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PCB Accumulation and Toxicokinetics in Amphibians: Accumulation Patterns' Regulation by Interspecific Differences and Toxicokinetics in Green Frogs (Rana clamitans) During Hibernation

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by

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CO-AUTHORSHIP STATEMENT

Chapters 2 & 3 in this thesis will be published as co-authored papers. In both cases, Dr. G.D. Haffner provided study ideas and consultation throughout the process of implementing and analyzing these studies. The experiment in chapter 2 was executed with the assistance of D. Wylie. The experiment in chapter 3 was executed by the thesis author, Robin Angell. All data were analyzed by the thesis author, Robin Angell. This thesis was written in its entirety by Robin Angell.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained permission from each of the co-author(s) to include the above material(s) in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University of Institution.

ABSTRACT

This thesis examined contaminant accumulation in newly transformed amphibian young of year and elimination kinetics during amphibian hibernation.

Chapter 2 evaluated PCB concentrations in four amphibian species at five different locations to determine the importance of species specific processes in environmental chemical exposure and accumulation in amphibians. Lipid levels between species were highly variable. Significant interspecific differences in PCB concentrations suggest contaminant accumulation is regulated in part by physiological and biological processes.

Chapter 3 examined PCB elimination rates in hibernating *Rana clamitans* to determine if changes in chemical activity occurred during hibernation. Significant PCB elimination rates were observed for low K_{ow} congeners, ranging from 0.0027 to 0.04 d^{-1}. A negative correlation was found between K_{ow} and elimination rate. There was an increase in fugacity of higher K_{ow} compounds corresponding to a decrease in lipid content. PCBs in metabolic group 2 were preferentially eliminated over those in metabolic group 3.

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LIST OF ABBREVIATIONS

ANOVA – Analysis of variance

C – Chemical concentration

C_{org} – Chemical concentration in organism

C_w – Chemical concentration in water

DCM – Dichloromethane

F – Fugacity

GC-ECD - Gas chromatography electron capture detector

GC-MSD – Gas chromatography mass selective detector

k_a – Elimination rate constant to air

k_f – Elimination rate constant to feces

k_m – Elimination rate constant for metabolism

K_{ow} – Octanol-water partitioning coefficient

k_w – Elimination rate constant to water

k_2 – Elimination rate constant

PCB – Polychlorinated biphenyl

POP – Persistent organic pollutant

SE – Standard error of the mean

t – Time

X_L – Fraction of lipid in organism

Z – Fugacity capacity

CHAPTER 1 – THESIS INTRODUCTION

1.1 General Introduction

Over the course of the last two decades a worldwide decline has been observed in amphibian species, and many stressors have been suggested as contributing factors in this decline including the introduction of chemical pollutants and pesticides into natural systems (Ankley et al. 2004; Wake, 1991; Collins, 2003). Previous elimination studies have demonstrated an increase in chemical fugacity of polychlorinated biphenyls (PCBs) during metamorphosis in green and leopard frogs (Leney et al., 2006), but there is little information on the uptake and elimination of hydrophobic organic chemicals during hibernation, or how biological processes such as life cycle differences, foraging strategies and latitudinal range distributions can affect species specific chemical accumulation patterns.

Chapter 2 of this thesis presents a study conducted to determine if amphibian species occupying common habitats had similar chemical accumulation patterns. Four species of native anuran amphibians were sampled just after metamorphosis at five sites along their overlapping species distributions.

Chapter 3 presents an elimination study conducted to determine whether chemical activity increases during hibernation in amphibians. In this chapter, green frogs (*Rana clamitans*) were dosed with a PCB mixture in order to determine chemical elimination rates during hibernation, or brumation; lipid levels were measured in order to assess bioenergetic demands of hibernation.

The hibernation and species specific differences studies presented in this thesis aid in the completion of kinetic models predicting metabolism of OCs during different amphibian life stages and activity periods.

1.2 Worldwide Amphibian Declines and Climate Change

Global minimum temperatures have been increasing at double the rate of maximum temperatures (Walther et al., 2002), with spring arriving a few days earlier every decade (Parmesan and Yohe, 2003).

Global warming is a potentially contributes to amphibian declines in both tropical and temperate regions (Pounds et al., 1999). In temperate regions, lipid declines are steeper during warmer winters as amphibians are forced to operate at a higher metabolic level with minimal available food resources to make up for this additional energy expenditure (Rose, 1967). PCB concentrations and congener ratios in amphibians are possibly regulated by global warming effects including changes in lipid content, contaminant distribution in precipitation, and changes in population size that can result from higher levels of winter mortality (Maniero and Carey, 1997).

1.3 Life History of Amphibian Species Used in Studies

Four amphibian species native to North America were used in this study. Chapter 2 involved sampling leopard frogs, green frogs, wood frogs (*Rana sylvatica*), and Eastern American toad (*Bufo americanus)* young of year. The elimination experiments in Chapter 3 were conducted using green frogs (*Rana clamitans)*.

Green frogs grow to an adult length of approximately 10 cm. Green frogs are aquatic and live, breed, and hibernate in bodies of water ranging from rivers to ponds. The breeding season for green frogs in Ontario, Canada lasts from May into early June, and once hatched, the tadpoles transform in approximately 16 weeks, although tadpoles hatched later in the breeding season will overwinter as tadpoles and transform the following year (Stebbins, 1951).

Leopard frogs are within the same size range as green frogs, with adult lengths ranging from 6 to 10 cm. Leopard frogs can be found in a very wide range of habitats and tend to favor

open areas including fields, marshes, and agricultural areas. Leopard frog habitat is only limited by access to permanent water sources. Leopard frogs breed for the entire spring/summer season (Stebbins, 1951) – in Ontario from April to August – and tadpoles transform 8-12 weeks after hatching but do not overwinter as tadpoles (Harding, 1997).

Wood frogs are the smallest of the amphibians used in this study, with a size range of approximately 4 to 7 cm. Wood frogs can be found in damp and shady woods. Wood frog breeding occurs in April, and tadpoles transform within 13 weeks of hatching (Stebbins, 1951). Wood frogs are biologically notable for their capacity to survive extracellular freezing (Story and Story, 1984).

Eastern American toads range in size from 5 to 11 cm and are terrestrial, living and hibernating on land. Eastern American toads breed in late May, with the exception of late season cold weather forcing breeding into June, and tadpoles transform 6 to 10 weeks after hatching. Eastern American toad young of year, or 'toadlets', are very small – approximately 1 cm long (Harding, 1997).

1.4 Polychlorinated Biphenyls (PCBs)

Polychlorinated Biphenyls, or PCBs, is the name applied to a large group of congeners comprised of different numbers of chlorine atoms substituted into biphenyl rings (Tanabe, 1988). PCBs are man-made, biphenyl-based, chlorinated organic compounds. PCBs were intensively used in industry as stable, heat resistant oils. PCBs were manufactured and sold for industrial use from 1929 until 1977, when production was banned due to toxicological effects of PCBs (Waid, 1986). PCBs are extremely persistent, and are still readily detected in the environment 3 decades after the cessation of production (Tanabe, 1988).

There are 209 PCB congeners, resulting from chlorine substitution patterns of the benzene rings. Each PCB congener is associated with an octanol-water coefficient, or K_{ow} value,

which indicates how readily a specific congener partitions between octanol and water when at chemical equilibrium.

$$K_{ow} = \frac{\text{concentration of chemical in octanol}}{\text{concentration of chemical in water}}$$

PCB chlorination is positively correlated to K_{ow} as higher chlorinated PCBs are increasingly hydrophobic. The K_{ow} values associated with each PCB congener cover a wide range, necessitating the general use of a log scale; with log K_{ow} values ranging from 4.09-8.18 (Hakwer and Connell, 1988).

PCBs were banned due to concern over environmental persistence (Waid, 1986), bioaccumulative concerns (Tanabe 1988) and possible toxic effects in humans including cancer (Cogliano, 1988), cardiovascular disease (Gustavsson and Hogstedt, 1997), and weakened immune system functioning (Chang et al., 1981). PCB toxicity has also been observed in many other animal species (Lind et al., 2004; Aulerich and Ringer, 1977; Jensen et al., 1977).

Amphibians are vulnerable to the toxic effects of PCBs. *Xenopus laevis* tadpoles have been shown to develop reproductive abnormalities, including the feminization of male gonads, when dosed with PCBs (Qin et al., 2003). Tadpoles exposed to PCBs have decreased mass and survival rates (Fisher et al., 2003; Jelaso et al., 2002). Exposure to PCBs has been shown to result in permanent scoliosis, or kinking, of the tail of tadpoles (Fisher et al., 2003; Jelaso et al., 2002), a toxicological effect that has direct implications for the ability of tadpole stage amphibians to forage for food and effectively escape predators. Jelaso, et al. (2002) additionally observed neurological impairment such as circular swimming patterns at both low and high dosing concentrations. PCB exposure can also directly result in significant changes in gene expression, with possible effects including delayed metamorphosis and interference in the development and regulation of the nervous system (Jelaso et al., 2005).

1.5 A Kinetic Model for Chemical Uptake and Elimination

The change in concentration of a chemical within an aquatic organism can be predicted using the equation:

$$\frac{dC_{org}}{dt} = k_w C_w + k_{food} C_{food} - k_{diff} C_{org} - k_{eg} C_{org} - k_{gro} C_{org} - k_{rep} C_{org} - k_{mer} C_{org}$$

where C_{org} equals chemical concentration in the organism;

k_w is uptake rate constant from water;

C_w is chemical concentration in water;

k_{food} is uptake rate constant from food;

C_{food} is chemical concentration in food;

k_{diff} is the elimination rate constant from the organism by way of diffusion;

k_{eg} is the elimination rate constant from the organism by way of fecal egestion;

k_{gro} is the elimination rate constant from the organism because of growth;

k_{rep} is the elimination rate constant from the organism because of reproduction;

k_{mer} is the elimination rate constant from the organism because of metabolism.

It is possible to model the metabolism of individual PCB congeners by dosing an organism with a known amount of chemical and measuring elimination over time. PCB elimination rate constants in frogs have previously been assessed by Leney, et al. (2006) using a first order, one-compartment model (Barron et al., 1990). This model encompasses all possible routes of chemical elimination from the organism and resolves congeners that are passively

eliminated from those that are biotransformed. For elimination experiments where the test organism is dosed with a chemical and placed into a clean system the following equation is used:

$$\frac{dC_{org}}{dt} = -k_2 C_{org}$$

and this equation can then be rearranged:

$$\ln C_{org(t)} = \ln C_{org(t=0)} - k_2 t$$

where $C_{org(t)}$ represents the mass of chemical in the organism at time *t*, $C_{org(t=0)}$ is the mass of chemical in the animal at the beginning of the elimination experiment, and k_2 is the total chemical elimination rate constant.

$$k_2 = \frac{\ln C_{org(t=0)} - \ln C_{org}}{t}$$

Once the k_2 value has been determined experimentally, the time to a 90% steady state can be calculated using the following equation:

$$t_{90} = \frac{\ln 10}{k_2}$$

1.6 Metabolic Biotransformation

Substances foreign to an organism's system (xenobiotics), such as PCBs, can be bio-transformed by enzyme systems. The function of biotransformation is to convert lipid-soluble chemicals into water-soluble metabolites which are readily excreted by the organism (Livingstone, 1998). Some of these water-soluble metabolites are much more toxic than the original compound (Livingstone, 1988).

Oxidative metabolism of xenobiotic chemicals is a function of the mixed function oxidase, or MFO, system. The enzymes functioning within the MFO system are cytochrome P-

450, or CYP enzymes. PCB congeners are metabolized by different enzymes according to chlorination pattern, and four metabolic groups are recognized. Metabolic group one consists of congeners lacking both *meta-para* and *ortho-meta* vicinal hydrogen atoms. Congeners with only *meta-para* vicinal hydrogen atoms are classified as group two PCBs. Congeners with only *ortho-meta* vicinal hydrogen atoms are classified as group three PCBs. Group four consists of congeners with both *meta-para* and *ortho-meta* vicinal hydrogen atoms. Group two (*meta-para*) congeners are metabolized as a result of cytochrome P-450 2B isozyme activity. PCBs in group three (*ortho-meta*) are metabolized by cytochrome P-450 1A enzyme activity. Group four congeners are considered very easily to metabolize as they can be metabolized by both P-4501 1A and 2B isozymes (Kannan, 1995).

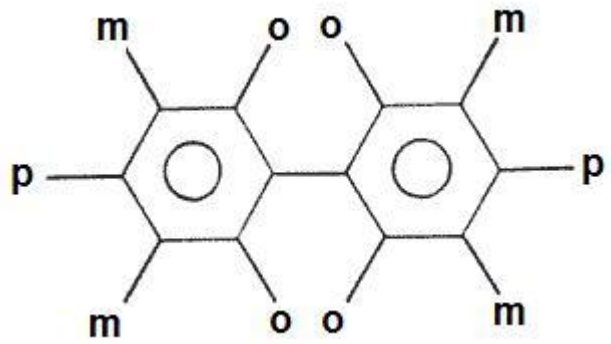

Figure 1.1 Diagram of basic PCB structure with letters depicting possible chlorine substitution patterns, with m=meta, o=ortho, and p=para.

P-450 activity can greatly differ according to amphibian species (Noshiro and Omura, 1984); animal species with low P-450 activity have been shown to bioaccumulate PCBs at high rates (Tanabe, 1988). Increased P-450 enzyme activity can also serve as a biomarker of high levels of PCB contamination in amphibians (Jelaso et al., 2005).

1.7 The Fugacity Concept

During times in which lipid levels decrease within an organism, the capacity to contain PCBs within lipid also decreases placing chemicals under greater escaping pressure. This chemical activity is called fugacity. Fugacity (*f* measured in Pa) describes the linear relationship between the whole body concentration of a chemical in mol x m^{-3} (C) and the fugacity capacity in mol x m^{-3} x Pa^{-1} (Z) so that:

$$f = \frac{C}{Z}$$

Fugacity capacity (Z) of an organism can be estimated from the concentration of lipid (X_L), the K_{ow} of the congener being assessed, and the Henry's law constant value in Pa x m^3 x mol^{-1} (H) so:

$$Z \sim \frac{X_L K_{OW}}{H}$$ (Mackay and Paterson, 1981)

thus:

$$f \sim \frac{CH}{X_L K_{ow}}$$

When fugacity is high, there is increased pressure for chemicals to move away from the lipid phase and into the circulatory fluids of an organism and potentially augment active toxicological stress.

1.8 Chemical Elimination and Metabolism of PCBs in Amphibians

Previous chemical elimination studies with amphibians have determined elimination rates for tadpole (average k_2=0.044 d^{-1}), metamorph (average k_2=0.170 d^{-1}), and adult stage amphibians (average k_2=0.017 d^{-1}; Leney et al., 2006a; 2006b; 2006c). It was determined that elimination rates were highest in metamorph stage amphibians (Leney et al., 2006b). Additionally, fugacity of PCBs increased up to a factor of four during metamorphosis, as the tail was adsorbed and the digestive tract and mouthparts morphologically adjusted to the shift from herbivore to insectivore

(Leney et al., 2006b). Adult amphibians at ambient temperatures had the slowest elimination rates for PCBs among the three life-stages (Leney et al., 2006b).

1.9 Study Objectives

1.9.1 Holistic Objective

The overall goal of this study was to provide critical information and data for the development of a model that predicts chemical dynamics and activity during the entire amphibian life cycle. Although the major morphological life stages (tadpole, metamorph, and adult) have been modeled (Leney, et al., 2006b) there has been very little work done towards understanding elimination processes during hibernation of amphibians in temperate regions. Furthermore, accumulation studies have focused only on a few species, and there is a need to assess if a general model describes PCB accumulation dynamics in different anuran species.

1.9.2 Chapter 2 Objectives

Chapter 2 set out to determine interspecific differences in lipid and PCB levels in field sampled amphibians. Previous studies (Beck and Congdon, 2003; DeGarady and Halbrook, 2006) have found inter-species differences in lipid and PCB levels in young of year. Interspecific differences provide evidence for the need to develop biological models to predict chemical fate and effects in amphibians.

The hypothesis tested in Chapter 2 was:

2.1. Fugacity models predict there will be no differences in contaminant lipid concentrations among amphibian species, and that life histories do not regulate contaminant accumulation patterns.

1.9.3 Chapter 3 Objectives

The purpose of the experiment in Chapter 3 was to determine elimination rate constants (k_2 values) for PCBs in hibernating adult green frogs. Previous studies by Leney, et al. (2006b)

showed an increase in fugacity in tadpole stage amphibians. Chapter 3 set out to determine whether a similar increase in chemical activity occurred for hibernating amphibians.

The hypotheses tested in Chapter 3 were:

3.1. There will be no significant changes in lipid in adult frogs during hibernation.

3.2. There will be no elimination of PCBs by adult frogs during hibernation.

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CHAPTER 2 – INTERSPECIES DIFFERENCES IN AMPHIBIAN PCB CONCENTRATIONS

2.1 Introduction

It is necessary to evaluate the effects of environmental chemicals in different species and life stages of amphibians in relation to the worldwide amphibian decline (Ankley 2004). PCBs continue to be observed in a wide range of environmental samples including amphibians (DeGarady and Halbrook, 2006; Kadokami et al., 2004). Chemical concentration in tadpoles and young of year amphibians is directly related to body burden and lipid levels of the parents prior to breeding (Kadokami et al., 2004). Aquatic organisms in temperate regions have been shown to experience seasonal shifts in elimination activity (Paterson et al., 2007). In yellow perch for example, PCBs were not readily eliminated in fall or winter, and elimination rates at the beginning of spring were extremely slow (Paterson et al., 2007). Amphibians eliminated persistent chemicals more slowly than other aquatic organisms, even at ambient temperatures (Leney, 2006a).

There is growing evidence that bioenergetic processes are as important as chemical properties when predicting the accumulation of persistent chemicals in organisms, and that simple thermodynamic models are not sufficient to explain exposure dynamics among species (Paterson et al., 2007). In amphibians these processes include overwintering strategies, foraging strategies, time spent in the larval form and where within the range of a species an individual is located. Fugacity models predict that all species of amphibians occupying the same habitat would have similar lipid concentrations of chemicals, thus the above bioenergetic processes and range effects would be rendered relatively unimportant.

The goal of this study was to evaluate PCB concentrations in four different amphibian species at five different locations along a latitudinal gradient to determine the relative importance

of species specific processes in the exposure and accumulation of environmental chemicals in anuran amphibians. The null hypothesis is that there are no latitudinal range effects on lipid concentration and no interspecific differences in PCB accumulation. If there are no inter-species differences in lipid or PCB concentrations, this would indicate that chemical properties such as K_{ow} dominate chemical accumulation. Little is known about interspecific differences in contaminant bioaccumulation in amphibian species even though species-specific differences in contaminant bioaccumulation could be a factor in the rapid decline of certain amphibian species.

2.2 Materials and Methods

2.2.1 Sample collection

Four native amphibian species, *Rana clamitans* (green frog), *R. sylvatica* (wood frog), *R. pipiens* (Northern leopard frog), and *Bufo americanus* (American toad), young of year were sampled at five locations where geographical ranges for each species overlap in Ontario, Canada (Figure 2.1). These species were chosen for sampling as they are common to Ontario and they have the largest range overlap. Samples were taken at a variety of latitudes to assess possible lipid differences within and among species along their range. Young of year frogs were chosen for sampling because chemical concentrations in newly metamorphosed amphibians have been recently influenced by hibernation, breeding, and metamorphosis - all of which feature distinct, species-specific time frames (Table 2.1). Sampling sites were coded as follows: Essex (≈42°), Wellington (≈43°), Frontenac (≈44°), Nipissing (≈45° & 46°), and Cochrane (≈49°). Site details including exact coordinates and sampling dates are available in Table 2.2. Sampling took place over the first two weeks of July, 2006. Amphibians were sampled from multiple locations within the same area due to differences in habitat preference. Five young of year amphibians of each species were sampled at each site, with the exception of American toad young of year. Due to the much smaller size of American toad young of year compared to other amphibian species young of year, approximately 35-40 American toads were collected at each site to meet the minimum

weight requirements for chemical analysis. After collection, samples were stored on dry ice until transfer to a -30° C freezer where they were stored pending analysis. Leopard frog young of year could not be located over an extensive 3 day sampling period at latitude ≈49°. Wood frogs were not available in Essex County (≈42°). Wood frogs have become rare in Essex County, presumably as a result of habitat destruction.

2.2.2 Analysis

Methods for PCB analysis are described in specific detail by Leney, et al. (2006). To summarize, whole organism amphibian samples were homogenized using hexane and acetone rinsed surgical scissors. Mortar and pestle were used to pulverize an approximately 2 gram subsample of each homogenized sample with sodium sulphate. The ground sample was then packed into a glass column with 50 mL of 1:1 dichloromethane: hexane. Each column was spiked with 100 µl of tri-bromo-benzene spiking standard at a concentration of 125 ng/g for recovery correction during analysis. Another 250 mL of 1:1 dichloromethane: hexane was added and the column stood undisturbed for a minimum of 1 hour before elution.

Collected extracts were concentrated to 10 mL and 10% of each extract was removed for lipid concentration analysis. The extract was then added to a glass column containing 6 grams of florisil and eluted with 50 mL of hexane for lipid cleanup. Extracts were concentrated and transferred to vials with a final volume of 1 mL in 2,2,4-trimethylpentane.

A method blank column and a column containing fish homogenate from the Detroit River were processed alongside each set of samples to provide a reference sample for quality assurance.

Chemical analyses were performed using an HP 5890 with gas chromatography electron capture detector (GC-ECD). For amphibians, whole body PCB concentrations were determined. American toad samples consisted of enough pooled organisms to meet minimum mass requirements for analysis. A secondary standard and spiking standard were run with each set of

samples. Congeners were identified by retention time and by molecular ion and quantified by comparison to the peaks in a secondary standard. Recovery of the tri-bromo-benzene spiking standard in the samples was 85±6.7% (mean± SE) of the recovery in the homogenate. Recovery of PCB congener 180 in the reference homogenate tissue extracted alongside each sample set was within two standard deviations of the mean value from the laboratory control charts maintained by the organic analytical laboratory at the Great Lakes Institute for Environmental Research, a Canadian Association for Laboratory Accreditation Inc. certified facility. PCB concentrations in method blanks were below machine detection limits (which range from 0.1 pg - 0.05 µg). Statistical analysis was performed using Kruskal-Wallis One-way Analysis of Variance (Systat 12 for Windows).

2.3 Results

There were no statistically significant differences in percent lipid found among species ($p=0.999$) or locations ($p=0.999$; Kruskal-Wallis One-way Analysis of Variance, SYSTAT 12). Lipid levels were highly variable within locations and species. American toad lipid levels were significantly higher than those of other species at the Wellington County sampling location ($p=0.004$; Figure 2.2, Lat. 44).

Wellington county amphibians (Lat. 43°) had significantly higher total wet weight PCB (Figure 2.3) and lipid corrected PCB concentrations (Figure 2.4) than amphibians sampled from all other sites. Average wet weight (Figure 2.4) and lipid corrected (Figure 2.5) concentrations of PCB 31/28, PCB 52, PCB 101, PCB 110, PCB 153, PCB 138, and PCB 180 were plotted individually by location as these PCB congeners are known to be generally resistant to being metabolized. Average concentrations of PCB congeners 153 and 180 were significantly higher in Wellington County frogs (Figure 2.5, lat. 43° PCB 153 $p=0.001$, PCB 180 $p=0.03$). Lipid corrected concentrations of PCB congeners 153, 138, and 180 were significantly higher in Wellington County frogs (Figure 2.6, lat. 43° PCB 153 $p=0.001$, PCB 138 $p=0.01$, PCB 180

p=0.01). There were no significant differences in congener concentration in frogs from the other four sites. Average concentrations of congeners 153 and 180 were significantly higher in leopard frogs (Figure 2.5, PCB 153 p=0.02, PCB 180 p=0.005). Lipid corrected concentrations of PCB congeners 153 and 180 were significantly higher in Northern leopard frogs (Figure 2.6, PCB 153 p=0.02, PCB 180 p=0.006). Lipid corrected concentrations of PCB 110, however, were significantly higher in American toads (Figure 2.6, p=0.01).

2.4 Discussion

Significantly higher levels of PCBs in Wellington County amphibians (Figures 2.2 and 2.3) indicated that the observed chemical concentrations in leopard frogs were in response to local contamination. A previous study calculating the concentrations of PCBs in mink collected in this area confirmed the elevated levels in Wellington County (Haffner, et al. 1998). This offers further support for the use of amphibians as biomonitoring organisms.

Fugacity models predict there will be no differences in contaminant lipid concentrations among amphibian species such that species specific processes do not regulate contaminant accumulation patterns. As lipid corrected concentrations of PCB 110 were significantly higher in American toads (Figure 2.6, p=0.01), and lipid corrected concentrations of PCB congeners 153 and 180 were significantly higher in Northern leopard frogs (Figure 2.6, PCB 153 p=0.02, PCB 180 p=0.006) the accumulation of PCBs in part is driven by biological processes, and this was observed at all sampling sites.

Biological processes are concluded to be important in regulating contaminant accumulation in frogs as evidenced by interspecies differences in amphibians within the same location. Furthermore, the interspecific differences do not appear to be physiologically regulated – in that species accumulation patterns are not consistent between sites (Figures 2.4 and 2.6). The interspecific differences in PCB concentration in this study indicate that amphibian species exhibit species specific exposure patterns that might be related to bioenergetic processes

associated with different ecological efficiencies in the different sampling areas. There is not a common pattern for the most contaminated species among sites suggesting that resource utilization by a species might be site dependent. Future efforts to develop a life cycle model for amphibians need to address species specific differences in contaminant accumulation and metabolism.

Differences in bioenergetic processes could account for interspecific differences observed in this study. Leopard frogs, wood frogs, and green frogs remain in the tadpole stage for a much longer time than American toads (Table 2.1). Additionally, it has been shown that length of tadpole stage is positively correlated with the length of time it takes for a species to make post-metamorphosis behavioural and physiological changes, such as increased aerobic capacity (Pough and Kamel, 1984). Green frogs remain in tadpole stage the longest, while American toads and wood frogs develop at such a rapid rate that they still contain some immature organs after undergoing metamorphosis (Pough and Kamel, 1984; Table 2.1). Metamorphic differences, such as organ development and functioning, have implications for chemical accumulation and exposure; depending on how long it takes for post-metamorphosis organ functioning to resume, amphibians such as American toads and wood frogs could be relying more on stored energy after transformation. At latitude 44 newly transformed American toads had significantly ($p=0.004$) higher lipid concentrations compared to other species tested (Figure 2.2) and also higher average PCB concentrations (Figure 2.3). The shorter tadpole stage could have direct effects on PCB accumulation. Habitat effects could also result in differences in PCB accumulation; for example, American toads are largely terrestrial post-metamorphosis and are thus exposed to aquatic contaminants for a much shorter time period than species such as green frogs.

Leopard frogs have an intermediate length tadpole stage but participate in a much longer breeding season than the other three amphibian species collected during this study (Table 2.1). Leopard frog young of year may transform as late as the end of the summer, resulting in a

metamorphic disadvantage compared to other species. Leopard frog lipid corrected PCB concentrations were higher than those of other species at latitudes 43 and 46. Wood frogs and American toads would have the last months of summer to store lipid, while green frogs are able to overwinter as tadpoles and reserve lipid stores for transformation at a more advantageous time the following year. Hibernation in amphibians results in significant costs to the lipid stores of adult amphibians; late emerging adults would be placed at a distinct disadvantage. Additionally, Northern amphibians breed immediately after emerging from hibernation, when lipid levels are already depleted (Donohoe et al. 1998). It has been observed that the wet weight PCB concentration in amphibian eggs is twice that of the mother (Kadokami, et al. 2004) and bioamplification of PCBs has been observed in yellow perch eggs (Daley et al., 2009). Thus, American toads would be transforming closer to the original maternal transfer of PCB body burden which could be directly influencing the high contaminant lipid levels found at latitude 44 (Figure 2.3). Due to a longer breeding season, adult leopard frog lipid levels should decrease and chemical activity, or fugacity, should increase at a greater rate than for the other amphibians collected, resulting in greater PCB concentration within the parents and greater fugacity, and lower lipid levels, in the eggs. Indeed, leopard frog lipid concentrations were below 2% at all sites (Figure 2.2), and lipid corrected PCB concentrations were higher than those of other species at several sampling sites (Figure 2.4). Significantly higher lipid levels in American toad young of year at one sampling site (Figure 2.2) are of interest, as American toads have the shortest breeding season and parents and eggs should emerge from breeding with higher lipid levels than the other three species collected for this study. Habitat and site which determine quality of foraging, cover, and weather combined with species specific differences in the bioenergetic mechanisms regulating growth, development and metabolism would account for the interspecific contaminant accumulation differences in this study.

Adult anuran amphibians eliminate POPs such as PCBs at extremely slow rates (Leney, 2006a). Adult chemical body burden could be determined by species-specific accumulation patterns occurring early in development. These findings have implications for contaminant life cycle modeling in amphibians. Future modeling work will need to take into account biological processes which result in significant differences in chemical accumulation between species and sites.

2.5 Conclusions

This study determined that there are inter-species differences in contaminant lipid concentrations and PCB mass in different frog species. Such differences cannot be explained by simple thermodynamic partitioning models, and provide strong support for the need of species specific models for anurans. As lipid and PCB concentration are the two factors directly regulating fugacity, these observations support previous research that suggest life cycle and ecological factors such as foraging strategies are regulating chemical accumulation dynamics in amphibians. PCB concentrations were not indicative of latitudinal effects due to processes such as cold condensation as other factors such as local contamination and biological processes were more dominant. Further inspection of interspecific differences in life cycle processes with relation to chemical activity could help to explain the species specific declines and extinctions of amphibians.

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Table 2.1 Species specific details for study organisms.

Species[1]	Adult Size[1]	Habitat[1]	Length of breeding Season[1]	Time to metamorphosis[1]
Rana clamitans (Green Frog)	≈10 cm	Aquatic	May to early June	≈16 weeks; tadpoles hatched later overwinter as tadpoles
R. pipiens (Leopard Frog)	≈6-10 cm	Variable; only limiting factor is access to water	April to August	8-12 weeks[2]
R. sylvatica (Wood Frog)	≈4-7 cm	Damp, shaded, wooded areas	April	≈13 weeks
Bufo americanus (Eastern American Toad)	≈5-11 cm[2]	Terrestrial; variable; only limiting factor is access to water[2]	late May[2]	6-10 weeks[2]

[1]All information from Stebbins (1951) except where otherwise noted.
[2]Harding (1997).

Table 2.2 Site details and collection dates for five amphibian species.

Species	Location and coordinates	Date
Rana clamitans (Green Frog)	Essex County 42° 5.91438, -82° 56.6424	June 29, 2006
	Wentworth County 43° 14.419, -79° 59.458	July 5, 2006
	Frontenac County 44° 43.623, -76° 48.095	July 8, 2006
	Nipissing County 46° 15.495, -78° 54.108	July 10, 2006
	Cochrane County 48° 17.887, -79° 50.965	July 13, 2006
R. pipiens (Leopard Frog)	Essex County 42° 5.91438, -82° 56.6424	June 29, 2006
	Wentworth County 43° 17.619, -79° 53.057	July 5, 2006
	Frontenac County 44° 31.376, -76° 36.636	July 6, 2006
	Nipissing County 46° 15.495, -78° 54.108	July 10, 2006
	No sample	
R. sylvatica (Wood Frog)	No sample	
	Wentworth County 43° 17.619, -79° 53.057	July 4, 2006
	Frontenac County 44° 30.465, -76° 33.280	July 7, 2006
	Nipissing County 46° 21.206, -78° 46.053	July 15, 2006
	Cochrane County 48° 17.887, -79° 50.965	July 13, 2006
Bufo americanus (Eastern American Toad)	Essex County 42° 5.91438, -82° 56.6424	June 29, 2006
	Wentworth County 43° 17.619, -79° 53.057	July 5, 2006
	Frontenac County 44° 23.456, -76° 34.328	July 6, 2006
	Nipissing County 46° 06.804, -78° 55.509	July 11, 2006
	Cochrane County 48° 11.723, -79° 51.720	July 13, 2006

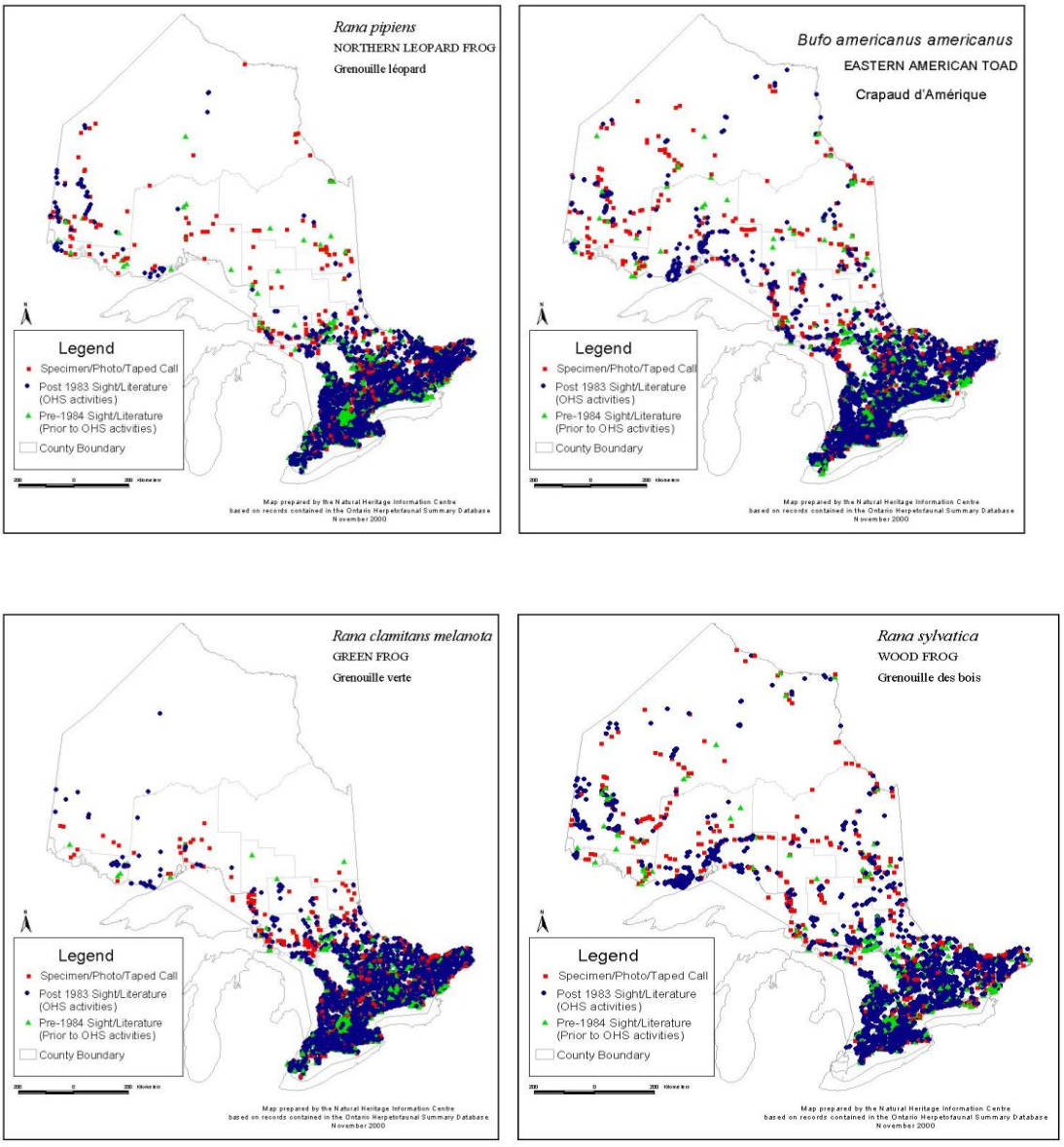

Figure 2.1 Distribution maps[1] depicting the ranges of each test species (Northern leopard frog, Eastern American toad, green frog, and wood frog).

[1]All distribution maps prepared by the Natural Heritage Information Centre and based on the November, 2000 records contained in the Ontario Herpetofaunal Summary Database.

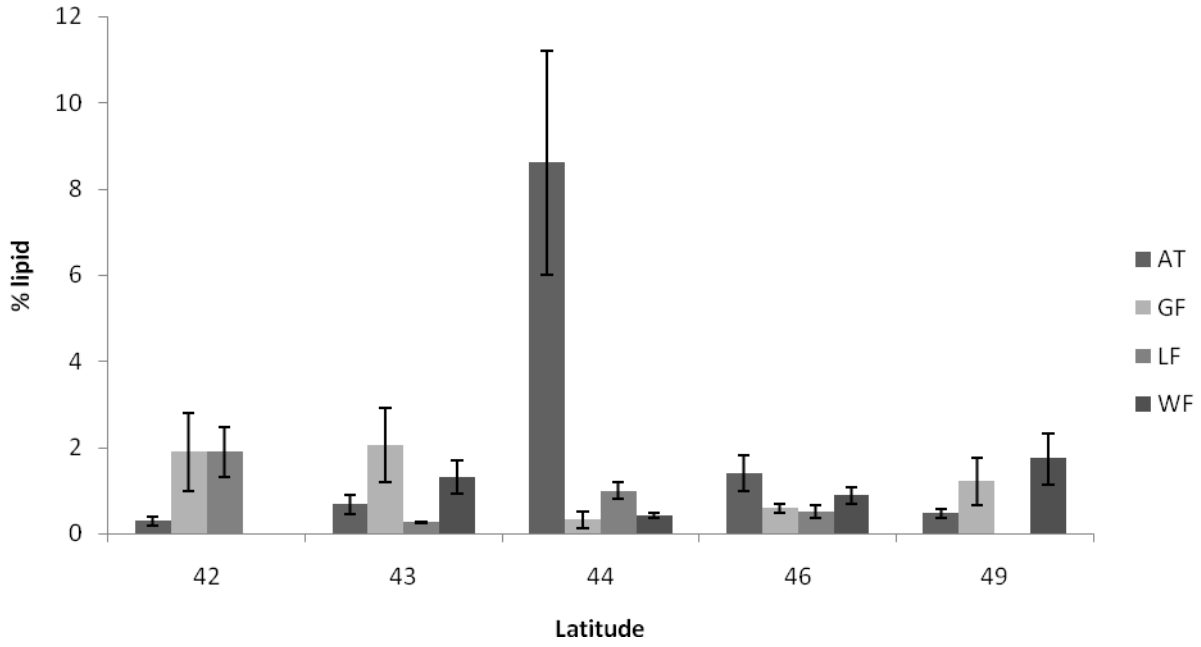

Figure 2.2 Average total body percent lipid by sampling location latitude and by species. Error bars represent standard error. American toad lipid levels were significantly higher than those of other species at latitude 44 (p=0.004).

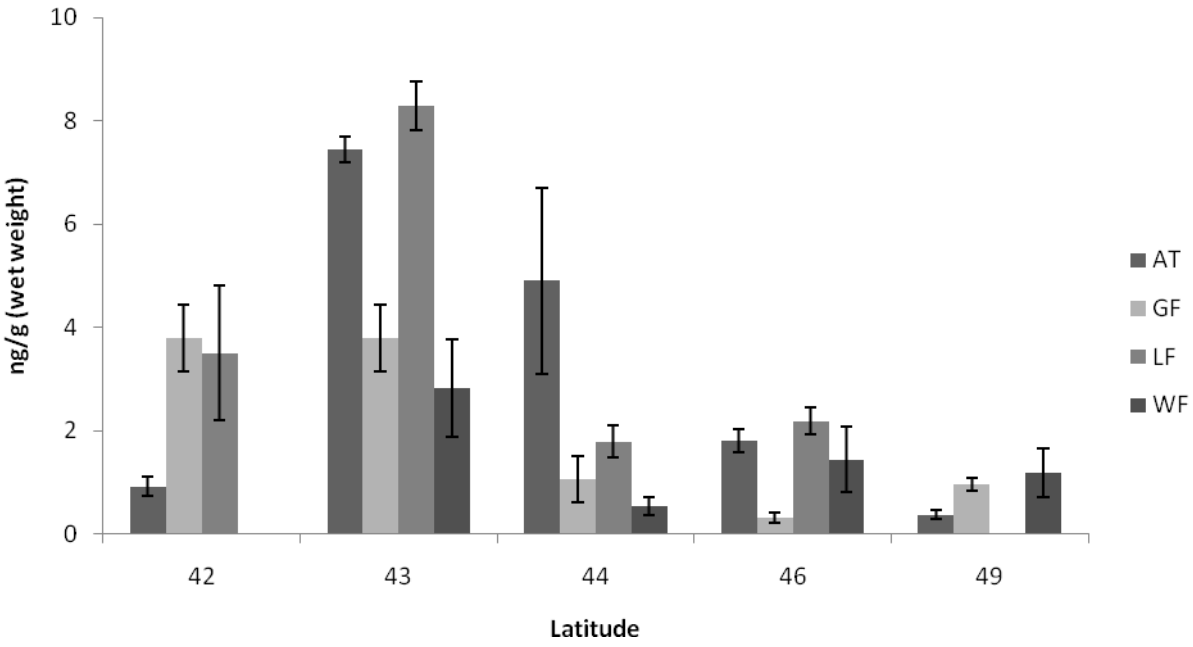

Figure 2.3 Average PCB concentration (ng/g wet weight) by amphibian species and sampling location. Error bars represent standard error. AT=American Toad, GF=Green Frog, LF=Leopard Frog, and WF=Wood Frog. Total PCB concentrations were significantly higher in Wellington County, lat. 43° (p=0.001).

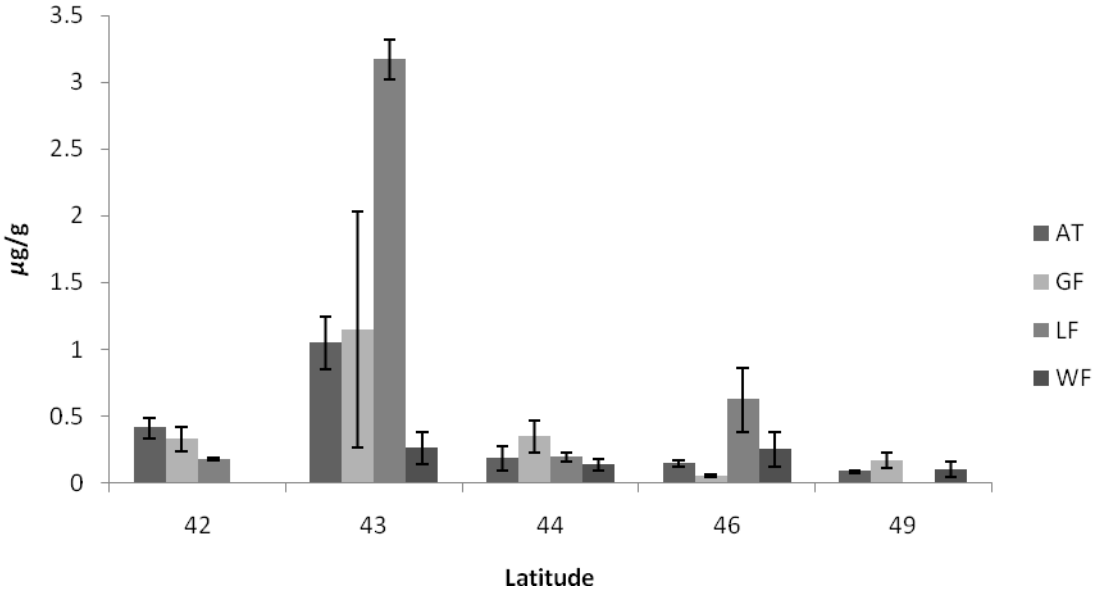

Figure 2.4 Lipid corrected PCB concentration (μg/g) by amphibian species and sampling location. Error bars represent standard error. AT=American Toad, GF=Green Frog, LF=Leopard Frog, and WF=Wood Frog. Lipid corrected total PCB concentrations were significantly higher in Wellington County, lat. 43° (p=0.002).

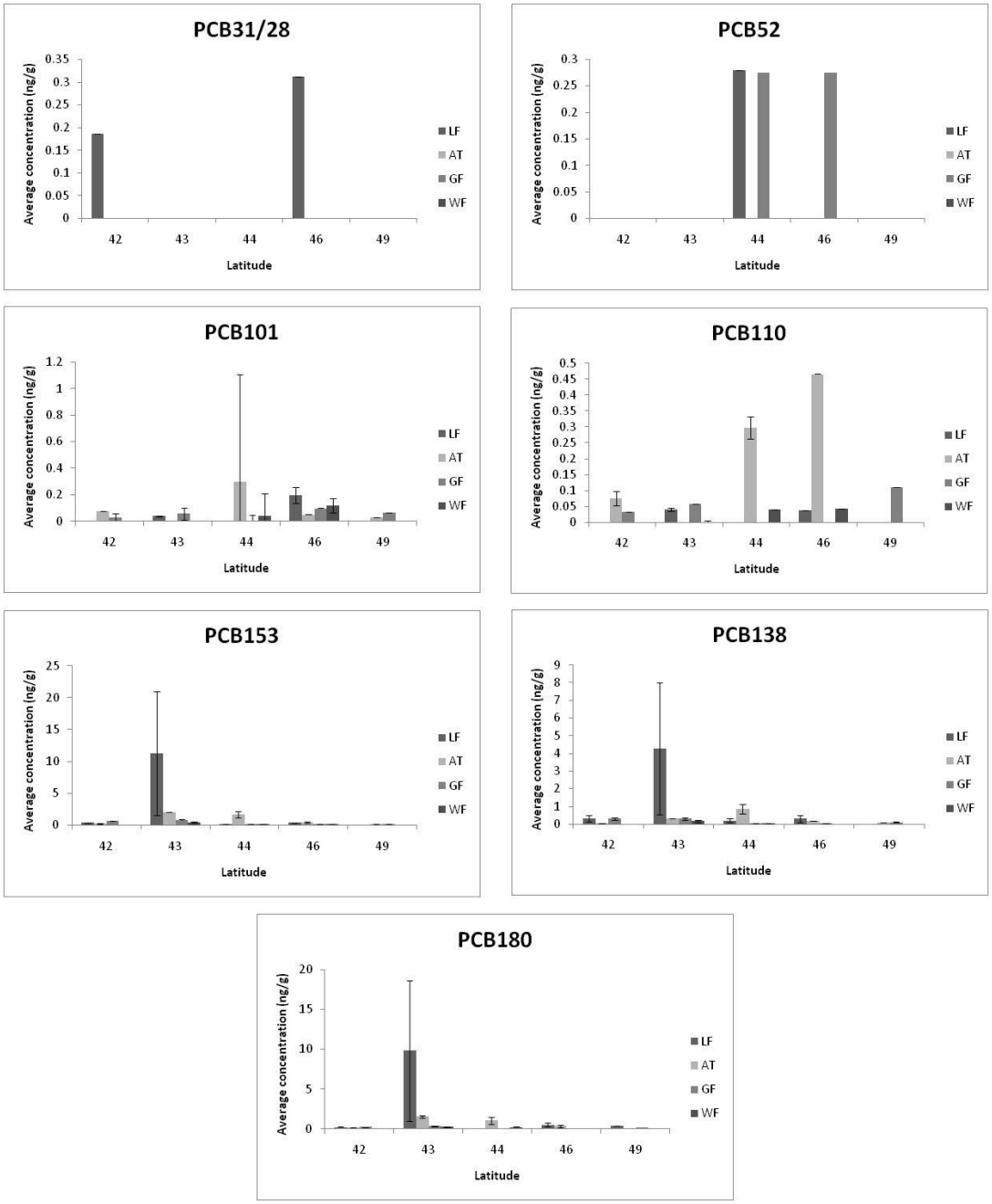

Figure 2.5 Average wet weight concentrations in ng/g for sample congeners (K_{ow}=low, medium, and high) for four amphibian species (LF=Leopard Frog, AT=American Toad, GF=Green Frog, WF=Wood Frog) at five different sampling sites in Ontario, Canada. Error bars represent standard error. Concentrations of PCB congeners 153 and 180 were significantly higher in Wellington County frogs (lat. 43° PCB 153 p=0.001, PCB 180 p=0.03). Concentrations of congeners 153 and 180 were significantly higher in leopard frogs (PCB 153 p=0.02, PCB 180 p=0.005).

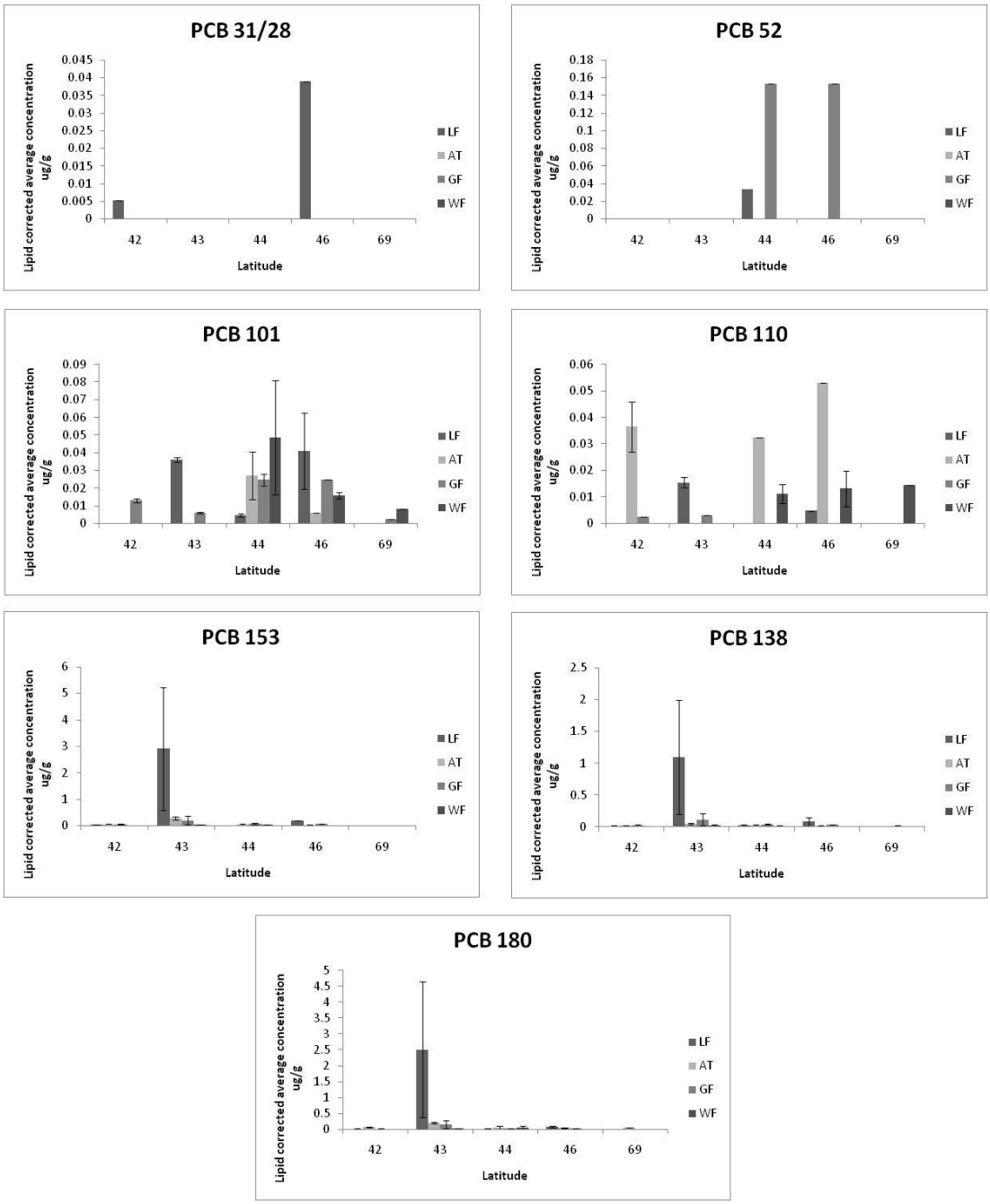

Figure 2.6 Lipid corrected average PCB concentrations in μg/g for sample congeners (K_{ow}=low, medium, and high) for four amphibian species (LF=Leopard Frog, AT=American Toad, GF=Green Frog, WF=Wood Frog) at five different sampling sites in Ontario, Canada. Error bars represent standard error. Concentrations of PCB congeners 153, 138, and 180 were significantly higher in Wellington County frogs (lat. 43° PCB 153 p=0.001, PCB 138 p=0.01, PCB 180 p=0.01). Concentrations of PCB 110 were significantly higher in American toads (p=0.01). Concentrations of PCB congeners 153 and 180 were significantly higher in Northern leopard frogs (PCB 153 p=0.02, PCB 180 p=0.006).

CHAPTER 3 – POLYCHLORINATED BIPHENYL ELIMINATION RATES AND CHANGES IN CHEMICAL ACTIVITY IN HIBERNATING AMPHIBIANS

3.1 Introduction

Many species worldwide are in decline, resulting in a loss of biodiversity. Over the course of the last 20 years, a major decline has been observed in amphibian species (Ankley et al., 2004). Many stressors have been suggested as contributing to this decline, including the introduction of chemical pollutants and pesticides into natural systems (Ankley et al., 2004). In order to resolve the relative potential of environmental chemicals affecting the observed decline in amphibian species, it is essential to develop life cycle models. These models require specific information on chemical uptake and elimination rates. Amphibian elimination studies, using polychlorinated biphenyls (PCBs), are able to provide a calibrated model for amphibian chemical metabolism at key lifecycle stages. Previous studies using frogs have shown an increase in the chemical activity, or fugacity, of PCBs during metamorphosis, due to the accelerated use of lipids being absorbed from the tail (Leney et al., 2006b). This same increase in fugacity is predicted to occur at other points of the amphibian lifecycle where stored lipids are being utilized as an energy source.

Hibernation, or burmation, is a critical period when amphibians are forced to rely on stored lipids. Hibernation is unique to cold adapted amphibians; hibernating amphibians are at increased risk of infection, predation, and death by hypoxia (Weber, 2009; Beebee, 1996). Frogs occur further North in all geographical areas than other ectotherms; aquatic ranid tadpoles are the only larval stage amphibians that have been found to consistently overwinter (Feder and Burggren, 1992). Some hibernating amphibians are able to adjust their blood chemistry to rely on

cutaneous gas exchange, but amphibians have generally been found to be less successful than other hibernating vertebrates in hypoxic conditions (Boutilier et al., 1997). Lipids are the primary stored energy source during hibernation, supplying a fuel reserve to last for up to 8 months and for immediate breeding activity in the spring (Fitzpatrick, 1976; Feder and Burggren, 1992). In the fall, lipid and glycogen are heavily stored in the liver and fat bodies of amphibians (Feder and Burggren, 1992). Cold adapted amphibians go underwater or bury underground or layers of leaf litter to overwinter (Beebee, 1996); hibernating on land avoids the risk of hypoxia, and hibernating in the water lessens the risk of freezing (Feder and Burggren, 1992). Amphibians enter a hypometabolic state of torpor, cued by epinephrine and thyroid hormonal signals, when submerged and exposed to low temperatures that lower metabolic rates and potentially regulate the biotransformation of PCBs (Feder and Burggren, 1992;Donohoe et al, 1998). Chemical elimination rates for PCBs during amphibian hibernation have yet to be quantified. The development of a life-cycle model for the elimination rates of pollutants such as PCBs is essential in predicting the hazard of manmade chemicals relative to the observed decline of amphibians.

The primary goal of this study was to quantify the rate that amphibians were able to eliminate and metabolize PCBs during hibernation. Previous studies of green frog tadpoles, metamorphs, and adults concluded that elimination rates were slowest in the adult phase and highest during the metamorph stage (Leney et al., 2006c). Leney, et al. (2006c) also concluded that fugacity increased during the metamorph stage of green frogs with lipid content decreasing relatively rapidly as a result of tail adsorption. We hypothesized that PCB elimination rates will be very slow for overwintering adult green frogs, and that chemical fugacity will increase due to a decline in stored lipids in hibernating frogs.

3.2 Materials and Methods

3.2.1 Experimental Design

An elimination experiment was performed using 67 adult green frogs (*Rana clamitans*) ranging in size from 15.28 to 41.97 grams (mean mass of 27.78 g). Frogs were collected during October, 2007 from ponds at Leadley Environmental Co., an aquaculture facility in Essex County near Windsor, Ontario, Canada. 57 frogs were dosed with 1 µg/g wet weight PCB mixture (1:1:1 ratio of Aroclors 1248:1254:1260 in sunflower oil) and sampled on days 0, 21, 62, and 85 with day 0 occurring October 21, 2007. 10 control frogs were injected with 1 µg/g clean sunflower oil and collected at the start (day 0) and termination (day 180) of the experiment. After dosing, frogs were split into two groups and transferred into cages in two separate ponds. To control for PCB recycling and contamination, control frogs were also placed into the same ponds as the experimental organisms in a separate caged area. The two experimental ponds contained sediment from local ponds where the frogs were collected, and floating plants and cinderblocks were provided for cover and to enable the frogs to climb out of the water before entering into the hibernation phase.

Frogs were immediately homogenized upon collection using hexane and acetone rinsed blenders and stored in hexane rinsed aluminum foil at -20°C until analysis. Samples were analysed following the termination of the experiment. Whole body PCB concentrations were analyzed.

3.2.2 Analysis

Methods for PCB analysis are described in specific detail in by Leney, et al. (2006a). To summarize, mortar and pestle were used to pulverize a 2 gram subsample of each homogenized sample with sodium sulphate. The ground sample was then packed into a glass column with 50 mL of 1:1 dichloromethane: hexane. Each column was spiked with 100 µl of tri-bromo-benzene spiking standard at a concentration of 125 ng/g in order to assess recovery correction during analysis. Another 250 mL of 1:1 dichloromethane: hexane was added and the column stood undisturbed for a minimum of 1 hour before elution. Collected extracts were concentrated to 10

mL and 10% of each extract was removed for lipid concentration analysis. The extract was then added to a glass column containing 6 grams of florisil and eluted with 50 mL of hexane for lipid cleanup. Extracts were concentrated and transferred to vials with a final volume of 1 mL in 2,2,4-trimethylpentane. A method blank column and a column containing fish homogenate from the Detroit River (reference sample for quality assurance) were processed alongside each set of samples.

Chemical analysis of extracted samples for PCBs was performed using a Hewlett-Packard 5890 gas chromatograph with a 5973 mass-selective detector and a 7673 autosampler. A secondary standard and spiking standard were run with each set of samples. Congeners were identified by retention time and by molecular ion and quantified by comparison to the peaks in a secondary standard. Recovery of the spiking standard calculated from (13C) PCB 153 was 73±3.4% (mean± SE). Recoveries of PCB congeners 180 and 138 in the reference homogenate tissue extracted alongside each sample set were within one and two standard deviations, respectively, of the mean value from the laboratory control charts maintained by the organic analytical laboratory at the Great Lakes Institute for Environmental Research, a Canadian Association for Laboratory Accreditation Inc. certified facility. PCB concentrations in method blanks were below the machine detection limit of 0.05 ng/g.

3.2.3 Modeling

Elimination rate constants for PCB congeners were assessed using the one compartment first order rate constant model previously described by Leney, et al. (2006a). This model encompasses all possible routes of chemical elimination from the organism. For elimination experiments where the test organism is dosed with a chemical and placed into a clean system (no uptake) the following equation is used:

$$\frac{dC_{org}}{dt} = -k_2 C_{org}$$

this equation can be rearranged to derive:

$$\ln C_{org(t)} = \ln C_{org(t=0)} - k_2 t$$

where $C_{org(t)}$ represents the mass of chemical (in ng) in the organism at time t (days), $C_{org(t=0)}$ represents the mass of chemical in the animal at the beginning of the elimination experiment, and k_2 is the total chemical elimination rate constant in units of d^{-1}. From this the equation to calculate the elimination rate constant, k_2, can be derived:

$$k_2 = \frac{\ln C_{org(t=0)} - \ln C_{org}}{t}$$

Elimination rate constant, k_2, values were determined by plotting the natural log of the chemical concentration of individual PCB congeners in each sample against sampling date in days. The slope of the regression line for each congener resulted in the k_2 value. Regression analysis of concentration against sampling day was performed to determine whether k_2 values were significant.

Once the k_2 value has been determined, the time to a 90% steady state .was calculated using the following equation:

$$t_{90} = \frac{\ln 10}{k_2}$$

Chemical activity, or fugacity, is inversely related to the capacity of an organism, which is inversely dependent on the amount of lipid, and directly related to chemical concentration. As lipid decreases within an organism, the capacity to contain PCBs within an organism decreases, and chemicals are placed under greater pressure to partition into other phases such as the circulatory system. Fugacity (f measured in Pa) of an organism can be measured as a function of

the whole body concentration of chemical in mol x m^{-3} (C) and the capacity of the organism in mol x m^{-3} x Pa^{-1} (Z) such that

$$f = \frac{C}{Z}$$

Fugacity capacity (Z) can be estimated as the concentration of lipids in the sample (X_L), the K_{ow} of the congener being assessed, and the Henry's law constant value in Pa x m^3 x mol^{-1} (H) .

$Z \sim \frac{X_L K_{ow}}{H}$ (Mackay and Paterson, 1981)

thus:

$$f \sim \frac{CH}{X_L K_{ow}}$$

Fugacity increases have been observed during hibernation, with increased toxic effects being a predicted outcome (Macdonald et al., 2002).

3.3 Results

Mortality rates for this experiment were greater than 50% as a result of a complete die off in one of the experimental ponds; as a result the experiment was ended after 85 days because of insufficient numbers of frogs available for day 180. As there were no surviving animals in the disease affected pond, only animals from one pond were sampled.

A regression analysis was performed to determine if the lipid content of sampled frogs changed during the experiment. A significant decrease in lipid mass of sampled frogs was observed over the sampling period. Mean lipid at the beginning of the experiment was 2.2 ± 1.0% of total mass (mean±standard error) and declined to 0.89±0.18 of total mass in the final control. Figure 3.2 illustrates the decline of lipid content over the duration of the experiment. The coefficient of determination for linear-regression analysis (r^2) value derived from regression

analysis of lipids over the course of the study indicated that 25% of the variability of lipid could be accounted for by time of hibernation.

Table 3.1 summarizes the elimination rate constants for individual PCB congeners as well as the corresponding t_{90} values. Significant ($p<0.05$) PCB elimination rate constants (k_2) ranged from 0.0027 to 0.04 d^{-1} and t_{90} were in the range of 61 to 7675 days, suggesting only a few low K_{ow} congeners had the potential to achieve steady state during the period of hibernation. Non significant and non-calculable k_2 values were the result of either fast elimination rates with too few time points (low K_{ow} congeners), or very slow elimination such that there was no significant loss of chemical over the sampling period (very high K_{ow} congeners).

Low (PCB 31/28, PCB52), medium (PCB 101, PCB 110), and high (PCB 153/132, PCB 138, PCB 180) K_{ow} congener concentrations were plotted vs. sampling date (Figure 3.3). These congeners are resistant to metabolism and revealed that for passive elimination there is a negative relationship between k_2 and K_{ow} There was, however, no significant relationship between elimination rate and K_{ow} for all congeners with significant elimination rates. Figure 3.4 suggests that biotransformation processes continued to operate during hibernation, and metabolic activity was sufficient in hibernating frogs to confound the relationship between k_2 and K_{ow} over the course of the experiment. Significantly eliminated congeners showed a negative correlation for k_2 vs. log K_{ow}, but the relationship was not significant ($p=0.49$; Figure 3.4) supporting the conclusion that biotransformation mechanisms of chemical elimination are relatively important in the hibernating frogs. These results indicate that elimination rates in hibernating amphibians are a result of both passive elimination and metabolism. In either case, elimination rates were very low compared with those observed in active adults (Leney et al., 2006a). It is not known if the metabolic activity was a response to a warm period during the winter, and might be an artifact of the winter warm spell experienced during this study. The relationship for k_2 vs. log K_{ow} for all measured congeners was significant ($p=0.002$; Figure 3.4).

Fugacities of congeners PCB 31/28, PCB 52, PCB 101, PCB 110, PCB 153/132, PCB 138, and PCB 180 at each sampling date were plotted as a ratio of fugacity (*f*) at Day 0 to illustrate relative change in fugacity during hibernation (Figure 3.5).

Fugacity increases were observed for the high K_{ow} congeners (PCBs 153/132, 138, and 180) which were being eliminated more slowly than lipid contents were being depleted during hibernation. Relative change in fugacity for these congeners was positively correlated to log K_{ow} (Figure 3.6; r^2=0.5918, p<0.001). Congeners with a Log K_{ow} value below 7.0 tended to decrease in fugacity while congeners with a Log K_{ow} over 7.0 tended to experience an increase in fugacity.

Relative fugacities of congeners belonging to metabolic groups 2 (r^2=0.6941, p<0.001) and group 3 (r^2=0.2165, p=0.04) were significantly related to K_{ow} (Figure 3.7). There was a significant difference (p<0.001) in relative fugacity between congeners belonging to metabolic group 2 and more recalcitrant congeners in group 3 (Figure 3.7).

3.4 Discussion

Although high hibernation survival (>80%) rates of amphibians have been observed in laboratory conditions at constant above freezing temperatures (James et al., 2004), mortality rates of close to 50% of a monitored Ranid frog population have been observed and attributed to the stresses related to amphibian hibernation in field conditions (Maniero and Carey, 1997). Similar mortality rates have been observed in salamander populations overwintering in caged habitats (Vernberg, 1953). Although relatively high mortality rates were anticipated in selecting the number of frogs to be used for this study, the actual mortality rate of approximately 70% limited the duration of the experiment. The unexpectedly high mortality rate was largely a result of the loss of 30 test organisms to disease during a mid-winter warm spell. During this period water temperatures rose to >4°C, and encouraged some individuals out of hibernation. Such an event places a high energetic cost on adult frogs resulting in increased lipid utilization and also making

them susceptible to disease as amphibian immune functioning is reduced at colder temperatures (Maniero and Carey, 1997). Low body temperatures during winter have been observed to result in delayed growth and spread of pathogenic organisms within amphibians (Ratkowsky et al., 1982). When temperatures fluctuate during this time period, or winter temperatures are warmer, the bacteria and fungus causing infection in amphibians are able to thrive while amphibian immune response is still compromised.

Elimination rates for hibernating adult green frogs in this study ranged from 0.0027 to 0.04 d^{-1} compared to 0.013 to 0.04 d^{-1} in adult green frogs under ambient conditions in a study by Leney, et al. (2006a; Figure 3.1). Elimination rate constants were lower for hibernating frogs than adult frogs under ambient temperatures, and much lower than tadpole and metamorph stage green frogs (Figure 3.1). The lower k_2 values observed during hibernation reflected lower metabolic rates, but as basal metabolism was still evident with the biotransformation of group 2 congeners during the study.

Lipid significantly decreased during hibernation ($P<0.05$; Figure 3.2) from 2.2 ± 1.0% of total mass at the beginning of the experiment (mean±standard error) to 0.89% of total mass on the last sampling day. Lipids are preferentially consumed during hibernation as lipid yields a higher energy return than carbohydrate or protein metabolism (Donohoe et al., 1998). Adult frogs start to metabolize carbohydrates rather than lipids in the latter stages of hibernation, saving remaining lipids for post-hibernatory breeding activities (Donohoe et al., 1998). Lipid declines are steeper at warmer winter temperatures as amphibians are operating at a higher metabolic level with few available food sources (Holenweg and Reyer, 2000). This is a concern as the planet is currently experiencing a period of global warming, with minimum temperatures increasing (Walther et al., 2002). The fugacity of environmental pollutants in cold blooded organism such as amphibian will be expected to vary with global temperature change due to changes in lipid dynamics (Macdonald et al., 2002). Amphibian weight in hibernating Ranid species is negatively correlated

to warmer winter temperatures, with female frogs losing more weight than male frogs (Holenweg and Reyer, 2000). Shifting winter and spring temperatures have been correlated with earlier spawning in Ranid frogs (Beebee, 1995; Terhivuo, 1988). The primary storage site for lipids in frogs is the abdomen, with these abdominal fat storage bodies directly storing energy for the gonads and use in reproduction. Reduction of this lipid store, as occured during hibernation in this experiment, severely handicaps yolk production (Rose, 1967) at the same time causing increased chemical activity in the amphibian at a critical point of its life cycle. Ranid amphibian eggs have been previously shown to have approximately 5x greater lipid concentrations and 2x greater PCB concentrations (by wet weight) compared to the female frogs that produced the eggs (Kadokami et al., 2004). This maternal transfer of PCBs has direct implications for the chemical activity in eggs and offspring; the increased chemical activity observed in hibernating amphibians in this study would be amplified within offspring. A global warming trend will result in increased rates of lipid loss during hibernation, augmenting toxic chemical stress at a critical point of the amphibian life cycle, reproduction.

Amphibian elimination studies conducted during tadpole and metamorph stages have found PCBs with *meta-para* vicinal hydrogens, commonly called group 2 PCBs, are more readily metabolized than congeners with *ortho-meta* vicinal hydrogens, group 3 PCBs (Leney et al., 2006c). Group 2 congeners are more readily metabolized due to the activity of the cytochrome P-450 2B isozyme (Kannan et al., 1995). The same trend of higher relative change in fugacity for group 3 PCBs than group 2 PCBs was observed in metamorphs (Leney, 2006c) and hibernating adult amphibians. The difference in relative change in fugacity between metabolic groups was significant for metamorphs (Leney et al., 2006c) and hibernating frogs (Figure 3.7). Generally, it would appear that during metamorphosis and hibernation, when anurans are relying on internal energy supplies, they become metabolically more active with respect to enzymatic processes (Leney et al., 2006c; Figure 3.7). There is little evidence of strong metabolic signals in

elimination by adult frogs when actively feeding, but there is gathering evidence that during periods of lipid utilization cytochrome P-450 (Phase 1) metabolic activities become pronounced. It is not known if this increase in Phase 1 activity is matched by an increase in the conjugation of cytochrome P-450 metabolites (Phase 2 metabolism).

3.5 Conclusions

This study determined that hibernating green frogs were metabolically active, resulting in reduced lipid concentrations. The negative correlation between K_{ow} and k_2 previously observed in tadpole, metamorph, and ambient temperature adult green frogs was observed in hibernating frogs in this study, but chemical elimination rate constants in hibernating adult green frogs were at least an order of magnitude lower than those observed during summer conditions. Fugacity increases as a result of lipid loss during hibernation were primarily observed in high K_{ow} congeners. The observed increase in chemical activity during hibernation has direct implications for increased contaminant exposures in offspring, and this phenomena is predicted to become more of an issue in Northern amphibians as a result of climate change.

3.6 References

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Table 3.1. Elimination rate constants (k_2) of polychlorinated biphenyl (PCB) congeners and time to 90% steady state (t_{90}) in hibernating adult green frogs[ab].

PCB	Metabolic group [c]	Log K_{ow} [e]	k_2 (d^{-1})[e]	r^{2f}	p values [g]	t_{90} [h]
19	4	5.02	-[i]	-	-	-
18	4	5.24	0.0285±0.0120*	0.38	0.042	80.8
17	4	5.25	0.0300±0.0086**	0.57	0.0070	76.8
24/27	4	5.44	0.0027±0.0010*	0.46	0.022	852.8
16/32	4	5.32	0.0164±0.0072*	0.37	0.048	140.4
26	4	5.66	0.0030±0.0105	0.01	0.78	767.5
25	4	5.67	0.0055±0.0071	0.06	0.46	418.7
31/28	3/4	5.67	0.0273±0.0054***	0.74	0.00073	84.3
33/20	4	5.6	0.0270±0.0081**	0.55	0.0087	85.3
45	4	5.53	0.0012±0.2966	0.01	0.81	1918.8
52	2	5.84	0.0253±0.0102*	0.41	0.035	91
49	4	5.85	-	-	-	-
47/48	3/4	5.82	-	-	-	-
44	4	5.75	0.0027±0.0099	0.01	0.79	852.8
42	4	5.76	0.0108±0.0097	0.12	0.30	213.2
64/41/71	4	5.92	0.0376±0.0059***	0.82	0.00013	61.2
40	4	5.66	0.0049±0.0014**	0.56	0.0077	469.9
74	3	6.2	0.0033±0.0029	0.13	0.28	697.8
70/76	4	6.2	0.0142±0.0095	0.20	0.17	162.2
66	3	6.2	0.0201±0.0040***	0.74	0.00066	114.6
95	2	6.13	0.0299±0.0094*	0.53	0.011	77
91	4	6.13	-	-	-	-
92	2	6.35	-	-	-	-
84	4	6.04	0.0086±0.0096	0.08	0.39	267.7
101	2	6.38	0.0269±0.0047***	0.78	0.00030	85.6
99	3	6.39	0.0056±0.0032	0.25	0.12	411.2
97	4	6.29	0.0125±0.0074	0.24	0.13	184.2

87	4	6.29	0.0222±0.0076*	0.48	0.018	103.7
85	3	6.3	-	-	-	-
110	4	6.48	0.0233±0.0095*	0.40	0.037	98.8
118	3	6.74	0.0037±0.0027	0.17	0.21	622.3
105	3	6.65	0.0179±0.0030***	0.79	0.0002	128.6
136	2	6.22	0.0036±0.0081	0.02	0.67	639.6
151	2	6.64	0.0158±0.0061*	0.42	0.030	145.7
144/133	1/2	6.81	0.0033±0.0073	0.02	0.66	697.8
149	2	6.67	0.0296±0.0048***	0.81	0.00016	77.8
134	4	6.55	0.0061±0.0074	0.07	0.43	377.5
146	1	6.89	0.0037±0.0029	0.15	0.24	622.3
153/132	1/4	6.85	0.0069±0.0030*	0.37	0.048	333.7
141	2	6.82	0.0003±0.0081	0.0001	0.97	7675.3
137	3	6.83	-	-	-	-
130	3	6.8	-	-	-	-
138	3	6.83	0.0044±0.0028	0.22	0.14	523.3
158	3	7.02	0.0048±0.0031	0.22	0.15	479.7
128	3	6.74	0.0116±0.0071	0.23	0.14	198.5
156	3	7.18	0.0045±0.0024	0.28	0.091	511.7
157	3	7.18	-	-	-	-
179	2	6.73	0.019±0.0046**	0.65	0.003	121.2
176	2	6.76	-	-	-	-
178	1	7.14	0.0016±0.0033	0.03	0.64	1439.1
187/182	1	7.17	0.0052±0.0027	0.29	0.09	442.8
183	1	7.2	0.0052±0.0026	0.30	0.08	442.8
185	2	7.11	-	-	-	-
174	2	7.11	0.0214±0.0038***	0.78	0.00032	107.6
177	2	7.08	0.0129±0.0039**	0.55	0.0088	178.5
171	3	7.11	0.0055±0.0026	0.33	0.063	418.7

172	1	7.33	-	-	-	-
180	1	7.36	0.0051±0.0026	0.29	0.085	451.5
170/190	3	7.31	0.0049±0.0026	0.29	0.087	469.9
202	1	7.24	-	-	-	-
200	1	7.27	-	-	-	-
199	2	7.2	-	-	-	-
201	1	7.62	0.0049±0.0027	0.27	0.10	469.9
196/203	1	7.65	0.0054±0.0024	0.35	0.054	426.4
195	3	7.56	0.0036±0.0017	0.32	0.071	639.6
194	1	7.8	0.0056±0.0026	0.33	0.063	411.2
208	1	7.71	-	-	-	-
207	1	7.74	-	-	-	-
206	1	8.09	-	-	-	-

[a]No asterisk indicates that no significant elimination of this chemical occurred during the experiment.

*** $p < 0.001$ (analysis of variance), ** $p<0.01$, * $p < 0.05$.

[b] n=11 for all congener analysis

[c]Values from Kannan, et al. (1995) and Leney, et al. (2006a).

[d] Values from Hawker and Connell (1988).

[e] Values are calculated as the mean ± standard error.

[f] Coefficient of determination for linear-regression analysis.

[g] P-value determined by ANOVA.

[h] Calculated as ln $10/k_2$.

[i] Unable to calculate elimination rate.

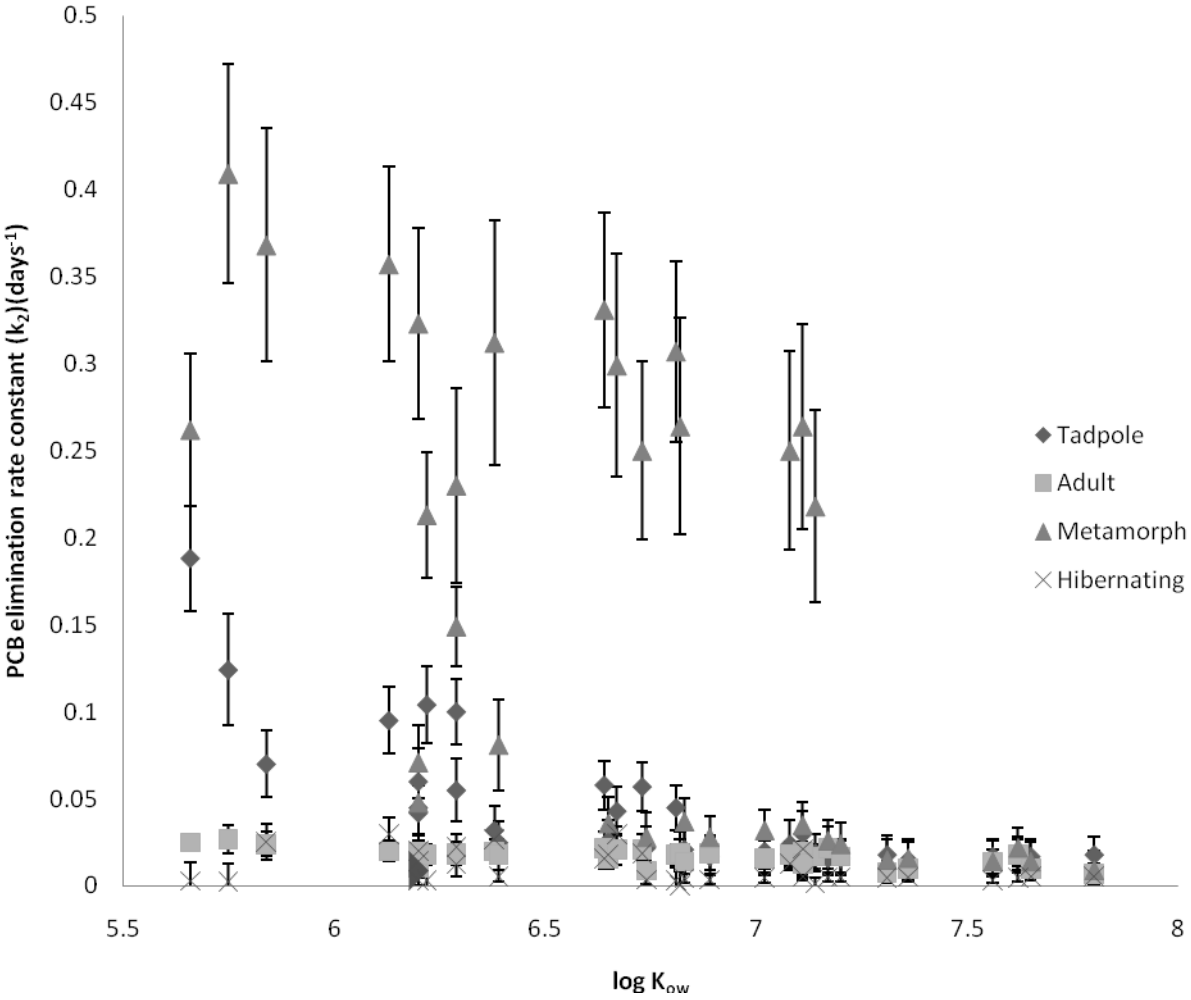

Figure 3.1 PCB elimination rate constants (k_2) for tadpole, metamorph, adult at ambient temperature, and hibernating adult green frogs. Error bars represent standard error. Figure includes only PCBs for which elimination rate constants could be calculated for all four life cycle stages. Tadpole, metamorph, and adult elimination rate constants from Leney, et al., 2006a and Leney, et al., 2006c.

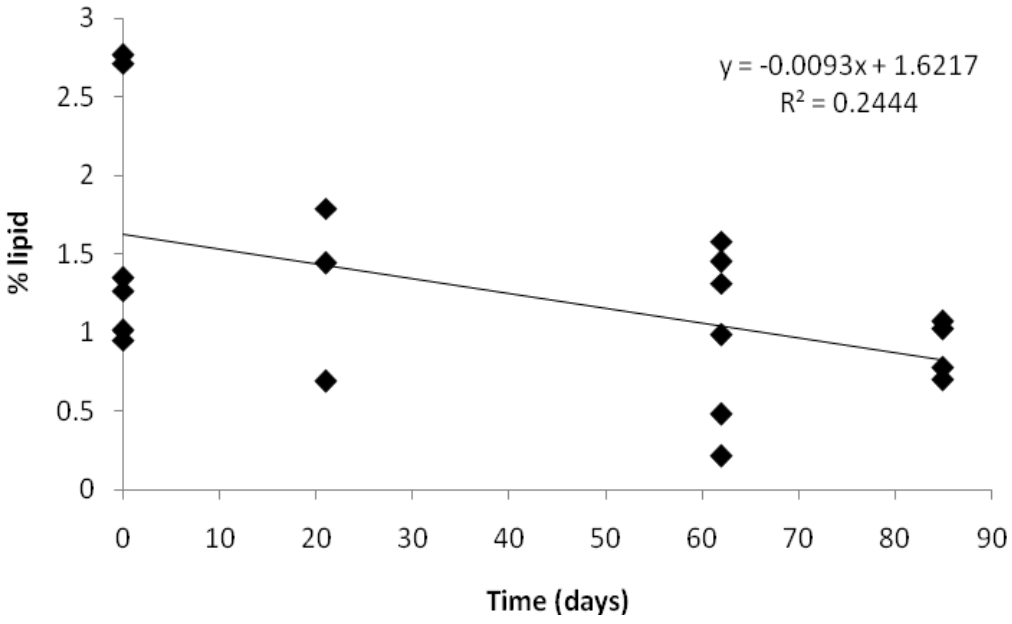

Figure 3.2 Regression analysis of lipid measured as percentage of total mass. The slope of the trendline for percent lipid vs. time (days) was -y = -0.0093x + 1.6217 (r^2= 0.2444, p=0.03).

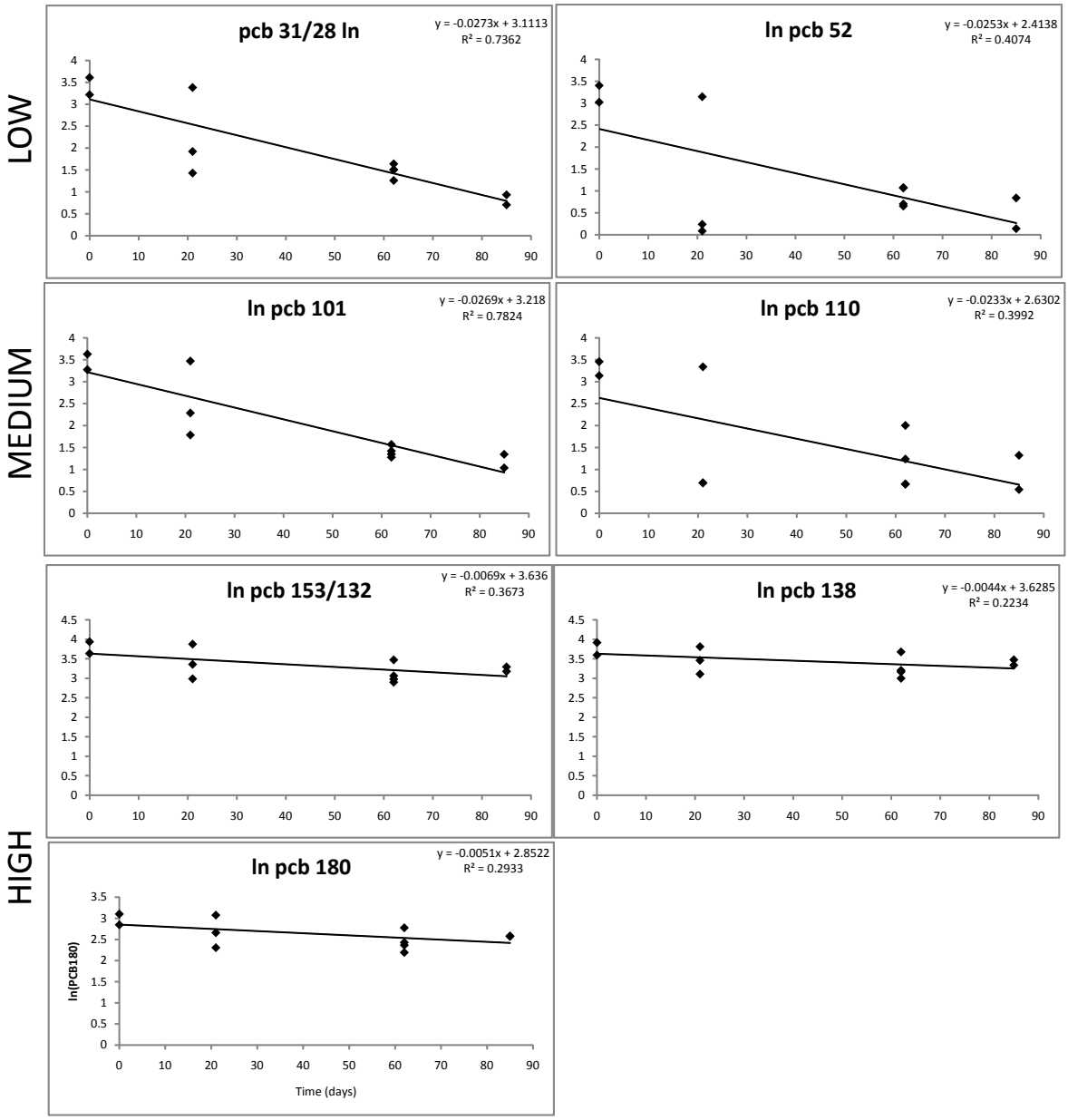

Figure 3.3 Example regressions for sample congeners (Kow =low, medium, and high). PCBs 101 p=0.0003, 31/28 P=0.0007, 52 p=0.03, 110 p=0.04, 153/132 P=0.048, 138 p=0.14, and 180 P=0.09.

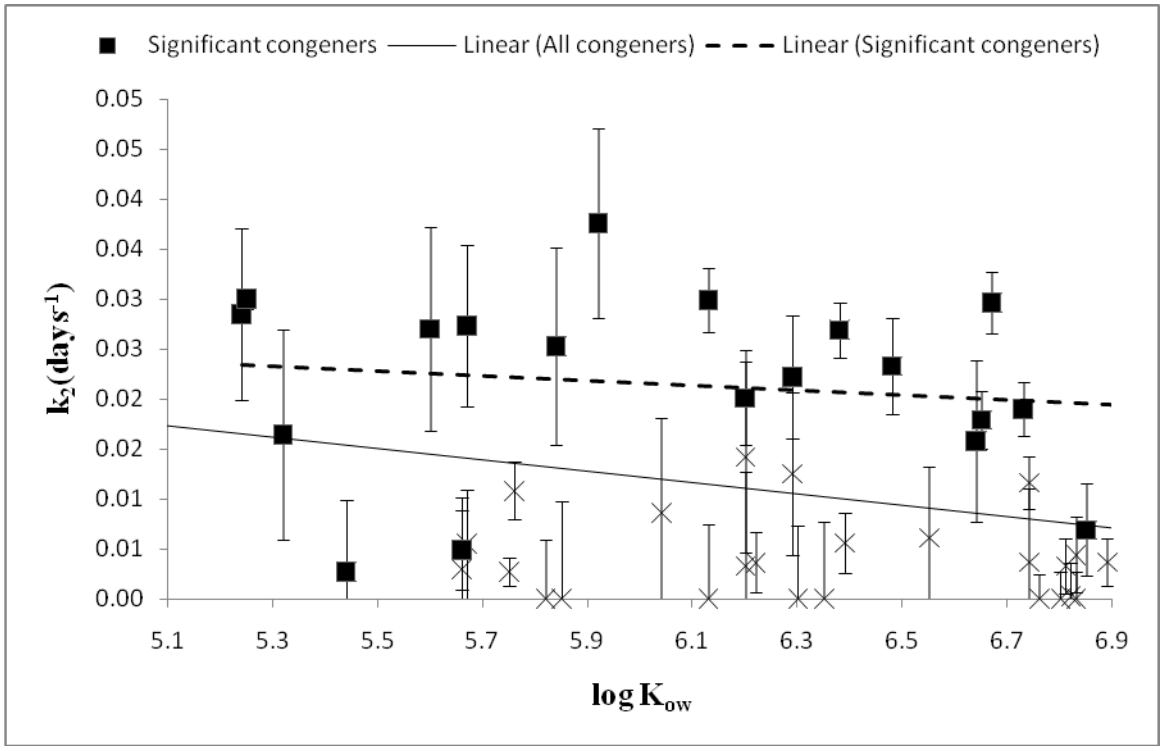

Figure 3.4 Chemical elimination rate constant (k2) vs. hydrophobicity (logKow) for both significantly eliminated (square markers) and all measured (cross markers and square markers) polychlorinated biphenyl (PCB) congeners in green frogs. The trendline (dashed line) slope for significantly eliminated congeners was y = -0.0024x + 0.0359 (r2=0.0257, p=0.49). The trendline (solid line) slope for all measured congeners was y = -0.0057x + 0.0465 (r2=0.1644, p=0.002).

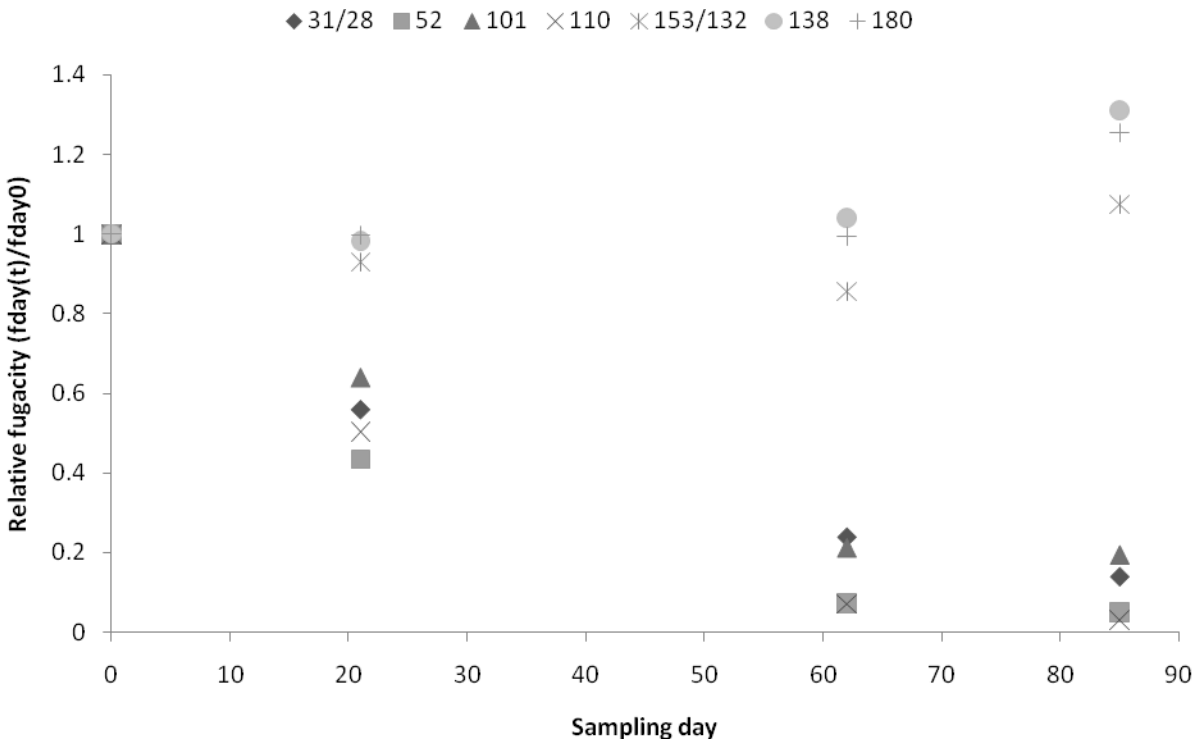

Figure 3.5 Relative fugacity ($f_{day(t)}/f_{day0}$) for select low (PCB 31/28, PCB52), medium (PCB 101, PCB 110), and high (PCB 153/132, PCB 138, PCB 180) K_{ow} congeners vs sampling day.

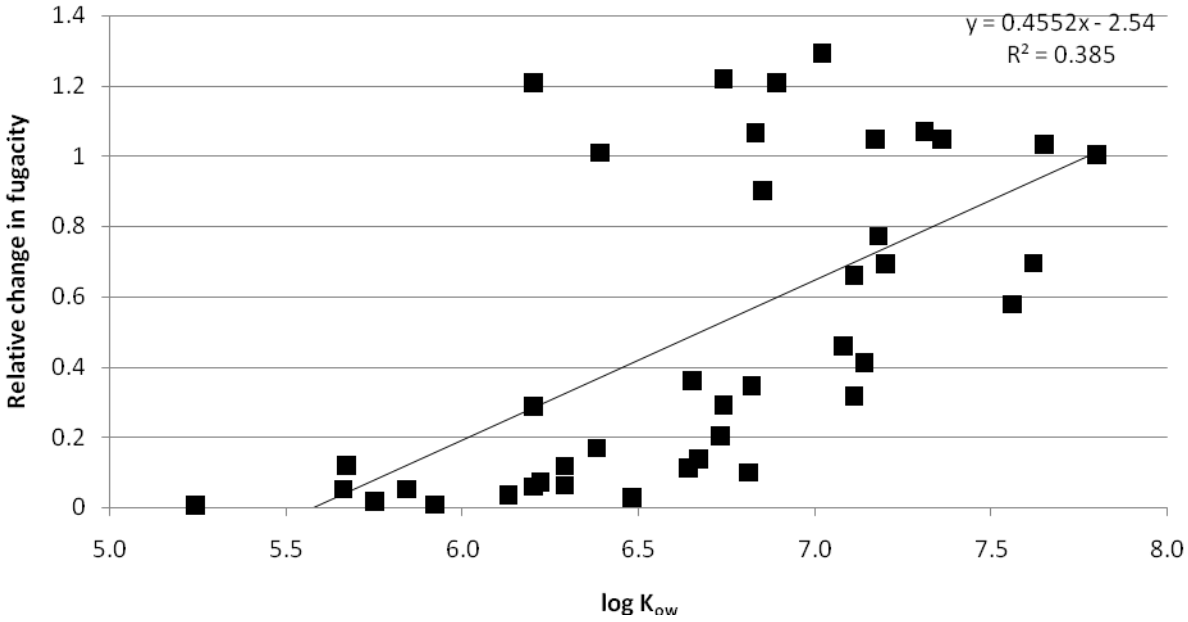

Figure 3.6 Relative change in fugacity of all PCB congeners for which elimination rate could be calculated (R^2=0.385, P= $1.5x10^{-5}$).

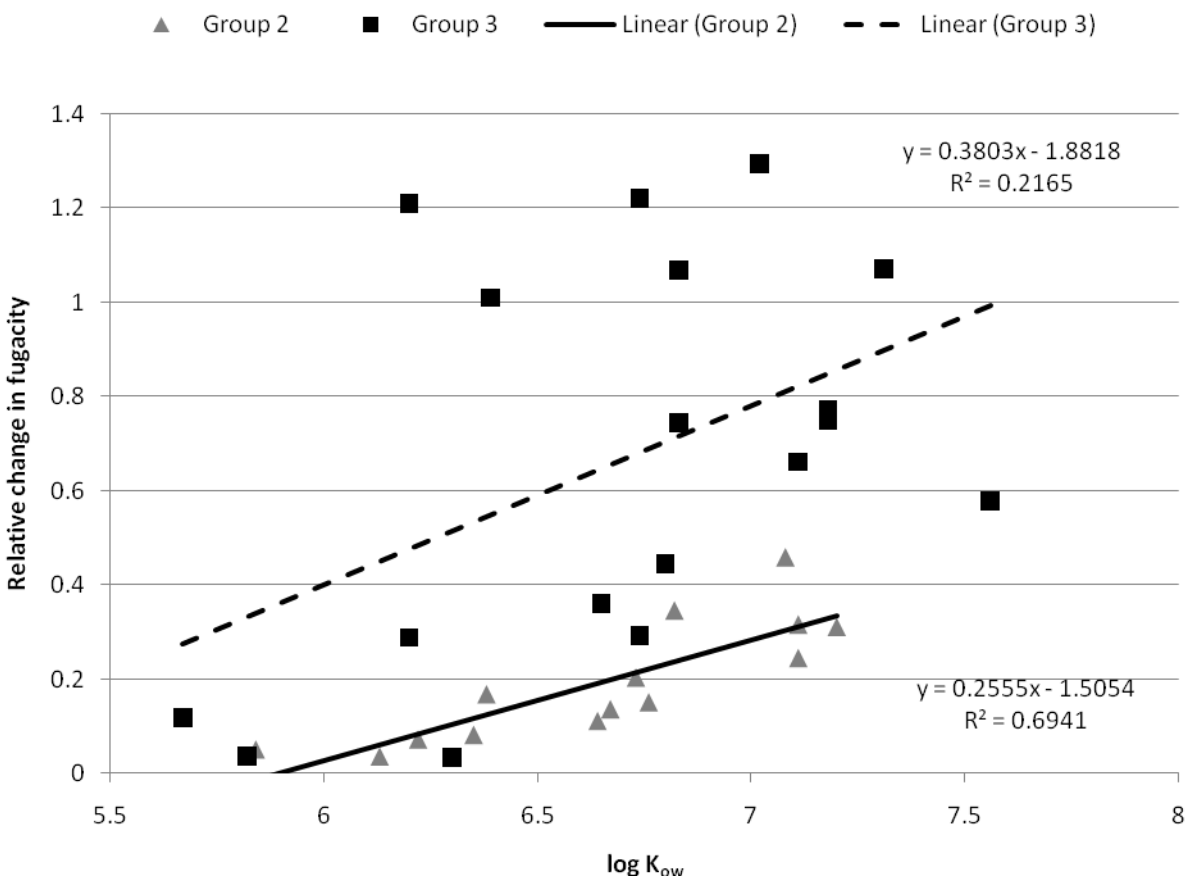

Figure 3.7 Relative change in fugacity of metabolic group 2 and metabolic group 3 PCB congeners. The trendline (dashed line) slope for group 3 was y =0.3803x – 1.8818 (r^2=0.2165, p=0.04). The trendline (solid line) slope for group 2 congeners was y = 2555x – 1.5054 (r^2=0.6941, p=5.0x10^{-6}). There was a significant difference (p=0.002, Kruskal-Wallis One-way Analysis of Variance Systat 12 for Windows) in relative fugacity between groups 2 and 3.

CHAPTER 4 – Conclusions

This thesis examined contaminant accumulation in newly transformed amphibian young of year and contaminant elimination during amphibian hibernation. Chemical concentrations and lipid levels were measured in anuran species to assess inter-specific differences in contaminant body burden. Elimination processes during the hibernation of amphibians in temperate regions were quantified.

Chapter 2 determined that there are inter-species differences in lipid and PCB mass in different frog species. As lipid and PCB concentration are the two factors directly regulating fugacity, these data support previous research suggesting that life cycle and related bioenergetic processes are regulating factors for chemical metabolism in amphibians. Thus, the hypothesis tested in Chapter 2 was that:

2.1. Fugacity models predict there will be no differences in contaminant lipid concentrations among amphibian species, and that life histories do not regulate contaminant accumulation patterns.

Hypothesis 2.1 was rejected; although few lipid variations were statistically significant, lipid mass was highly variable between species and between sampling locations. Total PCB concentrations and total lipid corrected PCB were highly variable between species. Interspectific differences in PCB accumulation were observed at all sampling sites.

Further inspection of interspecific differences in life cycle processes with relation to chemical activity could help to explain the species specific declines and extinctions of amphibians. Although disease is purported to be the major driver of extinctions, exposures to environmental chemicals have been demonstrated to lower immune responses making organisms more susceptible to disease.

Chapter 3 determined that hibernating green frogs were metabolically active, resulting in reduced lipid concentrations. Chemical elimination rate constants in hibernating adult green frogs were at least an order of magnitude lower than those observed during summer conditions. Fugacity increases as a result of lipid loss during hibernation were primarily observed in high K_{ow} congeners. The observed increase in chemical activity during hibernation has direct implications for increased contaminant exposures in offspring, and this phenomena is predicted to become more of an issue in amphibians as a result of climate change. Thus, the hypotheses tested in Chapter 3, that:

3.1. There will be no significant changes in lipid in adult frogs during hibernation.

3.2. There will be no elimination of PCBs by adult frogs during hibernation.

Hypothesis 3.1 was rejected; there was a significant decrease in total body lipid over the course of the study. Hypothesis 3.2 was partially rejected; elimination of low K_{ow} PCBs did occur during hibernation.

The two studies presented in this thesis add to the body of evidence for the importance of bioenergetic processes in chemical accumulation and elimination. In Chapter 3, the lipid utilized during hibernation directly contributed to the increase in fugacity, which was compounded by the state of hibernation decreasing the already low metabolism of PCBs in adult green frogs. Chapter 2 found highly variable PCB and lipid concentrations between species and sites. Simple fugacity models failed to explain the results of either study. Additionally, the results of Chapter 2 provide counter evidence to chemical activity models developed using a single species. The sum total of the observations contained within this thesis is that rather than chemical properties predicting accumulation, bioenergetic processes appear to be driving contaminant accumulation and activity.

In a case such as the worldwide amphibian decline, where a major cause has been pinpointed as disease driven by global warming, it is important to research the factors

contributing the decline and spread of disease. Especially so are the toxicokinetics of cold resistant Northern amphibians, whose fitness is in many respects most dramatically affected by global warming. As the mean cool temperatures rise faster than the warm, hibernation in warm years directly effects fitness of egg bearing amphibians. The results of the research presented in this thesis, that lipids are significantly decreasing during hibernation and PCB congeners are eliminated slower than in other life stages, would result in increased chemical fugacity for amphibians emergent from hibernation and increases in the chemical body burden of their offspring. If hibernation is disrupted by climate change and this effect is intensified, the end result will be chemicals which are known to suppress immune function in amphibians becoming more chemically active and more likely to bind to receptor sites. Further immune suppression combined with the effects of a global warming trend would intensify the already dramatic declines observed with the chytrid fungus. Taking into account the interspecific differences in contaminant accumulation observed in the course of the research for this thesis, the amphibian life cycle model developed from research on green frogs becomes more complicated and difficult to apply as a general model.

In conclusion, this thesis found that there are inter-species differences in both lipid concentration and PCB body burden for young of year anurans. These differences are concluded to be regulated by bioenergetic processes including length of breeding season, length of tadpole stage, and species differences in resource utilization during hibernation and metamorphosis. This thesis found that there is an increase in chemical activity (fugacity) during hibernation due to lipid utilization and lowered PCB elimination rates. This increase in fugacity poses a potential hazard for both the hibernating organism and its offspring. Bioenergetic processes emerged as the dominating factors influencing chemical dynamics in both studies presented in this thesis. It is apparent that bioenergetic modeling needs to be tied into amphibian kinetic models if a meaningful life cycle model is to be produced.

Further areas for future study include modeling chemical metabolism during the time leading up to hibernation in temperate zone amphibians. Also, very little is understood about chemical elimination and activity during aestivation, a state similar to hibernation that desert amphibians enter during extreme summer heat. Although it is accepted that there is a worldwide amphibian decline, the interactions between factors involved in this decline remain unclear but the effects of environmental chemicals cannot be ruled out.

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