Effects of Oil Sands Process-Affected Water and Substrates on Wood Frog (*Rana sylvatica*) Eggs and Tadpoles

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A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Toxicology Graduate Program at the University of Saskatchewan

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ABSTRACT

An essential element of the reclamation strategy proposed by the oil sands mining industry in northern Alberta, Canada, includes the creation of wetlands for the bioremediation of mining waste materials. The mining process used to extract oil from these deposits results in the production of large volumes of process-affected water (OSPW) and sediments (OSPS), which must be incorporated into wetlands as a component of the reclaimed landscapes. Wood frogs (*Rana sylvatica*) are an abundant native species that might be expected to inhabit these reclaimed wetlands. The objective of this study was to determine potential detrimental effects of OSPW and OSPS on the growth and development of wood frogs. Several morphological (weight, length, condition factor) and biochemical (whole body tadpole thyroid hormone and triglyceride concentrations and metamorph hepatic glycogen concentration) endpoints were assessed in conjunction with hatchability and survivability of wood frog eggs and tadpoles exposed to process-affected materials (OSPM) under field and laboratory conditions.

As part of this study, assay techniques were optimized to enable simultaneous measurement of whole body 3,5,3'-triiodothyronine (T_3), thyroxine (T_4) and triglyceride (TG) concentrations in wood frog tadpoles. These assays were used to monitor changes in T_3, T_4 and TG in wood frog tadpoles during development from hatching to metamorphosis (Gosner stages 19-46), to establish baseline levels for subsequent application of the assays to evaluate contaminant effects. The results indicated peak T_3 and T_4 concentrations occurred during metamorphic climax (Gosner stages 40-46) and prometamorphosis (Gosner stages 31-40), respectively. Maximal TG concentrations were also observed during prometamorphosis. These assays were further employed to assess body condition and

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development in wood frogs during a field study in 2005, and the following laboratory studies in 2006 and 2007.

In summer 2005, 29 reclaimed and five unimpacted wetlands were monitored for use by native amphibians, and tadpoles and newly-metamorphosed wood frogs were collected from a subset of sites as a preliminary assessment of contaminant effects. Endpoints such as metamorph hepatic glycogen and whole body tadpole T_3, T_4 and triglyceride concentrations were compared among six impacted and three reference wetlands. The surveys indicated 60% of OSPW-impacted wetlands were used by breeding adult amphibians, while wood frog tadpoles and newly-metamorphosed frogs were observed in 37 and 30% of OSPW wetlands, respectively. In general, lower whole body tadpole T_3 and triglyceride concentrations were observed in wood frogs from wetlands containing OSPM. In contrast, hepatic glycogen concentrations in newly-metamorphosed frogs and whole body tadpole T_4 and T_3/T_4 concentrations were comparable among the reference and impacted wetlands. In addition, the differences observed in total body weight and length of tadpoles and newly-metamorphosed wood frogs among OSPM and reference sites were likely due to minor differences in developmental stages of the animals collected from the various wetlands, rather than any contaminant effect.

In 2006 and 2007, wood frog eggs and tadpoles were exposed to several sources of OSPW and OSPS collected from reclaimed Suncor and Syncrude wetlands under controlled laboratory conditions. Hatchability was reduced in eggs exposed to water from only one of the OSPW sites, compared with the other process-affected ponds and the control water ($P<0.05$). In contrast, survivability of tadpoles was significantly reduced ($P<0.05$) in all the impacted sites in both years, with nearly all OSPW sites having <10%

survival. The exposure study evaluated the toxicity of five types of OSPS. Results indicated no impact of OSPS exposure on survivability of tadpoles, but showed reduced whole body weight (in three OSPS treatments), length (in two OSPS treatments) and body condition (in one OSPS) of tadpoles exposed to process-affected substrates tested ($P<0.05$). Whole body T_3 and T_4 concentrations in tadpoles from OSPS treatments were not different from the control treatment, but tadpole TG concentration was reduced in groups exposed to two impacted substrates ($P<0.05$). Water quality measurements, including determination of dissolved metals were conducted in an initial attempt to relate any potential toxic effect on wood frog growth and development to specific contaminants.

Results of the laboratory studies strongly suggest that exposure to OSPW and OSPS may adversely affect wood frog growth and survival. However, these findings were not entirely consistent with field observations and results of concurrent mesocosm studies. Further research is therefore needed to fully evaluate the suitability of reclaimed oil sands wetlands to support indigenous amphibian population. Future work should focus on the cumulative effects of water and substrates, as well as the effect of OSPM ageing on acute and chronic toxicity.

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LIST OF ABBREVIATIONS

AEPEA = Alberta Environmental Protection and Enhancement Act

ANOVA = Analysis of variance

BDL (*or* <LOD) = Below detection limit

Ca^{2+} = Calcium

Cl^- = Chloride

CT = Consolidated tailings

D_2 = Type II iodothyronine

D_3 = Type III iodothyronine

DDT = 1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane

DO = Dissolved oxygen

ELISA = Enzyme-linked immunosorbent assay

FT = Fine tails

HCO_3^+ = Bicarbonate

ICP-MS = Inductively coupled plasma mass spectrometer

K^+ = Potassium

K_{oc} = Sorption coefficient

LC_{50} = Median lethal concentration

MFT = Mature fine tails

Mg^{2+} = Magnesium

N_2 = Nitrogen gas

Na^+ = Sodium

NH_3 = Ammonia (un-ionized)

NH_4^+ = Ammonia (ionized)

o,p'-DDT = 1,1,1-trichorlo-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane

OSPM = Oil sands process-affected materials

OSPS = Oil sands process-affected sediments/substrates

OSPW = Oil sands process-affected water

PAHs = Polycyclic aromatic hydrocarbons

PGO = Peroxidase-glucose oxidase

ppb = Parts per billion

ppm = Parts per million

PTU = Propyl-thio-uracil

r = Correlation coefficient

SO_4^- = Sulfate

T_3 = 3,5,3'–triiodothyronine

T_4 = 3,5,3,5'-tetraiodothyronine or thyroxine

TG = Triglyceride

TH = Thyroid hormone

CHAPTER 1
1.0 GENERAL INTRODUCTION

1.1   Background: Athabasca oil sands

The Athabasca oil sands, located in northern Alberta, constitute one of the world's largest deposits of heavy crude oil (Figure 1.1). These oil sands cover an area of more than 42,000 km^2 and are estimated to contain over 700 billion barrels of bitumen (Madille *et al*., 2001). They are the only oil sands deposits shallow enough to be suitable for open pit mining. Open pit mines are used for extracting commercially useful mineral or rock deposits found near the surface, where the overburden (surface material covering the valuable deposit) is relatively thin or the material of interest is structurally unsuitable for tunneling, as is the case for sand, cinder and gravel. Underground mining methods are utilized to extract valuable materials where the overburden is thick or the mineral occurs as veins hosted in hard rock. Open-pit mines are generally enlarged until the mineral reserve is exhausted.

Figure 1.1: Map outlining the Athabasca oil sands and surrounding area in Alberta, Canada (modified from Tarbuck and Lutgens, 1999).

Mining of oil sand in Alberta yields bitumen, which is a semi-solid form of crude oil containing silica sand, clay minerals and water. On average, bitumen consists of 83.2% carbon, 10.4% hydrogen, 4.8% sulphur, 0.94% oxygen and 0.36% nitrogen (FTFC, 1995). It is extracted using the Clark Hot Water extraction process, which involves two main steps. During the first step, known as the conditioning stage, the slurry (a combination of oil sand and water) is mixed with hot water (79-93ºC) and caustic soda (sodium hydroxide) at a pH of approximately 8.2 (FTFC, 1995). This causes the bitumen and sand to separate due to their different masses, allowing the collection of bitumen from the surface of the mixture. The second step, known as the separation stage, involves a combination of hot water and agitation that results in the release of bitumen from the oil sand, and allows small air bubbles to attach to the bitumen droplets. The bitumen froth floats to the top of the separation vessel, and is further treated to remove residual water and fine solids. Together these stages are known as primary bitumen recovery. Crude bitumen is sticky and tar-like, which makes it much more viscous than traditional crude oil. Consequently, it must either be chemically split or mixed with lighter petroleum (either liquid or gas) before it can be transported by a pipeline and be upgraded into synthetic crude oil (Suddhasatwa *et al*., 1998).

1.1.1   Contaminants associated with oil extraction

The extraction procedure generates large volumes of liquid wastes, referred to as oil sands process-affected water (OSPW), along with a relatively stable suspension of solids and unrecovered bitumen called fine tails. Approximately 0.65 m^3 of wastewater is produced during extraction of each ton of oil sands (Matthews *et al*., 2000). Bitumen

itself contains saturated hydrocarbons with long chain alkyl groups attached to bi- to tetracyclic cores, polycyclic aromatic hydrocarbons (PAHs), and naphthenic acids (Madill *et al.*, 2001).

Naphthenic acids are composed of saturated aliphatic, monocyclic and polycyclic alkanes with carboxylated aliphatic side chains of various lengths. They are biodegradable despite exhibiting surfactant properties that make them acutely toxic to aquatic life (Headley and McMartin, 2004). Polycyclic aromatic hydrocarbons, on the other hand, are potentially more persistent, and are known mutagens and carcinogens. They are present at 1.5 - 150 times higher concentrations in wetlands containing oil sand process-affected sediments than in sediments from unaffected wetlands; and are 4 - 6 times higher in water from wetlands containing OSPW when compared with water from unaffected wetlands (Smits *et al.*, 2000).

1.1.1.1 Oil sand process-affected water (OSPW)

Fresh tailings water resulting from oil sands processing can be acutely toxic to fish (LC_{50} = 125ml of OSPW/L for rainbow trout) and aquatic invertebrates (LC_{50} = 980 ml of OSPW/L for *Daphnia magna*) (Mackay and Verbeek, 1993).

Pore water present between particles of fine tailings in the settling ponds is slightly saline with the main ions being sodium (Na^+), bicarbonate (HCO_3^+) and chloride (Cl^-). Other major ions such as potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}) and sulfate (SO_4^-) are found at low concentrations (<20 ppm). Nutrients (most significantly, ammonia) and trace elements (generally at concentrations below 50 ppb) are also present in the pore water, along with high concentrations of dissolved organic carbon (50-70 mg

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C/l) (Boerger *et al.*, 1992). Most of the dissolved organic matter (>80%) is in the acid extractable fraction, and includes carboxylic acids, humic and fulvic acids, surfactants and phenolic components.

Fine tailings pore water also contains simple phenols of low molecular weight at concentrations of less than 0.2 mg/l. Characterization of base/neutral organics extracted from the fine tails pore waters has shown concentrations of aromatic hydrocarbons, including PAHs, to be at or below detection levels (<1 ppb), except for certain low molecular weight organics (Boerger *et al.*, 1992). Metals, including cadmium, zinc, lead and vanadium have been measured at concentrations well above the levels needed to elicit a toxic response in aquatic invertebrates and other taxa (Barton and Wallace, 1979).

In addition to the complex mixture of organic and inorganic contaminants listed above, OSPW contains a variety of naphthenic acids in potentially high concentrations. This is not surprising, since naphthenic acids constitute as much as 50% by weight of the total acidic proportion in crude oil (Headley and McMartin, 2004). They are leached from the oil sands during the extraction process, and are present in the fine tails zone at a pH of 8-8.5 and concentrations of 10-25 mg/l (Boerger *et al.*, 1992). These compounds are responsible for most of the acute toxicity of OSPW to aquatic organisms (Mackay and Verbeek 1993). Although generally bioavailable, naphthenic acids can gather at aqueous/nonaqueous interfaces due to their surfactant-like properties, which can decrease their bioavailablity to a certain extent.

1.1.1.2 Oil sand process-affected substrates (OSPS)

Unextracted (or residual) bitumen accounts for approximately 1-9% of fine tails in tailings ponds (FTFC, 1995). Concentrations of PAHs and heterocyclic aromatic compounds from bitumen range from <0.01-10 mg/kg in fine tails (FTFC, 1995). Sediments can act as reservoirs for trace metals and PAHs due to their hydrophobic properties. Characteristically, four- to six-ring PAHs sorb strongly to sediment but have very low water solubilities (Neff, 1979). This leads to a concentration-dependent equilibrium between the sorbed and dissolved states in the water column. Low molecular weight PAHs (K_{oc} ~10^3-10^4) adsorb less strongly than high molecular weight PAHs (K_{oc} ~10^5-10^6) to organic carbon in sediments. In a wet landscape system (i.e., a lake or a pond), the relatively greater solubility of low molecular weight PAHs will allow them to cycle through the water-capping layer, while high molecular weight PAHs should remain bound to the fine tails (Madill *et al*., 2001). High organic carbon content in sediments is known to reduce PAH bioavailability.

Studies have shown lower species diversity in benthic invertebrates, such as insect larvae in wetlands containing OSPS compared to wetlands unaffected by mining (Bendell-Young *et al*., 2000; Leonhardt, 2003). The larvae (e.g. chironomids) live and feed on sediment that has accumulated significant amounts of contaminants related to the oil sands extraction process. Insectivorous vertebrates (fish, birds and amphibians) feed on these benthic larvae, consuming both the larvae and the gut contents (Bendell-Young *et al*., 2000). Chronic exposure to OSPS resulted in lower body mass in mallard ducklings (Gurney *et al.*, 2005) and greater mortality and stunted growth in Northern Canadian toad and wood frog tadpoles (Pollet and Bendell-Young, 2000). In addition to

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direct toxic effects on insectivores, the presence of potentially bioaccumulative compounds in the oil sands effluent may lead to the transfer of these substances to higher trophic levels.

1.1.2 Wetland reclamation strategy

Surface mining by oil sands companies such as Syncrude Canada Ltd. and Suncor Energy Inc. currently account for 65% of total production from the oil sands (Alberta Department of Energy, 2005). The Alberta Environmental Protection and Enhancement Act (AEPEA) prohibits the release of the potentially toxic fine tailings and OSPW into the Athabasca River, and requires these companies to remediate their leases to a state approximating the environment present before mining operations began (Madill *et al.*, 2001). The production and storage of large volumes of process-affected water from extraction of bitumen poses a challenge for site remediation.

Because the oil sands companies operate under a zero discharge policy, large tailings ponds are required to retain liquid wastes. The ponds function not only as clarifiers for tailings but also as temporary storage areas for the fine tails before reclamation. The slurry of water, solids and unrecovered bitumen resulting from the extraction of oil sand is transported to the tailings pond. Rapid settling of the coarser solids (>22 µm) from the discharged tailings slurry forms the dyke system around the tailings pond. Fine tails are formed when the solids (mostly kaolinite and illite clays) settle to the bottom. The surface water layer is recycled after a period of settling. Recycled water accounts for >70% of the water needed for bitumen extraction (Boerger *et al.*, 1992).

The "wet landscape" option for complying with the AEPEA involves transferring the fluid tailings into a mined-out pit and capping them with clean water, to create artificial lakes and wetlands with (theoretically) the appearance and biological productivity of natural lakes in the region. To satisfy regulatory demands, these reclaimed wetlands need to demonstrate the ability to support aquatic and semi-aquatic organisms.

1.2 Amphibian model species

Amphibians are a class of vertebrates that generally undergo dramatic metamorphosis involving the transformation from an aquatic larva to a terrestrial or a semi-aquatic adult. Modern amphibians can be classified into three orders: Anura (frogs and toads), Urodela (newts and salamanders), and Caecilia (legless, wormlike animals) (Shi, 2000).

There are a number of amphibian species that are indigenous to the Athabasca oil sands region. These species include: the western toad (*Bufo boreas*), the largest toad found in Alberta (55-125 mm in length); the boreal chorus frog (*Pseudacris triseriata*), Alberta's smallest amphibian (20-40 mm in length); the Canadian toad (*Bufo hemiophrys*), Alberta's smallest toad (37-75 mm in length); and the wood frog (*Rana sylvatica*), the smallest true frog in Alberta (30-60 mm in length).

1.2.1 Amphibians as indicator species in ecotoxicology studies

Amphibians can be extremely useful sentinel species in the assessment of contaminant effects in wetland habitats. They are important components of many different ecosystems worldwide. Many amphibians are herbivorous as tadpoles and

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carnivorous as adults (Burger and Snodgrass, 1998). This accords them the status of both an important predator and a prey species. In many forest habitats, for example, the numbers and biomass of amphibians exceed all other vertebrates (Stebbins and Cohen, 1995), such that declines in population can have severe implications for the whole community.

Amphibians are particularly good representatives of wetland environments, since they have a life cycle that generally includes both an aquatic and a terrestrial phase. Their life history is unique among vertebrates, with the deposition of unshelled eggs in aquatic environments, followed by a gill-respiring, swimming detritivore/herbivore larval stage, and a semi-aquatic hopping or climbing insectivorous adult stage. This makes them potentially highly vulnerable to numerous stressors in both aquatic and terrestrial environments (Wassersug, 1997).

Amphibians are sensitive to many pollutants, including metals such as aluminum, cadmium, iron, lead and zinc, and pesticides, such as DDT and atrazine. Birge *et al*. (2000) compared the sensitivity of fish species commonly used in toxicity tests to a variety of indigenous amphibian species. A total of 694 amphibian/fish comparisons were done to test 50 metals and inorganic chemicals, as well as 13 organic compounds. Results demonstrated that in 64% of all tests amphibians had lower LC_{50} values than fish. Amphibians are also potentially more likely than other vertebrates to accumulate significant body burdens, since toxicants can be readily taken up by both dietary ingestion and dermal absorption. Their highly permeable skin not only facilitates dermal respiration, but also potential uptake of contaminants.

1.2.2 Amphibian (anuran) development

Amphibian metamorphosis is one of the oldest and best studied hormone-regulated developmental processes. Amphibian larvae hatch as embryos from eggs and quickly transform into free swimming tadpoles. These larvae then undergo many changes themselves, in order to transform into a frog (Figure 1.2).

(a)   (b)

(c)   (d)
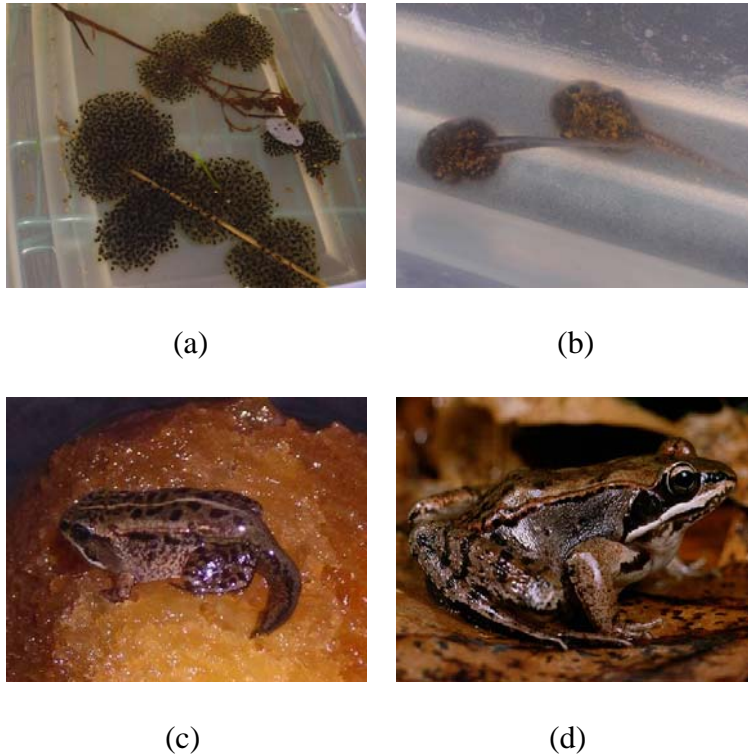
Figure 1.2: Life cycle of an amphibian: (a) clustered egg masses; (b) free-swimming tadpoles; (c) froglet with remnant tail; (d) fully metamorphosed frog.

Anuran metamorphosis has three specific phases: premetamorphosis, prometamorphosis and metamorphic climax. Premetamorphosis includes embryogenesis and early tadpole growth and development. Some morphological changes, such as the initial development of the hind limbs, occur during this phase. During prometamorphosis, the hind limbs undergo rapid and extensive growth. Metamorphic climax is the period in

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which the most rapid and significant morphological changes take place. These include the complete resorption of the tadpole's tail, forelimb development, and dramatic changes in internal organs (Shi, 2000).

1.2.2.1 Gosner stages

In 1960, Kenneth L. Gosner devised a comprehensive system to classify the several stages of anuran embryonic and larval development. These "Gosner stages" demonstrate the complex changes a tadpole undergoes to complete metamorphosis in 46 distinct steps.

The embryonic or prefeeding phases are defined by Gosner stages 1 through 20. Embryos hatch between stages 17 - 20, and the external gill filaments develop fully between stages 21 and 23. The transition to a feeding and free-swimming tadpole occurs between stages 21 - 25. The hind limb bud develops between the stages of 26 and 30. From stages 31 - 37, there is the appearance of individual toes. Stages 38 - 40 are delineated by changes in the length of individual toes and the appearance of tubercles. Metamorphic climax, which heralds the drastic final changes in metamorphosis, extends from stage 41 to stage 45. The resorption of the tail results in a decrease of total length. Forelimbs appear in stage 42 and metamorphosis is complete at stage 46 (Gosner, 1960).

1.2.2.2 Metamorphic climax

Metamorphosis involves systematic, coordinated transformations of numerous structures and organs, which culminate in metamorphic climax (Figure 1.3). These final changes include remodeling of the digestive system along with resorption of the gills and

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the tail, differentiation of the hind limb toes, and the appearance of forelimbs (Cai and Brown, 2004). Prior to the development of lungs, the gills are the main respiratory organs. As the lungs develop, the tadpole begins to swim to the surface of the water to breathe. Changes to the digestive system include shortening of the intestines to accommodate a carnivorous diet (Shi, 2000). The complex, highly integrated process of amphibian metamorphosis is vulnerable to disruption by environmental stressors, including chemical contaminants (Chinathamby *et al*., 2006; Gutleb *et al*., 2000)

(a)   (b)

(c)   (d)
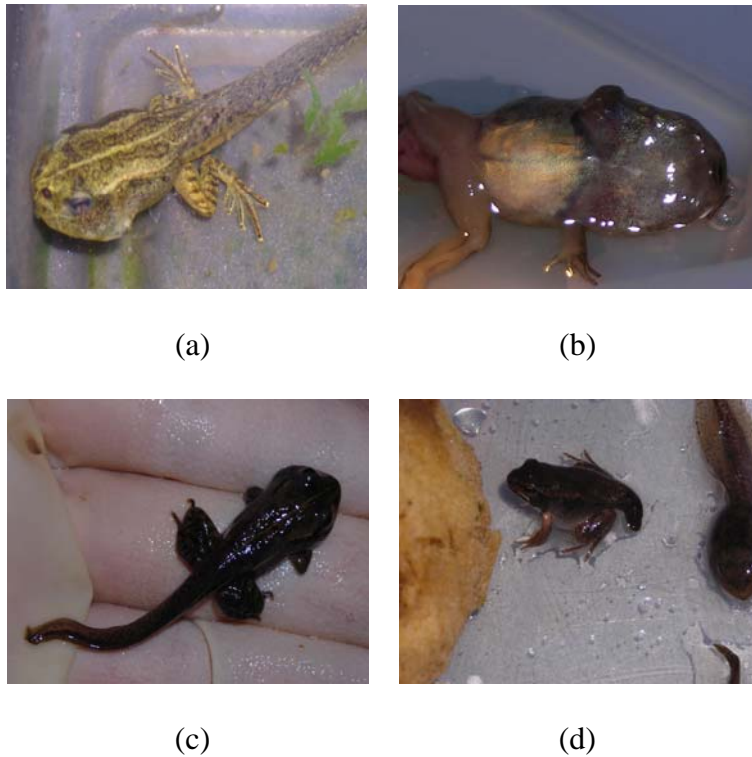
Figure 1.3: Changes that occur during metamorphic climax in anuran metamorphosis. (a) Differentiation of hind limb toes (Gosner stage 41); (b) Appearance of forelimbs (Gosner stage 42); (c) Resorption of the tail (Gosner stage 43); (d) Remnant tail (Gosner stage 44-45).

1.2.3 Wood frog natural history and distribution

Rana sylvatica, commonly known as the wood frog, is recognizable by its characteristic black "mask" across the eyes, and may have a thin white stripe down its back (Government of Alberta, 2002) (Figure 1.4). Females are a few millimetres longer than males. Males have paired vocal sacs that inflate when they call. Tadpoles have a very short, round body and an arched tail fin that begins high on the back. They are uniformly dark with gold flecks in lines around the mouth. Wood frog tadpoles are omnivores, feeding on algae and bacteria, as well as on other amphibian eggs and hatchlings, including the American toad (*Bufo americanus*), gray treefrog (*Hyla chrysoscelis*), pickerel frog (*Rana palustris*), and spotted salamander (*Ambystoma maculata*). The adult wood frog diet includes insects, worms, snails, millipedes, molluscs, and other small invertebrates (Morin and Johnson, 1988; Petranka *et al*., 1994; Petranka *et al*., 1998).

Figure 1.4: A wood frog (*Rana sylvatica*) (Government of Alberta, 2002).

Breeding can occur from late April to June, with males calling during daylight or at night for one to two weeks. During breeding, the males have enlarged thumbs and toe webs to facilitate amplexus (the mating position of frogs and toads, in which the male

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grasps the female with his limbs and waits for her to lay eggs so that he can fertilize

them; fertilization is external). Females lay 2000-3000 eggs in large round masses of

jelly. Masses from several females are usually laid together, either attached to submerged

sticks and plants or free floating in seasonal pools, shallow ponds, marshy lake edges,

flooded meadows, and quiet stretches of streams. An egg is about 1.5 mm in diameter and

hatches in approximately three weeks (Government of Alberta, 2002). Tadpoles usually

live in the shallowest and warmest parts of the wetland. Males mature one year after

metamorphosis, while females reach maturity in two years. Wood frogs seldom live more

than three or four years.

Wood frogs are largely terrestrial, but are not usually found far from water. They

inhabit marshes, riparian areas, wet meadows, moist brush, and open grassy areas

adjacent to such habitats. Wood frogs hibernate in the soil, under logs, leaves or tree

stumps covered by an insulating layer of snow. They do not go very far underground to

hibernate and, as such, are exposed to freezing temperatures in winter. Their survival

depends on the production of high levels of glucose as a cryoprotectant, which enables

them to survive the freezing of up to 65 - 70% of their body water (Muths *et al.*, 2005).

When frozen, blood flow, pulmonary breathing and cardiac activity essentially cease.

After thawing, these physiological functions are rapidly restored.

Since wood frogs are able to withstand freezing, they are the only amphibians in

North America that are found north of the Arctic Circle. Specifically, their range extends

from Alaska to Labrador in northern North America (Chubbs and Phillips, 1998),

reaching south to New Jersey, northern Georgia and northern Idaho. There are also

disjunctive populations in northern Colorado and Arkansas-Missouri (Stebbins, 1985, Conant and Collins, 1991) (Figure 1.5).

Figure 1.5: Map of wood frog (*Rana sylvatica*) distribution in North America.

1.3   Physiological endpoints and biomarkers in amphibian studies

Environmental contamination and the potential adverse effects of chemical exposure have been proposed as contributing factors in the decline of some amphibian populations (Blaustein *et al.*, 1994). Various physiological endpoints and biochemical biomarkers are being used as tools to assess the health of amphibians exposed to contaminants worldwide. Reproductive endpoints such as hatching rate and tadpole growth and survival can provide valuable insight into population sustainability in the face of environmental stressors. Biochemical biomarkers, such as determination of energy stores (triglycerides, hepatic glycogen) and hormone concentrations (thyroid hormones),

can provide useful information concerning the health and fitness of individual animals, and insight into the effects of exposure on survival potential.

1.3.1 Reproductive endpoints

Environmental contaminant exposure has the potential to affect amphibian reproductive success at several levels. Contaminant exposure of adult frogs can inhibit breeding behaviour, production of gametes, fertilization or offspring sex ratio (Carey and Bryant, 1995). Toxicants can also disrupt embryonic and larval development and growth. Hatching success, tadpole survival and growth, and time to metamorphosis are common indicators used to demonstrate the effects of contamination on amphibian population productivity.

1.3.1.1 Hatching success

Amphibian eggs possess a permeable membrane that allows uptake of chemicals from the water by the developing embryo (Dunson *et al.*, 1992). Unfavourable conditions, such as dissolved metals (Horne and Dunson, 1995), low pH (Sadinski and Dunson, 1992) and/or high levels of organic carbon (Freda *et al.*, 1990), can decrease amphibian egg hatching rate. Embryos of some species can take several weeks to hatch, which can increase the exposure period to any toxicants present in the water column.

Studies with Spotted (*Ambystoma maculatum*) and Jefferson salamanders (*Ambystoma jeffersonianum*) have shown that low pH can cause thoracic swelling in the hatchlings. Low pH, high cation concentrations, or high dissolved metal concentrations

can also cause curling deformities in the exposed embryo (Laposata and Dunson, 1998).

These deformities can affect hatching success and subsequent survival.

1.3.1.2 Tadpole growth and survival

Alterations in environmental factors such as resource limitation, predation, crowding and habitat dessication can affect tadpole growth and survival. These factors can either stimulate growth if present during prometamorphosis, or inhibit it if present during premetamorphosis (Shi, 2000).

Anuran larvae can adapt to the varying availability of water. They can either prolong their aquatic phase, maximizing growth but risking desiccation, or escape the drying conditions, thus metamorphosing below optimal size. Juveniles that metamorphose at a small size have lowered ability to withstand desiccation and take longer to reach reproductive maturity (DiMauro and Hunter, 2002). Evaporation of the pond may also lead to increased concentration of nonvolatile compounds and metabolic wastes, which represent additional stressors to developing tadpoles.

Most anurans do not ingest food at metamorphosis due to the magnitude of physiological and anatomical changes occurring in the final transition from aquatic larval stage to a semi-aquatic adult. While these changes take place, xenobiotic compounds taken up during the larval stage can either be eliminated or retained and redistributed in the tissues. Due to the loss of mass resulting from lack of feeding and the high energetic costs associated with metamorphosis, environmental contaminants that have been retained may become concentrated in tissues (Snodgrass *et al*., 2003).

1.3.2 Biochemical endpoints

Changes in biochemical parameters (biomarkers) in response to environmental stressors can provide information which is more sensitive and specific for particular contaminant exposures than alterations in morphological or behavioral characteristics. Contaminant-induced changes in thyroid hormones and stored energy reserves in amphibian tadpoles may have significant consequences for successful metamorphosis and subsequent survival.

1.3.2.1 Thyroid hormones

The two major thyroid hormones responsible for metabolic regulation in vertebrates are 3,5,3'–triiodothyronine (T_3) and 3,5,3,5'-tetraiodothyronine (T_4). T_4 is commonly known as thyroxine. These hormones act on the liver, kidney, heart, nervous system and skeletal muscle in order to stimulate cellular respiration, oxygen consumption and metabolic rate. The increase in metabolism, stimulated by thyroid hormones, generates heat which is of major importance in the thermoregulation of many vertebrates (Randall *et al*., 2002). T_4 is the circulating hormone produced in the thyroid gland. It is converted into the active hormone T_3 by the enzyme type II iodothyronine deiodinase (D_2) in peripheral tissues. The location of the enzyme D_2 plays a role in a tissue's response to the circulating hormone (Cai and Brown, 2004).

Thyroid hormones are essential in vertebrate development in general and play a pivotal role in the stimulation of amphibian metamorphosis in particular. Thyroid hormone levels usually rise significantly during metamorphosis in anurans. For example, studies with *Xenopus laevis* demonstrate steadily rising plasma thyroid hormone

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concentrations as the tadpoles undergo both growth and morphological transformation (Leloup and Buscaglia, 1977; White and Nicoll, 1981).

Because of their critical role, any stimulatory or inhibitory effects on thyroid hormone status may result in changes in larval morphology or the timing of metamorphosis. Increased levels of thyroid hormones accelerate metamorphosis of larvae in early developmental stages, leading to smaller juveniles with reduced fitness. Conversely, decreased thyroid hormone concentrations inhibit metamorphosis, prolonging the aquatic lifetime with associated increased chances for predation or desiccation.

There are an increasing number of substances present in the environment thought to interfere with critical endocrine systems and developmental processes in vertebrate populations. These chemicals include insecticides, herbicides, pharmaceuticals and many industrial chemicals (Cheek *et al.*, 1998; Danzo, 1997; Brucker-Davis, 1998; Sonnenschein and Soto, 1998). Several of these xenobiotics have the potential to interfere with various aspects of thyroid hormone function by disrupting secretion and/or distribution of endogenous hormone. For instance, the herbicide acetochlor can accelerate T_3 induced precocious metamorphosis in the northern leopard frog, *Rana pipiens* (Cheek *et al*., 1999) and in the African clawed frog, *Xenopus laevis* (Crump *et al.,* 2002). Methoxychlor, an estrogenic organochlorine pesticide, has been known to reduce and delay T_3 surge during metamorphic climax (Fort *et al*., 2004).

1.3.2.1.1 Body condition indices

Evaluation of amphibian body condition can serve as an indication of overall fitness and predict overwinter survival (Congdon *et al.*, 2001). Contaminants can directly or indirectly increase metabolic rate, which can induce biochemical changes that, in turn, can adversely affect the amphibian's ability to store energy. Lipids are mainly stored as triglycerides, while carbohydrates are stored as glycogen (Nelson and Cox, 2005).

1.3.2.1.2 Triglyceride stores

Fats are composed of triglyceride molecules which typically accumulate in the fat vacuoles of specialized adipose cells in vertebrates. Triglycerides in lipid tissue represent a primary form of energy storage. They are rendered highly compact by the relatively high proportions of hydrogen and carbon and low proportions of oxygen in the molecule. Consequently, 1 g of triglyceride yields about twice the energy upon oxidation as 1 g of carbohydrate. Triglycerides can be stored in high concentrations in the body since they have low solubility in water (Randall *et al*., 2002). Lipids in anuran amphibians can be found in fat bodies, liver, subcutaneous tissue, muscle, gonads and the tail (Sheridan and Kao, 1998).

Stress induced by contaminant exposure can cause decreased levels of triglyceride storage in fat tissue, which can adversely affect critical energy-demanding processes, such as metamorphosis. Triglycerides are utilized by amphibians not only for metabolic maintenance during dormancy, but also for the production of gametes. Fat bodies are also shown to be essential for gonadal maintenance (Fitzpatrick, 1976). The highest

concentration of stored lipids in most adult amphibians occurs in early fall preceding hibernation, while the lowest concentration is in the spring and early summer.

Triglycerides are also the principal form of stored energy in fish (Sheridan 1988; Jobling *et al.,* 1998). Measurement of whole body triglyceride concentration has been used as an index of body condition in small fish, and have been shown to be sensitive to the energetic demands of environmental stressors, including contaminants. For example, reduced triglyceride levels were measured in sailfin mollies (*Poecilia latipinna*) exposed to increasing concentrations of 1,1,1-trichorlo-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane (*o,p'*-DDT), an agricultural pesticide (Benton *et al*., 1994). Triglyceride levels were also seen to be depleted in the livers of cod (*Gadus morhua*) and winter flounder (*Pseudopleuronectes americanus*) from the Northwest Atlantic, following long-term exposure to crude petroleum (Dey *et al*., 1983).

1.3.2.1.3 Hepatic glycogen

In animals, glycogen is an essential form of stored energy rapidly available in response to a stressor (Campbell *et al.*, 1999). It is primarily stored in the liver in most species, but also found at lower concentrations in muscles (Nelson and Cox, 2005).

Glycogen reserves are essential to ensure overwinter survival of freeze-tolerant anuran species. It is converted to glucose which acts as a "cryoprotectant", facilitating survival during slow cooling and thawing. Glycogen phosphorylase breaks up glycogen into glucose subunits. The active form of this enzyme, known as glycogen phosphorylase *a*, cleaves glycogen to form glucose 1-phosphate. In cells, glucose 1-phosphate is readily converted to glucose-6-phosphate by the enzyme phosphoglucomutase. Glucose 6-

phosphate enters the glycolytic pathway, or is dephosphorylated to glucose, which is transported across the plasma membrane into the bloodstream (Randall *et al*., 2002).

Mobilization and metabolization of liver glycogen reserves initiates an increase in blood and tissue glucose concentrations. The excess glucose promotes hydrogen bonding with water molecules which prevents cellular dehydration by decreasing water activity when extracellular spaces freeze. Wood frogs in particular utilize this approach to survive freezing up to -5ºC (Willens *et al*., 2005).

1.4   Research objectives and hypotheses

Athabasca oil sands mining companies are required by environmental legislation to restore impacted areas. Constructed wetlands are an essential component of current reclamation strategies. These wetlands contain OSPW and OSPS. There is considerable uncertainty regarding their suitability as habitats for indigenous amphibians, including wood frogs. Wood frogs are an abundant native species in the Athabasca oil sands region which are likely to attempt to use these reclaimed landscapes as their habitat. This study represents an initial attempt to determine if OSPW and OSPS exposure adversely affects growth and development of wood frog embryos and larvae.

It was hypothesized that contaminants associated with oil sand effluent present in these wetlands would adversely affect developing wood frogs. To test this general hypothesis, wood frog at different life stages with OSPW and OSPS exposure were monitored to assess potential toxicity. Physiological and biochemical endpoints, such as hatchability, early life stage survival, tadpole growth rate, time to metamorphosis, and

measurement of hepatic glycogen reserves, tadpole whole body triglyceride and thyroid hormone (T_3 and T_4) concentrations were used to assess contaminant effects.

In addition, a laboratory study was performed to assess triglyceride and thyroid hormone (T_3 and T_4) concentrations in wood frog tadpoles at different stages of metamorphosis (Gosner stages 19 through 46). Consequently, a method for measuring whole body triglyceride and thyroid hormone concentrations in fish was modified for use in tadpoles. This method was further utilized to evaluate the effects of oil sands process-affected materials (OSPM) on triglyceride and thyroid hormone concentrations in wood frogs.

Specific objectives:

 (i) Determine the relationship between whole body triglyceride and thyroid hormone concentrations during normal wood frog development (Chapter 2).

(ii) Assess amphibian use of selected reclaimed and reference wetlands in the Athabasca oil sands region, and collect biological samples from tadpoles and emergent froglets for initial health evaluation (Chapter 3).

(iii) Determine the effects of exposure to OSPW and OSPS on the hatchability, growth and survival of wood frog eggs and tadpoles (Chapter 4).

(iv) Determine the effects of exposure to OSPS on whole body triglyceride and thyroid hormone concentrations in wood frog tadpoles (Chapter 4).

CHAPTER 2
2.0 Development of whole body tadpole bioassays and their application to stages in wood frog metamorphosis

2.1   Abstract

This study was designed to assess the usefulness of whole body thyroid (TH) and triglyceride (TG) concentrations as tools to evaluate the effect of environmental stressors on tadpole thyroid gland function and energy stores, respectively. Thyroid hormone concentrations are potentially useful biomarkers because these hormones appear to be essential initiators of amphibian metamorphosis. Triglyceride stores reflect general health and nutritional status. Both endpoints may be sensitive to environmental stressors, such as chemical contaminants. In this study, whole body concentrations of thyroid hormones, 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4), and triglycerides were measured in developing wood frogs (*Rana sylvatica*) during development and metamorphosis (Gosner stages 19 through 46). A new method was employed, which utilizes the same whole body tadpole homogenate to measure all three biomarkers. Results indicated that the highest concentration of T_3 occurred during metamorphic climax (3.69 ng/g tadpole), while T_4 concentrations were highest during prometamorphosis (57.6 ng/g tadpole). The T_3/T_4 ratio was also greatest during metamorphic climax (0.105), consistent with increased conversion of T_4 to T_3 during this period of development. Whole body triglyceride concentrations were generally greatest during early prometamorphosis (1.44 mg/g tadpole), just before metamorphic climax. Although somewhat preliminary, these baseline results may enable the future application of these whole body assays to the assessment of potential impacts of environmental contaminants on amphibian populations.

2.2 Introduction

Approaches to evaluate the effects of environmental contaminants on amphibian health should include tools to assess potential effects on growth and development of the aquatic larval life stage, and the process of metamorphosis. Two potential biomarkers of toxicological significance to amphibian growth and development are thyroid hormone concentrations, such as 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4), as well as triglyceride (TG) stores.

Amphibian metamorphosis constitutes three main segments: Premetamorphosis occurs prior to the formation of a functional thyroid gland, and signifies initial tadpole growth. In this study, this period is defined by Gosner stages 19 through 31. Prometamorphosis (Gosner stages 31 through 40) includes the period of thyroid gland development, as well as development of the hind limbs and differentiation of the toes (Leloup and Buscaglia, 1977). Metamorphic climax is characterized by the most dramatic internal and external transformations of the tadpole, culminating in the final transition to the adult body form. This period coincides with Gosner stages 40 through 46, until completion of metamorphosis. Amphibian larval development and metamorphosis is initiated and controlled by T_3 and T_4 (Shi, 2000), such that changes in their status may be a useful biomarker of adverse effects.

Previous studies in *Rana*, *Bufo* and *Xenopus* have demonstrated that elevations in circulating plasma concentrations of the thyroid hormones T_3 and T_4 correlate with metamorphosis (Leloup and Buscaglia, 1977; Miyauchi *et al*., 1977; Regard *et al*., 1978; Suzuki and Suzuki, 1981; Weil, 1986). Prometamorphosis is generally characterized by rising concentrations of endogenous T_3 and T_4, with peak plasma levels usually seen at

metamorphic climax (Shi, 2000). Studies of *Xenopus laevis* development revealed very low levels of T_3 and T_4 during premetamorphosis, when tadpoles grow rapidly but exhibit little morphological change. During prometamorphosis, synthesis of endogenous thyroid hormones increase (coincident with thyroid gland development and growth), and concentrations of plasma T_3 and T_4 rise, as the tadpole undergoes both growth and morphological transformations. Finally, at the climax of metamorphosis, plasma T_3 and T_4 concentrations peak and the tadpole stops feeding and undergoes a rapid metamorphic transition. Plasma T_3 and T_4 concentrations typically decline again on completion of metamorphosis (Leloup and Buscaglia, 1977; White and Nicoll, 1981), perhaps in part due to partial regression of the gland itself (Shi, 2000).

Metamorphosis leads to a wide range of morphological and biochemical changes. The brain undergoes restructuring, and genetic reprogramming leads to the appearance of digestive enzymes in the pancreas, urea cycle enzymes and serum albumin in the liver, and the keratinization of the larval skin. The most dramatic modifications, however, are the almost simultaneous emergence of limbs and total loss of larval tails, gills and the digestive system (Tata, 2006).

Both thyroid hormones (T_3 and T_4) are synthesized in the thyroid gland. T_4 is either released directly into the circulating plasma or converted to T_3 (the active hormone) in the thyroid gland through the action of the enzyme type II iodothyronine deiodinase (D_2); although T_4 can be converted to T_3 in other organs as well. T_3 is generally present at much lower concentrations than T_4 in plasma. Expression of D_2 enzyme is upregulated in tissues undergoing metamorphic change. Disruption in the function of this enzyme could prevent the conversion of T_4 to T_3, thereby delaying

metamorphosis and making the larvae more susceptible to predation and/or dessication (Cai and Brown, 2004).

Although thyroid hormones play an essential role during metamorphosis, their synthesis in the thyroid gland is under complex neuroendocrine control. Thyroid hormones in turn can influence neuroendocrine function during metamorphosis. These interactions are manifested in the hypothalamus-pituitary-thyroid axis through the actions of several hormones. The pituitary gland positively regulates the thyroid gland whereas the thyroid gland negatively feeds back to regulate the pituitary gland secretion. The consequence of this interaction is that surgical removal of the pituitary gland inhibits frog metamorphosis. Metamorphic stasis can also be induced by blocking the influence of the hypothalamus on the pituitary (Dodd and Dodd, 1976; White and Nicoll, 1981; Kikuyama *et al*., 1993; Kaltenbach, 1996; Denver, 1996). Therefore, thyroid hormone profiles in amphibians are the net result of numerous regulatory mechanisms acting at two levels: the neuroendocrine axes that control hormone production, and the peripheral tissues that control hormone processing and hormone removal (Shi, 2000).

Determination of total body triglyceride concentrations may also be a valuable biomarker for evaluating the health of developing tadpoles. Like most vertebrate animals, adult anurans store fat, an important energy source, when there is an abundance of food. The developing tadpole's ability to store fat may also reflect the health of their environment as it relates to food abundance and the presence of specific stressors. For example, tadpoles that are subjected to chronic contaminant exposure may be required to expend additional energy to metabolize xenobiotics or maintain homeostasis, and consequently store less energy in fat bodies (Rowe *et al*., 2009).

Lipids play a major role in fueling the energetic demands associated with metamorphosis. Generally, the concentration of whole body lipid is low in premetamorphic tadpoles, increases during prometamorphosis, and declines during metamorphic climax as fat stores are utilized and tadpole's food intake declines (Sheridan and Kao, 1998).

The objective of this study was to assess changes in whole body thyroid hormone (T_3 and T_4) and triglyceride concentrations in the same tadpole during the course of normal development and metamorphosis of wood frog (*Rana sylvatica*) tadpoles, in order to evaluate the potential usefulness of these biomarkers in the assessment of amphibian population health. This has not previously been done in this species. Methods originally developed to measure whole-body thyroid hormones and triglycerides in fish (Weber *et al*., 2003) and lizards (Brasfield *et al*., 2004) were modified for application to wood frog tadpoles.

It was hypothesized that, similar to other anuran species, wood frog tadpole T_3 and T_4 concentrations would gradually increase during development, reaching their highest concentrations during metamorphic climax. Triglyceride concentrations were expected to increase during pre- and prometamorphic phases and decline during metamorphic climax, coincident with a reduction in body mass.

2.3   Materials and Methods

2.3.1   Method development for whole body wood frog tadpole assays

One of the goals of this assay development effort was to determine whether both the triglyceride and the thyroid hormone assays could be determined in the same tissue

homogenate from a single tadpole. This required the investigation of any potential effect

of propyl-thio-uracil (PTU) on the triglyceride assay. Propyl-thio-uracil is an

iodothyronine deiodinase (D_2) enzyme inhibitor used in thyroid hormone assays to inhibit

the *in vitro* activity of D_2 in assay mixtures. It was also necessary to identify a solvent

that was compatible both the triglyceride and thyroid hormone assays. Ethanol has been

successfully used as a solvent in the thyroid hormone assay, but it was not known if it

was lipophilic enough to extract triglycerides.

2.3.1.1 Tissue homogenization and extraction

The homogenization method was modified from whole body methods developed

in fish (Weber *et al.*, 2003) and lizards (Brasfield *et al.*, 2004). Whole frozen tadpoles

were thawed and minced on ice in 95% ethanol containing 1 mM 6-*N*-propyl-2-thiouracil

(PTU, Sigma, Oakville, ON, Canada). Advanced preparation of this ethanol-PTU

solution was done by dissolving 0.017g PTU in 100ml 95% ethanol. The minced sample

was transferred into a glass vial and another 1X volume of ethanol-PTU solution was

added. A smooth homogenate was then obtained using a Tissure Tearor® homogenizer

(BioSpec Products, Bartlesville, OK, USA) at three 15 second intervals. The glass vials

were capped and centrifuged at 2900 rpm for 10 min. at 4°C. After centrifugation, the

supernatant was removed and a 50µL aliquot was collected and stored at –80 °C for

triglyceride analysis. The pellet was extracted a second time with another 2X volume of

ethanol-PTU solution and centrifuged as before. The combined supernatant from the two

extractions was placed under a stream of N_2 to evaporate the ethanol and reduce the

volume to the aqueous portion of the homogenate. The final extract was aliquoted and stored at –80 °C pending T_3 and T_4 analysis.

2.3.1.2   Whole body tadpole triglyceride assay development

The effect of PTU on the triglyceride assay was evaluated by dividing three large tadpoles into equal halves lengthwise and recording the mass of each half. One half of each tadpole was subsequently homogenized in a standard 95% ethanol solution, and the other half was homogenized in 95% ethanol-PTU solution. To assess any interference of PTU with the triglyceride assay, the amount of total triglyceride measured in each half of the tadpoles was compared.

2.3.1.2.1   Triglyceride assay

The tadpole whole body triglyceride assay was based on a method developed in juvenile fish by Weber *et al.* (2003), which uses a commercially available kit (Sigma, Saint Louis, MO, USA). The initial step in the triglyceride assay produces free glycerol from acyl glycerides (mono-, di- and triglycerides) with lipase. Glycerol content is then ascertained in samples and glycerol standards spectrophotometrically via a colourimetric reaction after the addition of glycerol kinase.

2.3.1.3   Whole body tadpole thyroid hormone assay development

Spike recoveries were performed to evaluate the extraction efficiency of the thyroid hormone assays. After tadpole homogenization, the homogenate was divided into two equal volumes. The first aliquot (volume within a range of approximately 250μL –

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510μL) was spiked with 50μL of 7.5 ng/mL T_3 and 50μL of 250 ng/mL T_4, obtained from

enzyme-linked immunosorbent assay (ELISA) kits (BioQuant, San Diego, CA, USA)

(spike and endogenous T_3 or T_4). An equivalent amount of water (100μL) was added to

the second aliquot (endogenous T_3 or T_4 only). A third sample consisted of 50μL of 7.5

ng/mL T_3 and 50μL of 250 ng/mL T_4 added to reagent-grade water (spike amount). The

T_4 and T_3 hormone concentrations were measured in all three samples. The average

percent recovery, measured as [(spike and endogenous T_3 or T_4 - endogenous T_3 or

T_4)/spike amount] x 100%, for T_3 from the spiked samples was 51.3% ($\pm$ 2.9%).The

average recovery for T_4 was higher at 69.7% ($\pm$ 5.5%). Percent recovery increased with

the amount of tissue used, such that 0.50g was determined to be the minimum amount of

tissue required.

2.3.1.3.1   Thyroid hormone assays

Whole-body tadpole T_3 and T_4 concentrations were measured using commercial

enzyme-linked immunosorbent assay (ELISA) kits (BioQuant, San Diego, CA, USA).

2.3.2   Association of triglyceride and thyroid hormone concentrations with tadpole development

After assay optimization, they were used to measure whole body T_3 and T_4 and

triglyceride concentrations in developing wood frog tadpoles, to evaluate changes in

these critical endpoints throughout the course of development and metamorphosis.

2.3.2.1 Animal husbandry and sampling of wood frog tadpoles

Newly-fertilized wood frog egg masses were collected from local Saskatchewan water bodies with minimal exposure to agricultural chemicals or other environmental contaminants in April-May of 2006. Each egg mass was placed in four litres of dechlorinated tap-water in a 11.7x20.8x34.0 cm plastic container in one of two controlled environmental chambers at 10ºC. The containers were continuously aerated using an air stone, and subjected to 16 hours of full spectrum light and eight hours of darkness per day. Once the eggs started to hatch, tadpoles from different egg masses were randomly allocated to different containers at a density of 15 tadpoles per four litres. There were 7 replicate containers in each environmental. The temperature of the environmental chamber was gradually increased from 10ºC to 22ºC over the course of development to mimic water temperature in the source ponds. Once the tadpoles started feeding, their diet consisted of boiled green lettuce and ground Tetramin® tropical fish flakes. The food was replaced every second day along with half of the water volume. Basic water quality parameters such as conductivity (517-589 µs/cm), pH (7.24-8.18), hardness (132-143 mg $CaCO_3/L$), alkalinity (92-94 mg/L), ammonia (0.06-0.17 mg/L) and dissolved oxygen (DO) (6.0-8.74 mg/L) levels were monitored on a weekly basis to ensure that they remained within acceptable ranges.

Tadpoles were collected at specific developmental stages (Gosner stages 19 to 46) from containers in both environmental chambers, weighed and stored in cryovials at -80°C pending analysis. Because newly-hatched tadpoles were too small to effectively be used for the whole body tadpole triglyceride and thyroid hormone assays, individual animals were pooled to achieve a minimum mass of 0.50g per replicate.

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2.3.3 Statistical analyses

Samples obtained from the two environmental chambers were pooled for both the triglyceride and thyroid hormone assays in order to increase the sample size, n, for a given Gosner stage. To determine if samples from the different chambers could be pooled, a t-test was conducted to compare sample means from T_3, T_4 and triglyceride assays at each Gosner stage from the two environmental chambers. Log transformation was used for data that failed normality.

Parametric one-way ANOVA and non-parametric Kruskal-Wallis one-way ANOVA on ranks were done to test significant differences. Multiple comparisons were performed with Tukey's (parametric ANOVA) and Dunn's (non-parametric ANOVA) test. Where needed, data was normalized using log and arcsine transformations. To assess the relationship between endpoints (TG and T_4; TG and T_3), Pearson product moment correlations were used. The correlation coefficient (r) was given at $p < 0.05$ for all correlations. The results are expressed as mean $\pm$ SEM.

Six determinations of a pooled sample were used to assess intra-assay variability for each of the three endpoints (triglycerides, T_3 and T_4). In addition, the same pooled sample was run six more times on separate occasions to evaluate inter-assay variability.

2.4 Results

2.4.1 Whole body tadpole thyroid hormones and tadpole development

Figure 2.1 shows the concentrations of both T_3 and T_4 hormones (graph A), as well as the ratio of T_3 to T_4 concentrations (graph B) measured in whole body homogenates of wood frog tadpoles over the course of development. Samples for T_4 analysis collected from the two environmental chambers were pooled after results of a t-test on log-transformed data indicated a probability of no difference between chambers (P=0.774). Samples for T_3 analysis collected from both chambers were also pooled after results of a t-test indicated a probability of no difference between the means (P=0.253).

There were significant differences in tadpole whole body T_3 and T_4 concentrations over time. Whole body tadpole T_3 concentrations were lowest during premetamorphosis, at Gosner stages 22-24 (0.68 ng/g tadpole). The T_3 concentrations increased significantly (P<0.05) during prometamorphosis, with concentrations of 2.96 and 3.40 ng/g tadpole at Gosner stages 31-32 and 38-39, respectively. The highest T_3 concentration was observed during metamorphic climax at Gosner stage 43-44 (3.69 ng/g tadpole), although this value was not significantly greater than that measured at the end of prometamorphosis. The lowest whole body tadpole T_4 concentration was also observed during premetamorphosis at Gosner stage 22-24 (17.1 ng/g tadpole). The T_4 concentrations increased significantly to peak during prometamorphosis, at Gosner stages 31-32 (53.4 ng/g tadpole) and 34-35 (57.6 ng/g tadpole). T_4 concentrations decreased during early metamorphic climax, followed by a second spike near completion of metamorphosis, at Gosner stages 45-46 (44.0 ng/g tadpole). There was a gradual increase in whole body T_3/T_4 ratio over the course of wood frog development. The lowest value

was observed during premetamorphosis at Gosner stages 22-24 (0.040), with the highest T_3/T_4 ratio measured during the metamorphic climax at Gosner stages 41-42 (0.105) and 43-44 (0.100) ($P<0.05$).

Mean values from the individual developmental stages comprising each metamorphic period were grouped to produce a composite picture of the distribution of whole body tadpole T_3 and T_4 concentrations (Figure 2.2) and T_3/T_4 ratio (Figure 2.3) during wood frog metamorphosis. The grouped data demonstrate that mean T_3 concentration increased significantly ($P<0.05$) from premetamorphosis through metamorphic climax (graph A). Conversely, mean T_4 concentration peaked during prometamorphosis and declined during metamorphic climax (graph B). A similar trend to T_3 concentration was observed in whole body tadpole T_3/T_4 ratio (Figure 2.3). The ratio was lowest during premetamorphosis and increased steadily to peak during metamorphic climax ($P<0.05$).
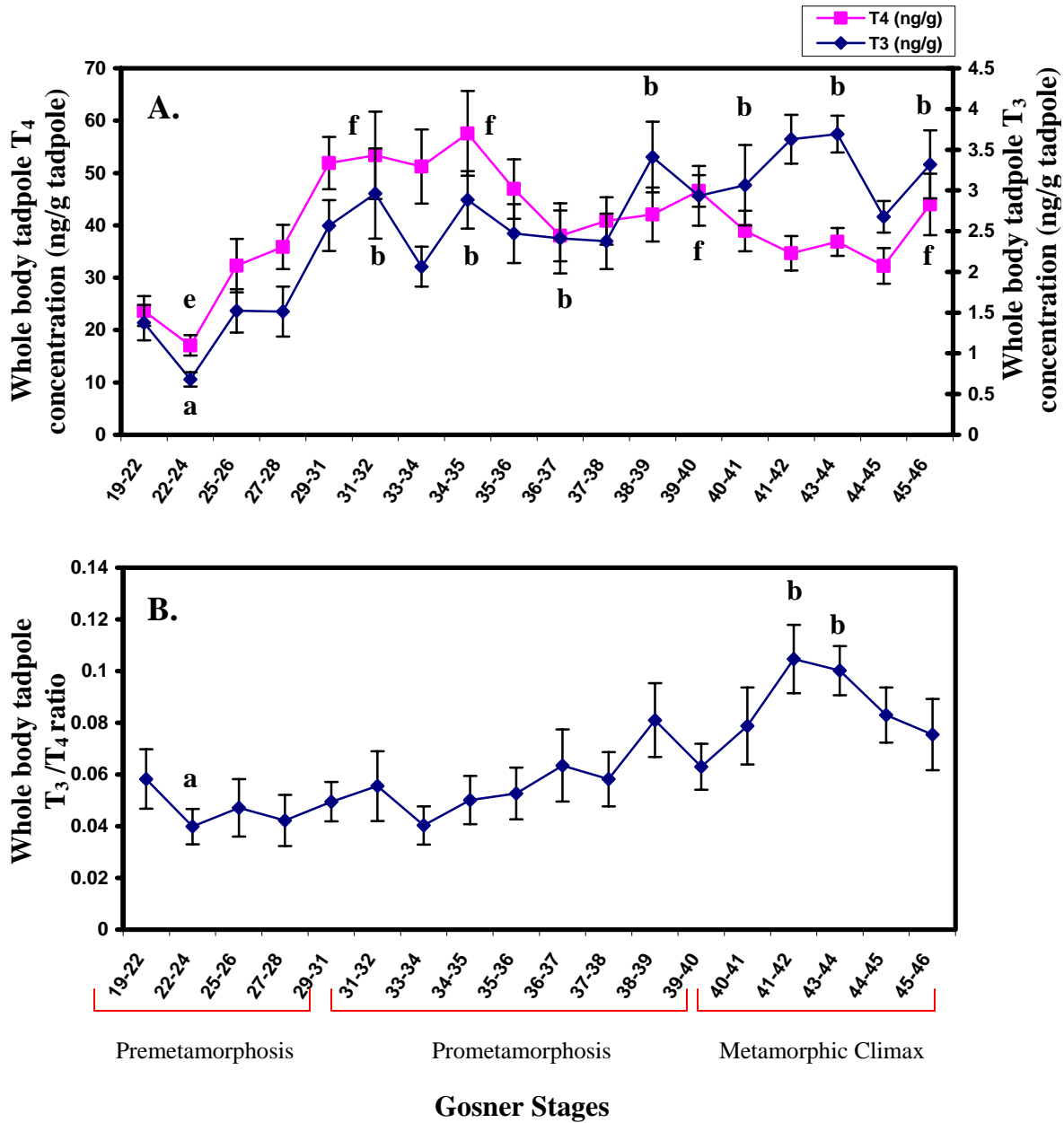
Figure 2.1: Whole body tadpole 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4) concentrations (ng/g tadpole tissue) (A) and T_3/T_4 ratio (B) in relation to wood frog (*Rana sylvatica*) metamorphosis in Gosner stages. Data shown are mean ± standard error of the mean. n=4-10 tadpoles per Gosner stage. Data were analyzed using parametric ANOVA followed by Tukey's post-hoc test. Values marked with different letters were significantly different from each other (P<0.05) ('a' is significantly lower than 'b' and 'e' is significantly lower than 'f').
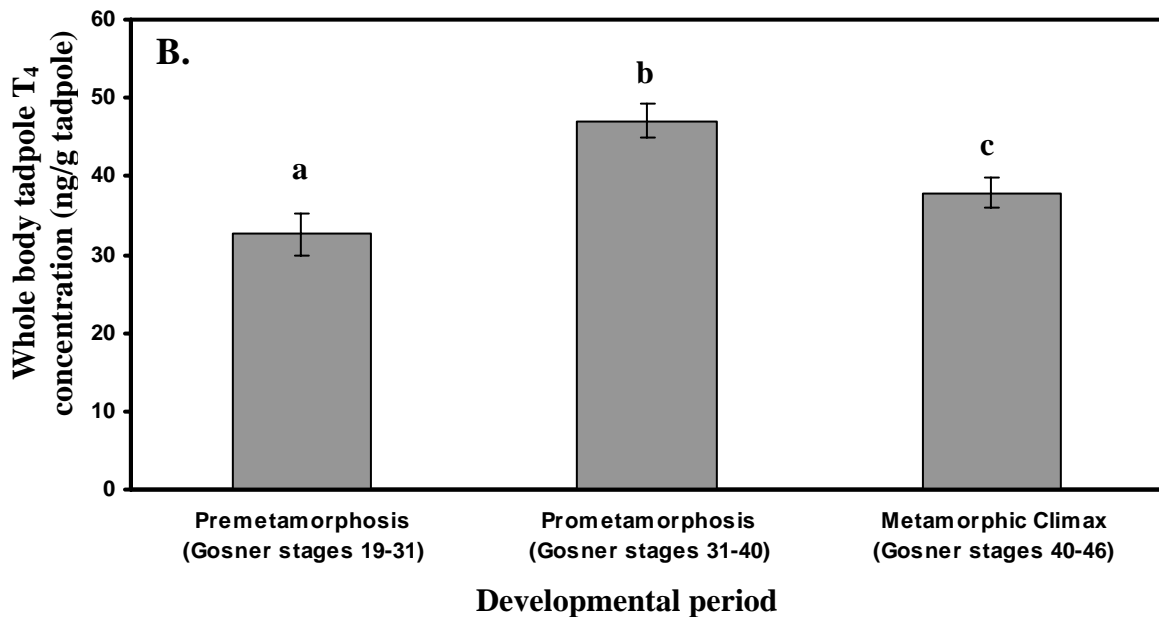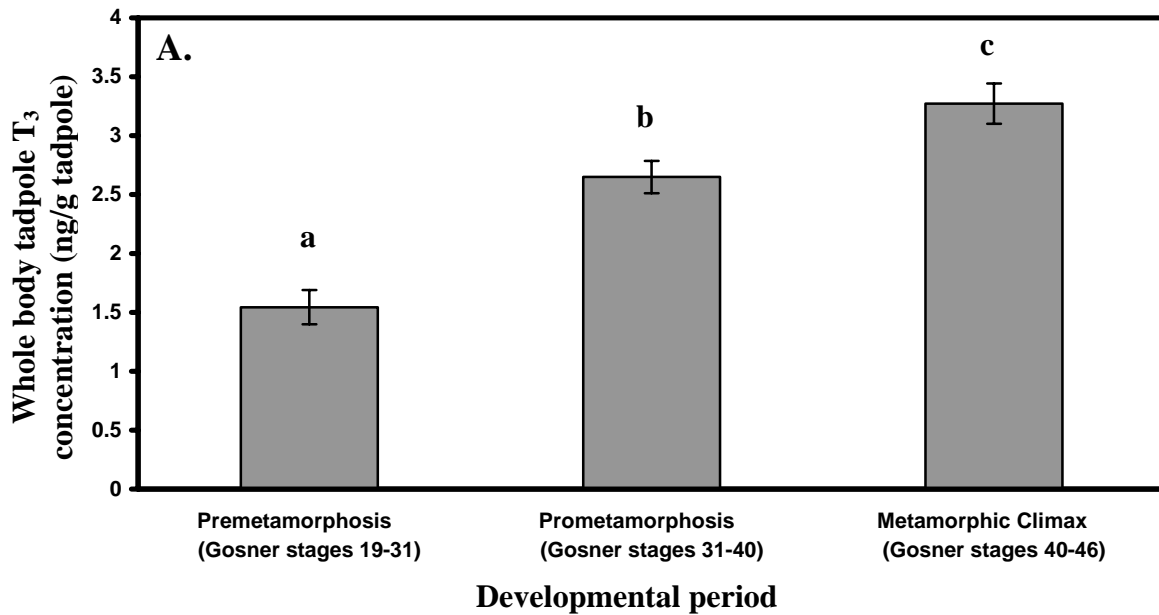
Figure 2.2: Whole body tadpole 3,5,3'-triiodothyronine (T_3) (A) and thyroxine (T_4) (B) concentrations (ng/g tadpole tissue) during premetamorphosis (Gosner stages 19-31), prometamorphosis (Gosner stages 31-40) and metamorphic climax (Gosner stages 40-46) stages of wood frog (*Rana sylvatica*) development. Data shown are mean ± standard error of the mean. n=40-74 tadpoles per developmental period. Data were analyzed using parametric ANOVA followed by Tukey's post-hoc test. Values marked with different letters were significantly different from each other ($P<0.05$).
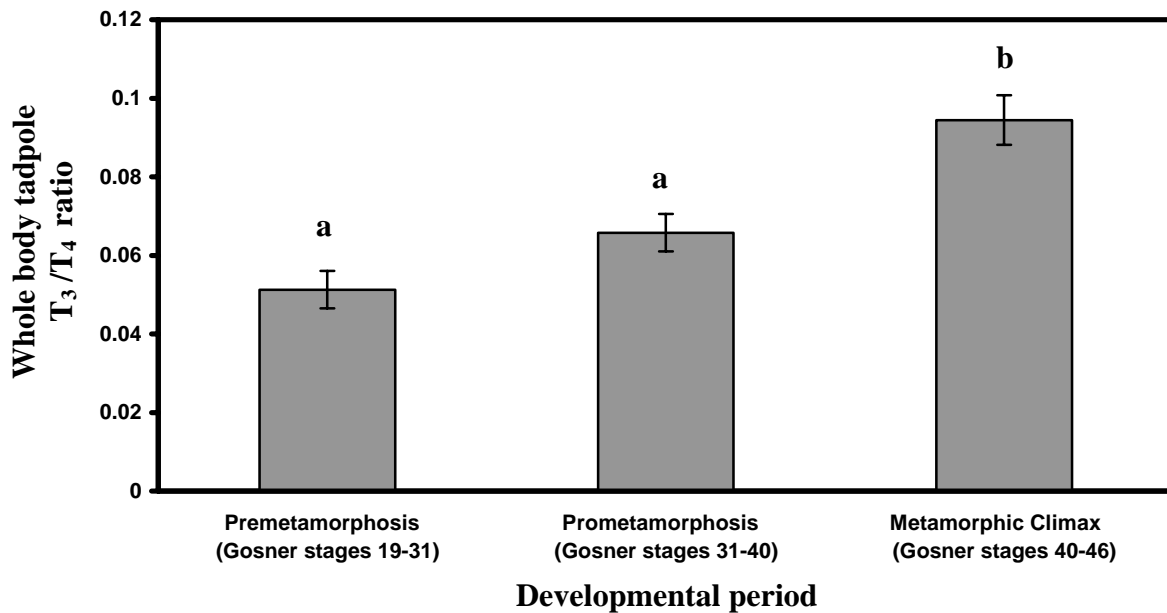
Figure 2.3: Whole body tadpole 3,5,3'-triiodothyronine (T_3):thyroxine (T_4) ratio during premetamorphosis (Gosner stages 19-31), prometamorphosis (Gosner stages 31-40) and metamorphic climax (Gosner stages 40-46) stages of wood frog (*Rana sylvatica*) development. Data shown are mean ± standard error of the mean. n=40-74 tadpoles per developmental period. Data were analyzed using parametric ANOVA followed by Tukey's post-hoc test. Values marked with different letters were significantly different from each other ($P<0.05$).

2.4.2 Whole body tadpole triglyceride concentration and tadpole development

Results of the preliminary triglyceride assay demonstrated that ethanol was a suitable solvent for extraction purposes and that homogenization in PTU did not affect the triglyceride assay. Figure 2.4 illustrates the change in wood frog whole body tadpole triglyceride concentrations during development and metamorphosis. Samples collected from the two environmental chambers were pooled after results of a t-test on log-transformed data indicated a probability of no difference between chambers ($P=0.171$). Results indicate that mean whole body triglyceride concentrations were greater during prometamorphosis than during premetamorphosis and metamorphic climax ($P<0.05$). The highest triglyceride concentration was observed at the beginning of prometamorphosis at Gosner stages 31-32 (1.44 mg/g tadpole), with the lowest values occurring at metamorphic climax (Gosner stage 43-44) (0.55 mg/g tadpole).

Mean values from the individual developmental stages comprising each metamorphic period were grouped to produce a composite picture of the distribution of whole body tadpole triglyceride concentrations during wood frog metamorphosis (Figure 2.5). The grouped data clearly demonstrate a significant increase in whole body triglyceride stores from pre- to prometamorphosis ($P<0.05$), with a subsequent decrease to minimal levels during metamorphic climax.
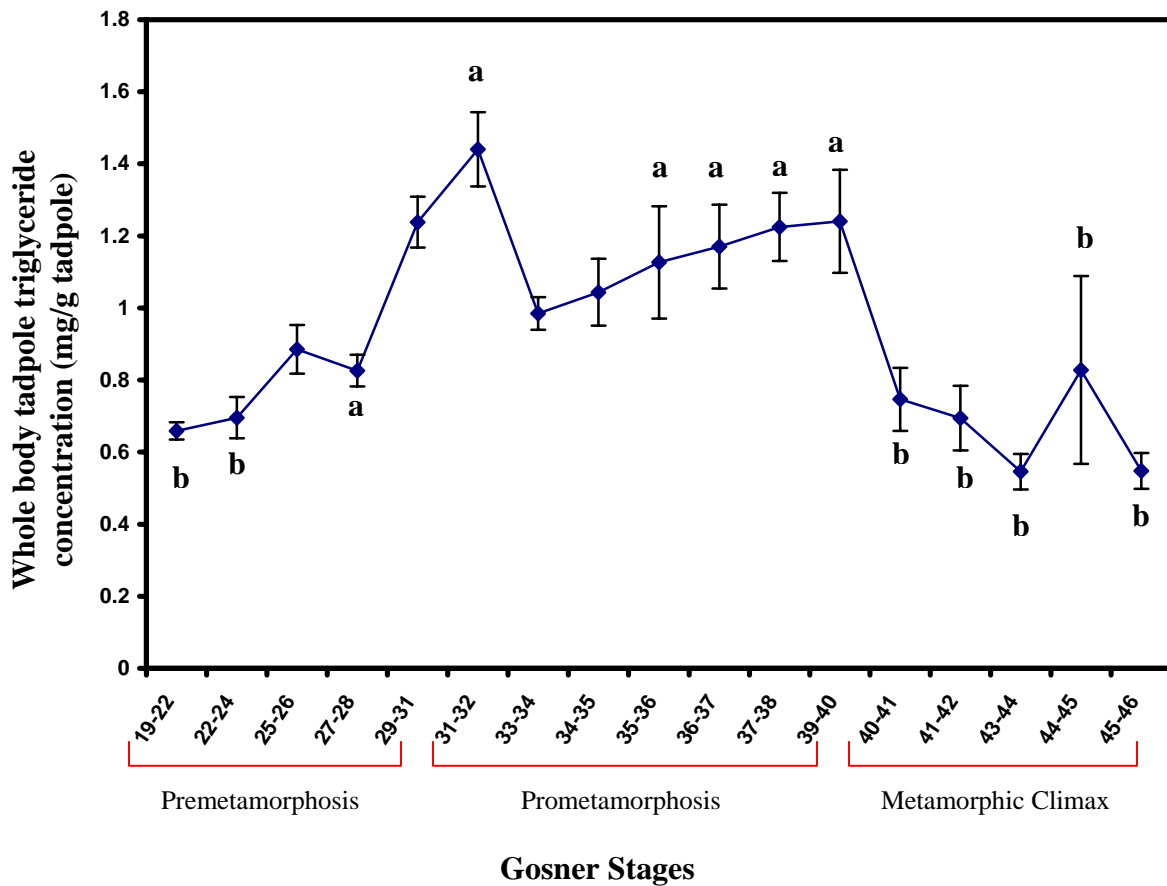
Figure 2.4: Whole body tadpole triglyceride concentrations (mg/g tadpole tissue) in relation to wood frog (*Rana sylvatica*) metamorphosis in Gosner stages. Data shown are mean ± standard error of the mean. n=8-13 tadpoles per Gosner stage. Data were analyzed using non-parametric Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's post-hoc test. Values marked with different letters were significantly different from each other ($P<0.05$) ('a' is significantly lower than 'b').
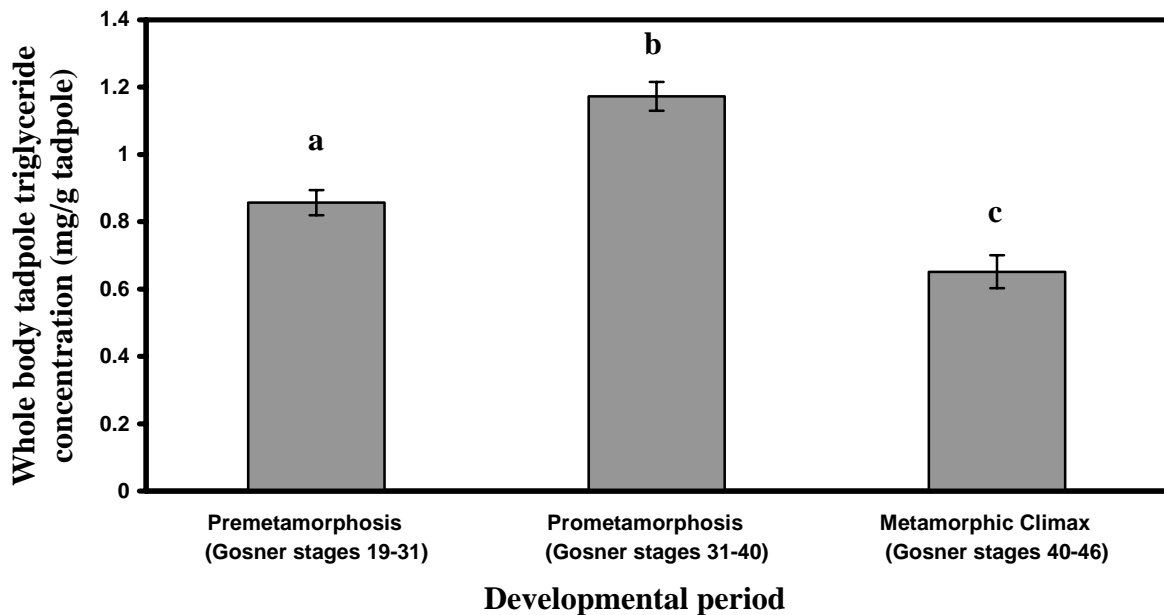
Figure 2.5: Whole body tadpole triglyceride concentrations (mg/g tadpole tissue) during premetamorphosis (Gosner stages 19-31), prometamorphosis (Gosner stages 31-40) and metamorphic climax (Gosner stages 40-46) stages of wood frog (*Rana sylvatica*) development. Data shown are mean ± standard error of the mean. n=50-76 tadpoles per developmental period. Data were analyzed using non-parametric Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's post-hoc test. Values marked with different letters were significantly different from each other (P<0.05).

2.4.3 Comparison of whole body tadpole triglyceride concentration with whole body tadpole thyroid hormone (T_3 and T_4) concentrations

Whole body tadpole T_3 and T_4 concentrations were compared with whole body tadpole triglyceride concentration to investigate a potential relationship between these endpoints. No significant relationship was found between whole body triglyceride and T_3 concentrations ($r = 0.148$, $p > 0.05$). However, whole body triglyceride concentrations were correlated positively with whole body T_4 concentrations ($r = 0.641$, $p < 0.05$) (Figure 2.6).
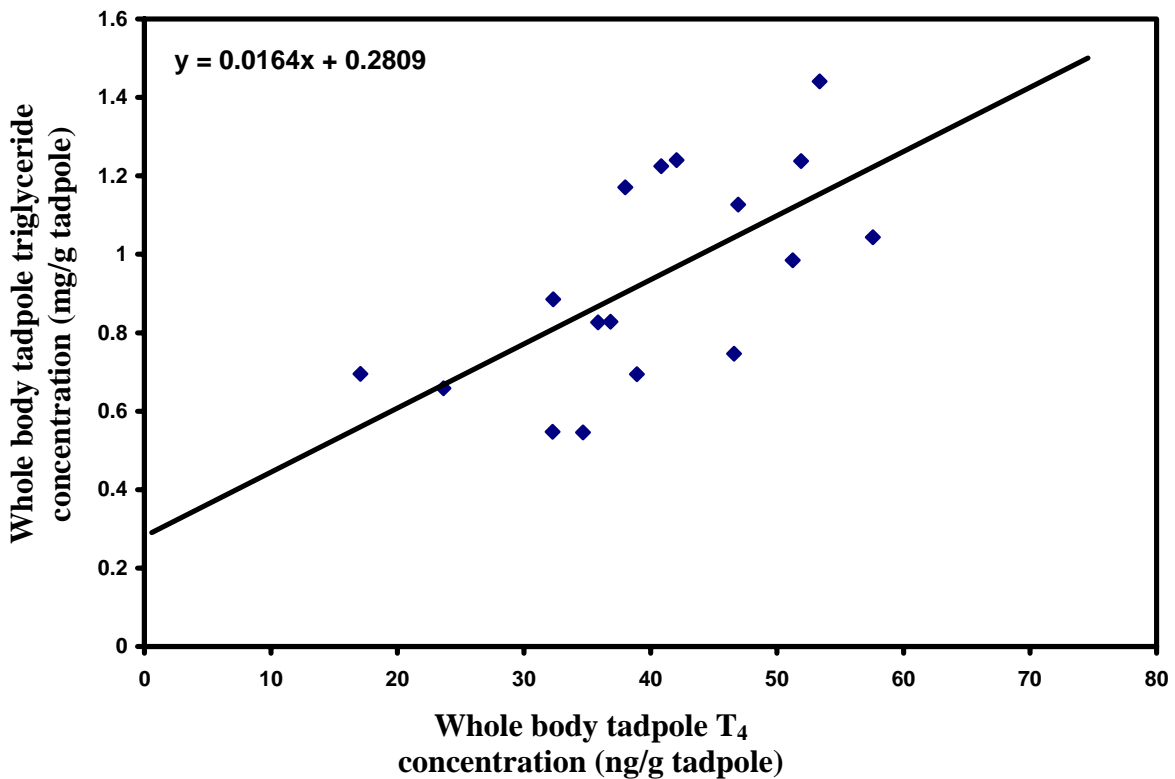
Figure 2.6: Comparison of whole body tadpole triglyceride concentrations (mg/g tadpole tissue) and thyroxine (T_4) concentrations (ng/g tadpole tissue) for wood frog (*Rana sylvatica*) development. Data were analyzed using Pearson product moment correlation, where the correlation coefficient (r) was given with $p < 0.05$.

2.5   Discussion

Whole body T_3 and T_4 and triglycerides were measured in wood frog tadpoles from hatching until completion of metamorphosis (Gosner stage 19 through 46). The objective of this study was to evaluate the association of these biomarkers with tadpole development, with a goal of producing baseline data that may provide a starting point in the application of these biomarkers to contaminant site assessment.

It was hypothesized that the concentration of whole body T_3 and T_4 would peak during metamorphic climax, around Gosner stages 40-43, consistent with other amphibian species. Studies with *Xenopus laevis* suggest that plasma levels of both T_3 and T_4 hormones were highest during metamorphic climax (Leloup and Buscaglia, 1977). Regard *et al.* (1978) measured T_3 and T_4 hormone concentrations in the plasma of *Rana catesbeiana* tadpoles, which demonstrated peak T_3 and T_4 values during Gosner stages 42-44. This result was similar to Mondou and Kaltenbach (1979), who also reported maximum T_4 concentrations in *Rana catesbeiana* at Gosner stage 42-44. In addition, Weber *et al.* (1993) observed peak whole body T_3 and T_4 concentrations in *Bufo marinus* at Gosner stage 43.

In the present study, whole body tadpole T_3 concentration followed the expected trend, generally increasing to peak during metamorphic climax. The increasing concentration of T_3 observed during premetamorphosis may reflect the increased energy requirements for rapid growth and initial development of structures such as the mouth for ingestion of food, muscle tissue for transforming into a free-swimming larva, and initial hind limb development between Gosner stages 26-30.

In contrast to the pattern observed with T_3, the change in T_4 concentration relative to development observed in this study was different than those reported for other frog species. Whole body T_4 concentrations peaked during prometamorphosis, not metamorphic climax, as originally predicted. The observed differences in the timing of thyroid hormone peaks in this species may reflect different targets for T_3 and T_4, depending on the developmental stages. Of the two thyroid hormones, T_3 binds to thyroid hormone receptors with about 5- to 10-fold higher affinity than T_4. However, both T_3 and T_4 can activate thyroid hormone-dependent target genes and induce metamorphosis (Shi, 2000).

The T_3/T_4 ratio was highest during metamorphic climax, since increases in T_3 levels exceeded those for T_4. Cai and Brown (2004) demonstrated that during metamorphic climax, expression of enzyme type II iodothyronine deiodinase (D_2) is concentrated in target tissues that will undergo extensive remodeling, such as the intestine and the tail, where T_4 is converted into T_3. Both T_3 and T_4 can also be inactivated in tissue through the action of type III iodothyronine deiodinse (D_3), thus D_2 and D_3 are deiodinase enzymes that regulate the local concentration of the active hormone T_3, in order to control the timing of developmental events in metamorphosis (Cai and Brown, 2004). In *Rana catasbeiana*, D_2 activity correlates strongly with metamorphosis, whereas the peak levels of the D_3 activity do not. Therefore, T_3 and T_3/T_4 ratio are expected to be highest during metamorphosis. High levels of D_2 activity have been shown to be present in a specific organ undergoing metamorphosis, with low or undetectable levels present at other stages (Brown and Cai, 2007). For example, D_2 activity is high in the hind limb during early stages of metamorphosis when limb

morphogenesis takes place, but it is low at a later developmental stage when the limb merely increases in size. Likewise in the tail, little D_2 is present in pre- and prometamorphic tadpoles, but high levels are present at the climax when the tail resorbs rapidly. In contrast, high levels of D_3 activity are achieved at earlier stages before tail metamorphosis (Becker *et al*., 1997). Thus, an appropriate balance of the deiodinase activities is needed to coordinate tissue specific metamorphosis. This is consistent with the findings of this study, where highest whole body tadpole T_3 concentrations were observed during metamorphic climax, possibly due to the D_2-mediated conversion of T_4 to T_3. In contrast, highest whole body tadpole T_4 concentration was seen during prometamorphosis, when D_2 activity is generally lower. Consequently, the T_3 and T_4 ratio can vary from tissue to tissue, depending upon the levels of various deiodinases (Shi, 2000).

Maximum triglyceride levels were observed during prometamorphosis, with the lowest concentrations occurring during metamorphic climax. The increasing concentrations observed during premetamorphosis can be attributed to uptake of stored triglycerides from consumption of the egg yolk. The yolk lipid provides not only the major energy source but also a supply of nutritionally essential tissue components (Noble and Moore, 1964; Manolis *et al.*, 1987). Yolk-derived triglycerides may be used for energy until Gosner stage 25, which marks the beginning of feeding for the tadpole. The gradual increase in triglyceride concentration observed during prometamorphosis in preparation for metamorphic climax would be physiologically beneficial, since tadpole mouthparts undergo drastic changes such that there is no feeding during metamorphic climax. The animal must therefore rely on stored energy.

The high triglyceride concentrations observed in this study during prometamorphosis were consistent with measures of fat concentrations in other anurans. Gramapurohit *et al.* (1998) observed increased fat storage during pre-metamorphic climax in other species, including *Rana curtipes, Rana cyanophylycitus, Rana tigrina and Polypedatus maculatus*. Concentrations of whole body lipid were reported to be lower during premetamorphosis, increasing during prometamorphosis, and declining during metamorphic climax in *Rana catesbeiana* (Blem, 1992).

Previous work indicates that thyroid hormones tend to favour lipogenesis in anuran amphibians. Lipogenesis encompasses the processes of fatty acid synthesis and subsequent triglyceride synthesis. Treatment of *Rana temporaria* with T_4 decreased plasma fatty acid levels of cold-acclimated frogs but had no effect on warm-acclimated animals (Harri and Puuska, 1973). Both T_3 and T_4 enhanced *in vivo* lipogenesis in the liver and fat body of *Rana esculenta* (Kasprzyk and Obuchowicz, 1980). In the present study, whole body tadpole triglyceride concentrations were correlated with T_3 and T_4 concentrations to evaluate this potential relationship in developing wood frogs. Results indicated a positive relationship between triglyceride and T_4 concentrations, but no relationship was seen between triglyceride and T_3 concentrations. The positive correlation between T_4 and triglycerides may be due to their presence in relatively high amounts at the same stages of wood frog metamorphosis. Amphibian larvae need increased lipid reserves as they enter metamorphic climax and stop feeding due to changes in oral and digestive morphology (Beck and Congdon, 2003). Therefore, in this instance, increasing T_4 concentrations did appear to induce lipogenesis, which was

supported by the similarity between distribution of triglycerides and of T_4 concentrations during wood frog development.

The present study examined changes in whole body concentrations of thyroid hormones (T_3 and T_4) and triglycerides from early development through metamorphosis of wood frog tadpoles. No previous studies of anuran tadpoles have measured both whole body triglyceride and thyroid hormones concurrently, using the same sample. Therefore, this study describes a novel application of the multiple biomarker approach to evaluating the health of amphibians during a critical stage in their life history. This approach is useful for reducing sample-to-sample variability and correlating the two measures.

This approach to tadpole biomarker measurement was developed to provide tools to evaluate the potential adverse effects of exposure to water and sediment from reclaimed oil sands wetlands on wood frog growth and development. Wood frogs are native to the oil sands ecosystem of northern Alberta, and were therefore chosen as the species in which to develop the triglyceride and thyroid hormone assays. Contaminant induced changes in tadpole thyroid hormone or triglyceride levels could delay or prevent successful metamorphosis and adversely impact frog populations.

CHAPTER 3
3.0 Occurrence and overall body condition of wood frogs in reclaimed oil sands wetlands

3.1   Abstract

Extraction of oil from Athabasca oil sands deposits produce large volumes of contaminated process-affected water (OSPW) which must be retained on site. Efforts are underway to reconstitute the OSPW into reclaimed wetlands after mining is complete. These wetlands need to provide suitable habitats for aquatic and semi-aquatic organisms such as wood frogs (*Rana sylvatica*), an abundant native species. The objective of this study was to determine potential detrimental effects of OSPW and process-affected sediments on the growth and development of wood frogs. In summer 2005, surveys were conducted to assess the presence of wood frogs in 29 reclaimed wetlands containing OSPW and five unimpacted reference sites. Tadpoles and newly-metamorphosed wood frogs were collected from three unimpacted reference ponds and six ponds containing OSPW in an initial attempt to evaluate contaminant effects on amphibian health. Endpoints evaluated included hepatic glycogen concentration in newly-metamorphosed frogs, and whole body triglyceride and thyroid hormone (3,5,3'-triiodothyronine [T_3] and thyroxine [T_4]) concentrations in tadpoles collected at Gosner stage 37-43. Morphometric endpoints such as weight, length and overall condition of wood frogs were also assessed.

From the surveys conducted in the field, it was determined that 60% of OSPW-impacted wetlands registered use by the adult amphibian population in the region. Wood frog tadpoles and newly-metamorphosed frogs were also collected from 37% and 30% of the OSPW sites, respectively. Significant differences ($P<0.05$) were observed among OSPW sites with regard to mean hepatic glycogen concentrations in newly-

metamorphosed wood frogs (n=6-17 frogs/site). However, frogs from most OSPW sites were not different from those from reference ponds. Tadpoles from two OSPW sites had decreased whole body triglyceride concentrations (1.11 and 0.998 mg/g tadpole) (n= 8-19 tadpoles) compared to one reference pond (2.10 mg/g tadpole) (P<0.05), but there were no other significant differences between OSPW-exposed and unexposed tadpoles. There was no consistent exposure-related effect on tadpole whole body T_3 concentration in tadpoles (n=16-20 tadpoles/site). Tadpoles from one reference site had greater T_3 concentrations (2.19 ng/g tadpole) than two impacted sites (1.50 and 1.42 ng/g tadpole) (P <0.05), but one of the other reference sites was also low (1.60 ng/g tadpole). No differences in mean whole body T_4 concentrations (P=0.11) and the ratio of T_3/T_4 (P=0.154) concentrations were observed among tadpoles from any of the wetlands.

Significant differences were observed in mean body weight and total length of newly-metamorphosed frogs from reference and OSPW wetlands, but the differences were not related to contaminant exposure. Similarly, significant differences were observed in mean body weight, total length and body condition of wood frog tadpoles from reference and OSPW wetlands, but no consistent exposure related effect was detected.

3.2   Introduction

Containment and elimination of process-affected materials as well as restoration of mined land are the greatest environmental challenges facing oil sand mining companies. Oil sands process-affected waters (OSPW) resulting from oil extraction are known to contain elevated levels of dissolved organic matter (naphthenic acids), salts and

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hydrocarbons (polycyclic aromatic hydrocarbons). Concentrations of sodium, sulfate and chloride ions are often particularly high, and may represent the greatest stressor for many native fresh-water plants and invertebrates, resulting in potential changes in aquatic communities (Hart *et al.*, 1990). Pollet and Bendell-Young (2000) noted reduced survival and growth, as well as increased incidence of deformities in *Rana sylvatica* tadpoles exposed to wetlands most heavily impacted by oil sands effluent. Studies with fish exposed to OSPW showed adverse affects on growth and survival. Peters (1999) observed increased mortalilty and deformities in Japanese Medaka (*Oryzias latipes*) embryos with increasing concentrations of OSPW, whereas Colavecchia *et al.* (2004) reported decreased hatching success and increased mortality and malformations in fathead minnow (*Pimephales promelas*) eggs and larvae exposed to process-affected materials.

Process affected water that cannot be reused in the extraction process is released into settling basins, which allow the settling of larger suspended sand grains as well as the smaller clays, silts, fine sands and other particulates. The result is a watery mixture referred to as fine tails (FT). Mature fine tails (MFT) is a term referring to fine tails that are older and contain less water. Reclamation of oil sands properties will include incorporation of large volumes of OSPW and tailings into man-made wetlands. The suitability of these wetlands for use by native wildlife is uncertain.

Amphibians may be good indicators to evaluate the viability of these wetlands as successful habitats. Shell-less eggs laid in water hatch into gill-breathing larvae with intimate contact with contaminated sediment, which metamorphose into semi-aquatic adults with permeable skin. Therefore, amphibians are vulnerable to toxicants via

multiple exposure pathways throughout development. Sublethal concentrations of OSPW-associated toxicants may increase the susceptibility of frog eggs and larvae to pathogenic organisms and disease, or reduce larval survival by retarding growth and metamorphosis, such that tadpoles are unable to metamorphose and depart breeding ponds at the appropriate time. Contaminants that affect behaviour may inhibit the ability of larvae to avoid predators. Furthermore, toxicants that have estrogenic, antiestrogenic, thyroid-disrupting, androgenic, or anti-androgenic properties may either impair or totally inhibit future reproduction by disrupting developmental processes. Higher concentrations of toxicants might directly cause mortality of the eggs, larvae or metamorphosing individuals (Venturino *et al.*, 2003).

Wood frogs (*Rana sylvatica*) are widely distributed in many ecoregions across North America. Their ability to withstand freezing enables the northern extension of their range farther than any other amphibian in the Western Hemisphere, and they are an abundant native species in the Athabasca oil sands.

Morphometric (weight, length and condition factor) and biochemical (whole-body tadpole triglyceride and thyroid hormone concentrations, and hepatic glycogen content) measurements may be useful indicators of adverse affects of oil sand process materials to developing wood frogs using reclaimed wetlands. External measurements including weight, length and condition factor offer a rough assessment of overall condition of an animal. Weight can be an indicator of energy storage, whereas length relates to growth. Generally, better body condition is associated with greater weight for a given length. Fulton's condition factor has been used previously to measure fitness level in fish (Bolger and Connolly, 1989) and amphibians (Gendron *et al.*, 2003).

Thyroid hormones (3,5,3'-triiodothyronine [T_3] and thyroxine [T_4]) are critical in controlling the sequential anatomical and physiological changes that occur during amphibian metamorphosis (Shi, 2000). All organ systems, specifically the skin, sense organs, blood, musculoskeletal, immune, gut, and excretory systems, are known to undergo thyroid-mediated changes at larval and/or metamorphic stages (Gilbert *et al*., 1996; Shi, 2000). Stress induced by contaminants can cause disruption of thyroid hormone production (Fort *et al*., 2004) and can prevent or retard typical development and/or metamorphosis (Crump *et al.,* 2002).

Total body triglyceride concentration is a useful indicator of body condition in many species including fish (Adams, 1999; Bennett, 2007) and amphibians (Ryuzaki and Oonuki, 1990). Deposition and utilization of fat in anuran tadpoles may be dependent on the stage and duration of larval development, food abundance, expenditure of energy in search of food and predator avoidance. Fat bodies in adult anurans store excess energy during the period of food abundance (Gramapurohit *et al*., 1998). If adults are subject to contaminant stress and require more energy to metabolize xenobiotics or maintain homeostasis, they may store less energy in fat bodies (Venturino *et al.*, 2003).

In addition to triglycerides, glycogen also acts as an essential source of energy in amphibians. Glycogen can be mobilized and converted to glucose to provide immediate energy more readily than lipids (Rocha-Leao, 2003), but it is also depleted at an increased rate, and yields less energy per gram than fat (Wells, 2007). Glycogen is stored mainly in the liver, but is also found in muscle and fat cells. Muscle glycogen in particular serves as an energy source during mechanical work. Hepatic glycogen may act as an important energy source during tadpole development. In metamorphosing *Xenopus laevis* larvae,

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liver glycogen content increased from 0.2 to 10% of liver weight from prometamorphosis until the end of metamorphic climax (Fox, 1984).

Wood frogs hibernate at the soil surface in sites with a good cover of damp leaf litter to prevent desiccation. When ice penetrates these sites, frogs cannot avoid freezing because their highly water permeable skin presents no barrier to the propagation of ice. Freezing for the wood frog begins when body fluids are seeded across the epidermis when in contact with environmental ice at or below the freezing point of body fluids (Storey and Storey, 1996a). Glycogen (approximately 180mg/g) stored in the liver preceding hibernation is essential for cryoprotectant synthesis in wood frogs. Once the freezing begins, glucose concentrations in blood and liver increase by 3.3 and 6.6 fold, respectively, as glycogen reserves are metabolized. Glucose production continues until it is stopped following freezing of the central circulation (Storey and Storey, 1996b). Exposure to any stressor, including environmental contaminants that decrease pre-hibernation glucose storage, could adversely affect the overwintering survival of wood frogs.

This study represents an initial assessment of the use of reclaimed oil sands wetlands by native wood frogs, and a preliminary evaluation of the ability of these wetlands to support tadpole growth and development. The specific wetlands evaluated were created on reclaimed areas, made out of tailings, or had potential OSPW seepage from various sources. Wood frogs inhabiting these wetlands were exposed to a complex mixture of potentially toxic compounds, the composition (and likely toxicity) of which varied with the type of effluent and history of each site.

Selected reclaimed and unimpacted (reference) wetlands on Suncor and Syncrude lease holds were surveyed for amphibian use in the spring and summer of 2005. Wood frog tadpoles and newly-metamorphosed frogs were collected from the surveyed wetlands to evaluate relative body condition of tadpoles (whole body triglyceride concentration) and frogs (hepatic glycogen), and potential impact on thyroid function (whole body tadpole T_3 and T_4 concentrations). It was hypothesized that whole body tadpole thyroid and triglyceride concentrations, hepatic glycogen concentration, and overall body condition of animals collected from wetlands with oil sands process-affected materials would be less than specimens from reference wetlands.

3.3   Materials and Methods

3.3.1   Site selection and water quality

A number of process-affected (OSPW) and unimpacted wetlands were selected for a preliminary assessment of their suitability as habitats for wood frog tadpoles and froglets. These wetlands were characterized based on their status (OSPW or reference) and their history (Table 3.1). Wetland age was also determined by assigning them the status of "young" or "old". Young wetlands were seven years old or less and old wetlands were eight years or older. Water quality variables including conductivity, pH and ionic content were determined for each wetland by collecting water samples in close proximity to where amphibian life was noted or suspected. Water quality analysis was conducted by the oil sand companies, Syncrude and Suncor, on whose lands the wetlands were situated.

Table 3.1: Description of unimpacted reference and process-affected (OSPW) wetlands in the Athabasca oil sands from which wood frog (*Rana sylvatica*) tadpoles and froglets were collected during the summer of 2005.

Name and Company Lease	Age	OSPW or Reference	History and Description
Senor Frog (Syncrude)	Young	Reference	- 80% cattail cover - contains no OSPW
SCL 1 (Syncrude)	Old	Reference	- 10% cattail cover - Contains natural surface water from an unimpacted site to a maximum depth of 5 meters - Minnows and White Suckers were added in 1990 - A well developed aquatic macrophyte community is present.
Highway (None)	Old	Reference	- 30% cattail cover - Unknown history
Golden (Syncrude)	Young	OSPW	- 2% cattail cover - A man-made wetland completed in 2002 - It receives runoff water from adjacent hill slopes and another OSPW site - Usually has a small pond attached on the north and south ends that tend to attract amphibian life

Table 3.1: Description of unimpacted reference and process-affected (OSPW) wetlands in the Athabasca oil sands from which wood frog (*Rana sylvatica*) tadpoles and froglets were collected during the summer of 2005. (Continued…)

Name and Company Lease	Age	OSPW or Reference	History
SCL 13 (Syncrude)	Old	OSPW	- 20% cattail cover - Approximately 70,000 m^3 of water from another OSPW site was transferred in 1993 - Intended to be storage area for the capping water for another OSPW site
B1 (Syncrude)	Old	OSPW	- 15% cattail cover - Seepage of oil sands process-affected materials from other OSPW sites
B2 (Syncrude)	Old	OSPW	- 75% cattail cover - Seepage of oil sands process-affected materials from other OSPW sites.
V-Notch Weir (Suncor)	Old	OSPW	- 30% cattail cover - Made up with mainly surface runoff water from the adjacent peat mineral stockpiles - Also some ground discharge from other OSPW sites
Weir 11 (Suncor)	Young	OSPW	- 5% cattail cover - Amphibian life had not been noted since approximately 2003. - Contains runoff water

3.3.2 Surveys of oil sands process-affected and reference wetlands for amphibian use

A series of visual encounter surveys were conducted in the spring and summer of 2005 to identify the presence of adult wood frogs, boreal chorus frogs and Canadian toads, as well as the location of egg masses and tadpoles. Visual encounter surveys were performed by walking the perimeter of the wetland (or representative section of wetland when perimeter was greater than approximately 1.5 miles) and noting any indicators of amphibian use (Droege, USGS Patuxent Wildlife Research Center). Amphibian use was further assessed by use of call surveys, which were conducted by listening carefully for 5-10 minutes and rating the intensity of the calling according to the Wisconsin Frog and Toad Survey (Appendix 1) (Droege, USGS Patuxent Wildlife Research Center).

3.3.3 Collection of biological samples

Minnow traps baited with cat food were placed in OSPW and reference wetlands when wood frog tadpole development progressed to the point of hind limb emergence. Wood frog eggs were laid earlier and tadpoles hatched and developed ahead of boreal chorus frogs and Canadian toads so species were easy to differentiate. Minnow traps were left in each pond and checked approximately every three days from June 13 through July 8, 2005 (Figure 3.1), when it was rare to catch a single tadpole. Tadpoles collected from the minnow traps were euthanized by overdose of 20% benzocaine gel (Orajel®) applied to the tops of their heads. Tadpoles were measured to determine total body length and weight, and immediately placed in cryovials and frozen in liquid nitrogen for subsequent analysis of whole body triglyceride and thyroid hormone concentrations.

Figure 3.1: Checking a minnow trap for wood frog (*Rana sylvatica*) tadpoles. Tadpoles reaching Gosner stages 37-43 (start of hind limb development) were collected.

Newly-metamorphosed wood frogs were captured as they emerged from their natal wetlands using a series of pit fall traps and drift fences located along the perimeter of the pond (Herpetological Animal Care and Use Committee, 2004). Pitfall traps were dug into the ground approximately 1.5 m apart from one another and located along drift fences placed about two m. from the littoral zone of the wetland. Newly-metamorphosed froglets were chosen to relate water quality of wetlands to the health of amphibians because their origin and exposure history could be established. The objective of the pitfall traps was to capture wood frogs, not estimate populations. Therefore, the location, number of traps, and length of drift fences were chosen based on observations and visual encounter surveys, and consequently were not consistent among wetlands (Figure 3.2). Pit fall traps were checked every 24 hours, and newly-metamorphosed wood frogs were euthanized by applying 20% benzocaine gel (Orajel®) on the dorsal aspect of the cranium. Total body length and weight were measured for each froglet, and the liver was removed immediately and the right lobe frozen in liquid nitrogen for subsequent glycogen measurement.

<center>(a)   (b)</center>

Figure 3.2: Pit fall traps (a) and a representative length of drift fence (b) located along the perimeter of a wetland to trap newly-metamorphosed emerging wood frogs (*Rana sylvatica*).

3.3.4 Biological assays

A newly developed technique (modified from Weber *et al*. 2003 and Brasfield *et al*. 2004) was applied to the wood frog tadpoles for measuring whole body tadpole triglyceride and T_3 and T_4 concentrations.

3.3.4.1 Thyroid Hormone Assays

Whole-body T_3 and T_4 concentrations were measured in wood frog tadpoles using a commercial enzyme-linked immunosorbent assay (ELISA) kit (BioQuant, San Diego, CA, USA) as described in section 2.3.1.3.1 of Chapter 2.

3.3.4.2 Triglyceride Assay

The triglyceride assay was based on a method developed in juvenile fish by Weber *et al*. (2003). Tadpole whole body triglyceride concentrations were measured using a modification of a commercial kit protocol (Sigma, Saint Louis, MO, USA) as described in section 2.3.1.2.1 of Chapter 2.

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3.3.4.3 Glycogen Assay

The glycogen assay was based on a method developed by Gómez-Lechón *et al.* (1996) and used in juvenile fish by Weber *et al.* (2008). Hepatic glycogen concentrations in newly-metamorphosed wood frogs were determined using purified Type IX bovine liver glycogen (Sigma-Aldrich) as a standard (0.05-20 µg/ml for standard curve).

A motorized Teflon pestle (Glas-Col®) was used to homogenize the liver samples (weighing 10 mg or more) with 3X volume of ice cold citrate buffer (tri-sodium citrate EM omnipure, VWR). Another 2X volume of buffer was added to the homogenate (final homogenate had 5X volume buffer) once homogenization was complete. This homogenate was then heated for five minutes in a heat block at 100 ºC to inactivate endogenous amylase. It was stored at -80ºC until assayed for glycogen. During the assay, two microcentrifuge tubes were labeled per sample; one received the 50µl citrate buffer and the other received 50µl amylase (amyloglucosidase solution, Sigma-Aldrich). Amylase (50µl) was also added to each of the standards. All the tubes were then incubated at 37ºC for two hours. The standards and the samples were centrifuged (8000 rpm for 10 minutes at 4ºC) and pipetted (40µl) onto a microplate. 10µl of 0.125 M NaOH and 200µl of PGO (peroxidase-glucose oxidase) enzyme solution (Sigma-Aldrich) were added to each well in the microplate. The PGO enzymes are useful for the quantitative enzymatic determination of glucose in aqueous solutions. Glycogen content was determined spectrophotometrically at 440nm (SpectraMax® 190) by deducting the value obtained without amylase from that obtained with amylase for each sample.

3.3.5   Statistical analyses

Significant differences were tested using parametric one-way ANOVA followed by Tukey's test for multiple comparisons, or non-parametric Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test for multiple comparisons. Log and arcsine transformations were used to normalize the data, where required. To evaluate the relationship between endpoints (TG and T_4; TG and T3), Pearson product moment correlations were used. The correlation coefficient (r) was given at $p < 0.05$ for all correlations. The results are expressed as mean $\pm$ SEM.

Intra-assay variability was assessed for each of the four tests (T_3, T_4, triglycerides and glycogen), by making six determinations of a pooled sample. The same pooled sample was measured six more times on separate occasions to evaluate inter-assay variability.

3.4   Results

3.4.1   Site selection and water quality

Water samples were collected from the three reference and six OSPW-impacted wetlands from which most of the biological samples were obtained. The samples were analyzed for major ions and other water quality variables commonly measured on oil sands sites (Table 3.2). Total conductivity was generally higher in the impacted sites, such as, B1, B2 and Golden Pond, although Weir 11 and SCL 13 were exceptions. The pH values were similar among the wetlands, ranging from 7.11 to 8.19. Ammonia (NH_3) concentrations were below detection limit (BDL) for some wetlands, and, where measurable, were highest in oil sands process-affected wetlands (V Notch Weir, Weir 11,

B1 and B2). Naphthenic acids were measured in only four wetlands. However, where

naphthenic acid concentrations were determined, the oil sands process-affected wetlands

(V Notch Weir, Weir 11 and SCL 13) demonstrated significantly higher values than the

reference wetland (Highway Pond). Concentrations of most major ions (Na^+, Cl^-, Mg^{2+}

and HCO_3), were higher in wetlands with process-affected materials.

Table 3.2: Summary of water quality measurements obtained by Syncrude Canada Ltd.
and Suncor Energy Inc. in summer 2005, for oil sands process-affected and reference
wetlands in Athabasca oil sands, Alberta.

SITE	COND. (µs/L) [i]	pH	NAPH. ACIDS [ii] (mg/L)	NH4 (ppm)	Na+ (mg/L)	K+ (mg/L)	Mg2+ (mg/L)	Ca2+ (mg/L)	Cl- (mg/L)	SO4 (mg/L)	HCO3 (mg/L)
SCL1 (Ref.)	811	7.73	NA [iii]	0.13	70.1	0.1	27.8	66.8	13	292	185
Senor Frog (Ref.)	519	7.40	NA	BDL [iv]	8.1	17.9	13.2	68.1	8.1	93.5	222
Highway (Ref.)	444	7.11	1.0	BDL	44.4	0.1	11.7	36.7	53	44.7	152
Golden (OSPW)	1939	7.59	NA	BDL	122	0.1	70.3	249	37	875	353
V Notch Weir (OSPW)	1289	7.68	7.0	0.47	180	12.7	36.3	70.5	38	451	293
Weir 11 (OSPW)	368	8.19	6.0	0.57	735	0.1	22.8	49.5	1200	125	231
SCL13 (OSPW)	595	8.13	8.0	BDL	55.8	5.3	21.3	39.3	15.0	106	239
B1 (OSPW)	2720	7.98	NA	0.2	567	0.1	36.9	83.3	520	77.5	897
B2 (OSPW)	2220	7.20	NA	0.2	363	0.1	42.1	119	330	179	746

i) Cond. (µs/L) = Conductivity (microsiemens/litre)
ii) Naph. Acids (mg/L) = Napthenic Acids (milligrams/litre)
iii) NA = Not available
iv) BDL = Below Detection Limit

3.4.2 Presence of wood frogs on reclaimed, oil sands process-affected and reference sites

Amphibian species reported from call surveys and visual encounter surveys were wood frogs, boreal chorus frogs and Canadian toads. Western toads and northern leopard frogs were initially thought to be observed in this area but none were observed. Table 3.3 summarizes the results of visual encounter and call surveys, indicated as presence (+) or absence (-) of adults at each wetland, and the number of wood frog tadpoles and newly-metamorphosed froglets collected from a total of 29 OSPW and five reference sites. Adult frogs were noted on 62% of OSPW and 60% of reference sites. Wood frog tadpoles were collected from 34% of OSPW and 80% of reference wetlands, whereas 31% of OSPW and 60% of reference sites had newly-metamorphosed froglets.

Table 3.4 summarizes the collection of wood frog samples from a subset of process-affected and unimpacted wetlands for the measurement of whole body tadpole T_3 and T_4 and triglyceride concentrations, and hepatic glycogen concentration from newly-metamorphosed froglets.

3.4.3 Morphometric measurements in newly-metamorphosed wood frogs and tadpoles

Weight and total length of Gosner stage 37-43 tadpoles trapped in reference and OSPW-impacted wetlands were compared using non-parametric Kruskal-Wallis ANOVA on ranks followed by Dunn's post-hoc test. Tadpoles from Senor Frog (reference), V Notch Weir (OSPW) and B2 (OSPW) wetlands had significantly lower average body weights and total lengths than tadpoles from the other reference and OSPW wetlands ($P<0.05$) (Table 3.5). Tadpole condition factors were compared using the same statistical

analysis. Condition factor for tadpoles from B1 and B2 (OSPW) were significantly higher than tadpoles from the other OSPW and reference wetlands ($P<0.05$).

Body weight of newly-metamorphosed wood frogs was compared using parametric ANOVA followed by Tukey's post-hoc test, while total length of frogs was compared using non-parametric Kruskal-Wallis ANOVA on ranks followed by Dunn's post-hoc test. Wood frogs from Golden Pond (OSPW) and SCL 1 (reference) had significantly higher average weight and body length compared with frogs collected from Senor Frog (reference) and V Notch Weir (OSPW) ($P<0.05$). Newly-metamorphosed frogs collected from Highway (reference) and SCL 13 (OSPW) also had significantly higher average body weights as compared to frogs from Senor Frog and V Notch Weir (Table 3.6). Condition factors of newly-metamorphosed frogs were compared using parametric ANOVA with log transformed data, followed by Tukey's post-hoc test. Frogs from Senor Frog and Highway reference wetlands appeared to have the highest condition factors, but no significant differences were detected among any of the wetlands ($P=0.081$).

Table 3.3: Observed habitat use by wood frogs (*Rana sylvatica*) of reclaimed (OSPW) and unimpacted (reference) wetlands on Suncor and Syncrude lease lands in the Athabasca oil sands, Alberta.

Company Sites	Wetland	Visual encounter/Call survey results [presence (+) and absence (-) of adult frogs]	Number of tadpoles collected (Gosner stage 37-43)	Number of newly-metamorphosed frogs collected
Off-Site	Highway (Ref.)	-	22	17
Suncor	Loon Lake (Ref.)	+	0	0
Suncor	Senor Frog (Ref.)	+	20	8
Suncor	Natural (OSPW)	+	9	1
Suncor	V Notch Weir (OSPW)	-	20	19
Suncor	Weir 11 (OSPW)	-	20	20
Suncor	Weir 7 (OSPW)	-	0	0
Suncor	Tar Island (OSPW)	+	0	0
Suncor	Poplar (OSPW)	+	0	0
Syncrude	SCL1 (Ref.)	+	20	6
Syncrude	WID (Ref.)	-	4	0
Syncrude	Golden (OSPW)	+	20	11
Syncrude	Bill's (OSPW)	+	0	1

Table 3.3: Observed habitat use by wood frogs (*Rana sylvatica*) of reclaimed (OSPW) and unimpacted (reference) wetlands on Suncor and Syncrude lease lands in the Athabasca oil sands, Alberta. (Continued…)

Company Sites	Wetland	Visual encounter/Call survey results [presence (+) and absence (-) of adult frogs]	Number of tadpoles collected (Gosner stage 37-43)	Number of newly-metamorphosed frogs collected
Syncrude	Peat (OSPW)	+	0	0
Syncrude	South Bison (OSPW)	-	0	0
Syncrude	SCL2 (OSPW)	+	0	0
Syncrude	SCL3 (OSPW)	+	0	0
Syncrude	SCL4 (OSPW)	+	0	0
Syncrude	SCL5 (OSPW)	+	5	1
Syncrude	SCL6 (OSPW)	+	0	0
Syncrude	SCL7 (OSPW)	+	0	0
Syncrude	SCL8 (OSPW)	+	0	0
Syncrude	SCL9 (OSPW)	+	0	0
Syncrude	SCL10 (OSPW)	-	0	0
Syncrude	SCL11 (OSPW)	-	0	0
Syncrude	SCL12 (OSPW)	-	0	0

Table 3.3: Observed habitat use by wood frogs (*Rana sylvatica*) of reclaimed (OSPW) and unimpacted (reference) wetlands on Suncor and Syncrude lease lands in the Athabasca oil sands, Alberta. (Continued…)

Company Sites	Wetland	Visual encounter/Call survey results [presence (+) and absence (-) of adult frogs]	Number of tadpoles collected (Gosner stage 37-43)	Number of newly-metamorphosed frogs collected
Syncrude	SCL13 (OSPW)	+	22	14
Syncrude	SCL14 (OSPW)	-	0	0
Syncrude	South Black (OSPW)	-	4	4
Syncrude	Seepage (OSPW)	-	0	0
Syncrude	B1 (OSPW)	+	20	1
Syncrude	B2 (OSPW)	+	12	0
Syncrude	BBQ Beach (OSPW)	-	7	0
Syncrude	Middle BC (OSPW)	+	0	0

Table 3.4: Summary of wood frog (*Rana sylvatica*) tadpoles and newly-metamorphosed wood frogs collected from oil sands process-affected and reference wetlands on Suncor and Syncrude lease lands in the Athabasca oil sands, Alberta, for the measurement of whole body tadpole thyroid hormone (TH) and triglyceride (TG) concentrations, and frog hepatic glycogen concentration.

Company	Site	Reference/OSPW	Number of Tadpoles Analyzed		Number of Frogs Analyzed
			TH	TG	
Syncrude	Senor Frog	Reference	19	18	8
Syncrude	SCL 1	Reference	19	17	6
None	Highway	Reference	20	19	16
Syncrude	Golden	OSPW	17	16	11
Syncrude	SCL 13	OSPW	20	17	14
Syncrude	B1	OSPW	18	16	0
Syncrude	B2	OSPW	0	8	0
Suncor	V-Notch Weir	OSPW	16	14	15
Suncor	Weir 11	OSPW	18	17	17

Table 3.5: Morphometric (weight, total length, condition factor) measurements determined in wood frog (*Rana sylvatica*) tadpoles, collected from several unaffected and oil sands process-affected wetlands in the Athabasca oil sands in Alberta in summer 2005. Data shown are mean ± standard error of the mean. Values marked with different letters were significantly different from each other (p<0.05). Condition factor = (weight/total length^3)*100.

	Unaffected/Reference Wetlands			Oil Sands Process-Affected Wetlands					
	SCL 1	Senor Frog	Highway Pond	Weir 11	V Notch Weir	SCL 13	Golden Pond	B1	B2
Sample Size	17	19	22	19	17	21	19	19	11
Weight (g)	2.03 ± 0.07^a	1.46 ± 0.06^b	1.77 ± 0.13^a	2.07 ± 0.10^a	1.08 ± 0.06^b	2.12 ± 0.06^a	1.74 ± 0.09^a	2.25 ± 0.18^a	0.22 ± 0.46^b
Total Length (cm)	5.34 ± 0.14^a	4.74 ± 0.13^b	5.11 ± 0.16^a	5.68 ± 0.10^a	4.39 ± 0.12^b	5.77 ± 0.08^a	5.18 ± 0.17^a	5.19 ± 0.17^a	2.05 ± 0.07^b
Condition Factor	1.33 ± 0.06^b	1.43 ± 0.09^b	1.51 ± 0.29^b	1.13 ± 0.04^b	1.30 ± 0.08^b	1.13 ± 0.06^b	1.42 ± 0.25^b	1.63 ± 0.10^a	3.26 ± 1.37^a

Table 3.6: Morphometric (weight, total length, condition factor) measurements determined for newly-metamorphosed wood frogs (*Rana sylvatica*), collected from several unaffected and oil sands process-affected wetlands in the Athabasca oil sands in Alberta in summer 2005. Data shown are mean ± standard error of the mean. Values marked with different letters were significantly different from each other ($p<0.05$). Condition factor = (weight/total length^3)*100.

	Unaffected/Reference Wetlands			Oil Sands Process-Affected Wetlands			
	SCL 1	**Senor Frog**	**Highway Pond**	**Weir 11**	**V Notch Weir**	**SCL 13**	**Golden Pond**
Sample Size	6	8	17	21	18	14	11
Weight (g)	1.16 ± 0.05^a	0.85 ± 0.06^b	1.08 ± 0.04^a	$1.03 \pm 0.03^{a,b}$	0.87 ± 0.04^b	1.11 ± 0.03^a	1.19 ± 0.07^a
Total Length (cm)	2.32 ± 0.06^a	1.98 ± 0.08^b	$2.12 \pm 0.04^{a,b}$	$2.04 \pm 0.10^{a,b}$	2.01 ± 0.02^b	$2.22 \pm 0.03^{a,b}$	2.35 ± 0.08^a
Condition Factor	9.28 ± 0.42^a	11.5 ± 1.45^a	11.5 ± 0.68^a	10.6 ± 0.28^a	10.6 ± 0.33^a	10.3 ± 0.42^a	9.37 ± 0.52^a

3.4.4 Whole body tadpole thyroid hormone analyses

The results indicate that whole body wood frog tadpole T_3 hormone concentrations were significantly lower in B1 (OSPW), Golden (OSPW) and Highway (reference) wetlands ($P<0.05$) compared to tadpoles from SCL 1, a reference site, which had the highest whole body tadpole T_3 concentration (Figure 3.3, graph A). The T_3 concentrations in tadpoles from the other OSPW sites were not significantly different from the reference wetlands. No significant differences in whole body wood frog tadpole T_4 hormone concentrations were observed among the unaffected and process-affected wetlands ($P=0.110$), although SCL 1 had the maximum whole body tadpole T_4 concentration (Figure 3.3, graph B). Data were log transformed to fit a normal distribution for T_4. Figure 3.4 shows the whole body tadpole T_3/T_4 ratio. No significant differences were found among all sites, ($P=0.154$) although tadpoles collected from the SCL 1 wetland demonstrated the highest T_3/T_4 ratio. Arcsine transformation of data was performed for statistical analysis.
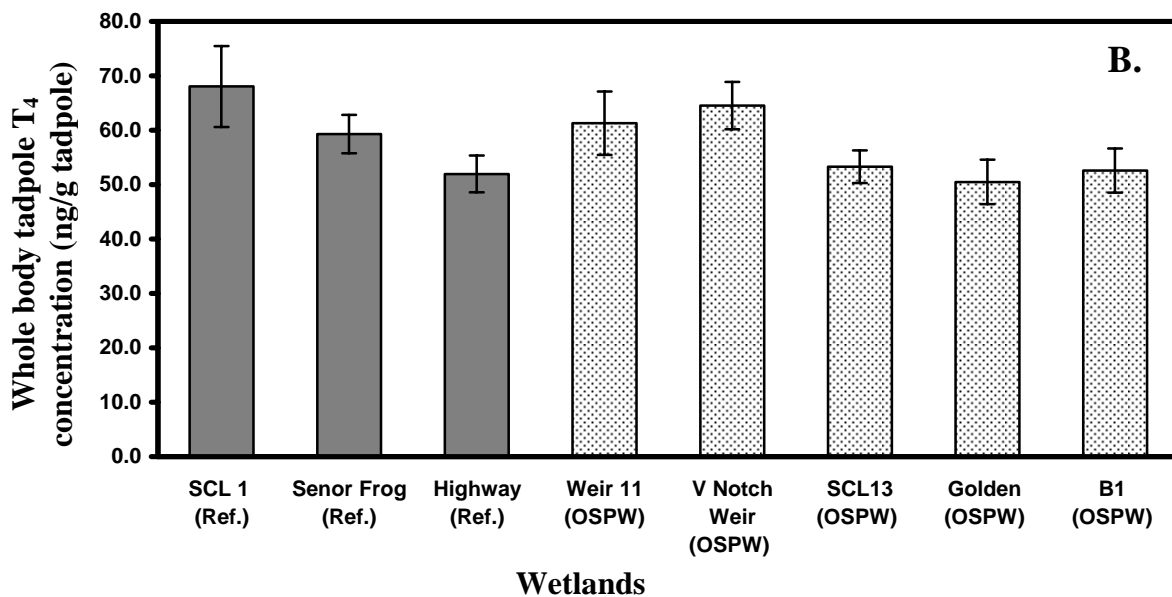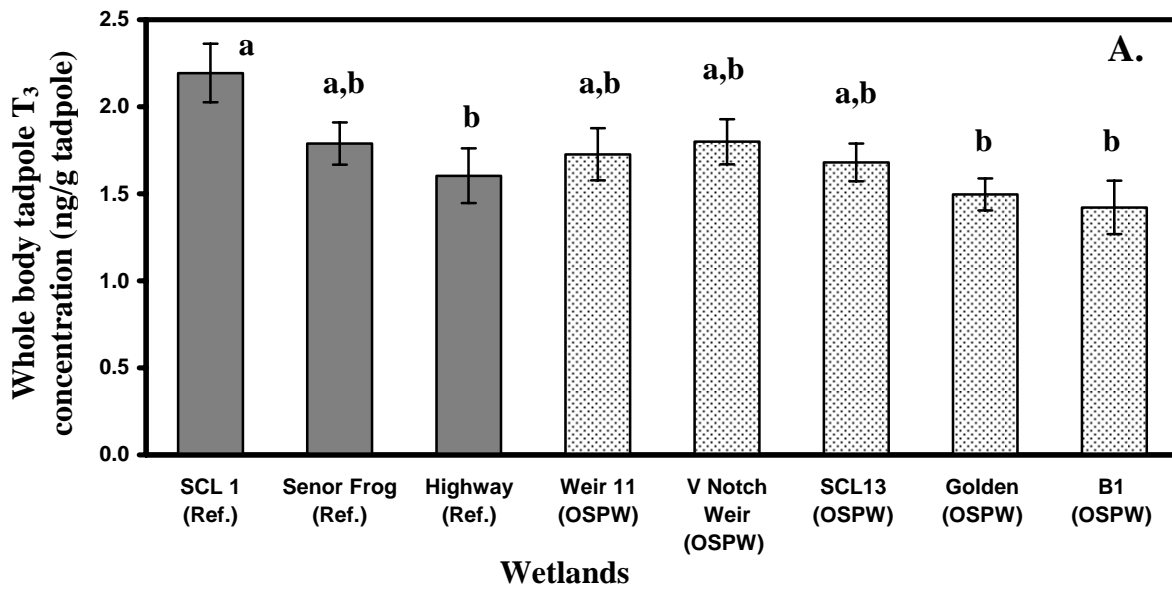
Figure 3.3: Whole body tadpole 3,5,3'-triiodothyronine (T_3) (A) and thyroxine (T_4) (B) concentrations (ng/g tadpole tissue) in wood frog (*Rana sylvatica*) tadpoles collected in 2005 from oil sands process-affected and reference sites in the Athabasca oil sands. Data shown are mean ± standard error of the mean. n=16-20 tadpoles per wetland. Data were analyzed using parametric ANOVA followed by Tukey's post-hoc test. Values marked with different letters were significantly different from each other ($P<0.05$). No significant differences in whole body tadpole T_4 hormone concentrations were observed ($P=0.110$).
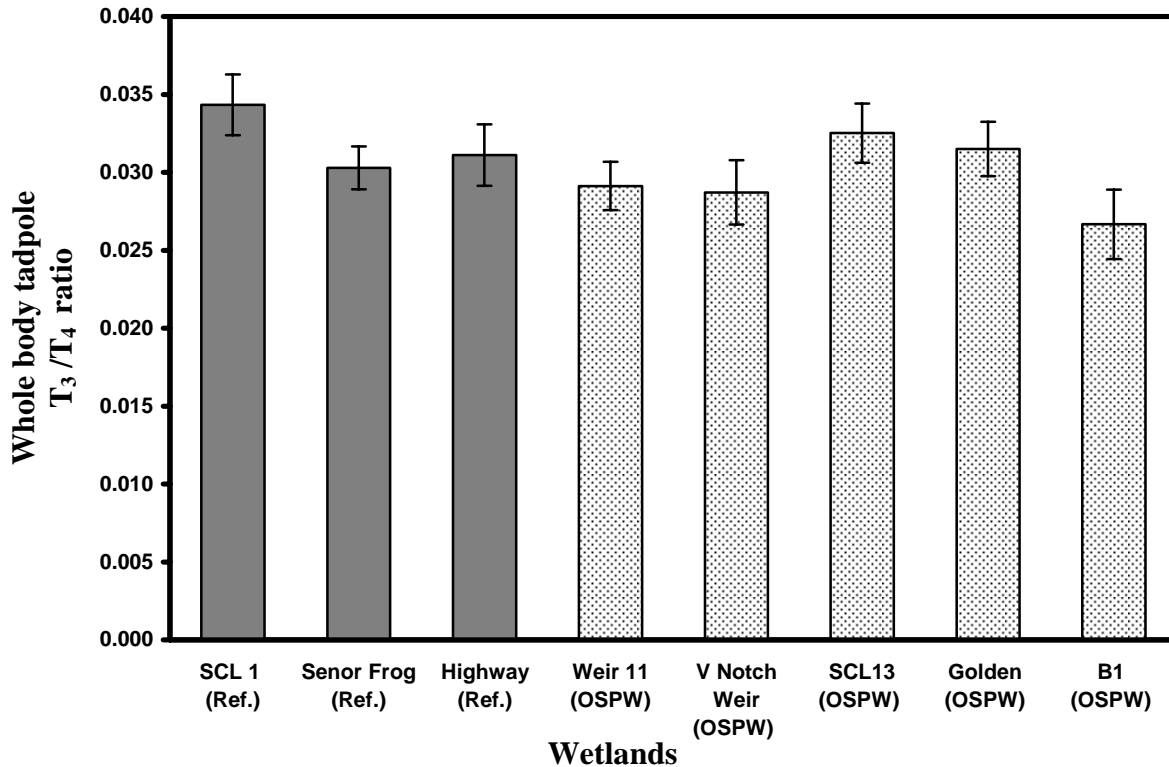
Figure 3.4: Whole body tadpole 3,5,3'-triiodothyronine (T_3):thyroxine (T_4) ratio in wood frog (*Rana sylvatica*) tadpoles collected in 2005 from oil sands process-affected and reference sites in the Athabasca oil sands. Data shown are mean ± standard error of the mean. n=16-20 tadpoles per wetland. Data were analyzed using parametric ANOVA with arcsine transformation of data. No significant differences were observed between treatments (P=0.154).

3.4.5 Whole body tadpole triglyceride analysis

Mean whole body tadpole triglyceride concentrations were significantly lower in OSPW sites B1 and B2 ($P<0.05$) than in tadpoles from Senor Frog, a reference site, which had the highest whole body tadpole triglyceride concentration (Figure 3.5). Triglyceride concentrations in tadpoles from other OSPW sites were not significantly different from the reference sites.
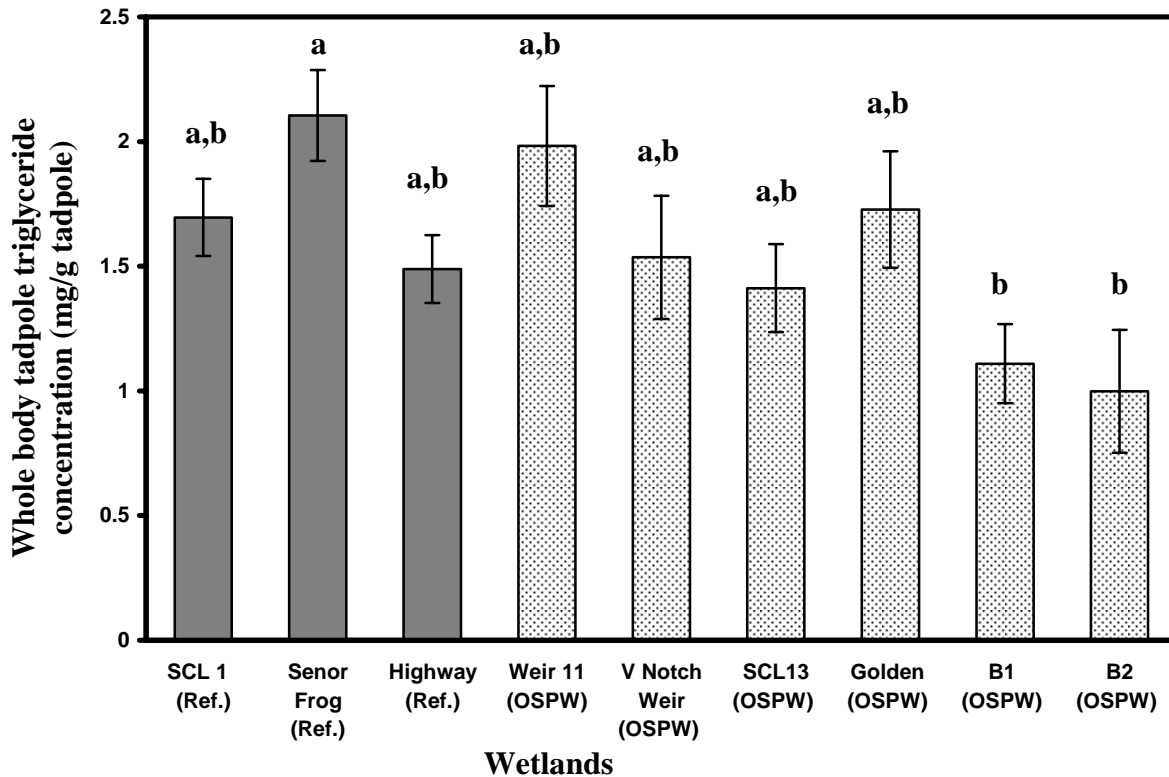
Figure 3.5: Whole body tadpole triglyceride concentrations (mg/g tadpole tissue) in wood frog (*Rana sylvatica*) tadpoles collected in 2005 from oil sands process-affected and reference sites in the Athabasca oil sands. Data shown are mean ± standard error of the mean. n=8-19 tadpoles per wetland. Data were analyzed using parametric ANOVA followed by Tukey's post-hoc test. Values marked with different letters were significantly different from each other ($P<0.05$).

3.4.6 Hepatic glycogen analysis in newly-metamorphosed froglets

Results indicate that hepatic glycogen concentrations were significantly reduced in newly-metamorphosed froglets from three OSPW sites, Weir 11, SCL 13 and Golden pond ($P < 0.05$) compared to froglets from V Notch Weir, another OSPW site, which had the highest hepatic glycogen concentration (Figure 3.6). However, no significant differences were found in hepatic glycogen concentrations of frogs from OSPW sites compared to frogs from the reference ponds.
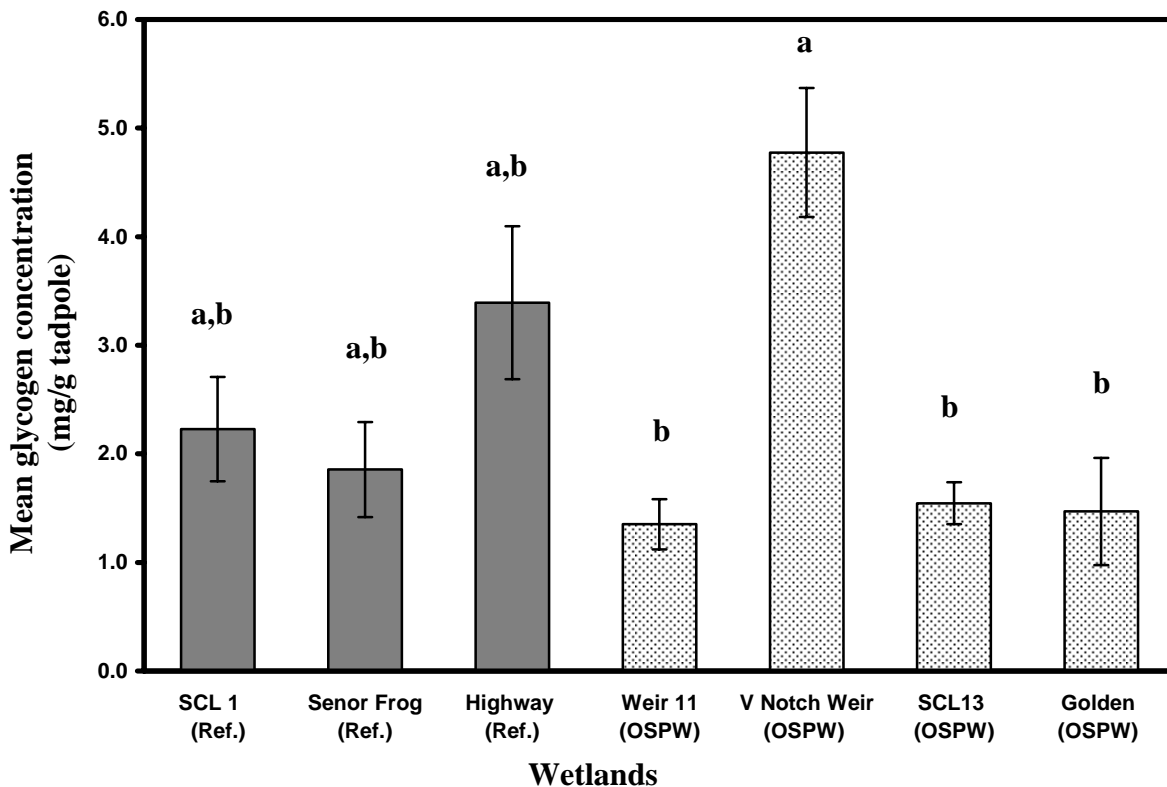
Figure 3.6: Hepatic glycogen concentrations (mg/g liver tissue) in young of the year wood frogs (*Rana sylvatica*) collected in 2005 from oil sands process-affected and reference sites in the Athabasca oil sands. Data shown are mean ± standard error of the mean. n=6-17 tadpoles per wetland. Data were analyzed using non-parametric Kruskal-Wallis ANOVA on ranks followed by Dunn's post-hoc test. Values marked with different letters were significantly different from each other (P<0.05).

3.5   Discussion

It was hypothesized that tadpoles and newly-metamorphosed frogs would demonstrate reduced growth and body condition, and thyroid gland dysfunction potentially associated with delayed metamorphosis. Wetlands on both Suncor and Syncrude lease lands with a wide range of site histories and water and sediment chemistries were surveyed for amphibian use, by assessing the presence of breeding adults, egg masses, developing tadpoles and emerging, newly-metamorphosed froglets. Gosner stage 37-43 tadpoles and newly-metamorphosed frogs were collected using minnow traps and pit fall traps, respectively, in both unimpacted reference and OSPW wetlands. Visual encounter and call surveys and trapping results demonstrated use of many OSPW-impacted Syncrude and Suncor sites by wood frogs. There were no readily discernible differences between wetlands with evidence of wood frog use and those without frogs. Previous studies by Bendell-Young *et al*. (2000) showed that although oil sands based wetlands would support a chironomid invertebrate community, fish would have difficulty surviving. This could indicate that if fish would not survive, organisms such as amphibians with aquatic life stages would also be adversely affected. The present study, although preliminary, indicates that many OSPW wetlands with diverse water chemistry and effluent history are being used by local populations of amphibians.

Whole body tadpole triglyceride results indicated lower triglyceride concentrations in animals from B1 and B2 OSPW-impacted wetlands compared with one of the reference sites. Tadpoles from B2 wetland were also significantly smaller than those from other OSPW and reference sites. They appeared to be developmentally delayed, and were just beginning to develop hind limbs (Gosner 26-28); the

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premetamorphic developmental stage that is associated with relatively lower energy stores. Previous studies have shown that amphibians undergoing premetamorphosis (Gosner stages 19-31) have reduced lipid concentrations as compared with lipid levels during prometamorphosis (Gosner stages 31-40) and metamorphic climax (Gosner stages 40-46) (Blem, 1992). These results were also corroborated in Chapter 2 of this thesis, when whole body tadpole triglyceride concentrations were measured in wood frog tadpoles undergoing metamorphosis. Wood frog tadpoles from site B1 were relatively large in contrast to tadpoles from site B2, but animals from both sites had high condition factors in spite of lower triglyceride stores. Water from both B1 and B2 was characterized by high conductivity and high bicarbonate content compared to the unimpacted sites. High conductivities in the range of 500-2000µS have been known to cause reduced survival in *Rana sylvatica* embryos and larvae (Karraker, *et al*., 2008). High salt concentrations can also result in reduced growth in amphibians. Studies with *Rana sylvatica* have shown that elevated salinity can decrease developmental rates and levels of stored glucose and total proteins, in association with increased internal osmolality in larvae (Karraker, *et al*., 2008). Resource limitation can also obviously play a part in the inhibition of larval growth, resulting in smaller metamorph sizes and longer larval periods relative to other source ponds. Reduced tadpole body size may be associated with lower energy storage and diminished reproductive fitness in the adults (DiMauro and Hunter, 2002).

Hepatic glycogen is an essential, rapidly mobilizable energy source, as well as the primary source of cryoprotectant in wood frogs. The extent of the hepatic glycogen reserve is a major determinant of the quantity of glucose produced during freezing

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(Costanzo and Lee, 1993). Hepatic glycogen concentrations in newly-metamorphosed wood frogs collected from three OSPW sites, Weir 11, SCL 13 and Golden Pond, were significantly lower than one other OSPW site, namely V Notch Weir which had frogs with the highest glycogen stores. This pattern was not consistent with the body condition of tadpoles collected from those sites, as represented by whole body triglyceride concentrations or condition factors, or with the body condition factor index of the frogs themselves. Previous work with amphibian tadpoles (*Ptychadena bibroni*) exposed to organophosphate pesticides have shown decreased body glycogen levels with increasing concentrations of chemical and exposure duration (Ezemonye and Ilechie, 2007). Studies with fish have shown depleted glycogen reserves with exposure to contaminants such as metals (Levesque *et al*., 2002; Teh *et al*., 2004) and pesticides (Nivedhitha *et al*., 1998). Therefore, the increase in hepatic glycogen concentration in wood frogs from an OSPW site was not expected. A possible explanation for reduced growth, but high glycogen concentration in frogs from an OSPW site could be the presence of metals in water and sediments from the wetland. Metals such as lead, mercury, cadmium, chromium, manganese, molybdenum, nickel and cobalt have been known to cause hepatic glycogenolysis (Goodman and Ishak, 1999; Gill and Pant, 1981). However, studies done by Peplow and Edmonds (2005) demonstrated that exposure of rainbow trout (*Oncorhynchus mykiss*) to elevated levels of copper in sediments resulted in a metabolic disorder where food was converted to liver glycogen, but the glycogen was not converted back into glucose for normal distribution to the tissues. This effect resulted from inactivation of the glycogen branching enzyme leading to synthesis of an abnormal insoluble glycogen molecule with decreased branch points and increased chain length.

The inability to mobilize this stored glycogen could lead to reduced growth in the animals.

Tadpoles collected from SCL 1, an unimpacted site, had higher whole body tadpole T_3 concentration compared with the other wetlands. This observation may reflect the developmental stage of these tadpoles, rather than specific environmental factors, such as contaminant exposure. Tadpole age varied from Gosner stages 37-43 among the different sites, which could have contributed to the elevated T_3 concentration, since T_3 concentrations vary with the developmental stage. As seen in Chapter 2 of this thesis, whole body tadpole T_3 concentrations were elevated during prometamorphosis (Gosner stages 31-40) and highest during metamorphic climax (Gosner stages 40-46). In addition, previous studies have reported highest T_3 concentrations in amphibians during metamorphic climax (Weber *et al.,* 1993). Tadpoles from this site were otherwise unremarkable, and the limited data available on water chemistry indicate that water quality was similar to other sites.

Highway Pond, another reference site, had a high condition factor for newly-metamorphosed frogs, but a low condition factor for tadpoles, as well as low whole body tadpole T_3 concentrations. As the name suggests, Highway Pond is located near a highway (not on Suncor or Syncrude lands), and this proximity may have adversely affected the developing tadpoles. In field studies, high abnormality prevalence has been correlated with human activities such as urbanization (Taylor *et al.*, 2005T; Vershinin, 2002). Specimens collected from the Golden Pond had relatively lower body condition and whole body tadpole T_3 concentrations, as well as lower hepatic glycogen levels. Water from the Golden Pond showed high conductivity, as well as high levels of

magnesium, calcium and sulfate ions as compared to the unimpacted sites. This could once again be attributed to varying stages of development of the specimens collected and not necessarily be due to any one water quality parameter.

Tadpoles from V Notch Weir (OSPW) exhibited lower body weight, total length and condition factor. However, this wetland supported newly-metamorphosed wood frogs with relatively high hepatic glycogen concentrations. Both Weir 11 and SCL 13 (OSPW) had lower condition factors for wood frog tadpoles and lower concentrations of hepatic glycogen in newly-metamorphosed frogs. Waters from V Notch Weir and Weir 11 had high ammonia levels when compared with other wetlands, which could have adversely affected body condition of wood frogs inhabiting these wetlands. Furthermore, water from both Weir 11 and SCL 13 had relatively high pH. Previous work with fish exposed to water with high pH has shown inhibition of ammonia excretion and subsequent increase in plasma ammonia, which can be potentially lethal (Wilson *et al*., 1998; Laurent *et al*., 2000). Ammonia is toxic to many aquatic organisms, and occurs in two forms in aqueous solution: the un-ionized form (NH_3) and the ionized form (NH_4^+). An increase in NH_3 results from increased carbonate hardness, and subsequent increased pH, as NH_4^+ ions are converted to toxic NH_3 molecules. At lower pH levels, NH_3 converts to NH_4^+ (Boyer and Grue, 1995).

Comparison between whole body tadpole triglyceride and thyroid hormone (T_3 and T_4, separately) concentrations among the various wetlands yielded no relationships between these endpoints. This result, which is different than that observed in Chapter 2, could be attributed to the range of developmental stages of tadpoles collected from each wetland (Gosner stages 37-43).

In the Athabasca oil sands, wood frog eggs, tadpoles and new metamorphs are at risk due to predation, dessication (due to evaporation of water from source ponds) or low temperatures. Only 4%, 4.4% and 3.3% of wood frog (*Rana sylvatica*), spotted frog (*Rana pretiosa*) and tiger salamander (*Ambystoma tigrinum*) eggs, respectively, survive to metamorphosis in the field under normal conditions (Venturino *et al*., 2003). Although this mortality is usually assumed to be due to natural environmental stressors, the role of contaminants in decreased overall body condition and survivability cannot be overlooked.

Wetlands that support diverse, sustainable populations of indigenous amphibians usually have many of the characteristics of a healthy ecosystem. This study shows that amphibians are present in and around many of the water bodies containing oil sands process-affected water on Syncrude and Suncor leases. Obviously, the presence of adults and evidence of efforts at reproduction (i.e., egg masses and tadpoles) is not sufficient to demonstrate that reclaimed wetlands are capable of sustaining viable populations. Amphibians are frequently attracted to water bodies that are not conducive to reproductive success. Local populations in poor quality habitat that experience recruitment failure due to high embryonic or larval mortality can, within limits, be sustained by in-migration of adults from surrounding higher quality habitat (Pulliam, 1988). Therefore, population surveys alone are not adequate to predict habitat suitability in a heterogeneous landscape.

This study reports preliminary findings about wood frog tolerance to a range of OSPW and substrates in reclaimed wetlands. The study design does not permit rigorous conclusions about the toxicity of OSPW and sediments to native amphibians. Future work with exposure of wood frog eggs and tadpoles to OSPW and substrates under laboratory

conditions to assess the effects on wood frog development and growth can perhaps help

ascertain individual contributions of OSPW and substrates to any potential toxicity.

CHAPTER 4
4.0 Laboratory exposure of wood frog eggs and tadpoles to oil sands process-affected waters and substrates

4.1   Abstract

The Athabasca oil sands in northern Alberta represent one of the largest known oil deposits in the world. The process used to extract oil from these deposits results in the production of large volumes of process-affected water (OSPW) and substrates (OSPS). These effluents will be incorporated into wetlands as a component of current landscape reclamation strategies. Wood frogs (*Rana sylvatica*) are an abundant native amphibian likely to inhabit these reclaimed wetlands.

The objective of this study was to evaluate potential detrimental effects of OSPW and OSPS from existing reclaimed wetlands with different histories and contaminant profiles on the growth and development of wood frogs. In 2006, wood frog eggs collected from uncontaminated wetlands were exposed to OSPW from five different OSPW-impacted wetlands or water from one unimpacted wetland (reference) and dechlorinated tap water (control) under laboratory conditions. Endpoints evaluated included percent hatchability and tadpole survival. Hatchability was reduced in eggs exposed to water from one of the OSPW sites (25.7%), compared with the other process-affected ponds and the control water (P<0.05). Tadpole survival was significantly affected (<20%) by exposure to water from all the OSPW-impacted sites (P<0.05). In 2007, newly hatched wood frog tadpoles (Gosner stage 19-21) were exposed to water from six different wetlands containing OSPW or a tap water control. Results showed <10% survivability of tadpoles in five of the six OSPW sites relative to control animals (P<0.05).

A substrate exposure study was also conducted in 2007, in which older wood frog tadpoles (Gosner stages 27-30) were exposed to five process-affected substrates or a control substrate overlaid with dechlorinated tap water. Endpoints evaluated included survivability (to Gosner stage 37-39), body weight, total length, condition factor and whole body tadpole thyroid hormone and triglyceride concentrations. The OSPS exposure did not affect tadpole survivability, but tadpoles exposed to two of the impacted substrates demonstrated significant changes in morphological endpoints and whole body triglyceride concentrations ($P<0.05$). Water chemistry and metals concentrations were compared with egg hatchability and tadpole survival, growth and development.

4.2   Introduction

Reclamation strategies in the Alberta oil sands include the bioremediation of water (OSPW) and sediments (OSPS) produced by the oil sand extraction process. The oil sand mining companies (including Syncrude Canada Ltd. and Suncor Energy Inc.) are required by provincial environmental legislation to demonstrate that reclaimed wetlands containing OSPW and OSPS are capable of sustaining populations of indigenous aquatic and semi-aquatic organisms. Wood frogs (*Rana sylvatica*) are an abundant native amphibian representative of these populations.

Amphibians as a group are important keystone species in many habitats, and can be used as models in ecotoxicology studies to evaluate the impact of many environmental stressors, including acute and chronic chemical toxicity (Sparling *et al*., 2000). The presence of wood frogs (and other amphibians) has been noted in and around many water bodies containing oil sands process-affected materials on Syncrude and Suncor lease

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lands. However, the presence of egg masses and young larvae at these sites is not indicative of habitat capable of sustaining amphibian populations long term, because breeding adults can be drawn towards wetlands that are, in actuality, unfavorable for reproductive success, and act as population sinks (Pollet and Bendell-Young, 2000).

The OSPW and OSPS are known to contain elevated levels of dissolved organic matter (e.g. naphthenic acids), hydrocarbons, including polycyclic aromatics, various metals and salts. The toxicity of reclaimed wetland water and sediments to amphibians will likely vary as a function of developmental stage. Unshelled wood frog eggs are directly exposed to sediments and water, and may readily absorb toxic substances. Amphibian embryos are very sensitive to acidic water (pH 4-5) (Freda, 1986), but developing larvae become tolerant of increases in acidity after hatching (Pierce *et al.,* 1984; Freda & Dunson, 1985). Certain metals such as aluminum can also become toxic to developing amphibian larvae in conjunction with low pH. Aluminum sensitivity varies according to the developmental stage. Newly hatched tadpoles are extremely sensitive, followed by embryos and then older tadpoles. The 96 hour LC_{50} for Leopard frogs (*Rana pipiens*) exposed to monomeric aluminum was less than 250 µg/L for newly hatched tadpoles, 403 µg/L for embryos, and greater than 1000 µg/L for 3-week old tadpoles (Freda and McDonald, 1990). Concentrations of sodium, sulfate, chloride and bicarbonate ions are often particularly high in OSPW, resulting in high levels of salinity. Previous work with amphibian eggs (Haramura, 2007) and larvae (Gomez-Mestre and Tejedo, 2004) have shown decreased hatching and rate of development with increased salinity.

Consequently, although there are indications of amphibian use of reclaimed oil sands wetlands, little work has been done to determine the sensitivity of developing embryos and larvae to the complex mixtures present in OSPW and OSPS. Controlled, laboratory-based studies are needed to assess potential adverse effects on eggs and tadpoles. Laboratory-based studies allow control of environmental variables, such as unfavourable weather, risk of predation and exposure to infections that may affect physiological and biochemical endpoints. In addition, laboratory studies allow assessment of any potential toxic affects of oil sands process-affected water (OSPW) and substrates (OSPS), independent of each other, to wood frog eggs and tadpoles.

The objective of this study was to determine the impact of exposure to OSPW and OSPS on hatchability, survivability, growth and development of wood frog eggs and tadpoles. In tadpoles that survived to metamorphosis, potential adverse effects on whole body triglyceride and thyroid hormone (3,5,3'-triiodothyronine [T_3] and thyroxine [T_4]) concentrations were also determined. It was hypothesized that wood frog eggs and tadpoles exposed to OSPW or OSPS would demonstrate lower hatchability and survivability, and reduced growth and delayed time to metamorphosis than those exposed to control water. In addition, wood frog tadpoles exposed to OSPW and OSPS would demonstrate reduced whole body triglyceride (TG), T_3 and T_4 concentrations compared to control tadpoles.

4.3   Materials and Methods

Water was collected from three different sites each on Suncor and Syncrude oil sands leases in 2006 (Table 4.1), beginning as soon as the wetlands were ice free in the spring. One of the Suncor wetlands (Loon Lake) is considered to be an on site reference wetland, with no process-affected materials. In 2007, water was collected from six different OSPW-impacted wetlands on Syncrude lease lands in the spring, once the weather conditions were favourable (Table 4.2). Dechlorinated aged tap water was used as control water during both years. A sediment toxicity study was also carried out in 2007 to evaluate five different types of substrates (OSPS) relevant to the oil sands reclamation efforts. Clean silica sand was used as the control sediment (Table 4.3). Water and substrates were shipped in 20L containers to the Toxicology Centre at the University of Saskatchewan in Saskatoon.

All OSPS were obtained from Syncrude Canada Ltd. Consolidated tailings (CT) are produced when gypsum ($CaSO_4 \bullet 2H_2O$) is added to fine tailings, in order to expedite the process of settling. The CT in tailing ponds settles much more rapidly than fine tailings (FTFC, 1995). Saline-sodic overburden is characterized by properties such as a pH of ≤ 8.5 and a high concentration of salts. Peat mineral mix consists of salvaged mineral soil materials, tailings sand and surface organic materials. A peat mineral mix is generally abundant in mining areas and is used as a surface treatment (Fung and Macyk, 2000). Coke, generally a blackish-grey, porous solid, is a waste product produced during heavy oil upgrading processes at Syncrude Canada Ltd. Clean silica sand (425-850 μm) was used as a control sediment (Unimin Corporation, Connecticut, USA).

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4.3.1 Water sources for 2006

Table 4.1: Summary of water sources from the Athabasca oil sands, Alberta, used for the laboratory exposure of wood frog (*Rana sylvatica*) eggs and tadpoles in 2006. Five of the sources represented oil sands process-affected waters (OSPW), with one on-site reference water source and tap water acting as control.

Company	Site	Treatment
Suncor	Loon Lake	Reference
Suncor	Natural Wetland	OSPW
Suncor	Jan's Pond	OSPW
Syncrude	Peat Pond	OSPW
Syncrude	Beaver Creek	OSPW
Syncrude	Test Pond 7	OSPW
	Dechlorinated aged tap water	Control

4.3.2 Water sources for 2007

Table 4.2: Summary of water sources from the Athabasca oil sands, Alberta, used for the laboratory exposure of wood frog (*Rana sylvatica*) tadpoles in 2007. Six of the sources represented oil sands process-affected waters (OSPW), with tap water acting as a control.

Company	Site	Treatment
Syncrude	Peat Pond	OSPW
Syncrude	Test Pond 9	OSPW
Syncrude	Test Pond 5	OSPW
Syncrude	Mike's Pond	OSPW
Syncrude	Bill's Lake	OSPW
Syncrude	Demonstration (Demo) Pond	OSPW
	Dechlorinated aged tap water	Control

4.3.3 Substrate types tested in 2007

Table 4.3: Summary of substrate sources (OSPS) from the Athabasca oil sands, Alberta, used for the laboratory exposure of wood frog (*Rana sylvatica*) tadpoles in 2007, with clean silica sand acting as a control.

Company	Substrate Type	Treatment
Syncrude	Consolidated tailing	OSPS
Syncrude	Plain tailings sand	OSPS
Syncrude	Saline sodic overburden	OSPS
Syncrude	Peat mineral mixture	OSPS
Syncrude	Coke	OSPS
Unimin Corporation	Clean silica sand	Control

4.3.4 Collection of egg masses

Amplexed pairs of wood frogs and newly fertilized egg masses were collected from several different wetlands near Saskatoon, Saskatchewan in April 2006. Only newly laid wood frog egg masses were collected in April 2007 (Figure 4.1). Breeding sites were located using frog calling surveys.

(a)   (b)

Figure 4.1: (a) Collection of wood frog egg masses from local Saskatchewan water bodies; (b) amplexed pair of wood frogs

Amplexed pairs and egg masses were transported to the laboratory at the Toxicology Centre, University of Saskatchewan, and put in a controlled environmental chamber at 10ºC. Eggs from several different sources were mixed prior to being allocated to the specific treatments to minimize genetic variability among treatments.

4.3.5   Tadpole husbandry and monitoring

Procedures for tadpole husbandry were based on <u>Amphibians: Guidelines for breeding, care and management of laboratory animals</u> (National Research Council, 1974). Containers with eggs and tadpoles were continuously aerated using an air stone and aquarium pumps. Containers were exposed to 16 hours of full spectrum light and eight hours of darkness per day. The temperature of the environmental chamber was increased gradually from 10ºC to 22ºC over the course of development, to match the water temperature in the source ponds. Once the tadpoles started feeding, their diet consisted of boiled green lettuce and ground Tetramin® tropical fish flakes. One half of water volume, as well as residual food was replaced every second day.

4.3.6   Experimental design for oil sands process-affected water exposure (2006 and 2007)

For the OSPW exposure study in 2006, pooled, newly fertilized eggs were randomly allocated to specific treatments by placing 30 eggs each in an 11.7x20.8x34.0 cm plastic container with two L of OSPW, site-specific control water or dechlorinated tap-water. There were ten replicates for each treatment (total of 300 eggs/treatment) (Figure 4.2). Percent hatchability and percent survivability of wood frog eggs and tadpoles were monitored daily through Gosner stages 26-27.

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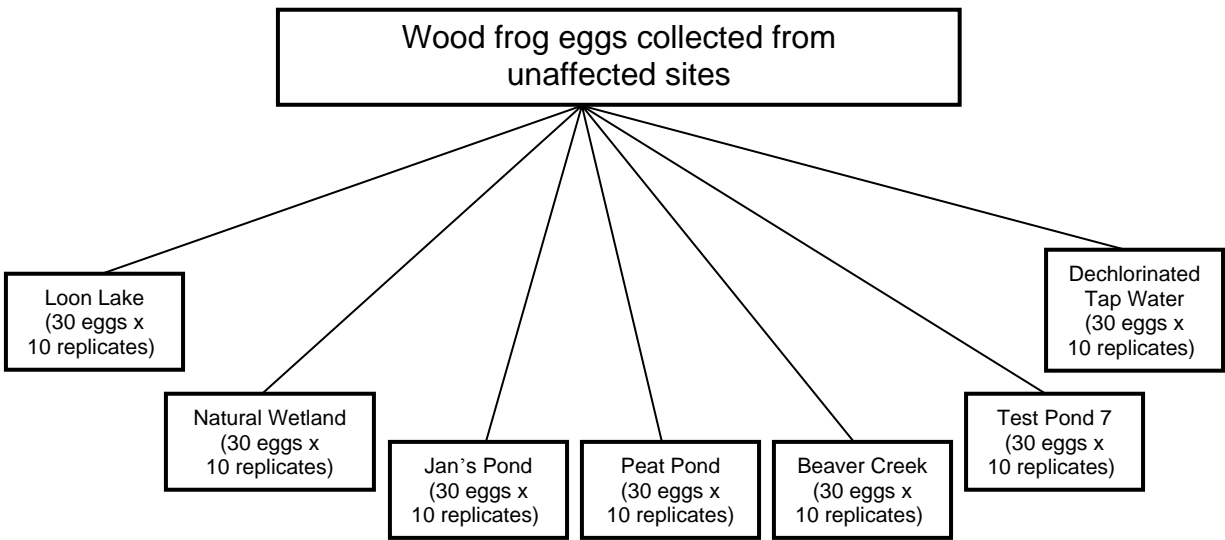
Figure 4.2: Experimental design for exposure of wood frog (*Rana sylvatica*) eggs and tadpoles in 2006 to dechlorinated tap water (control), reference water and oil sands process-affected water (OSPW) from five different reclaimed wetlands in the Athabasca oil sands, Alberta.

In 2007, newly fertilized wood frog eggs were collected from several water bodies around Saskatoon and transported to the laboratory as before. After hatching, fifteen newly hatched tadpoles from different egg masses (Gosner stages 19-21) were randomly allocated to the various treatments and placed in a 11.7x20.8x34.0 cm plastic container with one cm of clean silica sand substrate (Unimin Corporation, Connecticut, USA), overlaid with two L of OSPW or dechlorinated tap-water. There were 10 replicates for each treatment (total of 150 tadpoles/treatment) (Figure 4.3). Percent survivability of wood frog tadpoles was monitored daily through Gosner stages 26-27.
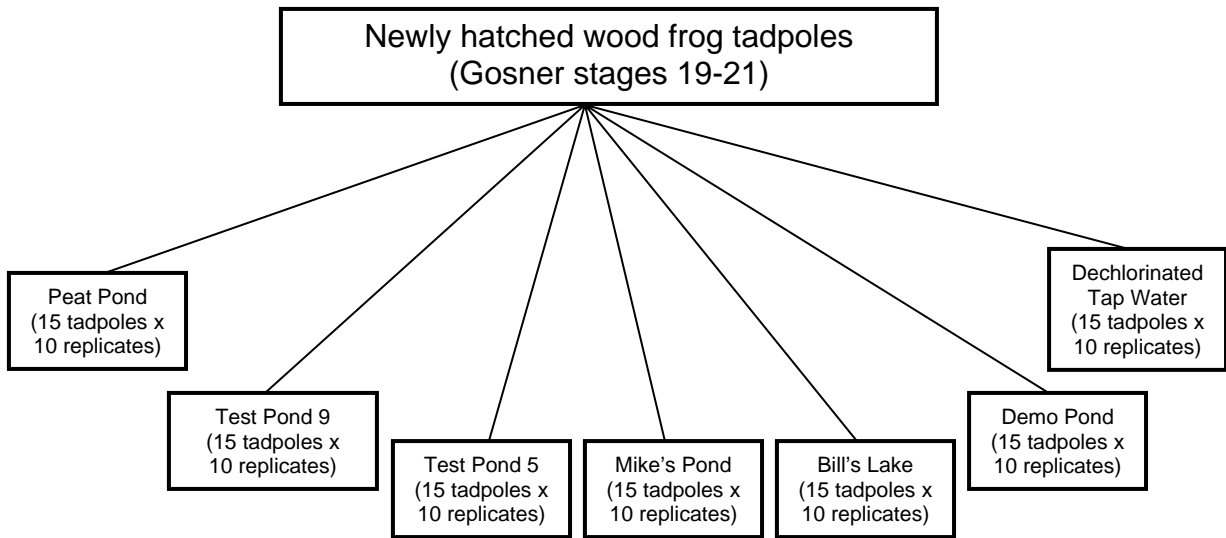
Figure 4.3: Experimental design for exposure of newly-hatched wood frog (*Rana sylvatica*) tadpoles in 2007 to dechlorinated tap water (control) and oil sands process-affected water (OSPW) from six different reclaimed wetlands in the Athabasca oil sands, Alberta.

4.3.7 Experimental design for oil sands process-affected substrate exposure (2007)

In 2007, wood frog tadpoles hatched from eggs collected from unaffected sites near Saskatoon were exposed to oil sands process-affected substrates or clean silica sand as control. Six tadpoles at Gosner stages 27-30 were randomly allocated to OSPS treatments, and placed in a 11.7x20.8x34.0 cm plastic container with two cm of substrate overlaid with two L of dechlorinated tap water. There were 10 replicates per treatment (total of 60 tadpoles/treatment) (Figure 4.4). The OSPS exposure experiment was not initiated with newly hatched tadpoles similar to OSPW studies because the substrate materials to be tested were not shipped to the University of Saskatchewan at the appropriate time.

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┌─────────────────────────────────────────┐
│  Wood frog tadpoles at Gosner stages 27-30 │
│          (hind limb bud emergence)         │
└─────────────────────────────────────────┘
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Consolidated Tailing
(6 tadpoles x
10 replicates)

Plain Tailings Sand
(6 tadpoles x
10 replicates)

Saline Sodic
Overburden
(6 tadpoles x
10 replicates)

Peat Mineral
Mixture
(6 tadpoles x
10 replicates)

Coke
(6 tadpoles x
10 replicates)

Clean Silica Sand
(6 tadpoles x
10 replicates)
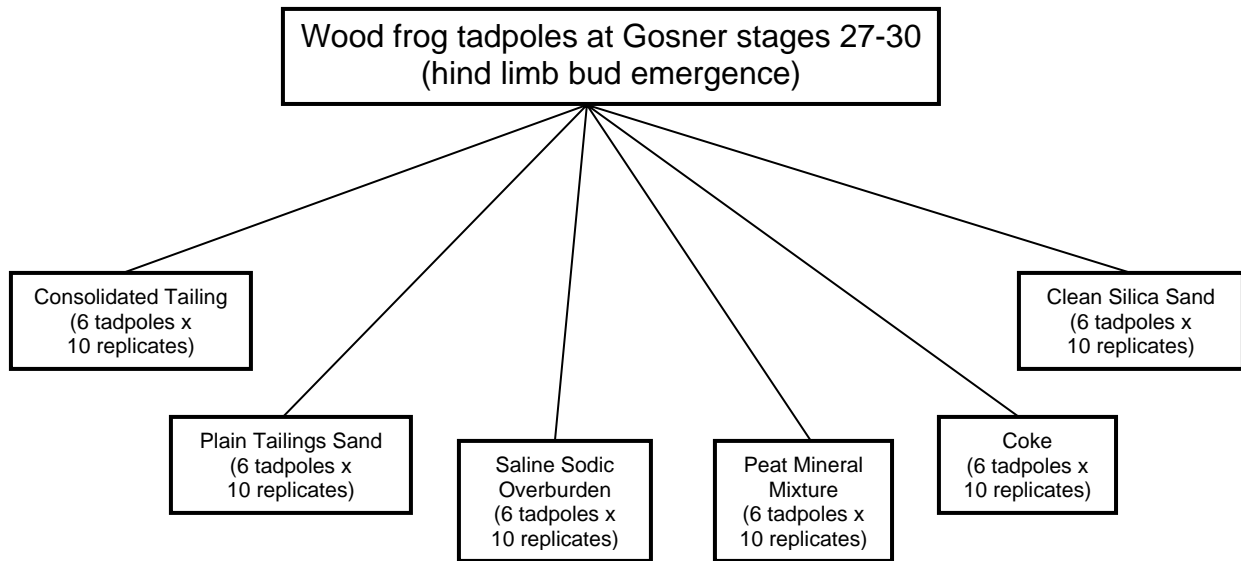
Figure 4.4: Experimental design for exposure of wood frog (*Rana sylvatica*) tadpoles (at hind limb development) in 2007 to clean silica sand (control) and oil sands process-affected substrates from five different sources in the Athabasca oil sands, Alberta.

4.3.7.1 Biological Assays

A modified method from Weber *et al*. 2003 and Brasfield *et al*. 2004 was applied to wood frog tadpoles to evaluate whole body tadpole triglyceride and T_3 and T_4 concentrations.

4.3.7.1.1   Thyroid hormone assays

Commercial enzyme-linked immunosorbent assay (ELISA) kits (BioQuant, San Diego, CA, USA) were used to measure whole-body tadpole T_3 and T_4 concentrations in wood frog tadpoles as described in section 2.3.1.3.1 of Chapter 2.

4.3.7.1.2   Triglyceride assays

The triglyceride assay was based on a method developed in juvenile fish by Weber *et al*. (2003). As described in section 2.3.1.2.1 of Chapter 2, tadpole whole body

triglyceride concentrations were measured using a modification of a commercial kit

protocol (Sigma, Saint Louis, MO, USA).

4.3.8 Water and substrate chemistry testing

Water samples were analyzed for basic water quality variables (conductivity, pH,

hardness, alkalinity, ammonia and dissolved oxygen), and OSPW and OSPS were also

assayed for metal concentrations. Metal analysis was conducted by filtering water

samples at 0.45μm, and acidifying them with 12.5μL of 2% nitric acid (69% Omni-Trace,

Merck, NJ, USA) per one ml of sample. Each substrate sample (0.1g) was microwave

digested using nitric acid, hydrogen peroxide and hydrogen fluoride. Samples were

subsequently analyzed with a Thermo X Series inductively coupled plasma mass

spectrometer (ICP-MS) (Thermo Electron Corporation, MA, USA).

4.3.9 Statistical analyses

Statistical analyses included parametric and non-parametric Kruskal-Wallis one-

way ANOVA followed by post hoc tests (Tukey's and Dunn's) for multiple comparisons.

Specifically, weight measurements were analyzed by parametric ANOVA with Tukey's

post-hoc test, whereas the length and condition factor measures were evaluated using

non-parametric Kruskal-Wallis ANOVA, followed by Dunn's post-hoc test. Since

animals were not exposed to treatment solutions individually, the potential tank effect

was tested for all variables using one way ANOVA, but was always found to be not

significant. Pearson product moment correlations were used to evaluate the relationship

between triglyceride and T_3 and T_4 concentrations. The correlation coefficient (r) was

given at $p < 0.05$ for all correlations. The results are expressed as mean ± SEM.

Intra-assay variability was assessed for each of the three tests (T_3, T_4 and triglycerides), by making six determinations of a pooled sample. The same pooled sample was measured six more times on separate occasions to evaluate inter-assay variability.

4.4   Results

4.4.1   Hatchability and survival of wood frog tadpoles exposed to oil sands process-affected water in 2006 and 2007

Figure 4.5 illustrates the percent hatchability of wood frog eggs exposed to dechlorinated aged tap water (control) and several water sources from the Athabasca oil sands. Percent hatchability was significantly lower in eggs exposed to water from Jan's Pond, an OSPW site, compared to the reference source, Loon Lake, and dechlorinated tap water control ($P<0.05$). Jan's Pond hatchability was also significantly lower than OSPW-impacted Beaver Creek and Peat Pond results ($P<0.05$). With the exception of Jan's Pond, there were no significant differences in hatchability between other OSPW sites and the unimpacted sources.

Figure 4.6 (graph A) shows percent survivability of wood frog tadpoles exposed to control, reference and process-affected waters over a period of 28 days after hatching in 2006. Survivability of tadpoles from one OSPW site (Jan's Pond) and the on-site reference source (Loon Lake) dropped dramatically within 1-2 weeks of hatching. Tadpoles exposed to OSPW from Jan's Pond succumbed faster than all other treatments except Loon Lake. Relative to the other water treatments, tadpoles in dechlorinated aged tap water had significantly higher survivability over the course of the observation period ($P<0.05$). Survivability of tadpoles exposed to OSPW sources Test Pond 7 and Peat Pond also showed significantly higher survivability than Jan's Pond and Loon Lake ($P<0.05$).

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Figure 4.6 (graph B) illustrates percent survivability of wood frog tadpoles following exposure to OSPW from six impacted sites over a period of 21 days in 2007. Tadpole survival in all the water treatments declined over time. However, survival decreased most rapidly in tadpoles exposed to OSPW sources, Peat Pond and Demo Pond. In contrast, survivability of tadpoles in dechlorinated aged tap water remained high throughout the exposure period ($P<0.05$).

Figure 4.7 (graph A) summarizes survivability of the tadpoles after 28 days of exposure in 2006. Survivability was zero among tadpoles exposed to water from Loon Lake (reference), Natural Wetland (OSPW), Jan's Pond (OSPW) and Beaver Creek (OSPW). The dechlorinated aged tap water control had the highest percent survivability among the remaining treatments ($P<0.05$). Figure 4.7 (graph B) summarizes survivability of wood frog tadpoles exposed to OSPW or tap water after 21 days in 2007. Tadpole survivability was significantly higher in aged tap water control ($P<0.05$) than that of the tadpoles in all of the OSPW treatments with the exception of Bill's Lake. Tadpole survivability in Bill's Lake water was also significantly greater than in the other OSPW sources ($P<0.05$).
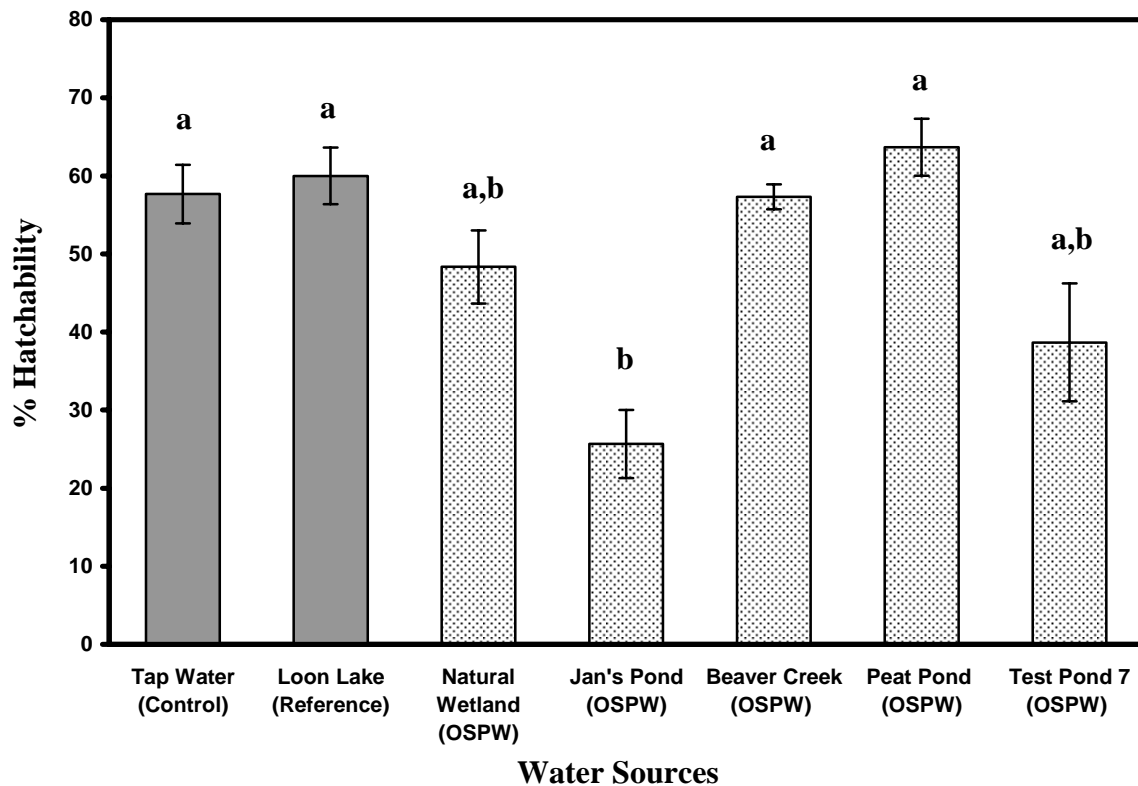
Figure 4.5: Percent hatchability of wood frog (*Rana sylvatica*) eggs exposed to dechlorinated tap water (control), on-site reference, and five different oil sands process-affected water sources (OSPW) from the Athabasca oil sands, Alberta, in 2006. Data shown are mean ± standard error of the mean. n=300 eggs per water source. Data were analyzed using non-parametric Kruskal-Wallis ANOVA on ranks followed by Tukey's post-hoc test. Values marked with different letters were significantly different from each other (P<0.05).
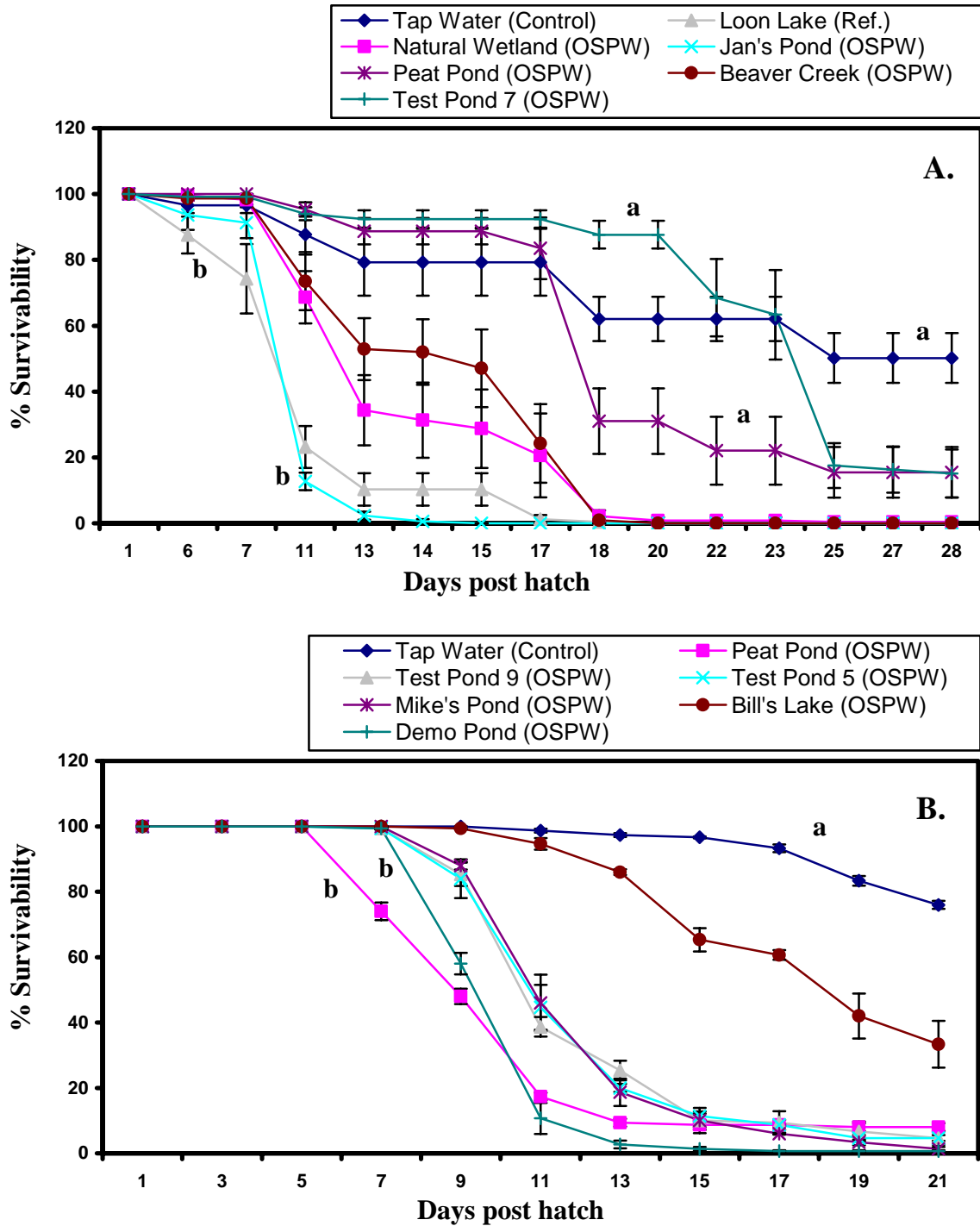
Figure 4.6: Percent survivability of wood frog (*Rana sylvatica*) tadpoles exposed to dechlorinated tap water (control), on-site reference and oil sands process-affected water sources (OSPW) from the Athabasca oil sands, Alberta, in 2006 (A) and 2007 (B). Data shown are mean ± standard error of the mean. n=173-261 tadpoles for 2006 and 150 tadpoles for 2007, per water source. Data were analyzed using non-parametric Kruskal-Wallis ANOVA on ranks followed by Tukey's post-hoc test. Values marked with different letters were significantly different from each other (P<0.05).
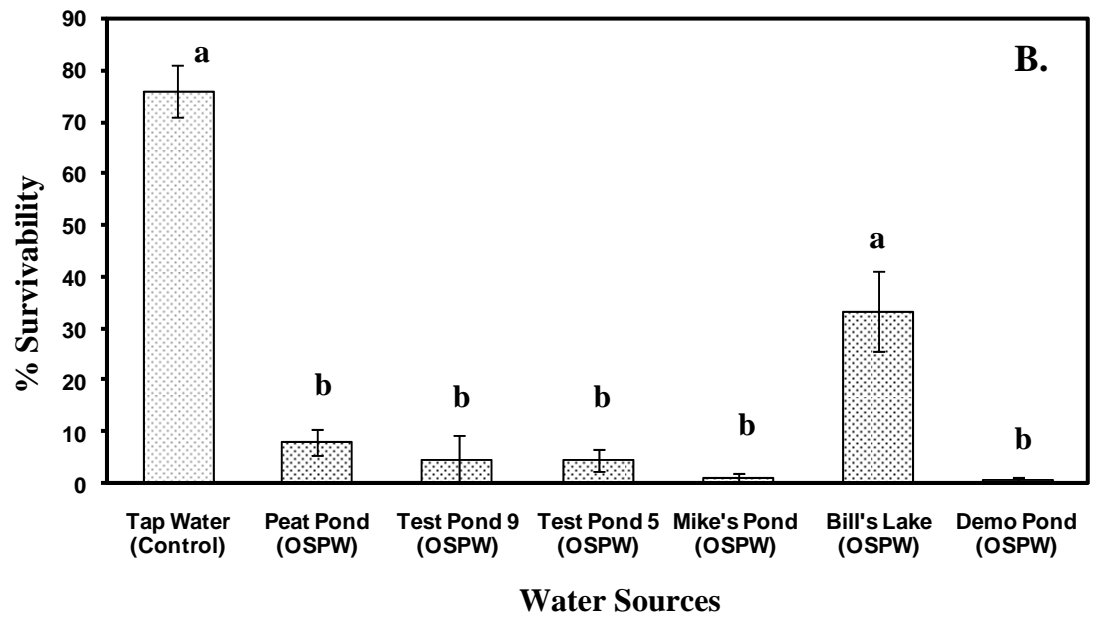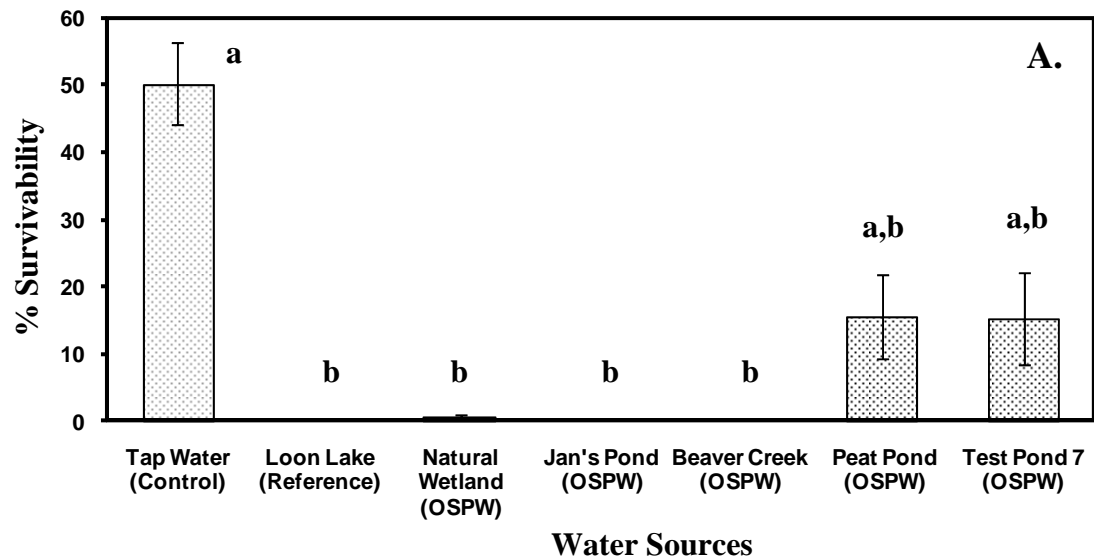
Figure 4.7: Summary of percent survivability of wood frog (*Rana sylvatica*) tadpoles, exposed to dechlorinated tap water (control), on-site reference and oil sands process-affected water sources (OSPW) from the Athabasca oil sands, Alberta, in 2006 (A) and 2007 (B). Data shown are mean ± standard error of the mean. Sample sizes included 10 replicates per water source. Data were analyzed using non-parametric Kruskal-Wallis ANOVA on ranks followed by Tukey's post-hoc test. Values marked with different letters were significantly different from each other (P<0.05).

4.4.1.1 Water Chemistry for oil sands process-affected water, reference and control water (2006 and 2007)

Results of basic water chemistry and metals analyses of sources used in the OSPW study in 2006 showed particularly high conductivity and total water hardness for Jan's Pond among all the treatments. Water from Natural Wetland had high pH, alkalinity and high dissolved oxygen content, whereas Loon Lake water, a reference source, had the highest ammonia level compared to the rest of the water sources (Table 4.4). Concentrations of boron, strontium, uranium, lead, nickel, cobalt, chromium and vanadium were elevated in Jan's Pond water. High concentrations of molybdenum were observed in Natural Wetland, while Beaver creek had elevated manganese and zinc, and Loon Lake had a high barium concentration. Test pond 7 had highest aluminum and iron concentrations among the OSPW sources (Table 4.5).

Results of water chemistry and metals analyses of the water sources used in the OSPW study in 2007 showed the highest pH, alkalinity and dissolved oxygen content in water from Demo Pond; the highest conductivity and ammonia in Mike's pond; and elevated hardness in dechlorinated tap water control (Table 4.6). Elevated concentrations of boron, strontium, molybdenum and uranium were observed in Mike's Pond, compared to other water sources. Test Pond 9 had highest concentrations of aluminum, vanadium, chromium, nickel and arsenic, whereas, dechlorinated tap water had relatively higher levels of zinc and lead (Table 4.7).

Table 4.4: Summary of water quality measurements for oil sands process-affected (OSPW), reference and control water used for laboratory exposure of wood frog (*Rana sylvatica*) eggs and tadpoles in 2006.

Water Source	Conductivity (µs/cm)	pH	Hardness (mgCaCO$_3$/L)	Alkalinity (mg/L)	Ammonia (mg/L)	Dissolved Oxygen (mg/L)
Loon Lake (Reference)	853 ± 3.0	7.95 ± 0.03	307 ± 1.5	164 ± 1.5	1.72 ± 0.19	9.27 ± 0.33
Natural Wetland (OSPW)	754 ± 2.0	8.70 ± 0.02	78.7 ± 0.7	294 ± 1.0	1.50 ± 0.42	10.0 ± 0.10
Peat Pond (OSPW)	974 ± 2.5	7.93 ± 0.19	289 ± 3.5	17.0 ± 2.1	0.176 ± 0.02	7.95 ± 0.02
Test Pond 7 (OSPW)	194 ± 3.5	7.84 ± 0.22	17.0 ± 2.2	7.00 ± 0.7	0.809 ± 0.04	6.29 ± 0.31
Jan's Pond (OSPW)	2511 ± 6.0	8.58 ± 0.01	522 ± 2.0	56.7 ± 1.3	0.716 ± 0.04	5.17 ± 0.70
Beaver Creek (OSPW)	1330 ± 3.5	7.93 ± 0.24	369 ± 3.5	24.3 ± 1.2	0.700 ± 0.03	8.13 ± 0.04
Dechlorinated Tap Water (Control)	400 ± 5.5	8.30 ± 0.25	138.7 ± 1.6	6.00 ± 0.7	0.263 ± 0.05	7.02 ± 0.05

Table 4.5: Metal analysis for water sources from impacted (oil sands process-affected water) and reference wetlands in the Athabasca oil sands, Alberta, that were used for laboratory exposure of wood frog (*Rana sylvatica*) eggs and tadpoles in 2006.

Element (µg/L)	Canadian Water Quality Guidelines**	Loon Lake (Reference)	Jan's Pond (OSPW)	Natural Wetland (OSPW)	Peat Pond (OSPW)	Test Pond 7 (OSPW)	Beaver Creek (OSPW)
Boron	1200	128	1808	1539	73.2	1120	61.7
Aluminum	1010	9.42	9.72	18.2	9.39	52.7	5.69
Titanium	NA*	0.413	0.464	0.879	0.400	1.70	0.00
Vanadium	100	0.283	0.904	0.632	0.279	0.299	0.253
Chromium	8.90	0.187	0.575	0.182	0.176	0.251	0.264
Manganese	0.600	1.04	0.874	1.02	23.5	22.6	690
Iron	NA	5.85	66.8	97.1	2019	4049	677
Cobalt	0.900	0.014	0.440	0.343	0.042	0.050	0.100
Nickel	NA	0.815	6.63	2.25	0.528	0.872	1.84
Copper	2.00	0.344	0.614	0.711	0.472	0.792	0.363
Zinc	55.0	12.9	11.9	13.4	80.9	52.2	525
Arsenic	5.00	0.361	1.11	2.51	0.540	1.25	1.089
Strontium	NA	273	1262	369	382	255	189
Molybdenum	73.0	1.32	35.5	93.6	1.13	1.58	1.06
Antimony	NA	0.619	0.749	0.736	0.866	0.654	0.827
Barium	NA	111	36.5	51.8	18.7	32.2	48.9
Lead	35.0	0.085	1.39	0.165	0.114	0.507	0.104
Uranium	200	0.993	1.91	1.46	0.557	0.382	0.132

*NA = Not available   **Canadian Water Quality Guidelines for the Protection of Aquatic Life

Table 4.6: Summary of water quality measurements for oil sands process-affected (OSPW) and control water used for laboratory exposure of wood frog (*Rana sylvatica*) tadpoles in 2007.

Water Source	Conductivity (µs/cm)	pH	Hardness (mgCaCO$_3$/L)	Alkalinity (mg/L)	Ammonia (mg/L)	Dissolved Oxygen (mg/L)
Test Pond 5 (OSPW)	1014 ± 4.5	7.82 ± 0.14	13.0 ± 1.1	90.0 ± 0.9	0.039 ± 0.001	10.0 ± 0.14
Test Pond 9 (OSPW)	1447 ± 4.0	8.72 ± 0.01	6.00 ± 0.1	306 ± 0.9	0.009 ± 0.001	10.1 ± 0.07
Mike's Pond (OSPW)	3577 ± 21.5	8.32 ± 0.02	14.0 ± 0.4	152 ± 1.5	0.186 ± 0.007	10.5 ± 0.15
Peat Pond (OSPW)	1320 ± 3.5	7.99 ± 0.25	31.0 ± 2.1	202 ± 4.0	0.101 ± 0.010	10.4 ± 0.28
Demo Pond (OSPW)	1882 ± 6.0	8.90 ± 0.04	10.0 ± 0.3	414 ± 2.5	0.004 ± 0.002	10.9 ± 0.30
Bill's Pond (OSPW)	560 ± 2.0	7.63 ± 0.02	13.0 ± 0.7	116 ± 3.0	0.032 ± 0.009	10.1 ± 0.25
Dechlorinated Tap Water (Control)	513 ± 2.0	7.40 ± 0.035	136 ± 2.0	88.3 ± 1.9	0.102 ± 0.021	6.60 ± 0.26

Table 4.7: Metal analysis for water sources from impacted (oil sands process-affected water) wetlands in the Athabasca oil sands, Alberta, and control water that were used for laboratory exposure of wood frog (*Rana sylvatica*) tadpoles in 2007.

Element (µg/L)	Canadian Water Quality Guidelines**	Bill's Lake (OSPW)	Peat Pond (OSPW)	Demo Pond (OSPW)	Mike's Pond (OSPW)	Test Pond 5 (OSPW)	Test Pond 9 (OSPW)	Dechlorinated Tap Water (Control)
Boron	1200	88.2	80.2	1082	1744	470	972	85.1
Aluminum	1010	6.12	7.94	45.2	29.9	15.5	136	8.02
Titanium	NA*	0.567	0.280	1.18	0.456	0.788	3.99	0.399
Vanadium	100	0.326	0.848	1.40	0.559	0.540	1.86	0.852
Chromium	8.90	0.143	0.198	0.130	0.158	0.202	0.262	0.146
Manganese	0.600	3.41	2.47	0.564	0.392	0.536	1.63	2.87
Iron	NA	126	10.0	10.7	11.9	12.5	76.8	6.71
Cobalt	0.900	0.108	0.108	0.114	0.027	0.022	0.099	0.113
Nickel	NA	1.60	0.767	1.72	1.27	0.722	1.83	0.563
Copper	2.00	2.55	0.497	0.635	0.714	0.772	1.02	1.38
Zinc	55.0	7.71	3.35	5.79	2.74	5.05	4.07	13.5
Arsenic	5.00	1.25	1.15	3.01	0.530	1.28	3.76	0.335
Strontium	NA	245	418	223	433	213	95.8	195
Molybdenum	73.0	0.763	0.292	1.64	32.8	1.14	2.84	0.990
Antimony	NA	0.682	0.643	0.685	0.692	0.718	0.669	0.700
Barium	NA	50.8	40.9	34.3	26.9	14.4	22.9	35.6
Thallium	170	0.002	0.002	0.002	0.004	0.003	0.003	0.003
Lead	35.0	0.593	0.549	0.227	0.239	0.219	0.738	1.45
Uranium	200	0.199	1.00	2.28	4.06	0.566	2.04	0.015

*NA = Not available   **Canadian Water Quality Guidelines for the Protection of Aquatic Life

4.4.2 Oil sands process-affected substrate exposure study (2007)

4.4.2.1 Survivability and time to metamorphosis

Survivability was determined for wood frog tadpoles exposed to five different oil sands substrates compared with clean silica control (Figure 4.8). Tadpoles were exposed for 30 days, from Gosner stage 27-30 to Gosner stage 37-39 (pre-metamorphic climax). Although mean survival was not significantly different among the treatments (P=0.794), it ranged from 65% in tadpoles exposed to consolidated tailings to 41.7% in tadpoles exposed to coke.

Wood frog tadpoles exposed to most OSPS and the silica control reached metamorphic climax roughly around the same time (within 30-33 days). Tadpoles exposed to coke sediment took somewhat longer to reach metamorphic climax (approximately 38 days). Tadpoles exposed to the coke treatment were also smaller in size than those in the other treatment groups.

4.4.2.2 Morphometric endpoints (weight, length and condition factor)

Table 4.8 summarizes the morphometric measurements (weight, total length and condition factor) obtained for wood frog tadpoles exposed to the OSPS and clean silica sand (control). Tadpoles exposed to saline sodic overburden had significantly lower mean body weight, whereas tadpoles maturing in consolidated tailings and coke treatments had significantly lower body weights and total length (P<0.05). Tadpoles exposed to peat mineral mixture, on the other hand, had the highest mean body weight and length measurements. The condition factor calculated for tadpoles exposed to saline sodic overburden was significantly lower than the tadpoles exposed to other substrates. Sample

sizes for the tadpoles collected for morphometric measurements were less than those that

survived due to difficulties in collecting the animals from their substrate treatments. The

tadpoles were often hidden within the substrate, which made them difficult to scoop up

and collect from the substrates.
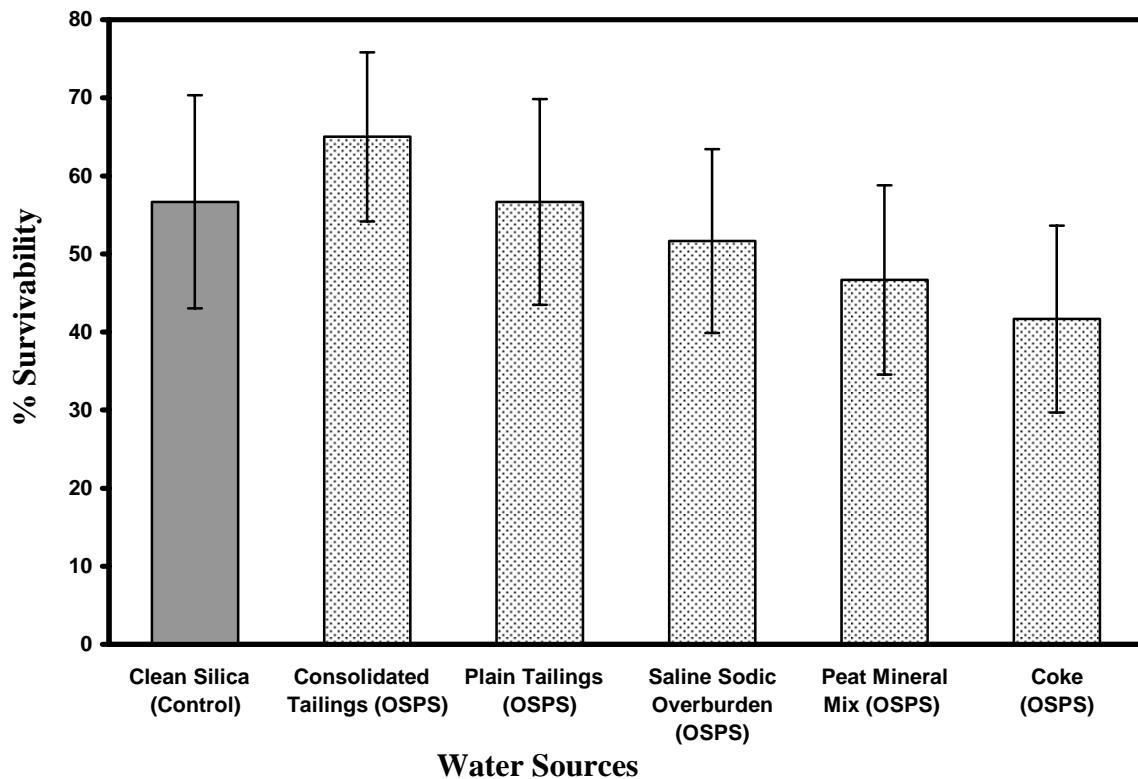
Figure 4.8: Percent survivability of wood frog (*Rana sylvatica*) tadpoles exposed to clean silica sand (control) and oil sands process-affected substrates (OSPS) from five different sources in the Athabasca oil sands, Alberta, in 2007. Data shown are mean ± standard error of the mean. n=60 tadpoles per substrate treatment. Data were analyzed using non-parametric Kruskal-Wallis ANOVA on ranks. No difference was observed among treatments (P=0.794).

Table 4.8: Morphometric (weight, total length, condition factor) measurements determined for wood frog (*Rana sylvatica*) tadpoles exposed to several oil sands process-affected substrates (OSPS) collected from the Athabasca oil sands in Alberta in summer 2007. Data shown are mean ± standard error of the mean. Values marked with different letters were significantly different from each other (P<0.05). Condition factor = (weight/total length^3)*100.

	Clean Silica Sand (control)	Consolidated Tailings (OSPS)	Plain Tailings (OSPS)	Saline Sodic Overburden (OSPS)	Peat Mineral Mixture (OSPS)	Coke (OSPS)
Sample Size	23	28	20	20	17	12
Weight (g)	1.43 ± 0.05[a]	1.24 ± 0.04[b]	1.47 ± 0.06[a]	1.23 ± 0.05[b]	1.49 ± 0.04[a]	0.86 ± 0.08[b]
Total Length (cm)	4.70 ± 0.07[a]	4.33 ± 0.08[b]	4.74 ± 0.08[a]	4.70 ± 0.09[a]	4.89 ± 0.07[a]	4.03 ± 0.12[b]
Condition Factor	1.39 ± 0.05[a]	1.57 ± 0.08[a]	1.39 ± 0.05[a]	1.23 ± 0.10[b]	1.30 ± 0.07[a,b]	1.29 ± 0.06[a,b]

4.4.2.3 Whole body tadpole triglyceride and thyroid hormone analyses for wood frog tadpoles exposed to oil sands process-affected substrates in 2007

Whole body T_3 concentrations were measured in wood frog tadpoles exposed to OSPS or silica control (Figure 4.9, graph A). Whole body T_3 concentrations in tadpoles exposed to saline sodic overburden were significantly lower than tadpoles exposed to plain tailings ($P<0.05$). However, there were no significant differences in mean T_3 concentrations of tadpoles exposed to OSPS compared to silica control, or among any other treatments. Whole body T_4 concentrations were also assessed in tadpoles exposed to OSPS or silica control (Figure 4.9, graph B). None of the treatments were significantly different ($P=0.062$), although tadpoles exposed to silica sand (control) had the highest mean T_4 concentration, with the lowest concentration observed in tadpoles exposed to coke (OSPS).

In addition to whole body T_3 and T_4 concentrations, whole body T_3/T_4 ratio was also determined in the wood frog tadpoles exposed to OSPS (Figure 4.10). There was no significant difference among tadpoles exposed to the various substrates ($P=0.072$). Highest T_3/T_4 ratio was observed in tadpoles exposed to plain tailings sand, while the lowest T_3/T_4 ratio was observed in saline sodic overburden tadpoles.

Whole body triglyceride concentrations measured in wood frog tadpoles exposed to OSPS were not different than results of the control treatment. However, significantly lower whole body triglyceride concentrations were observed in tadpoles exposed to saline sodic overburden and coke OSPS treatments, compared to plain tailings sand, another OSPS treatment ($P<0.05$).
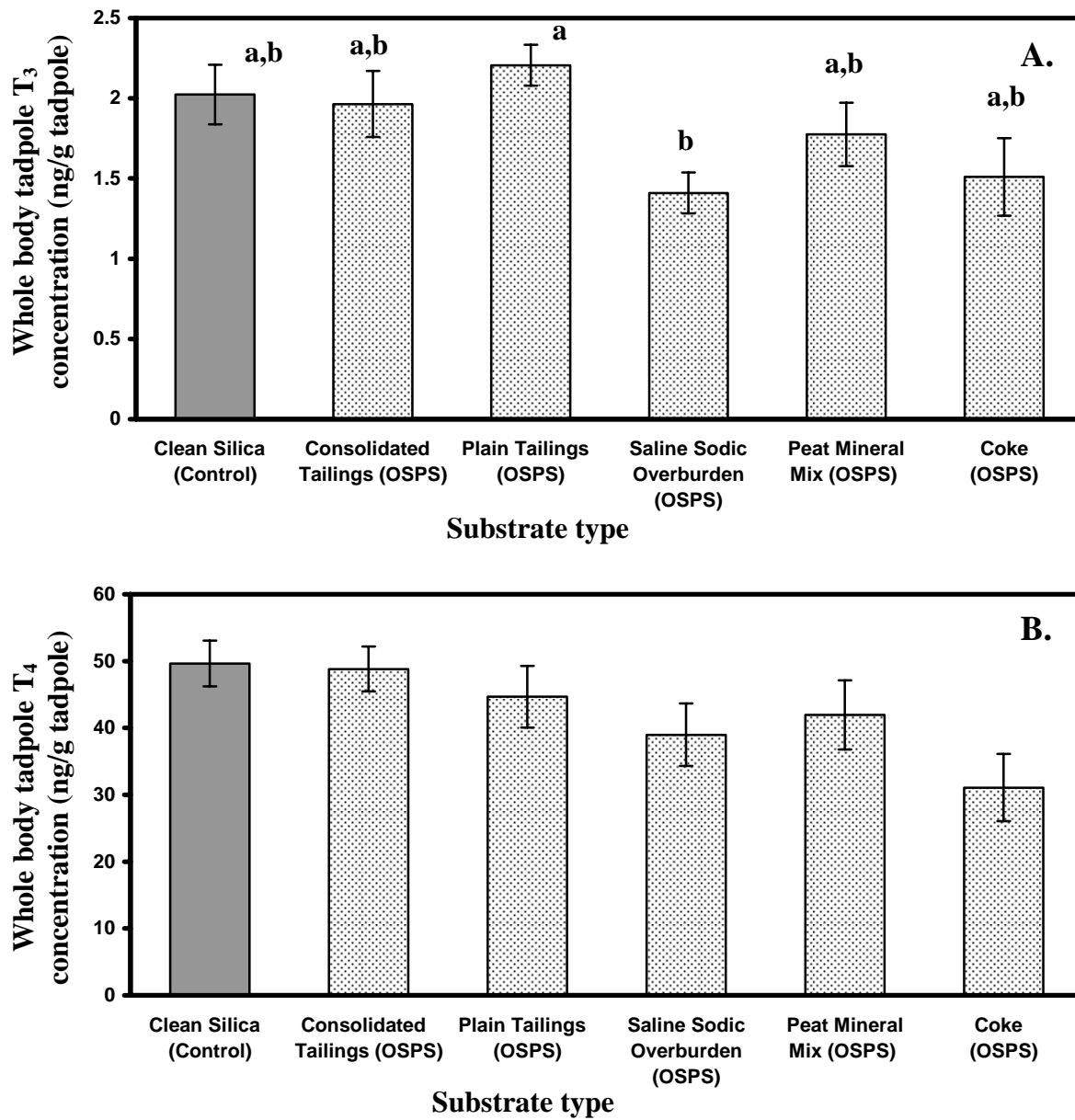
Figure 4.9: Whole body tadpole 3,5,3'-triiodothyronine (T_3) (A) and thyroxine (T_4) (B) concentrations (ng/g tadpole tissue) in wood frog (*Rana sylvatica*) tadpoles at pre-metamorphic climax (Gosner stages 37-39), exposed to clean silica sand (control) and oil sands process-affected substrates (OSPS) from five different sources in the Athabasca oil sands, Alberta, in 2007. Data shown are mean ± standard error of the mean. n=9-23 tadpoles per substrate treatment. Data were analyzed using parametric ANOVA followed by Tukey's post-hoc test. Values marked with different letters were significantly different from each other ($P<0.05$). No significant differences in whole body tadpole T_4 hormone concentrations were observed ($P=0.062$).

Figure 4.10: Whole body tadpole 3,5,3'-triiodothyronine (T_3):thyroxine (T_4) ratio in wood frog (*Rana sylvatica*) tadpoles at pre-metamorphic climax (Gosner stages 37-39), exposed to clean silica sand (control) and oil sands process-affected substrates (OSPS) from five different sources in the Athabasca oil sands, Alberta, in 2007. Data shown are mean ± standard error of the mean. n=9-23 tadpoles per substrate treatment. Data were analyzed using non-parametric Kruskal-Wallis ANOVA on ranks. No difference was observed among treatments (P=0.072).
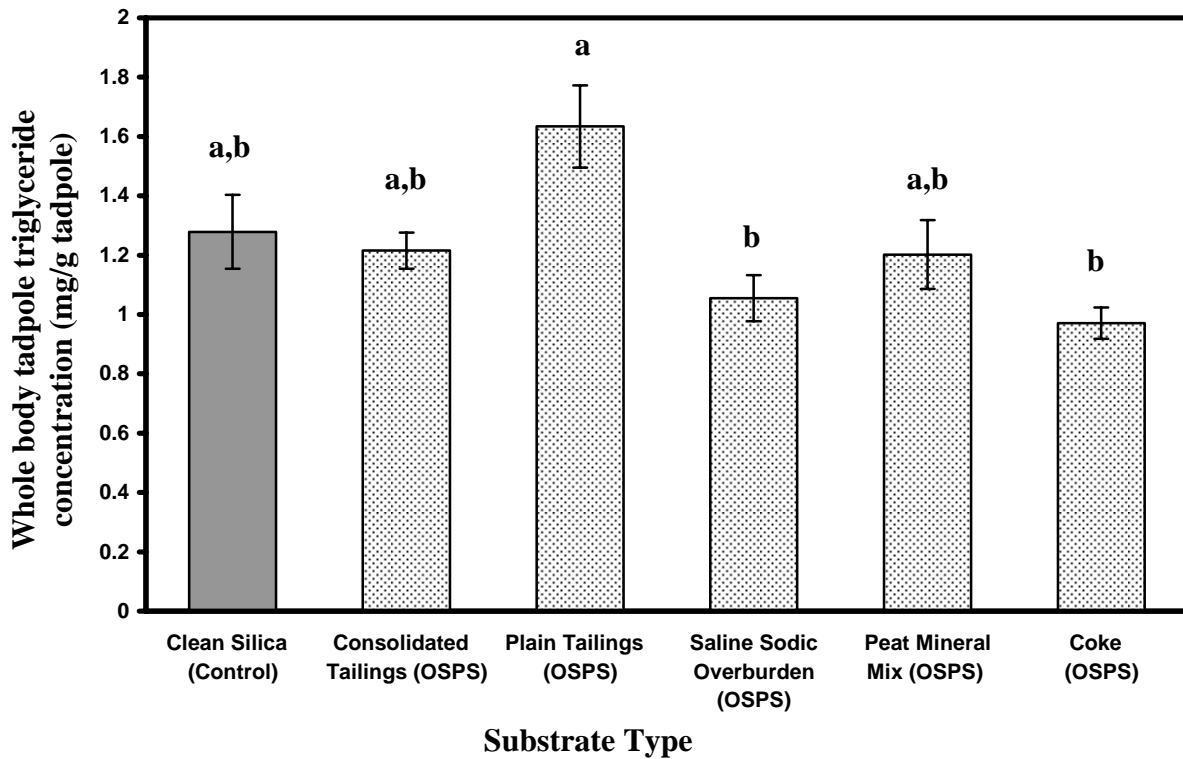
Figure 4.11: Whole body tadpole triglyceride concentrations (mg/g tadpole tissue) in wood frog (*Rana sylvatica*) tadpoles at pre-metamorphic climax (Gosner stages 37-39), exposed to clean silica sand (control) and oil sands process-affected substrates (OSPS) from five different sources in the Athabasca oil sands, Alberta, in 2007. Data shown are mean ± standard error of the mean. n=12-27 tadpoles per substrate treatment. Data were analyzed using non-parametric Kruskal-Wallis ANOVA on ranks followed by Dunn's post-hoc test. Values marked with different letters were significantly different from each other ($P<0.05$).

4.4.2.4 Water and substrate chemical analyses (2007)

Chemical analyses of water overlaid on the OSPS substrates and the substrates themselves are illustrated in Tables 4.9 and 4.10, respectively. Water overlaid on the coke substrate contained high concentrations of vanadium and copper. Samples of coke OSPS were high in vanadium, nickel, molybdenum and mercury, compared to the other substrates. Water overlaid on consolidated tailing had high concentrations of tin and barium. Substrate chemical analysis showed high concentration of manganese in the consolidated tailing. Metal analysis of water overlaid on saline sodic substrate showed elevated concentrations of boron, nickel, zinc, strontium, molybdenum, cadmium, antimony, thallium and uranium. Substrate chemical analysis of saline sodic overburden revealed the highest concentrations of most of the metals among all the OSPS treatments, including aluminum, iron, copper, zinc, strontium, cadmium, tin, antimony and lead. Metals concentrations in clean silica sand (control) were mostly below the detection limit.

Table 4.9: Metal analysis of water overlaid on five different oil sands process-affected substrates (OSPS) from the Athabasca oil sands, Alberta, and clean silica sand (control) used for laboratory exposure of wood frog (*Rana sylvatica*) tadpoles in 2007.

Element (µg/L)	Canadian Water Quality Guidelines**	Coke (OSPS)	Consolidated Tailings (OSPS)	Peat Mineral Mix (OSPS)	Plain Tailings Sand (OSPS)	Saline Sodic Overburden (OSPS)	Clean Silica Sand (Control)
Boron	1200	69.3	415	80.2	109	1077	82.8
Aluminum	1010	56.2	49.1	129	29.5	10.4	7.26
Titanium	NA*	1.08	2.32	4.85	1.05	0.309	0.392
Vanadium	100	25.7	0.713	0.516	0.611	1.21	1.06
Chromium	8.90	0.236	0.162	0.313	0.233	0.111	0.177
Manganese	0.600	18.0	27.9	385	2.95	1.94	1.86
Iron	NA	29.8	32.4	128	41.5	10.6	8.35
Cobalt	0.900	0.185	0.195	0.367	0.077	0.288	0.055
Nickel	NA	4.13	1.42	1.01	1.14	6.73	1.09
Copper	2.00	2.31	0.677	0.498	0.675	0.993	2.19
Zinc	55.0	2.43	2.42	2.85	3.48	9.71	6.66
Arsenic	5.00	0.342	0.148	1.25	0.234	0.683	0.427
Strontium	NA	202	316	191	219	2461	215
Molybdenum	73.0	1.70	1.51	1.96	1.40	3.09	1.89
Cadmium	0.017	<LoD*	<LoD	0.033	0.038	0.058	0.044
Tin	NA	0.110	0.328	0.101	0.160	0.224	0.217
Antimony	NA	0.751	0.699	0.676	0.755	1.17	0.770
Barium	NA	16.9	50.0	44.6	38.1	20.6	37.7
Thallium	170	0.002	0.006	0.002	0.009	0.028	0.008
Lead	35.0	0.165	0.430	0.248	0.248	0.232	0.432
Uranium	200	0.069	0.636	2.52	0.205	3.55	0.231

<LOD – Below the limit of detection   **Canadian Water Quality Guidelines for the Protection of Aquatic Life
*NA – Not available

Table 4.10: Chemical analysis of five different oil sands process-affected substrates (OSPS) from the Athabasca oil sands, Alberta, and clean silica sand (control) used for laboratory exposure of wood frog (*Rana sylvatica*) tadpoles in 2007.

Element (mg/kg)	Coke (OSPS)	Consolidated Tailings (OSPS)	Peat Mineral Mix (OSPS)	Plain Tailings Sand (OSPS)	Saline Sodic Overburden (OSPS)	Clean Silica Sand (OSPS)
Aluminum	4292	37736	32510	6342	51756	155
Titanium	999	3435	2554	588	3477	34.7
Vanadium	1225	59.6	64.7	6.13	141	0.38
Chromium	13.7	49.7	40.5	3.12	70.8	< LoD*
Manganese	84.4	269	195	18.8	182	3.02
Iron	2769	9179	14697	1128	25620	89.9
Cobalt	8.56	11.3	6.17	1.23	13.8	0.06
Nickel	516	20.4	15.2	1.93	33.0	< LoD
Copper	14.1	6.13	6.93	< LoD	27.3	< LoD
Zinc	17.5	28.8	17.6	1.84	85.5	7.33
Strontium	56.8	67.8	120	26.7	189	3.06
Molybdenum	79.3	0.83	< LoD	< LoD	1.20	< LoD
Silver	0.068	0.049	0.043	0.022	0.148	< LoD
Cadmium	< LoD	0.140	0.110	0.030	0.160	0.03
Tin	0.74	0.920	0.450	< LoD	0.940	< LoD
Antimony	0.269	0.401	0.425	0.047	1.03	< LoD
Barium	45.1	295	288	184	499	9.12
Thallium	0.054	0.338	0.321	0.089	0.503	0.003
Lead	7.79	14.6	10.7	3.58	15.6	0.430
Uranium	0.920	1.74	3.51	0.320	2.72	0.290

* <LOD – Below the limit of detection

4.5   Discussion

Early life stages of amphibians are generally more vulnerable to environmental stressors than adults (Ortiz-Santaliestra *et al*., 2006). Adverse effects of stressors such as environmental contaminants include lower survival and growth rates, as well as alterations in physiological endpoints that may impact fitness, such as endocrine disruption and reduced energy stores. Consequently, the ability of oil sands process-affected waters and substrates to support amphibian populations was tested by exposing wood frog eggs and tadpoles to a variety of OSPW and OSPS. Potential effects of oil sands contaminants on hatchability, tadpole survival, growth and development, and biomarkers associated with metamorphosis and body condition were evaluated.

4.5.1   The effect of oil sands process-affected waters (OSPW) and substrates (OSPS) on the hatchability, survival and growth of wood frogs in 2006 and 2007

Hatchability of wood frog eggs was not affected by exposure to most of the OSPW treatments, with the exception of Jan's Pond. Hatchability in this treatment group was reduced to less than half of the average of the reference treatments. The source of this adverse effect is uncertain, but Jan's Pond exhibited the highest concentrations of many potentially toxic analytes of all water treatments. The list includes the metals boron, vanadium, chromium, cobalt, nickel, strontium, lead and uranium, as well as conductivity and hardness. Wood frog eggs are deposited in water. The embryos are surrounded by a vitelline membrane and a jelly capsule composed of mucopolysaccharides and mucoproteins (Freda, 1991). The absence of a hard shell makes the eggs potentially vulnerable to even the slightest changes in their environment, and may also facilitate the

uptake of contaminants present in the surrounding water and sediment (Greulich and Pflugmacher, 2004; Bridges, 2000).

Survivability of wood frog tadpoles was drastically reduced by exposure to OSPW in both years (2006 and 2007). Four OSPW sources tested in 2006 and five OSPW sources evaluated in 2007 had less than 10% tadpole survivability compared to >50% and 76% in control treatments for 2006 and 2007, respectively. Previous work by Pollet and Bendell-Young (2000) also showed reduced survivability of wood frog tadpoles in OSPW. In both studies, tadpole mortality was not only greater but much more rapid in OSPW treatments compared to reference treatments. These results strongly suggest that OSPW is toxic to young wood frog tadpoles. Between the 2006 and 2007 studies, a total of 10 different OSPW sources were evaluated for toxicity. The reference water (Loon Lake) showed drastic die-offs during the OSPW study in 2006. During the field study in 2005 (Chapter 3; Table 3.3), there were no tadpoles and/or newly-metamorphosed froglets collected from Loon Lake. Pollet and Bendell-Young (2000) observed a similar trend in their site-specific reference waters. Their study showed high tadpole mortality and reduced weight and length when exposed to unimpacted sources. These OSPW-impacted and the reference sites represent a range of different effluent compositions and chemistries such that it is difficult to identify a specific toxic component.

Survivability of wood frog tadpoles was not affected by exposure to OSPS. The tadpoles in all the substrate treatments were weighed and their total length determined at Gosner stage 37-39. Tadpole size and growth rate are important determinants in the timing and success of metamorphosis, which in turn is linked to fitness (Morey and

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Reznick, 2001). Larger juveniles and those metamorphosing early are known to have a higher survival rate and earlier age at first reproduction (Gomez-Mestre and Buchholz, 2007). Tadpoles exposed to OSPS had lower mean body weights, length and condition factors compared with the control substrate. Snodgrass *et al*. (2004) compared the effects of exposure to coal combustion wastes on *Rana clamitans* and *Rana sylvatica* larvae. Although, both species experienced decreased growth and developmental rates, *Rana clamitans* larvae were seen to have reduced survival and metamorphic success when compared with *Rana sylvatica*. These interspecies differences could very well be attributed to the longer larval period for *Rana clamitans* (~70 days), compared to *Rana sylvatica* (~40-90 days). Other studies with *Rana sylvatica* (Savage *et al*., 2002) have shown that direct contact with sediments contaminated with polychlorinated biphenyls may also result in reduced growth or body condition of tadpoles.

4.5.2 The effect of oil sands process-affected substrates (OSPS) on whole body tadpole T_3, T_4 and triglyceride concentrations in 2007

Whole body concentrations of T_4 and the T_3/T_4 ratio were not significantly different among tadpoles exposed to any OSPS sources. Tadpoles exposed to saline sodic overburden (OSPS) exhibited lower whole body T_3 concentrations compared with plain tailings sand (OSPS), but results were not significantly different from the control substrate. Tadpoles exposed to saline sodic overburden and coke treatments (OSPS) showed lower whole body triglyceride concentrations than tadpoles exposed to plain tailings sand (OSPS). These findings are consistent with the relatively lower body weight of the tadpoles in the former treatment groups. However, triglyceride concentrations in tadpoles exposed to the control substrate were not significantly different from tadpoles

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exposed to any of the OSPS treatments. These results indicate that exposure of tadpoles to OSPS did not follow the expected trend for thyroid hormone function or energy storage when compared with the control substrate.

These differences in thyroid hormones and triglyceride content could have resulted from varying salt, metal, hydrocarbon and naphthenic acid concentrations and/or the substrate type itself. Previous work by Sanzo and Hecnar (2006) showed that larval wood frogs exposed to salt concentrations between the range of 2636 and 5109 mg/L NaCl, exhibited reduced activity and feeding. The ecological implications of these effects can translate to higher susceptibility to predation, since with reduced feeding tadpoles require more time to metamorphose or metamorphose at a smaller size. Extended time to metamorphosis can reduce survival in wood frogs.

There were no correlations between whole body triglyceride stores and T_3 and/or T_4 concentrations in tadpoles exposed to the various treatments, unlike results observed in Chapter 2 of this thesis.

4.5.3   The impact of water and sediment chemistry on wood frog development
4.5.3.1 Oil sands process-affected water exposure study

Water quality and chemical contaminants can adversely effect the development, growth and subsequent survival of amphibian eggs and tadpoles. Sensitivity to specific contaminants and water quality variables varies with the stage of development. For instance, even though metals can adversely affect amphibian embryos, sensitivity to most metal exposure is far greater in the larval stage (Gross *et al.*, 2007; Horne and Dunson, 1995). In contrast, sensitivity to environmental pH is more pronounced in embryos than

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in larvae (Freda, 1991). Other water quality factors are also known to reduce hatching success. For instance, low pH resulted in thoracic swelling and increased larval mortality in hatchlings of Jefferson and spotted salamanders (Pough and Wilson, 1977). In addition, low pH, increased cation levels or high dissolved metal concentrations may cause curling of the embryo within the vitelline membrane (Freda and Dunson, 1985; Clark and LaZerte, 1985), which can result in embryo mortality. In contrast, increased water hardness can ameliorate the adverse effects of both low pH and toxic metals (Freda *et al.*, 1991). Gill surface permeability of larvae is reduced by increased water hardness, which acts to protect against whole body sodium loss, a principal mechanism of low pH and metal-induced toxicity (McDonald *et al.*, 1991; McDonald and Wood 1993).

Generally speaking, analysis of water used in the 2006 study showed high water hardness, alkalinity and dissolved oxygen content for the OSPW sources, whereas the reference source (Loon Lake) had the highest ammonia level. The OSPW samples also tended to have elevated metal concentrations, including boron, strontium, uranium, lead, nickel, cobalt, chromium, molybdenum, manganese, zinc and vanadium, but the range observed for most these analytes was large. Analyses of water sources used in the 2007 study showed high pH, alkalinity, ammonia and dissolved oxygen content in OSPW from oil sands wetlands compared to the control. High levels of metals including boron, strontium and molybdenum were also found in 2007 OSPW samples.

4.5.3.2 Oil sands process-affected substrate exposure study

Some sediment-associated toxicants, including metals, can be absorbed through an amphibian's water permeable skin and enter circulation for distribution to target

organs or sites of accumulation (Stolyar *et al*., 2008). Oral ingestion is another route of exposure to sediment contaminants. Physical examination of the wood frog tadpoles revealed the presence of sediments in the gut.

High concentrations of vanadium and copper were found in the water overlaid on the coke substrate, whereas the coke itself contained elevated levels of vanadium, nickel, molybdenum and mercury. Coke is known to contain several classes of contaminants including numerous metals and polycyclic aromatic hydrocarbons (PAHs). In previous studies, coke obtained from Syncrude Canada Ltd. demonstrated low levels of leaching on exposure to different acids and a range of pH. Nickel and vanadium were the only metals removed, whereas molybdenum was concentrated in water associated with coke storage (Komex International Ltd., 1998).

Overlay water analyses showed high concentrations of tin and barium in the consolidated tailings treatment while the substrate itself contained high manganese concentrations. In order to de-water the fine tails for settling, gypsum is added to produce consolidated tailings. The addition of gypsum results in a major accumulation of ions (SO_4^{2-} from the gypsum itself, and Na^+ and Ca^{2+} from the sediments). In addition to these ions, leaching from the oil sand ore itself during extraction releases Na^+ and Cl^-, which can result in high ionic content in CT waters (Renault *et al*., 1998).

Water overlaid on saline sodic substrate had high concentrations of boron, nickel, zinc, strontium, molybdenum, cadmium, antimony, thallium and uranium. Chemical analysis of the substrate revealed that saline sodic overburden had the highest concentrations of most of the metals among all the treatments. Saline sodic materials contain high salt levels and the overburden usually consists of organic and glacial

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material. Reclamation with saline sodic overburden may result in salt release, and potentially salt migration and salinization of groundwater, reclamation soils, and surface water (Oddie and Bailey, 1988).

4.5.3.3 Effect of metals on amphibians

Metals contamination can have severe impacts on aquatic ecosystems. Metals are found in water, suspended sediments and bottom sediments and they are not easily eliminated from aquatic environments (Forstner and Wittman, 1981). The magnitude of metal contamination is a good indicator of water quality, as well as the extent of potential anthropogenic contamination (Singh *et al*., 1997).

Teratogenicity of various metals including cobalt, nickel, cadmium, copper and zinc in frogs has been assessed in previous studies (Luo *et al*., 1993a,b; Plowman *et al*., 1991, 1994). Malformities such as intestinal and cardiac deformities have been observed in frog embryos exposed to cobalt (Plowman *et al*., 1991). Zinc and copper also produce malformations of the eye, gut, facial structure, notochord and cardiac anomalies (Luo *et al*., 1993a), while nickel exposure caused ocular, skeletal and intestinal deformities in frog embryos (Hopfer *et al*., 1991). Elevated boron concentrations adversely affect the development of amphibian embryos. Embryos of two species of salamanders (Jefferson salamander, *Ambystoma jeffersonianum* and spotted salamander, *Ambystoma maculatum*), wood frogs (*Rana sylvatica*) and American toads (*Bufo americanus*) were exposed to waste water effluent containing boron concentrations of 50 and 100 mg/L. Results indicated significant increases in the frequency of deformed larvae and reduced hatching rate (Laposata and Dunson, 1998).

Amphibian metamorphosis is regulated by and dependent on the action of thyroid hormones, T_3 and T_4. Consequently, disruption of thyroid function by environmental contaminants has significant impacts on amphibian populations. Metals such as cadmium have been reported to cause structural and functional damage to thyroid follicular cells in female rats (Pilat-Marcinkiewicz *et al*., 2003) and fish (Ricard *et al*., 1998). Cadmium may hinder thyroid function by inhibiting the conversion of T_4 to T_3 (Chaurasia *et al*., 1996; Gupta *et al*., 1997; Gupta and Kar, 1999; Paier *et al*., 1993). Accumulated cadmium in the mitochondria of thyroid follicular cells is thought to cause inhibition of the synthesis and release of thyroid hormones (Yoshizuka *et al*., 1991). A decrease in whole body T_3 levels was reported in tadpoles of *Xenopus laevis* following cadmium exposure (Fort *et al*., 2000). Other metals and salts such as magnesium, copper and calcium in their divalent forms can also chelate to thyroid hormones and alter their function (Norris and Carr, 2005; Hoch, 1962; Wahlborg and Frieden, 1965).

Fats and their component triglycerides are primary forms of energy storage in frogs. Several studies demonstrate the effect of metals in reducing lipid stores in amphibians (Vogiatzis and Loumbourdis, 1999, 2001; Rowe *et al*., 2009). A study conducted by Rowe *et al*. (2009) of dietary exposure to vanadium in southern leopard frogs (*Rana sphenocephala*) revealed lower growth rates, survival and reduced ability to store lipids in tadpoles. Exposure to cadmium resulted in depleted hepatic fat and general lipid stores in the marsh frog (*Rana ridibunda*), an indication of energy reserve exhaustion.

4.5.4   Future work

The exposure of wood frog eggs and larvae to a variety of OSPW in both 2006 and 2007 produced similar results for tadpole survival and growth. The rapid mortality of young tadpoles and very low survival in water from all sites indicates that OSPW may not be suitable for sustaining viable amphibian populations. However, these results are not necessarily consistent with field observations as reported in Chapter 3 and in a mesocosm study conducted in oil sands wetlands concurrent with this lab-based experiment (Hersikorn, 2009). The present OSPW-only exposure study did not take into account potential buffering effects of the sediments/substrates that are also present in the oil sand process-affected wetlands.

The sediment-only (OSPS) exposure study reported here was conducted with tadpoles that had reached more advanced developmental age (Gosner stages 27-30). Consequently, those animals may have been relatively more tolerant of OSPS contaminants and water quality parameters such as low pH than newly hatched tadpoles. In addition, the OSPS study did not represent a "real" scenario as encountered in field situations, since substrates and water from the same sources were not tested simultaneously. However, this study was a useful first attempt at interpreting the role of substrates separately from the natural overlaying water component in a wetland system.

A useful extension of this work would involve exposing wood frog eggs to various OSPS treatments and monitoring the hatchability and survival of newly-hatched larvae. In addition, it would be ideal to test the effects of OSPW and OSPS together on wood frog morphological and biochemical endpoints. Additional work is needed to

identify toxic constituents and evaluate the potential role of wetland sediments in buffering the effect of OSPW water on wood frog eggs and tadpoles.

CHAPTER 5
5.0 General Discussion

5.1 Project rationale and summary

The oil sands industries in the Athabasca region of northern Alberta face the unique challenge of incorporating both reclaimed aquatic and terrestrial habitats into self-sustaining ecosystems. To meet this challenge, a wetland-based approach is being strongly considered to reclaim oil sands process-affected materials (OSPM) and provide a suitable environment for species indigenous to the area. The large volumes of OSPM currently held within large tailings ponds are in keeping with the industry's zero-discharge policy. Seeping from these ponds has caused pooling in areas where obligate wetland species have become established (Crowe *et al*., 2001). The objective of this study was to evaluate the effects of OSPM on the survival of wood frog (*Rana sylvatica*) eggs and larvae, as well as on several morphological and biochemical endpoints related to tadpole growth and development.

Chapter 2 of this thesis outlined the development of two novel biomarkers for wood frog tadpoles, and the application of those biomarkers in an experiment to establish changes in baseline values over the course of metamorphosis. Changes in whole body thyroid hormones (3,5,3'-triiodothyronine [T_3] and thyroxine [T_4]) and triglyceride (TG) concentrations were measured at regular intervals during tadpole development from embryonic Gosner stage 19 through to completion of metamorphosis at Gosner stage 46. Peak levels of T_4 observed during the prometamorphosis (Gosner stage 31-40) did not follow the expected trend. In contrast, as predicted, T_3 and triglyceride concentrations were highest at metamorphic climax (Gosner stage 40-46) and prometamorphosis,

respectively. Alterations in whole body thyroid hormone and triglyceride concentrations during wood frog development may be useful indicators of the condition of the thyroid gland and the overall health of the developing tadpole. Furthermore, these results illustrate the importance and role of each endpoint at different stages of wood frog metamorphosis. These newly developed biomarkers were subsequently used to measure whole body T_3, T_4 and triglyceride concentrations in tadpoles exposed to OSPM in the field and the laboratory, to determine potential contaminant effect on these biomarkers.

Chapter 3 outlined a field study conducted in summer 2005 to assess the presence of wood frogs in the Athabasca oil sands region. Several OSPW-impacted and unimpacted sites were surveyed and wood frog specimens were collected on Syncrude and Suncor lease lands. Morphometric and biochemical endpoints were assessed in this study to evaluate the overall body condition of wood frogs susceptible to potential adverse effects of exposure to the OSPW wetlands. Although no discernible differences were seen between impacted and unimpacted wetlands regarding wood frog growth and body condition, the presence of this species was established in these wetlands via trapping and visual surveys.

Chapter 4 described the exposure of wood frog eggs and tadpoles to OSPW and substrates under controlled laboratory conditions. Endpoints reflecting larval growth and development were assessed to ascertain the individual contributions of OSPW and OSPS to potential toxicity. Although, hatchability was not severely affected by OSPW exposure, survivability of newly-hatched tadpoles was adversely affected in most of the OSPW treatments when compared with the control water. Older tadpoles (post-feeding

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stage) exposed to OSPS had overall lower mean body weight, length and condition factor when compared to clean sand control.

5.2   Endpoints evaluated

Thyroid hormones (T_3 and T_4) are essential physiological triggers of metamorphosis in larval amphibians (Fort *et al*., 2007; Shi, 2000). Environmental chemical contaminants can alter thyroid hormone function and adversely affect metamorphosis (Degitz *et al.*, 2005; Opitz *et al.*, 2005). During the field study (Chapter 3), whole body tadpole T_3 concentrations in tadpoles from one reference wetland was significantly higher than OSPW sites. This observation may reflect a contaminant effect, but the effect of variation in tadpole age among wetlands may confound this result. Results of the experiment reported in Chapter 2 demonstrated that T_3 concentrations can vary depending on the stage of tadpole development. Thus the minor differences in developmental stages of field-collected tadpoles may have obscured any contaminant effect on T_3 status. During laboratory exposures (Chapter 4), no consistent differences in T_3 were found between tadpoles exposed to OSPS and the control substrate. In both the field and laboratory-based studies, T_4 and T_3/T_4 concentrations in tadpoles exposed to process-affected materials were not different from the control substrate. These results suggest that exposure to the substrate component (at least) did not alter thyroid hormone status in developing tadpoles.

Hepatic glycogen stores are essential to enable freeze tolerance and consequent winter survival of wood frogs (Storey and Storey, 2004). Studies with wood frogs have shown that animals with larger hepatic glycogen stores have the capacity to synthesize

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greater amounts of cryoprotectant than frogs with smaller hepatic glycogen reserves (Costanzo and Lee, 1993; Costanzo *et al*., 1993). During the 2005 field study, hepatic glycogen concentrations were not different among newly-metamorphosed wood frogs from reference and OSPW wetlands (Chapter 3), although the highest glycogen concentration was observed in frogs from an OSPW site. The field study was a very preliminary effort, so results have to be interpreted with caution. At this point, it appears that wood frogs that are able to reach metamorphosis in most OSPW wetlands do not have lower energy stores than frogs from unimpacted wetlands.

Total body triglyceride concentration is another potentially useful biomarker to evaluate the health of developing tadpoles. Amphibians may experience periods of energy deprivation due to resource-poor habitats or during seasonal periods of limited food availability. In such cases, energy stores become vital for survival (Rowe *et al*., 2003). Amphibian lipid levels at metamorphosis correlate to post-metamorphic terrestrial survival (Scott *et al*., 2007). During the 2005 field study (Chapter 3) triglyceride levels generally were found to be higher in tadpoles from reference wetlands than in OSPW-impacted sites. However, during the 2007 OSPS-exposure study (Chapter 4) there were no differences between the tadpoles exposed to OSPS and control treatments. Thus, the lower lipid content of metamorphs in the field study may reflect the habitat quality of OSPW sites as well as contaminant stress.

Morphometric indices such as body weight and length, and estimates of body condition derived from these measures, can be correlated with fitness. Advantages of metamorphosing at a larger size include higher survival, earlier maturity, larger size at first reproduction and greater clutch size in females (Scott *et al*., 2007). In the field study

(Chapter 3), observed differences in mean body weight and length of tadpoles among OSPW and reference wetlands were not consistently related to contaminant exposure. Differences in habitat quality and food availability, as well as variability in stage of development at collection (Gosner stage 37-43) may have confounded potential contaminant effects. Tadpole growth is known to be limited by temperature, food availability, toxicant exposure and other forms of stress (Shi, 2000). In the laboratory study (Chapter 4), lower mean body weights, length and condition factors were observed in tadpoles from OSPS treatments when compared with the control substrate. Tadpoles from one of the OSPS treatments with lower body weight and length also exhibited delayed metamorphosis compared with other substrates. Previous studies have shown that time to metamorphosis increases in the presence of some xenobiotics, and this delay may be accompanied by a reduction in tadpole weight and size (Venturino *et al*., 2003).

Hatchability of wood frog eggs was reduced with exposure to one OSPW source compared with other water treatments (Chapter 4). Although a specific cause could not be identified, chemical analysis indicated that this OSPW had highest concentration of several metals, as well as total conductivity and hardness of any source tested. Previous work has indicated that several of these contaminants, including boron (Laposata and Dunson, 1998), cobalt (Plowman *et al*., 1991) and nickel (Hopfer *et al*., 1990) cause increased deformities and mortality in amphibian embryos. Wood frog tadpole survival was affected by exposure to all OSPW in both 2006 and 2007 such that tadpoles were unable to complete metamorphosis. Survival of tadpoles during the 2006 and 2007 OSPW exposure studies was not high enough to assess thyroid hormone and triglyceride concentrations. Study by Pollet and Bendell-Young (2000) supports these findings as

amphibians exposed to OSPW experience higher mortality rates and reduced growth. The substrate exposure study involved exposure of pre-feeding tadpoles (Gosner stages 27-30) to OSPS and control substrate overlaid with clean water. Unlike the OSPW exposure, OSPS exposure alone did not reduce survivability compared to control substrate. The experiments are difficult to compare, because tadpoles were introduced to OSPW earlier in development (as eggs in 2006 and newly hatched larvae in 2007) than with the OSPS exposure study, and earlier life stages may be more susceptible to acute toxic effects. Comparisons of metals concentration of OSPS overlay water with OSPW concentrations reveal potential differences in metals exposure. The contribution of the substrates and of any organic contaminant or salts, are unknown.

Limited water and substrate chemistry analyses were carried out for OSPW and substrates used for wood frog exposures. Water chemistry provided in support of the 2005 field study did not include metal analysis or organic contaminants, and provided only limited insight into contaminant-specific interpretation of observations on the presence and condition of tadpoles and newly-metamorphosed wood frogs. Elevated metal and salt concentrations were observed in OSPW and substrates used in laboratory exposures in 2006 and 2007. Metal contamination of aquatic environments can lead to lower survival and growth rate of amphibian larvae, as well as adverse effects on thyroid hormone status and energy stores with attendant impacts on fitness (Chen *et al*., 2007; Pilat-Marcinkiewicz *et al*., 2003; Rowe *et al*., 2009). Alterations in salinity, hardness and pH can be equally detrimental to tadpole development (Gomez-Mestre and Tejedo, 2004; Freda *et al*., 1991). However, since OSPW and OSPS represent complex mixtures of numerous potentially toxic compounds, characterizing the toxic effects of one or a few

individual chemicals alone is insufficient to assess the suitability of reclaimed wetlands as a habitat (Pollet and Bendell-Young, 2000). Studies of the specific mixtures themselves with ecologically relevant model species are essential. The study reported here represents a preliminary effort to apply this approach.

5.3   Conclusion

The production of oil from oil sands can be divided into three main activities, namely mining, extraction, and upgrading, each process having the potential to affect the environment in different ways. This study demonstrates that in a controlled laboratory setting oil sands process-affected materials have an adverse effect on wood frog survival, growth and development. Hersikorn (2009) conducted a semi-field study involving *in situ* exposure of wood frog tadpoles in mesocosms located in selected reclaimed wetlands, which was intended to complement the present laboratory-based exposure scenario. The results of the *in-situ* exposure study showed that OSPM-affected wetlands that were less than 7 years old (considered to be "young") would not support amphibian life, due to acute toxicity to tadpoles. In contrast, wetlands older than eight years (referred to as "old") containing OSPM show amphibian survival similar to unimpacted wetlands. Furthermore, tadpoles raised in young OSPM wetlands demonstrated high mortality, delayed metamorphosis and lower T_3/T_4 ratio when compared with reference or old OSPM-impacted sites. These mesocosm results are similar to the OSPW exposure study performed in the laboratory in that high mortality was encountered for tadpoles exposed to some OSPW sources. The experimental design for the laboratory exposures did not intentionally focus on the specific age of a wetland, although the sources tested represent

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at least a limited range. Previous work with OSPM-impacted wetlands has shown that natural ageing of these sites can reduce toxicity of OSPW as measured by the Microtox® bioassay (Quagraine *et al*., 2005). Degradation of polycyclic aromatic hydrocarbons and naphthenic acids has been shown to occur due to microbial communities in these wetlands, which can reduce toxicity over time (Madille *et al*., 2001; Lai *et al*., 1996).

Both field and laboratory studies provide unique information and have advantages and disadvantages. The mesocosm approach represents the "real-life" situation experienced by animals on site and takes into account interaction between various physical, chemical and environmental stressors. It allows researchers to control certain variables in the field, such as predation (to some degree), and at the same time allows for exposure to natural conditions such as variations in temperature, photoperiod and water quality (Harris *et al.*, 2001). Furthermore, the combination of food resources available to a tadpole in the field (periphyton and debris in the sediment, etc.) may provide a higher nutritional plane or enhance the immune response when contaminants are present.

The laboratory-based study, on the other hand enables control of environmental variables such as meteorological conditions, risk of predation and exposure to infectious agents that may affect survival, growth, and other physiological endpoints, and thus confound results of mesocosm studies. In addition, the laboratory approach allows separate evaluation of potential toxic effects of OSPW and various substrates to amphibian eggs and tadpoles. Separating the impacts of sediment from overlaying water is more difficult with *in situ* exposures.

The combination of both of these amphibian studies has left many unanswered questions. Future work should include laboratory exposures with both OSPW and OSPS

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to assess their impact on amphibians concurrently. Consideration may be given to obtaining eggs from local (but non-OSPM) wetlands for laboratory exposure, since local populations may exhibit increased tolerance. In addition, more extensive water chemistry analyses including naphthenic acids and other organics and hydrocarbons should be conducted on all OSPW and OSPS sources, to attempt to identify the most significant contaminants to each stage of development.

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APPENDIX 1

Summary of call ratings and their description from the Wisconsin frog and toad survey (Droege, USGS Patuxent Wildlife Research Center).

Call Rating	Call Description
0	No frogs or toads were heard
1	Individual calls were heard but were not overlapping
2	Overlapping calls but individuals were still distinguishable
3	Numerous frogs can be heard, chorus is constant and overlapping