterns emerge from the sub-lethal effects observed in these studies. First, there are changes in metabolic function and regulation. The most common of these effects include elevated glycogen storage, which was observed in marine fish (Dokholyan & Magomedov, 1983) and rats (Rogers *et al*., 2002b), as well as reduced gas exchange capacity, seen in aspen seedlings (Kamaluddin & Zwiazek, 2002) and juvenile yellow perch (Nero *et al*., 2005).

Second, immune suppression was observed in both fish and mammals. Mice showed lower expression of inflammatory cytokines, reduced respiratory burst responses in macrophages, and impaired phagocytosis after NA exposure (Garcia-Garcia *et al*., 2011). Similarly, goldfish exposed to NAs had reduced pro-inflammatory gene expression and increased susceptibility to infection (Hagen *et al*. 2012). Immune suppression after NA exposure has also been observed in rainbow trout (MacDonald *et al*., 2013; McNeill *et al*., 2012).

Third, impaired embryo growth and development occurs in several types of aquatic vertebrates. Slow embryo growth during NA exposure was seen in yellow perch and Japanese medaka (Peters et al., 2007), as well as the northern leopard frog and the western-clawed frog

(Melvin & Trudeau, 2012). Embryonic deformities have been observed in northern leopard frogs (Melvin & Trudeau, 2012), yellow perch, Japanese medaka (Peters et al., 2007), and fathead minnows (He *et al*., 2011).

A reduction in NA concentration in tailings pond water consistently correlates with a decrease in toxicity as measured with the microtox assay (Scott *et al*., 2008; Clemente *et al*., 2004; Madill *et al*., 2001). This assay uses the marine bacterium *Vibrio fischeri*, which produces bioluminescence. A population of these bacteria is exposed to a toxicant for a short duration (15 minutes in all of the above studies), with the luminescence measured before and after exposure. Thus, the microtox assay provides a quick and easy estimate of toxicity, which is useful in the context of NA toxicity to species in a full range of trophic levels.

Some studies assessing NA toxicity have not found toxic effects. For example, adult northern leopard frogs (*Lithobates pipiens*) exposed to NAs showed no detrimental effects despite accumulation of NAs in their muscle tissue (Smits *et al*., 2012). Similarly, no harmful effects were observed in tree swallow (*Tachycineta bicolor*) hatchlings during NA exposure (Gentes *et al*., 2007). Thus, NAs may not be toxic to all organisms exposed to them.

1.6 Athabasca Oil Sands

The oil sands deposits in northern Alberta contain the world's third largest oil reserves, after Venezuela and Saudi Arabia (Alberta Government, 2015). The largest of these deposits, the Athabasca deposit, is located on the Athabasca River north of Fort McMurray, Alberta (Alberta Government, 2015). The Athabasca oil sands extraction industry employs about 100 000 people and accounts for nearly $68 billion in energy resource exports per year (Alberta Government,

2014). It accounts for a substantial portion of Alberta's economy, and generated $3.56 billion in royalties for the Alberta Government in 2012-2013 (Alberta Government, 2014).

The Athabasca oil sands have generated nearly as much attention for their environmental impact as for their economic impact. The European Union considered singling out Athabasca oil sands products as more polluting than oils from other sources, but decided against the measure in 2014 (CBC News, 2014). This industry has also been the subject of protests by Greenpeace (CBC News, 2009), as well as other environmental organizations (Global News, 2013).

1.6.1 Athabasca Region

The Athabasca oil sands deposit is located under an area of 142,200 km^2, about half of which is currently cleared or disturbed (Alberta Government, 2013). This region is part of the circumpolar boreal forest, and includes many lakes and other bodies of fresh water. The Athabasca River, one of the largest rivers in the region, flows from Mount Athabasca in Jasper National Park, in western Alberta, through the oil sands deposit, to the Peace-Athabasca Delta in northern Alberta.

The Peace-Athabasca Delta is one of the largest freshwater deltas in the world (Environment Canada, 1993). It is an important habitat for migratory birds, with more than 1 million birds using the area every fall (Environment Canada, 1993). This region serves as a refuge for ducks in years when the climate in more southern habitats is too dry (Environment Canada, 1993), and this function will be increasingly needed due to the effects of habitat destruction and climate change (Monk *et al*., 2012; Timoney, 2009; Environment Canada, 1993). The Peace-Athabasca Delta is also a nesting ground for the whooping crane and peregrine falcon, both of which have been the subjects of re-introduction programs (Environment Canada,

1993). The area surrounding the delta is home to the largest herd of free-roaming bison in the world, living in North America's largest undisturbed grass and sedge meadows (Environment Canada, 1993).

The area overlying the Athabasca oil sands deposit is home to large areas of wetlands as well. The total area of wetlands in the Athabasca region decreased by approximately 5000 hectares between 1984 and 2005 (Gillanders *et al*., 2008). Remaining wetlands in the Athabasca region are subject to the effects of climate change, including increasing temperatures and reduced water availability (Monk *et al*., 2012; Timoney, 2009). These wetlands are also exposed to oil sands materials, via water and air, due to both natural and anthropogenic processes.

Most research on the effects of oil sands-derived material on wetlands species has focused on reclaimed environments and laboratory exposures, yet some information is available on existing natural Athabasca wetlands. Tree swallows living in wetlands within 5 km of oil sands mining operations had increased detoxification enzyme activity and decreased immune responses among hatchlings compared to those living more than 60 km away from mining sites, although adult reproductive output was the same in both site types (Cruz-Martinez *et al*., 2015). These birds were exposed to oil sands-derived material through the air, but not the water, although food sources cannot be ruled out (Cruz-Martinez *et al*., 2015).

Bioaccumulation of polycyclic aromatic hydrocarbons (PAHs) is an important consideration in Athabasca region wetlands ecosystems. Wayland *et al*. (2008) tested PAH accumulation in three types of Athabasca wetlands: wetlands containing tailings or tailings water, wetlands located on mine leases to which no tailings material had been added, and wetlands located close to mine leases. Oil sands-derived PAHs were found in sediments, insect larva, and adult insects in all types of wetlands tested (Wayland *et al*., 2008). PAH accumulation

was generally higher in wetlands containing tailings material than in the other types of wetlands, however adult insects had unexpectedly high levels of PAHs in all types of wetlands (Wayland *et al*., 2008).

Despite these challenges, wetland ecosystems persist in the Athabasca region. Natural selection has almost certainly driven population shifts towards greater hardiness in the face of environmental contamination. Thus, we can expect that, with sufficient resources and effort, sustainable wetlands ecosystems can be generated from tailings-affected landscapes.

1.6.2 Bitumen Extraction and Processing

The petroleum in the Athabasca oil sands deposits is extracted in the form of bitumen ore, a thick, near-solid mixture of bitumen and sand. Bitumen ore is obtained in two ways: mining and *in situ* methods (Alberta Government, 2015). Mining production is conducted in open pits from which the overlying vegetation and soil have been removed. Bitumen ore is dug out of the deposit and loaded onto trucks for transport to upgrader facilities. *In situ* extraction methods are conducted where the oil sands deposits are too deep for mining to be practical (Alberta Government, 2015). Instead, wells are drilled into the deposit, allowing steam to be pumped in to melt the bitumen (Alberta Government, 2015). The less viscous hot bitumen ore can then be pumped to the surface for processing (Alberta Government, 2015). Mining has been the most common method for most the history of the Athabasca oil sands industry (Alberta Government, 2015). *In situ* extraction surpassed mining extraction in 2012, and represented 53% of bitumen ore production in 2013 (Alberta Government, 2015).

1.6.3 Tailings Ponds

When bitumen ore arrives at an upgrader facility, the first step in its processing is to remove the sand, clay, naphthenic acids, metals, and other substances found in the ore that would damage production equipment. This is accomplished by the Clark hot water method (Clark & Pasternak, 1932). During this process, bitumen ore is mixed with hot water and sodium hydroxide. The viscosity of the bitumen increases and it floats on the water. The sand and clay sink to the bottom of the vessel or are suspended in the caustic water, and water-soluble components of the ore, including metal ions, naphthenic acids, and polycyclic aromatic hydrocarbons, dissolve in the water. Most of the bitumen is removed for processing, and the water, sand, clay, unrecovered bitumen, and water-soluble contaminants are stored in tailings ponds (Giesy *et al*., 2010; Holowenko *et al*., 2002). A diagram of the Clark hot water extraction process is shown in Figure 1.4. Active tailings ponds receive wastewater from upgrader operations, and wastewater is often transferred between active ponds. Active tailings ponds contain 20 to 120 mg/L naphthenic acids (Holowenko *et al*., 2002; Holowenko *et al*., 2000; Schramm *et al*., 2000).

1.6.4 Reclamation

When land disturbed by oil sands operations is no longer needed for industrial applications, oil sands operators are obligated to reclaim them according to the Environmental Protection and Enhancement Act (ESRD, 2013). Reclamation assessments by the Alberta government have three objectives. The first objective is to create reclaimed landscapes capable of supporting natural ecosystem function (ESRD, 2013). The second objective is for natural ecosystems to become established on the reclaimed landscapes (ESRD, 2013). The third

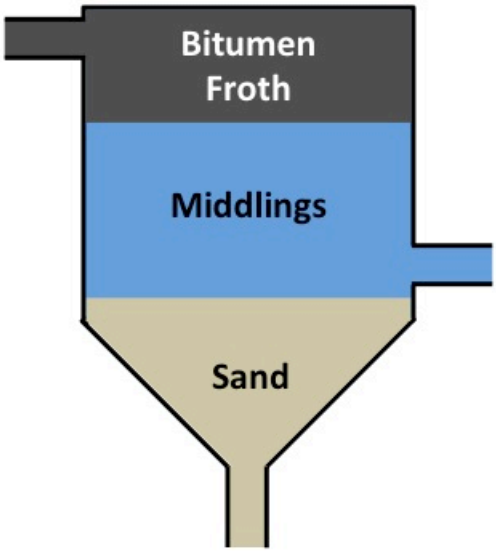

Figure 1.4 Clark Hot Water Method

The first step in bitumen processing is to separate the bitumen from the sand, clay, metals, aromatics, and naphthenic acids contained in the bitumen ore. This is done by mixing the ore with hot water and sodium hydroxide. The bitumen floats at the top as a froth, the sand and clay sink to the bottom, and the water-soluble components are dissolved in the water (this solution is called ‘middlings’). The bitumen is removed for further processing, and the sand, clay, water, and water-soluble components including naphthenic acids are stored in tailings ponds.

objective is for the reclaimed land to support a variety of land uses, but not necessarily the same land uses it supported before it was disturbed (ESRD, 2013). This presents numerous challenges, since ecosystems must be able to withstand whatever physical and chemical waste contents are still present after reclamation. As of September 2013, only 104 hectares (0.12% of the total area disturbed by oil sands operations) had been certified as reclaimed, although a further 6000 to 8000 hectares were undergoing reclamation efforts (Alberta Government, 2013).

Tailings pond remediation strategies fall into two main categories: wet, resulting in an aquatic environment, and dry, producing a terrestrial landscape (Leung *et al*., 2001). The simplest method of aquatic reclamation involves diluting the tailings water with clean water (Madill *et al*., 2011). This would reduce the concentration of aqueous toxicants, presumably reducing the toxicity of the water.

There are also a number of remediation strategies that act specifically on naphthenic acids, either to convert them to less toxic compounds, or to remove them from solution. Oxidation processes, including ozonation and UV radiation, accompanied by oxidizing agents, reduce the naphthenic acid concentration of tailings pond water (Liang *et al*., 2011; Scott *et al*., 2008). Microbial degradation of naphthenic acids, primarily by organisms found in the tailings pond water itself, has also been shown to reduce naphthenic acid concentrations (Quesnel *et al*., 2011; Rowland *et al*., 2011a; Clemente *et al*., 2004). Methods are also being developed to treat tailings water with ceramic ultrafiltration membranes, which would trap naphthenic acids, allowing them to be removed from solution (Hussein *et al*., 2011).

1.7 Project Overview

1.7.1 Project Goals

Naphthenic acids enter aquatic ecosystems from a variety of sources, and they are known to have detrimental effects on many different organisms. The effects of NAs have been well-studied in fish, but information about their effects on aquatic invertebrates is scarce. Thus, the overall purpose of my thesis research was to investigate the effects of NA exposure on freshwater mollusks. I hypothesized that naphthenic acid exposure would have detrimental effects on *Lymnaea* at both embryonic and adult life stages. I established two main goals for my thesis research. Firstly, I aimed to examine the effects of NA exposure, both acute and chronic, on *Lymnaea stagnalis* at multiple life stages. Within the context of this broader investigation, my second goal was to develop molluscan bioassays that could serve as relevant and cost-effective assessment tools for NA toxicity.

1.7.2 Project Design

Organisms in reclaimed ecosystems are exposed to remaining toxicants throughout their lives, for multiple generations. Thus, toxic effects must be examined at multiple life stages. In this project, I chose to focus on exposure during embryonic and adult life stages. Embryonic NA exposure focused on hatching success, but also assessed growth, development, and bioavailability of NAs. Adult NA exposure was investigated over both acute (3 weeks) and chronic (1 year) timescales. Since surviving animals in reclaimed wetlands will need to reproduce successfully to form healthy populations, I assessed not only lethal concentrations but also numerous fitness traits including growth and fecundity. As a whole, my research program

was designed to give a preliminary impression of the population stability of *Lymnaea stagnalis* in wetlands ecosystems contaminated with NAs.

1.7.3 Naphthenic Acids Used in this Project

My research goals were to investigate the effects of NA exposure and to develop bioassays. These goals require that toxicant compositions are consistent over time and between experiments. Since the composition and concentration of NAs in tailings ponds change over time, this condition is very difficult to attain through the use of tailings pond extracts. Instead, I used a mixture of three commercially available model NAs in all of my experiments. Model NAs are routinely used in toxicity experiments (e.g. Jones *et al*., 2011; Frank *et al*., 2010; Smith *et al*., 2008; Armstrong *et al*., 2008; Holowenko *et al*., 2001).

The model NAs used in the studies described in this thesis were cyclohexylsuccinic acid (CHSA), cyclohexanebutyric acid (CHBA) and 4-*tert*-butylcyclohexane carboxylic acid (4-TBCA) (Figure 1.5). Each of these NAs has a hydrogen deficiency of -2 due to a single ring structure. Moreover, all three classify as low-molecular weight NAs with carbon numbers of 10 (CHBA and CHSA) or 11 (4-TBCA). NAs with these combinations of carbon number and hydrogen deficiency are routinely detected in oil sands process-affected water (Clemente *et al*., 2003; Frank *et al*., 2006; Headley *et al*., 2013) and can persist for many years even in remediated ponds. For example, van den Heuvel *et al*. (2014) found NAs consistent with this study's model NAs in an experimentally reclaimed Syncrude pond (Demonstration Pond) 19 years after it was considered remediated by capping with surface water (McNeill *et al*., 2012). MacDonald *et al*. (2013) also detected similar NAs in an un-used Syncrude pond (Pond 10) 17 years after it was closed.

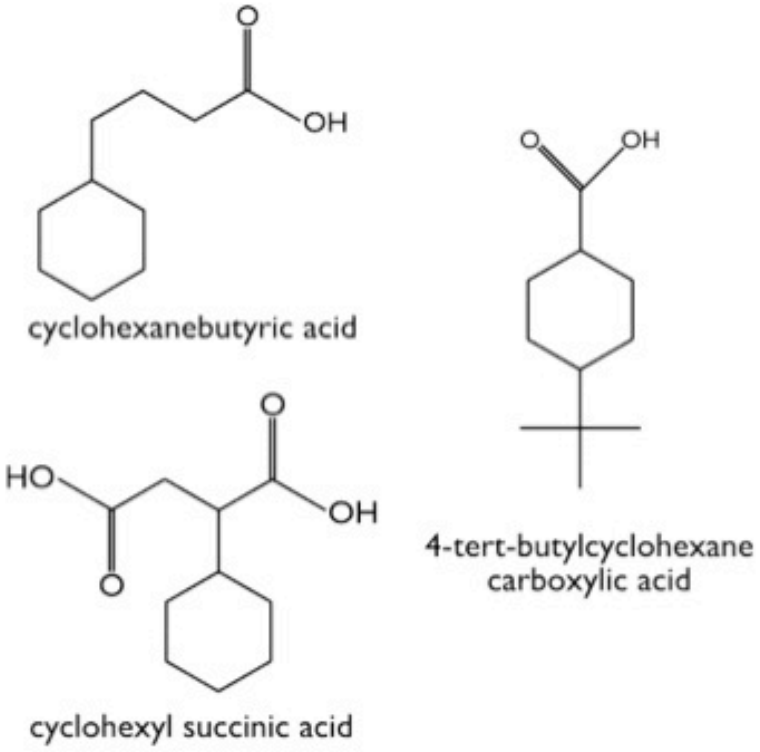

Figure 1.5 Model Naphthenic Acids

The three model naphthenic acids (NAs) used in this study were cyclohexanebutyric acid (CHBA), cyclohexyl succinic acid (CHSA), and 4-tert-butyl cyclohexane carboxylic acid (4-TBCA). Each of these NAs has a hydrogen deficiency of -2 due to a single ring structure. CHBA and CHSA have carbon numbers of 10, while 4-TBCA has a carbon number of 11.

Chapter Two: **Embryonic Naphthenic Acid Exposure**

2.1 Introduction

Naphthenic acids (NAs) are subject to the most remediation efforts among the toxic component of oil sands tailings pond water (Giesy *et al*., 2010; Clemente and Fedorak, 2005; Madill *et al*., 2001). NAs are carboxylic acids with large aliphatic tail groups, and are found in active tailings ponds at concentrations of 20 to 120 mg/L (Holowenko *et al*., 2002; Holowenko *et al*., 2000; Schramm *et al*., 2000). NAs are also present in most Athabasca region surface and ground waters. The Athabasca River contains 0.1 to 0.9 mg/L NAs (Schramm *et al*., 2000), while ground water near tailings ponds has been found to contain 0.4 to 51 mg/L NAs (Clemente and Fedorak, 2005). NAs are widely believed to act via membrane disruption, although I am not aware of any studies that have tested this hypothesis directly. This mechanism could affect any system in an organism, thus wide-ranging sub-lethal effects could result from long-term NA exposure. Some NAs can also act via endocrine disruption, generally acting as estrogen receptor agonists and androgen receptor antagonists (Thomas *et al*., 2009), although another study proposed non-receptor-mediated androgenic effects (Knag *et al*., 2013).

NAs have been shown to be acutely lethal to embryos of freshwater vertebrate species, including fathead minnows with an LC50 of 32.8 mg/L in NAs extracted from oil sands process-affected water (Kavanagh *et al*., 2012), the northern leopard frog with an LC50 of 4.1 mg/L in commercial NAs, and the western-clawed frog with an LC50 of 2.95 mg/L in commercial NAs (Melvin & Trudeau, 2012). Non-lethal effects have also been observed in embryos of these species, including deformities in the northern leopard frog at 4 mg/L commercial NAs (Melvin & Trudeau, 2012), yellow perch at 1.67 mg/L commercial NAs, Japanese medaka at 1.51 mg/L

commercial NAs (Peters *et al*., 2007), and fathead minnows in oil sands process-affected water containing 19.7 mg/L NAs (He *et al*., 2012). Small sized embryos were seen in both the northern leopard frog and the western-clawed frog at 2 mg/L commercial NAs (Melvin & Trudeau, 2012), in yellow perch at 1.12 mg/L commercial NAs, and in Japanese medaka at 1.44 mg/L commercial NAs (Peters *et al*., 2007). As yet, however, our knowledge regarding the toxic effects of environmentally relevant NA concentrations on the embryonic development of freshwater invertebrate species is still sparse.

In this study, I used the great pond snail *Lymnaea stagnalis* to assess the toxicity of NAs in aquatic environments. *Lymnaea*, a freshwater mollusk widely used as a toxicology model system, is native to much of the northern hemisphere, including most of Europe and North America (Segner *et al*., 2003; Janer & Porte, 2007; Prescott & Curteanu, 2004).

Embryonic development is a critical phase in animal life cycles, and can be particularly sensitive to toxicant exposure (Gomot, 1998). I therefore hypothesized that embryonic exposure to NAs would have detrimental effects on *Lymnaea* embryos, including slow growth, reduced hatching, and acute lethality.

In *Lymnaea*, embryonic development can be easily observed under a stereomicrosope, since their eggs are transparent capsules and are packaged in gelatinous egg masses that are also transparent. Thus, reproductive success in contaminated environments can be evaluated by observing the occurrence of developmental defects, and by counting embryo hatching and death rates. Embryonic development has been thoroughly documented in *Lymnaea* and can be broken down in easily recognizable anatomical stages (Filla *et al*., 2009; Morrill, 1982).

Throughout this study, I used a mixture of three model NAs: cyclohexylsuccinic acid, cyclohexanebutyric acid, and 4-*tert*-butylcyclohexane carboxylic acid. These are relatively low

molecular weight NAs, but NAs of this size are routinely found in both active (Clemente *et al*., 2003; Frank *et al*., 2006; Headley *et al*., 2013) and aged tailings ponds (van den Heuvel *et al*., 2014; McNeill *et al*., 2012, MacDonald *et al*., 2013). Indeed, low molecular weight NAs such as these are expected to be produced continually as tailings water ages due to microbial breakdown of high molecular weight NAs (Quesnel *et al*., 2011).

To test the effects of naphthenic acids on *Lymnaea* embryos, I exposed freshly-laid egg masses to varying NA concentrations (0.25 to 75 mg/L), extending well into the concentration range found in active tailings ponds. I conducted a similar experiment using tailings-derived NAs in concentrations up to 35 mg/L in order to compare the toxicity of our model NAs to those found in Athabasca tailings ponds. I also tested the effects of transient changes in NA concentrations by exposing fresh egg masses to 30 mg/L NAs for periods of 4, 12 and 28 days. To test whether the packaging of *Lymnaea* eggs into masses protects them from NAs, I exposed both isolated eggs and eggs in intact egg masses to varying NA concentrations (0.1 to 20 mg/L). I also analyzed the NA concentration in water, egg mass casings, and eggs (including embryos) by gas chromatography-mas spectrometry after 12 days of exposure to 30 mg/L NAs.

2.2 Materials and Methods

2.2.1 Animals and Housing Conditions

Animals were bred and raised in the laboratory as previously described (Hermann *et al*., 2007; Watson *et al*., 2012). Snails were housed at a density of no more than 1.5 animals per liter. Animals were fed *ad libitum* with lettuce and Aquamax-carniverous grower 400 trout pellets (Purina Mills LLC, St. Louis, MO, USA). Snails were kept in pond water produced from purified water reconditioned to a conductivity of approximately 450 µS/cm by the addition of Instant

Ocean salts (Aquarium Systems, Mentor, OH, USA) at 1 g per gallon. Calcium concentration was kept at a saturating level of ~60 mg/L by the addition of calcium carbonate. Adult snails (shell lengths 25 to 35 mm), never exposed to NAs, were randomly selected from a mixed-age population.

2.2.2 Model Naphthenic Acids

For all parts of this experiment, a mixture of equal parts by mass of cyclohexylsuccinic acid (CHSA), cyclohexanebutyric acid (CHBA), and 4-*tert*-butylcyclohexane carboxylic acid (4-TBCA) was used as model NAs. All NAs were obtained from Sigma Aldrich (St. Louis, MO, USA). The reported NA concentration represents the sum of the mass concentrations of the individual model NAs. For example, a solution of 30 mg/L NAs contains 10 mg/L CHSA, 10 mg/L CHBA, and 10 mg/L 4-TBCA.

2.2.3 Tailings Pond Naphthenic Acids

Water samples were collected from a closed pond that no longer receives fresh tailings in February, 2010. The acid-extractable organic fraction, containing the tailings-derived NAs, was prepared as follows. Water was acidified to a pH of 2 with 5.2M HCl, followed by centrifugation at room temperature at 2126g for 10 min. The supernatant was transferred to clean 4 oz Boston round bottles with Teflon cap liners. Two volumes of dichloromethane (Sigma–Aldrich, St. Louis, MO, USA) were added to the bottles. The supernatant was then extracted for 2 h on an orbital rotator (Boekel Scientific, Feasterville, PA, USA, Model: 260200). After extraction, samples were poured through 125 mm phase separator Whatman 220 filter papers previously rinsed with dichloromethane. The extraction bottle was rinsed with 10 mL of dichloromethane,

then 5 mL of dichloromethane, which was then poured through the phase separator. The filter paper was rinsed again with dichloromethane, and the volume of the flow through was reduced to 1.5 mL with a Rotovapor R II rotary evaporator (BÜCHI Labortechnik AG, Flawil, St. Gallen, Switzerland). Samples were then weighed and dissolved in DMSO. This procedure was adapted from Quesnel *et al*., 2011.

2.2.4 Embryonic Development

To assess dose-dependency of NA toxicity, a stock solution of 150 mg/mL NAs in dimethyl sulphoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was prepared. NA pond water solutions with concentrations of 5, 10, 15, 20, 25, 30, 40, 50, 60 and 75 mg/L NA were subsequently prepared. The pond water was pre-treated by aeration in the presence of excess calcium carbonate for several days to ensure sufficient carbonate buffering capacity. The DMSO concentration of all solutions was equilibrated at 500 ppm through supplementation of additional DMSO. A pond water plus DMSO solution (500 ppm; "DMSO control") and pond water only ("pond water control") were used as controls. Egg masses harvested within 24 hours after deposition were randomly assigned to the various test conditions or one of two control groups and maintained individually in covered 6-well plastic plates (Falcon, Franklin Lakes, NJ, USA) under those conditions for 20 or 28 days. Each well contained 7 mL of test solution. Egg masses were kept at 18°C on a 12:12h light:dark cycle. Development of the embryos was observed under a stereomicroscope on days 12, 14, 16, 18 and 20, and the number of hatched embryos was determined. An embryo was considered to have hatched when it emerged from its egg. Emergence from the egg mass was not considered a necessary condition for hatching. On day 20, un-hatched embryos were photographed, and their vitality status was assessed by watching for

signs of heartbeat and body movement. Embryos lacking both heartbeat and body movement were considered to be arrested. Advanced embryos (i.e. > day 12) displaying anomalous shell and/or visceral mass shapes were scored as abnormal. Anomalous development typically included a reduced shell with minimal torsion, and large cavities in the visceral mass (see Figure 2.4C and 2.4D).

The effects of tailings-derived NAs on hatching were determined as described above, except that day 28 was used as the endpoint. An identical experiment using model NAs was conducted immediately afterwards, also using day 28 as the endpoint, to be used to compare toxicities of model NAs to tailings-derived NAs.

Critical periods in the sensitivity of embryos to NA exposure were examined by exposing embryos during different developmental stages to 30 mg/L NAs as prepared above. Exposure periods were chosen to cover zygote to trochophore stages (days 0 to 4), the veliger stage (days 4 to 8), and the adult-like stage (days 8 to 12), as defined by Filla *et al*. (2009) and Morrill (1982) (see Figure 2.1A to 2.1D). Embryos were maintained in standard pond water before and/or after completion of the exposure period. Development and hatching of the embryos were observed on day 4, day 8, and every 2 days from day 12 to day 28. On day 28, un-hatched embryos were photographed and scored for heartbeat and movement. See Figure 2.2 for a schematic illustration of this experiment.

Potential interference of egg mass casings with the expression of NA toxicity to embryos was investigated by removing eggs from their egg masses and maintaining the isolated eggs under the following experimental conditions: pond water control, pond water + DMSO control, and NA solutions of 0.1, 1, 5, and 20 mg/L in pond water, prepared as described above. In each well with isolated eggs, the eggs were isolated from a single egg mass. Development and

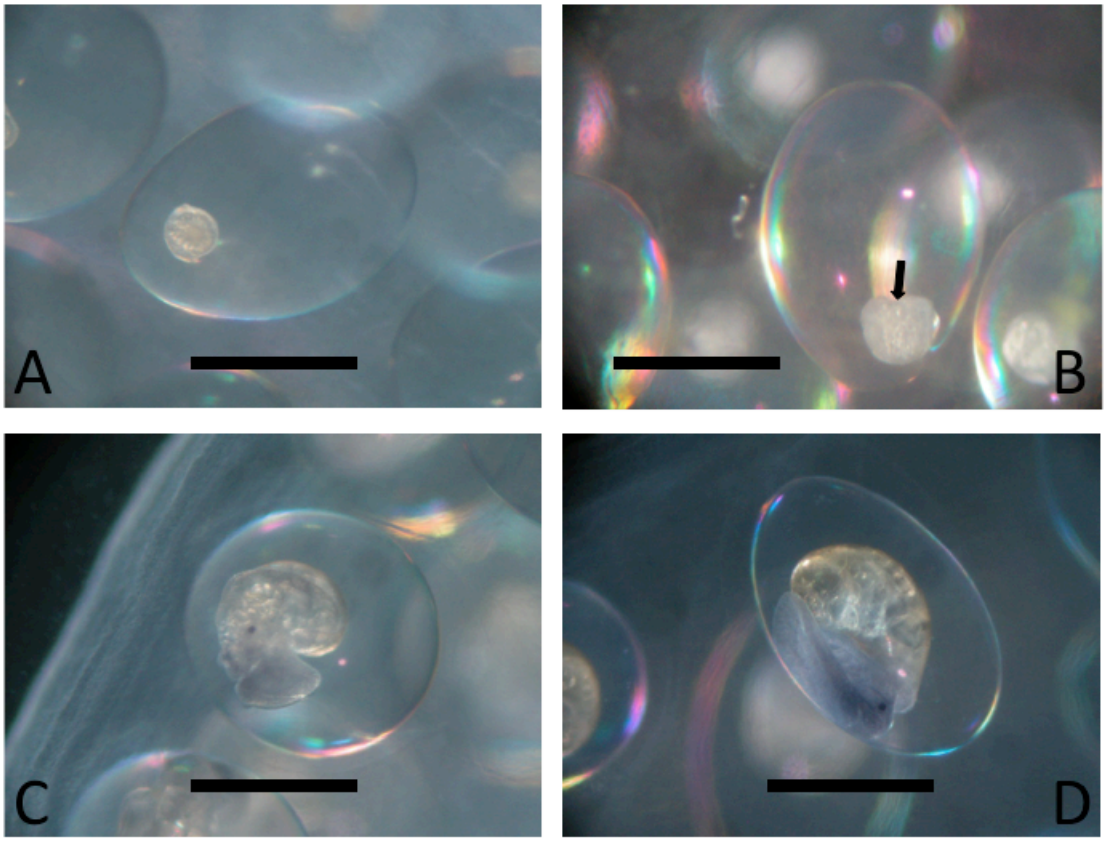

Figure 2.1 Developmental Stages of *Lymnaea* Embryos

A: Trochophore. The embryo is essentially spherical, with a few small indentations and protrusions. B: Veliger. The arrow indicates the indentation that will divide the foot from the visceral mass. C: Early adult-like. The embryo is snail-shaped, but the shell lacks torsion and the foot is small and unable to grip surfaces. Pigmented eyes are visible. D: Late adult-like. The embryo has reached the mature form it will have when it hatches. The foot is much larger and able to grip surfaces. Pigmentation in the head and foot, as well as shell torsion, are evident. A – D: Scale bars indicate 0.5 mm.

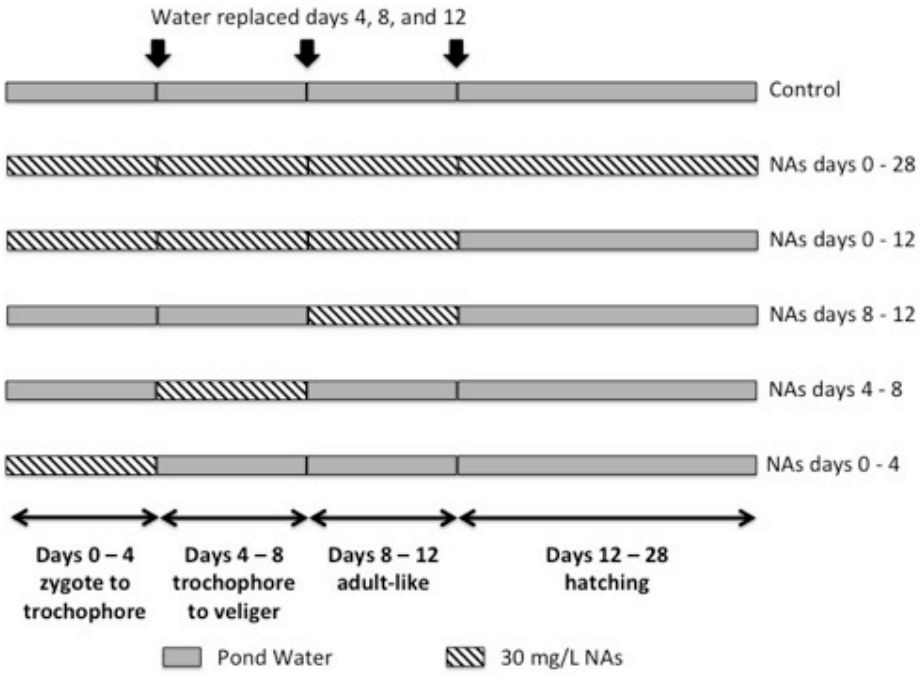

Figure 2.2 Schematic Diagram of Critical Exposure Period Experiment

Water was replaced on days 4, 8, and 12 in every treatment. Solid grey bars indicate pond water exposure. Black and white slashed bars indicate 30 mg/L naphthenic acid (NA) exposure.

hatching of the embryos were observed on day 4, day 8, and every 2 days from day 10 to day 20. On day 20, un-hatched embryos were photographed and scored for heartbeat and movement. All data collection was done without prior knowledge of experimental conditions.

2.2.5 Embryo-to-Egg Ratio

Embryo size was estimated by measuring the ratio of the embryo area to the area of its egg in photographs, all of which were taken at the same microscope magnification and camera settings. Embryo and egg capsule areas were determined by carefully tracing their outline in ImageJ (Version 1.45s, NIH, Bethesda, MD, USA), then having the program determine the area in pixels. The embryo-to-egg ratio was calculated for each embryo by dividing the embryo area by the egg area. Photographs were excluded if the egg or embryo could not be seen clearly, if there was more than one embryo in the egg capsule, or if the embryo displayed gross developmental abnormalities.

2.2.6 Naphthenic Acid Detection in Water and Egg Mass Samples

To investigate whether the model NAs used in this experiment are able to penetrate *Lymnaea* eggs, I incubated eggs in either an NA solution or a control solution, and analyzed the NA content of the solution, egg mass casing, and eggs. A solution of 30 mg/L NAs in pond water was prepared as described above. 6-well plastic plates were used to expose egg masses to the NA solution, to a pond water control as described earlier, or filled with 30 mg/L NAs without an egg mass. Four NA-exposed egg masses and the water they were incubated in were sampled after 4 days. In addition, 4 control and 2 groups of 4 NA-exposed egg masses and water were sampled after 12 days. Egg masses were removed from their incubation water and rinsed in pond water.

Subsequently, eggs were isolated from the egg mass and placed in a glass homogenization tube. Eggs were crushed with a glass rod, and 4.4 ml/egg of distilled water was added. The egg samples were pooled from the 4 egg masses in the same treatment, then 3 replicates per pooled sample were added to GC-MS vials (Agilent Technologies, Santa Clara, CA, USA). Egg mass casings were chopped into small pieces, and then transferred to a glass homogenization tube. Distilled water was added at a concentration of 1 µL/3 mg of casing, and the samples were crushed. The casing samples were pooled from the 4 egg masses in the same treatment, then 3 replicates per pooled sample were added to GC-MS vials. Water samples were taken from exposure wells, pooled with the water from other wells in the same treatment, then 3 replicates per pool were added to sample vials. All samples were prepared in triplicate. Samples were acidified with 1 drop of 2 M HCl, then dried overnight at 45ºC. Dried samples were frozen and stored at -20ºC before use.

Dried samples were prepared for GC-MS analysis by adding 300 µL dichloromethane (Sigma Aldrich, St. Louis, MO, USA), followed by 50 µL of N,O-bis(trimethylsilyl)Trifluoro acetamide (BSTFA) (Thermo Scientific, Waltham, MA). Samples were then incubated for 15 min in a 60ºC water bath.

1 µL of prepared sample was manually injected into an Agilent 7890A gas chromatograph equipped with an Agilent HP-5MS 50 m x 0.25 mm x 0.25 µm column and an Agilent 5975C mass selective detector (Agilent Technologies, Santa Clara, CA). Holding temperature for the oven was 50°C for the first 5 min, then increased to 250°C at a rate of 8°C/min, then held at 250°C for 5 min. Analysis was carried out with Enhanced MSD ChemStation E.02.00.493 RTE integrator software (Agilent Technologies, Santa Clara, CA).

2.2.7 Data Analysis and Statistics

Unless otherwise noted, statistical analysis was done using Statistica version 7.1 (StatSoft Inc., Tulsa, OK, USA). Data analysis was done by univariate analysis of variance (ANOVA), unless stated differently. Multiple comparisons of experimental groups were done using Dunnett's test using the pond water plus DMSO group as reference group in experiments where two controls were tested, unless stated differently. Tukey's test was used in the critical exposure period experiment, since it was important to know whether different stages of exposure differed from each other, not just whether each stage differed from controls. Effects of NAs on hatching proportions were treated as log(inhibitor) vs. response models, with NA concentration as the dependent variable. The bottom of the curve was fixed at zero, since zero hatching is observed at high NA concentrations. Modeling was done using Prism version 6.0e (GraphPad Software Inc., La Jolla, CA, USA), which applies least-squares loss functions. Analysis of developmental progression was done by means of Pearson's chi squared test, where the categories were the developmental stages and the counts were the total number of embryos in that stage in each treatment group. Graphic analysis of residuals and homogeneity of variances and parametric analyses (Levene's test, Hartley F-max test) confirmed that all data met parametric assumptions, unless stated otherwise. Egg sizes in the dose-response experiment had significantly different variances between groups (Levene's test, $F_{11,485} = 2.223$, $p = 0.0124$), so egg sizes were compared using the Kruskal-Wallis test. Figures were prepared using GraphPad Prism version 4.03 (GraphPad Software, Inc., La Jolla, Ca, USA). Averages are shown as arithmetic mean ± standard error.

2.3 Results

2.3.1 Dose-Response Curve

First, I explored the concentration dependency of NAs on embryonic development in *Lymnaea stagnalis*. To this end, egg masses were continuously exposed to concentrations of model NAs ranging from 5 to 75 mg/L. Developmental progression, hatching rates, embryo death and embryo size were monitored for 20 days.

2.3.1.1 Embryo Size and Development

Embryonic exposure to NAs had a significant concentration-dependent effect on the embryo-to-egg ratios of un-hatched embryos at day 20 (Figure 2.3; ANOVA $F_{11,485} = 20.321$, $p < 0.0001$). Specifically, embryos exposed to 30, 60, and 75 mg/L NAs were smaller than control embryos, whereas embryos in DMSO control and 5, 10, 15, 20, 40, and 50 mg/L NAs did not significantly differ in size from control embryos (Dunnett's test; $p = 0.0476$ for 30 mg/L, $p < 0.0001$ for 60 and 75 mg/L). The size difference observed at 30 mg/L was considered trivial, since the p value was barely below 0.05, and the effect was not continuous with respect to NA concentration. Areas of eggs did not differ significantly between treatments (Kruskal-Wallis test, $H_{11,486} = 15.93624$, $p = 0.1435$), so the embryo-to-egg ratio is a good estimate of embryo size.

Embryos showing developmental abnormalities were very rare (usually less than 1% of embryos) among controls and most NA concentrations. However, in the 75 mg/L treatment group, roughly 40% of embryos showed morphological abnormalities, including misshapen or absent shell, and a reduced or misshapen visceral mass (for examples, see Figures 2.4C and 2.4D). These embryos often lacked pigmentation in the skin as well.

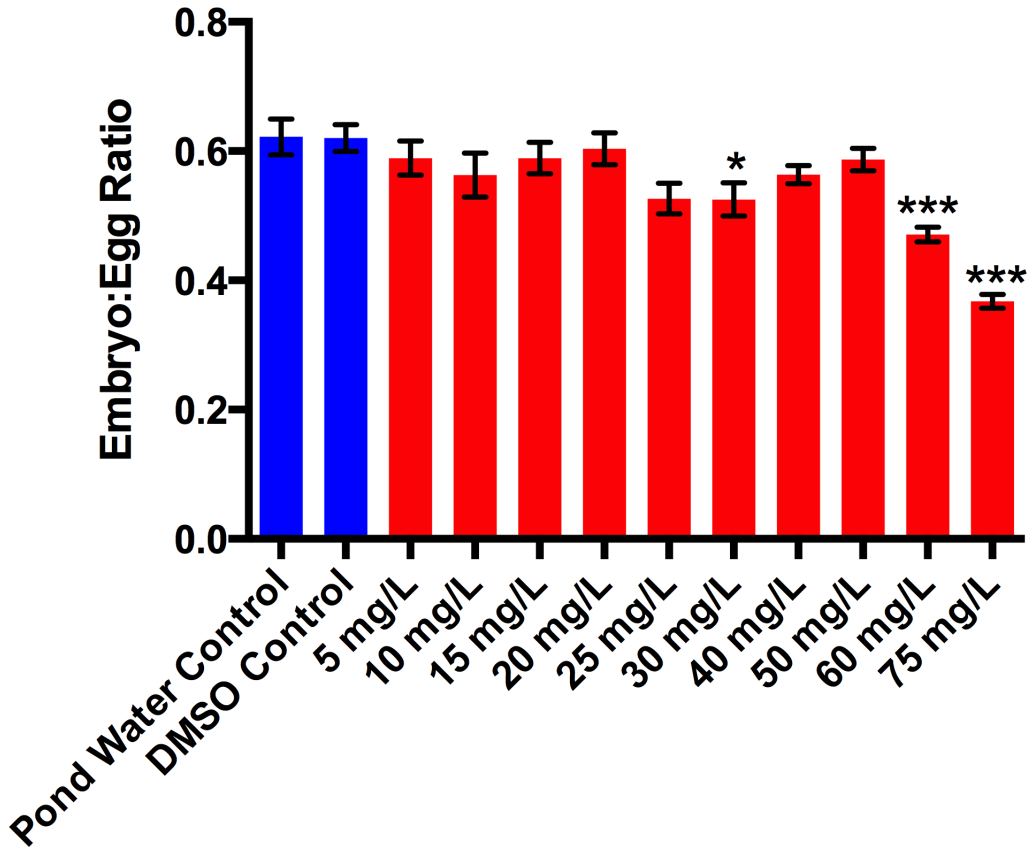

Figure 2.3 Concentration Dependence of *Lymnaea* Embryo Size

Embryo-to-egg ratios for embryos in pond water only ("Pond Water Control", blue bar), pond water with 500 ppm dimethyl sulphoxide (DMSO) ("DMSO Control", blue bar), or pond water with 500 ppm DMSO and varying concentrations of naphthenic acids (NAs) (NA concentrations, red bars). Data are shown as mean±SEM. Sample sizes range from 20 embryos for DMSO Control and 30 mg/L NAs to 78 embryos for 75 mg/L NAs. Data are shown as mean±SEM. Statistical significance is indicated with respect to DMSO control. * $p < 0.05$; *** $p < 0.0001$.

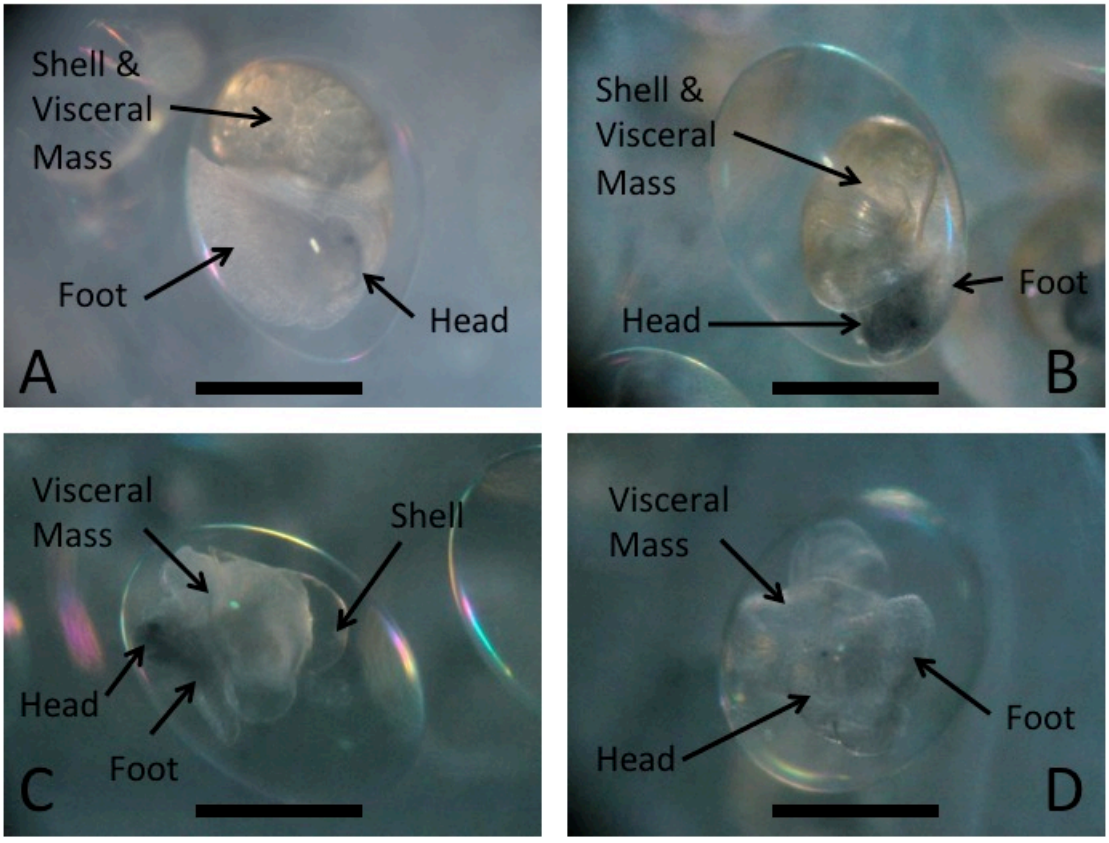

Figure 2.4 Day 20 *Lymnaea* Embryos

A: Normal late adult-like embryo from the DMSO control group. B: Small late adult-like embryo from the 60 mg/L naphthenic acid (NA) group. C: Abnormal embryo from the 75 mg/L NA group. The shell is not properly formed and not well attached. The visceral mass inside the shell has an abnormal shape and does not fill the shell like it does in normal embryos of this stage. The head and foot appear normal. D: Abnormal embryo from the 75 mg/L NA group. The shell is reduced or absent. The visceral mass, head, and foot are all abnormally shaped. A – D: The embryo's shell, visceral mass, head, and foot are indicated if they are present. Scale bars indicate 0.5 mm.

2.3.1.2 Hatching

In addition to its effect on embryo size, NA-treated embryos showed a significant dose-dependent decrease in total hatching proportions within 20 days when tested in 2012 (Figure 2.5B; ANOVA, $F_{11,107} = 57.046$, $p < 0.0001$). With hatching rates of 82.1±2.8% and 80.6±3.5% respectively, pond water and DMSO control groups were statistically indistinguishable ($F_{1,18} = 0.068959$, $p = 0.7935$). Multiple comparison revealed that, compared to the DMSO control group, hatching rates were lower for all NA-treated groups from 25 to 75 mg/L NAs (Dunnett's test, $p < 0.0001$ for each NA concentration from 25 to 75 mg/L). Hatching rates declined in a clear concentration-dependent manner from 47.7±5.6% for embryos in egg masses exposed to 25 mg/L to 0.1±0.1% for embryos in egg masses exposed to 75 mg/L.

In 2011, I conducted this assay on egg masses from different donor snails with NA concentrations of 0.25 to 50 mg/L, using day 28 as final census point. In this experiment, I also observed a concentration-dependent decrease in hatching (Figure 2.5A; ANOVA $F_{9,102} = 63.650$, $p < 0.0001$). Multiple comparisons showed significantly lower hatching rates in egg masses in NA concentrations from 20 to 50 mg/L compared to DMSO controls (Dunnett's test, $p = 0.00435$ for 20 mg/L, $p < 0.0001$ for 25 to 50 mg/L).

I compared these two hatching experiments using a log(inhibitor) vs. response model, considering NAs to be an inhibitor of hatching. The two curves were very similar to each other, having IC50 values of 32.05 mg/L for the 2011 experiment and 31.95 mg/L for the 2012 experiment. The R^2 values were 0.8743 for 2011 and 0.8617 for 2012 (Figure 2.5C).

Further visual inspection of un-hatched embryos revealed that a small fraction of these embryos in all treatment groups were arrested in trochophore or earlier stages (Figure 2.6A). These embryos typically appeared to be breaking apart and lacked clearly demarcated cells. This

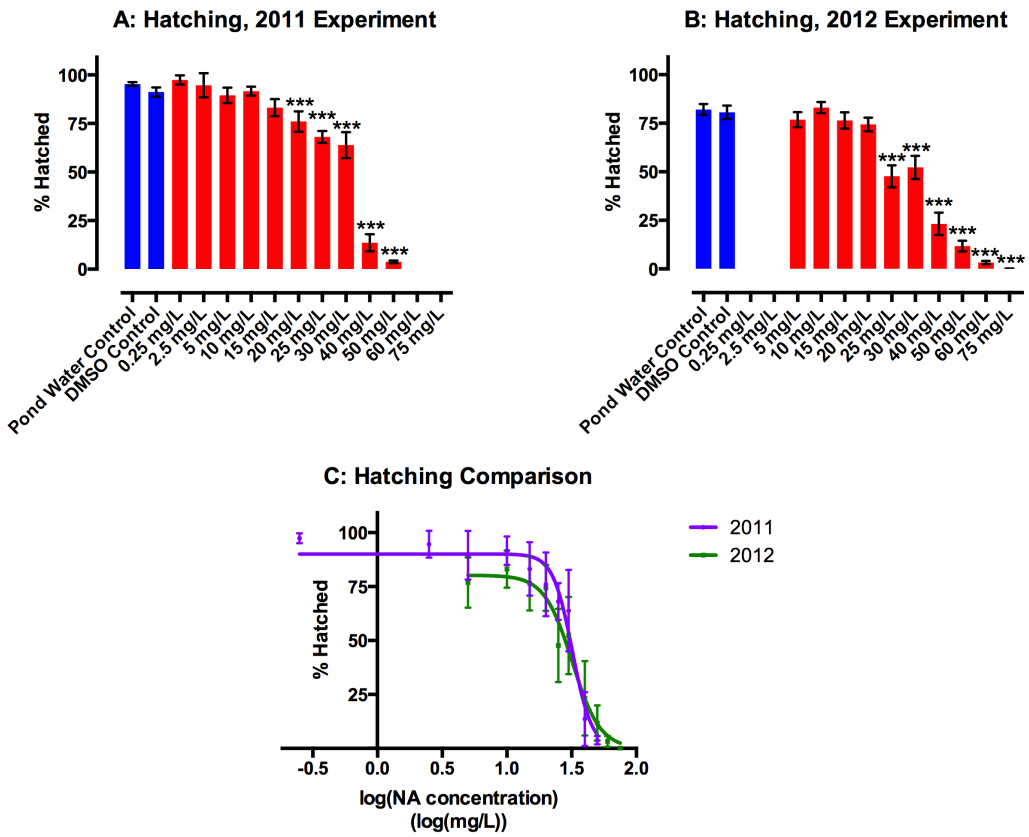

Figure 2.5 Concentration Dependence of *Lymnaea* Embryo Hatching

A: 2011 dose-response experiment. Hatching proportions at day 28 are shown. Note that hatching proportions are higher in A than in B due to observation at a later time point. B: 2012 dose-response experiment. Hatching proportions at day 20 are shown. These hatching proportions are for the same animals for which size data are shown in Figure 2.3. C: 2011 (purple) and 2012 (green) hatching proportions modeled as log(inhibitor) vs. response. Note that hatching proportions are higher in the 2011 data due to observation at a later time point. IC50 values were 32.05 mg/L and 31.95 mg/L for 2011 and 2012, respectively. R^2 was 0.8743 for 2011 and 0.8617 for 2012. Both A and B: Data represent the percentage of eggs that hatched in each egg mass. n = 9 egg masses for each group. A, B, and C: Data are shown as mean±SEM. Statistical significance is indicated with respect to DMSO control. *** $p < 0.0001$.

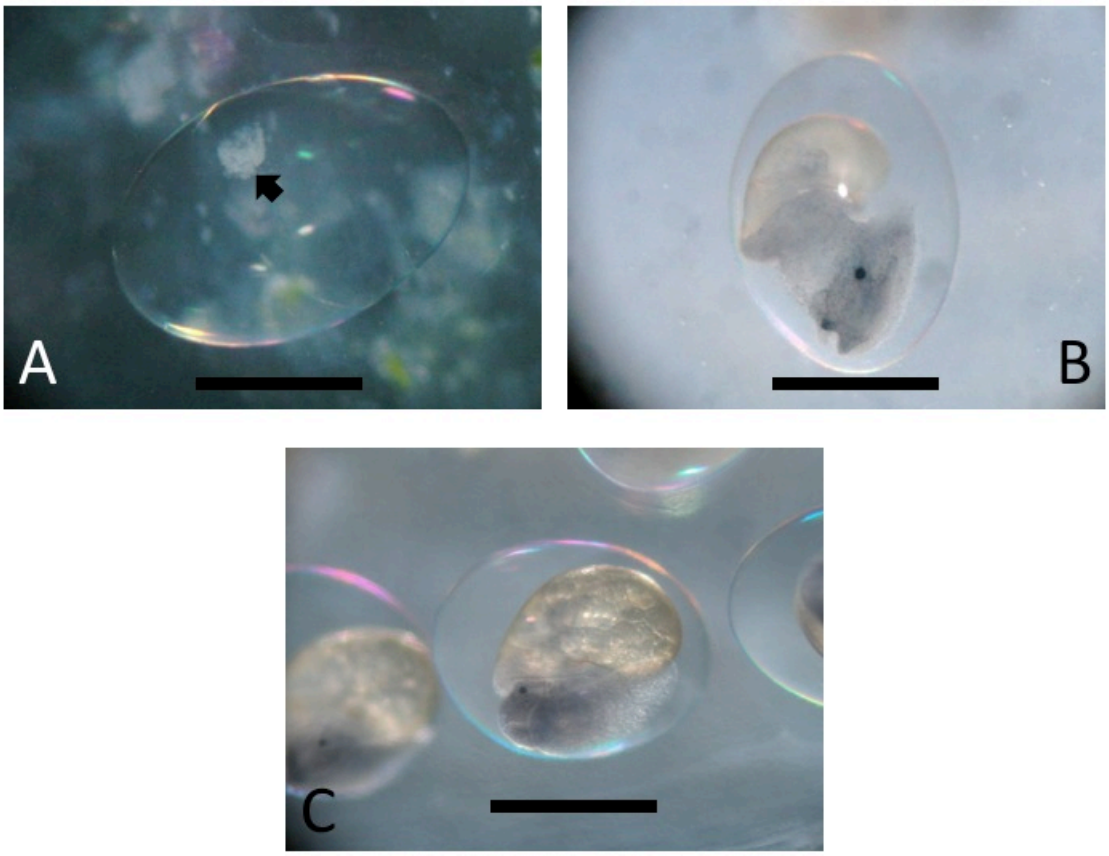

Figure 2.6 Arrested and Live *Lymnaea* Embryos

A: Early arrested embryo. The arrow points to the embryo within the egg capsule. The embryo is small, appears to be breaking apart, lacks easily distinguishable cells, and has a fuzzy, indistinct surface. B: Dead mature embryo. The embryo has dull, fuzzy skin and lacks organ definition in the visceral mass, which would be visible through the shell. Note also that the foot is not gripping the inside of the egg capsule. C: Live mature embryo. The embryo has smooth, shiny skin and visible organ definition in the visceral mass (visible through the shell). Note also that the embryo is gripping the inner surface of the egg capsule with its foot. A – C: Scale bars indicate 0.5 mm.

condition was observed in 1.83±0.61% of embryos in the pond water-only and 1.56±0.53% of embryos in the DMSO control. Very similar and statistically indistinguishable early stage embryonic arrest rates were found in all NA-exposed egg masses, including the highest concentrations (e.g., 0.44±0.30% for 20 mg/L NAs to 2.32±1.24% for 75 mg/L NAs; ANOVA, $F_{11,107} = 0.727$, $p = 0.710$). Among embryos in later developmental stages (i.e., >75% embryonic development, Figure 2.1D), a small proportion of embryos were arrested. Criteria for late stage arrest were lack of both *in ovo* heartbeat and embryo movement (i.e., rotation and gliding) at a developmental stage when they should display both of those phenomena. A late-stage arrested embryo can be seen in Figure 2.6B, and compared to a normal late-stage embryo in Figure 2.6C. There was no evidence that NAs promoted embryo death in later developmental stages (ANOVA, $F_{11,107} = 1.697$, $p = 0.0857$). The percentage of immobile and cardiac inactive late stage embryos was 0.13±0.13% for pond water control and 0.15±0.15% for DMSO control, while there were none in 5, 50, 60 and 75 mg/L NA test groups and 0.12±0.12% for 20 mg/L to 3.6±2.0% for the 25 mg/L among the remaining NA test groups.

2.3.1.3 Toxicity of Model NAs and Tailings-Derived NAs

To calibrate the effects of the mixture of 3 low molecular weight model NAs used in this study against a much more complex oil sands tailings pond sample, I conducted a hatching study using the acid-extracted organic fraction of tailings water from a closed pond that no longer receives fresh tailings (collected February 2010). The hatching rates of embryos exposed to the tailings sample are shown in Figure 2.7, along with the hatching rates from a similar experiment using model NAs, conducted in 2011, immediately following the hatching experiment using tailings-derived NAs (see Figure 2.5A). The effects on hatching are remarkably similar between

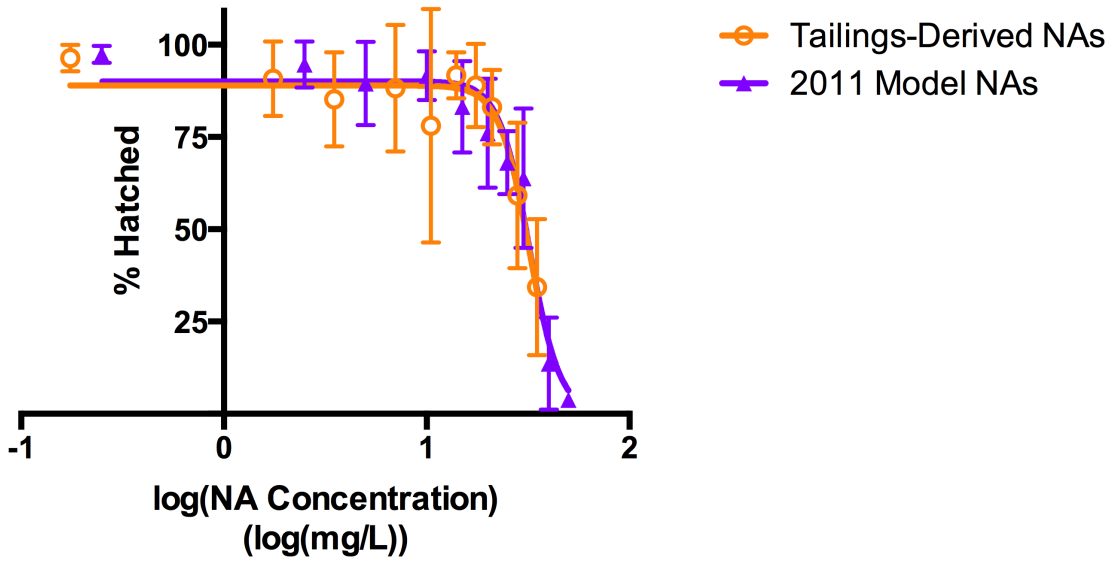

Figure 2.7 Model NA Toxicity Compared to Tailings-Derived NA Toxicity

Tailings-derived NAs were extracted from samples collected from a closed pond that no longer receives fresh tailings in February, 2010. Model NAs were cyclohexylsuccinic acid, cyclohexanebutyric acid, and 4-*tert*-butylcyclohexane carboxylic acid, used in equal parts by mass. Hatching proportions at day 28 are shown for egg masses exposed to model NAs (purple) and tailings-derived NAs (orange). Hatching proportions were fit to log(inhibitor) vs. response model. IC50 values were 32.05 mg/L for model NAs and 32.04 mg/L for tailings-derived NAs. R^2 values were 0.8743 for model NAs, and 0.5499 for tailings-derived NAs. The model NA data shown in this figure is the same as the 2011 model NA data shown in Figure 2.5A and 2.5C.

the two experiments. Indeed, the log(inhibitor) vs. response models fitted to both data sets were virtually indistinguishable from each other, having IC50 values of 32.05 and 32.04 mg/L, respectively (R^2 = 0.8743 for model NAs and 0.5499 for tailings-derived NAs). Thus, the model NAs used in this study appear to have similar embryonic toxicity to tailings-derived NAs on a mass concentration basis.

2.3.2 Critical Embryonic Exposure Period

Having found that NA exposure during embryonic development affects embryo size, I chose to investigate whether this effect was due to the overall pace of somatic growth, the timing of developmental milestones, or some combination of those factors. To this end, I investigated the effect of duration and timing of NA exposure on *Lymnaea* embryonic development by exposing 5 different sets of egg masses to 30 mg/L NAs over the following varying periods of embryonic development: three of the groups were exposed during, respectively, days 0 to 4, days 4 to 8, and days 8 to 12, one group was exposed during days 0 to 12, one group was exposed during days 12 to 28, and one group was exposed to NAs for the entire test period of 28 days (see Figure 2.2). Developmental progression, hatching rates, embryo death, and embryo size were monitored at regular intervals over a period of 28 days.

2.3.2.1 Embryo Size and Development

The duration and timing of NA exposure had a significant effect on the embryo-to-egg ratio of un-hatched embryos at day 12 (Figure 2.8; ANOVA, $F_{5,271} = 38.712$, $p < 0.0001$). Specifically, embryos exposed to NAs after day 4 were smaller than control embryos whereas embryos exposed to NAs on days 0 to 4 only were not different in size from control animals

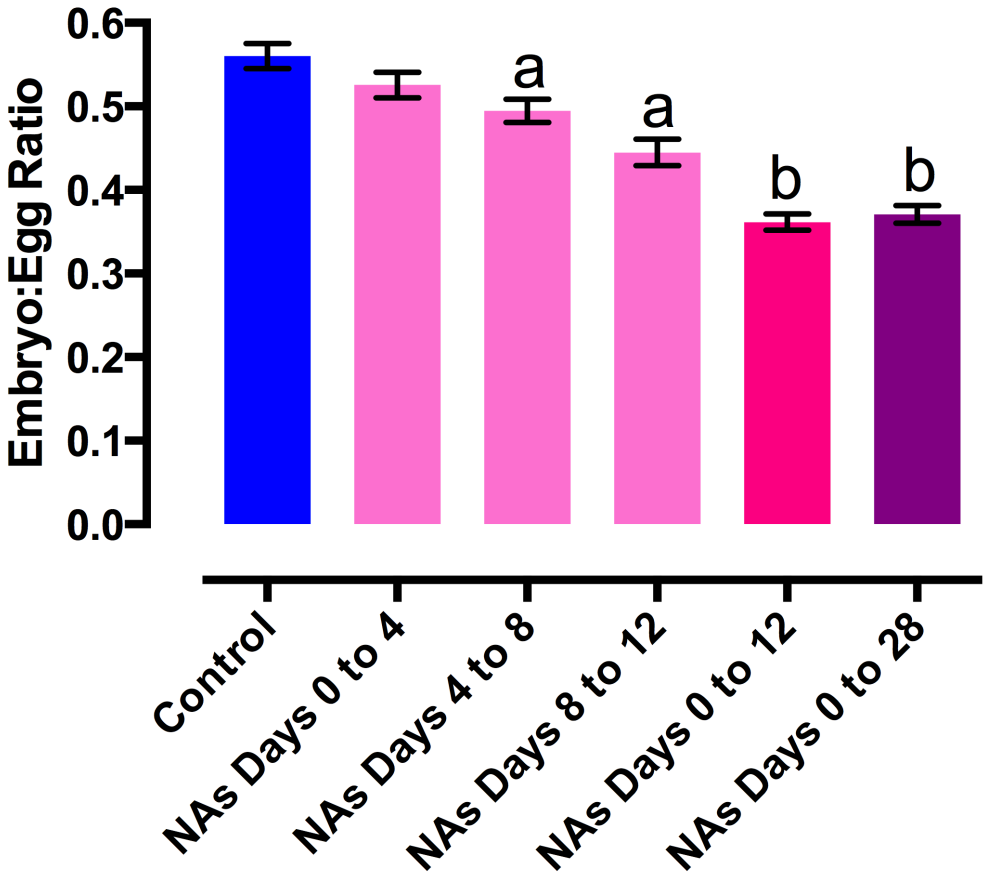

Figure 2.8 Growth during NA Exposure

Incubation media was either pond water or pond water with 500 ppm dimethyl sulfoxide (DMSO) and 30 mg/L naphthenic acids (NAs). Incubation media was changed on days 4, 8, and 12 for all groups. Treatment groups were pond water only ("Control"), or 30 mg/L NAs at various developmental stages and durations ("NAs Days a to b"). Embryo-to-egg ratios at day 12 are shown. Note that, at this stage in the experiment, the groups "NAs Days 0 to 12" and "NAs Days 0 to 28" had received identical NA exposure. Sample sizes range from 37 embryos for "NAs Days 0 to 4" to 60 embryos for "NAs Days 0 to 28". Data are shown as mean±SEM. Bars with letters differ from controls ($p < 0.05$). Bars with different letters are significantly different from each other ($p < 0.05$). Bars with the same letter do not differ from each other.

(Dunnett's test, $p = 0.00332$ for days 4 to 8, $p < 0.0001$ for days 8 to 12, 0 to 12, and 0 to 28). In addition, embryos exposed to NAs for 12 or 28 days were also smaller in comparison to embryos exposed to NAs for 4 days (Tukey's Test, $p < 0.0001$ for all relevant comparison pairs). Egg sizes differed marginally between treatments ($F_{5,266} = 2.521$, $p = 0.02989$), with only the days 0 to 4 NA group differing significantly from controls (Dunnett's test, $p = 0.016$). The average egg area was 1212±31.18 kilopixels for controls, and 1075±31.98 kilopixels for the days 0 to 4 NA group. The sizes of eggs in this group are not far from the egg sizes in the other NA-exposed groups, which ranged from 1145±28.81 kilopixels for the days 4 to 8 NA group to 1212±30.89 kilopixels for the days 0 to 28 NA group. Thus, although the sizes of the days 0 to 4 NA group may be slightly overestimated, the embryo-to-egg ratio can still be considered a reasonable estimate of embryo size.

Embryos in the 28-day exposure group that did not hatch during the 28 days of the experiment were larger on day 28 than they were on day 12 (ANOVA, $F_{1,112} = 111.451$, $p < 0.0001$). There were not enough un-hatched control embryos left at day 28 to allow for a comparison, so day 20 DMSO control embryos from the dose-response experiment were used instead. There was no size difference between control embryos and day 28 continuous NA-exposed embryos (ANOVA, $F_{1,84} = 1.141$, $p = 0.289$), suggesting that the surviving un-hatched 28-day NA exposed embryos had reached normal sizes for late adult-like embryos. Thus, despite the fact that they did not emerge from their egg capsules, these surviving embryos continued to grow during this period.

There was no evidence of differences in developmental stages between handling controls and any NA-exposed group at day 4 (Figure 2.9A; χ^2 test not possible due to lack of variation),

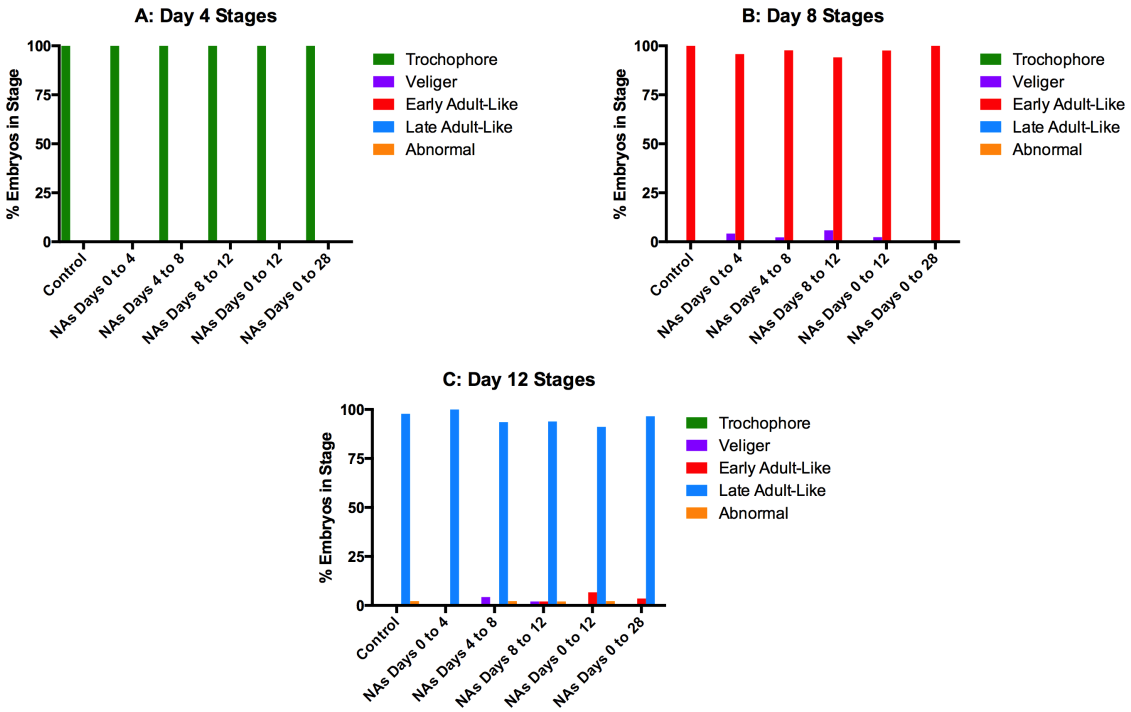

Figure 2.9 Development during NA Exposure

Developmental stages at days 4, 8, and 12. Stages are trochophore (green), veliger (purple), early adult-like (red), late adult-like (blue), and abnormal (orange). Data are shown as the fraction of scored embryos in each developmental stage for each treatment group. A: Day 4. Note that, at this stage of the experiment, there were essentially two treatment groups. “Control”, “NAs Days 4 to 8”, and “NAs Days 8 to 12” had been incubated in pond water only, while “NAs Days 0 to 4”, “NAs Days 0 to 12”, and “NAs Days 0 to 28” had been incubated in pond water with 500 ppm DMSO and 30 mg/L NAs. B: Day 8. C: Day 12. At days 8 and 12, both “NAs Days 0 to 12” and “NAs Days 0 to 28” had received identical NA exposures. Sample sizes range from 38 embryos for “Control” on day 8 to 57 embryos for “NAs Days 0 to 28” on day 12. There were no significant differences anywhere in the development data set.

day 8 (Figure 2.9B; χ^2 (5, n = 266) = 3.103, p = 0.684), or day 12 (Figure 2.9C; χ^2 (5, n = 288) = 10.558, p = 0.783).

2.3.2.2 Hatching

The timing and duration of NA exposure also affected hatching rates of NA-exposed embryos (Figure 2.10A; ANOVA $F_{5,71} = 42.421$, $p < 0.0001$). That is, embryos exposed to NAs for 12 or 28 days had lower hatching rates than controls, while hatching rates for the 4-day NA exposure groups were not different from controls (Dunnett's test, p = 0.0352 for days 0 to 12, p < 0.0001 for days 0 to 28). The 28-day continuous NA exposure group had a lower hatching rate than any other NA-exposed group (Tukey's test, p = 0.00013 for all relevant comparisons).

There was no evidence that NA exposure at any stage or duration promoted early stage arrest of embryonic development (ANOVA, $F_{5,71} = 0.652$, p = 0.661). Control embryos had an early stage arrest rate of 1.56±0.59%, while NA-exposed embryos had arrest rates ranging from 0.92±0.31% for the days 0 to 4 group, to 2.23±1.48% for the days 4 to 8 group.

In contrast, there was a significant effect of NA exposure duration on death rates of late adult-like embryos (Figure 2.10B; ANOVA $F_{5,71} = 10.663$, $p < 0.0001$). Embryos in the 28-day NA exposure group had higher rates of death observed on day 28 than control embryos, whereas embryos exposed to NAs for shorter durations did not differ from control embryos (Dunnett's test, $p < 0.0001$ for days 0 to 28).

2.3.3 Toxicokinetics of Lymnaea Eggs

Lymnaea eggs are enclosed by a membrane, and have roughly the same shape as a chicken egg. These eggs are packaged in groups of 10 to 100, and enclosed in a tough, gelatinous

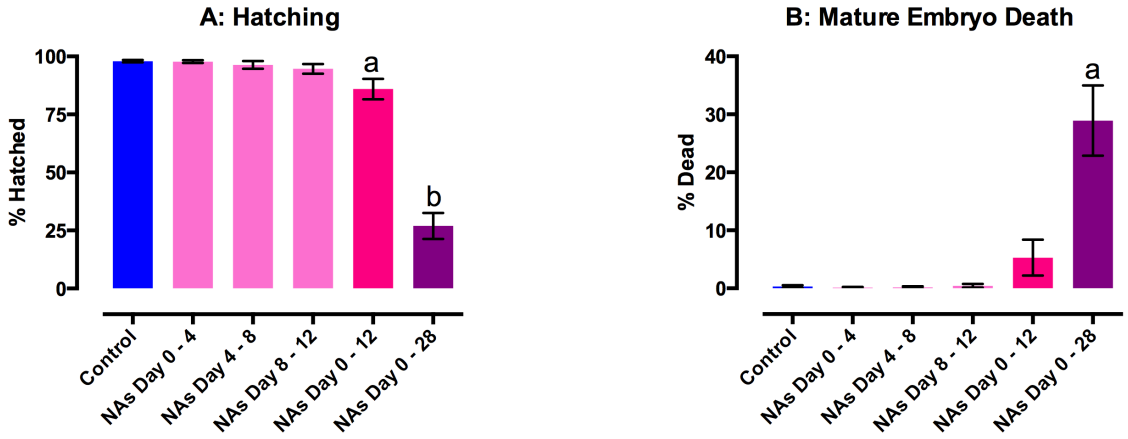

Figure 2.10 Hatching and Mature Embryo Death during NA Exposure

Incubation media was either pond water or pond water with 500 ppm dimethyl sulfoxide (DMSO) and 30 mg/L naphthenic acids (NAs). Incubation media was changed on days 4, 8, and 12 for all groups. Embryos were incubated in pond water only ("Control"), or 30 mg/L NAs at various developmental stages and durations ("NAs Days a to b"). A: Hatching proportions at day 28. n = 12 egg masses per group. Bars with letters differ from handling controls ($p < 0.05$). Bars with different letters are significantly different from each other ($p < 0.05$). Bars with the same letter do not differ from each other. Data are shown as mean±SEM. B: Rates of mature embryo arrest at day 28. Arrest was assessed by lack of movement and heartbeat. n = 12 egg masses per group. Data are shown as mean±SEM. Bars with letters differ from controls ($p < 0.05$). Bars with different letters are significantly different from each other ($p < 0.05$).

casing forming an egg mass. A recent study has shown that snail embryos (*Radix auricularia*) can be more vulnerable to aquatic toxins when eggs are isolated from their egg mass (Liu *et al*., 2013). Thus, to test the possibility that the egg mass casing protects *Lymnaea* embryos from NAs, I used gas chromatography-mass spectrometry to analyze the NA content of pond water, egg mass casings, and isolated eggs, after incubation of intact egg masses in 30 mg/L NAs.

All three model NAs used in this study were absorbed into *Lymnaea* eggs after 12 days of incubation, albeit at different rates. That is, the egg/water ratio was 83.2±11% for CHBA, 177±14% for 4-TBCA, and only 5.1±5.1% for CHSA (Figure 2.11).

Overall, the effective NA concentration detected in eggs after 12 days of exposure corresponds to 88.4% of the concentration in which NAs were added in the water, or about 26.5 mg/L when incubated in 30 mg/L NAs. No NAs were detected in eggs after 4 days of incubation, nor in egg mass casings at any exposure duration. The NA concentrations in water containing egg masses did not change over the duration of the experiment, thus NAs were not being depleted from the water due to diffusion into egg masses.

2.3.3.1 Compartmentalization of *Lymnaea* Egg Masses

To investigate whether the egg mass casings have a protective effect on development and hatching during NA exposure in water, I conducted an experiment with 0.1, 1, 5, and 20 mg/L NAs, as well as pond water and DMSO controls. For each treatment, half of the egg masses were left intact, while the other half of the egg masses had their eggs isolated. Developmental progression and hatching rates of embryos were monitored for 20 days.

Among embryos in intact egg masses, there was a significant dose-dependent hatching decrease after 20 days (Figure 2.12A; ANOVA, $F_{5,48} = 3.549$, $p = 0.008245$). Only the 20 mg/L

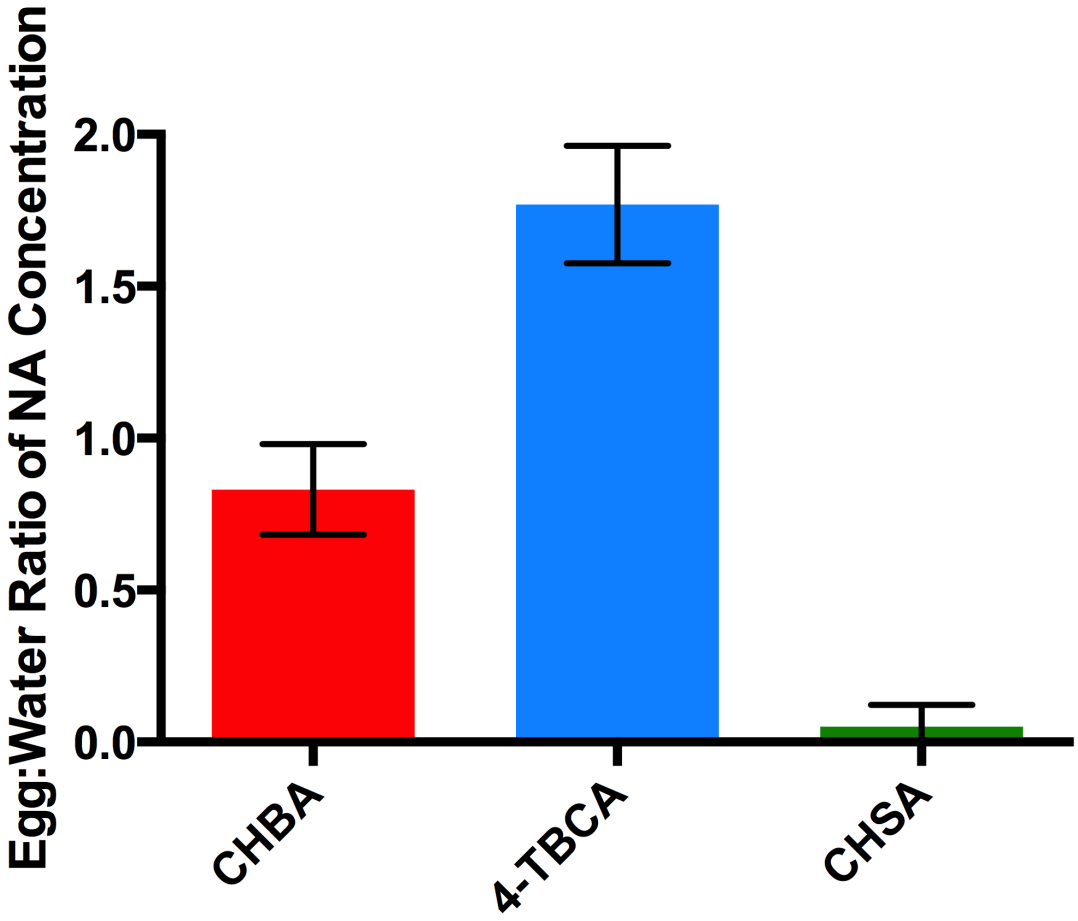

Figure 2.11 Model NA Penetration into *Lymnaea* Eggs

NA concentrations in eggs compared to the NA concentration in the water in which the eggs were incubated, as determined by gas chromatography-mass spectrometry analysis. Egg masses were incubated in NA solutions individually in 6-well plates. After 12 days of incubation, water samples were collected and eggs were removed from their egg masses. Water samples contained the pooled water from 3 wells, egg samples contained the pooled eggs from the egg masses in the same 3 wells as for the water samples, and casing samples contained the pooled egg mass casing from the same 3 egg masses. 2 sets of samples were tested (ie. 2 sets of 3 wells and egg masses), and each sample was analyzed in triplicate. Data are shown as mean±SEM.

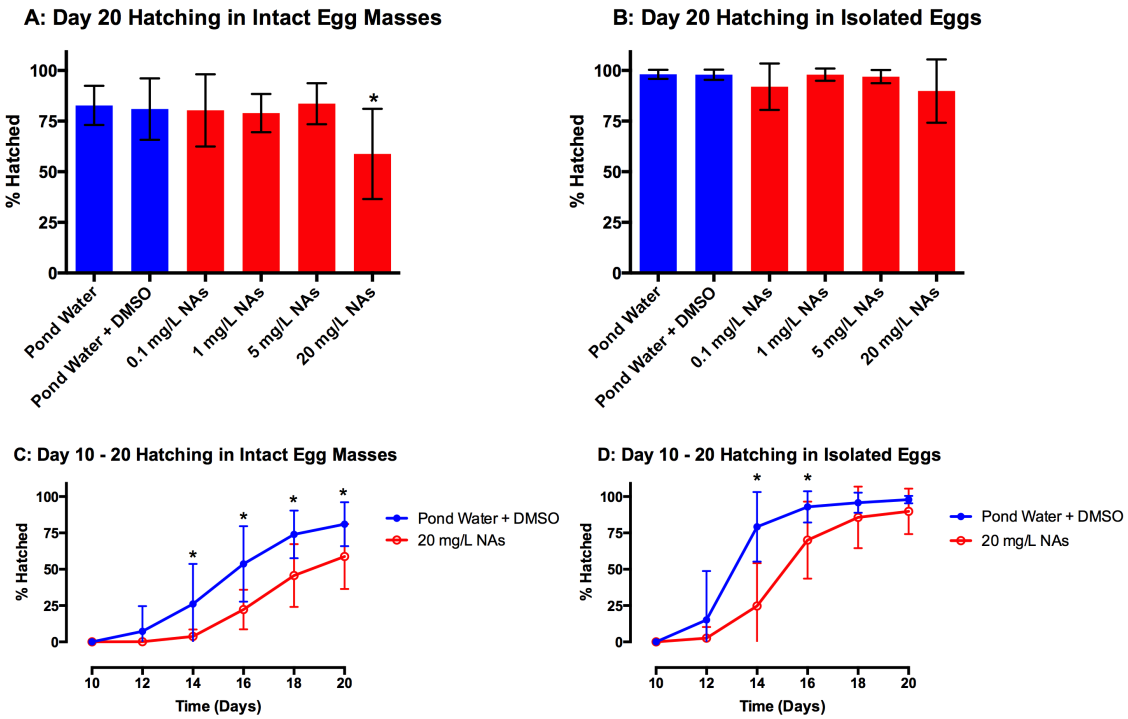

Figure 2.12 Effects of Egg Mass Casing on Hatching during NA Exposure

Effects of egg mass casing on hatching during NA exposure. Hatching rates are shown for *Lymnaea stagnalis* eggs that were exposed to NAs either in intact egg masses, or isolated and placed directly in the NA solution. A: Intact egg masses, % hatched after 20 days. B: Isolated eggs, % hatched after 20 days. Both A and B: Hatching rates for egg masses in pond water only ("Pond Water Control", dark grey), pond water with 500 ppm dimethyl sulphoxide (DMSO) ("DMSO Control", dark grey), or pond water with 500 ppm DMSO and varying concentrations of naphthenic acids (NAs) (NA concentrations, light grey). C: Intact egg masses, % hatched after 10-20 days. These are the same eggs for which day 20 hatching rates are shown in A. D: Isolated eggs, % hatched after 10-20 days. These are the same eggs for which day 20 hatching rates are shown in B. Isolated eggs perform better than eggs in intact egg masses, and are less susceptible to NA toxicity. A – D: Data are shown as mean±SEM. * $p < 0.05$.

group had a significantly lower hatching percentage compared to the DMSO control group (Dunnett's test, $p = 0.0154$), while the pond water, DMSO control, 0.1 mg/L, 1 mg/L, and 5 mg/L groups were statistically indistinguishable. The 20 mg/L NA group had a hatching percentage of 58.8±7.4, while the remaining groups had hatching rates ranging from 79.0±3.1% for 1 mg/L NA to 83.6±3.4% for 5 mg/L NA after 20 days. There was a significant effect of NA exposure and time on hatching rates throughout the observation period (Figure 2.12C; repeated measures ANOVA, $F_{20,192} = 2.7087$, $p = 0.003242$). Specifically, univariate results of the repeated measures ANOVA revealed significant effects of NA concentration at days 14 through 20 (day 14: $F_{5,48} = 2.535$, $p = 0.0409$; day 16: $F_{5,48} = 5.026$, $p = 0.00088$; day 18: $F_{5,48} = 4.459$, $p = 0.00204$; day 20: $F_{5,48} = 3.549$, $p = 0.00825$).

Embryos in isolated eggs generally hatched faster than embryos in intact egg masses, reaching 50% hatching after 13 days, compared to 15-16 days for intact egg masses (see Figure 2.12C and 2.12D). The number of hatchlings at 20 days was also higher for isolated eggs, which generally reached over 90% hatching. Unlike intact egg masses, the percentage of hatched embryos at day 20 in isolated eggs was not significantly affected by NA exposure (Figure 2.12B; ANOVA, $F_{5,46} = 1.614$, $p = 0.175$). The 20 mg/L NA group had a hatching rate of 89.9±5.2%, not far from controls at 98.1±0.8% for pond water control and 97.9±0.8% for DMSO control. Despite similar final hatching percentages, there was a significant effect of time and NA concentration on hatching rate (Figure 2.12D; repeated measures ANOVA; $F_{20,192} = 2.424$, $p = 0.000920$). This effect was apparent early in the hatching stage at days 14 and 16, but not at days 18 or 20 (univariate results of repeated measures ANOVA, day 14: $F_{5,46} = 6.655$, $p < 0.0001$; day 16: $F_{5,46} = 3.047$, $p = 0.0186$; day 18: $F_{5,46} = 1.654$, $p = 0.165$; day 20: $F_{5,46} = 1.614$, $p = 0.175$).

Thus, embryos in isolated eggs in 20 mg/L NAs showed delayed hatching early in their normal hatching period, but hatching caught up with controls well within the normal hatching period.

2.4 Discussion

In this study, I showed that a mixture of three model NAs have a number of detrimental effects on *Lymnaea stagnalis* embryos. A consistent, dose-dependent decrease in the number of hatchlings occurred from 25 mg/L to 75 mg/L, at which point hatching was essentially zero. *Lymnaea* embyros withstood exposure to 30 mg/L NAs for nearly 12 days with only small decreases in hatching success, but exposure for 12 to 28 days resulted in substantial decreases in hatching. Somatic growth was delayed at 60 and 75 mg/L NAs, but the pace of development was unaffected. Thus, the effects of these model NAs on snail embryos include growth and hatching delays. I did not observe any differences in embryo survival during the first 20 days of development, thus no direct lethal effects were seen during development. Embryos that failed to hatch during the first 20 days of development generally died in their egg, thus failure to hatch within 3 to 4 weeks is a lethal outcome. The model NAs used in this study displayed very similar mass concentration/effect relationship in the hatching assay as the tailings-derived NA mixture, even though the total molar concentrations of NAs in both mixtures were likely quite different. Toxic effects of the model NAs on *Lymnaea* embryos were similar to those observed in fish and frog embryos (Peters *et al*., 2007; He *et al*., 2012; Melvin and Trudeau 2012), but occurred at much higher mass concentrations in *Lymnaea*. This apparent difference in susceptibility is not due to better protection from the materials surrounding eggs in their egg masses, suggesting that *Lymnaea* embryos are more resilient to NA exposure than other aquatic species tested thus far.

2.4.1 Effects of Naphthenic Acids on **Lymnaea** *Embryos*

2.4.1.1 Decreased hatching rates are consistent and repeatable

At 25 mg/L NAs and above, a clear dose-dependent decrease in hatching rate was observed, both during the initial run in the summer and fall 2011, and when it was repeated with a different cohort of egg-laying snails in the spring of 2012. The range of NA concentrations used in the present study is consistent with those found in active tailings ponds (Holowenko *et al*., 2002; Holowenko *et al*., 2000; Schramm *et al*., 2000). At these relatively high concentrations, the toxic effects of NAs on hatching are sufficient to overwhelm any inherent variation in hatching time or NA susceptibility, leading to consistent hatching decreases. Thus, with slight differences at moderate NA concentrations (20 to 25 mg/L), this assay is highly repeatable in its ability to detect detrimental effects of NAs on hatching.

2.4.1.2 Failure to hatch is a lethal outcome

The mixture of model NAs used in the current study was not acutely lethal to *Lymnaea* embryos during their development (within 2-3 weeks of egg deposition). NAs are acutely lethal to fathead minnow embryos, with an estimated LC_{50} of 32.6 mg/L during embryonic exposure to NAs extracted from oil sands process-affected water (OSPW) (Kavanagh *et al*., 2012). Japanese medaka embryos had 100% mortality in 20 mg/L commercial naphthenic acids, as well as in untreated OSPW containing 71.03 mg/L NAs, while Yellow perch embryos showed 100% mortality in 20 mg/L commercial NAs (Peters *et al*., 2007). The northern leopard frog embryos had an LC_{50} of 4.1 mg/L in commercial NAs, while the western-clawed frog had an LC_{50} of 2.95 mg/L during embryonic exposure to commercial NAs (Melvin & Trudeau, 2012). By contrast, *Lymnaea* embryos showed no increased mortality during development (2 to 3 weeks post-

deposition) at any NA concentration tested (up to 75 mg/L). A summary of the effects of NAs on aquatic species during embryonic exposure can be seen in Table 2.1.

Lymnaea embryos that had not hatched by the end of the experiment (20 or 28 days) typically did not hatch at all. Tested egg masses were observed 1 to 3 months after the conclusion of the hatching experiments, and little additional hatching had occurred. Un-hatched embryos were immobile and showed no heartbeat, and were therefore considered dead. Higher rates of late adult-like embryo death were observed at day 28 in the critical exposure period experiment, while no increase in embryo death was observed at day 20 in the dose-response experiment. This suggests the existence of a critical hatching timeframe where embryo death occurs if hatching does not take place within this period. Therefore, failure to hatch within 4 weeks appears to be a lethal outcome for *Lymnaea* embryos. Death might result from nutrient depletion, since most of the embryo's nutrition comes from the perivitelline fluid inside its egg capsule (Nagle *et al*., 2001; Wijsman & van Wijk-Battenburg, 1987), while waste product build-up might contribute as well. Thus, even though the mixture of model NAs used in the present study do not have direct, acutely lethal effects on *Lymnaea* embryos during their development, delayed hatching in itself appears to have lethal consequences.

The effects of this mixture of model NAs on hatching was indistinguishable from the effects of NAs extracted from a closed pond that no longer receives fresh tailings, both having IC50 values of about 32 mg/L. Since egg masses exposed to both types of NAs had similar hatching rates after 28 days of exposure, they may also have the same effect on late-stage embryo death.

Table 2.1 Effects of Embryonic NA Exposure on Aquatic Animals

Threshold concentrations for the effects of embryonic NA exposure in various aquatic animal species are shown. NA mixtures differed between experiments, thus comparisons are reasonable but not direct. OSPW: oil sands process-affected water. CNAs: commercial naphthenic acids. MNAs: model naphthenic acids.

Species	Small Size Threshold (mg/L)	Deformity Threshold (mg/L)	Lethality Threshold (mg/L)	Reference
Yellow Perch *Perca flavescens*	1.92 (OSPW) 0.88 (CNAs)	7.52 (OSPW) 1.67 (CNAs)	--- 4.89 (CNAs)	Peters *et al*., 2007
Japanese Medaka (Rice Fish) *Oryzias latipes*	6.18 (OSPW) 1.44 (CNAs)	30 (OSPW) 1.51 (CNAs)	--- 4.49 (CNAs)	Peters *et al*., 2007
Fathead Minnow *Pimephales promelas*	---	1.9 (OSPW)	19.7 (OSPW)	He *et al*., 2012
Northern Leopard Frog *Lithobates pipiens*	2.0 (CNAs)	4.0 (CNAs)	4.10 (CNAs)	Melvin & Trudeau, 2012
Western Clawed Frog *Silurana tropicalis*	2.0 (CNAs)	---	2.95 (CNAs)	Melvin & Trudeau, 2012
Great Pond Snail *Lymnaea stagnalis*	60 (MNAs)	75 (MNAs)	>75 (MNAs)	Current Thesis

2.4.1.3 NA exposure slows somatic growth, not development

NA-exposed embryos in the critical exposure experiment reached the same developmental stages as each other and as controls at days 4, 8, and 12, regardless of the duration or developmental period of NA exposure. Thus, NA-exposed embryos did not show any delays in reaching developmental milestones. Embryos in 30 mg/L NAs were smaller than controls at day 12 and day 20, but reached normal size by day 28. Therefore, *Lymnaea* embryos grow continuously during NA exposure, although this somatic growth occurs at a slower rate than in controls.

The apparent size reductions of embryos were also concentration-dependent. In the highest concentrations tested, 60 and 75 mg/L, embryos were much smaller than controls and than embryos in lower NA concentrations after 20 days. This suggests that the somatic growth rate is substantially slowed in NA-exposed embryos at these high concentrations. Small sized embryos were seen at 2 mg/L commercial NAs in both the northern leopard frog and the western-clawed frog (Melvin & Trudeau, 2012). Small yellow perch embryos were observed at 1.12 mg/L commercial NAs and in OSPW containing 7.52 mg/L NAs, while small Japanese medaka embryos were seen at 1.4 mg/L commercial NAs and in OSPW containing 6.18 mg/L NAs (Peters *et al*., 2007). With *Lymnaea*, however, reductions in embryo size began at 60 mg/L model NAs. Thus, *Lymnaea* embryos appear to be less sensitive to growth impairments during NA exposure than freshwater vertebrate embryos (see Table 2.1).

At 75 mg/L, I also observed a large increase in embryos with anatomical abnormalities, with roughly 40% of embryos showing abnormalities, compared to less than 1% of embryos at all other concentrations. Deformities were observed at 4 mg/L commercial NAs in northern leopard frog embryos (Melvin & Trudeau, 2012). Deformities were seen in yellow perch

embryos at 1.67 mg/L commercial NAs and in OSPW containing 7.52 mg/L NAs, and in Japanese medaka embryos at 1.51 mg/L commercial NAs and in OSPW containing 30 mg/L NAs (Peters *et al*., 2007). Deformities were observed in fathead minnow embryos exposed to OSPW containing 20 mg/L NAs (He *et al*., 2011). *Lymnaea* embryos, however, showed morphological abnormalities only at 75 mg/L. Thus, as I also saw with lethality and growth, *Lymnaea* embryos show the same detrimental effects from NA exposure as freshwater vertebrate embryos, but only at much higher concentrations (see Table 2.1).

2.4.1.4 Embryonic NA toxicity is duration- and developmental stage-dependent

When embryos were exposed to NAs for different durations and developmental stages, hatching success declined only in groups exposed to NAs for 12 or 28 days. Size declined only in embryos exposed to NAs after day 4, when they were in the early veliger stage. Thus, *Lymnaea* embryos appear to be more sensitive to NA exposure during the middle and later developmental stages than during early development.

NA exposures of *Lymnaea* embryos for longer durations are associated with more pronounced detrimental outcomes. None of the 4-day exposure treatments showed a decrease in hatching. The hatching rate in the 12-day exposure treatment (85.9±4.4% hatched) was lower than in any of the 4-day exposure treatments, but significantly higher than the 28-day exposure treatment (27.0±5.6% hatched). Thus, hatching attenuation only occurs with NA exposures longer than 4 days. Under the environmental conditions used in the current study, hatching of embryos typically occurred between day 12 and day 20. Thus, my data indicate that hatching attenuation is greatly increased if NAs are present in the environment during the period during

which hatching would normally occur. This suggests that NAs interfere with mechanisms by which embryos emerge from their eggs and egg masses.

2.4.1.5 *Lymnaea* eggs are selectively permeable to model NAs

Lymnaea eggs and egg masses were readily permeable to two of the model NAs used in this study, CHBA and 4-TBCA. However the third model NA, CHSA, was almost completely excluded from eggs. All three model NAs were detected in the water and eggs, but no NAs were detected in the gelatinous material in the egg mass surrounding the eggs, suggesting that the outer casing of *Lymnaea* egg masses is selectively permeable to different NA structures. A notable difference between the almost impermeable CHSA and the readily permeable CHBA and 4-TBCA is that CHSA has two carboxyl groups, while CHBA and 4-TBCA have only one carboxyl group each. CHSA molecules would therefore be more hydrophilic and more bulky, either of which could contribute to its lower permeability. Young *et al*. (2011) found that NAs are able to enter the eggs of exposed adult rainbow trout, but did not address the effects of NA structure on egg permeability.

The total NA concentration within the eggs reached 88.4% of the NA concentration in the water after 12 days of exposure. This suggests that the NA concentration in the eggs could reach the same level as the surrounding water by the time most embryos are ready to hatch, but the composition of NAs in the eggs would be different from the NA composition in the water. Although this suggests that not all NAs have the same toxic effects on embryos, once hatchlings emerge from their egg masses they will be exposed to all NAs present in the water. Thus, any analysis of egg mass permeability to NAs must be considered in the context of NA toxicity to hatchlings. I am not aware of any studies of the effects of NA exposure on *Lymnaea* hatchlings.

2.4.1.6 Egg mass casings do not protect *Lymnaea* embryos at low NA concentrations

Since *Lymnaea* embyros appeared to be less susceptible to model NA toxicity than fish and frog embryos in every category where comparisons were available (lethality, growth, and deformities), I hypothesized that snail embryos may be better protected from their chemical environment than their vertebrate counterparts. Pond snail eggs can be removed from their protective egg mass with no noticeable detrimental effects on their development, and this method has been used to assess the toxicity of cadmium to *Radix auricularia* embryos (Liu *et al*., 2013), and to investigate the effects of solvents and tributyltin on *Lymnaea* embryos (Bandow & Weltje, 2012).

Egg mass content can act as a barrier between the embryo and its environment, which comes with benefits and costs for snail embryos. For instance, this barrier can have protective effects against predators (Marois & Croll, 1991), to keep eggs and hatchlings in a suitable habitat (Marois & Croll, 1991), and in some cases to keep toxicants away from embryos, as Liu *et al*. (2013) found with cadmium exposure. By contrast, egg masses can also act as a barrier to oxygen diffusion, slowing the development of embryos. Marois and Croll (1991) showed that hatching time in *Lymnaea* embryos was strongly correlated with position in the egg mass, with embryos at the ends and upper surface (in closer contact with their external environment) hatching faster than embryos on the bottom surface (more isolated from their external environment). Removing eggs from their casings resulted in faster and more synchronous hatching, likely due to increased gas exchange between the fluid inside the eggs and the medium housing the eggs (Marois & Croll, 1990). All of these hatching patterns are consistent with those observed in the present study. *Lymnaea* embryos also take up sodium, potassium, and calcium ions from the water surrounding their egg mass (Taylor, 1977), thus uptake of these ions may

also play a role in the hatching asynchrony seen in *Lymnaea* embryos. Since day 20 hatching rates in isolated eggs were not affected by NA exposure, but eggs in intact egg masses did have lower hatching rates in the presence of 20 mg/L NAs, the protective effects of the egg mass material against NAs is uncertain. Inside the egg masses, however, the embryos had to deal with reduced gas exchange capacity, and perhaps reduced ion uptake as well, in addition to the toxic effects of NAs, slowing their progress towards hatching.

The data presented here suggests that *Lymnaea* embryos in isolated eggs were less susceptible to the mixture of model NAs than embryos in intact egg masses. Lower hatching rates were only seen in 20 mg/L NA-exposed isolated eggs at 14 and 16 days, but hatching had essentially caught up with controls after 18 days. By contrast, intact egg masses exposed to 20 mg/L NAs had lower hatching rates than controls at every time point from day 14 to day 20. Thus, instead of imparting protection from NAs to *Lymnaea* embryos, my data seem to suggest that the *extra-ovo* structures of the egg mass may enhance the toxic effects of the model NA mixture. Noisette *et al*. (2014) concluded that encapsulation did not protect embryos of the marine snail *Crepidula fornicata* from acidification due to elevated CO_2 levels.

2.4.2 Mechanism of NA Toxicity

My study did not directly address mechanisms of NA toxicity, but their toxicity is generally believed to result from membrane disruption (He *et al*., 2011; Frank *et al*., 2009). NAs have a hydrophilic region and a hydrophobic region, just as phospholipids in biological membranes have. Because of this structural similarity, NAs in aqueous solutions will preferentially become incorporated into biological membranes with their aliphatic tails in the hydrophobic region of the membrane and their carboxyl group(s) in the hydrophilic region of the

membrane (Frank *et al*., 2009). This could alter local membrane architecture and fluidity, disrupting membrane-associated functions (Frank *et al*., 2009). The presence of a polar (hydrophilic) and non-polar (hydrophobic) region on NA molecules means that NAs act as surfactants (Frank *et al*., 2009; Schramm *et al*., 2000). This surfactant activity may act directly on embryonic tissues via membrane disruption, which could affect embryos by slowing cell division (Nethery, 1967), or impairing membrane-associated functions. NAs' surfactant activity could also affect embryos by disrupting protein folding. Indeed, surfactants are known to have the ability to interfere with protein structure and reduce enzyme activity (Ontzen, 2011). This mechanism would allow NAs to affect physiological functions that are not membrane-associated.

NAs have also been shown to have endocrine disrupting effects (Knag *et al*., 2013; Kavanagh *et al*., 2012; Thomas *et al*., 2009; Lister *et al*., 2008). Some of the endocrine disrupting effects of NAs have been attributed to aromatic carboxylic acids that mimic the structure of estrogens (Rowland *et al*., 2011b). Since none of my model NAs had aromatic structures, they are not likely to induce receptor-mediated endocrine disruption. Some of the endocrine disrupting effects seen in NA mixtures are believed to be non-receptor-mediated (Knag *et al*., 2013), and likely not dependent on an aromatic structure. This type of endocrine disruption may be possible with the model NA mixture used in this study.

While most non-hatching embryos appeared morphologically normal, my study was not designed to detect microstructural changes in *Lymnaea* embryos or eggs. Thus, it is possible that NA-exposed embryos failed to hatch due to anatomical defects in the embryos themselves, or changes in the structure or composition of the egg and egg mass materials, which I did not observe.

There is little information available on the mechanisms by which *Lymnaea* embryos escape from their eggs, so I will examine two possible scenarios based on what is known about *Lymnaea* embryos and the embryos of other mollusk species.

One mechanism by which some snail embryos (*Odontocymbiola magellanica* and *Chorus gigantus*) can escape from their egg capsules is by actively abrading the capsule membrane with their feeding structures (Bigatti *et al*., 2014; Leiva *et al*., 1998). *Lymnaea* embryos display rasping behavior beginning at 90% development (where 0% development represents fertilization and 100% development represents hatching) (Serfozo & Elekes, 2002; Voronzhskaya *et al*., 1999), and Voronzhskaya *et al*., (1999) suggest that this may be how *Lymnaea* embryos escape from their eggs. Marois and Croll (1991) found that faster embryonic development was correlated with earlier hatching within a single egg mass, suggesting that the hatching process could involve active participation from the embryo. Thus, if the embryos in this study had defects in their feeding structures, or if the egg material had become too tough for the embryos to rupture it, the embryos may have been unable to hatch for these reasons.

Besides the embryonic tissue itself, another possible target for NAs is the perivitelline fluid that surrounds the embryos inside the egg (Nagle *et al*., 2001; Wijsman & van Wijk-Battenburg, 1987). The environmental reservoir of NAs is the water surrounding the egg mass, and the embryo is the point farthest from this reservoir in the system consisting of the water, the egg mass content, the perivitelline fluid, and the embryo. Thus, the NA concentration in the perivitelline fluid would be higher than in embryonic tissue according to Fick's First Law of Diffusion. The perivitelline fluid may play an important role in the hatching process, since hatching can occur spontaneously in some mollusk species, such as the giant sea snail *Chorus giganteus*, without the active participation of the embryo (Leiva *et al*., 1998). The perivitelline

fluid contains a trypsin inhibitor that is likely able to inhibit the activity of both trypsin and chymotrypsin (Nagle *et al*., 2001). This suggests that the perivitelline fluid also contains proteases such as trypsin. This idea is supported by the notion that the presence of the trypsin inhibitor may reduce digestion of peptides and proteins that are important during embryonic development (Nagle *et al*., 2001). If these proteases are required to digest the egg capsule during hatching, a disruption in their activity by NA surfactant action might prevent embryos from hatching. The possibility of perivitelline fluid involvement with the hatching process does not preclude the possibility of egg mass materials being involved in the hatching process, nor the possibility of active participation of the embryo in its hatching.

Chapter Three: **Adult Naphthenic Acid Exposure**

3.1 Introduction

Naphthenic acids (NAs), carboxylic acids with saturated hydrocarbon tails, are generally believed to be the major toxic component of oil sands tailings ponds (Giesy *et al*., 2010; Clemente and Fedorak, 2005; Madill *et al*., 2001). NAs are acutely lethal in the milligram to tens of milligram per liter range to many species. For example, NAs are known to have lethal effects on juvenile and mature fish (e.g., Nero *et al*., 2005; Toor *et al*., 2013, Dokholyan & Magomedov, 1983), zooplankton (Dokholyan & Magomedov, 1983), and bacteria (Frank *et al*., 2008). However, even sub-lethal doses of NAs have been shown to affect a wide range of body functions, including disturbances in energy metabolism and host defense mechanisms, in many species.

As one of the main suspected toxicants of oil sands tailings ponds, much of the emphasis in the study of NA toxicity has focused on aquatic animals. This work has, however, very much focused on vertebrates. Comparatively little is known about the effects of NAs on adult aquatic mollusks, one of the largest and most diverse groups of aquatic biota. Cairns and Scheier (1962) observed acute lethal effects of commercial NAs (i.e., NAs produced by oxidizing petroleum distillates, see section 1.5.1 for more detail) in the freshwater snail *Physa heterostropha* below 20 mg/L. Dokholyan and Magomedov (1983) reported that no harmful effects were seen in several clam species up to 3 mg/L of commercial naphthenic acids, using survival and blood chemistry as endpoints (Dokholyan & Magomedov, 1983).

Since effective reclamation strategies must result in healthy ecosystems, the effects of toxicants must be examined at all trophic levels. To this end, I investigated chronic and acute NA

exposure of adult *Lymnaea stagnails*. They can live 2 to 2.5 years both in the wild (Lake Chany; Yorlova, 2003) and under laboratory conditions (Watson *et al*., 2014). As scavengers and primary consumers, they are an important link for energy transfer through freshwater ecosystems. I hypothesized that NA exposure would have detrimental effects on adult *Lymnaea*, focussing on the ecologically relevant endpoints of survival, growth and reproduction.

In this study, I exposed adult *Lymnaea* to 0.1, 1.0, and 4.0 mg/L NAs, comparable to NA concentrations found in the Athabasca River and ground waters in the Athabasca region (Clemente & Fedorak, 2005; Schramm *et al*., 2000). These concentrations are considered background concentrations, because they can be present throughout the Athabasca region, whether or not anthropogenic contamination has occurred. I observed their survival and reproduction during 13 months of NA exposure. This will be referred to as the chronic exposure study. After 8 to 10 months of NA exposure, I used a righting assay, which integrated the snails' ability to perceive a change in their special positioning, and their ability and motivation to respond to that change by returning to an upright position (Fei *et al*., 2007).

In addition, I exposed snails to higher NA concentrations of 10, 25, and 50 mg/L. These concentrations are higher than those found in Athabasca surface waters, and extend into active tailings pond NA concentrations (Holowenko *et al*., 2002; Holowenko *et al*., 2000; Schramm *et al*., 2000). Exposure in this study lasted 3 weeks, which was considered acute exposure. Acute exposure is usually limited to periods less than 2 weeks (IUPAC, 1997), but any short exposure duration can also be considered acute (IUPAC, 1997). Since 3 weeks is very close to the standard definition of acute exposure, and represents a very small proportion of *Lymnaea*'s lifespan, this experiment can reasonably be referred to as acute exposure.

In this acute exposure experiment, I quantified the number and timing of deaths during exposure to model NAs, while also assessing a number of fitness traits in surviving animals. I measured snails' ability to respond to a sugar stimulus to assess their ability to detect and respond to food signals, as well as their motivation to do so (Kemenes *et al*., 1986). I also quantified food consumption to see whether changes in evoked feeding responses were associated with changes in spontaneous feeding behavior. Food consumption rates also gave me insight into energy resources available for growth, reproduction, and immune responses. I observed the snails weekly and assigned them a score based on their spontaneous behavior, including locomotion, attachment to the substrate, emergence from the shell, and feeding movements. Like with the righting assay, the behavioral score is a method by which the snails' perception of and interaction with their environment can be investigated (Tuersley & McCrohan, 1987).

No studies have been conducted to date on the immune response of snails to NA exposure. *Lymnaea* has circulating blood cells called hemocytes, which perform similar functions to mammalian macrophages (Adema *et al*., 1993). These cells can phagocytize material and produce reactive oxygen species in response to foreign stimuli (Adema *et al*., 1993). The production of reactive oxygen species, with and without non-self stimulus, was used to investigate whether NA exposure affects *Lymnaea* immune function.

3.2 Materials and Methods

3.2.1 Animals and Housing Conditions

Animals were bred and raised in the laboratory as previously described (Hermann *et al*., 2007; Watson *et al*., 2012). Snails were housed at a density of no more than 1.5 animals per liter.

Animals were fed *ad libitum* with lettuce. Snails were kept in artificial pond water (APW) produced from purified water reconditioned to an initial conductivity of approximately 450 uS/cm by the addition of Instant Ocean salts (Aquarium Systems, Mentor, OH, USA) at 1 g per gallon. Calcium concentration was kept at a saturating level of ~60 mg/L by the addition of calcium carbonate (EMD analytics, Gibbstown, NJ, USA).

3.2.2 Model Naphthenic Acids

For all parts of this experiment, a mixture of equal parts by mass of cyclohexylsuccinic acid (CHSA), cyclohexanebutyric acid (CHBA), and 4-*tert*-butylcyclohexane carboxylic acid (4-TBCA) was used as model NAs. All NAs were obtained from Sigma Aldrich (St. Louis, MO, USA). The reported NA mass concentrations represent the sum of the mass concentrations of the individual model NAs. For example, a solution of 50 mg/L NAs contains 16.7 mg/L CHSA, 16.7 mg/L CHBA, and 16.7 mg/L 4-TBCA.

3.2.3 Population Experiment 1: Chronic, Low-Dose NA Exposure

Stock 4 mg/L naphthenic acid pond water (NAPW) was prepared in batches of 20, 25 or 50 L approximately every 3 weeks. NAPW was prepared using APW with 4 mg/L NAs (1.33 mg/L each of CHSA, CHBA and 4-TBCA) and 100 mg/L calcium carbonate. Since the saturation concentration of calcium carbonate is about 60 mg/L, not all calcium carbonate dissolved, however excess was added to ensure saturated buffering capacity in the water. Glass aquaria were filled with either undiluted NAPW (i.e., 4 mg/L NA tanks) or diluted with APW (for 1 mg/L NA and 0.1 mg/L NA tanks). Control tanks contained regular APW. Four aquaria with 15 snails each were assigned to each treatment, for a total of 240 snails in the experiment.

All snails were sexually mature adults (shell lengths 21 mm to 41 mm) aged 6 months and older. Size distribution was similar in all tanks and treatment groups. Over the course of the experiment, one control tank was excluded from the analysis due to an unusually high initial death rate. One third (5 L) of the water in each tank was replaced weekly, and excess calcium carbonate was added to each tank after water replacement. Any dead snails were removed daily. Egg masses were removed weekly, and eggs were observed and counted under a stereomicroscope.

3.2.4 Population Experiment 2: Acute, High-Dose NA Exposure

Since higher concentrations of the NAs are not water soluble, stock solutions of NAs were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA). Naphthenic acid pond water (NAPW) was prepared by adding 7.5 mL of NAs in DMSO to 15 L of APW in 20L glass aquaria providing final NA concentrations of 10, 25, and 50 mg/L, and a final DMSO concentration of 0.05 %v/v. Control tanks contained regular APW with 0.05 %v/v DMSO. The pH of the water in all treatments and controls was between 6.2 and 6.6. Four aquaria with 12 snails each were assigned to each treatment, for a total of 192 snails in the experiment. Snails were sexually mature adults (weights 1.0 to 2.5g). The exposure duration was 3 weeks. One third of the water (5L) in each tank was replaced halfway through the exposure time (1.5 weeks). Snails were weighed at the beginning of the experiment, and weekly thereafter using a Mettler PC 2000 balance (Mettler Toledo, Greifensee, Switzerland), readable to 0.01 g. Snails were fed *ad libitum* with Romaine lettuce. Lettuce fed to snails at any given time was kept as consistent as possible with respect to leaf and stem content, and radial position in the head. Lettuce

consumption was monitored by weighing lettuce before addition, then weighing uneaten lettuce removed 2 to 4 days later. Any dead snails were removed daily.

Egg masses were collected after 1.5 weeks and again after 3 weeks. Eggs were observed and counted under a stereomicroscope. Egg masses were analyzed for normal embryo packing as follows. Egg masses considered abnormal met at least one of the following criteria: 1. More than one egg containing multiple embryos, 2. Any egg containing more than three embryos, 3. An embryo not contained inside an egg, or 4. An egg located outside the egg mass casing, when the casing was undamaged. These eggs adhered to their egg mass by the gelatinous strings holding all of the eggs together before they were packaged into an egg mass, thus they were not dislodged from other egg masses.

3.2.5 Righting Response

A 14 cm diameter plastic dish (Falcon, Franklin Lakes, NJ, USA) was filled with 75 mL of regular APW (7.5 mm depth). Snails were tested for their righting response after 8 to 10 months of NA exposure according to protocol adapted from Fei *et al*. (2007). Snails were removed from their home tank and placed in the plastic dish. After a 5 min recovery period, each snail was manually flipped onto the back of its shell and a timer was started. The timer was stopped when the snail had returned to an upright position. After righting, snails were allowed to recover for 3 min before another righting trial was performed. A third righting trial was performed after another 3 min recovery period. If the snail took more than 5 min to right itself, and was not in the midst of a righting attempt at the 5 min mark, it was manually righted and a time of 10 min was recorded for that attempt. This was done to differentiate failures in righting

from successful righting attempts that took 5 minutes. After 3 righting trials, snails were returned to their home tank.

3.2.6 Behavioral Score

Each week animals were observed and assigned a behavioral score according to Tuersley and McCrohan (1987). Briefly, snails were placed individually in 200 mL of pond water in a 250 mL glass beaker and allowed to acclimatize for 5 min. Snails were then observed for 1 min and given a basic score out of 5 based on their general behavioral state. Snails fully retracted into their shell, including the mantle pulling away from the edges of the shell, were given a basic score of 0. Snails retracted into their shell with the head, foot, and mantle even with the aperture of the shell were given a basic score of 1. Snails withdrawn into their shells but with exploratory movements of tentacles received a score of 2. Snails whose heads emerged from their shell without head movement or locomotion were given a score of 3. Snails whose head and foot emerged from their shell, performing exploratory movements of the head and tentacles but without locomotion received a score of 4. Snails that had fully emerged from their shells and performed locomotor activity were given a score of 5. Snails were also given up to 5 bonus points for rasping (2 points), mouthing without radula movements (1 point), attaching to the substrate (1 point), floating (2 points), and rotating or shrugging the shell (1 point), for a maximum of 10 possible points. Since snails in poor physical condition can sometimes float involuntarily, snails were only given bonus points for floating if they had a high basic score of 4 or 5.

3.2.7 Sucrose Response

Lymnaea feed by scraping their radula along the substrate in movements called rasps (Kemenes *et al*., 1986; Dawkins, 1974), and rasping rates have been shown to increase in response to the presentation of sugar to the lip and tentacle area of the snails (Kemenes *et al*., 1986). Snails were placed in 80 mL of pond water in a plastic cup surrounded by mirrors and allowed to acclimatize for 6 minutes. Rasps ('bites') were then counted for 2 minutes with no stimulus. 10 mL of pond water was then added to the cup by syringe (water disturbance), and rasps were counted for a further 2 minutes. 10 mL of pond water with 40 g/L sucrose was then added by syringe (sucrose stimulus), and rasps were counted for a final 2 minute period in the final sucrose concentration of 0.4% The protocol was adapted from Hermann *et al*. (2013).

3.2.8 Oxidative Burst

The impact of NAs on Lymnaea's host defense system was assessed by assaying circulating hemocytes' ability to generate reactive oxygen species using a protocol adapted from Lacchini *et al*. (2006) and Hermann *et al*. (2013). After 3 weeks of NA exposure, hemolymph was collected from surviving snails by stimulating the foot with a pipette tip, triggering hemolymph ejection through the hemopore as the snail withdraws into its shell (Beaulieu *et al*. 2014; Hermann *et al*., 2013; Sminia 1972). Hemolymph was collected in 1.5 mL plastic microtubes and kept on ice. The volume of hemolymph collected depended on the size of the animal, and generally ranged from 200 to 600 µL. Samples were analyzed individually without pooling. Hydrogen peroxide production by hemocytes was quantified using a Life Technologies Amplex® Red Hydrogen Peroxide/ Peroxidase Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA; see Hermann *et al*. (2013) for details). Laminarin from *Laminaria digitata* (Sigma-

Aldrich, St. Louis, MO, USA) was used as an immune challenge (Hermann *et al*., 2013). Assays were conducted in saline containing (in mM): 51.3 NaCl, 1.7 KCl, 4.1 CaCl2, 1.5 MgCl2, and 10 HEPES at pH 7.9, in 96-well micro plates. Assay wells contained 33.3 µL of hemolymph in a total volume of 150 µL. Final concentrations of reagents were 33 µM amplex red and 0.066 U/mL horseradish peroxidase. Each sample was tested in 2 separate wells, one with 9 mg/mL laminarin and one with no laminarin. Fluorescence of the samples was determined with a SpectraMax 2Me multidetection microplate reader (Molecular Devices, Sunnyvale, CA, USA) at excitation 544 nm and emission 590 nm. Measurements were taken 2 hours after the addition of amplex red to samples. Amplex red fluorescence was measured in relative fluorescence units (RFUs) and shown as fractions of the signal produced by a 10 µM H_2O_2 internal standard.

3.2.9 Data Analysis and Statistics

Statistical analysis was done using Statistica version 7.1 (StatSoft Inc., Tulsa, OK, USA). Unless otherwise noted, analysis was done using a general linear model with NA concentration being treated as continuous variable. When a repeated measures ANOVA was used (weight and behavioral score over time), NA concentration was treated as a discrete variable. Survival was analyzed using the Log-rank test with a Bonferroni correction for multiple comparisons. Frequency of abnormal egg masses was analyzed by the Chi-square test on a 2 x 3 contingency table, with a Bonferroni correction for multiple comparisons. Graphic analysis of residuals and homogeneity of variances and parametric analyses (Levene's test, Hartley F-max test) confirmed that all data met parametric assumptions. Figures were prepared using GraphPad Prism version 4.03 (GraphPad Software, Inc., La Jolla, Ca, USA). Averages are shown as arithmetic mean ± standard error.

3.3 Results

3.3.1 Survival

To assess the potential lethal effects of exposure to various NA concentrations, I inspected the tanks every day for dead animals. During the 3-week acute exposure, survival among controls was 97.9±2.1%, while survival among NA-exposed snails was 95.8±2.4% for 10 mg/L, 77.1±20.2% for 25 mg/L, and 8.3±5.9% for 50 mg/L. Log-rank analysis showed a significant effect of NA concentration on survival (Figure 3.1A; χ^2 (3, n = 192) = 139.7, p < 0.0001). Multiple comparisons revealed that there was no difference in survival between the control and 10 mg/L NA groups (χ^2 (1, n = 96) = 0.3404, p = 0.5596). The 25 mg/L NA group had significantly lower survival than the control group (χ^2 (1, n = 96) = 84.66, p < 0.0001), as did the 50 mg/L NA group (χ^2 (1, n = 96) = 9.427, p = 0.0021). Most of the deaths in the 50 mg/L NA group occurred within the first week of exposure, while deaths in the 25 mg/L NA group were more evenly distributed throughout the 3-week exposure period. The LC50 for this mixture of NAs lies somewhere between 25 and 50 mg/L, but with only two data points in this range, the LC50 cannot be determined with more precision from this data.

During the 14-month chronic exposure, there was no significant effect of exposure to low NA concentrations on survival (Figure 3.1B; χ^2 (3, n = 225) = 3.928, df = 3, p = 0.2694). Survival declined to 31.1±5.9% for control, 23.3±5.8% for 0.1 mg/L NAs, 23.3±8.4% for 1.0 mg/L NAs, and 35.0±12% for 4.0 mg/L NAs. Deaths were fairly evenly distributed throughout the 13 month period in which survival was monitored. Considering that in our laboratory, *Lymnaea*'s maximal life expectancy is about 2 to 2.5 years (see Hermann *et al*., 2014), and experimental animals were already adults when the experiment began, these survival rates are

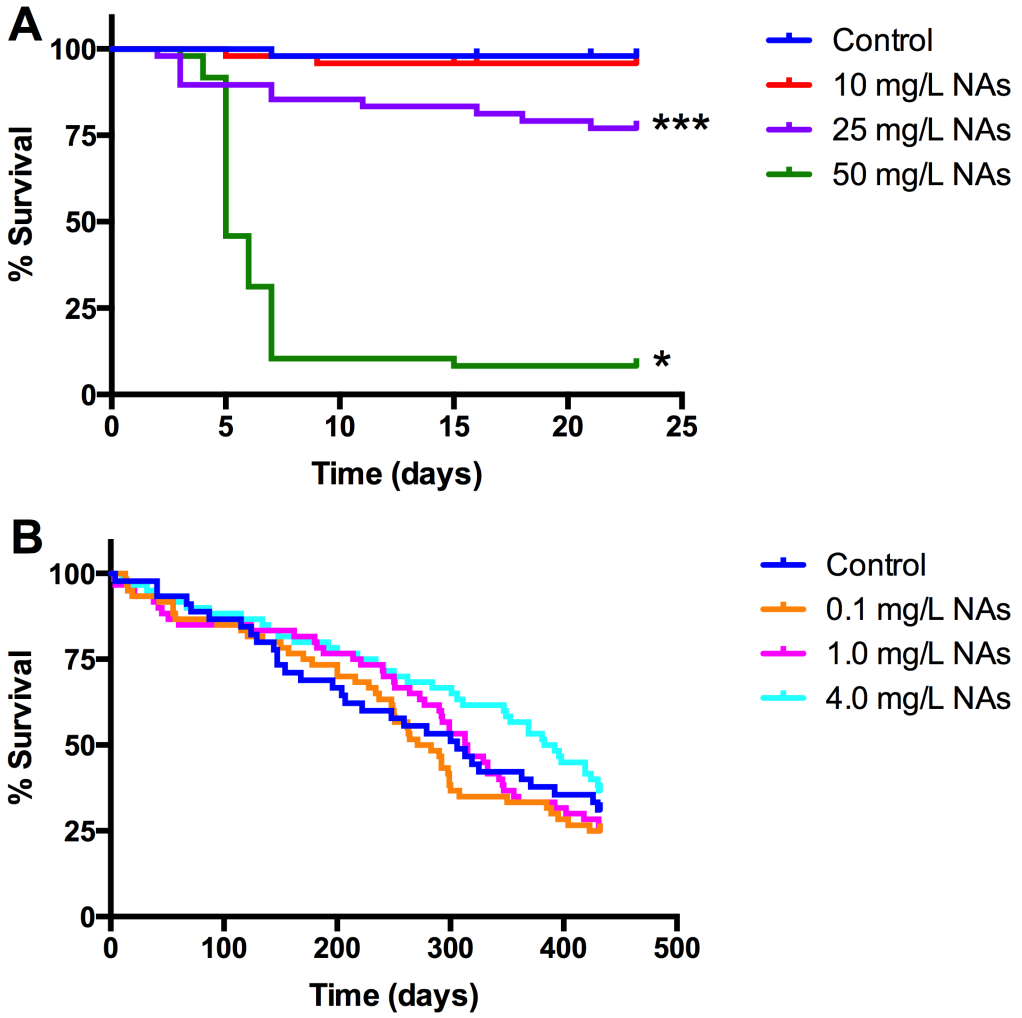

Figure 3.1 Survival during Adult NA Exposure

A: 21-day acute high-dose exposure. Blue line: solvent control. Red line: 10 mg/L NA. Purple line: 25 mg/L NA. Green line: 50 mg/L NA. Survival among 25 and 50 mg/L groups were significantly lower than control. n = 48 snails for each treatment group. .* $p < 0.05$; *** $p < 0.0001$. B: 14-month chronic low-dose exposure. Blue line: control. Orange line: 0.1 mg/L NA. Pink line: 1 mg/L NA. Turquoise line: 4 mg/L NA. There was no effect of NA concentration on survival. Survival proportions are consistent with those expected for an adult mixed-age population. n = 60 snails for each treatment group.

within normal expectations over this time frame. Thus, I did not observe any effect on survival for *Lymnaea* adults in background NA concentrations found in the Athabasca region.

3.3.2 Reproduction

Fecundity in *Lymnaea* was quantified using two distinct criteria: 1. the frequency of egg laying, during which a snail produces and deposits an egg mass containing multiple eggs, and 2. the number of eggs per egg mass, typically ranging from 10 to over 100. These endpoints were chosen because, although reproductive physiology and behavior is complex, offspring will be produced if these processes are successful. Since snails were housed together in tanks holding multiple animals, egg masses were collected from tanks, and an average egg-laying rate was determined for each tank based on the number of snails in the tank during the deposition period, and the interval between egg mass collections (usually 7 to 11 days).

3.3.2.1 Frequency of Egg Laying

During acute NA exposure, snails in most experimental groups laid an egg mass approximately once every 10 days. Specifically, control animals deposited an egg mass at a rate of 0.11±0.04 egg masses/snail/day, the 10 mg/L NA-exposed snails laid 0.12±0.02 egg masses/snail/day, and the 25 mg/L NA-exposed snails at a rate of 0.11±0.03 egg masses/snail/day. In contrast, when exposed to a 50 mg/L NA mixture, the egg laying rate dropped to 0.02±0.02 egg masses/snail/day. NA exposure had a significant effect on egg laying rates (Figure 3.2A; $F_{1,14} = 5.273$, $p = 0.038$), with the threshold for this effect above 25 mg/L. Snails generally laid more eggs during the second half of the exposure time, although the trends between treatment groups remained consistent throughout the exposure period.

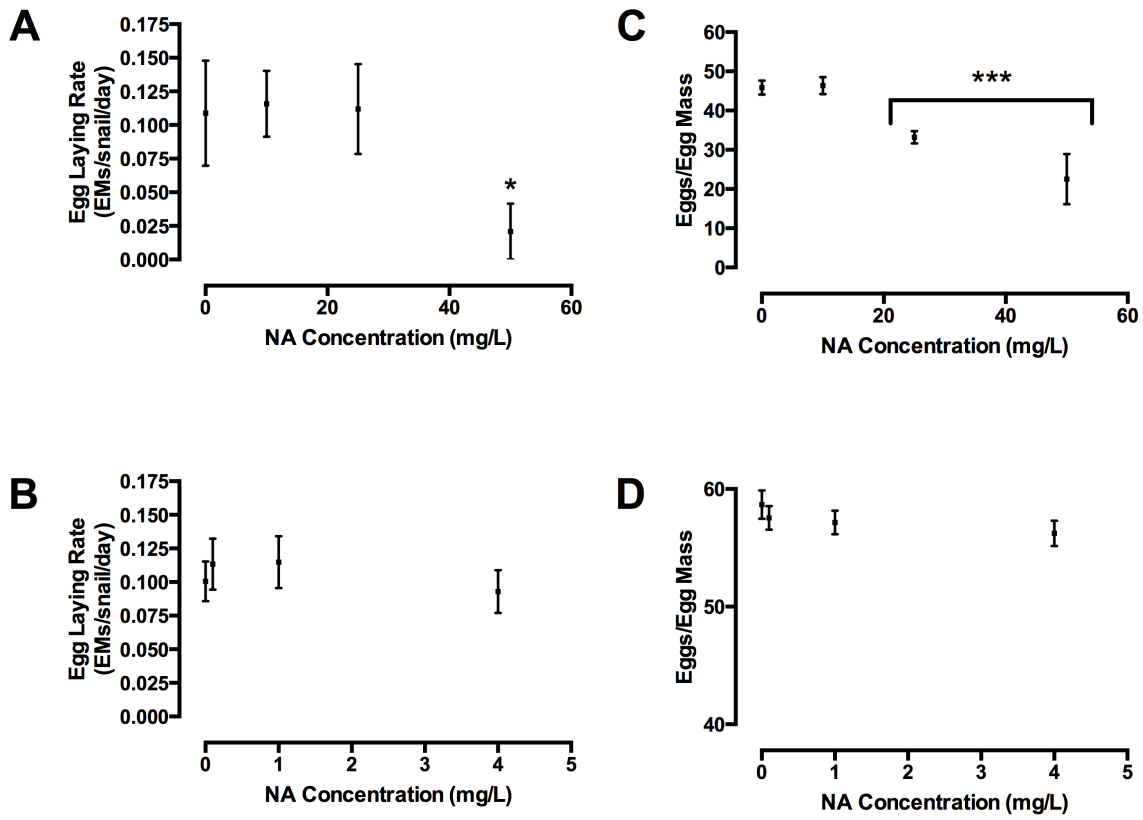

Figure 3.2 Fecundity during NA Exposure

A: Egg laying during 21-day acute high-dose exposure. NAs significantly reduced egg laying frequency above 25 mg/L. n = 4 tanks of 12 snails for each NA concentration. B: Egg laying frequency during 14-month chronic low-dose exposure. There was no effect of NA concentration on egg laying rate. n = 4 tanks of 15 snails for each NA concentration. C: Eggs per egg mass during 21-day acute high-dose exposure. Egg masses from NA-exposed snails contained fewer eggs in a dose-dependent manner. n = 4 tanks of 12 snails for each NA concentration. D: Eggs per egg mass during 14-month chronic low-dose exposure. There was no effect of NA concentration on eggs per egg mass. n = 4 tanks of 15 snails for each NA concentration. A, B, C, and D: Data are shown as mean±SEM. * $p < 0.05$; *** $p < 0.0001$.

During chronic NA exposure, all treatment groups had very similar oviposition rates over the 4 months during which egg masses were collected. These egg laying rates were 0.10±0.01 egg masses/snail/day for controls, 0.11±0.02 egg masses/snail/day for 0.1 and 1 mg/L, and 0.09±0.02 egg masses/snail/day for 4 mg/L. There was no significant effect of NA concentration on egg laying rate during the chronic exposure experiment (Figure 3.2B; $F_{1,238} = 1.8137$, $p = 0.179$). Thus, no effects were observed of background NA concentrations on egg laying rates of adult *Lymnaea*.

3.3.2.2 Eggs per Egg Mass

Above, I described a dose-dependent negative effect of higher concentrations of model NAs on the frequency of egg laying. The number of offspring produced depends not only on the oviposition frequency, but also on the number of eggs in each egg mass. Thus, I counted the number of eggs in each egg mass collected.

Regression analysis revealed that NAs had a significant effect on the number of eggs per egg mass laid by acutely exposed snails (Figure 3.2C; $F_{1,344} = 29.2283$, $p < 0.00001$). Control snails laid egg masses containing on average 45.8±1.8 eggs, while NA-exposed snails laid egg masses with 46.4±2.2 eggs for 10 mg/L, 33.2±1.6 eggs for 25 mg/L, and 22.5±6.4 eggs for 50 mg/L. The threshold for this effect appears to be between 10 and 25 mg/L.

Chronic exposure to low concentrations of NAs did not lead to significant differences in the number of eggs per egg mass (Figure 3.2D; $F_{1,2732} = 1.783$, $p = 0.182$). Snails in this experiment laid egg masses containing on average 58.7±1.2 eggs for control, 57.6±1.0 eggs for 0.1 mg/L, 57.2±1.0 eggs for 1 mg/L, and 56.2±1.1 eggs for 4 mg/L.

3.3.2.3 Embryo Packaging

The packaging of embryos into eggs and egg masses is under neuroendocrine control, and occurs in several glands as the embryos progress through the female reproductive tract (Dogterom *et al*., 1983). To investigate whether embryo packaging into eggs and egg masses was affected by NA exposure, I inspected egg masses under a stereomicroscope and counted the number of abnormal egg masses, which met at least one of the following criteria: 1. More than one egg containing multiple embryos, 2. Any egg containing more than three embryos, 3. An embryo not contained inside an egg, 4. An egg located outside the egg mass casing, when the egg mass casing was intact. The 50 mg/L NA group was excluded from formal analysis, since there were not enough egg masses produced in this treatment to conduct the analysis. Four egg masses were produced in the 50 mg/L treatment, all of which were normal.

Two of the 113 control egg masses were abnormal, representing 1.8% of the egg masses produced by this group. Each of the abnormal egg masses produced by the control group met only one criterion for abnormality, having two eggs with twin or triplet embryos. Twelve of the 116 egg masses laid by the 10 mg/L NA-exposed snails were abnormal, representing 10.3% of the egg masses produced by this group. Five of these abnormal egg masses met more than one criterion for abnormality. 21 of the 113 egg masses laid by the 25 mg/L NA-exposed snails were abnormal, representing 18.6% of the egg masses laid by this group. Seven of these abnormal egg masses met more than one criterion for abnormality. Abnormal egg masses occurred significantly more frequently among NA-exposed snails than control snails (Figure 3.3; Chi-square test; χ^2 (2, n = 342) = 17.39, p = 0.0002). Multiple comparisons revealed that the 10 mg/L NA-exposed group produced more abnormal egg masses than controls (χ^2 (1, n = 229) = 7.333, p = 0.0068), as did the 25 mg/L NA-exposed group (χ^2 (1, n = 226) = 17.47, p < 0.0001).

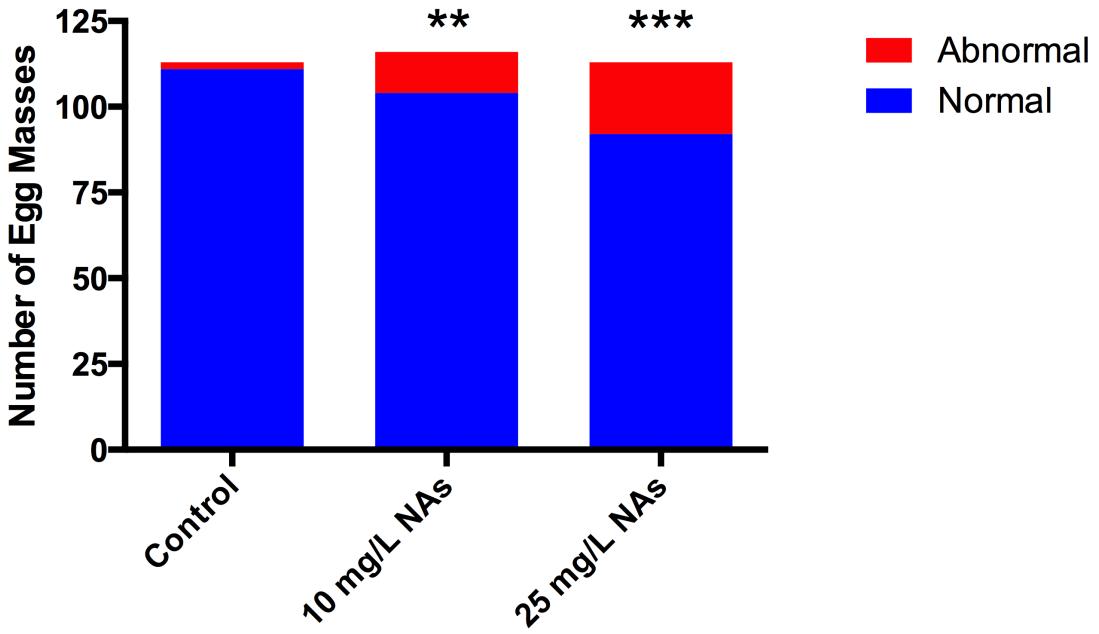

Figure 3.3 Embryo Packaging into Eggs and Egg Masses during NA Exposure

Numbers of abnormal egg masses are shown in red, and numbers normal egg masses are shown in blue. Egg masses scored as abnormal met at least one of the following criteria: 1. More than one egg containing multiple embryos, 2. Any egg containing more than three embryos, 3. An embryo not contained inside an egg, or 4. An egg located outside the egg mass casing, when the casing was undamaged. n = 342 egg masses (113 for control, 116 for 10 mg/L NAs, and 113 for 25 mg/L NAs). ** $p < 0.01$; *** $p < 0.001$.

3.3.3 Feeding and Growth

3.3.3.1 Growth

NAs have been reported to impair growth in a variety of organisms (Melvin & Trudeau, 2012; Peters *et al*., 2007), and some evidence of feeding impairment has been observed as well (Young *et al*., 2007; Rogers *et al*., 2002b). I therefore investigated whether this was also the case in *Lymnaea stagnalis* by measuring body wet weight and lettuce consumption in specimens housed in the presence of different concentrations of my model NA mixture.

Pond snails grow until they reach a maximum weight of 5 to 6 g at an age of about 300 days (Janse *et al*., 1989). Since all of the snails in the acute exposure experiment weighed less than 3 g at the beginning of the experiment, I expected their weight to increase over the duration of the experiment. To investigate whether NA exposure interferes with growth, I weighed the snails before exposure and weekly thereafter during 3 weeks of NA exposure. Before NA exposure, control snails weighed 1.61±0.04 g, snails in the 10 mg/L NA group weighed 1.65±0.04 g, snails in the 25 mg/L group weighed 1.64±0.04 g, and snails in the 50 mg/L group weighed 1.61±0.04 g. In the acute exposure experiment, control snails' weight increased by 13±1.7% to 1.81±0.05 g in 3 weeks, and average weight increased every week. In 10 mg/L NAs, snails grew by 7.0±1.2% to 1.76±0.04 g in 3 weeks, and average weight also increased every week. Snails in 25 mg/L did not grow at all on average (0±1.0%) in 3 weeks. This group of snails lost 3±0.9% of their weight in the first week, then grew in the remaining two weeks to return to their average initial weight. Snails in 50 mg/L decreased in weight by 8.0±0.9% over 3 weeks to a final weight of 1.47±0.06 g, with the average weight decreasing every week. Repeated measures ANOVA revealed a significant dose-dependent decrease in growth during acute NA exposure (Figure 3.4; $F_{6,252} = 5.50$, $p = 0.000023$).

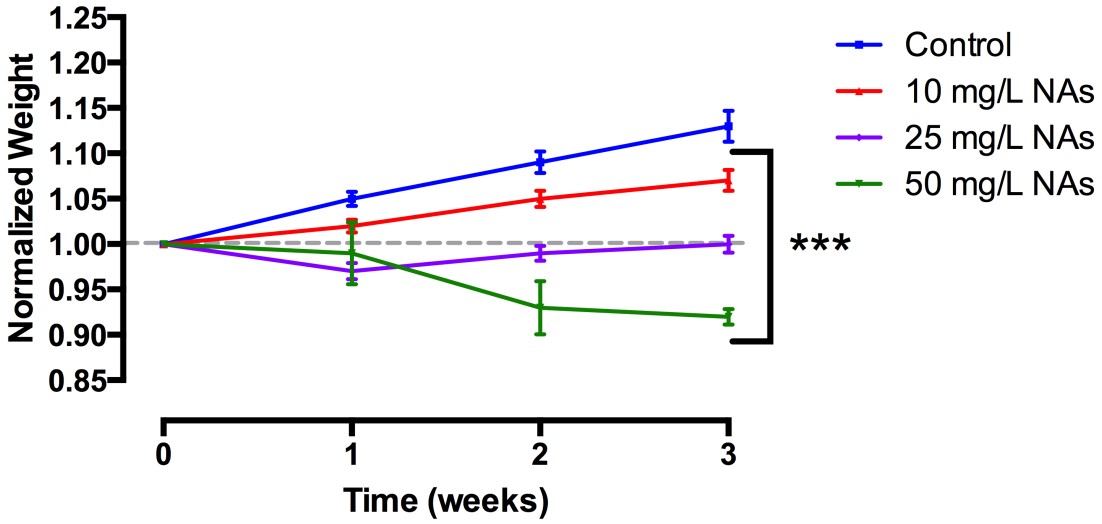

Figure 3.4 Growth during NA Exposure

Blue line: control (pond water + 500 ppm DMSO). Red line: 10 mg/L NAs. Purple line: 25 mg/L NAs. Green line: 50 mg/L NAs. Weights are presented as a proportion of initial weight. The grey dashed line represents no growth. n = 48 snails for each treatment group. Data are shown as mean±SEM. *** $p < 0.001$.

Weight was not quantified in the chronic exposure experiment.

3.3.3.2 Lettuce Consumption

To assess whether the effects of NAs on growth were due to reductions in food intake, lettuce consumption of groups of snails was quantified per tank and averaged for the tank based on the number of animals in the tank during the feeding period. Lettuce was weighed before addition to the tanks, and uneaten lettuce was removed and weighed every 2 to 4 days. Photographs of uneaten lettuce removed from these tanks after 2 days can be seen in Figure 3.5A to 3.5D. NA exposure affected lettuce consumption in a dose-dependent manner (Figure 3.5E; $F_{1,14} = 30.50238$, $p = 0.000075$). Control snails consumed 185±89 mg/snail/day, while NA-exposed snails consumed 157±23 mg/snail/day for 10 mg/L, 82.4±36 mg/snail/day for 25 mg/L, and only 7.6±7.6 mg/snail/day for 50 mg/L. Food intake was fairly consistent over time in all groups.

Lettuce consumption was not quantified during the chronic exposure experiment, however no obvious differences were observed by visual inspection.

3.3.3.3 Sucrose Response

The data presented in the previous section shows that model NAs suppress somatic growth and reduce food intake of adult *Lymnaea* in a dose-dependent manner. This reduction in food intake may have various origins, including a decline in metabolic demand. However, one other plausible possibility is that NAs incapacitate *Lymnaea*'s ability to eat. Feeding in *Lymnaea* is a rhythmic motor behavior consisting of a repetitive three-phase sequence of movements called rasps (Elliott & Benjamin, 1989; Dawkins, 1974). This behavior is generated by a well-

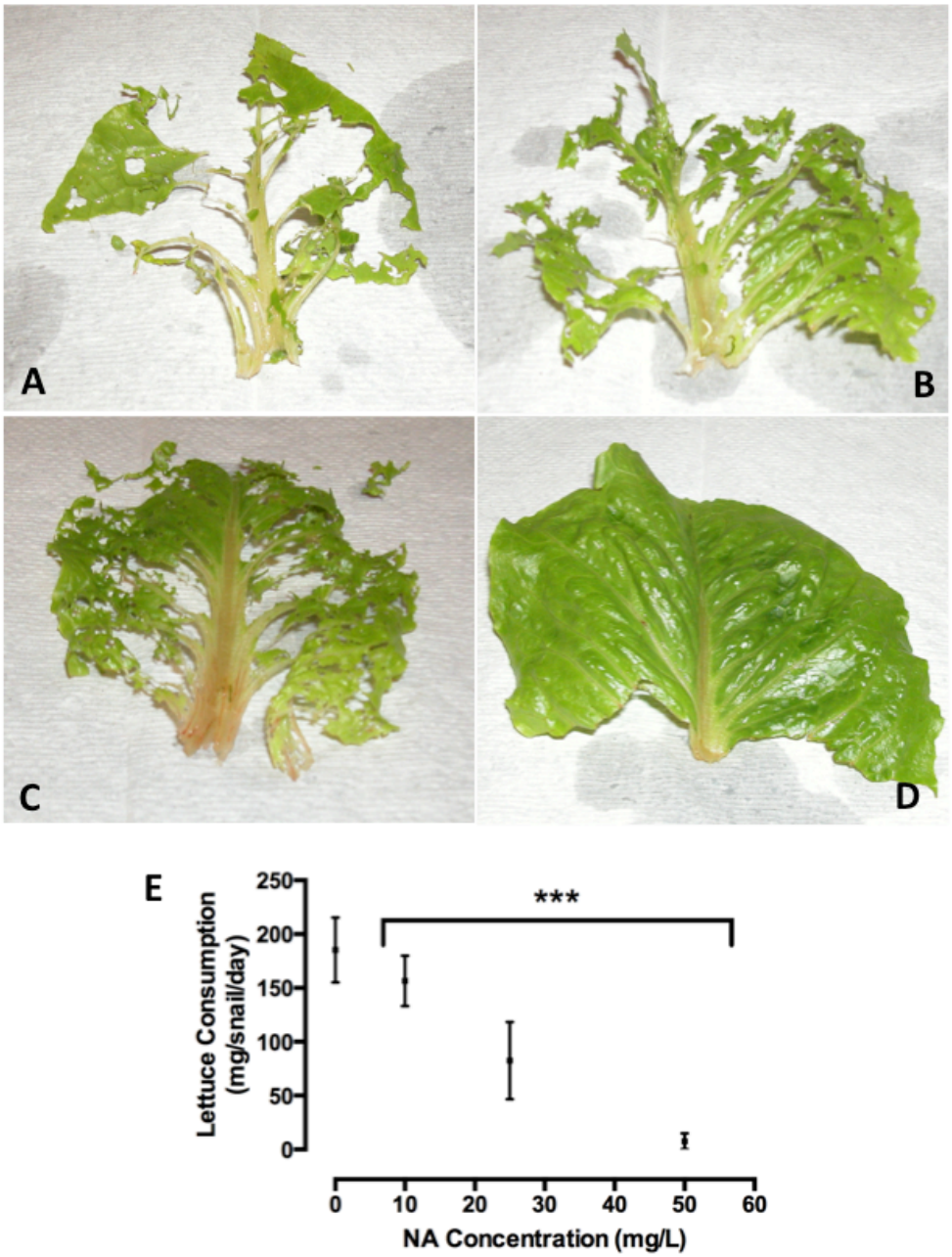

Figure 3.5 Spontaneous Feeding during NA Exposure

A – D: Uneaten lettuce after 2 days in a tank with 12 snails. A: control. B: 10 mg/L NAs. C: 25 mg/L NAs. D: 50 mg/L NAs. E: Lettuce consumption during NA exposure. Lettuce consumption declined in a dose-dependent manner with NA concentration. Data are shown as mean±SEM. *** $p < 0.001$.

defined neural circuit composed of several tens of neurons that together mediate multiple tasks, including sensation and integration of diverse external and internal sensory cues and information about the animals' physiological state, rhythm generation, and motor control (Kemenes *et al*., 1986). This feeding rhythm can be reliably triggered by exposing the animals to dilute sugar solutions (Kemenes *et al*., 1986). Therefore, a convenient way to assess fidelity of the feeding circuit is by testing the snails' appetitive behavioral responses to sugar stimuli.

During the acute exposure experiment, I measured the snails' ability to detect and respond to food stimuli by testing their response to a sucrose solution. Before exposure to NAs, all experimental animals showed a robust feeding response to the addition of sugar to their water, with rasping rates rising from 2-3 rasps/min without sugar to 20-21 rasps/min once sugar was added (Figure 3.6A). After 3 weeks, all NA-exposed snails showed much weaker responses to sugar than control snails. Control snails rasped at 16.7±1.0 rasps/min, while NA-exposed snails rasped at 10.3±1.4 rasps/min for the group exposed to 10 mg/L, 8.8±1.6 rasps/min for the animals exposed to 25 mg/L, and 4.2±2.5 rasps/min for the 50 mg/L exposed animals (Figure 3.6B). There was a significant effect of NA concentration on the sucrose response after 3 weeks of exposure ($F_{3,198} = 4.432$, $p = 0.00485$). There was also a significant effect of the sugar stimulus itself on rasping rates after NA exposure ($F_{2,198} = 320.03$, $p < 0.00001$), thus the snails were still able to respond to sucrose, albeit less than their control counterparts, and less than they did before NA exposure. The disturbance control (water) did not affect the rasping rate either before NA exposure ($F_{1,282} = 1.17$, $p = 0.2796$) or after NA exposure ($F_{1,204} = 0.141969$, $p = 0.7067$).

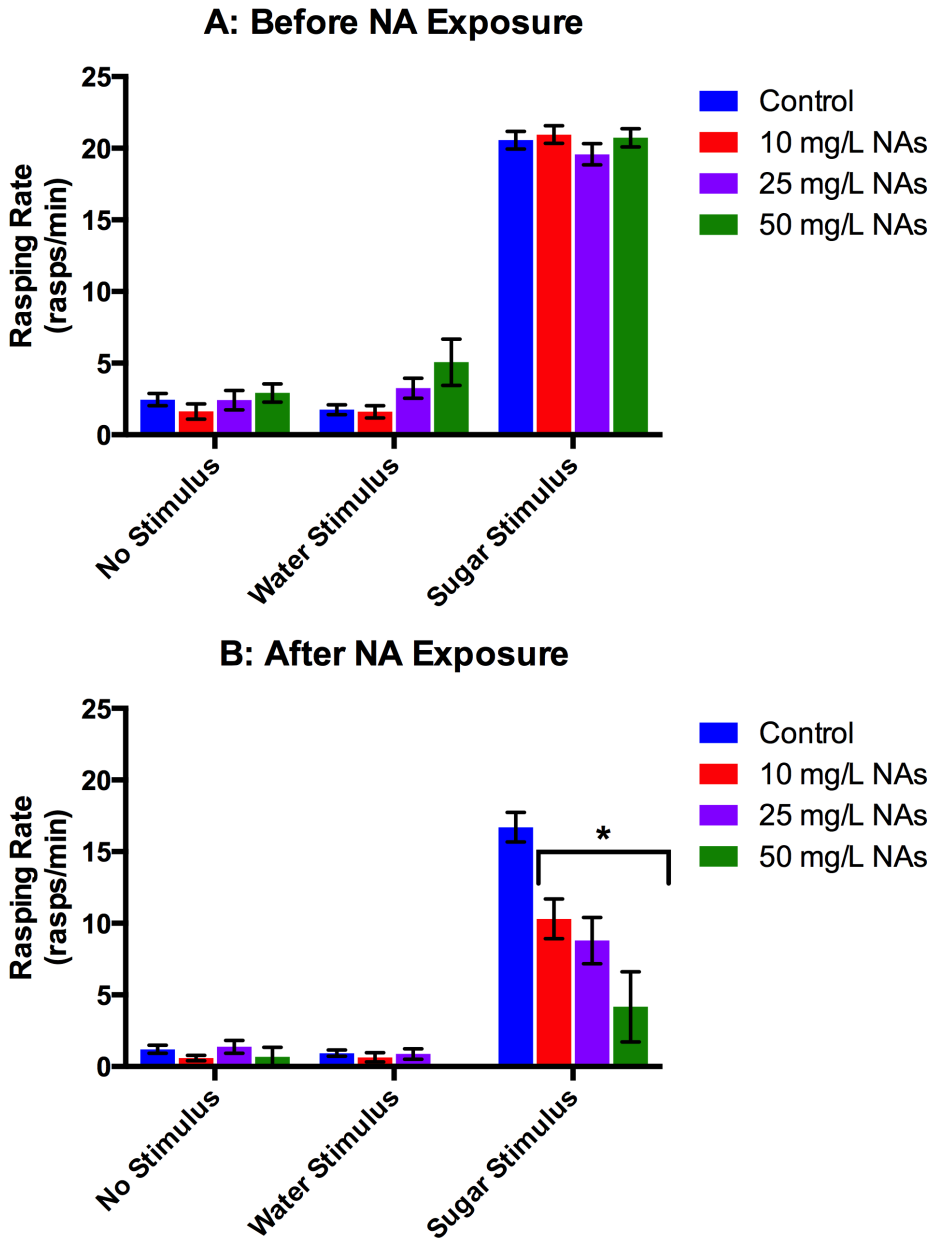

Figure 3.6 Evoked Feeding Responses during NA Exposure

A: Before NA-exposure, snails responded to sucrose with a robust increase in rasping rate. B: After 21 days of NA exposure, rasping rates in the presence of sucrose declined in a dose-dependent manner. A and B: See methods section for a detailed description of sucrose response testing procedure. Data are shown as mean±SEM. * $p < 0.05$.

3.3.4 Motor Behaviors

To investigate the possibility that NA exposure affects *Lymnaea*'s motivation and ability to interact with their environment, I conducted two types of behavioral assays. In the chronic exposure experiment, I implemented a righting assay in which snails were manually flipped upside down, and determined the time the snail took to return to an upright position. This assay, adapted from Fei *et al*. (2007), integrates snails' perception of spacial positioning with their motivation and ability to respond to perceived changes. In the acute exposure experiment, snails' perception and interaction with their environment was also investigated. To this end, snails were observed weekly and given a score based on their spontaneous behavior.

3.3.4.1 Behavioral Score

During the acute exposure experiment, snails were observed weekly and given a behavioral score according to Tuersley & McCrohan (1987). These scores have a maximum value of 10, and take into account factors such as emerging from or retracting into the shell, attaching to the substrate, locomotion and rasping. Scores for normal, active snails in this experiment typically ranged from 7 to 9.

There was a significant effect of NA concentration on behavioral score (Figure 3.7A; Repeated Measures ANOVA, $F_{3,66} = 5.9747$, $p = 0.0012$). There was no effect of time on behavioral score, nor was there an interaction between NA concentration and time. During the exposure phase of the experiment, control snails had an average score of 8.2±0.1, while NA-exposed snails had scores of 7.8±0.1 for 10 mg/L, 7.6±0.1 for 25 mg/L, and 6.8±0.2 for 50 mg/L. Although the NA-exposed snails had lower scores than control snails, it should be noted that the 10 and 25 mg/L groups still had scores in the range observed for active, normal snails. These

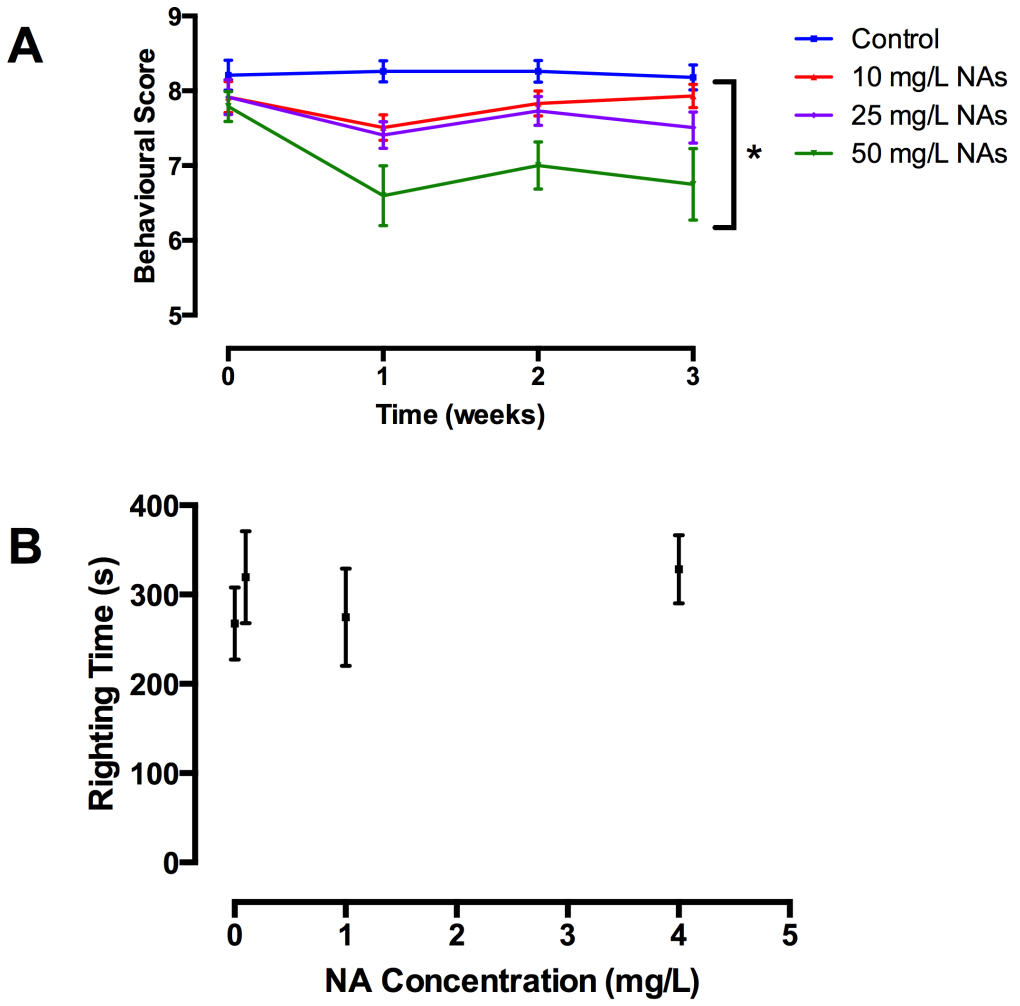

Figure 3.7 Behavioral Effects of NAs

A: Behavioral scores during 21-day acute high-dose exposure. These scores have a maximum value of 10, and take into account factors such as emerging from or retracting into the shell, attaching to the substrate, locomotion and rasping. Scores for normal, active snails typically ranged from 7 to 9. NA-exposed snails showed mild impairment. n = 4 tanks of 12 snails for each NA concentration. B: Righting time during 14-month chronic low-dose exposure. Snails were turned over onto their backs, and time to return to an upright position was measured. n = 4 tanks of 15 snails for each NA concentration. A and B: Data are shown as mean±SEM. * $p < 0.05$.

snails generally lost points for lack of spontaneous rasping, while their basic scores were usually the same as control snails. Scores for snails in 50 mg/L NAs were slightly lower than expected for unaffected snails. Snails in this treatment group generally lost points in the basic score category due to retraction into the shell and lack of locomotion, as well as lack of spontaneous rasping.

3.3.4.2 Righting Response

In the chronic exposure experiment, sensory-motor or geotactic coordination was quantified after 8 to 10 months of NA exposure by the righting response. Snails were placed in a plastic dish containing about 7.5 mm of pond water. After being allowed to recover from transportation and handling, snails were gently dislodged from the bottom of the dish and placed upside down on their shells, and the time taken to return to an upright position was recorded.

Snails in all treatment groups took similar times to right themselves. Control snails took 268±40 s, while NA-exposed snails took 320±51 s, 275±55 s, and 328±38 s for exposure to 0.1 mg/L, 1 mg/l and 4 mg/L NAs, respectively. There was no significant difference in righting time between control snails and any of the NA treatment groups (Figure 3.7B, $F_{1,46} = 0.47233$, $p = 0.495$). Thus, sensory-motor and geotactic functions, as measured by the righting response, are not affected by background NA concentrations.

3.3.5 Cellular Defense Mechanism

NAs have been shown to affect immune function in both mammals (Garcia-Garcia *et al*., 2011) and fish (McNeill *et al*., 2012; Hagen *et al*., 2012; Dokholyan & Magomedov, 1983). To investigate whether NAs affect immune function in *Lymnaea*, I collected hemolymph samples

after 3 weeks of NA exposure and tested the samples individually for hydrogen peroxide production by hemocytes.

In response to immune challenges, *Lymnaea's* blood circulating phagocytic cells called hemocytes produce reactive oxygen species while flattening and spreading in preparation for engulfing foreign matter (Hermann *et al*., 2013; Adema *et al*., 1991). I quantified the release of one of these reactive oxygen species, hydrogen peroxide, to assess the extent of hemocyte activation during NA exposure by means of a fluorescent respiratory burst assay (Amplex-Red). Blood samples from each snail were tested in two different conditions. One was given an *in vitro* immune challenge by the addition of laminarin, a polysaccharide from the algae *Laminaria digitata*, and the other sample was given a solvent control.

In samples that did not receive an immune challenge, hydrogen peroxide production increased in a dose-dependent manner with NA exposure (Figure 3.8; $F_{1,57} = 4.848$, $p = 0.0317$). In samples that were given an immune challenge, hydrogen peroxide levels were elevated with respect to un-challenged samples ($F_{1,112} = 6.12666$, $p = 0.014814$), but there was no significant effect of NA concentration on hydrogen peroxide concentration ($F_{1,57} = 3.418$, $p = 0.0697$). Thus, NA exposure triggers an enhanced activation of hemocyte response within 3 weeks, but immune cells are still able to increase hydrogen peroxide production in response to immune challenges after 3 weeks of exposure to intermediate and high concentrations of NAs.

3.4 Discussion

This study provides evidence that a mixture of low molecular weight NAs can have both lethal and sub-lethal toxic effects on adult pond snails ranging from reductions in female

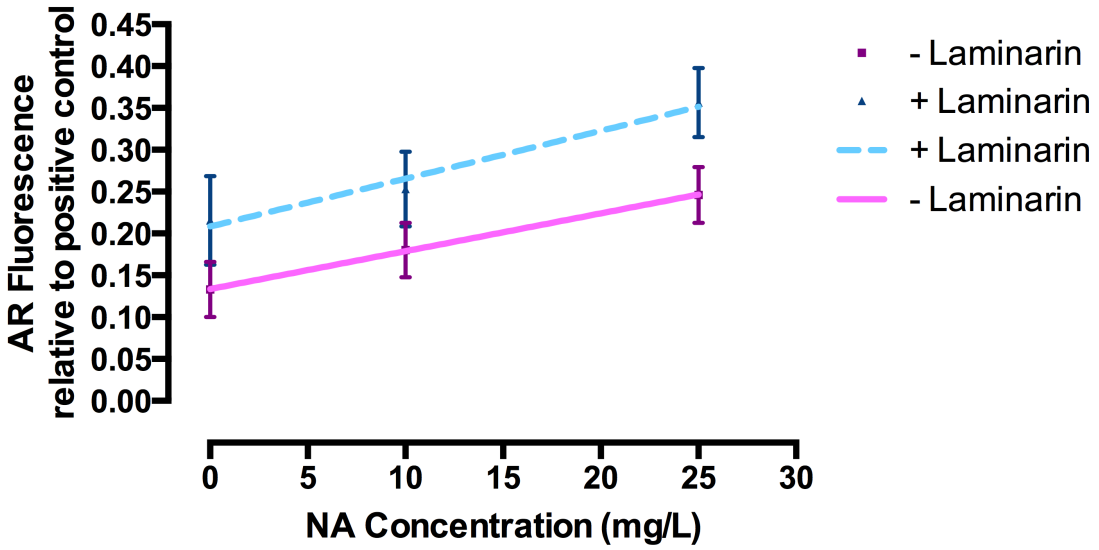

Figure 3.8 Immune Activation during NA Exposure

Amplex red fluorescence is a measure of hydrogen peroxide production. Pink solid line: linear regression for samples with no immune stimulus. H_2O_2 production increased in a dose-dependent manner. Blue dashed line: linear regression for samples with laminarin from *Laminaria digitata* as an immune stimulus. NA-exposed snails are still able to increase hydrogen peroxide production in response to an immune challenge after 3 weeks of NA exposure. Amplex red fluorescent signals are shown as fractions of the signal produced by a 10 µM hydrogen peroxide internal standard (positive control). n = 23 snails for control and 10 mg/L. n = 13 snails for 25 mg/L. Data are shown as mean±SEM.

reproductive output, body growth, and food intake, to alterations in immune status, and several indicators of sensorimotor function depending on their concentration and duration of exposure.

3.4.1 Effects of NAs on Fitness Traits of Adult Lymnaea stagnalis

3.4.1.1 Feeding behavior is impaired during NA exposure

3.4.1.1.1 Sucrose-Induced Feeding Movements

Feeding movements can be evoked in *Lymnaea* by adding sugar to their water, and the rasping rate has been shown to increase in a concentration-dependent manner during sucrose exposure (Kemenes *et al*., 1986). Without NA exposure, snails in this experiment rasped at a rate of 16 to 21 rasps/min. This corroborates various other studies with adult *Lymnaea* that observed comparable responses upon similar sucrose exposures (Kemenes *et al*., 1986; Hermann *et al*., 2007; 2013) Thus, the rasping rates observed for non-NA-exposed animals in this study are as expected for this species.

When I tested the snail's responses to the same sugar stimulus after exposing them to various concentrations of my NA mixture, I found that NA exposure diminished the sucrose-evoked rasping rate. This reduced responsiveness could be the result of sensory, motor, and/or motivation deficits. Non-NA-exposed snails typically began rasping at a high rate immediately upon addition of sucrose to their water. After NA exposure, some snails did not rasp at all when sucrose was added to their water. Other snails began rasping immediately, but their rasping rate was considerably slower than in normal snails. Still other snails began rasping at a normal rate, but only after a lag time with no rasping. This range of responses suggests that sensory, motor, and motivational deficits could all be occurring in NA-exposed snails, but manifesting themselves differently in different individuals.

3.4.1.1.2 Lettuce Consumption

During acute NA exposure, snails also consumed less lettuce in a dose-dependent manner. Dokholyan and Magomedov (1983) observed blood glucose at 50% below controls in sturgeon in 5 mg/L commercial NAs throughout their 30-day exposure. Glycogen storage also increased in these fish during this time period (Dokholyan & Magomedov, 1983). Food consumption was not quantified in this study, thus it is unclear whether the observed low blood sugar was a result of reduced food intake. Since increased glycogen storage would not be expected to accompany low blood sugar (Blasco *et al*., 2001; Fynn-Aikins *et al*., 1993), metabolic regulation could be impacted by NA exposure in these fish. Similarly, reduced feeding behavior would not be expected in snails that have increased energy demands due to increased need for detoxification processes. Thus, it is possible that impaired metabolic regulation is a symptom of NA exposure in aquatic animals.

3.4.1.2 NAs are acutely lethal to adults

Survival of *Lymnaea* was affected at NA high concentrations during acute exposure, but not during chronic exposure to background NA concentrations. The 3-week LC50 for this mixture of model NAs lies somewhere between 25 and 50 mg/L.

The freshwater snail *Physa heterostropha* exposed to a commercial NA mixture had 96-hour LC50 values between 6.6 and 15.6 mg/L, depending on temperature and water hardness (Cairns & Scheier, 1962). *Lymnaea*'s apparent greater resilience to NA exposure in the current study could be explained by differences in the NA mixtures used, as well as by the fact that the *Physa* snails were starved throughout their toxicant exposure and 36 hours before it (5.5 days

total) in Cairns and Scheier (1962), reducing their available energy resources, while snails were fed during NA exposure in the current study.

Adult marine fish (sturgeon, roach, and Caspian round goby) exposed to commercial NAs had 96-hour LC50 values of 50 to 75 mg/L, while these same species had 60-day LC50 values of 11 to 14 mg/L (Dokholyan & Magomedov, 1983). Fathead minnows were more susceptible to NA toxicity, having a 96-hour LC50 of 5.6 mg/L in commercial NAs, while the water flea *D. magna* had a 48-hour LC50 of 20 mg/L (Swigert *et al*., 2015). See Table 3.1 for a summary of lethal NA concentrations for aquatic animals. Although it is difficult to compare different exposure durations and different NA sources, our data suggest that *Lymnaea* survival is similar to survival among aquatic animals.

3.4.1.3 NA exposure decreases fecundity

During the acute and chronic exposure experiments, control snails laid eggs at rates of 0.10 to 0.12 egg masses/snail/day. This is well within the range of egg laying rates observed in *Lymnaea* (Giusti *et al*., 2014; Hermann et al., 2009; Ter Maat *et al*., 2007; Van der Schalie & Berry, 1973; Nowland & Carriker, 1946). Control snails produced egg masses containing, on average, 46 to 59 eggs per egg mass. These egg mass sizes are also as expected for this species (Janse *et al*., 1996; Dogterom *et al*., 1985; van der Schalie & Berry, 1973; Nowland & Carriker, 1946).

Chronic exposure to background NA concentrations had no effect on either the rate of egg mass production or the number of eggs in each egg mass. At higher NA concentrations, egg masses were produced less frequently and contained fewer eggs in a dose-dependent manner.

Table 3.1 Lethality of NAs to Various Aquatic Animal Species

NA mixtures differed between experiments, thus comparisons are reasonable but not direct. LC50: lethal concentration for 50% of test subjects. CNAs: commercial naphthenic acids. MNAs: model naphthenic acids.

Species	LC50 (mg/L)	Exposure Duration	Reference
Chum Salmon *Oncorhynchus keta*	25 (CNAs)	96 h	Dokholyan & Magomedov, 1983
	1.4 (CNAs)	60 d	
Russian Sturgeon *Acipenser gueldenstaedtii*	50 (CNAs)	96 h	Dokholyan & Magomedov, 1983
	11 (CNAs)	60 d	
Fathead Minnow *Pimephales promelas*	5.6 (CNAs)	96 h	Swigert *et al*., 2015
Water Flea *Daphnia magna*	20 (CNAs)	48 h	Swigert *et al*., 2015
Tadpole Snail *Physa heterostropha*	$6.6 \leq LC50 \leq 15.6$ (CNAs)	96 h	Cairns & Sheier, 1962
Great Pond Snail *Lymnaea stagnalis*	$25 < LC50 < 50$ (MNAs)	21 d	Current Thesis

Kavanagh *et al*. (2011) observed a decrease in egg production and egg laying events in fathead minnows exposed to oil sands process-affected water (OSPW) for 3 weeks. OSPW from five different ponds on Syncrude and Suncor leases were tested (Kavanagh *et al*., 2011). Reductions in spawning and number of eggs were observed in minnows exposed to water containing 28.6, 40.4, and 40.9 mg/L NAs, but not in water containing 10.9 or 19.2 mg/L NAs (Kavanagh *et al*., 2011). OSPW contains other toxic components besides NAs, but the same trend of decreasing fecundity was observed with exposure to the acid-extractable organic fraction of a different Syncrude pond (Kavanagh *et al*., 2012). Reductions in both spawning and number of eggs produced were observed in minnows exposed to 10 mg/L NAs, but not those exposed to 5 mg/L NAs (Kavanagh *et al*., 2012). These effects on female reproduction in minnows, and the concentrations at which they occur, are very close to those observed in *Lymnaea* in this study. Thus, this suggests that *Lymnaea* may have a susceptibility similar to minnows to female reproductive impairment during NA exposure. See Table 3.2 for a summary of the effects of NAs on female reproduction in aquatic animals.

The decrease in female reproductive activity observed in this study might be associated with reduced energy resources due to reduced food consumption. *Lymnaea* has been shown to produce fewer egg masses during reduced food intake and starvation (Ter Maat *et al*., 2007; Vandeven & Roubos, 1990; Bohlken *et al*., 1986; Dogterom *et al*., 1984; Ter Maat *et al*., 1982). This suggests that NA-exposed snails are prioritizing other energy demands, such as detoxification processes, over female reproduction.

In NA-exposed groups, I also observed numerous problems with the packaging of embryos and eggs into egg masses. These problems included multiple embryos per egg, empty eggs, embryos stuck to the outside of eggs, and eggs left outside of egg mass casings when these

Table 3.2 Reductions in Female Reproductive Output during NA Exposure

NA mixtures differed between experiments, thus comparisons are reasonable but not direct. OSPW: oil sands process-affected water. TNAs: tailings-derived naphthenic acids. MNAs: model naphthenic acids.

Species	Reduced Egg-Laying Events Threshold (mg/L)	Fewer Eggs per Laying Event Threshold (mg/L)	Reference
Fathead Minnow *Pimephales promelas*	28.6 (OSPW)	28.6 (OSPW)	Kavanagh *et al*., 2011
	10 (TNAs)	10 (TNAs)	Kavanagh *et al*., 2012
Great Pond Snail *Lymnaea stagnalis*	50 (MNAs)	25 (MNAs)	Current Thesis

casings were undamaged. Fertilization occurs in *Lymnaea* in the spermoviduct, just before the embryos enter the female part of the reproductive tract one at a time (Dogterom *et al*., 1983). Perivitelline fluid is secreted by the albumen gland, then the embryo and its perivitelline fluid is packed into an egg in the pars contorta (Dogterom *et al*., 1983). Gelatinous strings from the muciparous gland adhere to the eggs as they travel down the female reproductive tract (Dogterom *et al*., 1983). Finally, the mass of eggs and gelatinous material are packed into the egg mass capsule, called the ootheca, in the oothecal gland (Dogterom *et al*., 1983).

Since the most frequent egg mass abnormality observed was too many embryos in an egg, a likely target for NAs in the female reproductive tract is the first section from the spermoviduct to the pars contorta. The pars contorta is likely also where embryos adhered to the outside of their egg, rather than being packed inside it. Eggs that were left outside of their egg mass likely resulted from faulty packaging in the oothecal gland. These processes could be manifesting changes in signaling mechanisms, which could implicate either endocrine disruption, since this process is hormonally regulated (Koene, 2010; Vreugdenhil, 1988; Geraerts *et al*., 1985, 1983; Dogterom *et al*., 1983), or membrane disruption, related to membrane receptors for this peptide hormone (Ebberink *et al*., 1985). Abnormal egg packaging similar to what was observed in this study was also seen in *Lymnaea* exposed to testosterone and 17α-ethinylestradiol (Giusti *et al*., 2014). This suggests that the model NAs used in this study may act via similar mechanisms as the hormones used by Giusti *et al*. (2014). These model NAs are not expected to act via estrogen receptors since their structures are neither aromatic nor polycyclic (Rowland *et al*., 2011b), although non-receptor-mediated androgenic effects are possible due to exposure to NAs that do not mimic estrogen structure (Knag *et al*., 2013).

The observed decrease in fecundity could be compounded by environmental stresses that snails would encounter in reclaimed wetlands. Both egg laying and spermatogenesis ceased in *Lymnaea* populations in the Netherlands below 11°C (Joosse & Veld, 1972). Daily mean temperatures in Fort McMurray, Alberta, are above 11°C for only 3 months of the year, compared to 4 months in Calgary, Alberta, and 5 months in Algonquin Park, Ontario (Environment Canada, 2015), all of which are part of *Lymnaea*'s endemic range (Prescott and Curteanu, 2004). Predation stresses alone inhibit reproductive activity in many prey species (Latanville & Stone, 2013), but that effect can act synergistically with reproductive deficits due to toxicant exposure. Indeed, Latanville and Stone (2013) found that *Lymnaea* exposed to both predation signals and the pesticide malathion had much lower reproductive outputs than snails exposed to either stressor alone. *Lymnaea* have many predators in the Athabasca region, including carnivorous fish (Nero et al., 2006; Hershey, 1990), birds, and mammals. Thus, *Lymnaea* populations exposed to both NAs and environmental stresses could face major reproductive shortfalls.

3.4.1.4 Growth is hindered by NA exposure

Acute NA exposure impaired the growth of adult *Lymnaea* at all concentrations tested. Reduced growth could be linked to the diminished food consumption also observed in this study. Ter Maat *et al*. (2007) found that lower food availability did not affect growth in *Lymnaea* over an 80-day period when raised in a 12:12h light:dark regimen, as snails were in this study, but growth was hindered during food restriction on a 16:8h light:dark regimen. Zonneveld and Kooijman (1989) found that the weight of adult *Lymnaea* decreases during starvation due to use

of energy stores. Thus, the decrease in food intake likely played a role in reduced growth, but there could be other mechanisms involved.

I also observed slow somatic growth in *Lymnaea* embryos exposed to NAs during their development, when all necessary nutrition is pre-packaged in their eggs (see previous chapter). Although reductions in embryo growth could be associated with an impairment in nutrient uptake *in ovo*, there could also be an independent mechanism by which NAs inhibit growth in *Lymnaea*, besides reduced food intake.

NAs have surfactant activity due to their amphipathic structure, and surfactants have been shown to slow cell division (Nethery, 1967), thus NAs would be expected to slow the rate of cell division as well. Somatic growth requires both cell division and growth of existing cells (Pecl & Moltschaniwskyj, 1999), thus the NAs used in the current study may be able to slow somatic growth via their surfactant activity.

Considering the relatively short spring and summer seasons in the Athabasca region, these growth deficiencies might have detrimental effects of *Lymnaea* populations. Smaller snails produce fewer eggs per egg mass (Koene *et al*., 2007), which could compound the direct effects of NAs on egg production observed in this study.

3.4.1.5 Motor function is mildly impaired by NAs

To investigate the snails' ability to perceive and interact with their environment, I used two behavioral assays during the acute and chronic NA exposure experiments. After being flipped upside down onto their shells, control snails took on average 4.5 min to return to an upright position. This is considerably slower than control snails tested by Fei *et al*. (2007) and Orr *et al*. (2007), who on average righted themselves in 1.6 to 2 min. The snails tested by Fei *et*

al. (2007) were juveniles at 2 months old with shell lengths of 15 to 20 mm, while the snails used in the present study were approximately 14 to 22 months old when studied, with shell lengths of 27 to 43 mm. These mature snails also frequently had convolutions in their shells that would make righting difficult. Thus, the righting time taken by our snails is reasonable considering their age and size.

Snails chronically exposed to background NA concentrations showed no difference in time taken to right themselves from an inverted position, thus this study found no effects on sensory and motor control functions at NA concentrations below 4 mg/L.

During acute exposure, snails were given a weekly behavioral score according to Tuersley and McCrohan (1987). These scores take into account factors such as emerging from or retracting into the shell, attaching to the substrate, locomotion, and rasping. The maximum possible score is 10, and scores for normal, active snails in the current study received scores between 7 and 9.

Control snails in the current study had an average score of about 8. In the Tuersley and McCrohan (1987) study, average scores were usually 6 to 8. These scores are quite close, and differences can likely be attributed to different observers and handling practices.

NA concentration had a significant effect on behavioral scores, but time did not. Although the scores for snails in 10 and 25 mg/L NAs were lower than scores for control snails (just below 8), these NA-exposed snails still had scores that were typically associated with normal, healthy snails in this study. Only in the 50 mg/L NA group were scores slightly below the range of scores expected for active snails (just under 7). It is important to note that this scoring system was designed to quantify major behavioral and motor deficits such as retraction into the shell or failure to attach to the substrate, not to detect subtle differences between active snails. Thus, NA

exposure may be associated with mild sensory, motor, or motivational deficits at concentrations above 10 mg/L, but a different assay might be better suited to testing this possibility.

3.4.2 Effects of NAs on Cellular Defense Mechanisms

3.4.2.1 Elevated H_2O_2 Production by Hemocyte Activity After NA Exposure

After 3 weeks of NA exposure, elevated levels of hydrogen peroxide were produced by isolated hemocytes. When exposed to an immune challenge in the form of an algal polysaccharide, hemocytes were still able to increase hydrogen peroxide levels in response to this immune activator.

These elevated reactive oxygen species levels may allow these animals to eliminate infections more effectively in the short term, as Hagen *et al*. (2012) found with goldfish after 1 week of exposure to 20 mg/L commercial NAs. Immune induction was also seen in goldfish exposed to 20 mg/L NAs in fresh oil sands process-affected water for 30 days (Hagen *et al*., 2014). Similarly, Dokholyan and Magomedov (1983) observed that roach and sturgeon exposed to 5 mg/L commercial NAs had elevated leukocyte counts for the first 10 to 12 days of exposure. Thus, the enhanced immune activation observed in *Lymnaea* after 3 weeks of NA exposure is consistent with changes in immune responses seen in fish during 1 to 4 weeks of exposure to NAs from different sources.

This ROS production, however, is energetically costly, involving reduction of NADPH to produce reactive oxygen species by NADPH-oxidase (Adema *et al*., 1993). These reactive oxygen species might also result in oxidative stress (Hermann *et al*., 2013; Adema *et al*., 1993). Thus, given the limited energy resources available to NA-exposed snails, I would not expect this enhanced immune induction to continue indefinitely. Indeed, Hagen *et al*. (2012) showed that

goldfish had lower pro-inflammatory gene expression, increased levels of parasites in the blood, and much higher mortality from protozoan infections after 12 weeks of exposure to 20 mg/L commercial NAs. Roach and sturgeon showed decreased leukocyte counts during exposure to 5 mg/L commercial NAs for 1 month following an initial 10-12 day increase (Dokholyan & Magomedov, 1983). Hydrogen peroxide production by *Lymnaea* hemocytes was not quantified after long-term exposure, so I do not know if they follow the pattern of immune induction followed by immune suppression observed in these fish species.

3.4.3 Mechanisms of NA Toxicity

Reduced food consumption means fewer energy resources for these animals to allocate to developing and maintaining fitness traits. Zonneveld and Kooijman (1989) observed that food consumption, energy storage, growth, reproduction, and maintenance are balanced with each other in *Lymnaea*, and changes in resources or demands will alter the other categories. *Lymnaea* exposed to NAs could have enhanced ROS production, and may also have increased energy demands due to responses such as glutathione reduction and NA removal via glutathione. Glutathione is believed to bind and remove NAs due to their aliphatic structure and electrophilic carbonyl carbon, as opposed to cytochrome P450, which detoxifies aromatic molecules (MacDonald *et al*., 2013). They also have fewer energy resources due to reduced food consumption. The reductions in growth and fecundity observed in these snails could be directly linked to this scarcity of energy resources. Other effects observed in this study could be due to changes in signaling processes, such as the errors in egg packaging and the reduced food consumption itself. These signals could be associated with endocrine disruption, since hormones are involved in the regulation of both feeding (Li *et al*., 1996; Li *et al*., 1993) and egg mass

production (Roubos *et al*., 1994; Dogterom *et al*., 1983), or membrane disruption, related to hormone receptors or other membrane-associated functions. Membrane disruption could also affect the neural control of feeding (Benjamin, 2012; Straub *et al*., 2004; Straub & Benjamin, 2001) and egg mass production (Hermann *et al*., 1994; Roubos *et al*., 1994), perhaps via the localization and function of ion channels, or neurotransmitter vesicles or receptors.

Chapter Four: **General Discussion**

4.1 Summary of Observations

This thesis describes the effects of naphthenic acids (NAs) on different life stages of the pond snail *Lymnaea stagnalis*. Chapter 2 describes the effects of a range of environmentally realistic concentrations (from 0.25 to 75 mg/L) of a model naphthenic acid (NA) mixture on development of *Lymnaea* egg masses from deposition to juvenile hatching. During this embryonic NA exposure, I observed dose-dependent decreases in hatching rate and number of hatchlings, over a range of 20 to 75 mg/L NAs, and a reduction in embryo size, over a range of 60 to 75 mg/L NAs. The observed growth decreases were due to slow somatic growth, rather than a slow pace of morphological development. Deformed embryos were observed only in the highest concentration tested (75 mg/L). Embryos were resilient to NA exposure for 12 days in concentrations that were otherwise found to reduce hatching rates. NAs were not acutely lethal to *Lymnaea* embryos during their development (2 to 3 weeks), but failure to hatch within 3 to 4 weeks appears to be a lethal outcome. Egg mass materials were found to be selectively absorb the model NAs used in this study, with one of them (CHSA) almost completely excluded from *Lymnaea* eggs. Despite this apparent protection from NAs in surrounding water, embryos in isolated eggs performed better during NA exposure than embryos in intact egg masses exposed to a similar concentration of NAs.

Chapter 3 describes the effects of NAs on various aspects of the biology of adult *Lymnaea*. In these experiments adult *Lymnaea* were chronically exposed to background NA concentrations (0.1 to 4 mg/L) and acutely exposed to moderate and high NA concentrations (10 to 50 mg/L), using the same model NA mixture used during embryonic exposure. No detrimental

effects on survival, fecundity, or behaviour were observed at background NA concentrations. Acute lethality was observed at moderate to high NA concentrations, with the LC50 lying somewhere between 25 and 50 mg/L. Reductions in female reproductive output, quantified by rate of egg mass production and number of eggs in each egg mass, were observed at moderate to high NA concentrations, as were problems with packaging embryos into eggs. Reductions in food intake and evoked feeding behaviour were observed at moderate to high NA concentrations, along with growth deficits. Immune cells in NA-exposed animals showed increased activation without an immune challenge, while these cells were still able to increase reactive oxygen species production in response to a non-self immune challenge.

4.2 Mechanisms of NA Toxicity in *Lymnaea*

4.2.1 Metabolism and Energy Resources

The sub-lethal effects of model NA exposure in adult *Lymnaea* included reductions in food consumption, fecundity, behavioral performance and growth, as well as increased immune cell activation and problems with packaging embryos into eggs. Reduced food intake would not be expected in animals with increased energy demands due to oxidative stress and detoxification processes (Hermann *et al*., 2013; MacDonald *et al*., 2013; Adema *et al*., 1993). This corroborates a study by Dokholyan and Magomedov (1983), who observed both low blood sugar and increased glycogen storage in sturgeon during exposure to commercial NAs, a metabolic effect not expected in animals with normal metabolic regulation (Blasco *et al*., 2001; Fynn-Aikins *et al*., 1993). Thus, it is conceivable that metabolic dysregulation is a common effect of NA exposure.

This reduced food intake, in combination with increased energy demands by the innate immune system during toxicant exposure, might underlie the growth and fecundity impairments observed during acute NA exposure in adult *Lymnaea*. Animals with reduced energy resources will spend less of their available energy on growth and reproduction (Zonneveld & Kooijman, 1989).

4.2.2 Female Reproductive Output

The functional anatomy of *Lymnaea*'s reproductive tract and the processes and stages of egg mass formation are known in detail (Benjamin, 2012; Straub *et al*., 2004; Straub & Benjamin, 2001; Koene, 2010; Vreugdenhil, 1988; Geraerts *et al*., 1985, 1983; Dogterom *et al*., 1983). Problems with egg packaging could be associated with the hormonal control of the egg production system (Ebberink *et al*., 1985; Dogterom *et al*., 1983), or with changes in the function of parts of this system. One possible region in the female reproductive tract targeted by NAs is the pars contorta, the area in which embryos are packaged into their eggs (Dogterom *et al*., 1983). Malfunction in this area would conceivably lead to increasing frequency of multiple embryo eggs and extra-ovo embryos, which I observed at higher NA concentrations. Faulty packaging in the oothecal gland may be responsible for eggs left outside, but attached to, their egg mass (Dogterom *et al*., 1983). Changes in signaling mechanisms may explain these processing malfunctions. Signaling errors could implicate endocrine disruption, since egg production and packaging is hormonally regulated (Koene, 2010; Vreugdenhil, 1988; Geraerts *et al*., 1985, 1983; Dogterom *et al*., 1983). Membrane disruption could also be involved, perhaps affecting the number and localization of membrane receptors for the peptide caudodorsal cell hormones and neuropeptides, a collection of neuroendocrine modulators that control ovulation,

egg mass production and oviposition in *Lymnaea* (Ter Maat *et al*., 1989; Ebberink *et al*., 1985). NA surfactant activity could also be involved, by affecting the structure of membrane-bound receptors (Ontzen, 2011). Testosterone and 17α-ethinylestradiol have been shown to trigger abnormal embryo packaging similar to that seen in this study (Giusti *et al*., 2014). Thus, endocrine disruption may be a mechanism for this NA effect. However, the model NAs used in this study are neither polycyclic nor aromatic, thus they are not likely to interact with estrogen receptors (Rowland *et al*., 2011b). On the other hand, non-receptor-mediated androgenic effects have been proposed due to exposure to NAs that do not mimic estrogen structure (Knag *et al*., 2013), and this is the more likely mechanism in the current study.

4.2.3 Somatic Growth and Nutrient Uptake

Decreases in somatic growth during embryonic exposure were observed only at the highest NA concentrations tested (60 and 75 mg/L). This may be linked to NAs' surfactant activity, since surfactants have been shown to slow cell division (Nethery, 1967), which could in turn slow somatic growth (Pecl & Moltschaniwskyj, 1999). It is also possible that embryos were not taking up nutrients from the perivitelline fluid inside their eggs. Adult *Lymnaea* showed reduced food consumption in this study, which could suggest impairments in the neuropeptidergic control of feeding behaviour (Benjamin, 2012; Straub *et al*., 2004; Straub & Benjamin, 2001). Peters *et al*. (2007) observed less yolk utilization in Japanese medaka embryos exposed to commercial and tailings-derived NAs, which could suggest that NA exposure is associated with impaired nutrient uptake by embryos. *Lymnaea* embryos may have had similar deficits in nutrient uptake to those observed in adult *Lymnaea* in this study and in fish embryos in Peters *et al*. (2007). If such a nutrient deficit had occurred, it may have manifested itself as

reduced robustness in embryos that were the same size as unaffected embryos. This may have impacted the reduced hatching rates observed above 20 mg/L. Marois and Croll (1991) observed that faster embryonic development was correlated with earlier hatching within a single egg mass. Less robust embryos may therefore take longer to hatch, or may fail to hatch altogether.

4.2.4 Hatching Mechanisms

Reductions in hatching rates appear to be linked to failure to escape from the egg, since embryos developed normally except for small sizes beginning at 60 mg/L and deformities observed at 75 mg/L. Besides possible effects of nutrient uptake and robustness discussed above, NAs may have interfered with the embryos' ability to emerge from their eggs by microstructural changes to the egg mass contents or to embryo's feeding structures. Information on the mechanism(s) by which *Lymnaea* embryos escape from their eggs is difficult to find, so I will discuss hatching mechanisms in the context of what is known about embryos in *Lymnaea* and a variety of other snail species.

Active abrasion of the egg by means of feeding movements (rasping) is observed during hatching in some snail species (*Odontocymbiola magellanica* and *Chorus gigantus*) (Bigatti *et al*., 2014; Leiva *et al*., 1998), and this has been suggested to be the hatching mechanism in *Lymnaea* (Voronezhskaya *et al*., 1999). Thus it is possible that *Lymnaea* embryos failed to hatch due to changes in their feeding structures and/or impairments in the neuronal networks involved in feeding (Benjamin, 2012; Straub *et al*., 2004; Straub & Benjamin, 2001). Neurons involved the feeding processes develop after metamorphosis (the transition from the veliger stage to the adult-like stage, see Figure 2.1), connecting the lip to the cerebral ganglia, and the esophagus with the buccal ganglia (Voronezhskaya *et al*., 1999). Development of these neurons is

completed by 95% development (Voronezhskaya *et al*., 1999). Rasping rate in *Lymnaea* embryos beyond 90% development increases with exposure to serotonin, dopamine, and nitric oxide donors (Filla *et al*., 2009; Serfozo & Elekes, 2002). Nitric oxide synthase inhibitors have been shown to slow the rasping rate in *Lymnaea* embryos, as well as prolonging their time to hatch by several days (Serfozo & Elekes, 2002). Disruptions in any of these control systems could have delayed hatching in *Lymnaea* embryos without being directly observed in this study.

Some snail species, including the giant sea snail *Chorus giganteus*, are able to hatch without the active participation of the embryo (Leiva *et al*., 1998), suggesting that egg mass material could break down during embryonic development. Since a *Lymnaea* trypsin inhibitor homolog is known to be packaged in the perivitelline fluid surrounding the embryos (Nagle *et al*., 2001), it is tempting to speculate that proteases such as trypsin contribute to the hatching process in *Lymnaea stagnalis*. If proteases are necessary for hatching in *Lymnaea*, NAs may be interfering with the structure of proteases via their surfactant activity (Ontzen, 2011), thereby slowing the breakdown of egg mass material, preventing otherwise normal embryos from hatching.

It is also possible that NAs interacted with the egg's membrane in such a way that the membrane became tougher and more difficult to penetrate by whatever the hatching mechanism may be.

4.2.5 Naphthenic Acid Modes of Action

My thesis research did not directly address the mechanisms of NA toxicity in *Lymnaea*, but the effects that I observed in both the embryonic and adult life-stages could be linked to membrane disruption (Frank *et al*., 2009), or surfactant disruption of protein structure (Ontzen,

2011). Membrane disruption could interfere with the number and localization of membrane proteins (Frank *et al*., 2009), including membrane-bound receptors, which could interfere with peptide signaling processes like those associated with feeding (Li *et al*., 1996; Li *et al*., 1993) and egg laying (Koene, 2010; Vreugdenhil, 1988; Geraerts *et al*., 1985, 1983; Dogterom *et al*., 1983). Membrane disruption could also affect the function and localization of ion channels and neurotransmitter receptors, thereby affecting the neural control of feeding (Benjamin, 2012; Straub *et al*., 2004; Straub & Benjamin, 2001) and egg laying (Hermann *et al*., 1994; Roubos *et al*., 1994) as well. Disruption of protein structures by surfactant activity could influence the function of those membrane-bound receptors and ion channels (Ontzen, 2011), thereby also affecting metabolic and reproductive signaling systems.

Although a Lymnaeid snail (*Lymnaea ollula*) is known to express an estrogen receptor (Kumkate *et al*., 2009), and *Lymnaea stagnalis* are sensitive to both testosterone and 17α-ethinylestradiol (Giusti *et al*., 2014), endocrine disruption by receptor-mediated estrogenic or anti-androgenic actions are unlikely to have occurred in this study, since none of the model NAs used were aromatic, multi-ring structures (Thomas *et al*., 2009; Rowland *et al*., 2011b). A more likely mechanism is non-receptor-mediated endocrine disruptive effects observed in aliphatic NAs by Knag *et al*. (2013).

4.3 Model Naphthenic Acids

4.3.1 Molecular Weight and NA Toxicity

To compare my low molecular weight model NA mixture to NAs found in oil sands process-affected water, I conducted a hatching experiment using both my model NA mixture and an acid-extractable organic fraction that contained tailings-derived NAs obtained from an Athabasca

tailings pond. The two experiments rendered very similar results. That is, the concentration-dependent reductions in hatching rates were indistinguishable between the model NAs and NAs obtained from Suncor Pond 6.

The model NAs used in this experiment have molecular weights of 200.23, 170.25, and 184.28 g/mol, respectively. These molecular weights are within the range found in active tailings ponds, but they are below average (Frank *et al*., 2008; Holowenko *et al*., 2002; Holowenko *et al*., 2000). Considering that tailings-derived NAs typically have higher molecular weights than the model NAs used here, the similarity in their toxicities could be explained by the use of mass concentrations, rather than molar concentrations.

The relationship between molecular weight and NA toxicity depends on whether concentration is measured on a mass basis or a molar basis. Experiments using a mass basis conclude that lower molecular weight NAs are more toxic (Frank *et al*., 2008), while experiments using a molar basis find that higher molecular weight NAs are more toxic (Jones *et al*., 2011; Frank *et al*., 2009). Thus, lower molecular weight NAs are less toxic as individual molecules, but appear more toxic, due to their higher molar concentration, when mass alone is used to measure concentration.

Since my low molecular weight model NAs had the same apparent toxicity to *Lymnaea* embryos when compared on a mass basis to the higher average molecular weight tailings-derived NAs, my data supports the hypothesis that low molecular weight NAs are less toxic as individual molecules than higher molecular weight NAs. This is a promising finding for current oil sands process-affected water detoxification strategies. In addition to reducing overall NA concentrations, common detoxification strategies including ozonation (Vaiopoulou *et al*., 2015)

and algal biodegradation (Quesnel *et al*., 2011) also shift the composition of NA mixtures towards low molecular weight NAs, which may be less toxic molecules.

4.3.2 Molecular Structure and NA Toxicity

Molecular structure also plays an important role in determining NA toxicity. Bicyclic naphthenic acids are slightly more toxic than branched monocyclic acids (including 4-TBCA), although un-branched monocyclic acids (including CHBA and CHSA) are more toxic than both of those structures (Jones *et al*., 2011). NAs with two carboxyl groups (including CHSA) were found to be less toxic than similar NAs with only one carboxyl group, likely due to their decreased hydrophobicity (Frank *et al*., 2009). I also found that CHSA was nearly excluded from *Lymnaea* eggs, thus this compound likely made only a marginal contribution to the NA toxicity observed in this study. Due to its apparent enrichment in *Lymnaea* eggs, 4-TBCA may have been the major contributor to the toxicity of this model NA mixture. CHBA, as an un-branched monocyclic acid, may be more toxic than the un-branched 4-TBCA (Jones *et al*., 2001), although its concentration in *Lymnaea* eggs was only about half of the 4-TBCA concentration. Thus, CHBA likely also contributed to the toxic effects of this model NA mixture, although perhaps not as much as 4-TBCA.

The implications of this selective absorption of different NA structures are difficult to judge from this study. One model NA was nearly excluded while another was enriched, thus the toxicity profile of the NAs in *Lymnaea* eggs was different from that in the water surrounding them. However, embryos in isolated eggs performed better than eggs in intact egg masses, suggesting that the protection afforded to the embryos by the egg mass materials may be marginal. If the egg mass materials do protect *Lymnaea* embryos from NA toxicity, this

protection is transient, lasting only until the embryos hatch and emerge directly into the water with all NAs present. Thus, without studying the effects of these model NAs in isolation, on both embryos and hatchlings, it is difficult to make well-supported recommendations for NA detoxification goals from the NA permeability data presented in this study.

4.3.3 Comparisons Between Naphthenic Acid Mixtures

Since NAs exist in nature and tailings ponds as highly complex mixtures with a wide range of molecular weights (Vaiopoulou *et al*., 2015; Misiti *et al*., 2013; Frank *et al*., 2009, 2008b; Scott *et al*., 2008; Rogers *et al*., 2002a; Holowenko *et al*., 2002), their concentration, as a matter of practicality, is nearly always reported on a mass basis. Molar concentrations are only easily available for model NAs, while estimates of the average molecular weight for tailings-derived NA mixtures are only available if some form of spectroscopy analysis is performed on the sample (Vaiopoulou *et al*., 2015; Misiti *et al*., 2013; Frank *et al*., 2009, 2008; Scott *et al*., 2008; Rogers *et al*., 2002a; Holowenko *et al*., 2002). Considering that the toxicity of this model NA mixture was indistinguishable from the toxicity of a tailings-derived NA sample when compared on a mass basis, comparisons between this mixture of model NAs and tailings-derived NAs, on a mass concentration basis, are reasonable. As with any comparison between different NA mixtures, their toxicity profiles will differ due a number of factors, including the different molecular structures found in the mixtures as well as their average molecular weights. These different toxicity profiles cannot be avoided, thus comparisons between any two NA mixtures will never be perfect.

4.4 *Lymnaea* as a Model in Reclamation Assessment

4.4.1 Lymnaea *are resilient to background NA concentrations and short-term NA concentration increases*

Changes in NA composition and concentration can be expected in reclaimed freshwater environments due to rainfall, evaporation, and leaching from buried solid waste and groundwater. My data suggests that *Lymnaea* embryos should be able to withstand these transient concentration variations with only small changes in hatching success even into higher and otherwise dangerous NA concentrations for periods of nearly 12 days. Similarly, adult *Lymnaea* survived reasonably well at most NA concentrations where sub-lethal toxic effects were evident. Thus, *Lymnaea* populations may be able to withstand transient NA concentration variations with relatively minor effects. This temporal resilience makes *Lymnaea* a good model species for reclamation toxicology, since their survival is not dependent on consistent low NA concentrations. *Lymnaea* embryos are much more resilient to both lethal and sub-lethal effects of NA exposure than other types of freshwater animals (Melvin & Trudeau, 2012; He *et al*., 2012; Kavanagh *et al*., 2012, 2011; Peters *et al*., 2007), while adult *Lymnaea* show similar sensitivities to NA exposure as aquatic vertebrates with respect to survival (Dokholyan & Magomedov, 1983) and fecundity (Kavanagh *et al*., 2012, 2011). Considering that *Lymnaea stagnalis* is native to all of Alberta (Prescott & Curteneau, 2005; Clarke, 1981), and Lymnaeid snails have been found in wetlands on Athabasca oil sands leases (Baker *et al*., 2012), they are an ideal species to stock when building reclaimed wetlands in NA-contaminated areas. *Lymnaea* is, therefore, a species worthy of more attention when determining the effects of long-term exposure to low NA concentrations.

NAs are present in Athabasca-region surface and ground waters (Clemente & Fedorak, 2005; Schramm *et al*., 2000), and they will presumably still be present in low concentrations in remediated oil sands tailings water. For this reason, species that could live in reclaimed aquatic environments must be resilient to low concentration NA exposure. My results argue that *Lymnaea* is likely such a species. I found no detrimental effects on survival, reproduction, or locomotion in adults at NA concentrations up to 4.0 mg/L, more than four times the concentration found in the Athabasca River (Schramm *et al*., 2000). It is important to note, however, that growth and food consumption were not quantified during my chronic exposure experiment, thus the possibility remains that deficits occurred in these areas. Similarly, I did not see any detrimental effects on embryonic development or hatching below 20 mg/L. However, since I did not test the potential toxic physiological or behavioral effects on juveniles, my findings are currently not sufficient to consider this concentration range safe for early *Lymnaea* life stages.

4.4.2 High concentration NA exposure threatens **Lymnaea** *population stability*

I observed both lethality and detrimental effects on numerous fitness traits when adult animals were exposed to NA concentrations above background levels. Acute lethality was evident from 25 mg/L, and sub-lethal adverse effects were observed from 10 mg/L. Deficits in feeding might mean that animals are less robust and have fewer energy resources available (Ter Maat *et al*., 2007; Vandeven & Roubos, 1990; Zonneveld & Kooijman, 1989; Bohlken *et al*., 1986; Dogterom *et al*., 1984; Ter Maat *et al*., 1982). Reproductive output declined during NA exposure, and this effect might be compounded by both environmental stresses such as predation

and cold temperatures (Latanville & Stone, 2013; Joosse & Veld, 1972), as well as by the reduced growth also observed in this study (Koene *et al*., 2007; Zonneveld & Kooijman, 1989).

The Athabasca region presents additional challenges to existing *Lymnaea* populations due to its cold climate and short growing season. These challenges must be taken into account when studying the effects of tailings pond toxins on species that could live in reclaimed ecosystems. While *Lymnaea* appears to be a good candidate species to reside in reclaimed wetlands due to its resilience at background NA concentrations, populations would likely not be stable above 10 mg/L NAs. More research is necessary on the life cycle and multi-generational effects of NAs on pond snails below 10 mg/L, where this study did not find any detrimental effects of NAs. This information will be essential to establishing effective reclamation strategies for creating sustainable wetland ecosystems in NA-contaminated environments.

4.5 Limitations and Suggestions for Future Studies

While my work provides evidence that low molecular weight NAs can negatively impact survival, growth, fecundity, and hatching rates, it should be noted that I did not address possible adverse effects of NA exposure that may occur or manifest themselves after hatching. Similarly, my adult exposure tests at background NA concentrations did not quantify food intake or growth, endpoints that were among the most sensitive in the acute exposure study. Thus, the lack of observed detrimental effects on embryos below 20 mg/L NAs, and on adults below 10 mg/L NAs, cannot be taken as an indication that *Lymnaea* populations can be healthy in these NA concentrations. Rather, these results should be a starting point, providing a concentration range in which to investigate exposure to juveniles and mature animals, and to conduct experiments addressing individual fitness traits as well as population and ecosystem stability.

4.5.1 Naphthenic Acid Targets in Lymnaea

Like any other s*tudy*, the work presented here answered some questions but raised many other ones. For example, my data indicates that NAs affect *Lymnaea*'s feeding behavior, a phenomenon that could be rooted in changes in the functional status of the animal feeding apparatus and control circuitry. Since *Lymnaea*'s feeding behavior and its neurobiological substrate are very well defined, this idea could be readily investigated by a number of electrophysiological techniques. Sensory input and motor output could be tested by applying a sucrose stimulus to a semi-intact preparation of the lip, tentacles, and central nervous system (Staras *et al*., 1998) and monitoring the activity of identified neurons known to be associated with these functions (Benjamin, 2012; Straub *et al*., 2004; Straub & Benjamin, 2001).

My work also provides ev*i*dence that NAs cause disturbances in female reproductive activity, an effect that may or may not involve endocrine disturbances. Since the neuroendocrine basis of *Lymnaea* egg laying has as mentioned before been resolved in detail, this aspect of NA toxicity could also be investigated further. Hormonal regulation of egg laying during NA exposure could be investigated by measuring the activity of identified neurons known to be associated with control of female reproductive behavior, such as the caudodorsal cells, and cells in the dorsal bodies (Koene, 2010; Ter Maat *et al*., 1989; Dogterom *et al*., 1983). Sensitivity to hormones involved with female reproductive processes could be investigated by injecting snails with caudodorsal cell hormone (CDHC) or a tissue homogenate containing it, since this hormone has been shown to be sufficient to induce egg laying in *Lymnaea* (Ter Maat *et al*., 1989).

In the context of *Lymnaea'*s reproductive behaviors, it should be noted that NAs may also impact male reproductive activities in this simultaneous hermaphroditic snail, although I did not

address this question in my investigations. Observation of male reproductive behavior such as copulation as male could give insight into changes due to NA exposure, if there are any, and appropriate mechanistic tests could be determined from these observations.

4.5.2 Lifetime Effects of in Ovo *Naphthenic Acid Exposure*

The hatching assay may provide a relatively fast and reliable method for determining NA toxicity to *Lymnaea* embryos, but NA toxicity must be understood in a more comprehensive lifecycle context, thus including juveniles and adults. My GC-MS data suggest that *Lymnaea* eggs are selectively permeable to different NA structures, since one of my three model NAs was nearly excluded from the eggs. While this observation is interesting for embryonic development and from a biochemical perspective, in the context of reclamation, it is important to recall that any protection afforded by the egg or egg mass material ceases once the embryo hatches. I would therefore recommend a study that follows hatchlings from embryonic NA exposure at least until they reach sexual maturity. Their survival, growth, and fecundity during NA exposure at these life stages is necessary to understanding whether hatching rates after embryonic NA exposure are a reasonable representation of whole-life NA toxicity in *Lymnaea*.

4.5.3 Multi-Generational Effects of Naphthenic Acids

Finally, I would recommend incorporating multi-generational effects into further studies of *Lymnaea* during NA exposure. Detrimental effects of toxicants sometimes manifest themselves only after several generations of exposure (Tate *et al*., 1997). Sensitivities of organisms to toxicants can also either increase (Volker *et al*., 2013) or decrease (Vedamanikam & Shazilli, 2008) over multiple generations. This, the multi-generational effects of NAs on

Lymnaea populations will be important to understand in the context of reclamation ecology. The juvenile and adult studies proposed earlier will provide a supply of eggs whose parents were exposed to NAs. Following these offspring and assessing their fitness traits, for as many generations as is feasible, will provide insight into what might happen to *Lymnaea* populations during long-term exposure to NAs.

4.5.4 Multiple Toxicants and the Need for Realistic Exposure Scenarios

My work provides clear evidence for toxic effects of NAs on various aspects of *Lymnae*a biology. However, for methodological reasons, I restricted my studies to a simple mixture of NAs. I applied this simple NA mixture under otherwise ideal living conditions for *Lymnaea* rarely occur in real ecosystems. For instance, when considering the impact of toxicants associated with Athabasca oil sands activity, it is important to recall that naphthenic acids are not the only toxicant in wetlands reclamation sites. Metals such as nickel, iron, manganese, cadmium, chromium, lead, and vanadium are present in bitumen ore (Baker *et al*., 2012; Zhao *et al*., 2001; Smith & Schramm, 1992), and could enter reclaimed wetlands via process-affected water, tailings sediments, or petroleum coke buried during construction of the wetland landscape (Baker *et al*., 2012). Some of these metals, including manganese, lanthanium, yttrium, cobalt, and molybdenum, have been shown to leech into surface water from buried petroleum coke, tailings, and natural sediments at the bottom of wetland mesocosms (Baker *et al*., 2012). *Chara* species of algae accumulated both nickel and vanadium from buried petroleum coke and consolidated tailings sediments, while invertebrates, including Lymnaeaid snails, dragonfly larva, and midge larva, also accumulated metals in their tissues when exposed to these buried sediments (Baker *et al*., 2012).

Like most freshwater gastropods, *Lymnaea stagnalis* is very sensitive to metal ion contamination, and exposure to these metals is detrimental to *Lymnaea* in a number of ways. For example, cadmium exposure during embryonic development reduces hatching success at 200 µg/L, while adult exposure results in both a reduction in egg laying and acute lethality at 400 µg/L (Gomot, 1998). During nickel exposure, *Lymnaea* was found to be more sensitive than a crustacean (*Daphnia pulex*), an insect (*Chironomus riparius*), and an annelid (*Lumbriculus variegatus*) tested in the same conditions (Leonard & Wood, 2013). Nickel was acutely lethal to *Lymnaea* (the 96-hour LC50 was 8.2 µmol/L), and it accumulated in their tissues (Leonard & Wood, 2013). *Lymnaea* have been observed to accumulate mercury, cadmium, and lead in their tissues when exposed to these metals in pond sediments and vegetation (Sychra *et al*., 2011). Heavy metals have also been shown to act synergistically in *Lymnaea*. The combination of cadmium and zinc prevented memory formation in *Lymnaea* at concentrations considered safe in drinking water, when these metals did not have this effect alone (Byzitter *et al*., 2012). Thus, it is important to assess possible interactions between NAs and the metals found in the Athabasca region.

Similarly, polycyclic aromatic hydrocarbons (PAHs) are found in Athabasca region surface waters (Headley *et al*., 2001), as well as waste material from bitumen processing including pore water in mature fine tailings (Madill *et al*., 2001). Like metals, PAHs have been found to accumulate in invertebrate animals in the Athabasca region (Wayland *et al*., 2008). Relatively little is know about the effects of PAHs on mollusks, but they have been shown to reduce siphoning activity in mussels (Salanki *et al*., 2003). *Lymnaea* exposed to metals (copper and zinc), PAHs, and polychlorinated biphenyls in sediment showed DNA damage (single and double strand breaks), even when no detrimental effects were observed with respect to survival

and reproduction (Clement *et al*., 2004). Thus, although the effects of PAHs alone on *Lymnaea* deserve more attention, their effects in combination with NAs will be important for reclamation ecology in the Athabasca oil sands industry

Other chemical and physical parameters of oil sands process-affected water, such as high salinity and pH (Schramm *et al*., 2000), as well as suspended solids (Hall & Tollefson, 1982), will also pose challenges to organisms in aquatic ecosystems. Aged oil sands process-affected water from five ponds on Suncor and Syncrude leases had conductivities ranging from 1420 to 2780 µS/cm (Kavanagh *et al*., 2011). These salinity levels are within the range in which *Lymnaea* can act as osmoregulators (Wendelaar Bonga, 1972). These levels of salinity alone, therefore, would not be expected to have detrimental effects on *Lymnaea*, although the potential interaction between high salinity and other toxicants is unknown. The toxicity of naphthenic acids to *Lymnaea* should be tested in combination with these toxicants and stressors, especially at low concentrations, to provide a more complete picture of the toxicity profiles present in wetlands affected by the Athabasca oil sands industry.

4.6 The Great Pond Snail

My work provides evidence that the great pond snail *Lymnaea stagnalis* is resilient enough to NAs to be able to live in Athabasca surface waters, but sensitive enough to serve as a model in reclamation toxicology. This family of snails is already living on oil sands leases, and transfer energy through wetlands ecosystems from a wider variety of food sources than most pond snails. As embryos, they are much more robust during NA exposure than fish and frog embryos, while as adults they perform just as well as aquatic vertebrates. Healthy wetlands ecosystems in

contaminated oil sands sites will need a balance of organisms from all trophic levels, and *Lymnaea* are a promising piece of that puzzle.

References

Adema, C.M., and Loker, E.S. 2015. Digenean-gastropod host associations inform on aspects of specific immunity in snails. Developmental and Comparative Immunology 48(2): 275-283.

Adema, C.M., van Deutekom-Mulder, E.C., van der Knapp, W.P.W., and Sminia, T. 1993. NADPH-oxidase activity: the probable source of reactive oxygen intermediate generation in hemocytes of the gastropod *Lymnaea stagnalis*. Journal of Leukocyte Biology 54: 379-383.

Adema, C.M., van Deutekom-Mulder, E.C., van der Knapp, W.P.W., Meuleman, E.A., and Sminia, T. 1991. Generation of oxygen radicals in hemocytes of the snail *Lymnaea stagnalis* in relation to the rate of phagocytosis. Developmental and Comparative Immunology 15: 17-26.

Alberta Government. 2015. Alberta Oil Sands Industry Quarterly Report Winter 2015.

Alberta Government. 2014. Oil Sands Economic Benefits. http://www.oilsands.alberta.ca/FactSheets/Economic_FSht_Jan_2014.pdf. (ret. 12 Mar. 2015).

Alberta Government. 2013. Oil Sands Reclamation. http://www.oilsands.alberta.ca/FactSheets/Reclamation_FSht_Sep_2013_Online.pdf. (ret. 27 Jan. 2015).

Alberta Government. 2011. Alberta's Oil Sands: Facts about Tailings Management. http://www.oilsands.alberta.ca/FactSheets/Tailings_Management.pdf. (ret. 27 Jan. 2015).

Armstrong, S.A., Headley, J.V., Peru, K.M., and Germida, J.J. 2008. Phytotoxicity of oil sands naphthenic acids and dissipation from systems planted with emergent aquatic

macrophytes. Journal of Environmental Science and Health Part A – Toxic/Hazardous Substances & Environmental Engineering 43(1): 36-42.

Baker, F.C. 1905. Notes on the genitalia of *Lymnaea*. The American Naturalist 39(465): 665-679.

Baker, L.F., Ciborowski, J.J.H., and MacKinnon, M.D. 2012. Petroleum coke and soft tailings sediment in constructed wetlands may contribute to the uptake of trace metals by algae and aquatic invertebrates. Science of the Total Environment 414: 177-186.

Bandow, C., and Weltje, L. 2012. Development of an embryo toxicity test with the pond snail *Lymnaea stagnalis* using the model substance tributyltin and common solvents. Science of the Total Environment 435-436: 90-95.

Beadle, L.C. 1969. Salt and water regulation in the embryos of freshwater pulmonate molluscs. Journal of Experimental Biology 50: 473-479.

Beaulieu, E., Ioffe, J., Watson, S.N., Hermann, P.M., and Wildering, W.C. 2014. Oxidative-stress induced increase in circulating fatty acids does not contribute to phospholipase A(2)-dependent appetitive long-term memory failure in the pond snail *Lymnaea stagnalis*. BMC Neuroscience 15: 56-70.

Benjamin, P.R. 2012. Distributed network organization underlying feeding behavior in the mollusk *Lymnaea*. Neural Systems & Circuits 2(4): 1-16.

Benjamin, P.R., and Rose, R.M. 1980. Interneuronal circuitry underlying cyclical feeding in gastropod mollusks. Trends in Neurosciences 3(11): 272-274.

Bigatti, G., Giraud-Billoud, M., Vega, I.A., Penchaszdeh, P.E., and Castro-Vazquez, A. 2014. Embryonic development in the Patagonian red snail *Odontocymbiola magellanica*

(Neogastropoda: Volutidae): Morphology and biochemistry. Zoologischer Anzeiger 253: 372-381.

Blasco, J., Marimon, I., Viaplana, I., and Fernandez-Borras, J. 2001. Fate of plasma glucose in tissues of brown trout in vivo: effects of fasting and glucose loading. Fish Physiology and Biochemistry 24: 247-258.

Bohlken, S., Joosse, J., van Elk, R., and Geraerts W.P.M. 1986. Interaction of photoperiod and nutritive state in female reproduction of *Lymnaea stagnalis*, International Journal of Invertebrate Reproduction and Development 10: 151-157.

Bohman, A. 2012. The presence of the past: a retrospective view of the politics of urban water management in Accra, Ghana. Water History 4(2): 137-154.

Brient, J.A., Wessner, P.J., and Doyle, M.N. 2000. Naphthenic Acids. Kirk-Othmer Encyclopedia of Chemical Technology. Wiley Online Library.

Byzitter, J., Lukowiak, K., Karnik, V., and Dalesman, S. 2012. Acute combined exposure to heavy metals (Zn, Cd) blocks memory formation in a freshwater snail. Ecotoxicology 21(3): 860-868.

Cain, G.L. 1956. Studies on cross-fertilization and self-fertilization in *Lymnaea stagnalis appressa* Say. Biological Bulletin 111(1): 45-52.

Cairns, J. and Scheier, A. 1962. The effects of temperature and water hardness upon the toxicity of naphthenic acids to the common bluegill sunfish, *Lepomis macrochirus* Raf, and the pond snail *Physa heterostropha* Say. Notulae Naturae of the Academy of Natural Sciences of Philadelphia 353: 1-12.

CBC News. 2014. European Union drops plan to label oilsands crude 'dirty'. http://www.cbc.ca/news/business/european-union-drops-plan-to-label-oilsands-crude-dirty-1.2789868. Ret. 12 Mar. 2015.

CBC News. 2009. Greenpeace occupies Alberta oilsands site. http://www.cbc.ca/news/canada/edmonton/greenpeace-occupies-alberta-oilsands-site-1.819434. Ret. 12 Mar. 2015.

Clark, K.A., and Pasternak, D.S. 1932. Hot water separation of bitumen from Alberta bituminous sand. Industrial and Engineering Chemistry 24: 1410-1416.

Clarke, A. H. 1981. The freshwater mollusks of Canada. National Museum of Natural Sciences, Ottawa, ON.

Clement, B., Devaux, A., Perrodin, Y., Danjean, M., and Ghinidi-Fatus, M. 2004. Assessment of sediment ecotoxicity and genotoxicity in freshwater laboratory microcosms. Ecotoxicology 12: 323-333.

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

Clemente, J.S., MacKinnon, M.D., and Fedorak, P.M. 2004. Aerobic biodegradation of two commercial naphthenic acids preparations. Environmental Science & Technology 38(4): 1009-1016.

Clemente, J.S., Prasad, N.G.N., MacKinnon, M.D., and Fedorak, P.M. 2003. A statistical comparison of naphthenic acids characterized by gas chromatography–mass spectrometry. Chemosphere 50: 1265-1274.

Cort, W.W., Brackett, S., Olivier, L., and Nolf, L.O. 1945. Influence of larval trematode infections in snails on their second intermediate host relations to the strigeid trematode, *Cotylurus flabelliformis* (Faust, 1917). The Journal of Parasitology 31(1): 61-78.

Coutellec, M-A., and Lagadic, L. 2006. Effects of self-fertilization, environmental stress and exposure to xenobiotics on fitness-related traits of the freshwater snail *Lymnaea stagnalis*. Ecotoxicology 15: 199-213.

Cruz-Martinez, L., Fernie, K.J., Soos, C., Harner, T., Getachew, F., and Smits, J.E.G. 2015. Detoxification, endocrine, and immune responses of tree swallow nestlings naturally exposed to air contaminants from the Alberta oil sands. Science of the Total Environment 502: 8-15.

Czech, P., Weber, K., and Dietrich, D.R. 2001. Effects of endocrine modulating substances on reproduction in the hermaphroditic snail *Lymnaea stagnalis* L. Aquatic Toxicology 53: 103-114.

Dalesman, S., Rundle, S.D., Coleman, R.A., and Cotton, P.A. 2006. Cue association and antipredator behaviour in a pulmonate snail, *Lymnaea stagnalis*. Animal Behaviour 71: 789-797.

Davis, R.A., Welty, A.T., Borrego, J., Morales, J.A., Pendon, J.G., and Ryan, J.G. 2000. The UN-Water Status Report on the Application of Integrated Approaches to Water Resources Management. Environmental Geology 39(10): 1107-1116.

Dawkins, M. 1974. Behavioural analysis of coordinated feeding movements in the gastropod *Lymnaea stagnalis* (L.). Journal of Comparative Physiology 92: 255-271.

Dejoongbrink, M., Elsaadany, M., and Soto, M.S. 1991. The occurrence of schistosomin, an antagonist of female gonadotropic-hormones, is a general phenomenon in hemolymph of schistosome-infected fresh-water snails. Parasitology 103: 371-378.

de Szalay, F.D., Carrol, L.C., Beam, J.A., and Resh, V.H. 2003. Temporal overlap of nesting duck and aquatic invertebrate abundances in the grasslands ecological area, California, USA. Wetlands 23(4): 739-749.

Diaz, R.J., and Rosenberg, R. 2008. Spreading dead zones and consequences for marine ecosystems. Science 321: 926-929.

Dogterom, G.E., Thijssen, R., and van Loenhout, H. 1985. Environmental and hormonal control of the seasonal egg laying period in field specimens of *Lymnaea stagnalis*. General and Comparative Endocrinology 57: 37-42.

Dogterom, G.E., van Loenhout, H., Koomen, W., Roubos, E.W., and Geraerts W.P.M. 1984. Ovulation hormone, nutritive state, and female reproductive activity in *Lymnaea stagnalis*. General and Comparative Endocrinology 55: 29-35.

Dogterom, G.E., Bohlken, S., and Joose, J. 1983. Effect of the photoperiod on the time schedule of egg mass production in *Lymnaea stagnalis*, as induced by ovulation hormone injections. General and Comparative Endocrinology 49: 255-260.

Dogterom, A.A., and Robles, B.R. 1980. Stimulation of ornithine decarboxylase activity in *Lymnaea stagnalis* after a single injection with molluscan growth hormone. General and Comparative Endocrinology 40: 238-240.

Doi, H., Yurlova, N.I., Kikuchi, E., Shikano, S., Yadrenkina, E.N., Vodyanitskaya, S.N., and Zuykova, E.N. 2010. Stable isotopes indicate individual level trophic diversity in the freshwater gastropod *Lymnaea stagnalis*. Journal of Molluscan Studies 76: 384-388.

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

Duerr, F.G. 1967. Changes in the size-metabolic rate relationship of *Lymnaea stagnalis appressa* Say produced by digenetic trematode parasitism. Comparative Biochemistry and Physiology 20: 391-398.

Ebberink, R.H.M., van Loenhout, H., Geraerts, W.P.M., and Joose, J. 1985. Purification and amino acid sequence of the ovulation neurohormone of *Lymnaea stagnalis*. Proceedings of the National Academy of Sciences of the U.S.A. 82: 7767-7771.

Elbers, P.F. 1969. The primary action of lithium chloride on morphogenesis in *Lymnaea stagnalis*. Journal of Embryology and Experimental Morphology 22(3): 449-463.

Elliott, C.J., and Benjamin, P.R. 1989. Esophageal mechanoreceptors in the feeding system of the pond snail, *Lymnaea stagnalis*. Journal of Neurophysiology 61(4): 727-736.

Environment Canada. 2015. Canadian Climate Normals 1981-2010 Station Data.

Environment Canada. 1993. Peace-Athabasca Delta, Alberta, Information sheet on Ramsar wetlands. Environment Canada Environmental Conservation Branch, Edmonton AB.

ESRD. 2014. Environmental Quality Guidelines for Alberta Surface Waters. Alberta Environment and Sustainable Resources Development, Water Policy Branch, Policy Division. Edmonton, AB.

ESRD. 2013. Criteria and indicators framework for oil sands mine reclamation certification. Reclamation Working Group of the Cumulative Environmental Monitoring Association. Fort McMurray, AB.

Evans, K., and Hindell, M.A. 2004. The diet of sperm whales (*Physeter macrocephalus*) in southern Australian waters. ICES Journal of Marine Science 61: 1313-1329.

Fei, G., Guo, C., Sun, H.-S., and Feng, Z.-P. 2007. Chronic hypoxia stress-induced differential modulation of heat-shock protein 70 and presynaptic proteins. Journal of Neurochemistry 100: 50-61.

Filla, A., Hiripi, L., and Elekes, K. 2009. Role of aminergic (serotonin and dopamine) systems in the embryogenesis and different embryonic behaviors of the pond snail, *Lymnaea stagnalis*. Comparative Biochemistry and Physiology, Part C 149: 73-82.

Frank, R.A., Sanderson, H., Kavanagh, R., Burnison, B.K., Headley, J.V., and Solomon, K.R. 2010. Use of a (quantitative) structure-activity relationship [(Q)SAR] model to predict the toxicity of naphthenic acids. Journal of Toxicology and Environmental Health, Part A 73: 319–329.

Frank, R.A., Fisher, K., Kavanagh, R., Burnison, B.K., Arsenault, G., Headley, J.V., Peru, K.M., van der Kraak, G., and Solomon, K.R. 2009. Effect of carboxylic acid content on the acute toxicity of oil sands naphthenic acids. Environmental Science and Technology 43(2): 266-271.

Frank, R.A., Kavanaugh, R., Burnison, B.K., Arsenault, G., Headley, J.V., Peru, K.M., Van Der Kraak, G. Solomon, K.R. 2008. Toxicity assessment of collected fractions from an extracted naphthenic acid mixture. Chemosphere 72: 1309-1314.

Frank, R.A., Kavanagh, R., Burnison, B.K., Headley, J.V., Peru, K.M., Van Der Kraak, G., and Solomon, K.R. 2006. Diethylaminoethyl-cellulose clean-up of a large volume naphthenic acid extract. Chemosphere 68(8): 1346-1352.

Fynn-Aikins, K., Hung, S.S.O., and Hughes, S.G. 1993. Effects of feeding a high level of D-glucose on liver function in juvenile white sturgeon (*Acipenser transmontanus*). Fish Physiology and Biochemistry 12(4): 317-325.

Garcia-Garcia, E., Pun, J., Perez-Estrada, L.A., Gamal-El Din, M., Smith, D.W., Martin, J.W., and Belosevic, M. 2011. Commercial naphthenic acids and the organic fraction of oil sands process water downregulate pro-inflammatory gene expression and macrophage antimicrobial responses. Toxicology Letters 203: 62-73.

Gaunt, R. 1931. Effect of sulphydryl compounds and dl-alanine on rate of development of eggs of *Physa* and *Lymnaea*. Proceedings of the Society for Experimental Biology and Medicine 28(6): 660-663.

Gentes, M.-L., Waldner, C., Papp, Z., and 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 – Current Issues 70(13-14): 1182-1190.

Geraerts, W.P.M., Vreugdenhil, E., Ebberink, R.H.M., and Hogens, T.H.M. 1985. Synthesis of multiple peptides from a larger precursor in the neuroendocrine caudo-dorsal cells of *Lymnaea stagnalis*. Neuroscience Letters 56: 241-246.

Geraerts, W.P.M., Tensen, C., and Hogenes, T.H.M. 1983. Multiple release of peptides by electrically active neurosecretory caudo-dorsal cells of *Lymnaea stagnalis*. Neuroscience Letters 41: 151-155.

Giesy, J.P., Anderson, J.C., and Wiseman, S.B. 2010. Alberta oil sands development. Proceedings of the National Academy of Sciences 107(3): 951-952.

Gillanders, S.N., Coops, N.C., Wulder, M.A., and Goodwin, N.R.. 2008. Application of Landsat satellite imagery to monitor land-cover changes at the Athabasca Oil Sands, Alberta, Canada. The Canadian Geographer 52(4): 466-485.

Giusti, A., Lagadic, L., Barsi, A., Thome, J.-P., Joaquim-Justo, C., and Ducrot, V. 2014. Investigating apical adverse effects of four endocrine active substances in the freshwater gastropod *Lymnaea stagnalis*. Science of the Total Environment 493: 147-155.

Global News. 2013. Alberta oil sands, pipeline projects protested in rallies across Canada. http://www.theglobeandmail.com/news/national/alberta-oil-sands-pipeline-projects-protested-in-rallies-across-canada/article15476585/. Ret. 12 Mar. 2015.

Gomot, A. 1998. Toxic effects of cadmium on reproduction, development, and hatching in the freshwater snail *Lymnaea stagnalis* for water quality monitoring. Ecotoxicology and Environmental Safety 41: 288-297.

Graber, M., Euzeby, J.A., and Gevrey, J.P. 1981. Lutte biologique contre les mollusques vecteurs de Bilharziose. Action predatrice de *Tilapia rendalli*, Boulenger et de *Sarotherodon mossambica*, Peters a l'egard de *Biomphalaria glabrata*, Say. Hydrobiologia 78: 253-257.

Graham, A. 1955. Molluscan diets. Journal of Molluscan Studies 31(3-4): 144-158.

Grewer, D.M., Young, R.F., Whittal, R.M., and Fedorak, P.M. 2010. Naphthenic acids and other acid-extractables in water samples from Alberta: What is being measured? Science of the Total Environment 408: 5997-6010.

Habdija, I., Lajtner, J., and Belinic, I. 1995. The contribution of gastropod biomass in macrobenthic communities of a Karstic river. International Review of Hydrobiology 80(1): 103-110.

Hagen, M.O., Katzenback, B.A., Shahinoor Islam, M.D., Gamal El-Din, M., and Belosevic, M. 2014. The Analysis of Goldfish (Carassius auratus L.) Innate Immune Responses After Acute and Subchronic Exposures to Oil Sands Process-Affected Water. Toxicological Sciences 138(1): 59-68.

Hagen, M.O., Garcia-Garcia, E., Oladiran, A., Karpman, M., Mitchell, S., El-Din, M.G., Martin, J.W., and Belosevic, M. 2012. The acute and sub-chronic exposures of goldfish to naphthenic acids induce different host defense responses. Aquatic Toxicology 109: 143-149.

Hall, E.S., and Tollefson, E.L. 1982. Stabilization and destabilization of mineral fines-bitumen-water dispersions in tailings from oil sand extraction plants that use the hot water process. Canadian Journal of Chemical Engineering 60: 812-821.

Hamilton, D.J., Ankney, C.D., and Bailey, R.C. 1994. Predation of zebra mussels by diving ducks: An exclosure study. Ecology 75(2): 521-531.

He, Y., Patterson, S., Wang, N., Hecker, N., Martin, J.W., Gamal El-Din, M., Giesy, J.P., and Wiseman, S.B. 2012. Toxicity of untreated and ozone-treated oil sands process-affected water (OSPW) to early life stages of the fathead minnow (*Pimephales promelas*). Water Research 46: 6359-6368.

He, Y., Wiseman, S.B., Hecker, M., Zhang, X., Wang, N., Perez, L.A., Jones, P.D., Gamal El-Din, M., Martin, J.W., and Giesy, J.P. 2011. Effect of ozonation on the estrogenicity and androgenicity of oil sands process-affected water. Environmental Science & Technology 45: 6268–6274.

Headley, J.V., Peru, K.M., Mohamed, M.H., Frank, R.A., Martin, J.W., Hazewinkel, R.R.O., Humphries, D., Gurprasad, N.P., Hewitt, L.M., Muir, D.C.G., Lindeman, D.; Strub, R.,

Young, R.F., Grewer, D.M., Whittal, R.M., Fedorak, P.M., Birkholz, D.A., Hindle, R., Reisdorph, R., Wang, X., Kasperski, K.L., Hamilton, C., Woudneh, M., Wang, G., Loescher, B., Farwell, A., Dixon, D. G., Ross, M., Dos Santos Pereira, A., King, E., Barrow, M.P., Fahlman, B., Bailey, J., Mcmartin, D.W., Borchers, C.H., Ryan, C.H., Toor, N.S., Gillis, H.M., Zuin, L., Bickerton, G., Mcmaster, M., Sverko, E., Shang, D., Wilson, L.D., and Wrona, F.J. 2013. Chemical fingerprinting of naphthenic acids and oil sands process waters—A review of analytical methods for environmental samples. Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering 48(10): 1145-1163.

Headley, J.V., Akre, C., Conly, F.M., Peru, K.M., and Dickson, L.C. 2001. Preliminary characterization and source assessment of PAHs in tributary sediments of the Athabasca River, Canada. Environmental Forensics 2: 335-345.

Hermann, P.M., Watson, S.N., Wildering, W.C. 2014. Phospholipase A(2) - nexus of aging, oxidative stress, neuronal excitability, and functional decline of the aging nervous system? Insights from a snail model system of neuronal aging and age-associated memory impairment. Frontiers in Genetics 5.

Hermann, P.M., Park, D., Beaulieu, E., and Wildering, W.C. 2013. Evidence for inflammation-mediated memory dysfunction in gastropods: putative PLA_2 and COX inhibitors abolish long-term memory failure induced by systemic immune challenges. BMC Neuroscience 14: 83-97.

Hermann, P.M., Genereux, B., and Wildering, W.C. 2009. Evidence for age-dependent mating strategies in the simultaneous hermaphrodite snail, Lymnaea stagnalis (L.). Journal of Experimental Biology 212: 3164-3173.

Hermann, P.M., Lee, A., Hulliger, S., Minvielle, M., Ma, B., and Wildering, W.C. 2007. Impairment of long-term associative memory in aging snails (*Lymnaea stagnalis*). Behavioral Neuroscience 121: 1400-1414.

Hermann, P.M., Ter Maat, A., and Jansen, R.F. 1994. The neural control of egg-laying behaviour in the pond snail *Lymnaea stagnalis*: Motor control of shell turning. Journal of Experimental Biology 179: 79-99.

Hershey, A.E. 1990. Snail populations in arctic lakes: competition mediated by predation?. Oecologia 82: 26-32.

Hofkin, B.V., Mkoji, G.M., Koech, D.K., and Loker, E.S. 1991. Control of schistosome-transmitting snails in Kenya by the North American crayfish *Procambarus clarkii*. American Journal of Tropical Medicine and Hygene 45(3): 339-344.

Holowenko, F.M., MacKinnon, M.D., and Fedorak, P.M. 2000. Methanogens and sulfate-reducing bacteria in oil sands fine tailings waste. Canadian Journal of Microbiology 46(10): 927-937.

Holowenko, F.M., MacKinnon, M.D., and Fedorak, P.M. 2001. Naphthenic acids and surrogate naphthenic acids in methanogenic microcosms. Water Research 35(11): 2592-2606.

Holowenko, F.M., MacKinnon, M.D., and Fedorak, P.M. 2002. Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. Water Research 36(11): 2843-2855.

Hunter, R.D. 1980. Effects of grazing on the quantity and quality of freshwater aufwuchs. Hydrobiologia 69(3): 251-259.

Hussein, M.M., Deriszadeh, A., and Harding, T.G. 2011. Experimental and modeling study of MEUF removal of naphthenic acids. Desalination 273: 352-358.

IUPAC. Acute Toxicity. In Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). 1997 Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford.

Janer, G., and Porte, C. 2007. Sex steroids and potential mechanisms of non-genomic endocrine disruption in invertebrates. Ecotoxicology 16: 145-160.

Janse, C., van der Roest, M., Jansen, R.F., Montagne-Wajer, C., and Boer, H.H. 1996. Atrophy and degeneration of peptidergic neurons and cessation of egg laying in the aging pond snail *Lymnaea stagnalis*. Journal of Neurobiology 29(2): 202-212.

Janse, C., Wildering, W.C., and Popelier, C.M. 1989. Age-related changes in female reproductive activity and growth in the mollusc *Lymnaea stagnalis*. Journal of Gerontology 44(6): 148-155.

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

Jones, J.D. 1961. Aspects of respiration in *Planorbis corneus* L and *Lymnaea stagnalis* L (Gastropoda - Pulmonata). Comparative Biochemistry and Physiology 4(1): 1-29.

Joosse, J. and Veld, C.J. 1972. Endocrinology of reproduction in the hermaphrodite gastropod *Lymnaea stagnalis*. General and Comparative Endocrinology 18: 599-600.

Kamaluddin, M., and Zwiazek, J.J. 2002. Naphthenic acids inhibit root water transport, gas exchange and leaf growth in aspen (*Populus tremuloides*) seedlings. Tree Physiology 22: 1265-1270.

Karanova, M.V., and Gakhova, E.N. 2007. Biochemical strategy of survival of the freshwater mollusc *Lymnaea stagnalis* at near-zero temperatures. Journal of Evolutionary Biochemistry and Physiology 43(3): 310-317.

Kavanagh, R.J., Frank, R.A., Burnison, B.K., Young, R.F., Fedorak, P.M., Solomon, K.R., and van der Kraak, G. 2012. Fathead minnow (*Pimephales promelas*) reproduction is impaired when exposed to a naphthenic acid extract. Aquatic Toxicology 116: 34-42.

Kavanagh, R.J., Frank, R.A., Burnison, B.K., Young, R.F., Fedorak, P.M., Solomon, K.R., and Van Der Kraak, G. 2011. Fathead minnow (*Pimephales promelas*) reproduction is impaired in aged oil sands process-affected waters. Aquatic Toxicology 101: 214-220.

Kelly, E.N., Short, J.W., Schindler, D.W., Hodson, P.V., Ma, M.S., Kwan, A.K., and Fortin, B.L. 2009. Oil sands development contributes polycyclic aromatic compounds to the Athabasca River and its tributaries. Proceedings of the National Academy of Sciences of the United States of America 106(52): 22346-22351.

Kelly, P.M., and Cory, J.S. 1987. Operculum closing as a defence against predatory leeches in four British freshwater prosobranch snails. Hydrobiologia 144: 121-124.

Kemenes, G., Elliott, C.J.H., and Benjamin, P.R. 1986. Chemical and tactile inputs to the *Lymnaea* feeding system: Effects on behaviour and neural circuitry. Journal of Experimental Biology 122: 133-137.

Knag, A.C., Sebire, M., Mayer, I., Meier, S., Renner, P., and Katsiadaki, I. 2013. *In vivo* endocrine effects of naphthenic acids in fish. Chemosphere 93: 2356-2364.

Koene, J.M. 2010. Neuro-endocrine control of reproduction in hermaphroditic freshwater snails: mechanisms and evolution. Frontiers in Behavioral Neuroscience 4: 1-17.

Koene, J.M., Montaigne-Wajer, K., and Ter Maat, A. 2007. Aspects of body size and mate choice in the simultaneously hermaphroditic pond snail *Lymnaea stagnalis*. Animal Biology 57(2): 247-259.

Kumkate, S., Onmek, N., Boonburapong, B., Singhakaew, S., Leardkamolkarn, V. 2009. Estrogen-related fecundity reduction of *Lymnaea ollula* following *Fasciola gigantica* infection. Journal of Parasitology 95(6): 1391-1396.

Kuroda, R. 2014. How a single gene twists a snail. Integrative and Comparative Biology 54(4): 677-687.

Lacchini, A.H., Davies, A.J., Mackintosh, D., and Walker, A.J. 2006. Beta-1, 3-glucan modulates PKC signalling in *Lymnaea stagnalis* defence cells: a role for PKC in H2O2 production and downstream ERK activation. Journal of Experimental Biology 209: 4829-4840.

Latanville, J.K., and Stone, J.R. 2013. Pesticide and predation exposure effects on pond snail immune system function and reproductive output. Journal of Shellfish Research 32(3): 745-750.

Leishman, C., Widdup, E.E., Quesnel, D.M., Chua, G., Gieg, L.M., Samuel, M.A., and Muench, D.G. 2013. The effect of oil sands process-affected water and naphthenic acids on the germination and development of Arabidopsis. Chemosphere 93: 380-387.

Leiva, G.E., Munoz, J.E., and Navarro, J.M. 1998. Encapsulated development and hatching mechanism of the trumulco snail *Chorus giganteus* (Gastropoda: Muricidae), under laboratory conditions. Revista Chilena de Historia Natural 71: 157-167.

Leonard, E.M., and Wood, C.M. 2013. Acute toxicity, critical body residues, Michaelis-Menten analysis of bioaccumulation, and ionoregulatory disturbance in response to waterborne nickel in four invertebrates: *Chironomus riparius*, *Lymnaea stagnalis*, *Lumbriculus variegatus* and *Daphnia pulex*. Comparative Biochemistry and Physiology C 158(1): 10-21.

Leung, S.S-C., MacKinnon, M.D., and Smith, R.E.H. 2001. Aquatic reclamation in the Athabasca, Canada, oil sands: Naphthenate and salt effects on phytoplankton communities. Environmental Toxicology and Chemistry 20(7): 1532-1543.

Li, K.W., Van Minnen, J., Van Veelen, P.A., Van der Greef, J., and Geraerts, W.P.M. 1996. Structure, localization and action of a novel inhibitory neuropeptide involved in the feeding of *Lymnaea*. Molecular Brain Research 37: 267-272.

Li, K.W., Holling, T., de With, N.D., and Geraerts, W.P.M. 1993. Purification and characterization of a novel tetradecapeptide that modulates oesophagus motility in *Lvmnaea stagnalis*, Biochemical and Biophysical Research Communication 197(3): 1056-1061.

Liang, X., Zhu, X., and Butler, E.C. 2011. Comparison of four advanced oxidation processes for the removal of naphthenic acids from model oil sands process water. Journal of Hazardous Materials 190: 168-176.

Lister, A., Nero, V., Farwell, A., Dixon, D.G., and Van der Kraak, G. 2008. Reproductive and stress hormone levels in goldfish (*Carassius auratus*) exposed to oil sands process-affected water. Aquatic Toxicology 87(3): 170-177.

Liu, T., Koene, J.M., Dong, X., and Fu, R. 2013. Sensitivity of isolated eggs of pond snails: a new method for toxicity assays and risk assessment. Environmental Monitoring and Assessment 185(5): 4183-4190.

Lydeard, C., Cowie, R.H., Ponder, W.F., Bogan, A., Bouchet, P., Clark, S.A., Cummings, K.S., Frest, T.J., Gargominy, O., Herbert, D., Hershler, R., Perez, K.E., Roth, B., Seddon, M., Strong, E., and Thompson, F. 2004. The global decline of non-marine mollusks. Bioscience 54: 321-330.

MacDonald, G.Z., Hogan, N.S., Kollner, B., Thorpe, K.L., Phalen, L.J., Wagner, B.D., and van den Heuvel, M.R. 2013. Immunotoxic effects of oil sands-derived naphthenic acids to rainbow trout. Aquatic Toxicology 126: 95-103.

Madill, R.E.A., Orzechowski, M.T., Chen, G., Brownlee, B.G., and Brunce, 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(3): 197-208.

Marois, R., and Croll, R.P. 1991. Hatching asynchrony within the egg mass of the pond snail, *Lymnaea stagnalis*. Invertebrate Reproduction and Development 19(2): 139-146.

McKim, J.M. 1977. Evaluation of tests with early life stages of fish for predicting long-term toxicity. Journal of the Fisheries Research Board of Canada 34(8): 1148-1154.

McKim, J.M., and Benoit, D.A. 1974. Duration of toxicity tests for establishing "no effect" concentrations for copper with brook trout (*Salvelinus fontinalis*). Journal of the Fisheries Research Board of Canada 31(4): 449-456.

McNeill, S.A., Arens, C.J., Hogan, N.S., Kollner, B., and van den Heuvel, M.R. 2012. Immunological impacts of oil sands-affected waters on rainbow trout evaluated using an in situ exposure. Ecotoxicology and Environmental Safety 84: 254-261.

Melvin, S.D., and Trudeau, V.L. 2012. 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.

Misiti, T., Tandukar, M., Tezel, U., and Pavlostathis, S.G. 2013. Inhibition and biotransformation potential of naphthenic acids under different electron accepting conditions. Water Research 47: 406-418.

Monk, W.A., Peters, D.L., and Baird, D.J. 2012. Assessment of ecologically relevant hydrological variables influencing a cold-region river and its delta: the Athabasca River and the Peace–Athabasca Delta, northwestern Canada. Hydrological Processes 26: 1827-1839.

Morrill, J.B. 1982. Development of the pulmonate gastropod, *Lymnaea*. In: Harrison, F.W., Cowden, R.R. (Eds.), Developmental Biology of the Freshwater Invertebrates. Alan R. Liss., New York, pp. 399–483.

Munley, K.M., Brix, K.V., Panlilio, J., Deforest, D.K., and Grosell, M. 2013. Growth inhibition in early life-stage tests predicts full life-cycle toxicity effects of lead in the freshwater pulmonate snail, *Lymnaea stagnalis*. Aquatic Toxicology 128-129: 60-66.

Murakami, J., Okada, R., Fujito, Y., Sakakibara, M., Lukowiak, K., and Ito, E. 2013. Paired pulse ratio analysis of insulin-induced synaptic plasticity in the snail brain. Journal of Experimental Biology 216: 1771-1773.

Nagle, G.T., de Jong-Brink, M., Painter, S.D., and Li, K.W. 2001. Structure, localization and potential role of a novel molluscan trypsin inhibitor in *Lymnaea*. European Journal of Biochemistry 268: 1213-1221.

Nero, V., Farwell, A., Lee, L.E.J., Van Meer, T., MacKinnon, M.D., and Dixon, D.G. 2005. The effects of salinity on naphthenic acid toxicity to yellow perch: Gill and liver histopathology. Ecotoxicology and Environmental Safety 65: 252-264.

Nethery, A.A. 1967. Inhibition of mitosis by surfactants. Cytologia 32(3-4): 321-327.

Noisette, F., Comtet, T., Legrand, E., Bordeyne, F., Davoult, D., and Martin, S. 2014. Does encapsulation protect embryos from the effects of ocean acidification? The example of *Crepidula fornicata*. PLoS ONE 9(3): e93021

Nora, A., Szczepanek, A., and Koenen, G. 2001. Metallic Soaps. Ullman's Encyclopedia of Industrial Chemistry. Wiley Online Library.

Nowland, L.E., and Carriker, M.R. 1946. Observations on the biology of the snail *Lymnaea stagnalis appressa* during twenty generations in laboratory culture. American Midland Naturalist 36(2): 467-493.

O'Connor, T.P. 2002. National distribution of chemical concentrations in mussels and oysters in the USA. Marine Environmental Research 53: 117-143.

Ontzen, D. 2011. Protein-surfactant interactions: A tale of many states. Biochimica et Biophysica Acta 1814: 562–591.

Orr, M.V, Hittel, K., and Lukowiak, K. 2009. 'Different strokes for different folks': geographically isolated strains of *Lymnaea stagnalis* only respond to sympatric predators and have different memory forming capabilities. The Journal of Experimental Biology 212: 2237-2247.

Orr, M.V., El-Bekai, M., Lui, M., Watson, K., and Lukowiak, K. 2007. Predator detection in *Lymnaea stagnalis*. Journal of Experimental Biology 210: 4150-4158.

Pecl, G.T., and Moltschaniwskyj, N.A. 1999. Somatic growth processes: how are they altered in captivity?. Proceedings of the Royal Society of London B 266: 1133-1139.

Peters, L.E., MacKinnon, M., Van Meer, T., van den Heuvel, M.R., and 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.

Prescott, D.R.C., and Curteanu, M.M. 2004. Survey of aquatic gastropods in the central parkland subregion of Alberta. Alberta Sustainable Resource Development, Fish and Wildlife Division, Alberta Species At Risk Report No. 92.

Quesnel, D.M., Bhaskar, I.M., Gieg, L.M., and Chua, G. 2011. Naphthenic acid biodegradation by the unicellular alga *Dunaliella tertiolecta*. Chemosphere 84: 504-511.

Rittschof, D., and McClellan-Green, P. 2005. Molluscs as multidisciplinary models in environmental toxicology. Marine Pollution Bulletin 50: 369-373.

Rogers, V.V., Liber, K., and MacKinnon, M.D. 2002a. Isolation and characterization of naphthenic acids from Athabasca oil sands tailings pond water. Chemosphere 408: 519-527.

Rogers, V.V., Wickstrom, M., Liber, K., MacKinnon, M.D. 2002b. Acute subchronic mammalian toxicity of naphthenic acids from oil sands tailings. Toxicological Sciences 66: 347-355.

Rosza, K.S., and Nagy, I.Z.S. 1967. Physiological and histochemical evidence for neuroendocrine regulation of heart activity in the snail *Lymnaea stagnalis* L. Comparative Biochemistry and Physiology 23(2): 373-382.

Roubos, E.W., and van Heumen, W.R.A. 1994. Peptide processing and release by the neuroendocrine caudodorsal cells of *Lymnaea stagnalis* during an egg-laying cycle. Brain Research 644: 83-89.

Rowland, S. J., Jones, D., Scarlett, A.G., West, C.E., Hin, L.P., Boberek, M., Tonkin, A., Smith, B.E., and Whitby, C. 2011a. Synthesis and toxicity of some metabolites of the microbial degradation of synthetic naphthenic acids. Science of the Total Environment 409: 2936-2941.

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

Salakni, J., Farkas, A., Kamardina, T., and Rosza, K.S. 2003. Molluscs in biological monitoring of water quality. Toxicology Letters 140-141: 403-410.

Schramm, L.L., Stasiuk, E.N., and MacKinnon, M., 2000. Surfactants in Athabasca oil sands slurry conditioning, flotation recovery, and tailings processes. In: Schramm, L.L. (Ed.), Surfactants: Fundamentals and Applications in the Petroleum Industry. Cambridge University Press, Cambridge, pp. 365–430.

Scott, A.C., Zubot, W., MacKinnon, M.D., Smith, D.W., and Fedorak, P.M. 2008. Ozonation of oil sands process water removes naphthenic acids and toxicity. Chemosphere 71: 156-160.

Segner, H., Caroll, K., Fenske, M., Janssen, C.R., Maack, G. Pascoe, D., Schafers, C., Vandenbergh, G.F., Watts, M., and Wenzel, A. 2003. Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. Ecotoxicology and Environmental Safety 54: 302-314.

Serfozo, Z., and Elekes, K. 2002. Nitric oxide level regulates the embryonic development of the pond snail *Lymnaea stagnalis*: pharmacological, behavioral, and ultrastructural studies. Cell and Tissue Research 310(1): 119-130.

Sminia, T. 1972. Structure and function of blood and connective tissue cells of the fresh water pulmonate *Lymnaea stagnalis* studied by electron microscopy and enzyme

histochemistry. Zeitschrift für Zellforschung und mikroskopische Anatomie 130(4): 497-527.

Smit, A.B., Vreugdenhil, E., Ebberink, R.H.M., Geraerts, W.P.M., Klootwijk, J., and Joosse, J. 1988. Growth-controlling molluscan neurons produce the precursor of an insulin-related peptide. Nature 331: 535-538.

Smith, B.E., Lewis, C.A., Belt, S.T., Whitby, C., and Rowland, S.J. 2008. Effects of Alkyl Chain Branching on the Biotransformation of Naphthenic Acids. Environmental Science & Technology 42(24): 9323-9328.

Smith, R.G., and Schramm, L.L. 1992. The influence of mineral components on the generation of natural surfactants from Athabasca oil sands in the alkaline hot water process. Fuel Processing Technology 30: 1-14.

Smits, J.E.G., Hersikorn, B.D., Young, R.F., and Fedorak, P.M. 2012. Physiological effects and tissue residues from exposure of leopard frogs to commercial naphthenic acids. Science of the Total Environment 437: 36-41.

Staras, K., Kemenes, G., and Benjamin, P.R. 2000. Cellular traces of behavioral classical conditioning can be recorded at several specific sites in a simple nervous system. The Journal of Neuroscience 19(1): 347-357.

Staras, K., Kemenes, G., and Benjamin, P.R. 1998. Neurophysiological correlates of unconditioned and conditioned feeding behavior in the pond snail *Lymnaea stagnalis*. Journal of Neurophysiology 79(6): 3030-3040.

Straub, V.A., Styles, B.J., Ireland, J.S., O'Shae, M., and Benjamin, P.R. 2004. Central localization of plasticity involved in appetitive conditioning in *Lymnaea*. Learning & Memory 11: 787-793.

Straub, V.A., and Benjamin, P.R. 2001. Extrinsic modulation and motor pattern generation in a feeding network: a cellular study. The Journal of Neuroscience 21(5): 1767-1778.

Swigert, J.P., Lee, C., Wong, D.C.L., White, R., Scarlett, A.G., West, C.E., and Rowland, S.J. 2015. Aquatic hazard assessment of a commercial sample of naphthenic acids. Chemosphere 124: 1-9.

Sychra, J., Celechovska, O., Svobodova, Z., and Sychra, O. 2011. Lead, mercury and cadmium content in bottom sediments, reed (*Phragmites australis*) beds and great pond snails (*Lymnaea stagnalis*) in fishponds and the role of littoral zones in their accumulation. Acta Veterinaria Brno 80(3): 313-321.

Tate, T.M., Spurlock, J.O., and Christian, F.A. 1997. Effect of glyphosate on the development of *Pseudosuccinea columella* snails. Archives of Environmental Contamination and Toxicology 33: 286-289.

Taylor, H.H. 1977. The ionic and water relations of embryos of *Lymnaea stagnalis*, a freshwater pulmonate mollusc. Journal of Experimental Biology 69: 143-172.

Ter Maat, A., Zonneveld, C., Arjan, J., de Visser, G.M., Jansen, R.F., Montagne-Wajer, K., and Koene, J.M. 2007. Food intake, growth, and reproduction as affected by day length and food availability in the pond snail *Lymnaea stagnalis*. American Malacological Bulletin 23: 113-120.

Ter Maat, A., Pieneman, A.W., Goldschmeding, J.T., Smelik, W.F.E., and Ferguson, G.P. 1989. Spontaneous and induced egg laying behavior of the pond snail, *Lymnaea stagnalis*. Journal of Comparative Physiology A 164: 673-683.

Ter Maat, A., Lodder, J.C., Veenstra, J., and Goldschmeding, J.T. 1982. Suppression of egg-laying during starvation in the snail *Lymnaea stagnalis* by inhibition of the ovulation hormone producing caudo-dorsal cells. Brain Research 239: 535-542.

Thomas, K.V., Langford, K., Petersen, K., Smith, A.J., and 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.

Timoney, K.P. 2009. Three centuries of change in the Peace-Athabasca Delta, Canada. Climate Change 93: 485-515.

Tissot, B.P., and Welte, D.H. 1978. Petroleum Formation and Occurrence. Springer-Verlag, New York.

Toor, N.S., Franz, E.D., Fedorak, P.M., MacKinnon, M.D., and Liber, K. 2013. Degradation and aquatic toxicity of naphthenic acids in oil sands process-affected waters using simulated wetlands. Chemosphere 90: 449-458.

Tripet, F., and Perrin, N. 1994. Size-dependent predation by *Dugesia lugubris* (Turbellaria) on *Physa acuta* (Gastropoda): experiments and model. Functional Ecology 8: 458-463.

Tuersley, MD and McCrohan, CR. 1987. Food arousal in the pond snail, *Lymnaea stagnalis*. Behavioral and Neural Biology 48: 222-236.

UNEP. 2012. The UN-Water Status Report on the Application of Integrated Approaches to Water Resources Management. United Nations Environment Programme.

USEPA. 2013. Aquatic life ambient water quality criteria for ammonia – Freshwater 2013. U.S. Environmental Protection Agency Office of Water. Washington, D.C.

USEPA. 2007. Aquatic life ambient freshwater quality criteria – Copper. U.S. Environmental Protection Agency Office of Water. Washington, D.C.

USEPA. 2003. Ambient Aquatic Life Water Quality Criteria for Tributyltin (TBT) – Final. U.S. Environmental Protection Agency Office of Water. Washington, D.C.

USNRC. 2008. Urban Stormwater Management in the United States. United States National Research Council. The National Academies Press, Washington, D.C.

Vaentine-Darby, P.L., Kell, S.E., and Darby, P.C. 2015. Predation on Florida apple snails (*Pomacea paludosa*) by native and non-native aquatic fauna, and predator-prey size relationships. Florida Scientist 78(1): 47-56.

van den Heuvel, M.R., Hogan, M.S., MacDonald, G.Z., Berrue, F., Young, R.F., Arens, C.J., Kerr, R.G., and Fedorak, P.M. 2014. Assessing accumulation and biliary excretion of naphthenic acids in yellow perch exposed to oil sands-affected waters. Chemosphere 95: 619-627.

Vaiopoulou, E., Misiti, T.M., and Pavlostathis, S.G. 2015. Removal and toxicity reduction of naphthenic acids by ozonation and combined ozonation-aerobic biodegradation. Bioresource Technology 179: 339-347.

van der Schalie, H., and Berry, E.G. 1973. Effects of temperature on growth and reproduction of aquatic snails. Office of Research and Monitoring, U.S. Environmental Protection Agency.

van Duivenboden, Y.A., Pieneman, A.W., and ter Maat, A. 1985. Multiple mating suppresses fecundity in the hermaphrodite freshwater snail *Lymnaea stagnalis*: A laboratory study. Animal Behavior 33: 1184-1191.

Vandeven, A.M.H., and Roubos, E.W. 1990. Effects of diet on spontaneous and experimentally induced egg-laying of the fresh-water snail *Lymnaea stagnalis*. Invertebrate Reproduction & Development 18(3): 209-212.

Varro, P., Gyori, J., and Vilagi, I. 2009. *In vitro* effects of fipronil on neuronal excitability in mammalian and molluscan nervous systems. Annals of Agricultural and Environmental Medicine 16: 71-77.

Vedamanikam, V.J., and Shazilli, N.A.M. 2008. The effect of multi-generational exposure to metals and resultant change in median lethal toxicity tests values over subsequent generations. Bulletin Of Environmental Contamination And Toxicology 80(1): 63-67.

Vesprini, N.D., Dawson, T.F., Yuan, Y., Bruce, D., and Spencer, G.E. 2015. Retinoic acid affects calcium signaling in adult molluscan neurons. Journal of Neurophysiology 113(1): 172-181.

Vitousek, P.M., Aber, J.D., Howarth, R.W, Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger, W.H., and Tilman, D.G. 1997. Human alteration of the global nitrogen cycle: Sources and consequences. Ecological Applications 7(3): 737-750.

Volker, C., Boedicker, C., Daubenthaler, J., Oetken, M., and Oehlmann, J. 2013. Comparative toxicity assessment of nanosilver on three *Daphnia* species in acute, chronic and multi-generation experiments. Plos ONE 8(10).

Voronezhskaya, E.E., Hiripi, L., Elekes, K., and Croll, R.P. 1999. Development of catecholaminergic neurons in the pond snail, Lymnaea stagnalis: I. Embryonic development of dopamine-containing neurons and dopamine-dependent behaviors. The Journal of Comparative Neurology 404: 285-296.

Vreugdenhil, E., Jackson, J.F., Bouwmeester, T., Smit, A.B., Van Minnen, J., Van Heerikhuizen, H., Klootwijk, J., and Joose, J. 1988. Isolation, characterization, and evolutionary aspects of a cDNA clone encoding multiple neuropeptides involved in the stereotyped egg-laying behavior of the freshwater snail *Lymnaea stagnalis*. Journal of Neuroscience 8: 4184-4191.

Watson, S.N., Lee, J.R., Risling, T.E., Hermann, P.M., and Wildering, W.C. 2014. Diminishing glutathione availability and age-associated decline in neuronal excitability. Neurobiology of Aging 35(5): 1074-1085.

Watson, S.N., Nelson, M.A., and Wildering, W.C. 2012. Redox agents modulate neuronal activity and reproduce physiological aspects of neuronal aging. Neurobiology of Aging 33: 149-161.

Wayland, M., Headley, J.V., Peru, K.M., Crosley, R., and Brownlee, B.G. 2008. Levels of polycyclic aromatic hydrocarbons and dibenzothiophenes in wetland sediments and aquatic insects in the oil sands area of Northeastern Alberta, Canada. Environmental Monitoring and Assessment 136: 167-182.

Webber, L.M., and Lodge, D.M. 1990. Periphytic food and predatory crayfish: Relative roles in determining snail distribution. Oecologia 82: 33-39.

Wendelaar Bonga, S.E. 1972. Neuroendocrine involvement in osmoregulation in a freshwater mollusc, Lymnaea stagnalis. General and Comparative Endocrinology Supplement 3: 308-316.

Wijsman, T.C.M., and van Wijk-Battenburg, H. 1987. Biochemical composition of the eggs of the freshwater snail *Lymnaea stagnalis* and oviposition-induced restoration of albumen

gland secretion. International Journal of Invertebrate Reproduction and Development 12: 199-212.

Wilson, A. 2012. Water, power and culture in the Roman and Byzantine worlds: An introduction. Water History 4(1): 1-9.

Winiwarter, V., Schmid, M., and Dressel, G. 2013. Looking at half a millennium of co-existence: the Danube in Vienna as a socio-natural site. Water History 5(2): 101-119.

Yen, T.-W., Marsh, W.P., MacKinnon, M.D., and Fedorak, P.M. 2004. Measuring naphthenic acids concentrations in aqueous environmental samples by liquid chromatography. Journal of Chromatography A 1033: 83-90.

Yorlova, N.I. 2003. The influence of trematodes infections on reproductive potential of *Lymnaea stagnalis* (Gastropoda: Pulmonata) field population. Russian Journal of Zoology 82: 1027-1037.

Young, R.F., Michel, L.M., and Fedorak, P.M. 2011. Distribution of naphthenic acids in tissues of laboratory-exposed fish and in wild fishes from near the Athabasca oil sands in Alberta, Canada. Ecotoxicology and Environmental Safety 74: 889-896.

Young, R.F., Orr, E.A., Goss, G.G., and Fedorak, P.M. 2007. Detection of naphthenic acids in fish exposed to commercial naphthenic acids and oil sands process-affected water. Chemosphere 68: 518-527.

Zeinalov, E.B., Abbasov, V.M., and Alieva, L.I. 2009. Petroleum acids and corrosion. Petroleum Chemistry 49(3): 185-192.

Zhao, S., Kotlyar, L.S., Sparks, B.D., Woods, J.R., Gao, J., and Chung, K.H. 2001. Solids contents, properties and molecular structures of asphaltenes from different oil sands. Fuel 80: 1907-1914.

Zonneveld, C., and Kooijman, S.A.L.M. 1989. Application of a dynamic energy budget model to *Lymnaea stagnalis* (L.). Functional Ecology 3: 269-278.