Variation in Habitat Use and Its Consequences for Mercury Exposure in Eastern Ontario Bats (*Myotis lucifugus* and *Eptesicus fuscus*)

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Thesis submitted to the
Faculty of Science of the
University of Ottawa
In partial fulfillment of the requirements for the
M.Sc degree in Biology

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Table of Contents

Abstract……iv
Resumé……..vi
Acknowledgments…….....ix

Chapter 1: Introduction…….. 1
1.1.1 Forms of mercury and the environmental mercury cycle ……... 1
1.1.2. Human and ecotoxicology of mercury…….....3
1.1.3. Mercury in bats……... 5
1.1.4. Mercury in Eastern Ontario watersheds…….……..…6
1.2. Eastern Ontario bat species……...…… 7
1.2.1. *Myotis lucifugus*……...7
1.2.2. *Eptesicus fuscus*……... 9
1.3. Diet and foraging habit in *M. lucifugus* and *E. fuscus*……..... 10
1.3.1. Radiotelemetry as a tool to elucidate foraging habits in bats…….…… 10
1.3.2. Stable isotopes as a tool to trace dietary sources…….……... 11
1.4. DNA as potential molecular marker of mercury exposure in bats ……..……..……. 12
1.4.1. Global DNA methylation……... 12
1.4.2. Mitochondrial DNA……14
1.5. Objectives and hypothesis…….…..15

Chapter 2: Factors linked to mercury exposure in Eastern Ontario bats……..16
2.1. Introduction to the Determinants of Mercury Exposure…… 16
2.2. Materials and Methods……... 17
2.2.1. Bat captures and processing…… 17
2.2.2. Radiotracking bats……....18
2.2.3. Insect capture and processing……. 19
2.2.4. Total mercury analysis…… 20
2.2.5. Stable isotope analysis…… 21
2.2.6. Statistical analysis……22
2.3. Results…… 23
2.3.1. Total mercury fur concentration in Eastern Ontario bats is species-dependent…….. 23
2.3.2. Sex affects fur total mercury concentration in *E. fuscus* in Eastern Ontario ……. 25
2.3.3. Female reproductive status as determinant of fur total mercury concentration in Eastern Ontario bats…… 27
2.3.4. Age increases fur total mercury concentrations in Eastern Ontario bats…… 30
2.3.5. Geographical influence on fur total mercury concentration in Eastern Ontario bats.. 31
2.3.6. Geographical differences in fur total mercury concentration in Eastern Ontario bats are sex- and age-dependent……..……....33
2.3.7. Temporal influence on fur total mercury concentration in Eastern Ontario bats…..... 35
2.3.8. Dietary contributions to fur total mercury concentration in Eastern Ontario bats…...39
2.3.8.1. Total mercury concentration in aquatic emerging and terrestrial Eastern Ontario insects……39
2.3.8.2. Geographical influence on total mercury concentration in Eastern Ontario Insects...……..... 41

2.3.8.3. Isotope analysis in aquatic emerging and terrestrial Eastern Ontario insects……..42
2.3.8.4. Isotope analysis in Eastern Ontario bats……..…… 46
2.3.8.5. Correlative analysis of isotope and total mercury concentration in bats and insects……..….....51

Chapter 3: Assessment of DNA and RNA molecular markers as potential biomarkers of mercury exposure in *E. fuscus* in Eastern Ontario……...…….... 54
3.1. Introduction to DNA and RNA Biomarkers of Mercury Exposure……54
3.2. Materials and Methods……..……..55
3.2.1. DNA and RNA extractions…..…… 55
3.2.2. Quantification of DNA and RNA and assessment of RNA quality……55
3.2.3. Global DNA methylation assays ……57
3.2.4. Assessment of mitochondrial DNA abundance…….…….. 58
3.2.5. Quantification of metallothionein transcript abundance…..…….……..59
3.3. Results…….....60
3.3.1. Global DNA (hydroxy)methylation in internal tissues does not correlate with fur total mercury concentration in *E. fuscus*…….…….60
3.3.2. Mitochondrial DNA concentration does not correlate with fur total mercury concentration in tissues of *E. fuscus*…….....…. 61
3.3.3. Transcript abundance of metallothionein does not significantly correlate with fur total mercury concentration in *E. fuscus*……..…..62

Chapter 4: General discussion……63
4.1.1. Influences of environmental and biological factors on fur total mercury concentration in Eastern Ontario bats……63
4.1.2. Probing the potential contribution of aquatic emerging and terrestrial insect diet to fur total mercury concentration in Eastern Ontario bats……..72
4.2. Investigated mitochondrial and epigenetic DNA markers do not correlate with fur total mercury concentration in big brown bats in Eastern Ontario ……..…80
4.3 General Conclusions…….. 84

References……86

Abstract

Insectivorous bats have been found to have unusually high levels of mercury. While broad geographic scale studies have investigated factors contributing to mercury bioaccumulation in bats across Canada, studies investigating differences in regional scale bioaccumulation and the contributing factors remain scarce. Here, I comprehensively investigate the bioaccumulation of mercury in two insectivorous bats, the big brown bat (*Eptesicus fuscus*) and the little brown bat (*Myotis lucifugus*), collected over a period of ~20 years along the St. Lawrence River in Eastern Ontario, parts of which are historical hotspots of mercury, to address two objectives: First the determination of biological and environmental factors, including dietary sources, contributing to reported patterns of fur total mercury bioaccumulation, and second the investigation of DNA-based biomarkers as potential tools to assess internal tissue-responsiveness to mercury exposure, specifically global DNA methylation and expression levels of mitochondrial DNA. With regard to factors determining fur total mercury concentration in Eastern Ontario bats, significant differences between species exist, as higher concentrations were found in big brown bats compared to little brown bats. Sex contributed to differences in fur total mercury, however in a species-specific manner. Male fur contained higher total mercury concentrations compared to females in big brown bats, but not little brown bats. Female reproductive status differentially affected fur mercury concentrations between both species, reducing concentrations in pregnant little brown bats, while significantly increasing concentrations during lactation in big brown bats. Finally, fur total mercury concentration in adults was higher than that of juvenile bats (< 1 yr). To address the hypothesis that aquatic emerging and terrestrial insect diets differentially contribute to Eastern Ontario bat mercury concentration, I used stable isotope analysis and telemetry approaches and caught aquatic and terrestrial insects. While higher total mercury was identified in aquatic

compared to terrestrial insects, a high degree of variability in the isotope signature in insects and bats in Eastern Ontario did not allow to fully address this hypothesis. However, data pointed to a more specialized diet in big brown bats compared to a more generalist diet in little brown bats as well as a sex-specific correlation between dietary source and fur total mercury concentration in little and big brown bats. The evaluation of potential epigenetic and mitochondrial DNA-level molecular biomarkers in kidney, brain and liver (DNA methylation and assessment of relative mitochondrial DNA copy number) did not reveal significant correlations with fur total mercury concentrations. This may suggest that the mercury concentrations measured in this study were not high enough to elicit these specific DNA level responses or they do not represent relevant biomarkers of environmental methylmercury exposure, at least in big brown bats. Overall, this thesis contributes to our understanding of regional variability in fur total mercury concentration within and between Eastern Ontario bat species. These findings provide important insights for future targeted investigations of the contribution of aquatic emerging and terrestrial insect dietary sources on the one hand and underline the importance of accounting for regional variability in more global scale comparisons of bat mercury bioaccumulation on the other.

Résume

On a constaté que les chauves-souris insectivores présentaient des niveaux de mercure inhabituellement élevés. Bien que des études à grande échelle géographique aient examiné les facteurs contribuant à la bioaccumulation du mercure chez les chauves-souris du Canada, les études portant sur les différences de bioaccumulation à l'échelle régionale et sur les facteurs qui y contribuent demeurent rares. Ici, j'étudie de manière exhaustive la bioaccumulation du mercure chez deux chauves-souris insectivores, la grande chauve-souris brune (Eptesicus fuscus) et la petite chauve-souris brune (Myotis lucifugus), recueillies sur une période d'environ 20 ans le long du fleuve Saint-Laurent dans l'est de l'Ontario, dont certaines parties sont des points chauds historiques du mercure, afin d'atteindre deux objectifs : Premièrement, la détermination des facteurs biologiques et environnementaux, y compris les sources alimentaires, contribuant aux modèles rapportés de bioaccumulation du mercure total dans la fourrure, et deuxièmement, l'étude des biomarqueurs basés sur l'ADN comme outils potentiels pour évaluer la sensibilité des tissus internes à l'exposition au mercure, en particulier la méthylation globale de l'ADN et les niveaux d'expression de l'ADN mitochondrial. En ce qui concerne les facteurs déterminant la concentration totale de mercure dans la fourrure des chauves-souris de l'Est de l'Ontario, il existe des différences significatives entre les espèces, les concentrations étant plus élevées chez les grandes chauves-souris brunes que chez les petites chauves-souris brunes. Le sexe a contribué aux différences de concentration totale de mercure dans la fourrure, mais d'une manière spécifique à l'espèce. La fourrure des mâles contenait des concentrations de mercure total plus élevées que celles des femelles chez les grandes chauves-souris brunes, mais pas chez les petites chauves-souris brunes. Le statut reproductif de la femelle a eu un effet différent sur les concentrations de mercure dans la fourrure des deux espèces, réduisant les concentrations chez les petites chauves-souris brunes

enceintes, tout en augmentant de manière significative les concentrations pendant la lactation chez les grandes chauves-souris brunes. Enfin, la concentration de mercure total dans la fourrure des adultes était plus élevée que celle des chauves-souris juvéniles (<1 an). Pour répondre à l'hypothèse selon laquelle les régimes alimentaires des insectes aquatiques émergents et des insectes terrestres contribuent de façon différentielle à la concentration de mercure des chauves-souris de l'Est de l'Ontario, j'ai utilisé des approches d'analyse des isotopes stables et de télémétrie et j'ai capturé des insectes aquatiques et terrestres. Bien qu'un taux de mercure total plus élevé ait été identifié chez les insectes aquatiques par rapport aux insectes terrestres, le haut degré de variabilité de la signature isotopique chez les insectes et les chauves-souris de l'Est de l'Ontario n'a pas permis de répondre pleinement à cette hypothèse. Cependant, les données indiquent un régime alimentaire plus spécialisé chez les grandes chauves-souris brunes par rapport à un régime plus généraliste chez les petites chauves-souris brunes, ainsi qu'une corrélation spécifique au sexe entre la source alimentaire et la concentration de mercure total dans la fourrure des petites et des grandes chauves-souris brunes. L'évaluation de biomarqueurs moléculaires potentiels épigénétiques et au niveau de l'ADN mitochondrial dans les reins, le cerveau et le foie (méthylation de l'ADN et évaluation du nombre relatif de copies d'ADN mitochondrial) n'a pas révélé de corrélations significatives avec les concentrations de mercure total dans les fourrures. Cela peut suggérer que les concentrations de mercure mesurées dans cette étude n'étaient pas assez élevées pour provoquer ces réponses spécifiques au niveau de l'ADN ou qu'elles ne représentent pas des biomarqueurs pertinents de l'exposition environnementale au méthylmercure, du moins chez les grandes chauves-souris brunes. Dans l'ensemble, cette thèse contribue à notre compréhension de la variabilité régionale de la concentration de mercure total dans la fourrure au sein et entre les espèces de chauves-souris de l'Est de l'Ontario. Ces résultats fournissent des indications importantes pour de futures études

ciblées sur la contribution des sources alimentaires des insectes aquatiques émergents et terrestres, d'une part, et soulignent l'importance de tenir compte de la variabilité régionale dans les comparaisons à l'échelle mondiale de la bioaccumulation du mercure chez les chauves-souris, d'autre part.

Acknowledgements

I would first like to extend a special thanks to my supervisor Dr. Jan Mennigen. He took a chance taking me on as a M.Sc. student asking for his help to study a group of species in which he had no experience with and integrated his own ideas and expertise into the framework of the project to make for a very interesting thesis. I am so grateful for his enthusiasm and patience while learning about the ecology of the bats and my field work all the while sharing his expertise and teaching me the molecular side of things. There have been plenty of bumps in the road during this process and I am thankful for his positivity and encouragement along way, helping me to stay on track.

I would also like to thank my co-supervisor Dr. Brian Hickey of the St. Lawrence River Institute of Environmental Sciences. Not only did Brian introduce me to bat research and show me how interesting of a species bats are, but he gave me a better appreciation for all things nature during our field work through his fun facts and story-telling. The summers during my undergraduate degree working with Brian helped me to start applying what I was learning in the classroom to real-world scientific research. Without Brian, I wouldn't have been introduced to environmental research and be where I am today. His knowledge, patience, and humour are somethings I will always be appreciative of.

Next, I would like to show my gratitude to the Mennigen lab. Surrounded by great scientists who are always willing to help out where they can and learn about each other's research, they created a great environment to work in throughout the two years of my degree. Daniel Kostyniuk, thank you for introducing me into the lab, showing me around and giving me advice as I started out on this journey. Thank you to Elie Farhat for taking on the role of scheduling all of my qPCR timeslots. Divya Ramachandran, Giancarlo Giuseppe Mario Talarico, Jory Curry, and Tyler

Nguyen, thank you all for making the lab an enjoyable space to work in despite the restrictions we faced during the pandemic.

Another special thanks go out to scientists and volunteers of the St. Lawrence River Institute who were always willing to spend the night in search for bats and helping me collect data for this thesis. Thank you, Stephany Hildebrand, Lexy Harquail, Yanik Rozon, Emilie DeRouchie, and Harneet Cheema for all your help, support and sharing my love for bats with me along with the rest of the River Institute staff for their support and encouragement. An additional thank you to Stephany Hildebrand of the St. Lawrence River Institute for not only spending the summer chasing bats with me, but spending countless hours learning about and sorting through insects and for utilizing her outstanding photography skills to capture almost all aspects of my field work on camera.

To my family for always supporting me and being by my side throughout this whole process. My parents Shawn and Karen Bedard for always checking in, making sure I am on track and helping to keep my spirits high. To my siblings, Bryna and Brodie Bedard and sister-in-law France Bedard for always putting a smile on my face and making me laugh through it all. Finally, a special thank you to my best friend and roommate Lauren Love for always being understanding during my times of stress and celebrating every win with me, big or small. I am so grateful to have you sticking by my side.

Chapter 1 – Introduction

1.1.1. Forms of mercury and the environmental mercury cycle

Mercury is a naturally occurring chemical which is found globally in the environment. It can exist in different chemical species, specifically elemental mercury (Hg^0), reactive mercury (Hg^{2+}), inorganic mercury (cinnabar, HgS) and organic mercury (methylmercury, MeHg) (Gavis and Ferguson 1972). Each form of mercury is toxic to wildlife and humans however its toxicity is dependent on the mercury species, with MeHg being the most toxic (United States Environmental Protection Agency 2020). The transformations between mercury compounds in the environment are complex and need to be taken into consideration when investigating the impacts of mercury in an ecosystem.

Elemental mercury is highly volatile and thus easily deposited into the atmosphere through natural processes such as degassing of mercury from rocks, soil and surface water, volcanic emissions and forest fires (Environment and Natural Resources Canada 2013; United States Environmental Protection Agency 2020). Although mercury has always been ubiquitous in the environment, drastic increase in anthropogenic emission sources (Risher 2003) have rendered mercury more of an ecotoxicological concern in recent human history. Anthropogenic sources of mercury emissions include the combustion of fossil fuels, cement production, incineration of waste, gold amalgamation, mining operations, as well as industrial products and applications such as lamps, thermometers and dental fillings (Kazantzis 2002; Risher 2003).

Through both natural and anthropogenic sources, elemental mercury is released into the atmosphere due to its high volatility. In the atmosphere, elemental mercury can be transported globally and re-deposited into the lithosphere or hydrosphere (Risher 2003; Environment and Natural Resources Canada 2013). Atmospheric mercury can also be oxidized into the reactive

mercury form photochemically. Reactive mercury has low volatility properties but is very soluble in water, so it is readily absorbed into the hydrosphere. Reactive mercury combines with other compounds such as sulfur to form inorganic mercury which is commonly found in the environment (Risher 2003; Environment and Natural Resources Canada 2013). Once in aquatic ecosystems, reactive mercury and inorganic mercury compounds are readily converted into MeHg by the addition of a methyl group. The rate of this methylation process is dependent on environmental variables and typically occurs in acidic aquatic ecosystems with low oxygen levels and high concentrations of organic matter, for example stagnant wetlands and riverbed sediment (Environment and Natural Resources Canada 2013; Lin et al. 2021) through anaerobic bacteria such as *Deltaproteobacteria, Firmicutes,* and *Euryarchaeota* (McDaniel et al. 2020; Lin et al. 2021).

MeHg is known as the most toxic form of mercury and has the ability to not only bioaccumulate in tissues of organisms but also to biomagnify across trophic levels, meaning that MeHg increases in concentration moving up the food chain, with the apex predators having the highest concentrations of mercury in their tissues (Hong et al. 2012; Environment and Natural Resources Canada 2013; Lavoie et al. 2013). The biomagnification of MeHg is well described in aquatic ecosystems, where large quantities of primary producers such as phytoplankton absorb MeHg passively from water. MeHg then biomagnifies through different trophic levels of the food web and increases in concentration in zooplankton and aquatic invertebrates, small fish and finally aquatic and terrestrial top predators such as fish, bears, birds and humans (Lavoie et al. 2013). Key components of the environmental mercury cycle are summarized in **Fig. 1**.

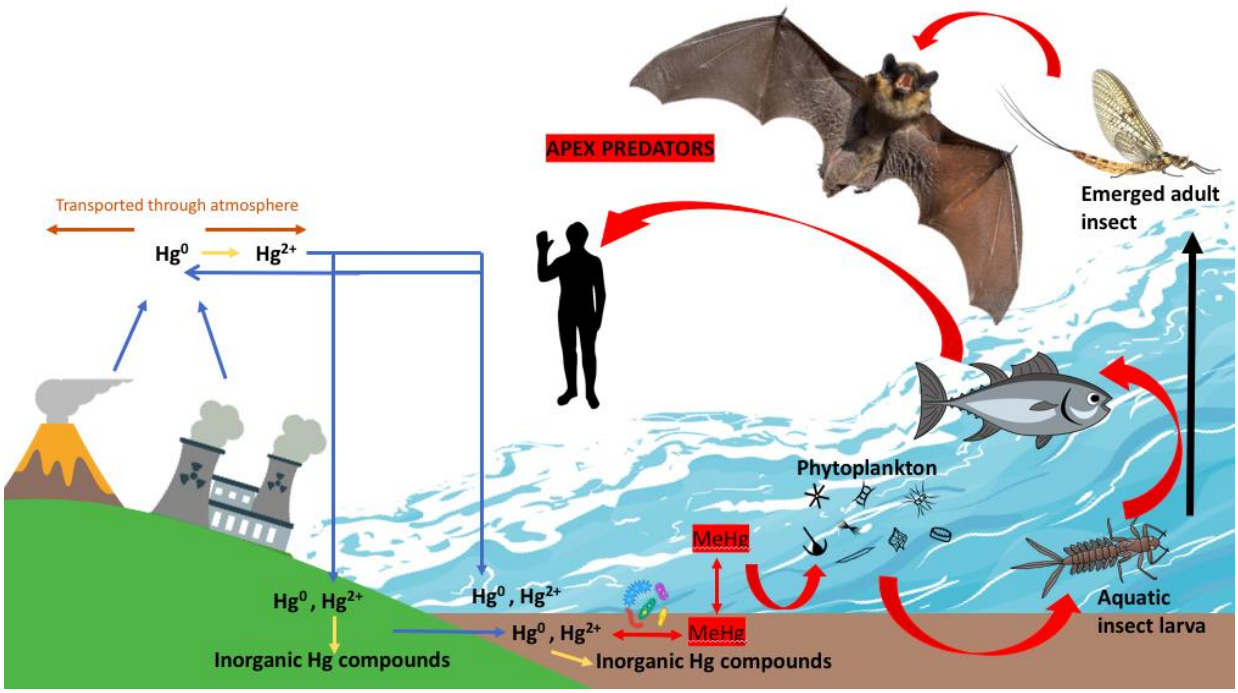

Fig. 1. *Schematic representation of the environmental mercury cycle in atmosphere, lithosphere and hydrosphere, where inorganic mercury is converted to MeHg and biomagnifies across trophic levels in the food web.*

1.1.2. The human and ecotoxicology of mercury

The example of Minamata, Japan is a widely cited example of the toxicological consequences of bioconcentration and biomagnification of MeHg from an anthropogenic source in the 1950's (Harada 1995). The Chisso Minamata industrial plant was responsible for contaminating coastal water by discharging waste from the production of chemical fertilizers, plasticizers and industrial chemicals directly into the water of Minamata Bay (Harada 1995). When children and adults began showing symptoms of neurological disorders, researchers began investigating potential causes of disease and poisoning. When it was discovered that aquatic life and top predators that feed on aquatic organisms, such as birds and cats were also showing adverse neurological symptoms, researchers determined that "Minamata disease" was a result of MeHg poisoning from ingesting fish that have been exposed to the contaminated water of Minamata Bay (Harada 1995). Since then, research has shown that MeHg has the capacity to readily cross the

blood-brain barrier causing accumulation in the central nervous system and therefore, neurotoxic effects (Aschner & Aschner 1990). It can also be transferred to a developing child as it can cross the placental barrier and accumulate in a women's breast milk (Health Canada Mercury Issues Task Group 2004). Concentrations of 10-20 μg/g of MeHg in maternal hair samples were determined to cause neurotoxic affects to the fetus during development (Grandjean & Herz 2011). Depending on exposure and the dose of MeHg, the consequences on health include extreme neurological damage; numbness in the hands and feet which eventually leads to the inability to coordinate muscles and difficulty speaking, hearing and seeing (Health Canada Mercury Issues Task Group 2004). Mechanistically, MeHg neurotoxicity has been linked to impairment of intracellular calcium homeostasis, alterations of glutamate homeostasis as well as oxidative stress and is dependent on the presence of potentially protective selenocompounds and selenoproteins (Farina et al. 2011).

Extensive research that has been done on the mercury cycle and its implications on human health and the environment has led to government-developed guidelines and regulations to protect humans and sensitive ecosystems. Guidelines on the levels of mercury in fish for consumption, drinking water, soil and tissue/blood have been stated based on this research. As such, blood mercury concentrations below 20 μg/L are considered acceptable and low risk (Health Canada Mercury Issues Task Group 2004). Additionally, hair mercury concentrations less than 6 mg/kg falls within the range of low risk (Health Canada Mercury Issues Task Group 2004). These guidelines are designed to assure that populations, including most vulnerable populations such as indigenous communities as well as developing children, are protected from the adverse effects of mercury.

1.1.3. Mercury in bats

Bats are, based on their body-size, comparatively long-lived (up to approximately 30 years) apex predators shown to have high levels of total mercury in their blood and fur. Bats possess unique and increasingly recognized roles in their ecosystems providing ecosystem services such as the control of insect populations including potential disease vectors and energy transfer to cave ecosystems (Kunz et al. 2011; Ghanem and Voigt 2012; Kasso and Balakrishnan et al. 2013). Because of their important ecosystem services, as well as their trophic position, bats, the second largest order of mammals, have been established as sentinel species to investigate mercury and metal pollution in general (Pikula et al. 2010; Zukal et al. 2015; Calao Ramos et al. 2021). Indeed, high total and/or MeHg concentrations have been reported in bat tissues in areas considered highly contaminated due to anthropogenic point-source emissions of mercury. For example, total mercury levels in fur and blood of 10 species of bats of all age classes collected near a mercury point source in the northeastern United States were eight and ten times greater than in bats collected at non-point sources averaging concentrations >50 μg/g total and MeHg in fur (Yates et al. 2014). In mercury hotspot areas bats often have mercury levels that exceed threshold concentrations of >10 μg/kg fresh weight (Chételat et al. 2018) which are known to cause neurochemical changes in bats (Nam et al. 2012; Yates et al. 2014; Little et al. 2015) and other similarly sized mammals such as wild mice (Burton et al. 1977). While point sources thus represent an important source of mercury exposure in bats, studies show that diet source, especially in designated hotspot areas, is an important parameter in determining mercury exposure. A recent study, for example showed that artisanal and small-scale gold mining in the Peruvian Amazon increased the exposure to mercury in specific bat species that foraged in close proximity to this gold mining point source, although feeding habits of different bat species played a large role in the mercury concentrations of bats in

this area (Monero-Brush et al. 2018). While the overall trophic position is well known to play a role in MeHg biomagnification, the importance of specific dietary sources has further been linked to total mercury levels in fur in a recent study comparing different species of bats in Belize characterized by broad ranges of feeding strategies (frugivores, carnivores, insectivores, piscivorous, and omnivores). This study found that higher mercury levels in bats were associated with feeding strategies that involved preying on species of aquatic ecosystems (Becker et al. 2018).

1.1.4. Mercury in Eastern Ontario watersheds

A particular section of the St. Lawrence River, from Lake Ontario to the section of the river that meets the border of Ontario, Quebec and New York, has been subjected to chemical pollution over the years via pulp and paper, aluminum and chemical production. This has led to high concentrations of mercury in this section of the river and, as a result, high concentrations of mercury in fish (Ridal et al. 2010). Indeed, sediment concentrations of total mercury in the contaminated zones of this section of the river have been found to exceed concentrations of 2000 ng/g of total mercury (mercury hotspots), while there are a few fairly uncontaminated sites upstream from the city of Cornwall (Ridal et al. 2010). The Raisin River, North-West of Cornwall, Ontario, Canada (75°44' W, 45°08' S) is surrounded by agriculture and empties into the Lake St. Francis portion of the St. Lawrence River. Susceptible to agricultural runoff, high levels of dissolved organic carbon (DOC) have been reported in the Raisin river (Cane & Clark 1999), which in turn, are generally shown to be highly correlated with increased rates of mercury methylation (Gill & Bruland 1990; Watras et al. 1995). Indeed, elevated concentrations of total mercury and MeHg correlating with high nutrient concentrations have historically been reported in the Raisin river (Maharaj et al. 2007). Thus, the Raisin River has historically been considered

as potential source for high sediment Hg concentrations in the St. Lawrence wetlands downstream from the Raisin River and thus outside of the described hotspots (Thompson-Roberts & Pick 2000). An overview of historical mercury hotspots in the St. Lawrence river basin as well as the location of the Raisin River tributary are presented in **Fig. 2**.

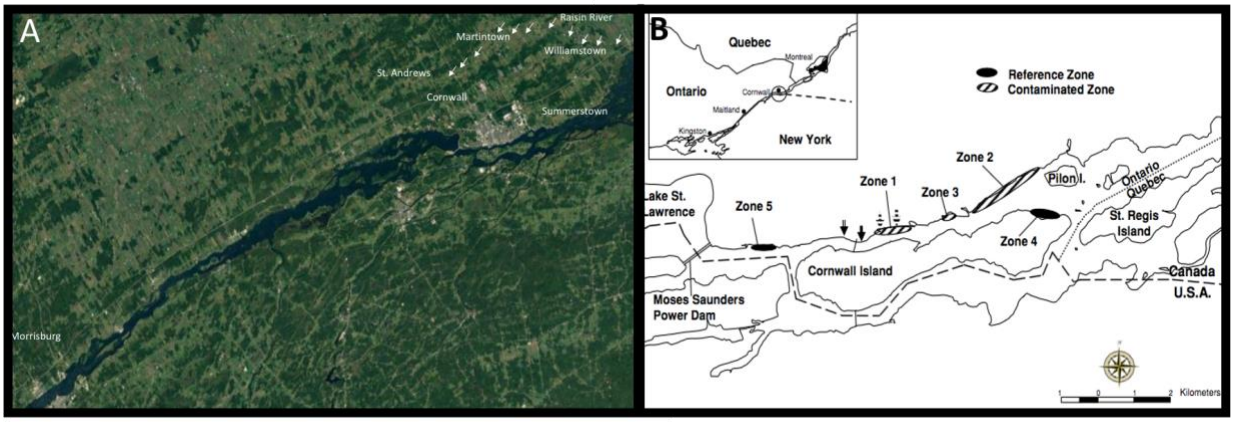

Fig. 2. *Map of parts of the St. Lawrence River basin including the Raisin River tributary (**A**) and detailed illustration of designated mercury contamination zones and reference zones in the Cornwall region of the St. Lawrence River (**B**) as described by according to Ridal et al. (2010).*

1.2. Eastern Ontario bat species

The little brown bat (*Myotis lucifugus*) and the big brown bat (*Eptesicus fuscus*) are two large populations of bat species that inhabit Eastern Ontario. While other species such as the Northern long-eared bat (*Myotis septentrionalis*) exist in Eastern Ontario, they are less abundant and only big and little brown bats relevant to the thesis are introduced in detail.

1.2.1. *Myotis lucifugus*

M. lucifugus have a widespread distribution across North America (**Fig. 3A**), roosting during summer days in buildings, bat boxes, and tree holes as colonies of 100-1000's of individuals. During the colder months, the little brown bats form larger hibernating colonies of more than 200,000 individuals in caves and mines (Taylor 2018). Unfortunately, the conditions of

these hibernacula chosen by the little brown bats are favourable conditions for the fungus *Pseudogymnoascus destructans,* which causes white nose syndrome (Brownlee-Bouboulis & Reeder 2013). The fungus invades the skin on the face and wings of bats and has been shown to increase disruptions in the bat's inactive state/rest. These interruptions are thought to increase energetic stress and prevent the bats from regaining homeostasis in the body (Brownlee-Bouboulis & Reeder 2013). This fungal infection has wiped many colonies of little brown bats across North America and the species has now been listed as endangered by the IUCN (2018), therefore protection and research on the little brown bats is crucial. The adult little brown bats weigh approximately 5-14 grams and females tend to be larger than males (Fenton & Barclay 1980; Taylor 2018). The females will give birth to pups in the early summer as fertilization occurs before hibernation and within the next four weeks, the pups become independent of their mother (Taylor 2018). Within this first summer, the pups can be identified by the lack of fully developed bone in their meta-carpal joints along the wings (Fenton & Barclay 1980). The little brown bats have been shown to have a generalist type diet feeding on a variety of insects as they rely heavily on mass emergences of aquatic insects like dipterans, trichopterans, and lepidopterans (Fenton & Barclay 1980; Clare et al. 2014). Although no territorial behavior has been observed in this species, little brown bats tend to return to the same feeding sites each night (Fenton & Barclay 1980). The type of prey being fed on is thought to be determined by what is available to the little brown bats in specific habitats and seasonal changes to prey populations (Clare et al. 2014). Lactating females tend to vary more in the composition of their diet likely due to the fact that their energy requirements are highest at this time (Fenton & Barclay 1980; Clare et al 2014).

1.2.2. *Eptesicus fuscus*

E. fuscus have a larger distribution than the little brown bats (**Fig. 3B**), covering almost all of North America and a portion of South America (Taylor 2018). This species roosts mainly in buildings and trees during the summer (Rancourt et al. 2007; Taylor 2018). Because of their sufficient fat reserves, big brown bats can withstand lower temperatures and typically hibernate in roosts that are exposed to the cold air (Phillips 1966; Taylor 2018). The size of the big brown bat colonies ranges from 10-100's of individuals (Phillips 1966) and as their name suggests, big brown bats are larger than the little brown bats, weighing around 14-20 g (Taylor 2018), females tending to be slightly larger than the males (Phillips 1966). Male and female big brown bats mate during the winter season and in the following summer, the females give birth to their pups early on in the season (Taylor 2018). The pups can be identified using the same unossified meta-carpal joint technique described previously (Brunet-Rossinni & Wilkinson 2009). Big brown bats are also known to be generalist insectivores where their diet varies based on insect abundance as it changes seasonally and spatially (Agosta 2002; Agosta & Morton 2003). Although they are seen as generalists, the larger jaw bones of the big brown bats allow them to feed on hard-bodied insects (i.e. coleoptera and hemiptera) for which there is less competition from other smaller bats, like the little brown bats (Phillips 1966; Agosta 2002; Agosta & Morton 2003). A difference between the little brown bats and the big brown bats most likely explained by their different body mass is their basal metabolic rate (BMR), the amount of energy needed to perform normal bodily functions at rest. The average BMR for big brown bats is approximately 0.113 Watts, significantly greater than the BMR of the little brown bats which is approximately 0.0510 Watts on average (Hock 1951; Willis et al. 2006; Cooper & Geiser 2008). This means that big brown bats require more energy at rest to maintain vital functions compared to the little brown bats.

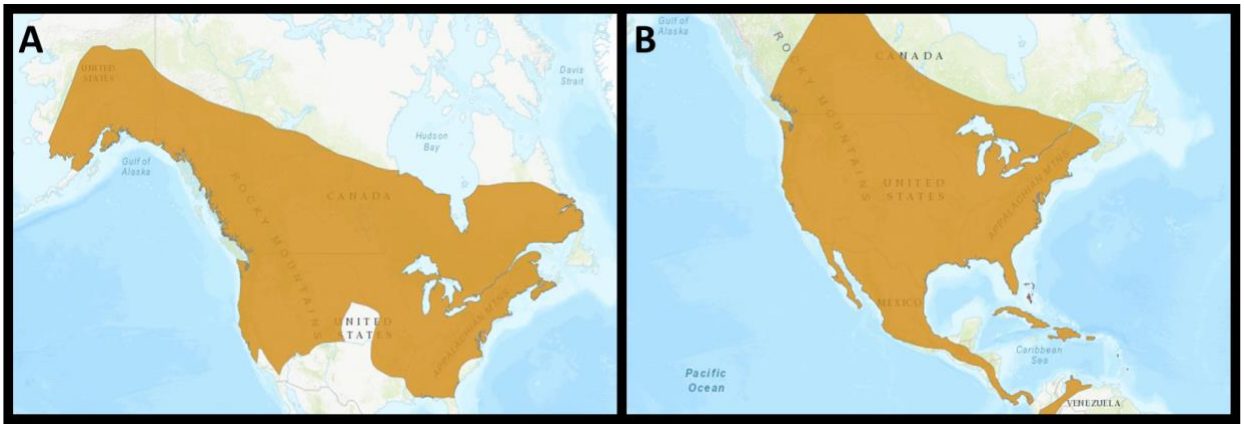

Fig. 3. *Distribution M. lucifugus (**A**) and E. fuscus (**B**) across North America (IUCN 2016).*

1.3 Diet and foraging habits in *M. lucifugus* and *E. fuscus*

As described, the diet of both bat species is principally based on a variety of insect groups present in different habitats at different times of the season. Relevant to bats whose habitats include both terrestrial as well aquatic systems, insects consumed by the bats may start their lifecycle in the water as an aquatic macroinvertebrate, while others exhibit an entirely terrestrial lifecycle. Aquatic invertebrates are potentially exposed to MeHg through aquatic food webs and thus, once emerged from aquatic systems as adults, may expose bats to MeHg biomagnified from the aquatic food chain. Importantly, as described, bats also have a very high metabolic rate, consuming up to 100% of their own body weight in insects in a single night increasing their potential for MeHg bioaccumulation (Hickey & Fenton 1996; Yates et al. 2014).

1.3.1. Radiotelemetry as a tool to elucidate foraging habits in bats

Radio telemetry is a useful tool that is used to determine foraging behaviours as well as roosting locations in bats (Willis et al. 2006; Olson & Barclay 2013; Randall 2014; Nelson & Gillam 2017). A radio transmitter that weighs < 5% of the bats' body mass (Aldridge & Brigham 1988) can be attached to the bat and allows individual tracking through a unique radio frequency

associated with the transmitter which can be pick up as a signal on a receiver attached to an antenna when the bat is within close proximity (approximately 0.5-2 km depending on terrain conditions). In the past, such studies have shown that little brown bats tend to return to the same foraging sites regularly (Fenton & Barclay 1980) and the use of radio telemetry data can thus be used to determine foraging behaviour and location in regional bat species.

1.3.2. Stable isotopes as a tool to trace dietary sources

Stable isotopes are an increasingly used tool investigate food-webs within ecosystems by determining the isotope composition, generally ^{15}N and ^{13}C of specific tissues (Jennings et al. 1997). A common application is to use the stable isotopic composition of consumers and their foods to make inferences about the composition of the animal's assimilated diet (Philipps et al. 2014). ^{15}N is enriched in the tissues of predators in comparison to their prey allowing to trace contaminant accumulation across trophic levels whereas, conversely, ^{13}C is only very weakly - if at all, enriched from prey to predator but acts as a proxy for carbon dietary source (i.e. primary producers, aquatic plants, terrestrial plants) (Peterson & Fry 1987; Hobson & Welch 1992; Bunn & Boon 1993; Jennings et al 1997). Measuring the abundance of these two stable isotopes in different tissues, but most often fur, has successfully been used to investigate food webs of different bat species across the world (Sullivan et al. 2006; Voigt & Kelm 2006; Painter et al. 2009; Broders et al. 2014; Reuter et al. 2016). Fur is an inert tissue that, which, when used for stable isotope analysis, will reflect isotope ratios associated with prey that had been consumed before or during the period of fur growth (Painter et al 2009).

1.4. DNA as potential molecular marker of mercury exposure in bats

To assess biological consequences of mercury exposure and bioaccumulation at the tissue level in bats, several molecular assays exist. For example, brain neurotoxicity has been quantified using enzyme assays probing the activity of neurotransmitter metabolizing enzymes (Nam et al. 2012), whereas gene expression of metallothionines, induced in response to heavy metal exposure have also been assessed (Pikula et al. 2010). The limitation of these approaches lies in the fact that tissue samples need to be quickly stored in appropriate conditions to minimize RNA or protein degradation (Tan & Yiap 2009). Especially in species sampled and collected over long periods of time, these assays may not be useful to assess tissue-level responses to mercury exposure. In contrast, both DNA as well as epigenetic DNA modifications, especially DNA methylation, are typically more inert (Tan & Yiap 2009; Li et al. 2018) and may, for this reason, represent potentially useful molecular level biomarkers for samples from species bank spanning a large period of sampling.

1.4.1. Global DNA methylation

Global DNA methylation/demethylation is a naturally occurring epigenetic modification that involves the addition or removal of a methyl group onto the fifth position of the cytosine ring (5-mC), almost exclusively in cytosine and guanine dinucleotides within regions termed CpG islands (Pilsner et al. 2010). DNA methylation dynamics are maintained by writers, notably maintenance and *de novo* DNA methyltransferases (Arita & Costa 2009; Shen et al. 2013; Tellez-Plaza et al. 2014), as well as erasers notably TET enzymes (Best et al. 2018). The process of demethylating 5-mC involves the intermediate generation of 5-hydroxymethylcytosine also termed 5-hmC (Branco et al. 2012; Tellez-Plaza et al. 2014). Through action of these enzymes,

changes in DNA methylation allow to regulate gene expression. DNA Methylation of CpG islands in promotor regions and first introns of genes generally correlate with repression of gene expression by limiting binding of transcription factors or enhancers (Rodenhiser & Mann 2006; Best et al. 2018). Any alterations to normal DNA methylation patterns, whether it be hypermethylation (increased amount of 5-mC) or hypomethylation (decreased amount of 5-mC), can cause irregular gene expression and lead to cancers, diseases, and neurological disorders (Rodenhiser & Mann 2006; Arita & Costa 2009). Alteration of methylation patterns can be caused by both environmental factors or inheritance, as DNA methylation patterns are mitotically and meiotically stable and can thus be transmitted to both somatic daughter cells as well as germ cells (Rodenhiser & Mann 2006; Best et al. 2018). Exposure to environmental contaminants, including heavy metals as well as persistent organic pollutants and endocrine disrupting chemicals (EDCs) have been shown to have the ability to disrupt regular DNA methylation patterns and thus potentially lead to disease (Head 2014). Indeed, heavy metals have been shown to affect DNA methylation by inhibiting DNA methyltransferase activity (Chen et al. 2004; Chen et al. 2006; Pilsner et al. 2007; Reichard et al. 2007; Jiang et al. 2008; Yamazaki et al. 2021). In line with these findings, investigation of global DNA methylation in mammals following exposure to MeHg, either environmentally relevant dietary exposures or measured mercury tissue burden, have found that higher mercury and/or MeHg concentrations are often associated with DNA hypomethylation (Pilsner et al. 2010; Bose et al. 2012; Basu et al. 2013; Goodrich et al. 2013; Cardenas et al. 2017). Thus, DNA methylation may represent a suitable and persistent biomarker of mercury exposure in bats.

1.4.2. Mitochondrial DNA

Another stable molecular biomarker that has potential to be used to assess tissue-level responses to mercury exposure and bioaccumulation is mitochondrial DNA (mtDNA) abundance. Methylmercury, like other neurotoxins, has been linked to increased production of reactive oxygen species (ROS) and/or a decrease in the production of antioxidant defence mechanisms thus resulting in increased oxidative stress (Antunes dos Santos et al. 2018). Mitochondria are not only sites of production of ROS such as O_2^-, NO, H_2O_2 during the electron transport chain stage of ATP production (Gomez-Cabrera et al. 2012), but their DNA is also highly susceptible to ROS induced damage to DNA (Lobo et al. 2010; Quiros et al. 2017). DNA repairing agents can typically mitigate DNA damage caused by oxidative stress, however DNA damage has been shown to accumulate with age (Gomez-Cabrera et al. 2012). Unlike nuclear DNA (nDNA), mtDNA lacks the protection of nucleosomes and DNA repair systems, rendering it thus susceptible to ROS induced damage and eventually resulting in a decrease in its abundance (Quiros et al. 2017). The comparative susceptibility of mitochondrial DNA to ROS induced oxidative stress is specifically thought to be linked to mutations in genes that are directly involved in replicating mtDNA (El-Hattab & Scaglia 2013), different diseases and neurological disorders (Kazachkova et al. 2013; Ramos et al. 2015; Abolhassani et al. 2017; Sepe et al. 2016), as well as ageing (Gomez-Cabrera et al. 2012). Importantly, recent studies have established that exposure to either mercury or MeHg alone or in combination with correlates of oxidative stress such as H_2O_2, result in a quantifiable decrease in mtDNA in the nematode *C. elegans* (Wyatt et al. 2017). Thus, the investigation of relative abundance of mtDNA relative to nuclear DNA may have the potential to be used as a molecular marker in response mercury exposure in bats.

1.5. Objectives and hypothesis

While studies investigating determinants of mercury burden in bats have been conducted at larger geographical scales in Canada (Chételat et al. 2018), fewer studies have investigated sources of variability within specific regions. Here I investigate in detail the contribution of specific factors such as species, sex, reproductive status, age, sampling time as well as location to fur total mercury burden in two species of Eastern Ontario, *M. lucifugus* and *E. fuscus*, sampled over a period of ~20 years at the St. Lawrence River Institute in Cornwall. Taking advantage of the presence of Eastern Ontario watersheds historically linked to high mercury concentrations, including the St. Lawrence River hotspot around Cornwall, as well as the Raisin River tributary, I furthermore aim to address the hypothesis that mercury load in bats feeding in proximity to these areas is affected by dietary contribution of insect with a partially aquatic lifestyle compared to an entirely terrestrial life-history. Specifically, I predict that insects emerging from aquatic systems in these watersheds will exhibit higher concentration of total mercury compared to fully terrestrial insects, and that increased consumption of aquatic system emerged insects will contribute to higher mercury concentration in bats. This first objective is addressed in **Chapter 2**.

The second objective will explore the utility of molecular DNA-based markers, specifically global DNA methylation as well as quantification of total mtDNA copy numbers to assess tissue-level responses to environmental mercury exposure in Eastern Ontario bats. These markers have, due to their stability compared to other current molecular markers, the potential to be applied to specimen collections sampled and stored over long periods of time. These objectives will be addressed in **Chapter 3**.

Chapter 2 - Factors linked to mercury exposure in Eastern Ontario bats

2.1. Introduction to the Determinants of Mercury Exposure

Several studies investigating mercury burden in North American bats have described patterns of mercury accumulation across large geographical scales, including comparisons of mercury burden in little brown bats and big brown bats across Canada, in which atmospheric mercury deposition has been identified as a key factor contributing to the bat species' mercury burden (Chételat et al. 2018). While regionalized studies exist, these are often limited to one or a few sampling seasons. Here, taking advantage of a ~20 year collection of little brown and big brown bats in the St. Lawrence watershed in Eastern Ontario, I investigate specific determinants of fur total mercury burden, a proxy of internal MeHg concentration, in detail. In addition to the relatively high abundance of big and little brown bats allowing for a species comparison, the ~20 yr species sample bank also allows to assess fur total mercury concentration over time. Furthermore, the St. Lawrence watershed and its tributaries have historically been monitored as mercury hotspots, allowing to explore whether diet and foraging habits are potentially linked to increased mercury exposure through aquatic emerging insects in these zones. Thus, in addition to assessing parameters which in the literature have been linked to fur total mercury burden in bats, such as species, sex, reproductive status, age as well as location and proximity to mercury hotspots, I here attempt to address the hypothesis that dietary contribution in the form of aquatic emerging insects contributes to fur total mercury burden in two predominant Eastern Ontario bat species.

Together, these findings will provide additional insight into mercury burden in Eastern Ontario bats and the degree of regional and temporal variability. Finally, these findings may offer potential insight into determining environmental and/or biological factors contributing to mercury burden in these species in Eastern Ontario.

2.2. Materials and Methods

2.2.1. Bat captures and processing

Between the years 1999 and 2020, live and deceased bats were collected at various locations between the Kingston, Ontario, Canada region and the border of Ontario and Quebec, Canada roughly following the St. Lawrence River focusing on five cities and towns: Morrisburg (44.8978° N, 75.1834° W), Cornwall (45.0213° N, 74.7303° W), St. Andrews West (45.0961° N, 74.7970° W), Martintown (45.1546° N, 74.7106°W) and Williamstown (45.1467° N, 74.5796° W). An overview of these locations is shown in **Fig. 2**. Fur samples were collected from the dorsal intra-scapular region of both live-caught and deceased bats (n=216 live-caught, n=32 deceased) and archived over a period of ~20 years by bat biologist Dr. M. Brian C. Hickey of the St. Lawrence River Institute of Environmental Sciences. Fur is often used as measure of chemical bioaccumulation in mammals because it is a non-lethal and stable measure that reflects chemical exposure at the time the fur/hair has grown (Chételat et al. 2018; Hickey et al. 2001; Little et al. 2015). All handling of bats followed guidelines approved of the St. Lawrence River Institute's Animal Care Committee conducted under scientific collector's provincial sampling permit to Dr. M. Brian C. Hickey. Live bats were captured using mist nets, hand nets or directly by hand. Fur samples were clipped using stainless steel scissors and then stored in clean cryovials at room temperature until further analysis. Supporting information was recorded for each captured bat when possible; species, location and time of capture, sex, age (young or adult) and weight. Sex was determined with the bat in hand by observing external sex organs. Age, or more precisely maturity stage, was determined by observing presence or lack of fully developed bone in their meta-carpal joints along the wings by holding the wings extended out over a flashlight. Adults have fully developed bone in the joint whereas pups lack the fully developed joint (Fenton &

Barclay 1980), making it transparent in some spots when held against light. On a small subset of live captured bats (big brown bats, n=3; little brown bats, n=5), 0.27-gram Holohil model LB2X radio-transmitters (Holohil Systems Ltd., Carp, Ontario, Canada) were fitted to the intra-scapular region using Skin Bond® (Smith and Nephew United Inc., Largo, Florida, United States of America) surgical adhesive before being released to track bats as they foraged.

Additionally, fur and tissue samples were obtained from the collected deceased bats preserved at -20° Celsius since their collection. After fur was obtained from the deceased bats, they were dissected using stainless steel tools that were cleaned with 90% ethanol in between dissections to harvest tissue samples. Tissues collected include the kidney, liver and brain which were then each placed in autoclaved plastic microtubes and kept at -20° C until further analysis. The total sample size for bat fur was 243 samples with a smaller portion represented the little brown bats (n=76) and a large portion of the sample size being big brown bats (n=167).

2.2.2. Radiotracking bats

Each bat that was radio tagged was tracked by tuning a hand-held telemetry receiver (Communication Specialist Inc., Orange, California, United States of America) attached to a 5-element Yagi antenna (Communication Specialist Inc., Orange, California, United States of America) to the channel that corresponded to the frequency emitted by the transmitter that was attached to the individual bat. A signal is picked up on the radio when in close proximity (varying from 0.5-2.0 kilometers depending on conditions such as terrain, surrounding buildings and whether the bat is flying or roosting) to the transmitter and therefore, the bat. All eight bats that were tagged were tracked during the following 5-7 days and nights on foot and by vehicle before the adhesive wore off and the transmitter would no longer be attached to the bat. During the day

signals that were picked up showed where the bats were roosting. Tracking at night would consist of picking up a signal from the bat at its roost and then following the signal as the bat exits the roost for its night of feeding to identify foraging sites. Total tracking efforts cumulated to approximately 31.03 hours over 423.4 km. Identifying foraging sites with the radio telemetry data was supported by the use of handheld superheterodyne Magenta 'Bat4' bat detectors (Magenta Electronics Ltd., Tutbury, Staffordshire, United Kingdom) and a Song Meter SM4BAT FS bioacoustics recorder (Wildlife Acoustics Inc., Maynard, Massachusetts, United States of America).

2.2.3. Insect captures and processing

Insect samples were collected from sites within the five districts that little brown bats and/or big brown bats bats were confirmed to be foraging at using three types of traps: Malaise trap, emergence trap and an ultraviolet light trap. Each trap would be set up overnight at foraging sites and collected the following day. Insects from the traps would be transported into pre-cleaned glass vials and stored at -20° C until further analysis. Samples collected on different nights but at the same foraging site were pooled together and given specific identification numbers. Each insect sample was then sorted using a dissecting scope by identifying each insect in the sample down to their order and then sorted further into aquatic emerging insects or terrestrial insects of each order using Bugs of Ontario (Sheldon & Acorn 2003), The Freshwater Animal Biodiversity Assessment (Balian et al. 2008), The Complete Illustrated World Encyclopedia of Insects (Walters 2012). The total sample size of insects was 90 samples (aquatic emerging Coleoptera, n=7; terrestrial Coleoptera, n=7; aquatic emerging Diptera, n=9; terrestrial Diptera, n=9; aquatic emerging Ephemeroptera, n= 6; aquatic emerging Heteroptera, n=5; terrestrial Heteroptera, n=2; terrestrial

Homoptera, n=5; terrestrial Hymenoptera, n=6; aquatic emerging Lepidoptera, n=5; terrestrial Lepidoptera, n=11; aquatic emerging Megaloptera, n=2; aquatic emerging Odonata, n=5; aquatic emerging Trichoptera, n=11). These 90 samples were then individually stored in pre-cleaned glass vials with Kimwipes® placed on top and secured with a rubber elastic as a cover and freeze dried for 24 hours using a Labconco FreeZone 2.5 litre freeze dry system, model 7670520 (Kansas, Missouri, United States of America) in the Laboratory for the Analysis of Natural and Synthetic Environmental Toxins (LANCET) at the University of Ottawa. Once freeze dried, each sample was placed in 2.5 mL microtubes and homogenized using a steel ball and mixer mill 301 machine from Retsch, Verder Scientific, Inc. (Newtown, Pennsylvania, United States of America) at 25 Hz for 15 min each and then stored at room temperature until further analysis.

2.2.4. Total mercury analysis

Using approximately 5 milligrams of the fur samples collected (n=243) total mercury content was measured at the LANCET using the MA-3000 Mercury Analyzer (Nippon Instruments Corporation, College Station, Texas, United States of America) following the United States Environmental Protection Agency Method 7473 (SW-846). These methods involve thermal decomposition, gold amalgamation and atomic absorption spectrometry to measure total mercury in the fur. Duplicate measurements were used every tenth sample to measure analytical precision with a mean relative standard deviation of 15 ± 11% (n=25). Total mercury recoveries of three certified reference materials from the National Research Council of Canada were 103 ± 1.4% for IAEA-085 (n=24) (human hair; International Atomic Energy Agency, Vienna, Austria), 95 ± 2.0% for NIMD-01 (n=23) (human hair; National Institute for Minamata Disease, Minamata City, Kumamoto, Japan), 105 ± 3.3% for DOLT-5 (n=24) (dogfish liver; National Research Council,

Ontario, Canada). No pre-treatment was used on the fur based pervious findings that washed and unwashed fur samples showed very little differences in total mercury concentrations (Chételat et al. 2018; Little et al. 2015).

Additionally, using 30-50 mg of the freeze dried, homogenized insect samples (n=54) total mercury content was measured using the MA-3000 Mercury Analyzer at the LANCET to measure total mercury concentration in the insect bodies of both aquatic emerging and terrestrial insects of different orders. Quality control measures included blanks, sample standards and duplicates for every tenth sample. The two standard reference materials used for insect samples were DOLT-5 (dogfish liver; National Research Council, Ontario, Canada), NIMD-01 (human hair; National Institute for Minamata Disease, Minamata City, Kumamoto, Japan).

2.2.5. Stable isotope analysis

Approximately 1 mg of the fur samples collected (n=104) was used to run ^{15}N and ^{13}C stable isotope analysis using the Thermo DeltaV+ Isotope Ratio Mass Spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States of America) at the Ján Veizer Lab of The University of Ottawa. Additionally, 1 mg of the freeze-dried, homogenized insect samples collected (n=90) was used to run ^{15}N and ^{13}C stable isotope analysis.

Both fur and insect samples were weighed and placed into 8 x 5 mm tin capsules that were then loaded into the mass spectrometer and flash combusted at 1800 °C (Dumas combustion method). Gaseous products were carried by helium to oxidizing and reducing chemicals that are optimized specifically for CO_2 and N_2. The gases were then separated by a trap and purge absorption column and sent to the isotope ratio mass spectrometry (IRMS) interface to the IRMS online elemental analyzer system. This interface allows the dual measurements of ^{15}N and ^{13}C.

Quality control measures included blanks, sample standards and duplicates on 10% of the samples. Measurements of ^{15}N were normalized to internal standards previously calibrated to International standards: IAEA-N1(+0.4‰), IAEA-N2(+20.3‰), USGS-40(-4.52‰) and USGS-41(47.57‰). Measurements of ^{13}C were normalized to internal standards previously calibrated to International standards: IAEA-CH-6(-10.4‰), NBS-22(-29.91‰), USGS-40(-26.24‰) and USGS-41(37.76‰). Internal standards used for both ^{15}N and ^{13}C (%^{15}N, %^{13}C) include C-51 Nicotiamide (0.07,-22.95), C-52 mix of ammonium sulphate + sucrose (16.58,-11.94), C-54 caffeine (-16.61,-34.46), blind std C-55: glutamic acid (-3.98, -28.53) which cover the naturally occurring ranges. The analytical precision of this methodology is approximately 0.2‰.

2.2.6. Statistical analysis

As the majority of the bat total mercury fur data set was limited by missing data for individual factors, no full model investigating all combined factors and their action simultaneously was run. Thus, once ANOVA-assumptions of normality (assessed by Shapiro-Wilk test) and homoscedasticity (assessed by Levene's test) were met in raw or transformed data, unpaired two-tailed t-tests, one- and two-way ANOVA analyses were conducted to assess the relevance of specific factors in fur total mercury concentration. Following significant findings in ANOVA omnibus tests, Tukey's post-hoc test was employed to identify significant differences between groups. In both cases, a $p<0.05$ significance cut-off was used. In cases where ANOVA conditions were not met, data were analyzed by Mann-Whitney U test or Kruskal-Wallis non-parametric test followed by Dunn's post-hoc test, again at significance cut-off of $p<0.05$. Linear correlations between total fur mercury and individual factors were conducted using linear regression analysis. Goodness of fit was evaluated using R_2 and slopes significantly different from 0 evaluated at

$p<0.05$ cut-off. All graphs and analyses were plotted and conducted using GraphPad Prims software v8 (Graphpad, San Diego, CA, United States of America).

2.3. Results

2.3.1 Total mercury fur concentration in Eastern Ontario bats is species-dependent

Fur total mercury concentration between Eastern Ontario bat species exhibit significant differences (df= 2, H=34.29, $p<0.001$; **Fig. 4A**). Specifically, fur total mercury concentration measured significantly higher in big brown bats compared to little brown bats ($p<0.0001$) but had no significant differences when compared to the northern long-eared bat ($p=0.060$). The difference in fur total mercury concentration between big brown bats and little brown bats is approximately 2-fold, reaching a median of 5125 µg/kg and 2594 µg/kg, respectively. 28% of bat fur measured exceeded the 10 ppm threshold. Similarly, body mass exhibited differences in the eastern Ontario bat species (df=2, H=107.9, $p<0.0001$; **Fig. 4B**). Body mass of big brown bats is significantly higher compared to little brown bats ($p<0.0001$) and the northern long-eared bats ($p<0.0001$) where the median body mass measured to be 14.5 g for big brown bats, 7.78 g for little brown bats and 5.7 g for northern long-eared bats. When fur total mercury concentration was normalized by body mass, we found similar differences between the species (df=2, H=20.08, $p<0.0001$; **Fig. 4C**). Big brown bats exhibit significantly greater fur total mercury concentration normalized by body mass than little brown bats ($p=0.006$) and northern long-eared bat ($p=0.0004$).

When correlating total fur mercury data across these eastern Ontario bat species with body mass, linear regression identifies a positive slope significantly different from zero with an $R^2=0.126$ (df=1, F= 23.33, $p<0.0001$; **Fig. 5A**). When considering the slopes within species (**Fig.**

5B), total fur mercury concentration does not significantly correlate with body mass in either little brown bats (df=1, F=0.023, p=0.87) nor big brown bats (df=1, F=0.1006, p=0.74).

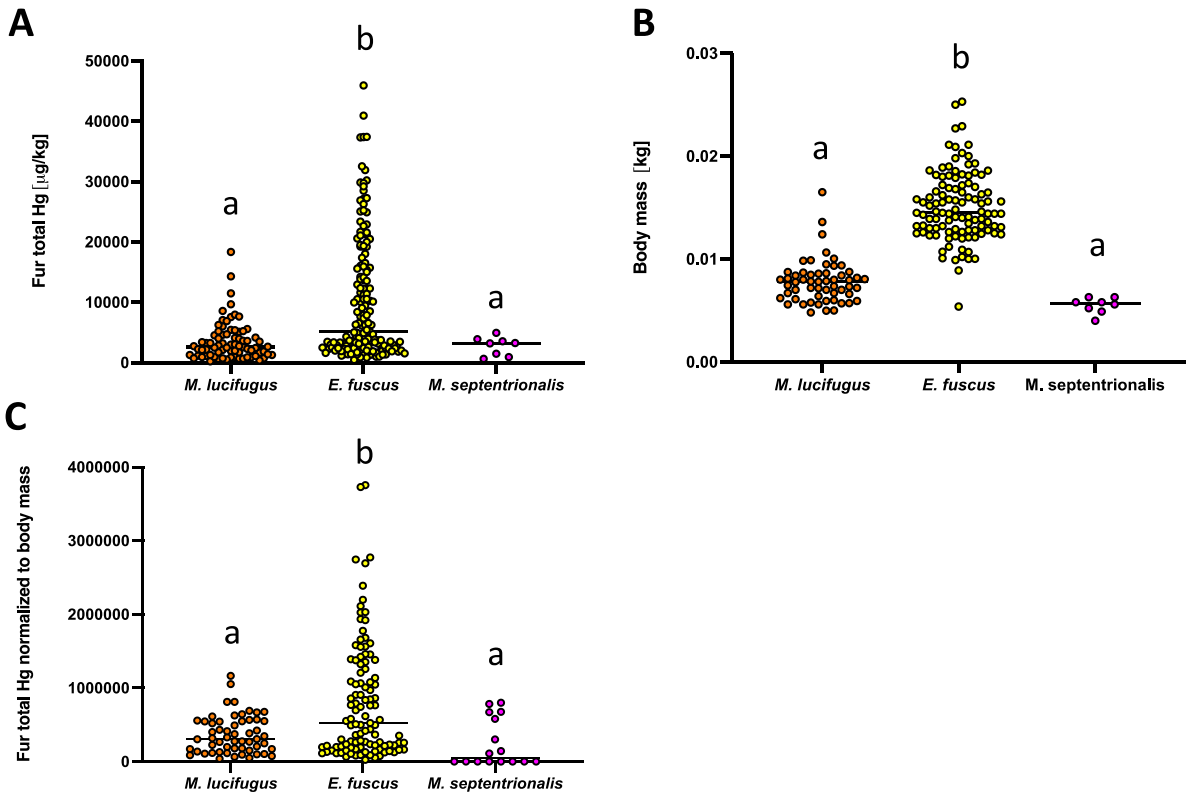

Figure 4. *Fur total mercury* (**A**) *body mass* (**B**) *and fur total mercury normalized to body mass* (**C**) *in Eastern Ontario bat species caught between 1999-2020. Individual observations for fur total mercury are as follows: M. lucifugus (n=76), E. fuscus (n=176) and M. septentrionalis (n=8). Available body mass data and therefore available data normalized by body weight are as follows: M. lucifugus (n=56), E. fuscus (n=107), M. septentrionalis (n=8). Here and throughout the thesis, M. lucifugus data points are presented in orange, E. fuscus data points are presented in yellow, and M. septentrionalis data points are presented in pink. All data were analyzed using a Kruskal-Wallis test and significant differences between groups (p<0.05) indicated by different letters.*

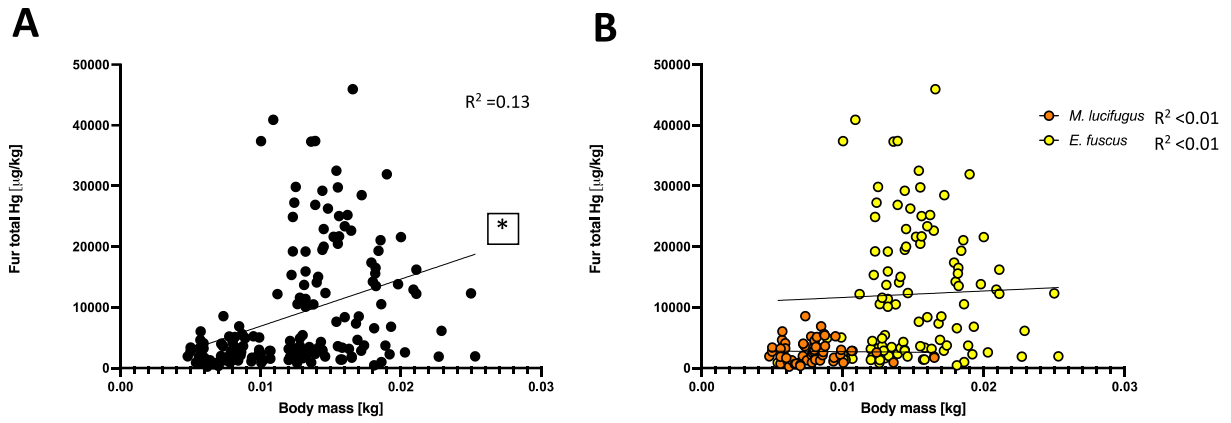

Figure 5. *Correlation between fur total mercury concentration and body mass in both M. lucifugus and E. fuscus combined (n=163)* (**A**) *and separated in M. lucifugus (n=56) and E. fuscus (n=107) separately* (**B**). *All data were analyzed by simple linear regression and significant slope deviations from zero (p<0.05) are indicated by asterisk.*

2.3.2. Sex affects fur total mercury concentration in *E. fuscus* in Eastern Ontario

When considering fur total mercury concentration within species by sex, little brown bats did not exhibit significant differences between females and males (df=60, t=1.147, p=0.26; **Fig. 6A**). There were no significant differences in little brown bats when comparing the body mass of females and males (df=54, t=0.079, p=0.94; **Fig. 6B**). When normalizing fur total mercury concentration to body mass, the female and male little brown bats did not exhibit significant differences in their fur total mercury concentration (df=54, t=0.73, p=0.47; **Fig. 6C**).

Conversely, within the big brown bats, males exhibit significantly higher fur total mercury concentration compared to females (U=1043, p=0.0075; **Fig. 6D**). The median fur total mercury concentration reaches 12 355 µg/kg in males, more than twice the median fur total mercury concentration of 4786 µg/kg in females. With respect to big brown bats, the body mass of females measured significantly higher than the males (df=104, t=4.97, p<0.0001; **Fig. 6E**). When fur total mercury concentration was normalized to body mass, the male big brown bats nevertheless still

exhibited significantly higher fur total mercury concentration than the female big brown bats (U=786, p=0.0007; **Fig. 6F**).

Fur total mercury concentration positively correlates with both female (df=1, F=23.96, p<0.0001) and male (df=1, F=13.93, p=0.0003) body mass when considering both eastern Ontario bat species combined (**Fig. 7A**). Within species (**Fig. 7B**), no correlations between fur total mercury concentration and body mass were observed for female (df=1, F=2.267, p=0.14) or male (df=1, F=0.37, p=0.55) little brown bats. While no significant correlation was observed between body mass and fur total mercury concentration for female big brown bats (df=1, F=2.54, p=0.12), these variables significantly and positively correlated within the male big brown bats (df=1, F=5.03, p=0.03).

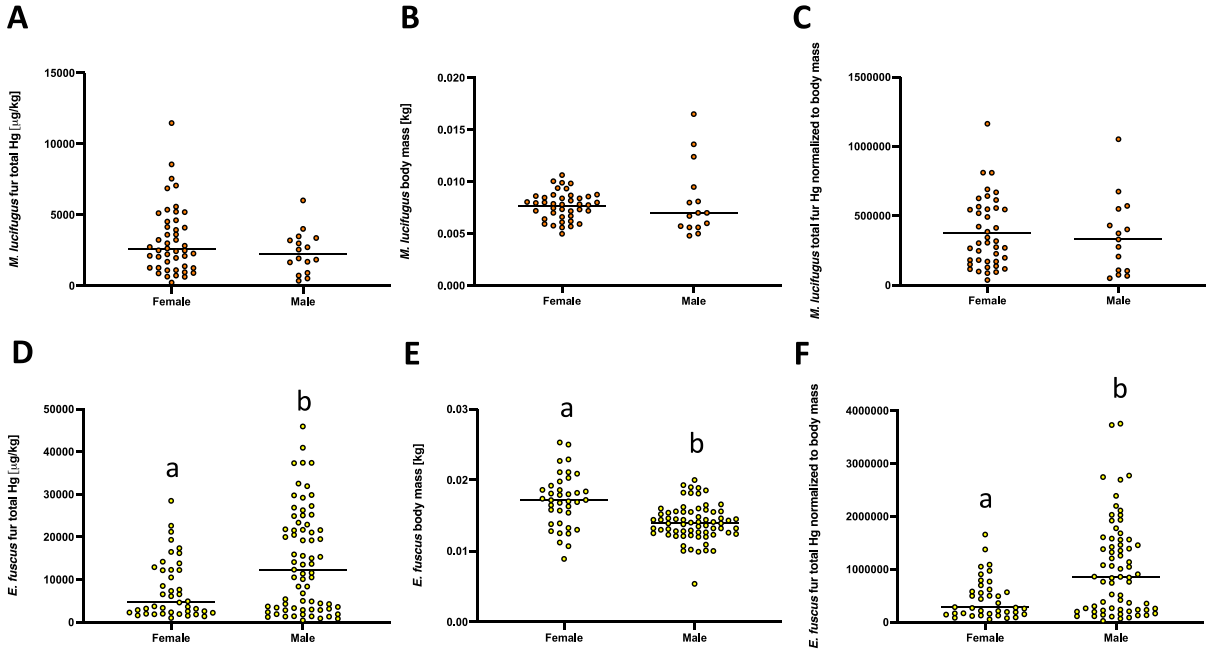

Figure 6. *Fur total mercury concentration by sex, body mass by sex and fur total mercury concentration normalized to body mass in M. lucifugus* (**A-C**) *and E. fuscus* (**D-F**). *Sample sizes for fur total mercury concentration are n=46 and n=15 for female and male M. lucifugus and n=42 and n=71 for female and male E. fuscus, respectively. Available weight data and thus for weight normalized fur total mercury for females and males was n=41 and n=15 for M. lucifugus and n=38 and n=68 for E. fuscus, respectively. In cases were data was normally distributed or could be transformed to fit a normal distribution, data were analyzed by unpaired t-test in cases were data was nonparametric by Mann-Whitney U test. All significant differences (<0.05) are indicated by different letters.*

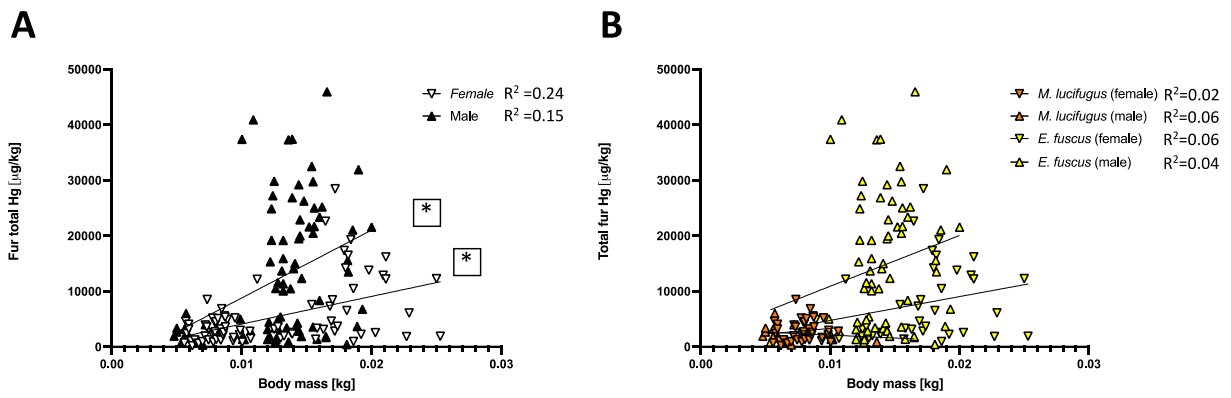

Figure 7. *Correlation between fur total mercury and body mass in both female (n=78) and male (n=84) M. lucifugus and E. fuscus combined (**A**) and separated by species (**B**). Within the M. lucifugus n=38 females and n=16 males were analyzed. Within E. fuscus n=40 females and n=68 males were analyzed. Data were analyzed by simple linear regression and significant slope deviations from zero (p<0.05) are indicated by an asterisk.*

2.3.3. Female reproductive status as determinant of fur total mercury concentration in Eastern Ontario bats

When comparing non-reproductive female adults, pregnant females, and lactating females within the little brown bats, significant differences in fur total mercury concentrations were observed (df=2, H=7.684, p=0.02; **Fig. 8A**), with significantly higher mercury concentrations in non-reproductive adult females compared pregnant females (p=0.02). When comparing body mass between reproductive status groups in female little brown bats (df=2, F=14.45, p<0.0001; **Fig. 8B**) a significant increase in body mass in pregnant females compared to non-reproductive adult females (p<0.0001) and lactating females (p=0.0061) was observed. Fur total mercury normalized to body mass in female little brown bats of each reproductive status showed significant differences (df=2, F=6.651, p=0.004; **Fig. 8C**) where the non-reproductive female adults measured significantly higher in fur total mercury concentration than the pregnant females (p=0.0042).

When comparing non-reproductive female adults, pregnant females, and lactating females within the big brown bats, significant differences in fur total mercury concentrations were

observed (df=2, H=7.7600, p=0.02; **Fig. 8D**), with significantly higher mercury concentrations in lactating females compared to non-reproductive adult females (p=0.03). Comparing body mass between reproductive status groups in female big brown bats showed significant differences (df=2, F=14.3, p<0.0001; **Fig. 8E**). Post-hoc analysis revealed significantly increased body mass in pregnant big brown bats compared to both non-reproductive adults (p<0.0001) and lactating females (p=0.01). Furthermore, lactating females had significantly higher body mass than non-reproductive adults (p=0.01). Fur total mercury concentration normalized to body mass in female little browns of the different reproductive statuses showed significant differences (df=2, H=7.563, p=0.02; **Fig. 8F**), with significantly higher normalized fur total mercury concentrations in lactating female big brown bats compared to pregnant female big brown bats (p=0.04).

When considering correlations between the combined eastern Ontario bat species reproductive status and body mass, a significant positive correlation was observed for lactating females (df=1, F=32.79, p<0.0001) but not for non-reproductive females (df=1, F=3.192, p=0.80) or pregnant females (df=1, F=3.043, p=0.12) (**Fig. 9A**). When considering the same correlations within species, no significant correlations were observed: non-reproductive female little brown bats (df=1, F=1.907, p=0.19), pregnant little brown bats (df=1, F=0.425, p=0.54), lactating little brown bats (df=1, F=0.057, p=0.82), non-reproductive female adult big brown bats (df=1, F=0.379, p=0.38), pregnant big brown bats (df=1, F=0.20, p=0.73), and lactating big brown bats (df=1, F=0.0154, p=0.76) (**Fig. 9B**).

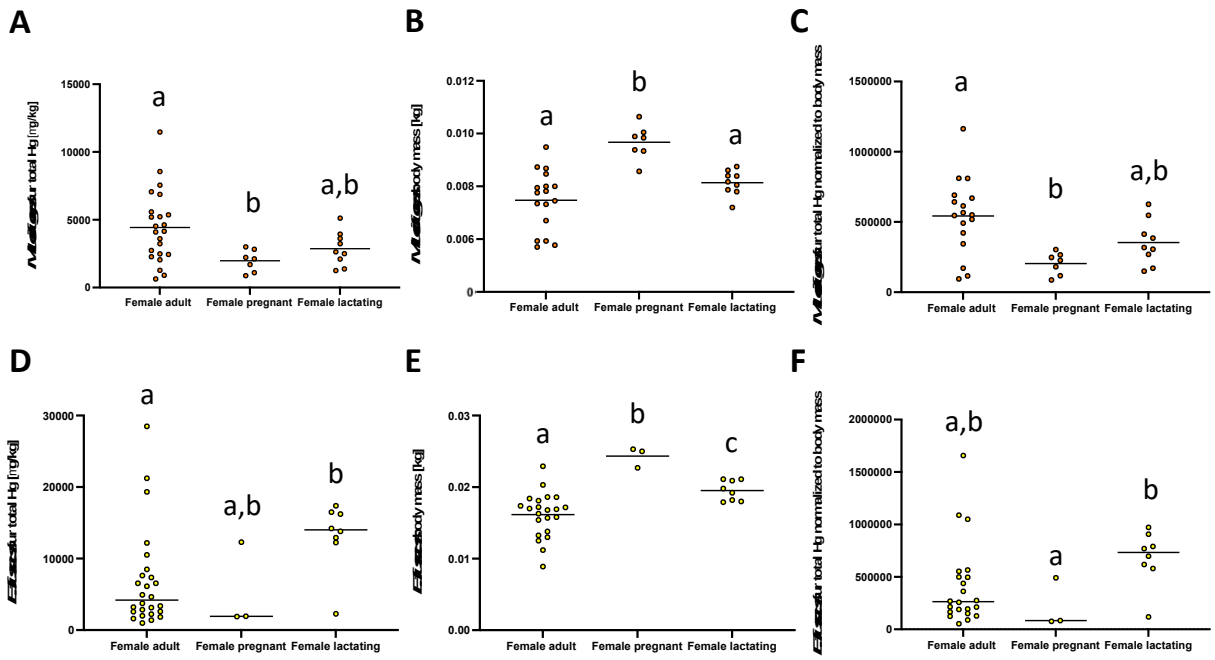

Figure 8. *Fur total mercury concentration, body mass and fur total mercury concentration normalized to body mass in non-reproductive adult females, adult pregnant females and adult lactating females in M. lucifugus* (**A-C**) *and E. fuscus* (**D-F**). *Sample sizes for fur total mercury concentration, available weight data and thus for weight normalized total fur mercury are n=23 for non-reproductive adult females, n=7 for pregnant females and n=9 for lactating females for M. lucifugus and n=26, n=3 and n=8 for the same groups in E. fuscus, respectively. Body mass data and therefore fur total mercury concentration normalized to body mass data were n=17 n=7 and n=9 for non-reproductive adult, pregnant and lactating females in M. lucifigus and n=22, n=3 and n=8 for the same groups in E. fuscus. In cases where data was normally distributed or could be transformed to fit a normal distribution, data were analyzed by one-way ANOVA. In cases where data were nonparametric and could not be transformed to meet normality criteria, a Kruskal- Wallis test was used. All significant differences ($p<0.05$) are indicated by different letters.*

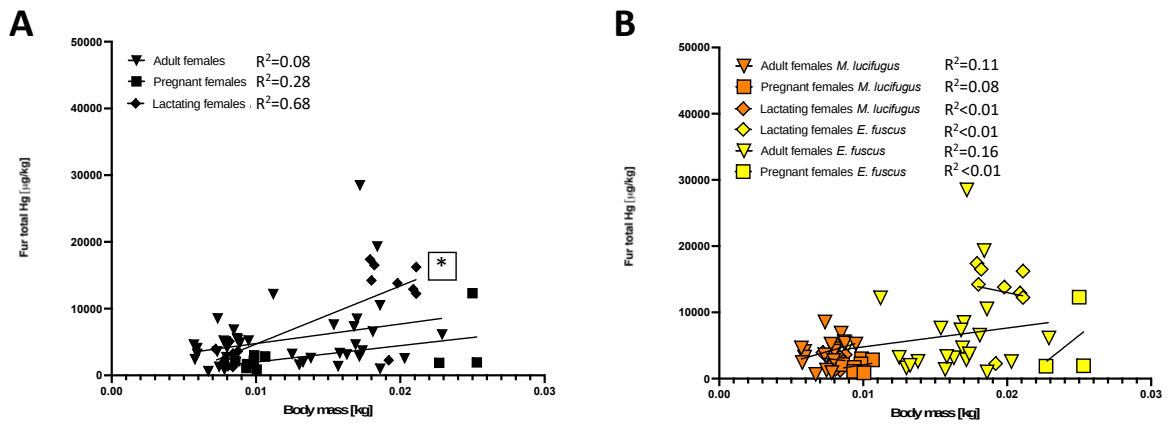

Figure 9. *Correlation between fur total mercury concentration and body mass in non-reproductive adult female (n=39), pregnant female (n=10) and lactating female (n=17) M. lucifugus and E. fuscus combined* (**A**) *and separated by species* (**B**). *Within M. lucifugus, n=17 non-reproductive adult females, n=7 pregnant females and n=9 lactating females were analyzed. Within E. fuscus n=22, n=3 and n=8 for were analyzed for the same respective groups. Data were analyzed by simple linear regression and significant slope deviations from zero (p<0.05) are indicated by asterisk.*

2.3.4. Age increases fur total mercury concentrations in Eastern Ontario bats

The effect of age on fur total mercury concentration between young and adult little brown bats showed significantly higher concentrations in adults compared to young (df=50, t=5.123, p<0.0001; **Fig. 10A**). The adult little brown bats measured significantly greater in terms of body mass than the young (U=17.50, p<0.0001; **Fig. 10B**). When normalizing the fur total mercury concentration to the body mass there was no significant difference between adult little brown bats and the young (df=12, F=1.399, p=0.42; **Fig 10C**).

The effect of age on fur total mercury concentration between young and adult big brown bats exhibited no significant differences in fur total mercury concentration (U=55.00 p=0.08; **Fig. 10D**) Similarly, the body mass showed to not be significantly different between the juvenile and adult bats (U=52.00, p=0.07; **Fig. 10E**). When fur total mercury concentration was normalized to body mass there was no significant difference exhibited between the total mercury concentration

in the fur of adult big brown bats and the young (U=66.00, p=0.13; **Fig. 10F**). These findings are likely due to the small sample size of juvenile big brown bats (n=3).

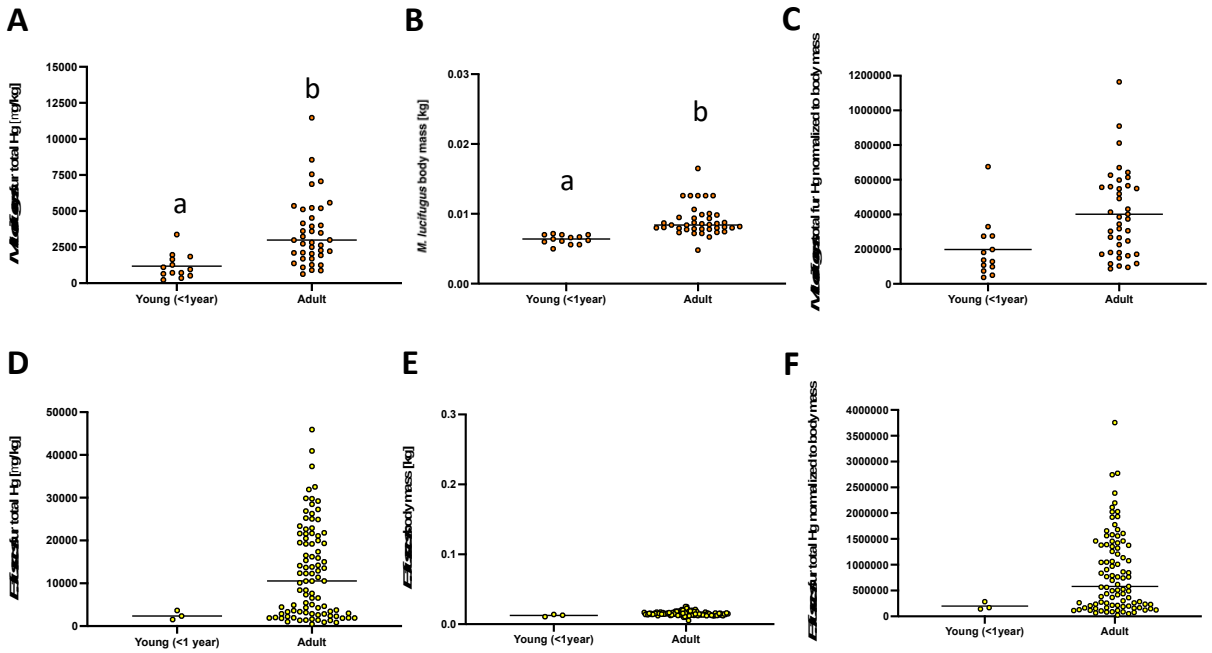

Figure 10. *Fur total mercury concentration by age, body mass by age and fur total mercury concentration normalized to body mass in M. lucifugus* (**A-C**) *and E. fuscus* (**D-F**). *Sample sizes for fur total mercury concentration, available weight data and thus for weight normalized total fur mercury for young and adults are n=13 and n=39 for young and adult M. lucifugus and n=3 and n=93 for E. fuscus, respectively. In cases where data was normally distributed or could be transformed to fit a normal distribution, data were analyzed by unpaired t-test. In cases where data were nonparametric and could not be transformed to meet normality criteria, a Mann-Whitney U test was used. All significant differences (p<0.05) are indicated by different letters.*

2.3.5. Geographical influence on fur total mercury concentration in Eastern Ontario bats

When comparing differences in fur total mercury concentration between little brown bats captured from different locations, there were no significant differences (df=2, F=1.673, p=0.20; **Fig. 11A**). The body mass of little brown bats differed significantly between locations (df=2, H=36.42, p<0.001; **Fig. 11B**) and little brown bats captured in Cornwall had a smaller body mass than those captured in Morrisburg (p<0.0001) and Williamstown (p<0.0001), respectively. The fur total mercury concentration normalized to body mass in the little brown bats exhibited no significant differences between capture locations (df=2, H=2.162, p=0.34; **Fig. 11C**).

Big brown bats captured from different locations had significantly different fur total mercury concentrations (df= 4, H= 13.50, p= 0.009; **Fig. 11D**), with higher fur total mercury concentrations being observed in big brown bats captured in Morrisburg compared to Cornwall (p=0.006). Big brown bats captured at different locations also measured significantly different in terms of their body mass (df=4, F=3.14, p=0.02; **Fig. 11E**), with big brown bats captured in Martintown measuring higher in body mass than big brown bats captured in Morrisburg (p=0.01). When normalizing fur total mercury concentration to body mass in the big brown bats, a significant difference in fur total mercury concentration (df=4, H=18.38, p=0.001; **Fig 11F**) exists, manifesting as a higher concentration in bats from Morrisburg compared to bats in Martintown (p=0.047) and Cornwall (p=0.001).

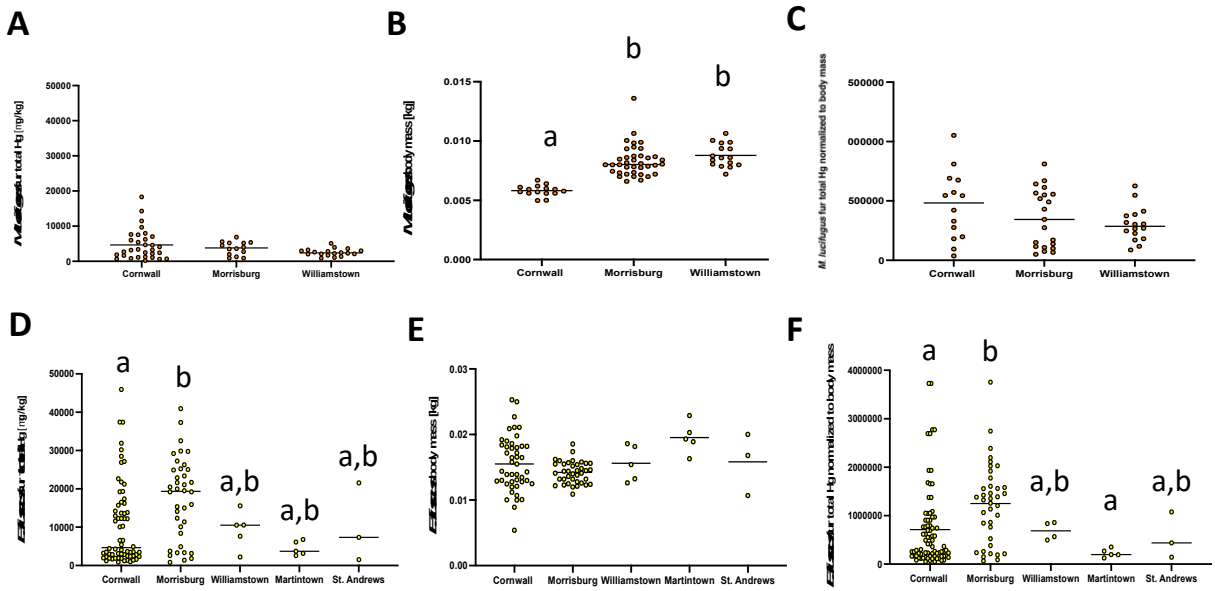

Figure 11. *Fur total mercury concentration by location, body mass by location and fur total mercury concentration normalized to body mass by location in M. lucifugus* (**A-C**) *and E. fuscus* (**D-F**). *Sample sizes for fur total mercury concentration for M. lucifugus were n=31 (Cornwall), n=14 (Morrisburg) and n=18 (Williamstown) as well as n=66 (Cornwall), n=38 (Morrisburg), n=5 (Williamstown), n=5 (Martintown), n=3 (St. Andrews) for E. fuscus. Weight data sample size and hence available weight normalized data sample size were n=15 (Cornwall), n=38 (Morrisburg), and n=16 (Williamstown) for M. lucifugus and n=46 (Cornwall), n=38 (Morrisburg), n=5 (Williamstown), n=5 (Martintown) and n=3 (St. Andrews) for E. fuscus. In cases where data was normally distributed or could be transformed to fit a normal distribution, data were analyzed by one-way ANOVA. In cases where data were nonparametric and could not be transformed to meet normality criteria, a Kruskal-Wallis test was used. All significant differences ($p<0.05$) are indicated by different letters.*

2.3.6. Geographical differences in fur total mercury concentration in Eastern Ontario bats are sex- and age-dependent

When further analyzing whether geographical effects are sex-dependent in little brown bats, neither location (df=1, F=0.296, p=0.59), nor sex (df=1, F=0.710, p=0.405) or their interaction (df=1, F=2.2, p=0.147) had a significant effect on the fur total mercury concentration of bats captured in Cornwall and Morrisburg (**Fig. 12A**). Fur total mercury concentration of the little brown bats normalized to their body mass (**Fig. 12B**) does also not change significantly by location (df=1, F=2.651, p=0.113) or sex (df=1, F=0.034, p=0.855). There is, however, a

significant effect on the fur total mercury concentration in little brown bats when investigating the interaction between location and sex (df=1 F=5.497, p=0.025), although the specific differences between groups could not be resolved by post-hoc analysis. When investigating whether the effects of geographical locations on fur total mercury concentration in the little brown bats are age-dependent (**Fig. 12C**), neither location (df=1, F=0.462, p=0.500), age (df=1, F=0.528, p=0.471), nor their interaction (df=1, F=0.010, p=0.921) exhibited a significant effect.

When analyzing whether geographical effects of total mercury concentrations are sex-dependent (**Fig. 12D**), there was no significant effect of location of capture (df=1, F=2.879, p=0.93). Conversely, significant effects of both sex (df=1, F=13.727, p=0.001) and location of capture and sex interaction (df=1 F=6.073 p=0.016) exist. These significant effects translate to male big brown bats measuring higher in fur total mercury concentration than female big brown bats across locations ($p<0.05$), driven by significant sex differences in Morrisburg ($P<0.05$) but not Cornwall. Big brown bats exhibited significant sex differences (df=1 F=9.422, p=0.003) but no significant effects of location (df=1, F=0.277, p=0.601) or the sex and location interaction (df=1, F=3.032, p=0.087) when fur total mercury concentration was normalized to body mass (**Fig. 12E**). Post-hoc analysis resolved sex differences as males measuring greater normalized fur total mercury concentration compared to females ($p<0.05$; **Fig. 12E**). Due to insufficient sample sizes of young big brown bats, a two-way ANOVA analysis could not be used when investigating the effect of age and location on fur total mercury concentration. However, a comparison between adult big brown bats collected from Cornwall and Morrisburg revealed significant differences in fur total mercury (df=82, t=3.683, $p<0.0001$; **Fig. 12F**) where adult big brown bats collected from Morrisburg measured higher in fur total mercury concentration than adult big brown bats collected in Cornwall (p=0.0004; **Fig. 12F**).

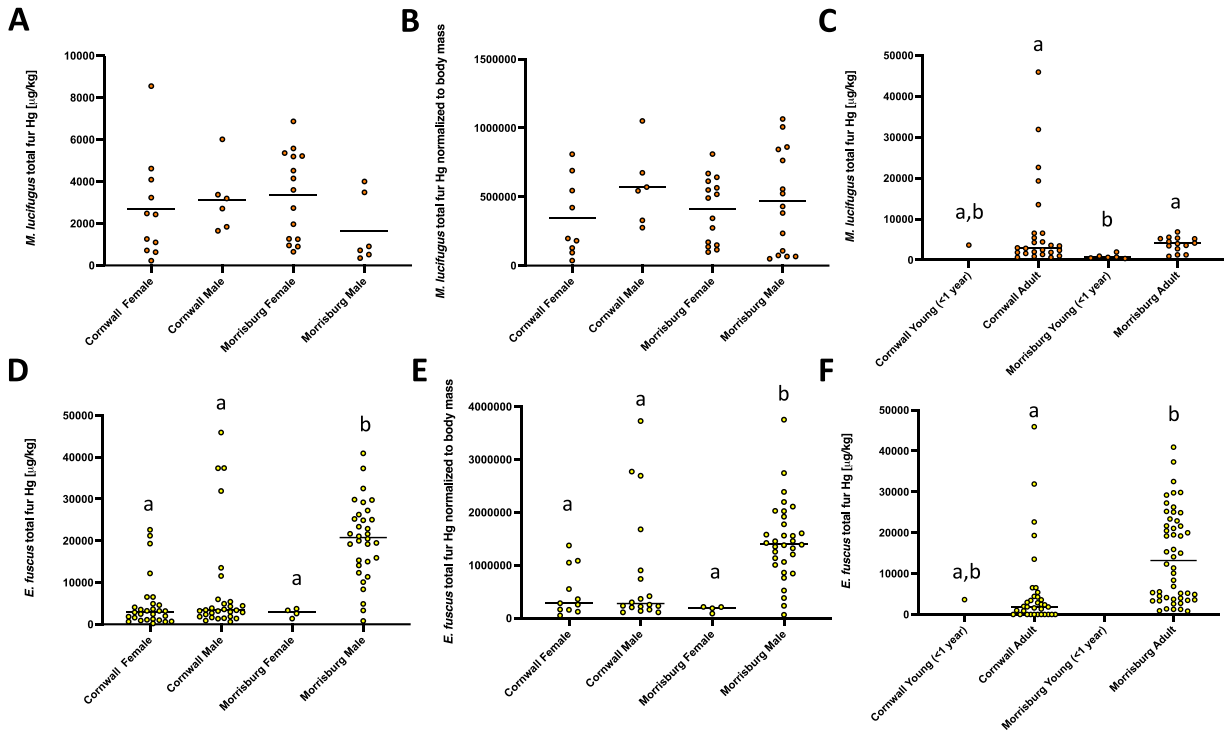

Figure 12. *Fur total mercury concentration by location and sex, location and sex normalized to body mass, and location by age in M. lucifugus* (**A-C**) *and E. fuscus* (**D-F**). *Sample sizes for fur total mercury concentration for M. lucifugus were n=11 females and n=6 males (Cornwall) and n=15 females n=6 males (Morrisburg). For E. fuscus, sample sizes for fur total mercury concentration were n=28 females and n=29 males (Cornwall) and n=4 females and n=32 males (Morrisburg). Sample sizes for fur total mercury concentration normalized to body mass were n=9 females and n=6 males (Cornwall), n=15 females and n=6 males (Morrisburg) for M. lucifugus and n=11 females and n=18 males (Cornwall), n=4 females and n=32 males (Morrisburg) for E. fuscus. Sample sizes for age-dependent analysis of fur total mercury in M. lucifugus were n=1 young and n=25 adult (Cornwall) and n=6 young and n=14 adult (Morrisburg). For E. fuscus, corresponding sample sizes were n=1 young and n=34 adult (Cornwall) and n=50 adult (Morrisburg). In cases where data was normally distributed or could be transformed to fit a normal distribution, data were analyzed by unpaired t-test. In cases where data were nonparametric and could not be transformed to meet normality criteria, a Kruskal-Wallis test was used. All significant differences ($p<0.05$) are indicated by different letters.*

2.3.7 Temporal influence on fur total mercury concentration in Eastern Ontario bats

There were significant differences in fur total mercury concentration between different years of capture in little brown bats (df=6, H=20.00, p=0.0028; **Fig. 13A**). Little brown bats captured in 2003 and 2005 measured significantly higher in fur total mercury concentrations than those captured in the year 2001 (p=0.049 and p=0.037, respectively). The body mass of little brown bats also showed significant differences between year of capture (df=3, F=11.48, p=0.0001; **Fig.**

13B), and little brown bats caught in 2019 measured higher in body mass than little brown bats caught in 1999 ($p<0.0001$). After normalizing the fur total mercury concentration in little brown bats to body mass, significant differences in fur total mercury concentration and year of capture were evident ($df=3$, $H=14.40$, $p=0.002$; **Fig. 13C**). Little brown bats captured in 1999 and 2019 measured greater in fur total mercury concentration that those caught in 2001 ($p=0.012$ and $p=0.03$, respectively).

With regard to big brown bats, the year of capture had a significant effect on fur total mercury concentrations ($df=10$, $H=44.43$, $p<0.0001$; **Fig. 13D**). Big brown bats collected in the year 2017 measured higher in fur total mercury concentration than those collected in 2013 ($p<0.05$) and big brown bats collected in 2016, 2017, and 2018 measured higher in fur total mercury concentration than those collected in 2015 ($p<0.05$ in all cases). The body mass of big brown bats differed significantly between years of collection ($df=8$, $F=7.141$, $p<0.0001$; **Fig. 13E**). Post-hoc analysis revealed that big brown bats collected in the years 2011 and 2016 measured higher in body mass compared to those collected in 2002, 2003, and 2013 ($p<0.05$ in all cases). When normalizing the fur total mercury concentration to body mass for big brown bats, a significant difference between years of collection was observed ($df=8$, $F=17.94$, $p=0.0216$; **Fig. 13F**), however, post-hoc analysis could not resolve differences between specific years of collection.

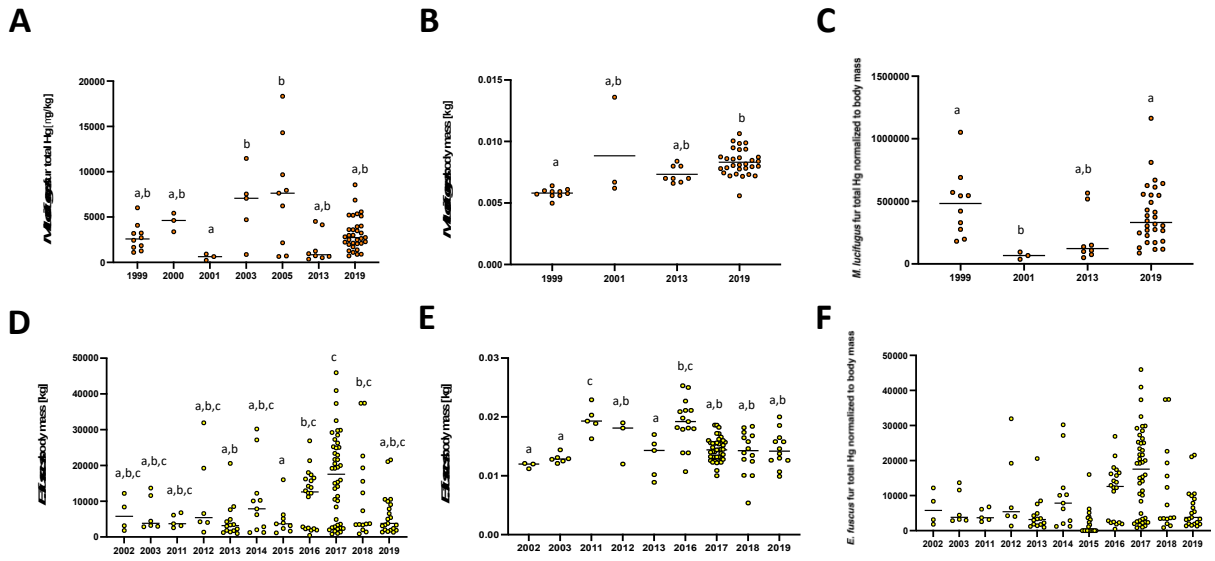

Figure 13. *Fur total mercury concentration by year of collection, body mass by year of collection, and fur total mercury concentration normalized to body mass by year of collection in M. lucifugus* (**A-C**) *and E. fuscus* (**D-F**). *Sample sizes for fur total mercury concentration for M. lucifugus are as follows: n=10 in 1999, n=3 in 2000, n=5 in 2001, n=9 in 2005, n=8 in 2013, and n=33 in 2019. Weight data sample sizes and hence available weight normalized data sample sizes for M. lucifugus are as follows: n=10 in 1999, n=3 in 2001, n=8 in 2013, and n=30 in 2019. Sample sizes for fur total mercury concentration for E. fuscus are as follows: n=4 in 2002, n=6 in 2003, n=5 in 2011, n=6 in 2012, n=14 in 2013, n= 11 in 2014, n=9 in 2015, n=20 in 2016, n=44 in 2017, n=15 in 2018, and n=22 in 2019. Weight data sample sizes and hence available weight normalized data sample sizes E. fuscus are as follows: n=3 in 2002, n=6 in 2003, n=5 in 2011, n=3 in 2012, n=5 in 2013, n=15 in 2016, n=34 in 2017, n=14 in 2018, and n=12 in 2019. In cases where data was normally distributed or could be transformed to fit a normal distribution, data were analyzed by unpaired t-tests. In cases where data were nonparametric and could not be transformed to meet normality criteria, a Kruskal Wallis test was used. All significant differences (p<0.05) are indicated by different letters.*

Investigating whether effects of collection time were dependent on sex (**Fig. 14A**), little brown bats revealed that there were no significant effects by year (df=2, F=2.618, p=0.84, sex (df=1, F=0.134, p=0.716), or the interaction between both factors (df=2, F=1.204, p=0.309). The body mass of little brown bats (**Fig. 14B**) differed significantly between years of collection (df=2, F=8.79, p<0.0001) where little brown bats collected during 1999 were significantly smaller in terms of body mass compared to those collected in 2013 and 2019 (p<0.05 in both cases). However, there were no significant effects of sex (df=1, f=1.988, p= 0.165) or the interaction of year and sex (df=2, f=0.128, p=0.881) on the body mass of little brown bats. After normalizing the

fur total mercury concentration to the body mass of little brown bats (**Fig. 14C**), there was a significant effect of the year of collection (df=2, f=3.462, p=0.040). However, specific effects could not be resolved by post-hoc test. There were no significant effects of sex (df=1, f=0.004, p=0.947) or the interaction of year and sex (df=2, F=1.381, p=0.262) on normalized fur total mercury concentrations for little brown bats.

Big brown bats exhibited a significant effect of sex (df=1, F=4.454, p=0.039; **Fig. 14D**) on fur total mercury concentration where males had higher concentrations compared to females ($p<0.05$). However, no significant effects were observed for the year of collection (df=2, F=0.0241, p=0.786) or the interaction of year of collection and sex (df=2, F=1.844, p=0.166). The body mass of big brown bats (**Fig. 14E**) differed significantly between years of collection (df=2, F=5.000, p=0.010) and sex (df=1, F=23.936, $p<0.0001$), but was not significantly affected by the interaction of collection year and sex (df=2, F=2.839, p=0.066). Post-hoc analysis revealed that in the year 2016, collected big brown bats had a higher in body mass compared to those collected in 2017 and 2018 ($p<0.05$ in all cases) and that across years, females were heavier than males ($p<0.05$). Fur total mercury concentration normalized to big brown bat body mass (**Fig. 14F**) showed a significant effect of sex (df=1, F= 7.714, p=0.007), and post-hoc analysis revealed that males measured higher in normalized fur total mercury than females ($p<0.05$). However, no significant effect of year of collection (df=2, f=0.413, P=0.664) or interaction between the year of collection and sex (df=2 1.116, p=0.334) was observed for the normalized fur total mercury concentrations of big brown bats.

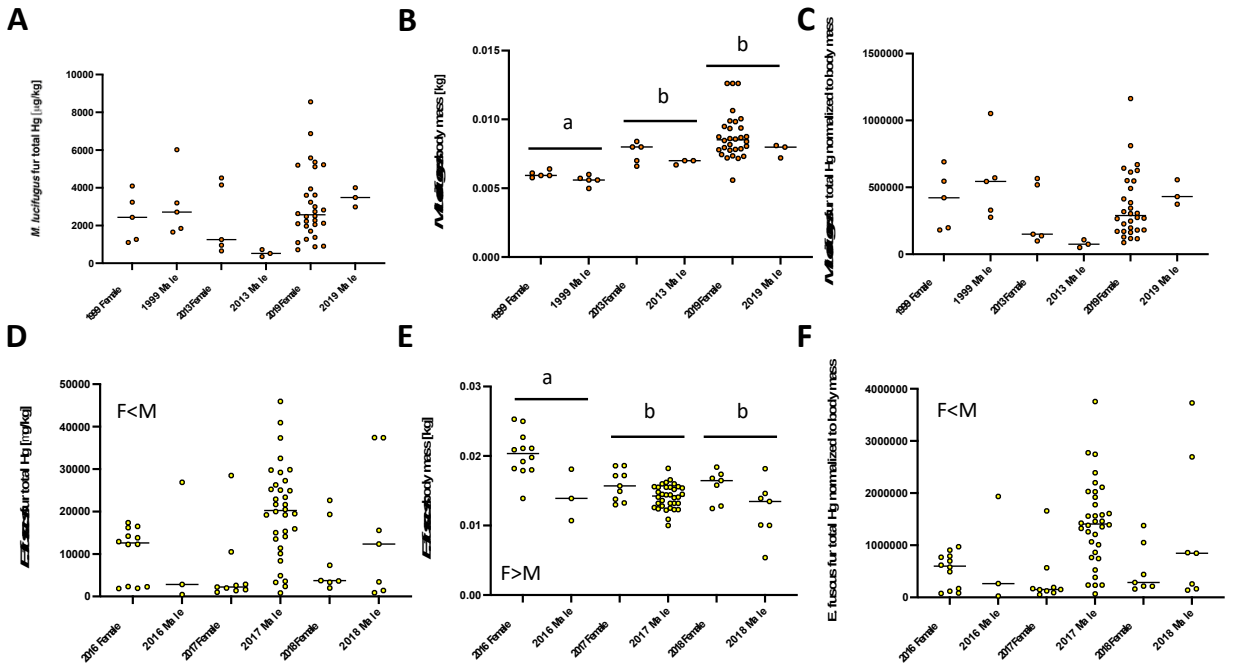

Figure 14. *Fur total mercury concentration by sex and year of collection, body mass by sex and year of collection, and fur total mercury concentration normalized to body mass by sex and year of collection in M. lucifugus* (**A-C**) *and E. fuscus* (**D-F**). *Sample sizes for fur total mercury concentration for M. lucifugus are as follows: n= 5 females and n=5 males in 1999, n=5 females and n=3 males in 2013, and n=29 females and n=3 males in 2019. Sample sizes for fur total mercury concentration for E. fuscus are as follows: n=13 females and n=3 males in 2016, n=9 females and n=33 males in 2017, and n=7 females and n=7 males in 2018. In cases where data was normally distributed or could be transformed to fit a normal distribution, data were analyzed by unpaired t-tests. In cases where data were nonparametric and could not be transformed to meet normality criteria, a Kruskal Wallis test was used. Significant differences between timepoints ($p<0.05$) are indicated by different letters and significant sex effects are indicated inside the relevant graphs using the letters F (female) and M (male).*

2.3.8. Dietary contributions to fur total mercury concentration in Eastern Ontario bats

2.3.8.1. Total mercury concentration in aquatic emerging and terrestrial Eastern Ontario insects

Total mercury concentrations exhibit significant differences between aquatic emerging insects and terrestrial insects ($df=50$, $t=4.706$, $p<0.0001$; **Fig. 15A**) with significantly higher in total mercury concentrations in aquatic emerging insects compared to terrestrial insects. When investigating differences in total mercury concentrations between insect orders irrespective of their status as aquatic-emerging species and terrestrial species (**Fig. 15B**), significant differences were

observed (df=7, H=36.29, $p<0.0001$). Post-hoc analyses revealed that lepidoptera measured lower in total mercury concentration compared to coleoptera, megaloptera and trichoptera ($p<0.01$). When total mercury concentrations between different orders of aquatic emerging species (**Fig. 15C**), significant differences in total mercury concentration were also identified (df=6, H=20.31, p=0.0024) with aquatic emerging lepidoptera measuring lower in total mercury concentration than aquatic emerging megaloptera ($p<0.05$). Among terrestrial insect orders (**Fig. 15D**), significant differences in total mercury concentration also exist (df=4, F=5.904, p=0.0032) with lower concentrations of total mercury in terrestrial lepidoptera compared to terrestrial coleoptera and terrestrial diptera ($p<0.05$).

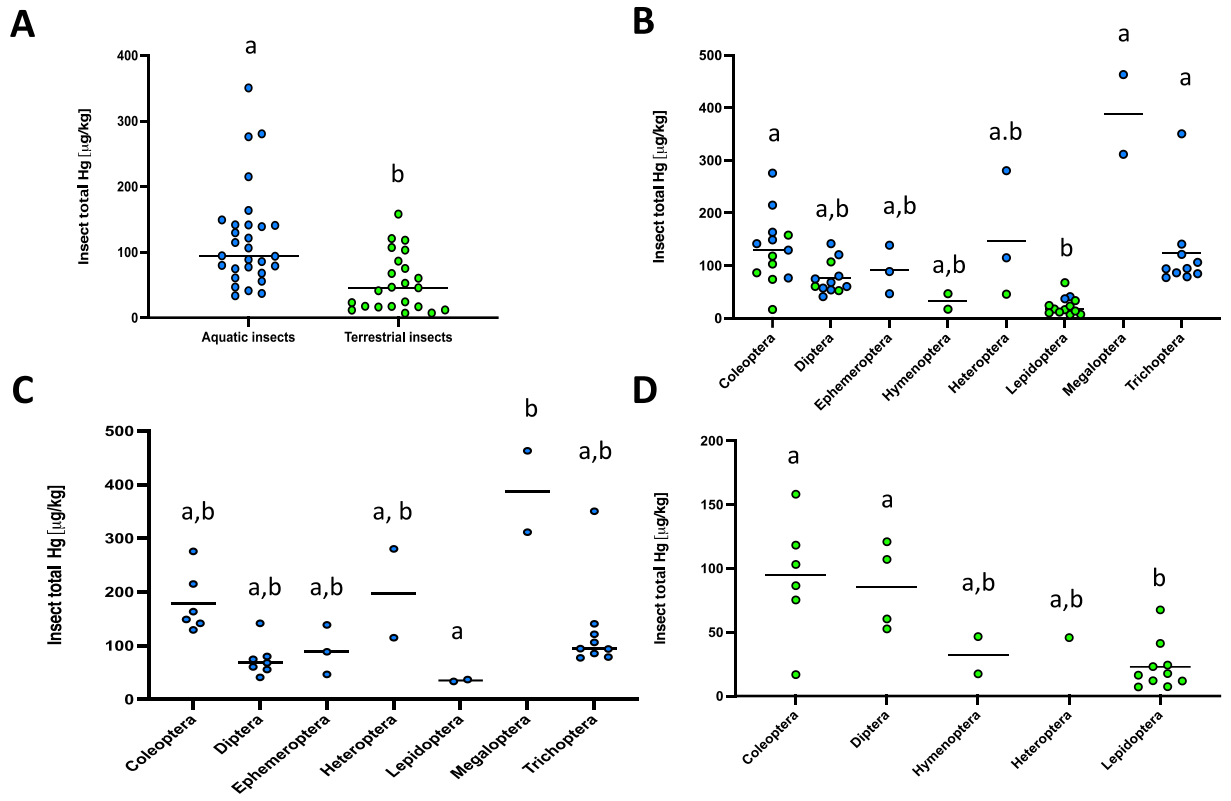

Figure 15. *Total mercury concentration in aquatic emerging and terrestrial insects* (**A**), *different orders of insects in aquatic emerging and terrestrial insects combined* (**B**), *aquatic emerging insect species* (**C**) *and terrestrial insect orders* (**D**). *Sample sizes are as follows: n=28 aquatic emerging insects and n=20 terrestrial insect; n=12 coleoptera, n=11 diptera, n=3 ephemeroptera, n=2 hymenoptera, n=3 heteroptera, n=11 lepidoptera, n=2 megaloptera, and n=8 trichoptera for combined aquatic emerging and terrestrial insect; n=6 coleoptera, n=7 diptera, n=3 ephemeroptera, n=2 heteroptera, n=2 lepidoptera, n=2 megaloptera, and n=8 trichoptera for aquatic emerging species; n=6 coleoptera, n=4 diptera, n=2 hymenoptera, n=1 heteroptera, and n=9 lepidoptera for terrestrial species. Here and throughout the thesis, aquatic emerging insects are presented in blue and terrestrial insects are presented in green. In cases where data was normally distributed or could be transformed to fit a normal distribution, data were analyzed by unpaired t-test and one-way ANOVA. In cases where data were nonparametric and could not be transformed to meet normality criteria, a Mann-Whitney U test or a Kruskal-Wallis test was used. All significant differences ($p<0.05$) are indicated by different letters.*

2.3.8.2. Geographical influence on total mercury concentrations in Eastern Ontario insects

Comparing total mercury concentration between insects collected at different locations (**Fig. 16A**) revealed significant differences (df=5 H=11.08, p=0.049), however, post-hoc analysis could not resolve the differences between specific locations. When comparing total mercury concentration in aquatic emerging insects collected from different locations, no significant

differences were found (df=4, F=2.0023, p=0.1206; **Fig 16B**). Comparing these same factors for terrestrial insects showed equally no significant differences in total mercury concentration between insects collected at different locations (df=5, F=1.319, p=0.3028; **Fig. 16C)**.

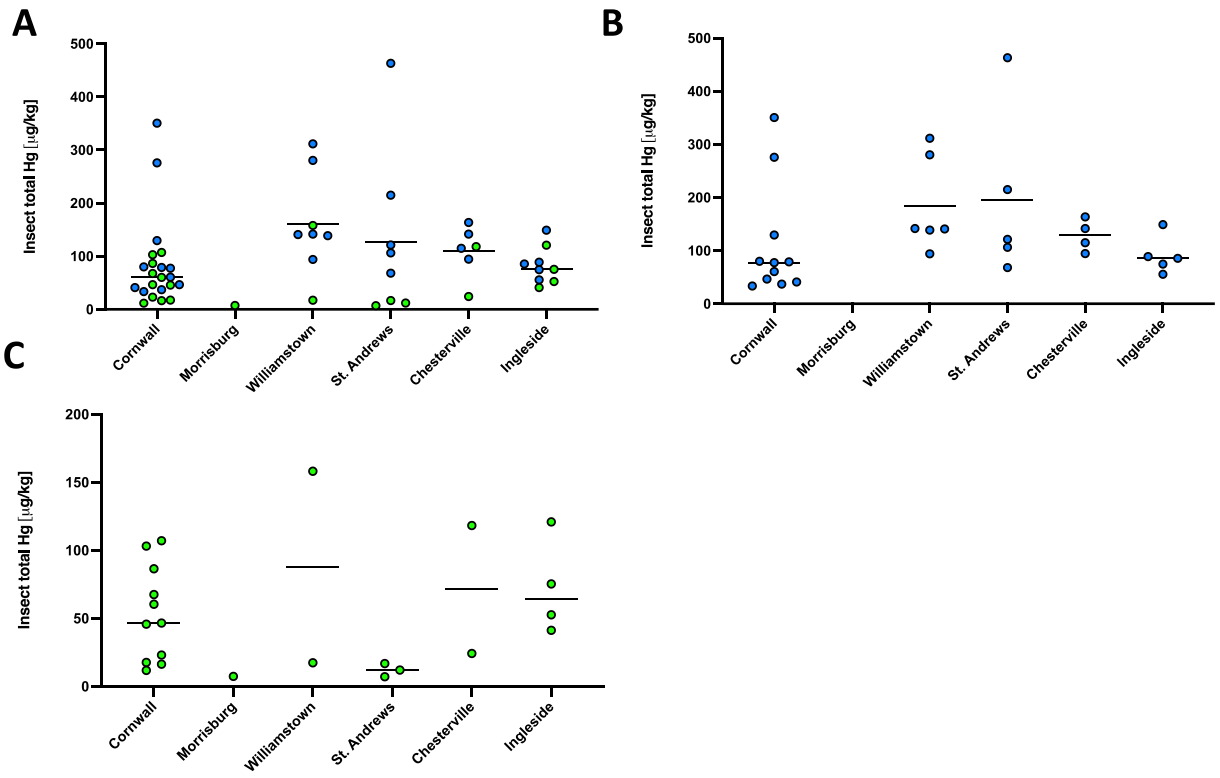

Figure 16. *Total mercury concentration in aquatic emerging insects and terrestrial insect combined* (**A**)*, aquatic emerging insects* (**B**) *and terrestrial insects* (**C**) *collected from different geographical locations. The sample sizes for individual observations are as follows: n=19 in Cornwall, n=1 in Morrisburg, n=8 in Williamstown, n=8 in St. Andrews, n=6 in Chesterville, and n=9 in Ingleside for aquatic emerging and terrestrial insect species combined; n=10 in Cornwall, n=6 in Williamstown, n=5 in St. Andrews, n=4 in Chesterville, n=5 in Ingleside for aquatic emerging insects; n=9 in Cornwall, n=1 in Morrisburg, n=2 in Williamstown, n=3 in St. Andrews, n=2 in Chesterville, n=4 in Ingleside for terrestrial insect. In cases where data was normally distributed or could be transformed to fit a normal distribution, data were analyzed by unpaired one-way ANOVA. In cases where data were nonparametric and could not be transformed to meet normality criteria, a Kruskal-Wallis test was used.*

2.3.8.3. Isotope analysis in Eastern Ontario aquatic emerging and terrestrial insects

When analyzing the δ^{13}C $^{0}/_{00}$ of the homogenized bodies of all orders of aquatic emerging insects and terrestrial insects (**Fig. 17A**), there were no significant differences between the two

groups (df=88, t=0.590, p=0.557). Contrarily, the $\delta^{15}N\ ^{0/}_{00}$ (**Fig. 17B**) differed significantly between the aquatic emerging and the terrestrial insect bodies (df=88, t=4.787, p<0.0001) where terrestrial insects measure lower in $\delta^{15}N\ ^{0/}_{00}$ in their bodies than insects that emerge from aquatic ecosystems during their life cycle.

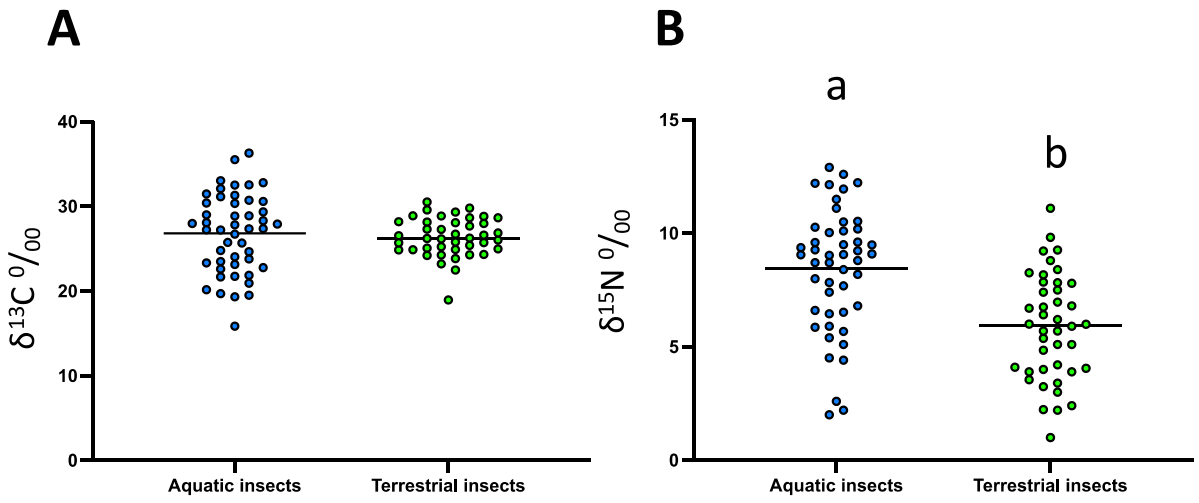

Figure 17. *$\delta^{13}C\ ^{0/}_{00}$* (**A**) *and $\delta^{15}N^{0/}_{00}$* (**B**) *measured in the bodies of aquatic emerging and terrestrial insects. n=48 for aquatic emerging insects and n=42 for terrestrial insects. In cases where data was normally distributed or could be transformed to fit a normal distribution, data were analyzed by unpaired t-tests. In cases where data were nonparametric and could not be transformed to meet normality criteria, a Kruskal Wallis test was used. All significant differences (p<0.05) are indicated by different letters.*

When investigating the $\delta^{13}C\ ^{0/}_{00}$ values of the homogenized bodies between different orders of aquatic emerging insects (**Fig. 18A)**, the results reflected no significant differences between orders (df=7, F=2.063, p=0.0712). The $\delta^{15}N\ ^{0/}_{00}$ values measured in the aquatic emerging insect bodies (**Fig. 18B**) differed significantly between orders (df=7, F=2.892, p=0.0156) and aquatic emerging trichoptera measured higher in their $\delta^{15}N\ ^{0/}_{00}$ values compared to aquatic emerging coleoptera (p=0.0434). When analyzing the same measurements between different terrestrial insect orders, the results show that there are no significant differences between their measured $\delta^{13}C^{0/}_{00}$ values in their bodies (df=7, H=8.105, p=0.323; **Fig. 18C**). The $\delta^{15}N\ ^{0/}_{00}$ values measured in terrestrial insects (**Fig. 18D**) did show significant differences between orders (df=7, F=5.182,

p=0.0005). $\delta^{15}N^{0/00}$ values were significantly higher in terrestrial diptera, orthoptera, hymenoptera lepidoptera, and aranae compared to homoptera ($p<0.05$ in all cases).

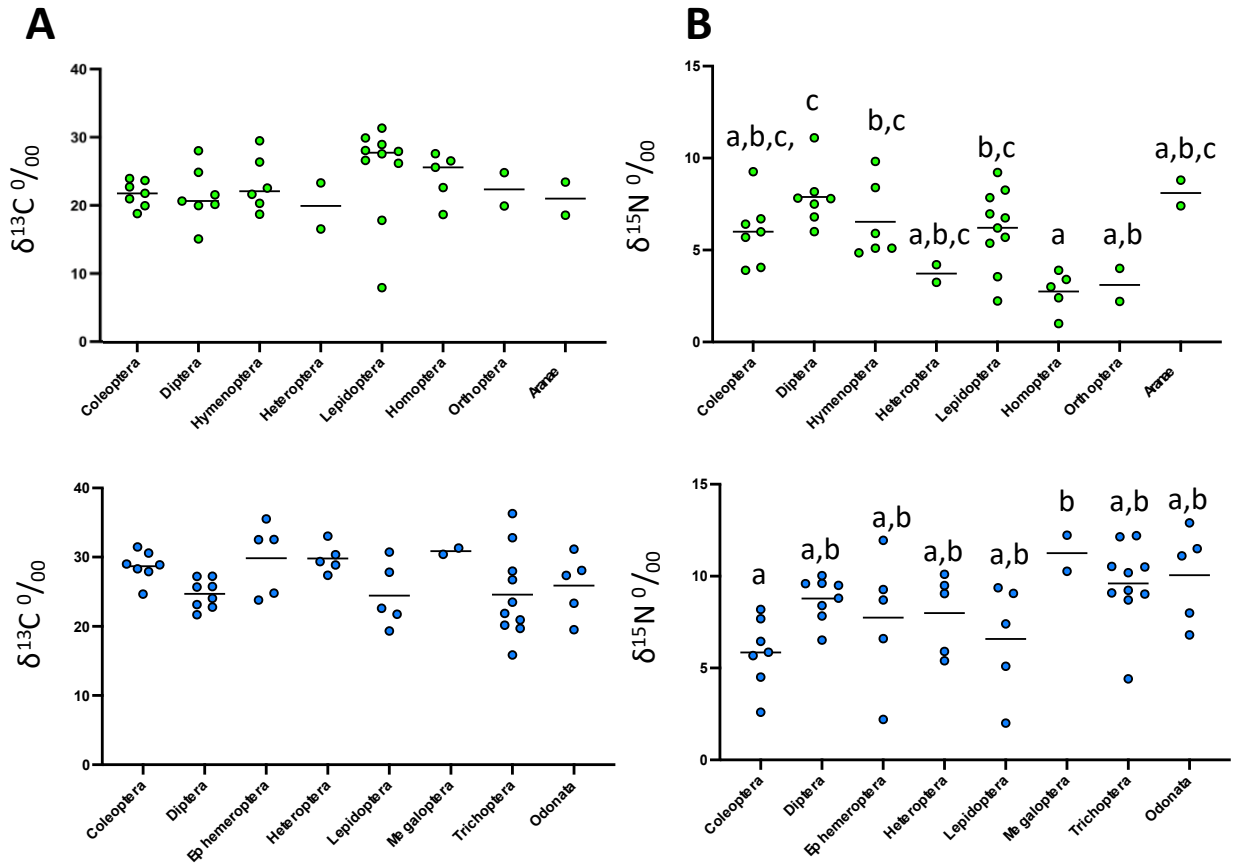

Figure 18. *$\delta^{13}C^{0/00}$ and $\delta^{15}N^{0/00}$ measured in the bodies between aquatic emerging insect orders* (**A, B**) *and between terrestrial insect orders* (**C, D**)*. The sample sizes for aquatic emerging insect orders are as follows: n=7 coleoptera, n=8 diptera, n=5 ephemeroptera, n=5 heteroptera, n=5 lepidoptera, n=2 megaloptera, n=10 trichoptera, and n=5 odonata. The sample sizes for terrestrial insect orders are as follows: n=7 coleoptera, n=7 diptera, n=6 hymenoptera, n=2 heteroptera, n=10 lepidoptera, n=5 homoptera, n=2 orthoptera, and n=2 aranae. In cases where data was normally distributed or could be transformed to fit a normal distribution, data were analyzed by one-way ANOVA. In cases where data were nonparametric and could not be transformed to meet normality criteria, a Kruskal-Wallis test was used. All significant differences (p<0.05) are indicated by different letters.*

When analyzing all aquatic emerging insects and terrestrial insects combined, there was no significant correlation between the total mercury concentration in the insect bodies and their $\delta^{13}C^{0/00}$ (df=1, F=2.553, p=0.1169; **Fig. 19A**). Similarly, in separate analyses of aquatic emerging insects (df=1, F=3.277, p=0.0818) and terrestrial insects (df=1, F=0.547, p=0.469), no significant

correlation between the total mercury concentration and $\delta^{13}C\ ^{0}/_{00}$ was found (**Fig. 19B**). When analyzing aquatic emerging insects and terrestrial insects together, a significant positive correlation between total mercury concentration and $\delta^{15}N^{0}/_{00}$ was identified (df=1, F=6.923, p=0.0115; **Fig. 19C**). However, these correlations were no longer significant when analyzing either aquatic emerging (df=1, F=0.737, p=0.399) or terrestrial insects (df=1, F=0.133, p=0.719) separately (**Fig. 19D)**.

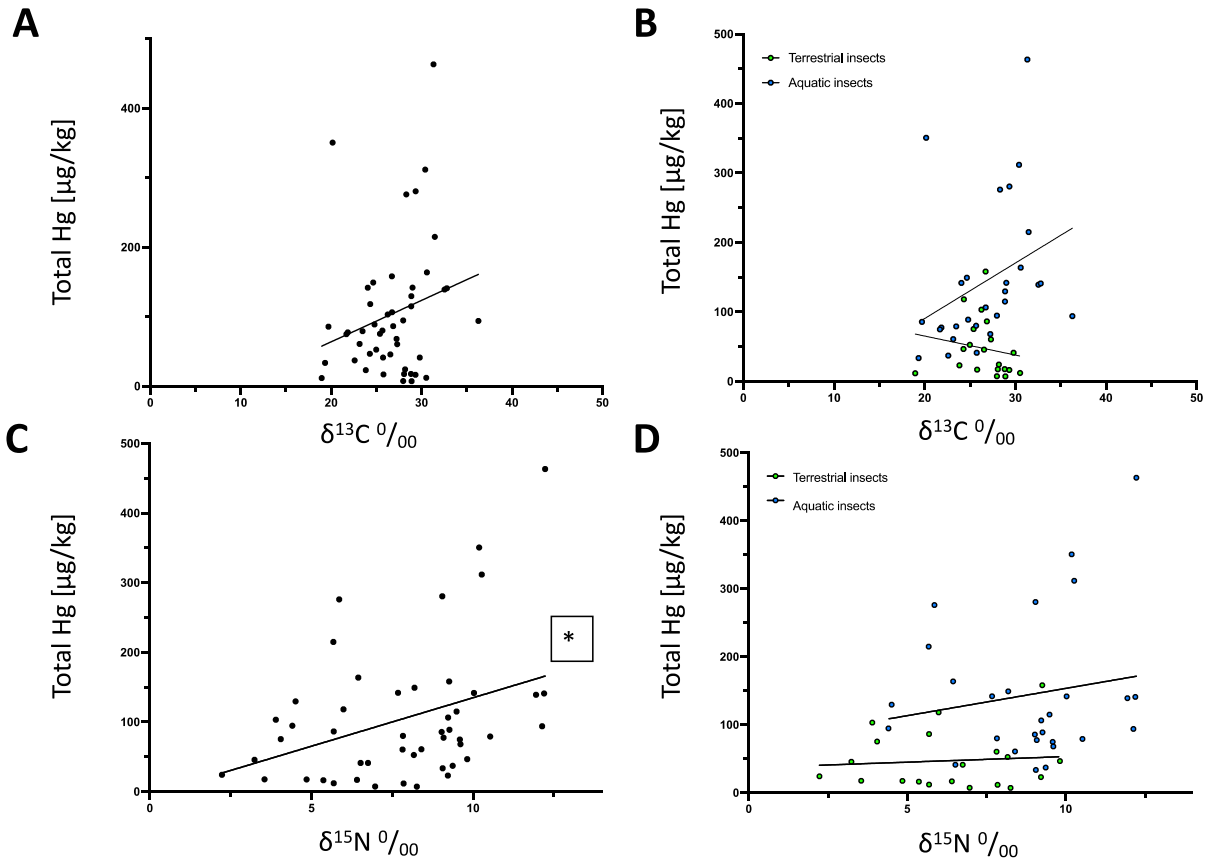

Figure 19. *Correlation between total mercury concentration and* $\delta^{13}C\ ^{0}/_{00}$ (**A**, **B**) *as well as total mercury concentration and* $\delta^{15}N\ ^{0}/_{00}$ (**C**, **D**) *in aquatic emerging and terrestrial insects combined (n=48) and aquatic emerging (n=28) and terrestrial species (n=20) separately. Data were analyzed by simple linear regression and significant slope deviations from zero (p<0.05) are indicated by asterisk.*

2.3.8.4. Isotope analysis in Eastern Ontario bats

Isotope measurements obtained from bat fur of *M. lucifugus* and *E. fuscus* showed that the $\delta^{13}C\ ^{0}/_{00}$ measurements in the bat fur (**Fig. 20A**) did not differ significantly between little brown bats and big brown bats (df=60, t=1.068, p=0.290). In contrast, $\delta^{15}N\ ^{0}/_{00}$ measurements (**Fig. 20B**) were significantly higher in *M. lucifugus* compared to *E. fuscus* (df=60, U=142.5, p<0.0001).

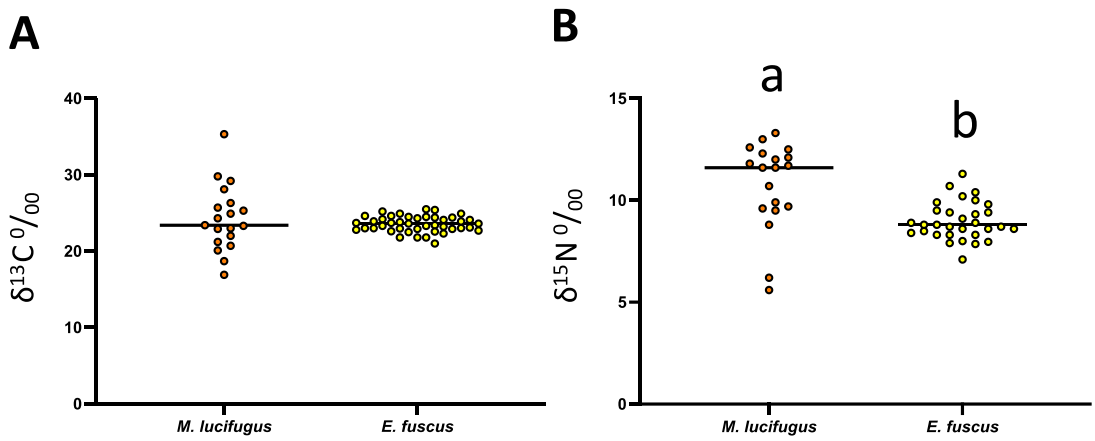

Figure 20. *$\delta^{13}C\ ^{0}/_{00}$* **(A)** *and $\delta^{15}N\ ^{0}/_{00}$* **(B)** *measured in the fur of M. lucifugus (n=19) and E. fuscus (n=43). In cases where data was normally distributed or could be transformed to fit a normal distribution, data were analyzed by unpaired t-tests. In cases where data were nonparametric and could not be transformed to meet normality criteria, a Mann-Whitney U test was used. All significant differences (p<0.05) are indicated by different letters.*

When investigating whether sex-dependent species-differences in $\delta^{13}C\ ^{0}/_{00}$ (**Fig. 21A**) or $\delta^{15}N\ ^{0}/_{00}$ isotopes (**Fig. 21B**) exist, neither species (df=1 F=0.460 p=0.500) nor sex (df=1 F=1.860 p=0.178) or their interaction (df=1 F=2.213 p=0.142) yielded significant differences for $\delta^{13}C\ ^{0}/_{00}$. Conversely, a species-specific difference (df=2 F=21.589 p<0.01), but no effect of effect of sex (df= 1F=1.206 p=0.277) or the interaction of species and sex (df=1 F=1.483 p=0.228) exist for $\delta^{15}N\ ^{0}/_{00}$, which exhibits significantly higher concentrations in *M. lucifugus* compared to *E. fuscus* (p<0.05).

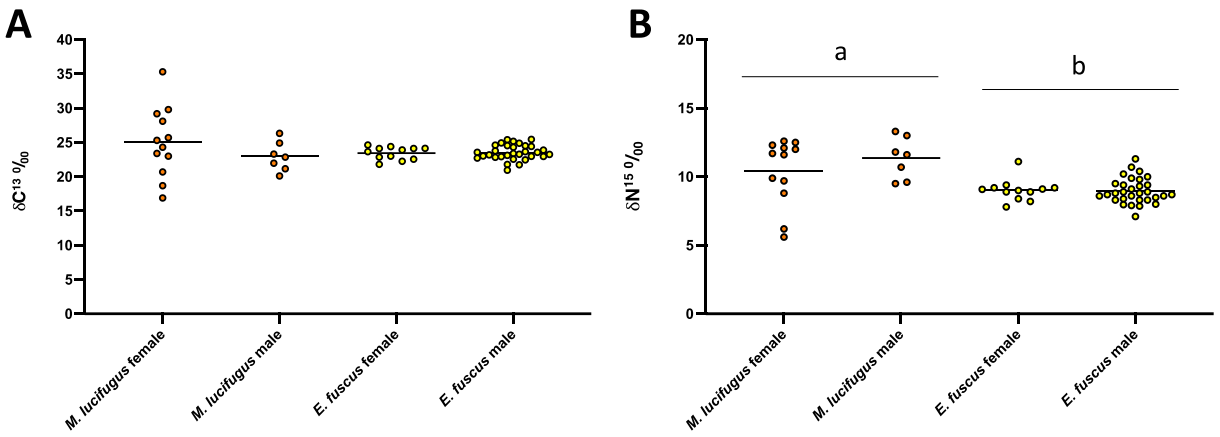

Figure 21. $\delta^{13}C\ ^{0/}_{00}$ **(A)** *and* $\delta^{15}N\ ^{0/}_{00}$ **(B)** *measured in the fur of female (n=12) and male (n=7) M. lucifugus and female (n=12) and male (n=31) E. fuscus. In cases where data was normally distributed or could be transformed to fit a normal distribution, data were analyzed by one-way ANOVA. In cases where data were nonparametric and could not be transformed to meet normality criteria, a Kruskal-Wallis test was used. All significant differences (p<0.05) are indicated by different letters.*

When analyzing both bat species combined, no significant correlations between fur total mercury concentration and $\delta^{13}C\ ^{0/}_{00}$ (df=1, F=0.0911, p=0.764; **Fig. 22A** or $\delta^{15}N\ ^{0/}_{00}$ (df=1, F=2.619, p=0.111; **Fig. 22B**) were identified. Investigating the same correlations in little brown bats showed a significant negative correlation between fur total mercury concentration and $\delta^{13}C\ ^{0/}_{00}$ measurements in the bat fur (df=1, F=5.029, p=0.0386; **Fig. 22C**) but no significant correlation between fur total mercury concentration and $\delta^{15}N^{0/}_{00}$ measurements (df=1, F=1.360, p=0.260; **Fig. 22D**). Investigating big brown bats individually showed that there is neither a significant correlation between fur total mercury concentration and $\delta^{13}C\ ^{0/}_{00}$ measurements (df=1, F=3.957, p=0.0534; **Fig. 22E**) nor $\delta^{15}N\ ^{0/}_{00}$ (df=1, F=0.617, p=0.437; **Fig. 22F**).

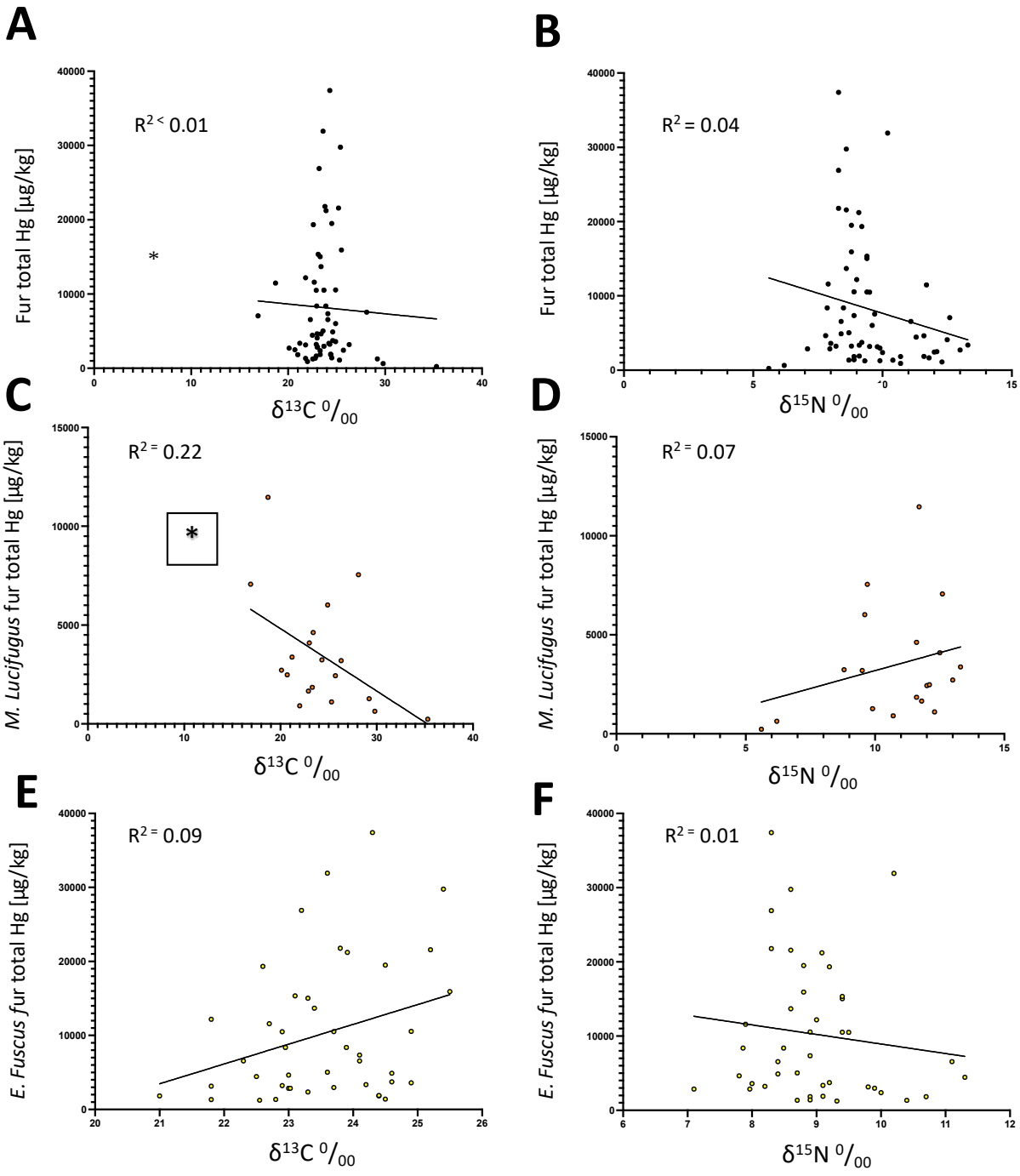

Figure 22. *Correlation between fur total mercury concentration and $\delta^{13}C^{0/00}$ and $\delta^{15}N^{0/00}$ for all bat specimens combined (n=62)* (**A**, **B**), *M. lucifugus alone (n=19)* (**C**, **D**) *and E. fuscus alone (n=43)* (**E**, **F**). *Data were analyzed by simple linear regression and significant slope deviations from zero ($p<0.05$) are indicated by an asterisk.*

The investigation of sex-specific correlations between isotope measures and fur total mercury concentrations in female little brown bats showed a significant negative correlation between fur total mercury concentrations and $\delta^{13}C\ ^{0/}_{00}$ (df=1, F=7.368, p=0.0218; **Fig. 22A**), but not $\delta^{15}N\ ^{0/}_{00}$ (df=1, F=2.317, p=0.159; **Fig. 23B**). In male little brown bats, no significant correlation between fur total mercury concentration and $\delta^{13}C\ ^{0/}_{00}$ (df=1, F=0.910, p=0.384; **Fig. 23C**) or $\delta^{15}N\ ^{0/}_{00}$ measurements (df=1, F=0.590, p=0.477; **Fig. 23D**) was identified.

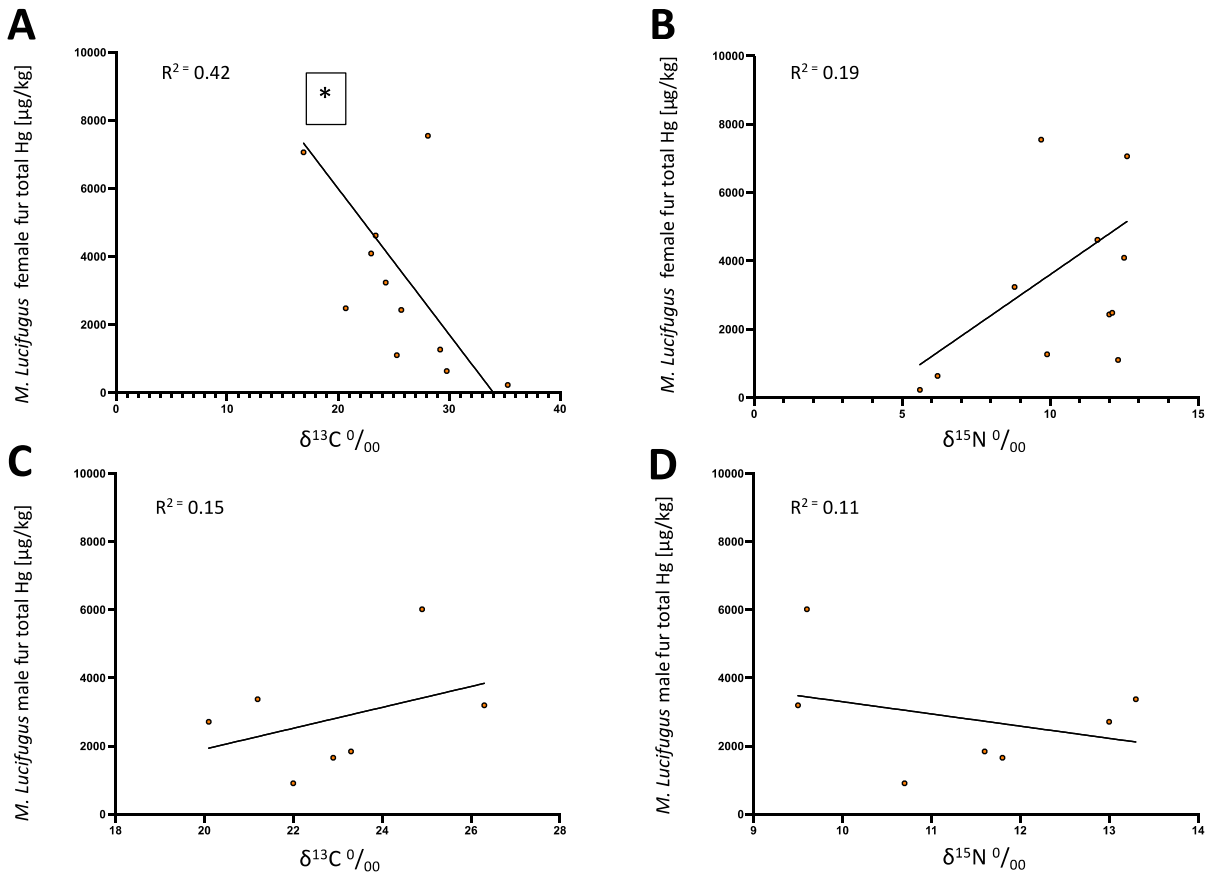

Figure 23. *Correlation between fur total mercury concentration and $\delta^{13}C\ ^{0/}_{00}$ and $\delta^{15}N\ ^{0/}_{00}$ measurements in female (n=12)* (**A,B**)*, and in male (n=7)* (**C,D**) *M. lucifugus. Data were analyzed by simple linear regression and significant slope deviations from zero (p<0.05) are indicated by an asterisk.*

The investigation of sex-specific correlations between isotope measures and fur total mercury concentrations in female big brown bats showed no significant correlation between fur

total mercury concentration and $\delta^{13}C\ ^{0/}_{00}$ (df=1, F=1.061, p=0.327; **Fig. 24A**) or $\delta^{15}N\ ^{0/}_{00}$ (df=1, F=0.285, p=0.605; **Fig. 24B**). In male big brown bats, a significant positive correlation between their fur total mercury concentration and $\delta^{13}C\ ^{0/}_{00}$ fur measurements (df=1, F=5.891, p=0.0217; **Fig. 24C**) but not $\delta^{15}N\ ^{0/}_{00}$ measurements (df=1, F=0.937, p=0.341; **Fig. 24D**) was found.

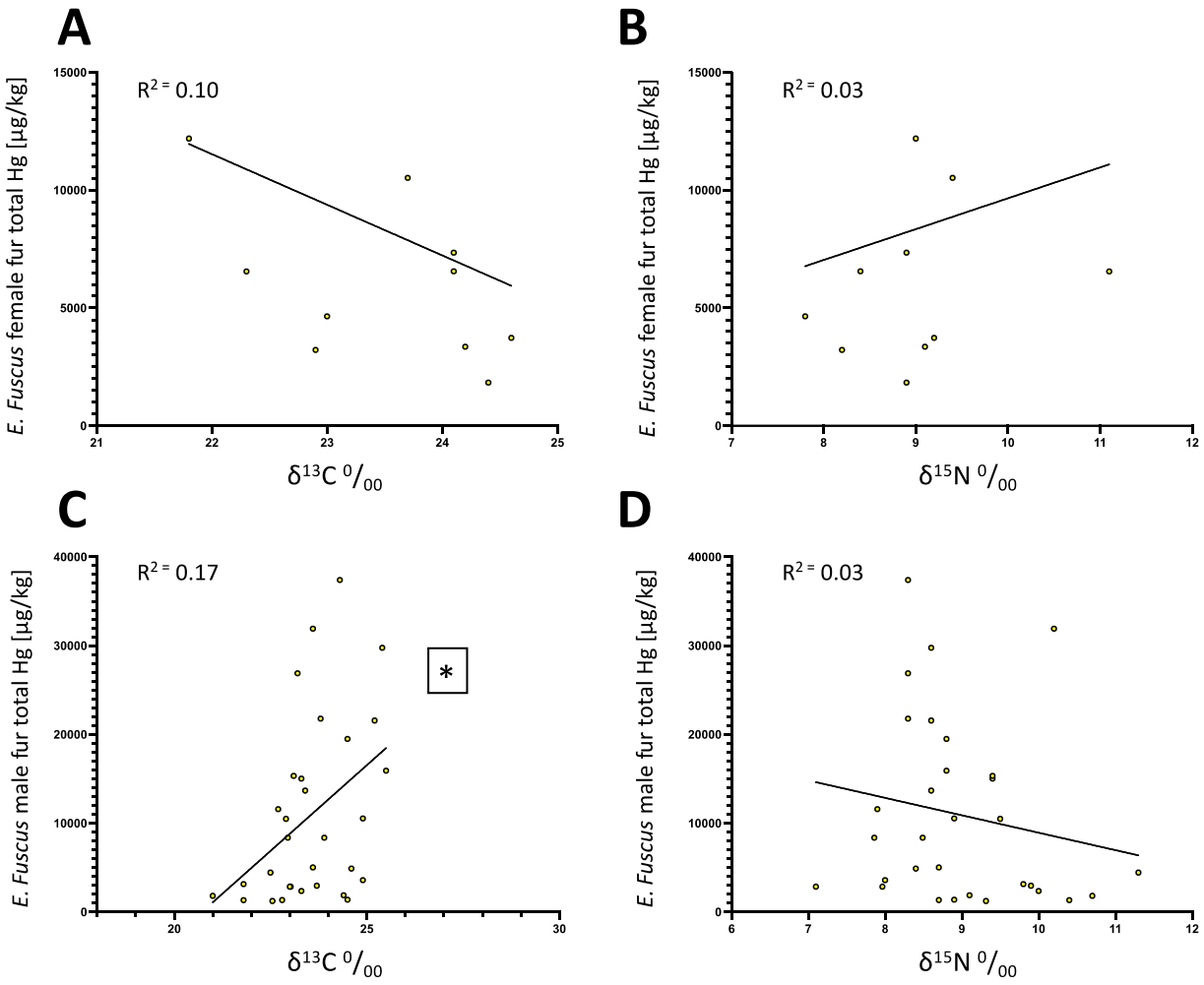

Figure 24. *Correlation between fur total mercury concentration and $\delta^{13}C\ ^{0/}_{00}$ and $\delta^{15}N\ ^{0/}_{00}$ measurements in female (n=12)* (**A,B**) *and in male (n=31)* (**C,D**) *E. fuscus. Data were analyzed by simple linear regression and significant slope deviations from zero (p<0.05) are indicated by an asterisk.*

2.3.8.5. Correlative analysis of isotope and total mercury concentration in bats and insects combined

The correlation between total mercury concentration and $\delta^{13}C\ ^{0/}_{00}$ measurements of all bat species and insects combined revealed a significant negative correlation (df=1, F=10.24, p=0.0017; **Fig. 25A**). To better visualize separation of specific groups, logarithmic values of total mercury concentration are plotted over $\delta^{13}C\ ^{0/}_{00}$ measurements (**Fig. 25B**). Contrarily, there was no significant correlation reflected between total mercury concentration and $\delta^{15}N\ ^{0/}_{00}$ measurements of all bat species and insects combined (df=1, F=1.852, p=0.176; **Fig. 25C**). Again, the logarithmic values of total mercury and the $\delta^{15}N\ ^{0/}_{00}$ measurements were plotted to allow for a visual discrimination of the data (**Fig. 25D**). To assess whether locally restricted insects and bat samples affect correlations, insect and bat samples from the site with the largest dataset of both insects and bats, Cornwall, was analyzed and visualized separately. As for all combined sites in Eastern Ontario, no significant correlations were identified for combined bat and insect total mercury data and $\delta^{13}C\ ^{0/}_{00}$ or $\delta^{15}N\ ^{0/}_{00}$ (**Fig. 26**).

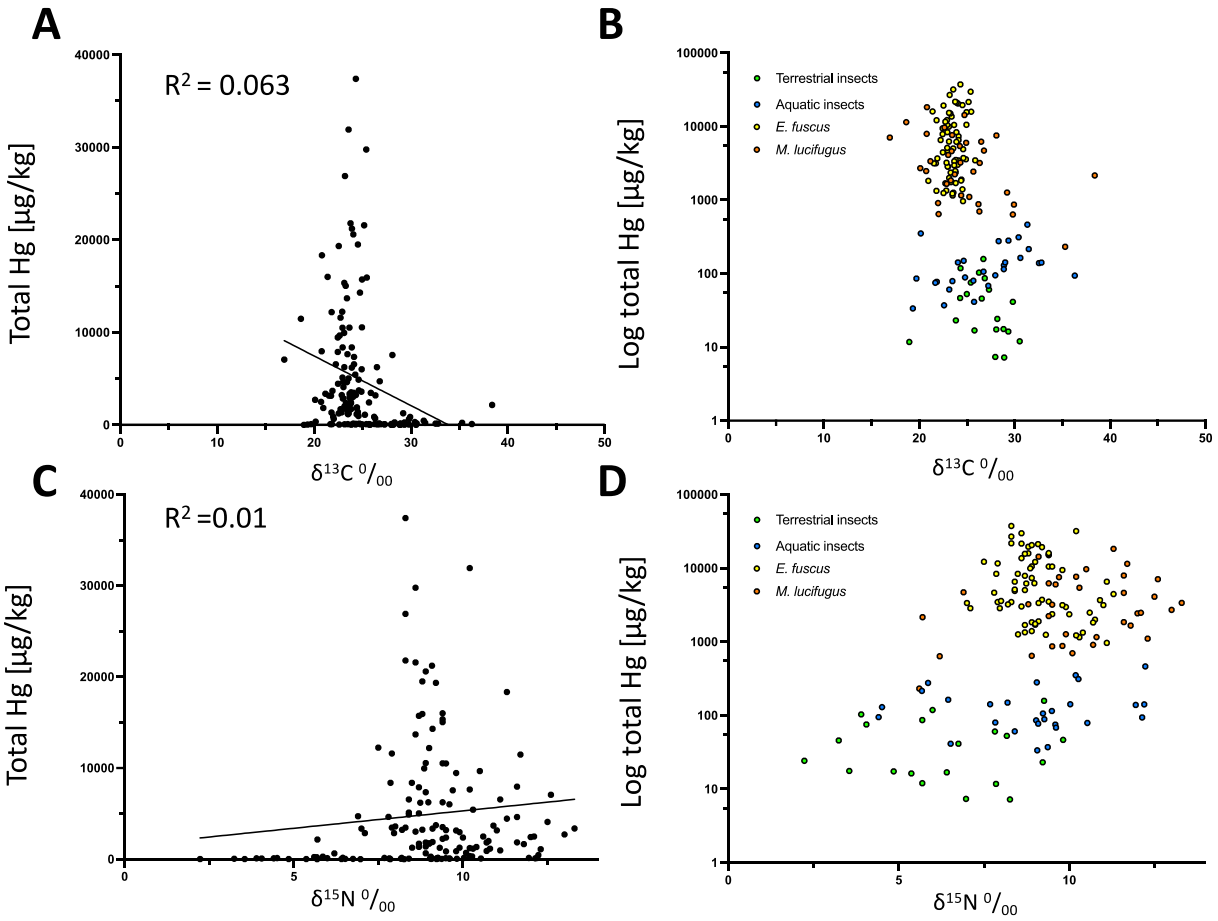

Figure 25. *Correlation between total mercury concentration and* $\delta^{13}C\ ^{0}/_{00}$ (**A**,**B**) *and* $\delta^{15}N^{0}/_{00}$ *(C,D) measurements of both bat species and all insects (n=110), and both bat species (M. lucifugus, n=19; E. fuscus, n=43) and aquatic emerging (n=28) and terrestrial (n=20) insects all separately. Data were analyzed by simple linear regression.*

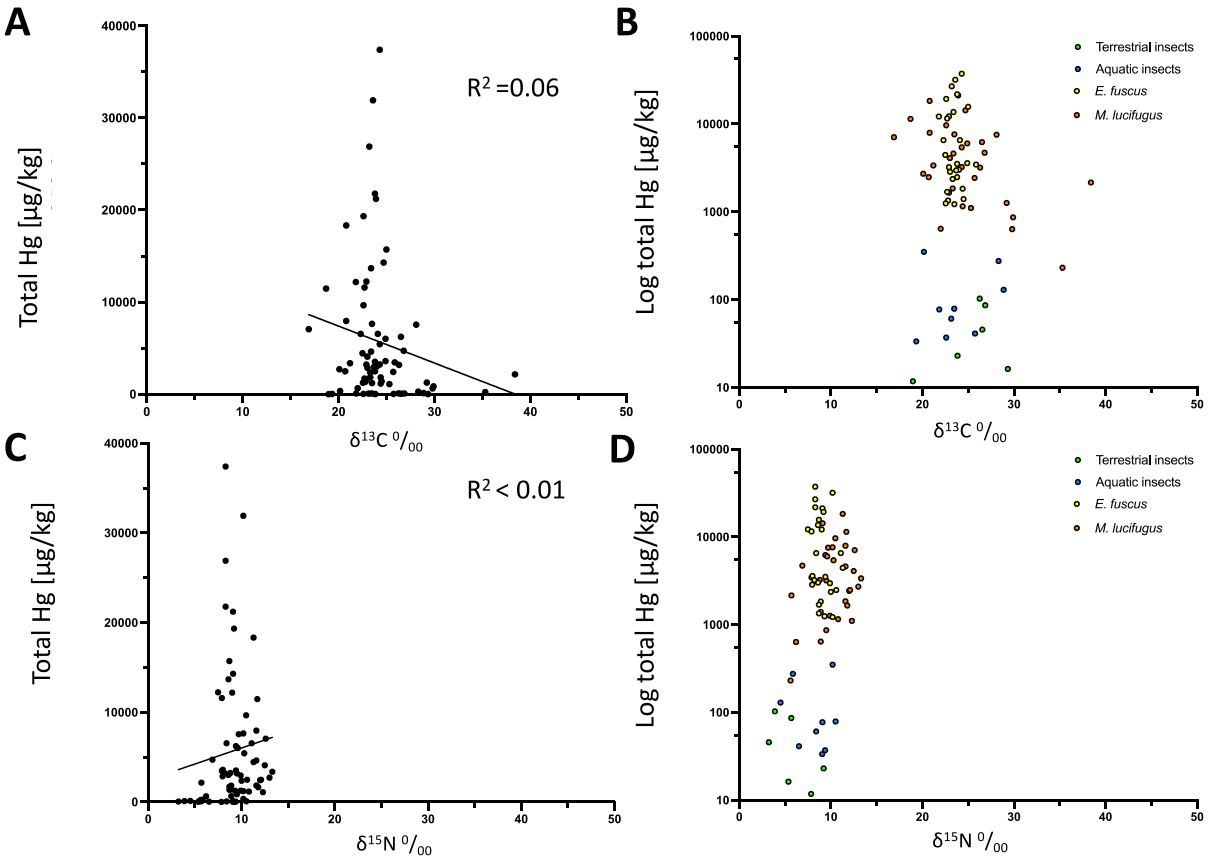

Figure 26. *Correlation between total mercury concentration and* $\delta^{13}C\ ^{0}/_{00}$ ***(A,B)*** *and* $\delta^{15}N\ ^{0}/_{00}$ *(C,D) measurements of both bat species and all insects (n=72), and both bat species (M. lucifugus, n=29; E. fuscus, n=28) and aquatic emerging (n=9) and terrestrial (n=6) insects all separated specific to the Cornwall region. Data were analyzed by simple linear regression.*

Chapter 3 - Assessment of DNA and RNA molecular markers as potential biomarkers of mercury exposure in *E. fuscus* in Eastern Ontario

3.1. Introduction to DNA and RNA Biomarkers of Mercury Exposure

Tissue-level biological effects of mercury exposure are well-documented and are generally measured in organs involved in detoxification and excretion, such as liver and kidney, as well as in tissues which represent principal sites of bioconcentration which are directly linked to known modes of actions of mercury, such as the brain. While acutely and terminally sampled bats allow for the utilization of molecular level assays used to measure biomarkers indicative of higher organismal mercury toxicity, such as neurotoxicological symptoms, the use of such assays is limited in sample collections not immediately stored under appropriate conditions or stored for long periods of time. For example, the of use gene expression assays to quantify, for example the induction of detoxification pathways exemplified by the evolutionarily highly conserved metal transcription factor-based induction of metallothionein transcripts or induction of oxidative stress enzymes (Aschner et al. 2006; Fujimura and Usuki 2020), are limited by the well-known rapid degradation of RNA molecules under ambient conditions if samples are not stored in protective solutions and/or in ultra-low freezing conditions of -80 °C. Similarly, enzyme activity assays such as, the quantification of enzymes involved in neurotransmission (Nam et al. 2012), or enzymes involved in the response to (MeHg-induced) oxidative stress (Fujimura and Usuki 2020), are contingent upon rapid and appropriate storage of tissue to conserve the structure and function of proteins and thus, their activity. Molecular analysis of tissue sample banks spanning sometimes several years, or decades of specimen collection are thus limited in terms of analyses. Taking advantage of the reported high stability of DNA as well as one of its epigenetic modifications, DNA methylation, I here investigate the utility of mitochondrial DNA quantity as well as global

DNA methylation quantification as biomarkers of mercury and MeHg exposure. Indeed, recent studies in model organisms such as the nematode *C. elegans* (Wyatt et al. 2017) point to a reduction of mtDNA while several studies have described DNA hypomethylation as molecular consequences of mercury and/or methylmercury exposure (Pilsner et al. 2010; Bose et al. 2012; Basu et al. 2013; Goodrich et al. 2013; Cardenas et al. 2017). I here test the potential relevance of these emerging biomarkers in big brown bats sampled in Eastern Ontario across a timespan of ~20 years.

3.2. Materials and Methods

3.2.1. DNA and RNA extractions

From the kidney, brain and liver tissues collected from each preserved bat, DNA and RNA was extracted using the TRIzol reagent (Invitrogen - Thermo Fisher Scientific, Waltham, Massachusetts, United States of America) according to the manufacturer's user guide. Using an ultrasonic convertor, 50-100 mg of each sample were homogenized in 100 mL of Trizol reagent. Chloroform was added next to separate the mixture into three layers: the top aqueous layer containing RNA, the middle interphase containing DNA, and the bottom organic layer containing proteins and lipids. Isopropanol was used to precipitate the RNA from the aqueous layer while 70% ethanol was used to precipitate DNA from the interphase. RNA was washed with ethanol and DNA with a sodium citrate and ethanol mixture removes any contamination. RNA was then resuspended in RNase-free water and DNA resuspended in sodium hydroxide for future analysis.

3.2.2. Quantification of DNA and RNA and assessment of RNA quality

To determine concentration of DNA and RNA extracted from each sample along with the purity of DNA/RNA, the samples were assessed using a Nanodrop 2000c Spectrophotometer

(Thermo Fisher Scientific, Waltham Massachusetts, United States of America). For DNA, an absorbance 260 nm/280 nm ratio of approximately 1.8 is indicative of "pure" DNA and a 260/280 ratio of approximately 2.0 is considered "pure" RNA. Of the DNA that measured relatively pure (260/280 of approximately 2.0 ± 0.2), most were from big brown bat carcasses, so the molecular biomarker endpoints were focused on big brown bats only (kidney, n=23; brain, n=20; liver, n=21). Because RNA is not as stable as DNA, additional quality control on the RNA samples was conducted using an Agilent 2100 Bioanalyzer from Agilent Technologies Inc. (Mississauga, Ontario, Canada) according to the manufacturer's user guide. This approach allows in addition to the quantification of RNA concentration the assessment of RNA quality, which is measured based on ribosomal fraction migration and quantification of specific bands. The ratio of these band allows for the calculation of the RNA integrity number (RIN). This number evaluates RNA integrity on a scale of 10, with 10 being a completely non-degraded RNA sample. Briefly, a ladder, a gel-dye mixture, and a marker solution from the Aligent RNA 6000 Nano Kit (Aligent Technologies Inc., Mississauga, Ontario, Canada), along with the 1 uL of the extracted RNA samples were loaded into the Bioanalyzer Chip and ran in the Aligent 2100 Bioanalyzer instrument designed to analyze RNA fragments. Electrophoresis is used to drive the fragments of RNA through interconnected microchannels, thereby separating the fragments by size. In mammals, rRNA is made up of 28s and 18s which measure approximately 5.0 kb and 2.0 kb, respectively. A 2:1 ratio of these bands in the gel are accepted as "intact" RNA, while a smear of bands shows that the RNA is fragmented and therefore not good quality for use in future analyses. Each sample is given an RNA integrity number (RIN) where samples with a RIN >9.2 are accepted as good quality. Of 7 tested RNA samples, the mean RIN was approximately 2.02 with a mean concentration of 724.51 ug/mL,

indicating that the RNA samples were degraded, and RNA transcript endpoints were thus not considered further for this thesis.

3.2.3. Global DNA methylation assays

Two DNA methylation marks, DNA hydroxymethylation, an intermediate step of the demethylation process, and DNA methylation and were quantified globally. The MethylFlash Global DNA Methylation and hydroxymethylation (5-hmC/5-mC) colorimetric ELISA Easy Kits (Epigentek Group Inc., Farmingdale, New York, United States of America) were used following the manufacturer's user guides. Each sample of extracted DNA was diluted to 100 ng DNA and loaded into wells that have been treated to have a high affinity for DNA using a binding solution. Capture and detection antibodies are then added to detect the methylated or hydroxymethylated portion of the DNA using a 5-mC detection complex solution. The addition of a developer solution causes an enzymatic reaction, turning the samples in the wells dark blue in the presence of methylated or hydroxymethylated DNA, whereas samples with insufficient amounts of methylated DNA remain uncoloured, including a negative control. A stop solution is added after the positive control becomes deep blue to stop the enzymatic reaction. The methylated or hydroxymethylated portions of the DNA samples were then quantified by spectrophotometry using a SpectraMax Plus 384 Microplate Reader (GMI, Ramsey, Minnesota, United States of America) at 450 nm, where optical density (OD) is directly proportional to the percentage of methylated or hydroxymethylated DNA. Both positive and negative controls, as well as a serially diluted standard were used to determine the percentage of methylated or hydroxymethylated DNA using the following formula.

$$\%\ \text{5-hmC or 5-mC} = \frac{\text{sample OD} - \text{NC OD}}{\text{slope} - \text{DNA input amount (ng)}}\ \text{X}\ 100\%$$

Samples sizes for the methylation assays were n=18 for kidney, n=16 for brain and n=18 for liver. For the hydroxymethylation assay the sample sizes were n=23 kidney, n=20 brain; n=21 liver. Obtained global methylation or hydroxymethylation values were then then plotted against quantified fur total mercury concentration and analyzed using linear regression analysis, as previously described.

3.2.4. Assessment of mitochondrial DNA abundance

Using the extracted DNA samples from *E. fuscus* kidney (n=23), brain (n=18) and liver (n=21) tissues, two-step Real-Time-PCR assays were performed on a BioRad CFX instrument (BioRad, Mississauga, Ontario, Canada) to quantify relative amount of *16S* mtDNA and *HK2* nDNA. A standard curve consisting of a 2x serial dilution of pooled DNA samples was used to quantify relative target DNA abundance. The total reaction volume was 20 µL which consisted of 1 µL of diluted DNA sample, 1 µL of 10 nM specific forward primer (**Table 1**), 1 µL of 10 nM specific reverse primer (**Table 1**), 7 µl of H_2O, and 10 µl of SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (BioRad, Mississauga, Ontario, Canada). For all reactions a no-Template control devoid of DNA was run to exclude DNA contamination. Real-time-PCR cycling parameters included a 2-minute activation step at 98 °C, 40 cycles of a 20 s denaturation step at 95 °C followed by a 30 s annealing and extension step at a primer-specific temperature (**Table 1**). Following each run, a melting curve was produced and monitored for a single peak to ensure specificity of the reactions. Amplification efficiencies were calculated from the standard curves and were between 75-100%, with R^2 values >0.90. The relative *16S* mtDNA level was normalized to the *HK2* nDNA level, as previously described in mice (Quiros et al. 2017). Normalized

mtDNA/nDNA ratios from each tissue were then plotted against quantified fur total mercury concentration and analyzed using linear regression analysis as previously described.

Table 1. *Real-time RT-PCR assay primer sequences and assay parameters.*

Target gene	Primer sequence (5' 3')	Annealing T [°C]	Efficiency %	R^2
16S	FW: AGCCTGGTGATAGCTGGTTG RV: ATTTGGTGGCTGCTTTTAGG	59	78.6	0.986
HK2	FW: GAACATGGCAGAAGCACAGA RV: TAACTTGGGTGAAGGGCAAG	60	92.9	0.989

3.2.5. Quantification of metallothionein transcript abundance

To assess relative transcript abundance of metallothionein in *E. fuscus*, the genome-derived predicted mRNA coding sequence retrieved from Pubmed Genbank (Acession number: XM_008152318.2). Real-time RT PCR primers designed were designed using the freely available primer 3 software, version 4.1.0. (https://primer3.ut.ee), using standard parameters with the following modifications: Amplicon length was limited to a size of 100-300 nt and primer size restricted to 19-21 nt. A primer T_m between 59 °C - 61 °C and a GC content between 45-55% was specified. Finally, allowable primer self-complementarity was restricted to 4 and allowable 3' self-complementarity restricted to 0. Using these parameters, primer sequences (listed below) were generated sand used to optimize real-time RT-QPCR reaction.

Using RNA extracted from kidney, brain and liver as previously discussed, cDNA synthesis was performed using a total RNA input of 1000 ng for the Quantitect reverse transcription kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's instructions. A negative control devoid of reverse transcriptase (noRT control) was included in cDNA synthesis

to assess possible DNA contamination in subsequent real-time RT-PCR runs. Reaction volumes of 20 µl were used and stored at -20 °C.

A semi-quantitative real-time-RT PCR assay probing relative abundance of the *MT-1* transcript was then performed using a two-step semiquantitative real-time RT-PCR assay on a BioRad CFX96 instrument (Bio-Rad, Mississauga, ON, Canada). Briefly, a standard curve consisting of serial dilutions of pooled cDNA, a negative noRT control consisting of cDNA generated in a reaction that did not include reverse transcriptase, and individual samples were run in duplicate for each experiment. For each individual reaction, the total volume was 20 µl, which consisted of 4 µl of diluted cDNA template, 0.5 µl of 10 nM specific forward (5' CATAATCAGGGGCAAAGGTG 3') and 0.5 µl of 10 nM specific reverse primer (5' GCAGAGATGAGGCAGTTGGT 3'), 10 µl of SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (Bio-Rad), and 5 µl of H_2O. Cycling parameters were a 5-min activation step at 95 °C, followed by 40 cycles consisting of a 20-s denaturation step at 95 °C and a combined 30 s annealing and extension step at primer specific temperature of 60 °C. After each run, melting curves were produced by gradually increasing temperature and the final curves were monitored for single peaks to confirm the specificity of the reaction and the absence of primer dimers. Relative mRNA abundance was the plotted over measured concentrations in fur corresponding to species from which specific tissue samples were derived.

3.3 Results

3.3.1 Global DNA (hydroxy)methylation in internal tissues does not correlate with fur total mercury burden in *E. fuscus*

Fur total mercury concentration showed no significant correlation to the percentage of global DNA hydroxymethylation in the kidney (df=1, F=3.12, p=0.092; **Fig. 27A**), brain (df=1,

F=0.255, p=0.620; **Fig. 27B**) or liver (df=1, F=0.1699, p=0.685; **Fig. 27C**) in the big brown tissue samples analyzed. Fur total mercury concentration also showed no correlation to the percentage of global DNA methylation in the kidney (df=1, F=0.592, p=0.453; **Fig. 27D**), brain (df=1, F=1.420, p=0.253; **Fig. 27E**) or liver (df=1, F=0.436, p=0.518; **Fig. 27F**) in the big brown bat tissue samples.

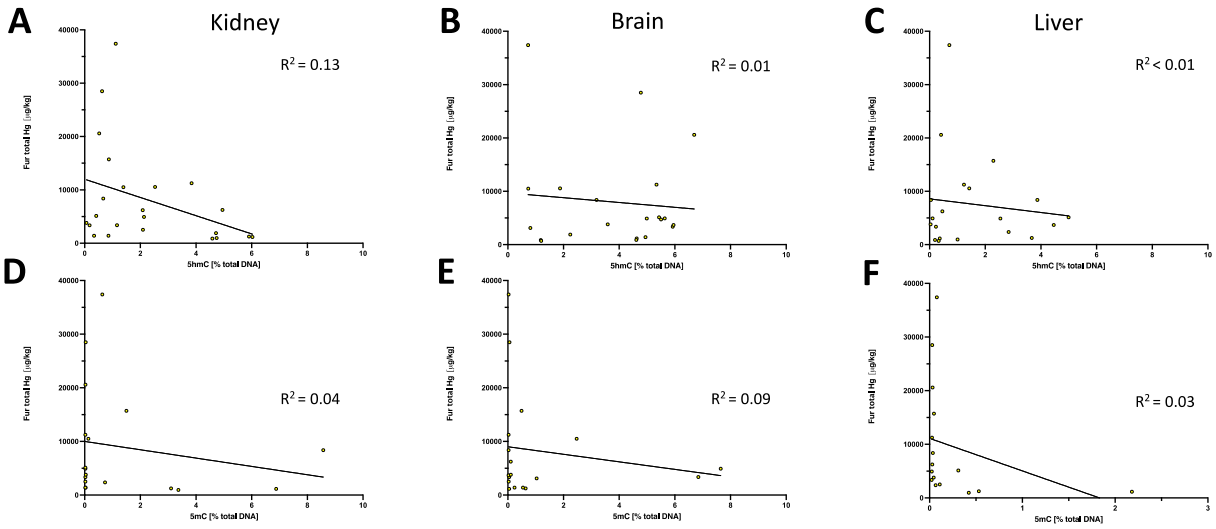

Figure 27. *Correlation between fur total mercury concentration and the percentage of global hydroxymethylated DNA* (**A-C**) *extracted from E. fuscus kidney (n=23)* (**A**)*, brain (n=20)* (**B**) *and liver (n=21*) (**C**) *and global methylated DNA* (**D-F**) *extracted from E. fuscus kidney (n=18*) (**A**)*, brain (n=16)*(**B**) *and liver (n=18*) (**C**)*. All data were analyzed by simple linear regression.*

3.3.2 Mitochondrial DNA abundance does not significantly correlate with fur total mercury concentration in *E. fuscus*

Fur total mercury concentration is not significantly correlated to the ratio of expression levels of the mitochondrial gene, *16S* and the housekeeping gene, *HK2* (*16S*:*HK2*) in the kidney (df=1, F=1.417, p=0.247; **Fig. 28A**), brain (df=1, F=0.221, p=0.645; **Fig. 28B**) or liver (df=1, F=0.615, p=0.443; **Fig. 28C**) tissue of big brown bats analyzed.

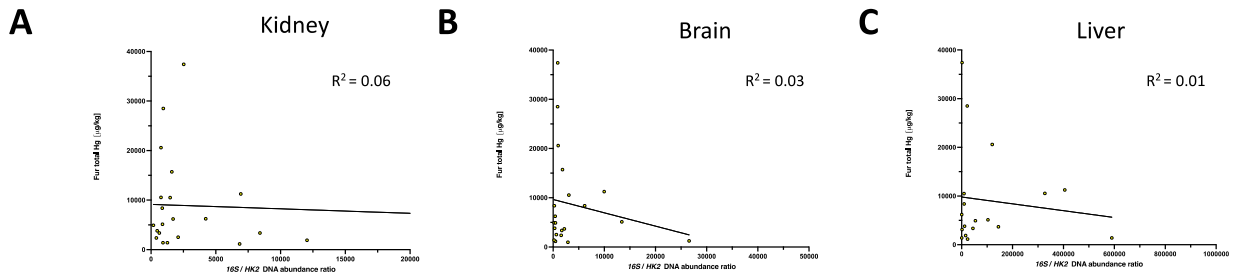

Figure 28. *Correlation between fur total mercury concentration and the ratio of expression levels of the mitochondrial gene, 16S and the nuclear housekeeping gene, HK2 (16S:HK2) in the kidney (n=23)(**A**), brain (n=18)(**B**) and liver (n=21)(**C**) tissue of E. fuscus. All data were analyzed by simple linear regression.*

3.3.3 Transcript abundance of metallothionein does not significantly correlate with fur total mercury concentration in *E. fuscus*

The fur total mercury concentration does not significantly correlate with the mRNA abundance of the metallthionein-1 (MT1) expressed in the kidney (df=1, F=0.247, p=0.628; **Fig. 30A**) brain (df=1, F=0.648, p=0.433; **Fig. 30B**) tissue of big brown bats analyzed.

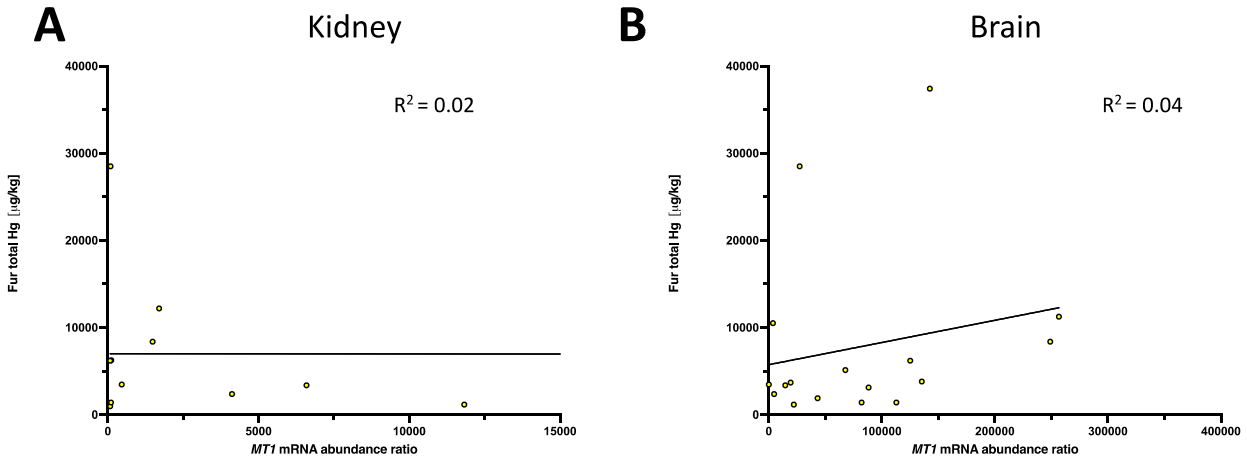

Figure 29. *Correlation between fur total mercury concentration and mRNA abundance ratio of hippocampal metallothionein-1 in the kidney (n=14)(**A**) and brain (n=17)(**B**) tissue of E. fuscus. All data were analyzed by simple linear regression.*

Chapter 4 – General discussion

4.1.1 Influences of environmental and biological factors on fur total mercury concentration in Eastern Ontario bats

The first objective of this study was to determine fur total mercury concentration concentrations in two abundant species of Eastern Ontario bats, *M. lucifugus* and *E. fuscus*, and to determine environmental and biological factors contributing to their variability. Given the reported history of specific areas of the St. Lawrence River, as well as its tributaries, such as the Raisin River as local hot-spots of mercury contamination, a particular objective with this context was to address the hypothesis that foraging and dietary factors and especially the contribution of aquatic insect diets contribute to elevated fur total mercury levels in Eastern Ontario bats. Specifically, within the Cornwall mercury "hotspot" section of the St. Lawrence river, along with other sites that run along the Raisin River, which have in the past reported to exhibit high mercury concentrations at least in part due to the high DOC levels (i.e. Williamstown, St. Andrews, and Martintown), being considered, I hypothesized that bats feeding over/nearby these sites would measure higher in their fur total mercury concentrations than compared to those feeding at upstream sites, uncontaminated zones (i.e. Morrisburg). This was further assumed to be true if bats feeding in the hotspot areas have a largely aquatic based diet, feeding more so on aquatic emerging insects rather than terrestrial insects.

Previous studies have shown that fur total mercury concentration measurements largely detect MeHg species contribution in fur, but furthermore also positively correlate with MeHg in the blood, brain, kidney and liver (Wada et al. 2010; Nam et al. 2012; Yates et al. 2014; Chételat et al. 2018). Specifically, fur total mercury is a validated marker allowing to extrapolate internal tissue burden of MeHg. As such, MeHg concentration in the fur was shown to positively correlate with total mercury concentrations in the fur where levels of methyl mercury made up 71-

95% of fur total mercury concentrations (Yates et al. 2014). These results show that fur total mercury concentration measures can be used as a relevant indicator of internal total mercury concentrations and fur methyl mercury concentrations. Fur is inert and represents the concentrations of total mercury in the body at the time of fur growth. For bats of North America this period spans the period between July and August (Fraser et al. 2013). Although a majority of bat fur collected for this thesis were from bats collected during the summer field seasons (May-September), a small subset of these bats was collected during time periods that fall outside of the field season. Bats that were collected during these time periods may differ in terms of their dietary exposure to mercury and so total mercury concentrations measured in their fur would likely cause slight variability to specific patterns I investigated throughout the thesis. Globally, I found that numerous individual bats acquired fur total mercury concentrations that exceed the 10 μg/kg threshold that was previously stated to cause neurochemical effects in bats and other mammals (Burton et al. 1977; Nam et al. 2012; Yates et al. 2014; Little et al. 2015). This is consistent with findings from other studies carried out in areas considered mercury "hotspots" (Wada et al. 2010; Nam et al., 2012; Little et al. 2015) in which bats collected from mercury contaminated sites often show elevated levels of fur total mercury and a majority of these individuals exceed the toxicity threshold.

Big brown bats tended to have higher fur total mercury concentrations than little brown bats but not northern long-eared bats. When considering if this result is an dependent on body mass differences, and hence scaled basal metabolic rate, between big brown bats (14-40 g) and the two *Myotis* species (*M. lucifugus*=5-14 g; *M. septentrionalis*=6-9 g) (Taylor 2018), I normalized total fur mercury concentrations to body mass where data was available for individuals. Big brown bats still exhibited significantly higher levels of fur total mercury normalized to body mass than the

little brown bat and now northern long-eared bats. This shows that inter-species size differences are not the driving factor behind fur total mercury differences between the three bat species. These results differ slightly from what has been previously found by Chételat et al. (2018) in which both the big brown bats and the northern long-eared bats showed higher average fur total mercury concentrations compared to the little brown bats across Canada. This difference may be linked to the geographical range difference between the Chételat et al. (2018) study and this thesis, where the former took place at sites across Canada, and the latter is a regional study focused only on sites in Eastern Ontario. A smaller sample size of the less abundant northern long-eared bats in this thesis may also be linked to statistical differences seen between the two studies. Given the inter-species differences that were exhibited in terms of fur total mercury concentration, I then focused on specific environmental and biological factors in each species. Northern-long eared bats were excluded from further analysis due to lack of data on biological factors of the very few individuals that were collected.

Consistent with previous studies (Yates et al. 2014; Korstian et al. 2018; Chételat et al. 2018), male and female little brown bats did not exhibit significant differences in their fur total mercury concentrations. However, I found that male big brown bats measured higher in fur total mercury concentration than female big brown bats. This novel finding cannot be explained by size differences between males and females considering our findings on sexual dimorphism which showed that female big brown bats are indeed larger in body mass than the males in our sample, and thus after normalizing the fur total mercury concentrations to body mass, the difference between males and females remains significant. The driving force behind this difference in fur total mercury concentration is unclear, however it may be evidence of sex related differences in feeding behaviours in the big brown bats and clearly warrants more research.

Narrowing in on the females of little brown bats and big brown bats, I investigated the biological factor of reproductive status to assess whether it is linked to fur total mercury accumulation in these females. For the little brown bats, I found that that non-reproductive female adults tend to have higher fur total mercury concentrations that pregnant females and despite the pregnant females having a greater body mass than both non-reproductive and lactating females, this effect was still exhibited after normalizing the fur total mercury concentrations to body mass, indicating that there is another driving force behind this difference in mercury accumulation. Similarly, Lisón et al. (2017) found that non-reproductive Schreiber's bent-winged bat of southeastern Iberian Peninsula exhibited higher total mercury concentrations in the brain and muscle compared to reproductive females (pregnant and lactating pooled). These findings may be caused by the tendency of mercury to cross the placental barrier and mobilize to the fetus or to the lactating mother's milk (Health Canada Mercury Issues Task Group 2004; Lisón et al. 2017). However, findings from a study in the Peruvian Amazon found that within 5 genera of bats from mercury contaminated and uncontaminated sites, the reproductive females (pregnant and lactating pooled) had higher levels of MeHg in their fur compared to non-reproductive females (Kumar et al. 2018). These findings are similar to our findings on reproductive status influence on fur total mercury concentration in big brown bats. Lactating big brown bats exhibits higher fur total mercury concentrations in comparison to non-reproductive female adults. The big brown bats from the three groups of reproductive status of focus differ in body mass, but after normalizing fur total mercury concentration to body mass, lactating big brown bats have significant higher fur total mercury concentration than the pregnant big brown bats, but not the non-reproductive adults. The effects of reproductive status on total mercury concentration in big brown bat fur could be linked to increased mobilization of mercury during lactation or an increase in prey consumption while

needing to provide enough nutrients to its pup. However, the power of these results is limited by the small sample sizes of some reproductive status groups. The fact that possible species-specific patterns in fur total mercury concentrations between females with different reproductive status exist is interesting, and future studies should investigate whether potential differences in energy reserve mobilization and dietary sources contribute to this phenomenon.

Age is a widely studied biological factor linked to persistent contaminant accumulation, and while a detailed information of age was not available from our samples, I analyzed the possible contribution of age to fur total mercury concentration by comparing individual bats younger than 1yr with adults defined as having an age >1yr. Little brown bats exhibited higher fur total mercury concentrations in adults compared to juvenile bats, a finding supported by other studies (Yates et al. 2014; Krostian et al. 2017; Chételat et al. 2018). With adult little brown bats being larger in body mass than the juveniles, the effect of age was no longer apparent after the fur total mercury concentration was normalized by body mass, suggesting higher dietary intake to meet higher metabolic demand which would introduce more MeHg to the organism over time is linked to this effect. In big brown bats, differences between fur total mercury concentration in the adults compared to young exhibited followed the same trend as the little brown bats with adults measuring higher in fur total mercury than the juveniles. However, these findings were not significant. The statistical power of this analysis was limited by sample size and would therefore likely increase with a supplemented sample size of the juvenile big brown bats. Given the lack of significance in age as an effect on the body mass normalized fur total mercury concentration, these results can overall largely be explained by adult bats having higher energetic requirements and larger body mass.

Previous studies have examined the spatial effects of mercury accumulation in bats. Particularly, these studies often focus on mercury concentrations in bats collected from mercury contaminated sites and compare them to bats from a reference site that is fairly uncontaminated, both across large geographical distances but also, to a lesser extent, in more regional contexts. The trend in each of these studies is that bats from mercury point-sources measure significantly higher in mercury concentration that bats collected from non-point source references sites (Wada et al. 2010; Nam et al. 2012; Yates et al. 2014; Korstian et al. 2018; Chételat et al. 2018; Monero-Brush et al. 2018). Cornwall, ON is a location used in this study that has been reported to be a mercury contaminated hotspot due to the high levels of mercury found in the fish and sediment (Ridal et al. 2010). The Raisin River is another body of water that empties into the St. Lawrence River downstream from the St. Lawrence River hotspot zones reported by Ridal et al. (2010). The Raisin River runs through inland sites that were used in this study (Williamstown, St. Andrews, Martintown, ON) and has been reported to have high levels of dissolved organic carbon (Cane & Clarke 1999) which is known to increase levels of MeHg in the body of water (Gill & Bruland 1990; Watras et al. 1995). Conversely, Morrisburg, ON, located west of Cornwall, is approximately 30 Km upstream from the mercury contaminated zones of the St. Lawrence reported by Ridal et al. (2010) and to my knowledge, has no reports of unusually high mercury concentrations in this area of the St. Lawrence River.

Little brown bats collected from Cornwall, Morrisburg and Williamstown exhibited fur total mercury concentrations that did not differ significantly between sites. We found that little brown bats captured from Cornwall tended to be smaller in body mass than those captured from Morrisburg and Williamstown, however, normalizing fur total mercury concentration to body mass exhibited the same results of little brown bats from the three sites having similar ranges of fur total

mercury concentration. Based on the results reported in previous studies (Wada et al. 2010; Nam et al. 2012; Yates et al. 2014; Korstian et al. 2018; Chételat et al. 2018; Monero-Brush et al. 2018), it was unexpected that little brown bats collected from sites with high concentrations of mercury in the local bodies of water, like Cornwall and Williamstown, did not differ significantly in fur total mercury concentrations from little brown bats collected from Morrisburg, a site not considered a mercury point-source.

In contrast, big brown bats captured from Morrisburg measured higher in fur total mercury concentration than big brown bats captured from Cornwall, and after normalizing the fur total mercury concentration to body mass, this effect persisted indicating that body mass and coupled energetic demands are not the principal contributor to this difference. Similar to the situation for bats from Cornwall, bats from Martintown also measured significantly less in normalized fur total mercury concentration than those from Morrisburg. Again, this outcome is unexpected and dissimilar to previous research (Wada et al. 2010; Nam et al. 2012; Yates et al. 2014; Korstian et al. 2018; Chételat et al. 2018; Monero-Brush et al. 2018) and the reason behind it remains unexplained. Higher levels of mercury in big brown bats from Morrisburg might suggest that the Morrisburg area had, over the period of sampling, higher levels of mercury than originally thought and may warrant further research.

Investigating spatial effects between Cornwall and Morrisburg further, I determined that sex does not differentially affect location-dependent fur total mercury concentration between the two locations for the little brown bats, in line with the fact that little brown bats from Cornwall and Morrisburg did not differ in fur total mercury concentration irrespective of sex either. However, male big brown bats from Morrisburg measured significantly higher in fur total mercury concentration than the female big brown bats from Morrisburg and both sexes of big brown bats

from Cornwall. This difference is also found when normalizing fur total mercury to body mass and it appears that the driving force behind this spatial difference is linked to sex in that male big brown bats explain a majority of the high levels of mercury concentration found in big brown bats from Morrisburg.

In addition to sex, I also investigated whether age differentially contributes to location-dependent differences in total fur mercury in bats caught or sampled in Cornwall and Morrisburg. Focusing age-dependent spatial effects, adult little brown bats collected from Cornwall and Morrisburg tended to have higher fur total mercury concentration than the young captured at those locations. This mirrors the age effects within the little brown bats discussed previously. Adult big brown bats from Cornwall and Morrisburg were compared in the same way, however, the lack of sample sizes of young big brown bats from both locations prevented comparisons of the young in Cornwall and Morrisburg. Adult big brown bats collected from Morrisburg showed significantly higher levels of fur total mercury concentration than those collected from Cornwall, confirming that adult male big brown bats are the majority of high fur total mercury containing individuals from Morrisburg.

The next biological factor that was examined was temporal differences in fur total mercury concentration within each bat species. There are very few studies that look at changes of mercury concentration in populations over time and none are focused on the region or species of bats that are of focus in this study. Furthermore, to my knowledge, no study spans the length of time comparable to my current study, roughly representing two decades. Small sample sizes in certain years limit the statistical analysis, however between years with sufficient sample sizes to allow for a meaningful comparison I found little brown bats captured in 2003 and 2005 had higher levels of fur total mercury compared to little brown bats captured in 2001. After normalizing fur total

mercury concentration to body mass, the results show that little brown bats captured in 1999 and 2019 measured higher in normalized fur total mercury concentrations than little brown bats captured in 2001, suggesting potential growth differences may contribute to temporal changes. The fur total mercury concentration of big brown bats also differed between years of collection with higher mercury measured in the fur of those collected in 2017 compared to 2013 and 2015. Big brown bats collected in 2016 and 2018 also measured higher in fur total mercury concentration than those collected in 2015. Linear regressions showed no significant changes overtime for either species (**data not shown**).

To determine if these temporal effects are driven by a particular sex, I examined the fur total mercury concentration differences between males and females within years we had sufficient sample sizes of both sexes within each bat species. Both the little brown bats and the big brown bats showed no significant differences in fur total mercury concentration based on sex and year interactions. Therefore, the fluctuations of mercury concentration in the bats in between specific years does not seem to be sex-dependent.

A study that examined heavy metal accumulation in bats from Great Britain, found that renal total mercury concentrations increased over time in bats (*Pipistrellus* spp.), although it was unknown what contributed to this increase (Walker et al. 2007). This trend is not apparent in the data presented in this thesis as it seems that fur total mercury concentration fluctuates between some years rather than increases steadily over time. The reasoning behind these fluctuations is unknown and it would be interesting to investigate data that might exist on atmospheric mercury deposition across time, as well as mercury concentrations in the St. Lawrence river, insects and other mammals during the years of high mercury concentration within these two bat species.

4.1.2 Probing the potential contribution of aquatic emerging and terrestrial insect diet to fur total mercury concentration in Eastern Ontario bats

Although I obtained successful radio tracking data for only a small subset of bat populations under study due to the nature of field data collection, my data supported the assumption that little brown bats and big brown bats typically follow a foraging routine, visiting the same sites to feed on most nights, which has been previously reported (Fenton & Barclay 1980). This allows me to confidently make the assumption that collecting insects at sites in which we have confirmed little brown bats and big brown bats to be feeding are a good representation on what insect populations make up the foraging sites of the bats that likely visit the sites every night to feed.

To my knowledge, this study is the first to examine total mercury levels in emergent insects and terrestrial arthropods of the Eastern Ontario region. We investigated differences in total mercury concentration of aquatic emerging insects and terrestrial insects to identify accumulation and potential differences of mercury in the little brown and big brown bats food web. The results show that globally, aquatic emerging insects measure higher in total mercury concentration than terrestrial insects, confirming this ancillary assumption. This finding is in line with the paradigm that inorganic mercury is methylated in aquatic ecosystem and then passed up the food web from primary producers to aquatic invertebrates, leading to biomagnification in the process (Lavoie et al. 2013). Some of these aquatic invertebrates are larval or nymph stages of certain insect species that emerge from bodies of water as they enter their adult stage while still likely carrying the mercury in their systems that they were exposed to in the body of water. Terrestrial insects are in contrast, not exposed to this level of mercury and it is not surprising that they measure lower in total mercury compared to aquatic emerging insects.

When looking at differences between orders of all insects collected, the orders coleoptera, megaloptera and trichoptera measured higher in total mercury concentration than lepidoptera with megaloptera measuring highest in their total mercury concentration, likely due to the longer aquatic larval stage of this order and carnivorous feeding behaviour (Rivera-Gasperín et al. 2019). When comparing aquatic emerging species of different orders, megaloptera again measure higher in total mercury than aquatic emerging lepidoptera. The same comparisons were investigated for terrestrial species of different orders and the results showed higher concentrations of total mercury in terrestrial coleoptera and diptera than terrestrial lepidoptera. Small sample sizes of specific insect groups limit the statistical power of analysis. Insect samples were also all collected during one field season, unlike the bat fur samples which were collected over two decades so any temporal variation in insect mercury data could not be accounted for or linked to the temporal variations in total mercury concentration in the bat fur.

Geographical location of insect collection was then examined, first between all insect samples collected at each site, and then with just aquatic emerging or just terrestrial species collected from each site. I found that insects did not differ in total mercury concentration between sites as a whole, and similarly no location dependent differences were found between aquatic emerging insects or terrestrial insects analyzed separately. Again, the lack of sample size from each insect limited the statistical power of the analysis and it prevented me from being able to compare total mercury concentration differences between each order and site as an interaction. With a sufficient sample size, a more detailed statistical analysis could be carried out.

To begin to approximate potential dietary contributions to mercury burden in bats, I first characterized the $\delta^{13}C$ and $\delta^{15}N$ isotope abundance in insect samples that represent described prey of insectivorous little brown and big brown diets. The $\delta^{13}C$ $^{0}/_{00}$ values act as a proxy of dietary

source of carbon dependent on C-fixating producers' signatures. For example, C4 plants such as corn or sugarcane that have an evolved mechanism of reducing water loss in their leaves have a tendency to have higher $\delta 13C^{0/}{}_{00}$ values (range of -20 to -10 $^{0/}{}_{00}$) compared to C3 plants such as wheat and rice) (range of -34 to -22 $^{0/}{}_{00}$) as their evolved water retaining mechanisms prevents the preference to the lighter ^{12}C isotope when fixing CO_2 like the C3 plants do (O'Brien 2015). $\delta^{15}N$ $^{0/}{}_{00}$ values are a proxy for trophic position, with top predators having increased $\delta 15N$ $^{0/}{}_{00}$ values compared to organisms lower on the food web based on protein intake and excretion of waste nitrogen which tends to be the lighter ^{14}N isotope (O'Brien 2015). When investigating $\delta^{13}C$ $^{0/}{}_{00}$ differences between aquatic emerging insects and terrestrial insects, we found that there were no significant differences between the two groups, indicating that the dietary source of carbon does not vary greatly between these two groups of insects. The terrestrial insects measured significantly lower in their $\delta^{15}N$ $^{0/}{}_{00}$ values compared to the aquatic emerging insects. However, without detailed additional measurements regarding other components of the food-web, the relevance of these results regarding trophic positioning remains to be fully explored. Since a degree of variability was observed for $\delta^{13}C$ $^{0/}{}_{00}$ and $\delta^{15}N$ $^{0/}{}_{00}$ data in both aquatic emerging and terrestrial insects a deeper analysis is warranted.

To further investigate these isotopic differences within the insects, we compared $\delta^{13}C$ $^{0/}{}_{00}$ and $\delta^{15}N^{0/}{}_{00}$ values between the different orders of aquatic emerging insects and between the different orders of terrestrial insects. The $\delta^{13}C^{0/}{}_{00}$ values did not differ significantly between either the aquatic emerging insect orders or between the terrestrial insects. In terms of the $\delta^{15}N$ $^{0/}{}_{00}$ values, aquatic emerging coleoptera had significantly lower values than trichoptera. There were significant differences in $\delta^{15}N^{0/}{}_{00}$ values between terrestrial orders as well with higher $\delta^{15}N$ $^{0/}{}_{00}$ values measured in diptera compared to homoptera and orthoptera, hymenoptera, lepidoptera and aranae

compared to homoptera. These results indicate that orders measuring higher in $\delta^{15}N\ ^{0}/_{00}$ values likely contain more predatory species, compared to those with lower $\delta^{15}N\ ^{0}/_{00}$ values which likely contain more species that feed on plants or filter feed. There is, however, a lot of variation in the $\delta^{13}C\ ^{0}/_{00}$ and $\delta^{15}N\ ^{0}/_{00}$ values for both aquatic emerging and terrestrial orders of insects. The insect samples were only sorted down to their order and aquatic or terrestrial life cycle, the small sample sizes prevented me from sorting the samples further into their collection sites. The different sites in which these insects were collected likely differ in plant populations (both aquatic and terrestrial plants), increasing the variation of carbon source between each site. Grouping the insects disregarding the site of collection is therefore a large confounding factor in this study. Increasing the sample size of insects at each site would not only allow us to make isotopic comparisons between sites but also within each site. Supplementing the sample sizes of insects may even allow for genus or species level comparisons considering within an order there tends to be differences in feeding behaviors (Walters 2012).

The isotope data for the little brown bats and the big brown bats showed that although there were no significant differences between the two species and their $\delta^{13}C\ ^{0}/_{00}$ values, the range and variation of the $\delta^{13}C\ ^{0}/_{00}$ measurements between the two species was different. The $\delta^{13}C\ ^{0}/_{00}$ measurements for the little brown bats varied greatly, with a wide range of values displayed. This is opposite of what the results reflect for the $\delta^{13}C\ ^{0}/_{00}$ measurements in big brown bats. The big brown bat $\delta^{13}C\ ^{0}/_{00}$ values do not vary greatly and fit on a much narrower scale. This suggests that the little brown bats have a more generalist type feeding behavior, whereas the narrow range in $\delta^{13}C\ ^{0}/_{00}$ values of big brown bats indicate that they likely depend on a more specific food source. This is consistent with reports that while little brown bats tend to have a more opportunistic approach to feeding (Belwood & Fenton 1976; Chételat et al. 2018), while big brown bats are

slightly more specialized in their feeding behavior (Moosman 2012). Our results showed that the $\delta^{15}N\ ^{0/}_{00}$ values in little brown bats were significantly greater than that of the big brown bats which is an interesting finding when compared to the $\delta^{15}N\ ^{0/}_{00}$ measurements reported in the insect samples. Dipterans and trichopterans have been reported to make up a large portion of little brown bats' diet, and as reported in our results, these two orders of insects fall higher in their $\delta^{15}N\ ^{0/}_{00}$ values compared to other orders, which is reflected in the little brown bats $\delta^{15}N\ ^{0/}_{00}$ measurements. Big brown bats, on the other hand, tend to feed largely on coleopterans, homoptera and heteroptera (two suborders of the hemiptera order) (Phillips 1966; Agosta 2002; Agosta & Morton 2003; Moosman et al. 2012) which again was reflected in our results where these three orders fell lower on the scale in terms of their $\delta^{15}N\ ^{0/}_{00}$. Terrestrial insects were found to have lower $\delta^{15}N\ ^{0/}_{00}$ values than aquatic emerging insects which is consistent with the $\delta^{15}N\ ^{0/}_{00}$ values found in big brown bats. Big browns have been reported to feed more on terrestrial insects compared to little brown bats who favor aquatic emerging insects (Phillips 1966; Agosta 2002; Agosta & Morton 2003; Moosman et al. 2012). This is a key finding of this study because despite big brown bats feeding on insects with lower $\delta^{15}N\ ^{0/}_{00}$ and therefore low total mercury concentrations, big brown bats typically measured higher in their fur total mercury concentrations. As previously discussed, given the variability in freshwater systems, additional analyses are required to substantiate inferences regarding the nature and diversity of bat food webs in Eastern Ontario.

With correlations between fur total mercury concentration and the $\delta^{13}C\ ^{0/}_{00}$ and $\delta^{15}N\ ^{0/}_{00}$ measurements for little brown bats and big brown bat, the only significant correlation that was reflected was a negative correlation within the little brown bats between their fur total mercury concentrations and $\delta^{13}C\ ^{0/}_{00}$ values. This is consistent with the large variation we reported in $\delta^{13}C$

$^{0/}_{00}$ values of little brown bats in comparison to big brown bats that seem to be more specialized in their feeding which could thus linked to the mercury content in their fur.

The influence of sex on differences in isotope measurements showed that there were no significant differences in $\delta^{13}C$ $^{0/}_{00}$ and $\delta^{15}N$ $^{0/}_{00}$ measurements between female and male little brown bats female and male big brown bats. The results did reflect, however, that female little brown bats are mostly responsible for the large variation in $\delta^{13}C$ $^{0/}_{00}$ previously described. This suggests sex differences within the little brown bats, such as nutritional needs during pregnancy and lactation or different baseline rates might lead to female little brown bats becoming less strict on what prey to depend on and being more opportunistic in their foraging. More research on this possible sexual dimorphism as well as consequences for mercury uptake is required.

The correlation between fur total mercury concentrations and $\delta^{13}C$ $^{0/}_{00}$ and $\delta^{15}N$ $^{0/}_{00}$ measurements showed that a significant negative correlation between fur total mercury concentration and $\delta^{13}C$ $^{0/}_{00}$ values within the female little brown bats and a significant positive correlation between that of the male big brown bats. The fact that these relationships are oppositely correlated suggests that there may be sex-specific feeding behavior differences linked to differential mercury uptake in small and big brown bats. The idea that the dietary carbon source may be linked to fur total mercury concentration in female little brown bats holds true with the support from the correlation between fur total mercury concentration and $\delta^{13}C$ $^{0/}_{00}$ values in the female little brown bats and not in the male little brown bats. Given the significant correlation between fur total mercury concentration and $\delta^{13}C$ $^{0/}_{00}$ measurements in the male big brown bats suggests that there may be sex differences in feeding behavior within the big brown bats as well. Wilkinson and Barclay (2016) reported that male big brown bats travel further from the roost during a night of foraging compared to reproductive females and suggested that the male big brown

bats likely tend to feed in areas of which are lower in quality in terms of prey abundance compared to the reproductive females. This may be linked to the correlation between fur total mercury concentration and $\delta^{13}C\ ^{0/}_{00}$ measurements in male big brown bats and not the females. This correlation might also be linked to the higher fur total mercury concentrations found within the male big brown bats compared to the females that was previously reported in this study, and suggest that prey location, rather than source, the latter of which was found to be highly homogenous in big brown bats, may contribute to higher mercury concentration in male big brown bats compared to females. Finally, when investigating temporal differences in and $\delta^{13}C\ ^{0/}_{00}$ and $\delta^{15}N\ ^{0/}_{00}$ values of bats, no significant differences were found between years of bat collection (**data not shown**). This was expected to be the case as $\delta^{13}C\ ^{0/}_{00}$ and $\delta^{15}N\ ^{0/}_{00}$ values should no differ greatly within a species between years. I nevertheless confirm this assumption as suggested by a review of best practices in isotope research (Philips et al. 2014).

Finally, I analyzed the correlation of total mercury concentration and $\delta^{13}C\ ^{0/}_{00}$ and $\delta^{15}N\ ^{0/}_{00}$ measurements of both bat species and both aquatic emerging and terrestrial insects. The results showed that when looking at all four groups of organisms as a whole, only $\delta^{13}C\ ^{0/}_{00}$ values and total mercury concentration had a significant negative correlation. The results reflect that the two bat species do tend to fall higher in $\delta^{15}N\ ^{0/}_{00}$ values than a majority of the aquatic emerging and terrestrial insects, which is expected considering the bats are higher on the food web than these insects. The great variability in insect $\delta^{13}C\ ^{0/}_{00}$ and $\delta^{15}N\ ^{0/}_{00}$ values are also reflected in these results and reiterate the need for more specific food web data to build this model in better resolution.

Due to the nature of field work data collection, there are limitations to this study. As previously mentioned, supplementing the insect sample sizes would allow for a much stronger statistical analysis and prevent the confounding factors of potential temporal, spatial or inter-

species differences within orders in terms of mercury concentration and isotope measurements. Constraints on field work and lab work during the COVID-19 pandemic resulted in losing one field season of data collection and pushed back a lot of lab work along the timeline of this study. With additional data obtained, it may be possible to run a mixed model on the data set potentially delineate dietary contributions through mixed modelling approaches (Philips et al. 2014). Given the inherent incertitude in such approaches (Philips et al. 2014), the current data set will need supplementation to increase sample size and account for local variability.

Supplementing the insect samples would also allow to run MeHg analysis on a subset of insects to validate the utility of insect total mercury as (cheaper) proxy for MeHg burden. This measurement is important because the percentage of MeHg mercury that makes up THg in insect might be highly variable. Similar validation for bat fur has been reported by Chételat et al. (2018), reporting that total mercury concentrations correlated very well to methyl mercury concentrations in the fur and internal tissues. Future work on investigating inter-species and intra-species sex differences in feeding behaviors is also warranted based of the results presented in this study. This could be carried out by focusing on a smaller regional scale, avoiding the confounds of spatial differences. Another method to add to the strength of isotope analysis in terms of mapping out food webs is analyzing bat guano or searching through stomach content which has been done several other studies in the past but have their share of limitations as well, such as the complete digestion of soft-bodied insects leading to bias toward hard-bodied insects (Arata et al. 1967; Belwood & Fenton 1976; Agosta & Morton 2003; Clare et al. 2014).

4.2. Investigated mitochondrial and epigenetic DNA markers do not correlate with fur total mercury concentration in big brown bats in Eastern Ontario

The second objective of this thesis was to investigate molecular level biomarker responses to fur total mercury concentration. Based on previous lab-based studies, I postulated that higher fur total mercury concentrations would be negatively associated with mitochondrial and epigenetic DNA level responses, specifically the amount of quantifiable mitochondrial DNA and global DNA methylation. Additionally, while *a priori* less suitable for long-term stored samples due to its high degradability, I also assessed the suitability of RNA-based methods, and specifically real-time RT-PCR expression analysis of known metal-inducible detoxification response genes (metallothioneins). Of the DNA that was extracted from the kidney, brain and liver tissue of little brown bats and big brown bats, the only sufficient sample sizes we were left with were for the big brown bat tissues, and so I focused only on big brown bat tissues in terms of the molecular biomarkers. While specific *MT1* expression was quantifiable, quality control of extracted RNA from long-term stored unsurprisingly revealed a high degree of degradation, thus RNA-based biomarkers were not further pursued.

Global DNA hydroxymethylation and methylation, mitochondrial DNA damage, and expression levels of genes involved in metal detoxification were the three molecular endpoints that were thus focused on for the purpose of this study. The process of DNA methylation is a normal physiological process involved in controlling gene expression and ageing (Rodenhiser & Mann 2006; Pilsner et al. 2010). The correlation between fur total mercury concentration and the percentage of global hydroxymethylated DNA, an intermediate step of DNA methylation (Branco et al. 2012; Tellez-Plaza et al. 2014) was investigated from extracted DNA of the three tissues collected from big brown bats. The results reflected that although there was a negative trend for

the correlation in all three tissues, there was no significant correlation between the two factors in neither the kidney, brain, nor the liver. The percentage of global DNA methylation also showed no significant correlation to fur total mercury concentration in any of the three tissues in big brown bats, although again the general trend was negative. To my knowledge this is the first study that looks at global DNA hydroxymethylation and methylation as a molecular response to mercury levels in bats. However, there have been reports of MeHg concentrations being associated with global DNA hypomethylation in other organisms (Pilsner et al. 2010; Bose et al. 2012; Basu et al. 2013; Goodrich et al. 2013; Cardenas et al. 2017) which follows the general trends reflected in our results, although again these correlations were not significant. Particularly interesting, a report by Basu et al. (2013) looked at this association within three species; mink, chicken and perch and found that within the minks with a MeHg dietary exposure of 1 ppm, there was a significant decrease in the percentage of methylated DNA, but this significant change was not seen in a group of mink that had a MeHg dietary exposure of 2 ppm. They also found variability in the effects on DNA methylation in the chicken and perch. This suggests that the effects of mercury on DNA methylation might be dose-dependent and that there is inter-taxa variability in the effects of mercury on global DNA methylation, which might explain why we do not see an association between total mercury concentration and global DNA hypomethylation as we expected.

Next, I looked at the expression of mitochondrial DNA expression as a molecular biomarker response to high fur total mercury concentration. Mitochondrial DNA lacks the repair system that nuclear DNA possesses. Without this repair system, mitochondrial DNA is susceptible to damage and a decrease in abundance following environmental stress, like mercury exposure (Quiros et al. 2017; Antunes dos Santos et al. 2018). I focused on the expression levels of mitochondrial gene *16S* in comparison to the expression levels of the housekeeping nuclear gene,

HK2 for the purpose of this study. There was no significant correlation between fur total mercury concentration and the ratio of *16S:HK2* expression in neither the kidney, brain, nor liver of big brown bats although there was a general negative trend in the three tissues. Karouna-Renier et al. (2014) reported that little brown bats collected nearby a well-known mercury hotspot (South River, VA, USA) only present very weak negative correlations between fur total mercury concentration and mitochondrial DNA damage in DNA samples extracted from wing tissue. Taking their results into consideration, along with the results presented in this thesis, there might be other environmental factors acting on mitochondrial DNA damage, or that due to the high mercury exposure bat species are faced with, they may have developed advanced tolerance levels or detoxification mechanisms. It is also possible that that mercury levels measured in the bat fur for this study were simply not high enough to elicit the molecular responses we were expecting to observe.

As previously mentioned, the focus of molecular biomarker responses to mercury concentration were mainly DNA based due the poor quality of RNA that was extracted from tissue samples after years of being initially collected and stored. However, it was decided that because all RNA samples extracted were deemed poor in quality, there would not be a bias during molecular biomarker analyses toward good quality RNA samples since there were none. Therefore, only a single RNA level molecular endpoint was investigated which was the expression of a *MT1* gene in the kidney and brain samples of the big brown bats. I found that there was no significant correlation between fur total mercury concentration and the MT1-hippocampal abundance in the big brown bat kidney samples. There was a very slight positive trend between the two factors in the big brown bat brain samples, although this correlation was also found to be not significant. There have been studies that report changes in metallothionein expression after

exposure to mercury (Zalups & Koropatnick 2000) but to my knowledge there has yet to be a study on the effects of metallothionein expression in bat species. Our results are confounded by uncertainty caused by the quality of extracted RNA and so more research on RNA level molecular responses to mercury exposure is warranted but must include field work methods that include proper preservation of tissues to allow extraction of high-quality RNA.

In terms of the molecular aspect of this thesis, most of the limitations involve the nature of field work. Field work data collection allows for the investigation of naturally relevant concentrations of mercury exposure, however there are many environmental factors that might affect the molecular level response that we cannot account for. When the results reflect no significant correlation between the fur total mercury concentration and the molecular biomarker response under investigation, it is difficult to determine if that is because the effects are diluted among the many other environmental and biological factors at play in the field-based study. Most studies that investigate these molecular biomarkers are lab-based studies with extremely controlled environments, allowing the effects of mercury exposure to be undiluted. Lack of correlations between fur total mercury concentration and the molecular responses might also suggest that bats have developed advanced detoxification systems or mercury tolerance levels based on their high level of exposure.

Relevant to the molecular approaches discussed, I conducted an *in-silico* exploration recently published bat genomes to identify transcription factors (MTF) and metallothionein genes (MT) in bat genomes. In short, my findings reflected that big brown bats had an expanded metallothionein network that compared to little brown bats and other similarly sized rodents (Günther et al. 2012; Schmidt et al. 2016), possibly linked to their increased metabolic rate compared to the little brown bats (Hock 1951; Willis et al. 2006; Cooper & Geiser 2008). The

results reflected in this term project suggest that big brown bats, which presented higher levels of fur total mercury concentrations, may have an expanded detoxification mechanism in comparison to little brown bats due to their higher metabolism and therefore, likely increased dietary exposure to mercury. Thus, future research exploring species-specific differences of bat metal detoxification systems is clearly warranted.

4.3 General conclusions

In my thesis, I characterized fur total mercury exposure in two local Eastern Ontario bat species, *M. lucifugus* and *E. fuscus* in known mercury hotspots and surrounding areas. We found that big brown bats tend to have higher levels of mercury in their fur compared to little brown bats. The high levels of fur total mercury found within the big brown bats is strongly driven specifically by male adult big brown bats. Further, the mercury concentration in male adult big brown bats were mainly driven by those collected from Morrisburg and during the year 2017. The reason for high levels of mercury exposure within the big brown bats in this area during this timeframe is unknown and it would be interesting to compare insect mercury data from the Morrisburg area during 2017 if that data exists. To my knowledge, I am the first to characterize fur total mercury in these two bat species over such a long timeframe in this regionalized area of eastern Ontario.

This is also the first study to measure total mercury levels in emergent insects of this eastern Ontario region. Our results showed that when looking at the total mercury concentrations of aquatic emerging insects in comparison to terrestrial insects, the aquatic emerging insects measure higher in mercury as expected when taking into account the mercury cycle. With this ancillary assumption met, I nevertheless identified great variation in total mercury concentration between

both aquatic emerging insect orders and terrestrial insect orders and indeed, it seems as though there is variation within the orders as well.

Isotope data collected from the insects and the bats supported previous reports that big brown bats tend to feed more on terrestrial insects while little brown bats feed more so on aquatic emerging insects. This, in conjunction with the mercury data showed that despite little brown bats feeding on the group of insects with higher total mercury concentrations, aquatic emerging insects, big brown bats tend to measure significantly higher in fur total mercury concentrations. Additional sampling is necessary to resolve species and location dependent variability between insects to fully address the hypothesis that preferential use of aquatic diets result in higher mercury burdens in bats. Furthermore, the isotope data uncovered important information providing circumstantial evidence for sex-specific diet-source contributions to mercury burden in bat species. My work represents the first attempt to characterizing the food web of these two local bat species on a regional scale in this area.

Although our work on molecular biomarker responses is novel for big brown bats, our findings showed that the endpoints we decided to focus on were not affected greatly by the levels of total mercury found in the bat fur measured. This warrants further investigation to determine if there are other biomarkers affected by mercury exposure in these bats or if, alternatively, they have developed a way to detoxify or tolerate such high fur total mercury concentrations.

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