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Compromised metamorphosis and thyroid hormone changes in wood frogs (*Lithobates sylvaticus*) raised on reclaimed wetlands on the Athabasca oil sands

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This work provides guidance for reclamation of oil sands tailings and shows the usefulness of frogs and caging studies in environmental toxicology.

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ABSTRACT

The wet landscape approach to oil sands tailings reclamation in the Athabasca Oil Sands region involves creating wetlands from fluid tailings in mined-out pits. We measured time to metamorphosis, thyroid hormone status, and detoxification enzyme (EROD) induction in Wood frog (*Lithobates sylvaticus*) tadpoles raised on reclaimed oil sands wetlands of different ages [young (≤7 yr) vs. old (>7 yr)] and compared data with tadpoles raised on reference (control) wetlands. Metamorphosis was delayed or never occurred in tadpoles raised in young tailings; those exposed to older tailings developed similarly to those in reference wetlands. Thyroid hormone disruption likely played an important role in the metamorphosis delay as the T3:T4 ratio was lowest in tadpoles raised in young, tailings-affected wetlands. Our findings suggest tailings wetlands become less toxic with age, and that these amphibians will be able to complete their life cycle in tailing wetlands that have sufficiently detoxified with age.

1. Introduction

The oil sands industry is undergoing rapid expansion and both solid and liquid tailings materials, called oil sand process-affected materials (OSPM), are accumulating in very large quantities (Madill et al., 2001; Quagraine et al., 2005). Government regulation requires liquid tailings be stored in large dyked tailings ponds until reclamation can take place (Crowe et al., 2001). One method for dealing with OSPM—the "wet landscape" approach—involves creating wetlands from fluid tailings in mined-out pits (Madill et al., 2001). To be viable, wetlands constructed with OSPM must be able to support a sustainable, functioning ecosystem. OSPM has proven toxic to many life forms (Clemente and Fedorak, 2005; Franklin et al., 2002; Leung et al., 2001; Rogers et al., 2002), but the toxicity appears to decrease over time (Lai et al., 1996).

In the past few decades, amphibians have been increasingly studied as indicators of environmental quality, and used as indicators of the toxicity of pesticides (Cooke, 1972; Diana et al., 2000; Fort et al., 2004), estrogenic compounds (Hogan et al., 2006), and contaminants from the petrochemical chemical industry (Huang et al., 2007; Pollet and Bendell-Young, 2000). Recently, Kerby et al. (2010) have published that amphibians may be only moderately sensitive to contaminants. However, as an important part of the local ecosystem, we determined them to be important to investigate. An ideal bioindicator is a species that acts as an early warning sign of damage to an ecosystem caused by pollution (McCarty et al., 2002). Amphibians are sensitive to environmental contamination (Cooke, 1981; Gupta et al., 2008) and undergo metamorphosis, a unique and readily identifiable transition between two distinct life stages—the aquatic tadpole stage, and the mainly terrestrial, adult stage—that contributes to their value as bioindicators. Tadpoles, being entirely aquatic, are exposed to toxicants through both dermal and dietary routes, and their survival and successful metamorphosis would provide an indication of the viability of reclaimed wetlands to support amphibians.

Metamorphosis, entailing the emergence of legs, resorption of the tail, and extensive remodeling of internal organs and physical structures (Shi, 2000), must be completed for amphibians to successfully reproduce. Amphibian metamorphosis has been extensively studied and is hormonally regulated, with thyroid hormones (TH) playing the most important role (Buchholz et al., 2007; Fort et al., 2007; Galton, 1992; Shi, 2000; Tata, 2006). These hormones are present in two forms (Denver, 1998), where thyroxine (T4) is converted to triiodothyronine (T3) to become physiologically functional (Shi, 2000), and with T3 having the greatest biological activity. Thyroxine produced by the thyroid

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gland is converted to T3 by deiodinase enzymes in several peripheral tissues, but most notably in the liver (Cai and Brown, 2004; Denver, 1998; Huang et al., 2001). Both T4 and T3 must be measured to understand thyroid status during amphibian development (Fort et al., 2007). Also, it may be of use to analyze thyroid stimulating hormone (TSH), particularly if T4 concentrations are different in animals exposed to contaminants versus reference animals. Subsequent to contaminant exposure, abnormalities may occur due to contaminants interfering directly with thyroid hormone production, or indirectly through action on target tissues, therefore disrupting metamorphosis.

Increased 7-ethoxyresorufin-o-dealkylase (EROD) enzyme activity is a well established biomarker of contaminant exposure in wildlife ranging from poikilotherms to mammals and birds (Whyte et al., 2000). EROD measures the activity of the cytochrome P450 1A (CYP 450) enzyme family, and has been employed to determine exposure to a variety of industrial contaminants (Havelkova et al., 2007). Notably, EROD induction has been seen in birds exposed to contaminants on these same areas of the oil sands (Gentes, 2006). Rogers (2003) showed naphthenic acids (NAs), one of the major toxic components of OSPM, induced EROD enzymes in rats. Oil sands tailings also contain polycyclic aromatic hydrocarbons (PAHs) (Madill et al., 2001), which induce CYP 450 enzymes and EROD activity in fish (van der Oost et al., 2003).

The purpose of this study was to investigate the biological effects of wetlands reclaimed with OSPM on amphibians. We chose Wood Frog (*Lithobates sylvaticus*; formerly *Rana sylvatica*) larvae as our test organism because this species is indigenous to the oil sands region, sensitive to contaminants, and has a life cycle is uniquely suited to assessment of toxicity of wetlands formed with OSPM. Metamorphosis and thyroid hormone status were used as indirect bioindicators and the EROD assay was included as an indicator of exposure to contaminants. Based on earlier work of Hersikorn et al. (2010), tadpoles have proven vulnerable to the chemical mixtures and salinity present in young tailings-based wetlands. Age and OSPM status of wetlands were hypothesized to affect tadpole health, with older OSPM-affected wetlands expected to be more similar to reference wetlands and less toxic than young OSPM-affected wetlands. EROD activity was expected to reflect exposure to higher concentrations of toxicants in wetlands formed with OSPM. The results offer insight regarding the sustainability of amphibian populations in wetlands formed as a result of the wet landscape approach to reclamation.

2. Materials and methods

2.1. Experimental design

2.1.1. Wetland and animal selection

Fourteen wetlands located on oil sands mine sites in northern Alberta, Canada, were selected for this study. They were grouped by age [young (≤7 yr) vs. old (>7 yr)] and OSPM status (reference vs. OSPM). The basis for using a cut off of7 years for "old" vs "young" was the information provided by Leonhardt (2003), which described zoobenthic abundance and richness reaching a plateau as the wetlands approach 7 years of age. Fig. 1 describes the wetlands used in this study. Due to the lack of availability of wetlands for some classes, groups contained unequal numbers.

Wood Frog (*L. sylvaticus*) tadpoles were obtained by collecting 25 egg masses from a reference wetland that was located in the oil sands region. All wetlands, including reference wetlands, were located on oil sands mine leases to provide security. For a list of wetlands used and other site information see Fig. 1. The egg masses were put in plastic buckets filled with water from the reference wetland, Bill's Lake, until the eggs hatched and tadpoles reached Gosner stage 23–25 (Gosner, 1960). Four enclosures as described in Hersikorn et al. (2010), each containing 50 tadpoles, were then placed in each wetland. Tadpoles were assigned randomly to the enclosures/wetlands and all tadpoles were placed in the wetlands on the same day.

2.1.2. Time to metamorphosis

Time to metamorphosis was the number of days for tadpoles to reach metamorphic climax (Gosner stage 42), with day zero as the day when early pre-metamorphic (stage 23–25) tadpoles were placed into enclosures. The study started on May 11, 2007, and concluded on July 24, 2007. For calculations of time to metamorphosis and statistics, tadpoles that had not completed metamorphosis by the study's end date were considered to have taken the entire study period of 75 days to complete metamorphosis, although, they may have completed metamorphosis at a much later date based on their development at this time.

2.2. Thyroid hormones

2.2.1. Whole-body thyroid hormone extraction

Thyroid hormone extraction was adapted from methods developed by Brasfield et al. (2004). At the termination of the study, whole tadpoles were frozen in liquid nitrogen (Praxair, Saskatoon, SK, Canada) immediately following euthanasia and measurement of body weight and length. Once returned to the Toxicology Centre in Saskatoon, SK, samples were transferred to a −80 °C freezer until analysis.

Tissue manipulations were done over ice. Homogenization buffer consisting of 1 mM 6-propyl-2-thiouracil (Sigma Aldrich, Oakville, ON, Canada) in 95% ethanol was made prior to extraction and stored at −20 °C in a glass bottle. Tadpoles were weighed in plastic weigh boats (VWR International, Mississauga, ON, Canada) and finely minced in an equal amount of homogenization buffer and transferred to 16 mm × 100 mm glass culture tubes (VWR International, Mississauga, ON, Canada). A second volume of homogenization buffer equal to the volume of the tadpole was then added to the tadpole homogenate prior to further homogenization using a Tissue Tearor (BioSpec Products, Inc., Bartlesville, OK, USA) in three bursts of 10 s each. Samples were then vortexed for 1 min, then stored on ice. Each sample was then centrifuged at 2900 rpm at 4 °C for 10 min in an Eppendorf 5810 R centrifuge and swinging bucket rotor [model A-4-62 (Eppendorf Canada, Mississauga, ON, Canada)]. Supernatant was transferred to a glass tube. This was repeated with the remaining pellet as described above. The resulting supernatant, which contained ethanol used in the extraction plus thyroid hormones, was combined with the previous supernatant. This sample was then evaporated under a stream of nitrogen in a water bath at 50 °C so the final volume was equal to that of the initial tadpole. The extract was divided into as many 150 μL aliquots as possible and stored at −80 °C until required.

2.2.2. Thyroid hormone quantification

Thyroid hormones were quantified in an extract produced from single whole tadpoles using commercially available competitive enzyme immunoassay kits. Individual kits specific for triiodothyronine (T3) and thyroxine (T4) were acquired from BioQuant Inc., San Diego, CA, USA (T3-BQ043T and T4-BQ044T). Samples were run in duplicate and any samples with a coefficient of variation above 20% were re-analyzed.

Wetland	OSPM status	Age	Area (m^2)	Location
Bill's Lake	Reference	Old	5821	56° 59′ 54.06″N, 111°36′9.79″W
Peat Pond	Reference	Young	6624	56°59′37.45″N, 111°37′24.81″W
Golden Pond	Reference	Young	5677	56°59′50.28″N, 111°37′28.44"W
Mike's Pond	OSPM	Young	16,920	57°6′39.49″N, 111°40′49.79″W
Test Pond 5	OSPM	Old	675	57°5′4.60″N, 111° 41′40.41″W
Test Pond 9	OSPM	Old	3732	57°5′3.58″N, 111°41′32.20″W
West Interceptor Ditch Wetland	Reference	Old	3155	57°6′35.34″N, 111°41′36.52″W
South West Sands Storage (Flood Wetland)	OSPM	Young	11,954	56°58′26.00″N, 111°47′40.00″W
Test Pond 14 (Shallow Wetland)	Reference	Old	35,000	57°4′52.53″N, 111°41′27.91″W
Natural Wetland	OSPM	Old	12,227	56°58′50.27″N, 111°30′35.31″W
High Sulfate Wetland	Reference	Old	2394	56°59′50.03″N, 111°33′10.32″W
Weir 1	Reference	Old	56,419	56°58′36.76″N, 111°27′57.26″W
4 Metre CT - No Peat Zone	OSPM	Young	4006	56°59′28.07″N, 111°31′55.42″W
4 Metre CT - Peat Zone	OSPM	Young	4006	56°59′28.42″N, 111°31′54.14″W

Fig. 1. Table of wetlands used in the study with descriptive information. These wetlands are the same as those used in Hersikorn et al. (2010).

2.3. Hepatic detoxification enzyme activity

2.3.1. Liver microsome production

Microsome production was determined using an adaptation of the methods described in Papp et al. (2005). This experiment was completed at the end of the study period on newly metamorphoseded tadpoles. Upon termination of the study, livers from tadpoles were removed from 5 tadpoles per enclosure and snap frozen in liquid nitrogen to prevent degradation of enzymes within 5 min of euthanasia, which was conducted by placing tadpoles in a 500 mg/L solution of MS222 (ethyl-*m*-aminobenzoate methanesulfonate salt, MP Biomedicals, Solon, OH, USA) dissolved in water. The livers of three to five Wood Frog larvae (depending on tadpole survival) from each enclosure were pooled and homogenized. All chemicals for preparation of buffers were acquired from Sigma Aldrich (Oakville, ON, Canada). During the entire microsome extraction process, tools, buffers, and samples were kept cool by working on crushed ice.

While still frozen, pooled livers forming each sample were removed from cryovials and placed in 2 mL glass homogenization tubes (Wheaton Science Products, Millville, NJ, USA). One mL of HEPES ((4-2-hydroxyethyl)-1-piperazineethanesulfonic acid) homogenization buffer (0.02 M HEPES: 0.15 M KCL; pH 7.5) was then pipetted into the glass tube and the sample homogenized by 20–30 up and down strokes of a hand homogenizer. The resulting homogenate was poured into a 36 mL centrifuge tube (VWR International, Mississauga, ON, Canada) and the glass homogenization tube then rinsed with homogenization buffer until all remaining particles were removed. The rinsate was also added to the centrifuge tube and the final volume was made to 35 mL with homogenization buffer. Six samples were then centrifuged (Sorvall WX90, Thermo Scientific, Waltham, MA, USA) for 20 min at 10,000 $\times$ *g* at 4 °C. The centrifuge rotor was cooled in a refrigerator prior to use. After centrifugation, the supernatant was carefully poured into a clean centrifuge tube, preventing the transfer of lipid that formed a layer on the surface. Lipid was removed before pouring by quickly inserting and removing a plastic micropipette tip, to which the lipids adhered. The volume in the new tubes was again made to 35 mL with homogenization buffer. These samples were centrifuged for 1 h at 100,000 $\times$ *g* at 4 °C. After this final centrifugation, supernatants were carefully poured off and the inside of the tubes dried using tissue paper with care taken not to touch the pellet at the bottom. The resulting microsome pellets were then resuspended in 600 μL of buffer [0.05 M tris (2-amino-2-hydroxymethyl-1, 3-propanediol); 1 mM EDTA (ethylenediamine tetraacetic acid); 20% v/v glycerol] and stored as 300 μL aliquots in cryovials at −80 °C until further analysis.

2.3.2. Ethoxyresorufin-o-deethylase activity (EROD)

EROD activity was measured using an adaptation of the method of Papp et al. (2005) and Gentes et al. (2007). Enzyme activity in hepatic tissue was quantified by measuring the production of the fluorescent compound, resorufin (Sigma Aldrich, Oakville, ON, Canada). Protein content was quantified using a fluorescamine method adapted from Kennedy and Jones (1994) and Olsgard (2007), where total protein in each sample is determined at the same time in the same well as production of resorufin.

The required substrate, 7-ethoxyresorufin (7-ER), (Sigma Aldrich, Oakville, ON, Canada), was prepared in methanol at a concentration of 207 mM and stored at −20 °C. At the time of the reaction, the 207 mM 7-ER was diluted to 11.7 mM with phosphate buffer (sodium phosphate 0.05 M, pH 8.0). The reaction was carried out in a 96-well, flat bottom, microtitre plate. All samples were run in triplicate, except in cases of insufficient sample when duplicates were run, with a specific blank used for all cases. The specific blank contained all reagents except for microsomes. The final volume in each well was 180 μL, and consisted of 40 μL phosphate buffer, 80 μL microsomes, and 30 μL 7-ER working solution, which was incubated for 10 min at room temperature, followed by addition of 30 μL of NADPH (2 mg/mL in phosphate buffer) to start the enzymatic reaction. The reaction was run for 40 min, and then stopped by the addition of 60 μL of a 0.6 mg/mL solution of fluorescamine (Sigma Aldrich, Oakville, ON, Canada) in acetonitrile; the addition of the fluorescamine allowed for the measurement of total protein simultaneously with the enzyme activity.

The fluorescence of the resorufin product was measured by excitation at 530 nm and emission at 590 nm using a microplate fluorometer (Dynex Technologies, Chantilly, VA, USA). Immediately thereafter, total protein was determined by reading the fluorescence at 390 nm excitation and 460 nm emission. A bovine serum albumin (BSA) standard curve was developed to quantify the concentration of protein in each sample well. This assay was carried out in a black, flat bottomed, 96-well microplate read using the microtitre plate fluorometer. Fluorescence was compared with the BSA standard curve and reported as total protein per mL of microsomes. The formation of resorufin was determined by subtracting the fluorescence of the specific blank from the mean of the triplicate sample. This value was compared to a standard curve generated from stock resorufin salt (Sigma Aldrich, Oakville, ON, Canada) solution. The EROD enzyme activity was reported as resorufin formed per mg protein per minute.

2.4. Statistics

We assumed wetlands within a group (young OSPM, old OSPM, young reference, old reference) had similar characteristics, such as concentration of naphthenic acids (NAs) and polycyclic aromatic hydrocarbons (PAHs). Our experimental unit was each enclosure, not individual frogs or wetlands. Statistics were carried out using SPSS statistical software (Version 16.0.1., SPSS Inc., Chicago, IL, USA) with $p \leq 0.05$ as the level of significance. All data were tested for normality and equality of variance assumptions using the Shapiro–Wilks and Levene's tests, respectively. If assumptions of normality and homogeneity of variance were met, a two-way ANOVA was completed with respect to wetland age (young or old) and OSPM status (reference or OSPM). The variables examined were time to metamorphosis, thyroid hormone status, and EROD activity. If assumptions of normality and homogeneity of variances were violated, data were transformed and retested for normality and homogeneity. If assumptions were met after transformation, a two-way ANOVA was performed. If assumptions were not met, the Scheirer–Ray–Hare extension of the Kruskal–Wallis [a non-parametric variation of a two-way ANOVA test (Sokal and Rohlf, 2003)], was performed as in Rickwood et al. (2008). A Tukey's post-hoc test was completed after the ANOVAs to determine where differences occurred. The results of post-hoc tests are presented on figures where a difference (significance <0.05) is denoted between superscript letters.

3. Results

3.1. Time to metamorphosis

Tadpoles raised in young OSPM-affected sites took significantly longer (up to 75 d) to complete metamorphosis or may not have completed metamorphosis at all, when compared with those in any other class of wetlands (shortest = 52 d, mature OSPM wetland) (Fig. 2). At the end of the 75 day study period, frogs that had not metamorphosed were considered not metamorphosed, even though they may have metamorphosed if the study was continued over a longer period. Age of wetland significantly affected time to metamorphosis while OSPM status did not; a significant interaction was found between these two factors (two-way ANOVA, age $F_{1,38} = 56.7$, $p < 0.001$; OSPM status $F_{1,38} = 0.335$, $p = 0.57$; interaction $F_{1,38} = 102.54$, $p < 0.001$). We believe the interaction between age and OSPM is a result of detoxification of wetlands as they age. This is likely the cause of the significant interactions found in this and subsequent analyses, while no significant main interactions are present.

3.2. Whole-body thyroid hormone concentration

Whole-body T3 concentrations in tadpoles did not differ among wetlands due to either age, OSPM status, or their interaction [(two-way ANOVA, age $F_{1,38} = 2.358$, $p = 0.13$; OSPM status $F_{1,38} = 0.524$, $p = 0.47$; interaction $F_{1,38} = 0.327$, $p = 0.57$) (Fig. 3)]. For whole-body

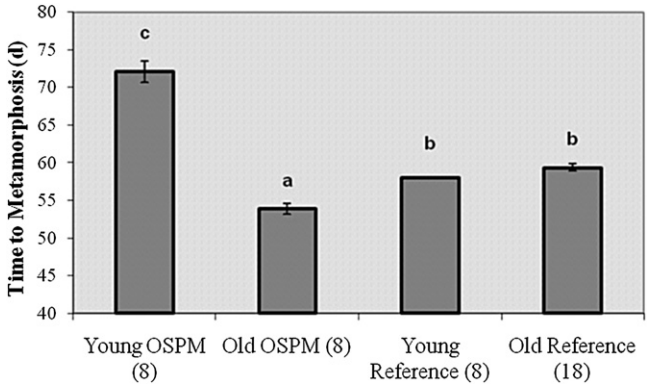

Fig. 2. Mean time to metamorphosis (d ± S.E.) of wetlands grouped by age (young or old) and OSPM status (reference or OSPM). The number of enclosures per type of wetland is shown in parentheses (n). Age of wetland significantly affected time to metamorphosis while OSPM status did not; a significant interaction was found between these two factors (two-way ANOVA, age $p < 0.001$, OSPM status $p = 0.57$, interaction $p < 0.001$). Different subscripts above bars indicate a significant difference between groups determined by a Tukey's post-hoc test ($p < 0.05$).

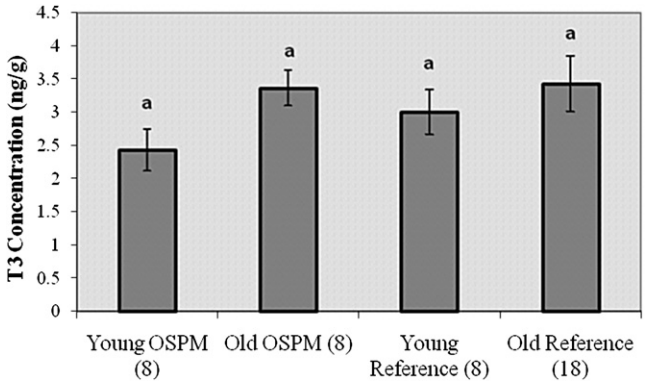

Fig. 3. Mean whole-body triiodothyronine (T3) (ng/g ± S.E.) concentration of *Lithobates sylvaticus* tadpoles raised in wetlands grouped by age (young or old) and OSPM status (reference or OSPM). The number of enclosures per type of wetland is shown in parentheses (n). T3 concentrations in tadpoles did not differ among wetlands due to either age, OSPM status, or their interaction (two-way ANOVA, age $p = 0.13$, OSPM status $p = 0.47$, interaction $p = 0.57$). Different subscripts above bars indicate a significant difference between groups determined by a Tukey's post-hoc test ($p < 0.05$).

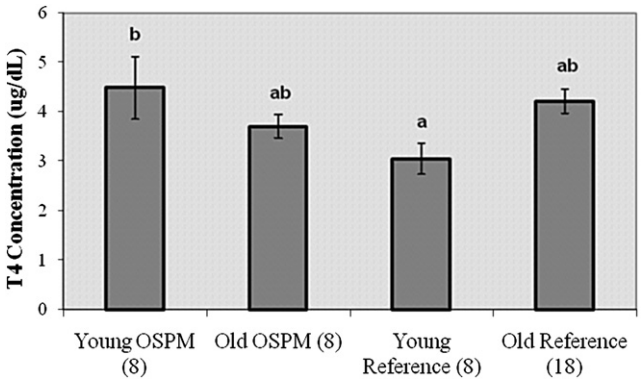

Fig. 4. Mean whole-body thyroxine (T4) concentration (μg/dL ± S.E.) of *Lithobates sylvaticus* tadpoles raised in wetlands grouped by age (young or old) and OSPM status (reference or OSPM). The number of enclosures per type of wetland is shown in parentheses (n). The effects of age and OSPM status were not significant, but a significant interaction was revealed (two-way ANOVA, age $p = 0.78$, OSPM status $p = 0.28$, interaction $p = 0.01$). Different subscripts above bars indicate a significant difference between groups determined by a Tukey's post-hoc test ($p < 0.05$).

concentrations of T4, the effects of age and OSPM status were not significant, but a significant interaction was revealed [(two-way ANOVA, age $F_{1,38} = 0.185$, $p = 0.78$; OSPM status $F_{1,38} = 1.197$, $p = 0.28$; interaction $F_{1,38} = 6.38$, $p = 0.01$) (Fig. 4)]. Tadpoles raised in young tailings-affected wetlands had the highest mean concentration of T4; tadpoles in young reference sites had the lowest. Neither age nor OSPM status affected the thyroid hormone ratio (T3:T4), but a significant interaction was detected [(two-way ANOVA, age $F_{1,38} = 1.039$, $p = 0.314$; OSPM status $F_{1,38} = 3.648$, $p = 0.06$, interaction $F_{1,38} = 8.640$, $p = 0.006$) (Fig. 5)]. The T3:T4 ratio was lowest in tadpoles raised in young tailings-affected wetlands.

3.3. Resorufin production (EROD activity)

The production of resorufin, and therefore EROD activity, was much higher in tadpoles raised in young OSPM-affected wetlands compared to all other wetland classes. No effects of age and OSPM status were detected, but the interaction between these two factors was significant [(two-way ANOVA, age $F_{1,37} = 2.455$, $p = 0.12$; OSPM status $F_{1,37} = 0.618$, $p = 0.44$, interaction $F_{1,37} = 11.694$, $p = 0.002$) (Fig. 6)].

4. Discussion

Results of this study are similar to those of Pollet and Bendell-Young (2000), who found Western Toad (*Anaxyrus boreas*; formerly *Bufo boreas*) tadpoles raised in reference wetlands completed metamorphosis faster than those raised in wetlands affected by OSPM. However, in the present study where wetlands were also classified by age (young or old), tadpoles raised in young OSPM-affected wetlands demonstrated significantly delayed metamorphosis. These observations along with the significant interaction between the treatments of age and OSPM status (shown for this endpoint and others), which seems to uphold the notion that tailings-based wetlands detoxify as they age due to natural processes such as biodegradation by microbes (Lai et al., 1996), as well as photolysis of contaminants such as NAs and PAHs. Due to the complex mixture of contaminants, identifying specific compounds as the cause of the delayed metamorphosis is not possible.

For frogs such as *L. sylvaticus* to be part of a functioning ecosystem, they must reproduce successfully, which entails completion of metamorphosis and survival until adulthood, which in this species occurs only by year two for all females and most males (Sagor et al., 1998). Amphibians completing metamorphosis later have decreased survival when they reach the young adult stage (Berven, 1990; Morey and Reznick, 2001). This is especially true for tadpoles inhabiting wetlands that are not permanent or at risk of drying before tadpoles can complete metamorphosis. Tadpoles completing metamorphosis earlier are larger once they reach the juvenile frog stage which, in turn, allows them to reproduce earlier (Berven, 1990; Goater and Vandenbos, 1997). However, tadpoles that complete metamorphosis too early (and at a smaller size) due to physical or chemical stressors can also be at risk of decreased fitness and increased predation. Our data imply viability is compromised in *L. sylvaticus* from young OSPM-affected wetlands, which ultimately may have negative effects on local amphibian populations.

Thyroid hormones have similar functions in a great range of vertebrates, including regulation growth, and development (Shi, 2000). Thyroid hormones are crucial to amphibian metamorphosis (Fort et al., 2007; Shi, 2000), and have complex functions controlling multiple events such as tail resorption and forelimb development. Metamorphosis is altered in the absence of normal TH levels, although arrested metamorphosis can be reversed if THs are administered at a later time (Rot-Nikcevic and

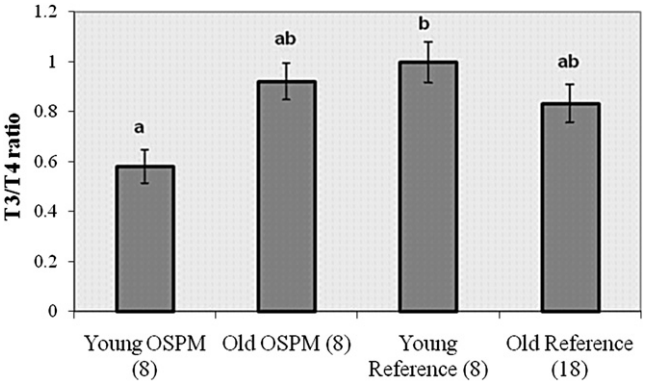

Fig. 5. Mean thyroid hormone ratio (T3/T4 ± S.E.) of *Lithobates sylvaticus* tadpoles raised in wetlands grouped by age (young or old) and OSPM status (reference or OSPM). The number of enclosures per type of wetland is shown in parentheses (n). Neither age nor OSPM status affected the thyroid hormone ratio (T3:T4), but a significant interaction was detected [(two-way ANOVA, age $p = 0.314$, OSPM status $p = 0.06$, interaction $p = 0.006$). Different subscripts above bars indicate a significant difference between groups determined by a Tukey's post-hoc test ($p < 0.05$).

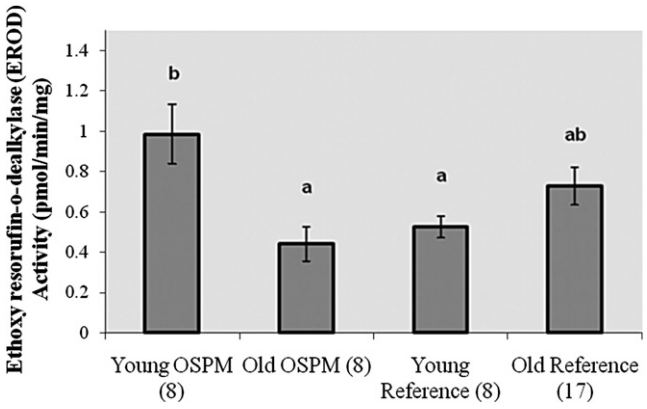

Fig. 6. Mean ethoxyresorufin-o-dealkylase (EROD) activity (pmol/min/mg ± S.E) in *Lithobates sylvaticus* tadpoles raised in wetlands grouped by age (young or old) and OSPM status (reference or OSPM). The number of enclosures per type of wetland is shown in parentheses (n). No effects of age and OSPM status were detected, but the interaction between these two factors was significant (two-way ANOVA, age $p = 0.12$, OSPM status $p = 0.44$, interaction $p = 0.002$). Different subscripts above bars indicate a significant difference between groups determined by a Tukey's post-hoc test ($p < 0.05$).

Wassersug, 2004). The current study found no difference in absolute T3 concentrations, but T4 was higher in tadpoles from the young, more toxic wetlands, relative to those in young reference wetlands. The ratio of T3:T4, which reflects the rate of conversion of T4 to T3 (Picard-Aitken et al., 2007), was also lowest in tadpoles in young OSPM-affected wetlands. While it may seem odd that a lower T3:T4 ratio was found in tadpoles from the most toxic wetlands, while no difference in T3 concentration was apparent, this finding could be a result of normal frog physiology being altered by contaminants. Two possible normal physiological occurrences that may have been affected by contaminants, as explained by Shi (2000), are the incomplete conversion of all T4 to T3, as well as the conversion of T3 to reverse T3 (rT3) by the enzyme deiodinase 3. Both of these processes could lead to lower concentrations of T3 in relation to T4. These alterations in thyroid status of *L. sylvaticus* are likely responsible for the delayed or arrested tadpole metamorphosis in the young OSPM-affected wetlands due to the strong link between THs and metamorphosis. Disrupted thyroid hormone metabolism may have other effects on frogs because TH is essential to normal, competent immunological function (Smits et al., 2002), and growth, along with other metabolic processes in many species.

Although the difference was not statistically recognized, the highest EROD activity was found in tadpoles caged in young OSPM-affected wetlands. Higher enzyme induction was expected considering the higher levels of NA and PAHs relative to the other types of wetlands in the study (Hersikorn et al., 2010), as well as in other wetlands in the oil sands region in general (Clemente and Fedorak, 2005; Gentes, 2006; Madill et al., 2001). When exposed to a toxicant such as a PAH, animals must redirect limited energy resources to production of detoxification enzymes. Therefore, less energy is available for other physiological processes, which could compromise fitness and survival.

Although elevated EROD activity confirmed increased detoxification efforts by these animals, levels here ($\leq$1 pmol/min/mg protein) were considerably lower than in amphibians exposed to river water from an industrial area [11.7 pmol/min/mg protein (control) versus 77.5 pmol/min/mg protein (exposure); Gauthier et al., 2004]. Many factors such as species, sex, age, different environmental factors, or differences in physiological factors among tadpoles can affect the degree of biotransformation efforts as indicated by EROD activity (Goksoyr and Forlin, 1992; Havelkova et al., 2007; Smits et al., 1995). These variables may explain the lower EROD activity found in our study when compared with that of Gauthier et al. (2004). Because the frogs used here have lived in the oil sands regions for many generations, the presence of naturally occurring PAHs in this region may result in higher background EROD activity in all animals. The relatively modest increase in EROD in our tadpoles in the most toxic environment may reflect toxicity to the liver, because injured organs may not function properly, which would compromise the liver's biotransformation capacity. No histopathology was done to confirm or refute this hypothesis, but the extremely high mortality in the young OSPM-affected wetlands group (Hersikorn et al., 2010) is evidence of exposure to high levels of contaminants. Hersikorn et al. (2010) reported that tadpoles in the young OSPM-affected wetlands had between 41.5% and 62.6% lower survival than in all other classes of wetlands (reference and old OSPM-affected). This led them to conclude, that because old OSPM-affected wetlands also had much higher survival rates than the old OSPM-affected wetlands, that the young wetlands would have the highest concentration of contaminants, but would detoxify as they age. For these reasons, direct comparison of EROD activity among studies may not be appropriate.

5. Conclusions

This 2007 field study of the native Wood Frog in the Athabasca Oil Sands region of northern Alberta, Canada, demonstrated that metamorphosis was delayed or not complete at the end of the 75 day study period, thyroid status was altered, and EROD enzymes were induced in tadpoles from the young wetlands formed with tailings from the oil extraction processes. Metamorphosis, thyroid status, and EROD activity of tadpoles in mature tailings-affected wetlands were similar to reference wetlands. These findings offer more insight into the results of decreased survival presented in Hersikorn et al. (2010) on the ecological sustainability of the wet landscape approach to reclamation. The results support the premise that wetlands become less toxic as they mature through a range of biological and physicochemical activities that result in degradation of contaminants. Further research on the adult life stage of the wood frog is yet required to evaluate the ability of this species to survive in reclaimed wetlands on the oil sands. Also, in the future, the use of tissue-specific, thyroid-dependent genes as a biomarker of thyroid axis dysfunction would be of use.

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