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Trophic Transfer of a Naturally Occurring Algal Toxin from a Freshwater Lake to Little Brown Bats

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Abstract

Microcystis aeruginosa is a species of cyanobacteria capable of producing a hepatotoxin called microcystin. As toxic *M. aeruginosa* overwinters in the sediments of lakes, it is ingested by some mayfly larvae, such as those of *Hexagenia* spp., and thus microcystin bioaccumulates in these insects. When *Hexagenia* emerge from lakes to reproduce, they provide an abundant, albeit temporary, food source for many terrestrial organisms such as bats. Little brown bats, *Myotis lucifugus*, likely feed opportunistically on aquatic insects. To test if microcystin moves from aquatic to terrestrial ecosystems via trophic transfer, we 1) tested bat feces for the presence of *Hexagenia* mayflies and 2) tested bat livers and feces for microcystin. In June 2014, in correspondence with the *Hexagenia* emergence, bat feces were collected from underneath a maternity roost near Little Traverse Lake (Leelanau County, MI). On 20 and 27 June we caught 19 female *M. lucifugus*, which were euthanized, and collected their livers and feces. DNA was extracted from feces, amplified with a Polymerase Chain Reaction (PCR), and sequenced. Concentrations of microcystin in liver tissue and feces were determined using an enzyme-linked immunosorbent assay (ELISA) and liquid chromatography with tandem mass spectrometry (LC-MS/MS). *Hexagenia* were present in the diet of *M. lucifugus* and the most likely source of microcystin. Our analyses reveal that microcystin was also present, with higher concentrations in the bat feces than the livers. Additionally, histopathology results of three bat livers with highest concentrations of microcystin show little to no cytological damage from the toxin. From these data, it appears that *M. lucifugus* are not highly affected by the ingestion of microcystin.

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Abbreviations

BOLD: Barcode of Life Database

CDC: Center for Disease Control

CEC: contaminants of emerging concern

DDT: dichlorodiphenyltrichloroethane

ELISA: enzyme-linked immunosorbent assay

HAB: harmful algal bloom

HSD: honest significant difference

IACUC: institutional animal care and use committee

LC-MS/MS: liquid chromatography with tandem mass spectrometry

MC: microcystin

MC-eq: microcystin equivalents, refers to all congeners of microcystin

MC-LR: microcystin-LR, one of the most toxic and commonly occurring congeners of microcystin

MID: multiplex identifier

MOTU: molecular operational taxonomic units

mtDNA: mitochondrial DNA

NGS: next generation sequencing

NOAEL: no observed adverse effect level

OUT: operational taxonomic unit

PBB: polybrominated biphenyl

PCB: polychlorinated biphenyl

PBDE: polybrominated diphenyl ether

PCR: polymerase chain reaction

SD: standard deviation

TDI: tolerable daily intake

WHO: World Health Organization

WNS: white-nose syndrome

Chapter 1

INTRODUCTION

Trophic interactions are defined by the transfer of energy between trophic levels. However, at the aquatic-terrestrial interface, these interactions may result in more than the movement of energy and nutrients between ecosystems. Emerging aquatic insects have been shown to be important prey items for riparian predators such as arthropods, birds, bats, reptiles, and amphibians (Baxter et al. 2005), but these aquatic insects have also been shown to be significant exporters of contaminants to terrestrial ecosystems (Menzie 1980; Runck 2007; Cristol et al. 2008). Of these predators, bats may be particularly important in the movement of aquatic subsidies, including contaminants, back to terrestrial ecosystems (Vander Zanden and Sanzone 2004).

Many studies have focused on the movement of both organic and inorganic contaminants from aquatic to terrestrial ecosystems, but few have assessed the transfer of naturally occurring algal toxins, such as microcystin (MC), into terrestrial ecosystems. As harmful algal blooms (HABs) increase in frequency and intensity due to human alterations such as climate change (Paerl and Huisman 2008), eutrophication (Huisman et al. 2005; Paerl and Fulton 2006), and invasive species (*e.g.*, zebra mussels, Vanderploeg et al. 2001), it is increasingly important that we understand the impact they may have on ecosystems. The effects that HABs have on aquatic ecosystems and drinking water quality are well studied, but few studies have focused on the movement of HAB-associated toxins into new

ecosystems specifically via trophic interactions.

Aquatic insects such as *Hexagenia* mayflies (Ephemeroptera) have been shown to contain high concentrations of MC at all life stages (Woller-Skar 2009; Woller-Skar et al. 2015). Little brown bats, *Myotis lucifugus,* are a common bat species in North America that consumes aquatic insects and may take advantage of emergences of Ephemeroptera (Clare et al. 2011). Thus, predation of *M. lucifugus* on *Hexagenia* may result in the trophic transfer of an algal toxin into the terrestrial ecosystem. Since MC can be harmful to mammals (Sivonen and Jones 1999), it is important to know what *M. lucifugus* are eating and if they are ingesting toxic *Hexagenia.*

My thesis will consist of two chapters focusing on the trophic transfer of microcystin from a freshwater lake to *M. lucifugus* specifically through *Hexagenia* mayflies. In chapter one, I use cloning to analyze the diet of *M. lucifugus* during mid-maternity season in order to determine what the bats are eating. Specifically, I am looking for the presence of *Hexagenia* in the diet of *M. lucifugus*, since we know this aquatic insect contains high concentrations of microcystin. This chapter is a manuscript to be submitted for publication in Acta Chiropterologica. In chapter three, I will quantify concentrations of microcystin in the livers and feces of *M. lucifugus* using LC-MS/MS and ELISA to determine if the bats are exposed to and bioaccumulating this toxin. Additionally, I will use histopathology to determine if the bats are suffering from liver damage that may be attributable to microcystin poisoning. Results from chemical analyses and histopathology will

provide insight into the degree of toxicity of microcystin to *M. lucifugus*. This chapter is a manuscript to be submitted for publication in PLoS ONE.

PURPOSE

The purpose of these studies is to determine if little brown bats (*Myotis lucifugus*) are being exposed to microcystin through trophic transfer. Using molecular techniques, I will determine if *M. lucifugus* are consuming *Hexagenia* mayflies, a potential source of microcystin. I will then determine if there is microcystin present in the livers and feces of *M. lucifugus* to better understand whether or not these bats are bioaccumulating this toxin. Because bats are suffering population declines due to many threats such as white-nose syndrome, it is important to understand if there are other potential threats to local populations of bats.

SCOPE

The scope of this study is limited to the time frame and location from which samples were collected. Because microcystin production varies temporally and spatially, bats may bioaccumulate different concentrations of microcystin and may be affected differently at different times of the year and in different locations. This study is also specific to one bat species and other species may be impacted differently. However, because this is the first study to examine the bioaccumulation of microcystin in bat livers, it provides insight into what other populations of bats may experience.

ASSUMPTIONS

This study assumes that the bats sampled are representative of the population as a whole. This study also assumes that concentrations of microcystin are distributed evenly throughout the bats' livers and feces since samples were divided for different uses (*i.e.,* feces were divided for dietary and chemical analyses and livers were divided for chemical and histopathological analyses). This study also assumes that microcystin concentrations measured with these techniques are representative of the true concentrations of microcystin present in each sample type.

Research Questions

1. Are *M. lucifugus* consuming *Hexagenia* mayflies?
2. Are *M. lucifugus* ingesting microcystin?
    a. Is microcystin present in the livers and/or feces of *M. lucifugus*?
3. Do *M. lucifugus* livers show histopathological damage due to microcystin?

Significance

This study is the first to determine if bats are ingesting microcystin specifically through trophic transfer and if bats are affected by the bioaccumulation of microcystin in their livers. Consequently, this study will determine if a naturally occurring algal toxin is moving from an aquatic ecosystem into a terrestrial ecosystem via trophic interactions. Although this study is limited in its scope, there are still implications for management of local bat populations. Evidence of movement of this hepatotoxin may prompt managers to dedicate more time, money, or resources to managing water quality in the area.

Chapter 2

Dietary flexibility of *Myotis lucifugus* during mid-maternity season

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RUNNING TITLE: Rapid shift in diet of *Myotis lucifugus*

ABSTRACT

Many chiropteran species are generalist predators that play key roles in ecosystems around the world. It is important to understand the diets of insectivorous bats in order to determine their potential impact on local insect populations. The little brown bat, *Myotis lucifugus*, is one of the most common and widely distributed bats in North America. Within the last decade, however, populations of *M. lucifugus* have suffered severe population declines due to white-nose syndrome (WNS) in the eastern U.S. and Canada. A large proportion of the *M. lucifugus* diet consists of aquatic insects, particularly in summer when aquatic insect species may undergo mass emergences from aquatic to terrestrial ecosystems. The purpose of this study is to determine which insect species constitute the diet of *M. lucifugus* during mid-maternity season in Michigan's lower peninsula. Molecular analyses of *M. lucifugus* feces collected over a two-week period revealed that these bats consume high proportions of Diptera, Ephemeroptera, and Lepidoptera. On the first sampling date, over 80% of the *M. lucifugus* diet consisted of Diptera and Ephemeroptera in approximately equal proportions. The second sampling date revealed a significant shift in *M. lucifugus* diet to over 50% Lepidoptera. Among these prey items were several lepidopteran pest species of agricultural and forest industries as well as dipterans of human and domestic animal health concern. Such characterizations of the *M. lucifugus* diet may lead to a better characterization of insects that may experience decreased predation as WNS spreads throughout North America and affects local bat populations.

KEYWORDS: diet, dietary analysis, insect pests, little brown bats, Michigan, *Myotis lucifugus*, predator-prey interactions, trophic interactions

INTRODUCTION

Predator-prey interactions contribute to defining the structure and function of ecosystems (Closs *et al.* 1999). Predators can impact the dynamics of prey populations either directly or indirectly by influencing parameters such as survival and distribution (Kalka *et al.* 2008; Williams-Guillén *et al.* 2008; Maine and Boyles 2015) and behavior (Tuttle *et al.* 1982; Belwood and Morris 1987). For example, bats have been shown to directly reduce the abundance of arthropods in a variety of ecosystems (Kalka *et al.* 2008; Williams-Guillén *et al.* 2008; Maine and Boyles 2015), thereby exhibiting top-down control on arthropod populations. Conversely, prey populations can affect the dynamics of their predators through their own reproductive success (*e.g.,* bats exploiting mass emergences of insects; Lee and McCracken 2002), temporal dynamics (Lang *et al.* 2006; McCracken *et al.* 2012), and spatial patterns (Rydell 1992; McCracken *et al.* 2012). Optimal foraging theory predicts that predators maximize food intake during their time spent feeding in order to minimize energy loss (Schoener 1971; Pyke *et al.* 1977); thus, if prey densities are low, some predators may change their behavior to spend less time foraging (Anthony *et al.* 1981). Consequently, dynamics of prey can have bottom-up impacts by influencing a predator's survival and reproductive success. Understanding these top-down and bottom-up interactions allows managers to better predict an ecosystem's response to disturbance.

One major disturbance to ecosystems throughout the United States and Canada is the novel fungal pathogen, *Pseudogymnoascus* (formerly *Geomyces*) *destructans*, which causes white-nose syndrome (WNS) in bats (Lorch *et al.* 2011). This disease has resulted in the deaths of millions of cave-roosting insectivorous bats and continues to spread

throughout the continent (Turner *et al.,* 2011, Coleman and Reichard, 2014). As populations of bats continue to decrease due to WNS, their insect prey have fewer potential predators and may therefore experience a relaxation of top-down control.

One species that has suffered significant population declines due to WNS is the little brown bat, *Myotis lucifugus* (Frick *et al.,* 2010; Langwig *et al.,* 2015). This species is distributed throughout most of North America (Jones *et al.,* 1977) and feeds opportunistically on aquatic insects (Belwood and Fenton, 1976; Clare *et al*., 2011) as well as other abundant arthropods. High geographic variation in the diet of *M. lucifugus* (Whitaker, 1972; Anthony and Kunz, 1977; Buchler, 1976; Clare *et al*., 2011, 2014) suggests that they are selective opportunists (*sensu* Fenton and Morris, 1976) that take advantage of variation in insect abundance and diversity. Several studies have shown temporal dietary shifts toward insects such as ephemeropterans and dipterans that correspond with mass emergences and swarms of these insects during summer (Buchler, 1976; Anthony and Kunz, 1977; Clare *et al*., 2011). Females face the additional constraint of needing to increase food consumption during this time due to high energy demand during pregnancy and lactation and therefore may become more selective in their feeding preferences when reproductively active in summer (Anthony and Kunz, 1977; Kurta *et al*., 1989). Because age, sex, and reproductive status may impact how *Myotis lucifugus* exploit prey, it is not surprising that Clare *et al*. (2014) found high levels of variation both spatially and temporally in the diet of *M. lucifugus* throughout Canada.

Due to ongoing population declines of WNS-affected species such as *M. lucifugus* (Frick *et al*., 2010; Langwig *et al.,* 2015), it is important to collect more information on dietary variation across numerous sites in order to understand the impacts of bat

population declines on insect abundance, community structure, and ultimately ecosystem functioning. Only a few studies (Clare *et al.* 2011, 2014) have used molecular techniques to determine the diet of this particular species. This study uses molecular techniques to characterize the diet of female *M. lucifugus* in the northern lower peninsula of Michigan (USA) during maternity season, a crucial time for adult and juvenile survival. We predict that female *M. lucifugus* feed heavily on ephemeropteran species known to experience mass emergences during this same time.

MATERIALS AND METHODS

Study Site and Sample Collection

Nineteen adult female *M. lucifugus* were caught on June 20, 2014 ($N = 9$) and June 27, 2014 ($N = 10$) at a maternity roost in a barn near Little Traverse Lake (Leelanau County, Michigan, USA; Fig 1). Bats returning from foraging were captured with a harp trap (G7 Cave Catcher, Bat Conservation and Management) that was placed in the open doorway of the barn. The trap was erected after adults left the roost to ensure that fecal samples represented prey items captured on the night of sampling. Captured bats were identified to species and held in cloth bags overnight (IACUC Approval # 14-08-A; Michigan Scientific Collector Permit SC-1489). Approximately 10% of the feces produced by each bat were subsampled and placed in 1.5 mL tubes with silica bead desiccant (Fischer Scientific) and then frozen at -20 °C. Fresh feces were also collected from roosting bats by placing aluminum foil directly underneath the roosting colony each trap night. Feces that accumulated on the foil were collected the next day, placed in 1.5 mL tubes with silica bead desiccant, and then frozen at -20 °C.

Molecular Methods

Intact fecal pellets were subsampled (as described above) from the feces produced by the 19 captured bats after each was held in cloth bags individually, yielding 19 individual samples. Feces collected from underneath the roost were pooled separately for each night and then divided into 7 subsamples per night, giving a total of 14 roost samples. DNA was extracted from these fecal samples using a PowerFecal® DNA Isolation Kit (MOBIO) according to the manufacturer's protocol.

After extraction, a portion of the mitochondrial cytochrome oxidase I gene was amplified via polymerase chain reaction (PCR) using LCO1490 and HCO2198 primers (Folmer *et al*., 1994) that yielded a fragment of approximately 650 bp. Each PCR was carried out using illustra PuReTaq Ready-to-go PCR beads (GE Healthcare Life Sciences) with 5 mM forward primer, 5 mM reverse primer, and 1 μL of template DNA in a 25 μL reaction. The thermal cycling conditions of this PCR were as follows: 10 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 48 °C, and 1.5 min at 72 °C with a final extension step at 72 °C for 7 min. All PCRs were visualized on a 1.5% agarose gel.

All PCRs that showed visible bands of the expected size were used as template DNA in a second PCR with the primers ZBJ-ArtF1c and ZBJ-ArtR2c (Zeale *et al*., 2011), using the same PCR profile as above and yielding a product of approximately 157 bp. Each PCR was carried out using 1 μL of product from the first PCR in a 25 μL reaction. All PCRs were again visualized on a 1.5% agarose gel. Samples with positive amplification success were cleaned using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol to excise fragments of approximately 157 bp.

The PCR products were cloned using the TOPO TA Cloning Kit (Life Technologies). Ligation was performed according to the manufacturer's protocol and using 4 μL of PCR product with a 3'-A overhang. Transformations were carried out using half the manufacturer's recommended amount of competent cells and SOC media. Colonies were selected using β-galactosidase blue/white screening and picked colonies were suspended in 20 μL of dH_20. Ligated plasmids were liberated by incubating the suspended cells at 95°C for 10 min. The cell lysate (2 μL) was used directly as template in a 24 μL PCR that contained 4.8 μL 10X PCR Buffer (with 15mM $MgCl_2$; Empirical Bioscience), 1.16 μL 25mM $MgCl_2$, 0.48 μL dNTPs, 1 μL of forward primer, 1 μL of reverse primer (Zeale *et al*., 2011), 2 U *Taq* (Empirical Bioscience), and 1 μL BSA and followed the thermal profile of Zeale *et al*. (2011). PCRs of positive clones were visualized on a 1.5% agarose gel.

Products consisting of only one band at the target size (~157 bp) were cleaned using ExoSAP-IT (Affymetrix) according to the manufacturer's instructions. Appropriately-sized products from PCRs with multiple bands were excised using a QIAquick Gel Extraction Kit (QIAGEN). Cleaned products were sent to the University of Arizona Genetics Core for unidirectional sequencing with either the forward or reverse primer. Following Zeale *et al*. (2011) and Clare *et al*. (2009), 16 clones were sequenced for each sample (accession numbers to be provided with manuscript acceptance).

Analysis of Sequence Data

Sequence data were compiled, aligned, and edited in Sequencher v.5.1 (GeneCodes). Samples that were sequenced with the reverse primer (ZBJ-Art R2c; Zeale *et al*., 2011) were reverse complemented to align with rest of the sequences. Query

sequences were then compared to known reference sequences in the Barcode of Life Data Systems (Ratnasingham and Hebert, 2007). Molecular Operational Taxonomic Units (MOTUs) were determined by creating contigs in Sequencher 5.1 using the criteria of a 98% match and 100 bp overlap. Due to the short length of the isolated fragments (~157 bp), some MOTUs had ≥98.5% matches to multiple species or genera. Thus, we categorized database matches as 1a = true species match (≥99%); 1b = good species match (≥98%); 2 = match to multiple species or genera, only one of which is located in sampling region; 3 = match (≥98%) to multiple species or genera, most conservative taxonomy kept (Krüger *et al*., 2014).

Because prey cannot be directly quantified as the number of individuals of each insect species consumed from our data (Clare, 2014), we defined the occurrence of prey species by its presence in an individual sample. Proportion of the diet was calculated by dividing the number of occurrences of a taxon by the total number of occurrences of all prey items detected in a sample (frequency of occurrence). For each sampling night, combining both individual and roost samples, the Shannon-Weiner Index (H') was calculated following the method of Razgour *et al*. (2011).

Statistical Analyses

To determine if there was a difference in the number of species detected in the feces between the two sampling dates (June 20 and June 27) and between the two sample types (feces collected from individuals and from the roost), data for the number of species were checked for homoscedasticity and normality using Bartlett's and Shapiro-Wilk's tests, respectively. Despite transformations, our data were not normal and had unequal variance; therefore we used non-parametric tests. We used Mann-Whitney tests

to determine whether the number of prey species detected in feces differed by sampling date or by types of fecal samples.

RESULTS

Sequence Processing

Of the 528 clones that were sequenced, 485 (92%) produced readable sequences. Of these readable sequences, 356 (73%) were identified to genus or species yielding 78 MOTUs, 35 (45%) of which were identified to species level and six (8%) to the genus level (Table 1). No sequences yielded non-target taxa (*e.g.*, bat, bacteria, or fungus), likely due to the specificity of the Zeale *et al.* (2011) primers. Apart from one roost sample that did not give any sequences that could be identified to order or family level with confidence, identifiable prey items per sample ranged from 1 to 6 with a mean of 2.41 identified taxa (SD ± 1.34). The average number of prey taxa per sample reported here is similar to that of Alberdi *et al.* (2012) who found an average of 2.75 (SD ± 1.6, range 1-8) taxa per sample.

There was no significant difference in the number of prey items detected in *M. lucifugus* feces between the two sampling dates (U = 104.5, $P = 0.25$). Likewise, there was no significant difference in the number of prey items detected in feces from individually caught *M. lucifugus* versus feces collected from underneath the roost (U = 182.5, $P = 0.064$).

Diet of M. lucifugus

From the 16 samples collected on June 20 (9 individual and 7 roost samples), we identified 18 MOTUs belonging to 3 different orders, 14 of which could be identified to species level. Detectable prey items in the *M. lucifugus* diet on the first sampling date (H' = 4.5) included members of the orders Diptera (40.6%), Ephemeroptera (43.8%), and Lepidoptera (15.6%, Fig. 2). From the 17 samples collected on June 27 (10 individual and 7 roost samples), we identified 27 MOTU's belonging to 5 orders, 24 of which could be identified to species level. On the second sampling date, the diversity of detected prey items (H' = 3.1) was dominated by Lepidoptera (52.4%), Diptera (21.4%), and Ephemeroptera (19%), while Coleoptera and Trichoptera constituted a relatively minor component of the diet (4.8% and 2.4%, respectively; Fig. 2). Overall, Diptera and Lepidoptera were represented by a large number of species (at least 14 and 18 species, respectively). Ephemeroptera had a high frequency of occurrence, but relatively low species diversity (3 confirmed species).

DISCUSSION

Our data demonstrate significant variation in the diet of *M. lucifugus* over a short time interval. In the span of seven days, the diet shifted from mostly Diptera and Ephemeroptera to primarily Lepidoptera. This variation suggests that *M. lucifugus* take advantage of temporal shifts in the local abundance of insects. Lee and McCracken (2005) showed high variation in the diet of the Brazilian free-tailed bat (*Tadarida brasiliensis*) from dusk to dawn hours, showing that other species of bats also take advantage of locally abundant insect emergences.

The decrease in Shannon-Weiner Diversity index from June 20 (H' = 4.5) to June 27 (H' = 3.1) suggests that the diet of *M. lucifugus* was more functionally diverse and even on the first sampling date. Although a greater number of species were detected in the diet of *M. lucifugus* on June 27, the higher Shannon-Weiner diversity index on June 20 is likely due to the evenness of species detected in the diet on that sampling date. This change in diversity also shows that *M. lucifugus* individuals exhibit high levels of dietary flexibility. Overall, our results show that pooling data from different sampling dates, even when they are separated by a short time period, can obscure changes in the diversity of the diet of *M. lucifugus*.

Our study identified the same five orders of insects that Clare *et al*. (2011, 2014) found most frequently in the diet of *M. lucifugus*. We also found patterns of abundance similar to those of Clare *et al.* (2011), whose dietary analysis of *M. lucifugus* showed that the proportion of Diptera decreased throughout the maternity season while the proportion of Lepidoptera increased over the same time interval. Like Clare *et al.* (2011), but unlike Clare *et al.* (2014), we found high proportions of Ephemeroptera in the bats' diet during the mid-maternity season (defined by Clare *et al*. (2011) as June 16-July 18). Because the sampling sites used by Clare *et al*. (2011) in southwestern Ontario were less than 400 km from our site, we expected to see similar results. Clare *et al*. (2014), however, sampled from *M. lucifugus* across Canada in locations varying widely in ecosystem, temperature and precipitation; differences with our study may thus reflect differences in the available insect fauna. However, while Clare *et al.* (2011, 2014) sampled approximately every 7 days, those samples were pooled for each part of the maternity season (early, mid, and

late), which may have obscured more fine-scaled variation in the diet of *M. lucifugus* over the short timespans explored here.

Although only 3 MOTUs of Ephemeroptera were identified in the diet of *M. lucifugus*, this order made up over a third of the diet on June 20, which corresponded with the peak of the *Hexagenia* emergence (Corkum *et al.,* 2006). *Hexagenia limbata* was the most frequently occurring prey species on June 20 at levels that exceeded the single-date occurrence of any other prey item in our study. Although no *H. limbata* were detected in the diet of *M. lucifugus* on June 27 (possibly due to the end of the emergence of this insect species; Corkum *et al.,* 2006), these data suggest that while *H. limbata* are emerging, they are a commonly consumed prey item. Another mayfly, *Stenonema femoratum,* was one of the most frequently consumed species on both dates. Ephemeroptera have transient emergences en masse, which appear to result in an abundant, but brief, food source for *M. lucifugus*.

Caveats

It is important to note that this type of molecular diet analysis through cloning is only semi-quantifiable in that we cannot determine the precise number of any given prey item consumed. We identified similar numbers of prey taxa at the species or genus level in bat feces (35 taxa) as did Zeale *et al.* (2011; 37 taxa) and Alberdi *et al.* (2012; 34 taxa), both of whom used similar techniques to determine the diets of temperate insectivorous bats. Although some authors (Deagle *et al*., 2005; Zeale *et al*., 2011) have shown that the use of cloning yields proportions that roughly correspond with dietary proportions through feeding trials, this may vary by predator and prey taxa. Results of this type of molecular analysis may also be influenced by differential survival of hard parts of prey

items (Rabinowitz and Tuttle, 1982) or prey DNA (Deagle and Tollit, 2007) or by competition during PCR due to the choice of primers, particularly the use of the LCO1490 and HCO2198 primers to amplify degraded DNA (Folmer *et al*., 1994).

In addition, not all insect orders are equally represented within the BOLD database. For example, lepidopterans tend to be highly represented; this overrepresentation may result in higher levels of observed lepidopteran species diversity in bats' diets. We were able to obtain higher resolution of lepidopterans than any other order, which may be a result of this increased representation. Biases in barcoding libraries may inhibit our ability to identify all prey taxa in the diet of *M. lucifugus* and increase the chance of type II error. Though sequences were called using BOLD, we also compared each sequence using the blast function in GenBank. Generally, GenBank yielded similar results to BOLD, but had higher sequence matches to a few dipteran and ephemeropteran species. This difference may have resulted in slightly different proportions of each insect order consumed.

Because we subsampled the feces taken from each individual bat, it is possible that not all prey items were detected. Furthermore, our analyses were limited by the number of clones sequenced per sample. Although the number of prey species we detected corresponded with other studies that analyzed bat diets using cloning (Zeale *et al.,* 2011; Alberdi *et al.,* 2012), studies using next generation sequencing (NGS) techniques have been able to identify approximately twice as many prey taxa (Bohmann *et al.,* 2011; Razgour *et al.,* 2011; Clare *et al.,* 2014; Krüger *et al.,* 2014). Thus, NGS appears to be better at capturing dietary α-diversity.

Myotis lucifugus *as a predator of pest insects*

We identified several notable constituents within the diet of *M. lucifugus.* Among these insects are two species of mosquito (*Aedes vexans* and *Ochlerotatus canadensis*; Diptera) that have been shown to be carriers of West Nile Virus (CDC, 2012), a zoonotic disease that has caused almost 2,000 human fatalities since 1999 (CDC, 2015). These mosquitos are also potential vectors of Eastern Equine Encephalitis virus (Vaidyanathan *et al*., 1997) and dog heartworm (*Dirofilaria immitis*; Ledesma and Harrington, 2011). Additionally, several species of agricultural and forestry pest insects were identified in our analyses as prey items of *M. lucifugus*. *Chrysoteuchia topiarias* (cranberry girdler; Lepidoptera: Crambidae) has been shown to feed on and cause significant damage to cranberries, Douglas fir, and turfgrass (Kamm *et al*., 1990). *Choristoneura rosaceana* (oblique banded leaf roller; Lepidoptera: Tortricidae) feeds on many fruiting plants, and causes significant economic losses to apple orchards (Ahmad *et al*., 2002). The removal of natural predators through the use of pesticides and the evolution of insecticide resistance in some strains of *C. rosaceana* has caused large outbreaks of this formerly minor pest, particularly in Michigan (Ahmad *et al*., 2002). *Choristoneura fumiferana* (spruce budworm; Lepidoptera: Tortricidae) is a softwood defoliator and has contributed to the loss of millions of hectares of North American spruce-fir forests, on which the paper industry depends (Miller and Rusnock, 1993). Similarly, *Phyllobius oblongus* (European snout beetle; Coleoptera: Curculionidae), an invasive weevil in the Great Lakes region, feeds on the roots and leaves of many hardwood species including, but not limited to, maple, birch, and many fruit trees, berries and shrubs (Pinski *et al.*, 2005). Clare *et al*. (2011) also found *P. oblongus* in the diet of *M. lucifugus* in southern Ontario.

Our results provide further evidence that bats such as *M. lucifugus* provide valuable ecosystem services. By consuming pests and thus reducing and delaying the application of pesticides, insectivorous bats have been estimated to provide services valued at $22.9 billion/year to agriculture in the United States alone (Boyles *et al*., 2011). However, this estimate does not include the value that bats have within forest ecosystems, and thus does not incorporate the impact they may have on reducing populations of forestry pest insects such as *C. fumiferana*. Bats have been shown to play a significant role in reducing herbivory and/or limiting arthropods in agricultural (Maine and Boyles, 2015), agroforest (Williams-Guillén *et al*., 2008), and tropical forest (Kalka *et al*., 2008) ecosystems. Because insectivorous bats exhibit top-down control of agricultural pest insects and arthropod vectors of human disease, these arthropods may experience a release from predation if WNS-affected bat species continue to experience population declines.

Impacts of Aquatic Prey on Bats

Although bats have significant top-down impacts on ecosystems, insect populations may have bottom-up effects on their predators as well. A large proportion of the diet of *M. lucifugus* consists of aquatic insects from the orders Diptera and Ephemeroptera. Insects in these orders are often used as environmental indicators because they can take up, tolerate, and transfer trace metals (Hare, 1992) and other environmental contaminants, such as polychlorinated biphenyls (PCBs; Walters *et al*., 2008), from the water to their terrestrial predators. Contaminants such as DDT have been shown to cause mortality in bats (Clark, 2001), although the source of this contaminant was unclear. Both Kannan *et al*. (2010) and Secord *et al*. (2015) found high

concentrations of several contaminants of emerging concern (CECs) in the tissues of dead or moribund bats affected by WNS. After Park and Cristinacce (2006) found that sewage treatment plants were significant foraging sites for bats, Park *et al*. (2009) showed that insects at these sites also had high concentrations of CECs, thus demonstrating possible trophic transfer of these environmental toxins.

Compared to other mammals of the same size, bats live approximately three times longer (Austad and Fischer, 1991) and have increased metabolic rates and high rates of food intake (Clark and Shore, 2001), likely as a result of the high energetic demands of flight. Increased longevity, metabolism, and food consumption may increase the susceptibility of bats to toxins in the environment. Furthermore, lactating females consume more prey due to the high energetic demands of milk production (Anthony and Kunz, 1977; Kurta *et al*., 1989). Because parturition of *M. lucifugus* is synchronous with emergences of aquatic Diptera and Ephemeroptera, the summer months may expose these bats and their developing offspring to additional health risks. Future work should examine the impacts of trophic transfer of aquatic toxins to bat populations.

Conclusions

Many bats in North America are facing anthropogenic threats such as habitat loss and fragmentation, and increased mortality due to wind turbines (Arnett and Baerwald, 2013). In addition, the fungal disease WNS has caused up to 90% mortality in several species of hibernating bats in eastern North America (Turner *et al*., 2011; Coleman and Reichard, 2014; Langwig *et al*., 2015). This study shows that the loss of *M. lucifugus* may have locally specific impacts on insect communities. Many agricultural and forestry

pests may be released from predation, which is likely to exacerbate the economic impact of these insects. Thus, it is crucial that we manage *M. lucifugus* both in areas affected by WNS and those that have not yet been impacted.

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LITERATURE CITED

AHMAD, M., R. M. HOLLINGWORTH, and J. C. WISE. 2002. Broad-spectrum insecticide resistance in obliquebanded leafroller *Choristoneura rosaceana* (Lepidoptera: Tortricidae) from Michigan. Pest Management Science, 58: 834–838.

ALBERDI A., I. GARIN, O. AIZPURUA, and J. AIHARTZA. 2012. The foraging ecology of the mountain long-eared bat *Plecotus macrobullaris* revealed with DNA mini-barcodes. PLoS ONE, 7: e35692.

ANTHONY, E. L. P., and T. H. KUNZ. 1977. Feeding strategies of the little brown bat, *Myotis lucifugus*, in southern New Hampshire. Ecology, 58: 775–786.

ANTHONY, E. L. P., M. H. STACK, and T. H. KUNZ. 1981. Night roosting and the nocturnal time budget of the little brown bat, *Myotis lucifugus*: Effects of reproductive status, prey density, and environmental conditions. Oecologia, 51: 151-156.

ARNETT, E. B. and E. F BAERWALD. 2013. Impacts of wind energy development on bats: implications for conservation. Pp. 435-456, *in* Bat evolution, ecology, and conservation (R. A. ADAMS and S. C. PEDERSEN, eds.). Springer, New York, xvi + 547 pp.

AUSTAD, S. N. and K. E. FISCHER. 1991. Mammalian aging, metabolism, and ecology: Evidence from the bats and marsupials. Journal of Gerontology: Biological Sciences, 46: B47–53.

BELWOOD, J. and M. FENTON. 1976. Variation in the diet of *Myotis lucifugus* (Chiroptera: Vespertilionidae). Canadian Journal of Zoology, 54: 1674–1678.

BELWOOD, J. and G. K. MORRIS. 1987. Bat predation and its influence on calling behavior

in neotropical katydids. Science, 238: 64-67.

BOHMANN, K., A. MONADJEM, C. L. NOER, M. RASMUSSEN, M. R. K. ZEALE, E. CLARE, G. JONES, E. WILLERSLEV, and M. T. P. GILBERT. 2011. Molecular diet analysis of two African free-tailed bats (Molossidae) using high throughput sequencing. PLoS ONE, 6: e21441.

BOYLES, J. G., P. M. CRYAN, G. F. MCCRACKEN, and T. H. KUNZ. 2011. Economic importance of bats in agriculture. Science, 332: 41–42.

BUCHLER, E. R. 1976. Prey selection by *Myotis lucifugus* (Chiroptera: Vespertilionidae). The American Naturalist, 110: 619–628.

CDC. 2012. Mosquito species in which West Nile Virus has been detected, United States, 1999-2012. Available from http://www.cdc.gov/westnile/resources/pdfs/mosquitospecies1999-2012.pdf. Downloaded on 1 April 2016.

CDC. 2015. West Nile Virus disease cases and deaths reported to CDC by year and clinical presentation, 1999-2014. Available from http://www.cdc.gov/westnile/resources/pdfs/data/1-wnv-disease-cases-by-year_1999-2014_06042015.pdf. Downloaded on 1 April 2016.

CLARE, E. L. 2014. Molecular detection of trophic interactions: emerging trends, distinct advantages, significant considerations and conservation applications. Evolutionary Applications, 7: 1144-1157.

CLARE, E. L., B. R. BARBER, B. W. SWEENEY, P. D. N. HEBERT, and M. B. FENTON. 2011. Eating local: Influences of habitat on the diet of little brown bats (*Myotis lucifugus*).

Molecular Ecology, 20: 1772–1780.

CLARE, E. L., E. E. FRASER, H. E. BRAID, M. B. FENTON, and P. D. N. HEBERT. 2009. Species on the menu of a generalist predator, the eastern red bat (*Lasiurus borealis*): Using a molecular approach to detect arthropod prey. Molecular Ecology, 18: 2532–2542.

CLARE, E. L., W. O. C. SYMONDSON, H. BRODERS, F. FABIANEK, E. E. FRASER, A. MACKENZIE, A. BOUGHEN, R. HAMILTON, C. R. WILLIS, F. MARTINEZ-NUÑEZ, A. K. MENZIES, K. J. O. NORQUAY, M. BRIGHAM, J. POISSANT, J. RINTOUL, R. M. R. BARCLAY, and J. P. REIMER. 2014. The diet of *Myotis lucifugus* across Canada: Assessing foraging quality and diet variability. Molecular Ecology, 23: 3618–3632.

CLARK, D. R. 2001. DDT and the decline of free-tailed bats (*Tadarida brasiliensis*) at Carlsbad Cavern, New Mexico. Archives of Environmental Contamination and Toxicology, 40: 537–543.

CLARK, D.R., and R. F. SHORE. 2001. Chiroptera. Pp. 159–214 , *in* Ecotoxicology of wild mammals (R. F. SHORE and B. A. RATTNER, eds). Wiley, London, 752 pp.

CLOSS, G. P., S. R. BALCOMBE, and M. J. SHIRLEY. 1999. Generalist predators, interaction strength and food-web stability. Pp. 93–126 in Advances in ecological research, vol. 28. Academic Press, London, viii + 224.

COLEMAN, J. T. H., and J. D. REICHARD. 2014. Bat white-nose syndrome in 2014: A brief assessment seven years after discovery of a virulent fungal pathogen in North America. Outlooks on Pest Management, 25: 374–377.

CORKUM, L., J. J. H. CIBOROWSKI, and D. M. DOLAN. 2006. Timing of *Hexagenia*

(Ephemeridae: Ephemeroptera) mayfly swarms. Canadian Journal of Zoology, 84: 1616-1622.

DEAGLE, B. E., and D. J. TOLLIT. 2007. Quantitative analysis of prey DNA in pinniped faeces: Potential to estimate diet composition?. Conservation Genetics, 8: 742-747.

DEAGLE, B. E., D. J. TOLLIT, S. N. JARMAN, M. A. HINDELL, A. W. TRITES, and N. J. GALES. 2005. Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. Molecular Ecology, 14: 1831–1842.

FENTON, M. B., and G. K. MORRIS. 1976. Opportunistic feeding by desert bats (*Myotis* spp.). Canadian Journal of Zoology, 54: 526–530.

FOLMER, O., M. BLACK, W. HOEH, R. LUTZ, and R. VRIJENHOEK. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3: 294–299.

FRICK, W. F., J. F. POLLOCK, A. C. HICKS, K. E. LANGWIG, D. S. REYNOLDS, G. G. TURNER, C. M. BUTCHKOSKI, and T. H. KUNZ. 2010. An emerging disease causes regional population collapse of a common North American bat species. Science, 329: 679–682.

HARE, L. 1992. Aquatic insects and trace metals: Bioavailability, bioaccumulation, and toxicity. Critical Reviews in Toxicology, 22: 327-369.

JONES, J. K., P. SWANEPOEL, and D. C. CARTER. 1977. Annotated checklist of the bats of Mexico and Central America. Occasional Papers, Museum, Texas Tech University, 47: 1-35.

KALKA, M. B., A. R. SMITH, and E. K. V. KALKO. 2008. Bats limit arthropods and

herbivory in a tropical forest. Science, 320: 71.

KAMM, J. A., G. C. FISHERT, L. M. MCDONOUGH, and C. H. SHANKS. 1990. Cranberry girdler as a pest of grass seed, cranberry and nursery tree production in the Pacific northwest. Pp. 141–150, *in* Monitoring and integrated management of arthropod pests of small fruit crops. Intercept Ltd., Andover, xvii + 301 pp.

KANNAN, K., S. H. YUN, R. J. RUDD, and M. BEHR. 2010. High concentrations of persistent organic pollutants including PCBs, DDT, PBDEs and PFOS in little brown bats with white-nose syndrome in New York, USA. Chemosphere, 80: 613–618.

KRÜGER, F., E. L. CLARE, S. GREIF, B. M. SIEMERS, W. O. C. SYMONDSON, and R. S. SOMMER. 2014. An integrative approach to detect subtle niche differentiation in the sympatric trawling bat species *Myotis dasycneme* and *Myotis daubentonii*. Molecular Ecology, 23: 3657-3671.

KURTA, A., G. P. BELL, K. A. NAGY, and T. H. KUNZ. 1989. Energetics of pregnancy and lactation in free-ranging little brown bats (*Myotis lucifugus*). Physiological Zoology, 62: 804–818.

LANG, A. B., E. K. V. KALKO, H. RÖMER, C. BOCKHOLDT, and D. K. N. DECHMANN. 2006. Activity levels of bats and katydids in relation to the lunar cycle. Oecologia, 146: 659-666.

LANGWIG, K. E., J. R. HOYT, K. L. PARISE, J. KATH, D. KIRK, W. F. FRICK, J. T. FOSTER, and A. M. KILPATRICK. 2015. Invasion dynamics of white-nose syndrome fungus, Midwestern United States, 2012–2014. Emerging Infectious Diseases, 21: 1023–

1026.

LEDESMA, N., and L. HARRINGTON. 2011. Mosquito vectors of dog heartworm in the United States: Vector status and factors influencing transmission efficiency. Tropical Review, 26: 178–185.

LEE, Y-F., and G. F. MCCRACKEN. 2002. Foraging activity and food resource use of Brazilian free-tailed bats, *Tadarida brasiliensis* (Molossidae). Ecoscience 9: 306-313.

LEE Y-F., and G. F. MCCRACKEN. 2005. Dietary variation of Brazilian free-tailed bats links to migratory populations of pest insects. Journal of Mammalogy, 86: 67–76.

LORCH, J. M., C. METEYER, M. J. BEHR, J. G. BOYLES, P. M. CRYAN, A. C. HICKS, A. E. BALLMANN, J. T. H. COLEMAN, D. N. REDELL, D. M. REEDER, and D. S. BLEHERT. 2011. Experimental infection of bats with *Geomyces destructans* causes white-nose syndrome. Nature, 480: 376-379.

MAINE, J. J., and J. G. BOYLES. 2015. Bats initiate vital agroecological interactions in corn. Proceedings of the National Academy of Sciences of the USA, 112: 12438–12443.

MCCRACKEN, G. F., J. K. WESTBROOK, V. A. BROWN, M. ELDRIDGE, P. FEDERICO, and T. H. KUNZ. 2012. Bats track and exploit changes in insect pest popluations. PLoS ONE, 7: e43839.

MILLER, A., and P. RUSNOCK. 1993. The rise and fall of the silvicultural hypothesis in spruce budworm (*Choristoneura fumiferana*) management in eastern Canada. Forest Ecology and Management, 61:171–189.

PARK, K.J., and A. CRISTINACCE. 2006. Use of sewage treatment works as foraging sites by insectivorous bats. Animal Conservation, 9: 259–268.

PARK, K. J., C. Y. MÜLLER, S. MARKMAN, O. SWINSCOW-HALL, D. PASCOE, and K L. BUCHANAN. 2009. Detection of endocrine disrupting chemicals in aerial invertebrates at sewage treatment works. Chemosphere, 77: 1459–1464.

PINSKI, R. A., W. J. MATTSON, and K. F. RAFFA. 2005. Composition and seasonal phenology of a nonindigenous root-feeding weevil (Coleoptera: Curculionidae) complex in northern hardwood forests in the Great Lakes region. Environmental Entomology, 34: 298–307.

PYKE, G. H., H. R. PULLIAM, and E. L. CHARNOV. 1977. Optimal foraging: A selective review of theory and tests. The Quarterly Review of Biology, 52: 137-154.

RABINOWITZ, A. R., and M. D. TUTTLE. 1982. A test of the validity of two currently used methods of determining bat prey preferences. Acta Theriologica, 27: 283–293.

RATNASINGHAM, S., and P. D. N. HEBERT. 2007. BOLD: the barcode of life data system (http://www.barcodinglife.org). Molecular Ecology Notes, 7: 355–364.

RAZGOUR, O., E. L. CLARE, M. R. K. ZEALE, J. HANMER, I. B. SCHNELL, M. RASMUSSEN, T. P. GILBERT, and G. JONES. 2011. High-throughput sequencing offers insight into mechanisms of resource partitioning in cryptic bat species. Ecology and Evolution, 1: 556–570.

RYDELL, J. 1992. Exploitation of insects around streetlamps by bats in Sweden. Functional Ecology, 6: 744-750.

SCHOENER, T. W. 1971. Theory of feeding strategies. Annual Review of Ecology and

Systematics, 2: 369-404.

SECORD, A. L., K. A. PATNODE, C. CARTER, E. REDMAN, D. J. GEFELL, A. R. MAJOR, and D. W. SPARKS. 2015. Contaminants of emerging concern in bats from the northeastern United States. Archives of Environmental Contamination and Toxicology, 69: 411-421.

TURNER, G. G., D. M. REEDER, and J. T. H. COLEMAN. 2011. A five-year assessment of mortality and geographic spread of white-nose syndrome in North American bats and a look to the future. Bat Research News, 52: 13-27.

TUTTLE, M. D., L. K. TAFT, and M. J. RYAN. 1982. Evasive behavior of a frog in response to bat predation. Animal Behavior, 30: 393-397.

VAIDYANATHAN, R., J. D. EDMAN, L. A. COOPER, and T. W. SCOTT. 1997. Vector competence of mosquitoes (Diptera: Culicidae) from Massachusetts for a sympatric isolate of Eastern Equine Encephalomyelitis Virus. Journal of Medical Entomology, 34: 346-352.

WALTERS, D. M., K. M. FRITZ, and R. R. OTTER. 2008. The dark side of subsidies: Adult stream insects export organic contaminants to riparian predators. Ecological Applications, 18: 1835–1841.

WHITAKER, J. O. 1972. Food habits of bats from Indiana. Canadian Journal of Zoology, 50: 877–883.

WILLIAMS-GUILLÉN, K., I. PERFECTO, and J. VANDERMEER. 2008. Bats limit insects in a neotropical agroforestry system. Science, 320: 70.

ZEALE, M. R. K., R. K. BUTLIN, G. L. A. BARKER, D. C. LEES, and G. JONES. 2011. Taxon-

specific PCR for DNA barcoding arthropod prey in bat faeces. Molecular Ecology Resources, 11: 236–244.

TABLE 1. Prey items detected in the diet of *M. lucifugus*. Occurrence refers to the presence or absence of a species in a sample. The confidence levels from Krüger *et al.* (2014) indicate 1a = true species match (≥99%), 1b = good species match (≥98%), 2 = species match to multiple species or genera, but only one is located in the sampling region, 3 = match (≥98%) to multiple species or genera, most conservative taxonomy kept and considered provisional.

				Frequency of Occurrence		
Order	**Family**	**Species**	**Conf**	**20-Jun**	**27-Jun**	**Total**
Coleoptera	Ptinidae	*Eucrada humeralis*	1b		1	1
	Curculionidae	*Phyllobius oblongus*	1a		1	1
Diptera	Chaoboridae	*Chaoborus punctipennis*	1a	3		3
	Chironomidae	*Paracladopelma winnelli*	1a	1	2	3
	Chironomidae	*Pseudochironomus sp.*	1a	3		3
	Culicidae	*Aedes vexans*	1a	1		1
	Culicidae	*Ochlerotatus canadensis*	1a, 1b		2	2
	Hybotidae	*Platypalpus major*	1a	1		1
	Muscidae	*Helina depuncta*	2		1	1
	Psychodidae	*Psychoda cinerea*	1b		1	1
	Sciaridae	*Scatopsciara atomaria*	1b	1		1
	Sphaeroceridae	*Leptocera erythrocera*	1a		1	1
	Tachinidae	*Cryptomeigenia sp.*	1a		1	1
	Tipulidae	*Tipula entomophthorae*	1a		1	1
	Tipulidae	*Tipula hermannia*	1a	1		1
	Tipulidae	*Tipula sp.*	1a	1		1
	Tipulidae	*Tipula monticola*	1a	1		1
Ephemeroptera	Caenidae	*Caenis sp.*	1a		2	2
	Caenidae	*Caenis youngi*	1a, 1b	1	2	3
	Ephemeridae	*Hexagenia limbata*	1a	6		6
	Heptageniidae	*Stenonema femoratum*	1a, 1b	4	4	8
	Heptageniidae	*Stenonema sp.*	1a	2		1
	Heptageniidae	*Unknown sp.*	3	1		1
Lepidoptera	Blastobasidae	*Holcocera chalcofrontella*	1a, 2		2	2
	Crambidae	*Chrysoteuchia topiarius*	1a		1	1
	Depressariidae	*Agonopterix pulvipennella*	1a	1		1
	Depressariidae	*Psilocorsis quercicella*	1a		1	1
	Gelechiidae	*Chionodes mediofuscella*	1a		2	2
	Gelechiidae	*Chionodes praeclarella*	1a	2		2
	Gelechiidae	*Glauce pectenalaeella*	1a		3	3

	Gelechiidae	*Pseudotelphusa quercinigracella*	1a		1	1
	Gelechiidae	*Xenolechia ontariensis*	1a		1	1
	Gracillariidae	*Caloptilia bimaculatella*	1a		1	1
	Gracillariidae	*Caloptilia packardella*	1a		1	1
	Tortricidae	*Choristoneura conflictana*	1a	1		1
	Tortricidae	*Choristoneura fractivittana*	1a		1	1
	Tortricidae	*Choristoneura fumiferana*	1a		4	4
	Tortricidae	*Choristoneura parallela*	1a		1	1
	Tortricidae	*Choristoneura rosaceana*	1a		1	1
	Tortricidae	*Clepsis virescana*	1a	1		1
	Tortricidae	*Eucosma sp.*	3		1	1
Trichoptera	Leptoceridae	*Triaenodes injustus*	1a		1	1

FIGURE CAPTIONS

FIG 1. Map of Little Traverse Lake in Leelanau County, Michigan. The sampled *Myotis lucifugus* roost is indicated with a star next to Little Traverse Lake (44.923580, -85.821265).

FIG 2. Frequency of occurrence of each order found in the diet of *M. lucifugus* on each sampling day.

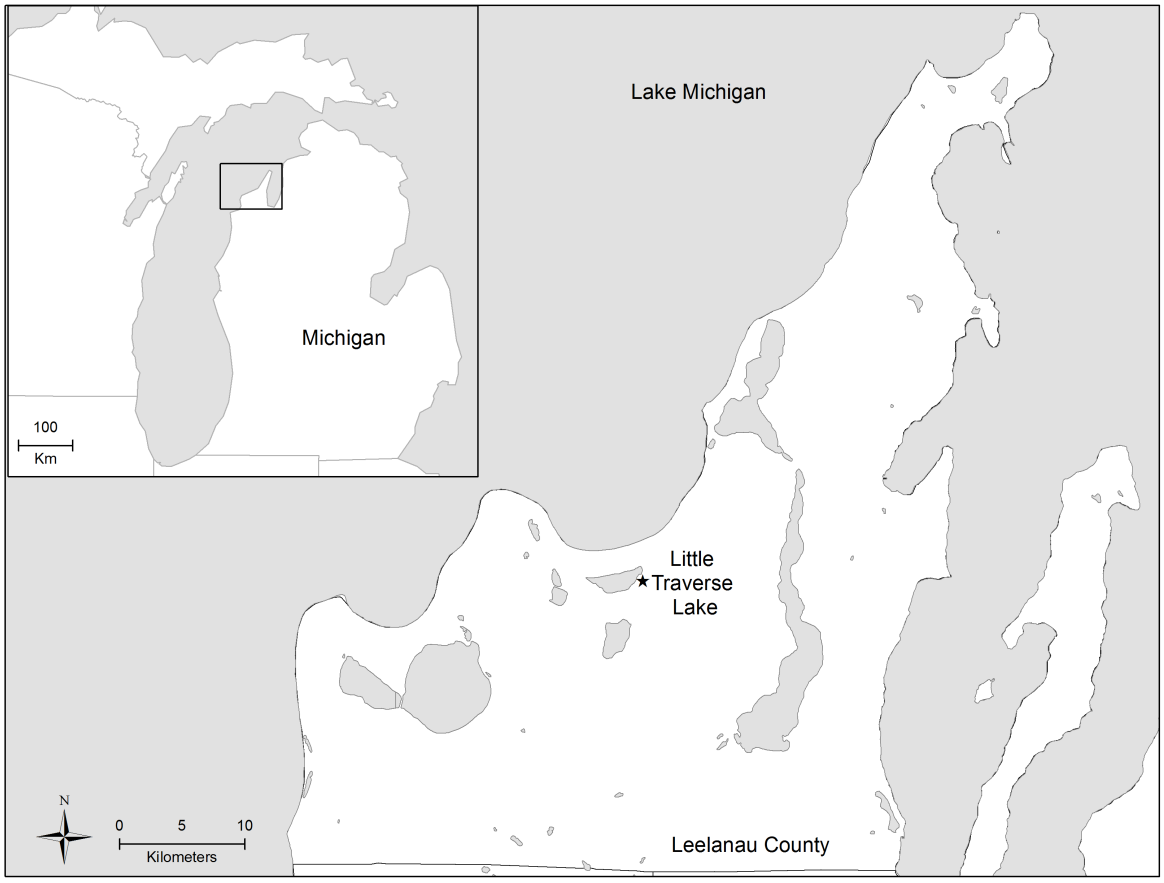
Lake Michigan
Michigan
100
Km
Little
Traverse
Lake
N
0
5
10
Kilometers
Leelanau County

FIG. 1.

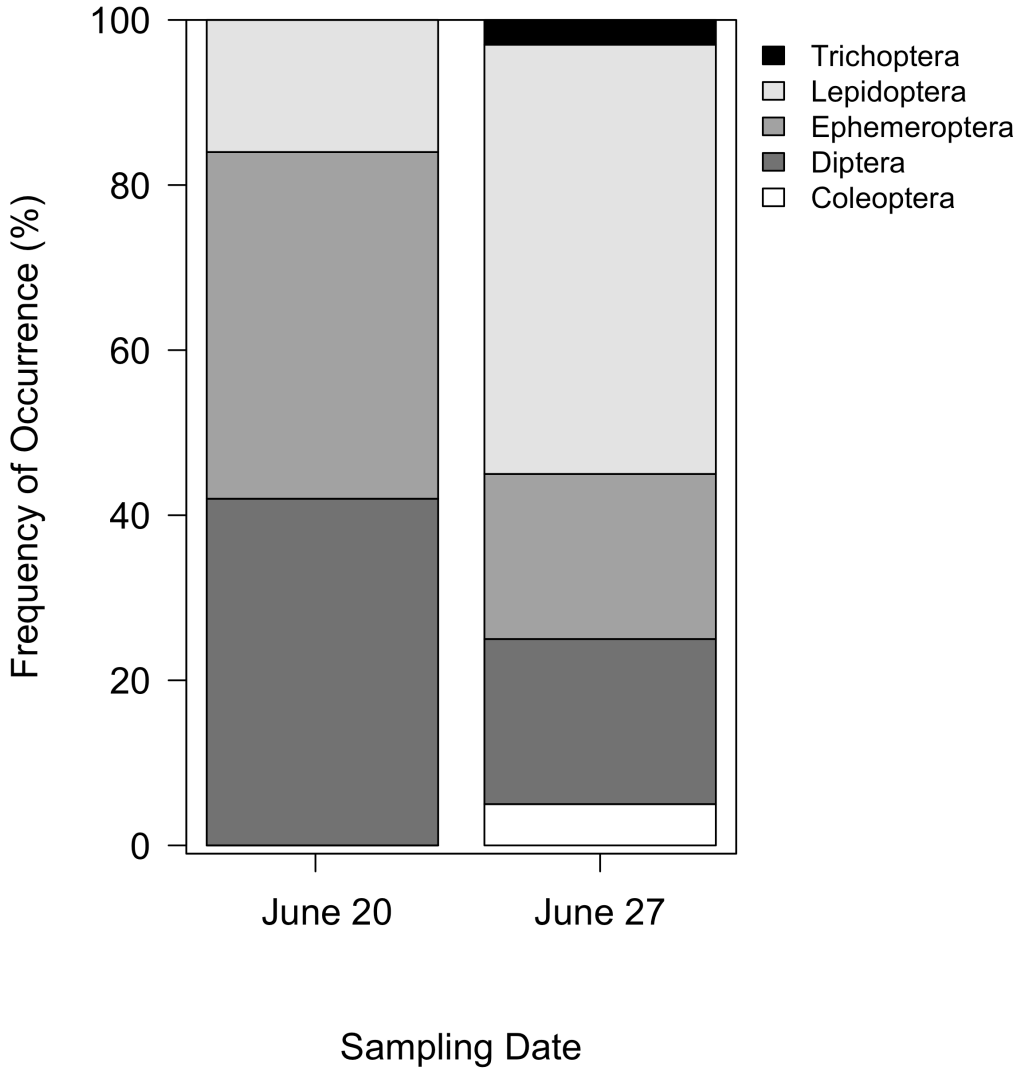
100
80
60
40
20
0
Frequency of Occurrence (%)
June 20
June 27
Sampling Date
Trichoptera
Lepidoptera
Ephemeroptera
Diptera
Coleoptera

FIG. 2

Chapter 3

Trophic Transfer of Microcystin from a Freshwater Lake to Little Brown Bats (*Myotis lucifugus*)

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Running Title: Trophic transfer of microcystin to *Myotis lucifugus*

Abstract

Microcystis aeruginosa is a species of cyanobacteria that is capable of producing a hepatotoxin called microcystin. When toxic *M. aeruginosa* overwinters in the sediments of lakes, it may be ingested by some aquatic insects, such as the nymphs of *Hexagenia* spp. mayflies, and thus bioaccumulate in these insects. When *Hexagenia* subimagos and imagos emerge from lakes to reproduce, they provide an abundant, albeit temporary, food source for many terrestrial organisms including bats. Little brown bats, *Myotis lucifugus*, have been shown to feed opportunistically on aquatic insects including *Hexagenia*. To test whether microcystin moves from aquatic to terrestrial ecosystems via trophic transfer, we tested bat livers and feces for microcystin. In June 2014, coincident with the local *Hexagenia* emergence, bat feces were collected from underneath a maternity roost near Little Traverse Lake (Leelanau County, Michigan, USA). On 20 and 27 June we caught 19 female *M. lucifugus*, and collected their livers and feces. We measured concentrations of microcystin in the liver tissue and feces using an enzyme-linked immunosorbent assay (ELISA) and liquid chromatography with tandem mass spectrometry (LC-MS/MS). Our analyses reveal that microcystin was present at high concentrations in the bat feces, but not the livers. Additionally, histopathological examination of three bat livers with the highest concentrations of microcystin show little to no cytological damage from the toxin. From these data, it appears that *M. lucifugus* may not be immediately affected by the ingestion of microcystin. Future work should examine whether bats suffer delayed physiological effects from ingestion of microcystin, either by ingesting contaminated insects or by drinking microcystin-tainted water during or after late summer blooms of *M. aeruginosa*.

Keywords: algal toxin, *Hexagenia*, microcystin, *Microcystis aeruginosa*, *Myotis lucifugus*, trophic interactions

Introduction

As blooms of cyanobacteria become more common in freshwater ecosystems due to global climate change [1], eutrophication [2,3] and invasive species (*e.g.,* zebra mussels [4]), these harmful algal blooms pose a threat to both aquatic and terrestrial ecosystems. Several genera of cyanobacteria produce neurotoxins and hepatotoxins that have resulted in poisonings of both humans and animals [5]. *Microcystis aeruginosa* is a species of cyanobacteria that is typically found in phosphorus-rich (eutrophic) fresh water [6]. However, research conducted in Michigan lakes has shown that invasive zebra mussels can promote the growth of *M. aeruginosa* [4], particularly in nutrient-poor (oligotrophic) basins [7]. Filtering by zebra mussels reduces chlorophyll and total phosphorus [8], consequently limiting nutrients in the water. Zebra mussels consume algal species and reduce their population sizes, giving *Microcystis* spp. a competitive advantage because the zebra mussels selectively reject these toxic species [4]. As a result of this advantage, *M. aeruginosa* can form unsightly blooms that reflect rapid increases in their population density, and result in decreases in water transparency and increases in foam production [6].

In addition to forming blooms, *M. aeruginosa* is capable of producing a hepatotoxin called microcystin. This algal toxin has been shown to cause vomiting, skin irritation, liver cancer, and even death in humans, pets, livestock, and various aquatic organisms [5,9]. Microcystin has been found to bioaccumulate in many aquatic organisms including zooplankton, crustaceans, mussels, and fish [10–13]. Of the few species of aquatic insects that have been tested for microcystin, Woller-Skar [6] found the

highest concentrations of microcystin in species of *Hexagenia,* which are burrowing mayflies.

After hatching, *Hexagenia* nymphs burrow in the sediments of freshwater sources [14]. While the nymphs overwinter in the lake sediments, they consume detritus and algae [14]. There, the nymphs likely ingest toxic *M. aeruginosa*, which also overwinters in lake sediments [15]. *Hexagenia* spend 1-2 years as nymphs, then move to the surface of the water and within a few days, undergo their first molt to become subimagos (subadults [16]). Shortly thereafter, the subimagos undergo a second molt to become imagos (adults [14]). The imagos mate during flight, and the females lay their eggs in the water to produce a new generation (cohort) and then die [16].

Throughout their lives, these mayflies are a part of the aquatic food web by both consuming and being consumed by aquatic organisms. However, the subimagos and imagos are also part of the terrestrial food web as potential prey for organisms such as birds and bats. Although this terrestrial existence is brief, lasting only a few weeks, it provides copious amounts of food for insectivorous animals. Thus, *Hexagenia* serves as a link between aquatic and terrestrial food webs and therefore has the potential to transfer aquatic toxins such as microcystin to terrestrial insectivores including bats.

One of the most common bats in Michigan is the little brown bat, *Myotis lucifugus*. Little brown bats feed opportunistically on aquatic insects such as mayflies and chironomid flies [17–20]. Although *M. lucifugus* do not feed exclusively on aquatic insects, a dietary analysis in southwestern Ontario found that approximately 66% of the diet of little brown bats consisted of mayflies (Ephemeroptera) during the summer maternity season [19]. Similarly, Jones et al. [20] found that little brown bats in Michigan

had diets consisting of over 50% aquatic insects, including *Hexagenia*. Due to their reliance on aquatic insects, little brown bats may be at particular risk of exposure to aquatic ecosystem toxins. Furthermore, the timing of parturition in *M. lucifugus* corresponds to the mass emergences of many aquatic insects. Lactating females consume more insects than non-reproductive females or males [18], further increasing the likelihood of toxin ingestion.

Here, we use chemical and histopathological analyses of *M. lucifugus* livers and feces to determine whether the bats are ingesting microcystin by consuming contaminated insects. We hypothesize that 1) microcystin is present in both bat livers and feces, and 2) high levels of microcystin are associated with liver damage in wild bat populations. From these results, we can more fully understand the health risks facing little brown bats, and determine whether new management strategies are required to address these risks.

Methods

Study site and sample collection

All samples were collected near Little Traverse Lake (Leelanau County, Michigan, USA, Fig. 1). Zebra mussels were introduced into this lake during the mid-1990's and have been established since the early 2000's [6]. Since the establishment of zebra mussels, *M. aeruginosa* has become the dominant algal species with blooms each year in August-September [6]. During and after bloom events, this lake has been shown to have concentrations of total microcystin (MC-eq) exceeding the World Health

Organization's guidelines for safe drinking water (1 μg/L or 1 ppb; [6, 21]). Furthermore, *Hexagenia* emerging from this lake have been shown to have high concentrations of microcystin at the nymph stage [6] and volant stages (subadult and adult, [22]. Because *M. lucifugus* have been shown to reduce their home-range during summer due to reproduction [23], it is likely that bats from a maternity colony peripheral to Little Traverse Lake will feed on the aquatic insects emerging from this body of water.

A total of 19 *M. lucifugus* were collected on June 20 ($N = 9$) and 27 ($N = 10$) of 2014. After adult bats had left the roost, all exits to the barn were blocked off and a harp trap was placed in the open doorway to catch bats returning from foraging. Captured *M. lucifugus* were identified and placed individually in cotton bags. After being held overnight, individuals were humanely euthanized with isoflurane followed by cervical dislocation (IACUC Approval #14-08-A, Michigan Scientific Collector Permit #SC1498). Approximately half of the liver tissue was placed in foil and stored at -20 °C for microcystin analysis. Approximately 10% of the feces from each bat were placed in 1.5 mL tubes with silica beads for dietary analysis [20]. The remaining feces were placed in individual foil packets for analysis of microcystin. Fresh feces were also collected from roosting bats by placing foil directly underneath the roosting colony each trap night. Feces accumulated on the foil were collected the next day. Approximately half of the feces from the roosting colony were used for microcystin analysis and half were used for dietary analysis [20].

Hexagenia mayflies were collected June 21 and 28, 2014, by hanging a black light in front of a white sheet [24]. Attracted subimagos and imagos were picked off the sheet or nearby ground, placed in foil, and frozen at -20 °C.

Ethics statement

Bats were captured under Michigan Scientific Collector Permit #SC1498. Captured individuals were humanely euthanized with isoflurane followed by cervical dislocation in accordance with guidelines of the GVSU Institutional Animal Care and Use Committee (IACUC Approval #14-08-A).

Microcystin analysis

Livers and feces from the 19 individual *M. lucifugus* and fresh feces from the roosting bats were freeze-dried (Labco Lyophilizer) for 24-48 hrs at -53 °C (pressure = 0.002 mbar). Liver tissue and fecal samples were then ground with a mortar and pestle and weighed to approximately 100 mg. Because most of the samples weighed less than 100 mg, livers and feces from 2-4 individuals were pooled to decrease the probability of false negatives for microcystin. To serve as positive controls, nine fecal samples and two pooled liver samples were spiked with 0.2 μg of nodularin after initial processing to determine recovery rates of the following extraction method. The 100 mg of tissue or feces were added to a centrifuge tube with 5 mL of 80% MeOH, then sonicated on ice for two 30-second pulses with 30 seconds between pulses. Samples were stored at -20 °C for 30 min, then centrifuged for 15 min at 14,000 rpm and -5 °C. The supernatant was decanted into an 8 mL glass tube using a glass pipet and speed vacuumed until dry. Samples were reconstituted in 1 mL of 80% MeOH and vortexed for 20 seconds, then stored at -20 °C for 30 min. After cold storage, samples were centrifuged for 10 min at 3,000 rpm at room temperature. The supernatant was transferred to a 1 mL autosampler vial and kept at -20 °C until analysis.

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used to quantify MC-LR, the most toxic and commonly reported variant [9,25], in our bat liver and fecal samples. MC-LR was detected in the samples using the transitions m/z 995.2→107.0; 134.5; 155.0, where MC-LR was quantified using the 107.0 m/z ion and confirmed using the 134.5 m/z and 155.0 m/z ions. Positive controls had the correct retention time (13.35 min) for nodularin and transitions m/z 825.2→135.4; 163.4; 226.4.

In addition to LC-MS/MS, enzyme-linked immunosorbent assay (ELISA) was used to quantify total MC (MC-eq) concentrations in *Hexagenia,* bat liver, and fecal samples with the QuantiPlate Kit for Microcystins (Envirologix). ELISA also has a much lower limit of detection (0.06 ppb) than LC-MS/MS (average limit of detection = 149.8 ppb), and is therefore more likely to detect lower concentrations of microcystin, especially given the small size of each sample. The increased sensitivity protocol was used for all bat liver and fecal sample extractions, which were diluted 1:16 to decrease the MeOH concentrations from 80% to 5%. All samples not spiked with nodularin were run in duplicate, triplicate, or quadruplicate to test the replicability of the ELISA. We then took the mean of these readings as the final concentration for each sample. Any replicate that yielded results differing by an order of magnitude or more were excluded from the mean. In order to test the recovery rates of ELISA, we also ran three positive controls of 0.5 ppb MC-LR standard in 5% MeOH. Optical density was determined at 450 nm on an iMark microplate reader (BioRAD).

Negative controls

Although ELISA is useful in detecting low concentrations of MC, samples extracted from tissues (as opposed to water or algal samples) can have matrix effects that

may lead to false positives for samples with MC concentrations below 6 ppb [26,27]. To avoid false positives due to matrix effects, we used negative controls for bat liver and fecal samples that were attained opportunistically. Because livers from captive-bred *M. lucifugus* were not available, we used livers from two North American vespertilionid bats (*Eptesicus fuscus* and *Nycticeius humeralis*) that were raised in captivity and therefore not exposed to MC. We also obtained feces from a colony of *M. lucifugus* that had been kept in captivity for approximately 8 months before the feces were collected. All bats used in the negative controls consumed mealworms and/or wax worms and drank tap or deionized water while in captivity, thereby minimizing the chance of exposure to MC.

Statistical analyses

To test for significant differences among the concentrations of MC-eq in each sample type, we initially tested the data for homoscedasticity and normality using Bartlett's and Shapiro-Wilk's tests respectively. We log-transformed the data to make it normal with equal variance, therefore allowing the use of parametric tests. We used an ANOVA to test for significant differences among the concentrations of MC in each sample type, then used Tukey's honest significant difference (HSD) post-hoc test to determine which groups differed from each other.

Histopathology

Halves of livers from three individuals with the highest concentrations of microcystin (measured via ELISA) were tested to determine if *M. lucifugus* livers showed physical signs of damage consistent with exposure to microcystin. Liver tissue was stored in RNAlater and was washed with PBS then fixed in 10% buffered formalin before processing for histopathological analysis using the standard protocol from [28]. Briefly,

tissue samples were dehydrated, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for light microscopic examination.

Results

Controls

Mean extraction efficiencies from the samples spiked with nodularin were 107.3% (± 10.8) for feces and 106.0% (± 21.1) for livers, and therefore did not warrant correction of MC concentrations with LC-MS/MS. Positive controls of MC-LR yielded a mean recovery rate of 90.1% (± 44.3), and therefore also did not warrant correction of MC concentrations for ELISA results.

Microcystin Concentrations

LC-MS/MS

All bat liver and fecal samples contained concentrations of MC-LR below the detectable limit of LC-MS/MS except for one liver. This liver yielded a concentration of 93.6 ppb MC-LR. However, while the quantification ion of MC-LR was present in this single liver sample, neither confirmation ion for MC-LR was present; thus, this result was determined to be a false positive.

ELISA

All samples, including the negative controls, contained concentrations of MC within the limit of detection of the ELISA (Fig. 2). Concentrations of MC in individual *Hexagenia* ranged from approximately 31.2- 636.4 ppb with a mean concentration of 152.8 ppb (± 125.4; Fig. 2). Feces collected from each individual bat contained a mean

MC-eq concentration of 107.7 ppb (± 16.8; Fig. 2) and the feces collected from underneath the roost contained a mean of 61.7 ppb MC-eq (± 22.9; Fig. 2). However, livers from *M. lucifugus* contained a much lower concentration of MC-eq than the feces with a mean of 14.1 ppb MC-eq (± 7.8; Fig. 2). Similarly, the negative controls for both feces ($\mu = 8.2 \pm 4.3$; Fig. 2) and livers ($\mu = 19.6 \pm 9.0$; Fig. 2) contained very low concentrations of MC-eq, although they fell within the limit of detection of the ELISA.

The ANOVA revealed significant differences in MC-eq concentrations among sample types ($F = 44.3$, $P < 2 \times 10^{-16}$). Tukey's HSD (Table 1) showed no significant difference between the feces collected from individuals and feces collected from underneath the roosting colony ($P = 0.534$). Concentrations of MC-eq in *Hexagenia* were not significantly different from those found in the feces collected from individuals ($P = 0.999$), but were significantly higher from all other sample types, including feces collected from underneath the roosting colony ($P = 4.17 \times 10^{-3}$). Concentrations of MC-eq in feces collected from underneath the roost and feces from individuals were significantly greater than concentrations of MC-eq detected in the livers of *M. lucifugus* ($P = 1.4 \times 10^{-6}$ and $P = 5.6 \times 10^{-6}$, respectively). Furthermore, there were no significant differences between the concentrations of MC-eq in the livers collected from *M. lucifugus* and either the negative control livers ($P = 0.867$) or the negative control feces ($P = 0.452$). The negative controls (livers and feces) were also not significantly different from each other ($P = 0.077$).

Table 1: P-values from Tukey's HSD pairwise comparisons of mean concentrations of MC-eq.

Histopathology

Histopathology of the livers revealed minimal changes, specifically dilation of sinusoids around central veins, in all three samples that could potentially be attributed to MC exposure. However, these changes are non-specific and may be caused by hepatic congestion, which can commonly occur in "healthy" animals.

Discussion

Our hypotheses were that 1) microcystin is present in both bat livers and feces, and 2) high levels of microcystin are associated with liver damage in wild bat populations. The presence of MC-eq in bat feces, but not their livers, suggests that *M. lucifugus* are exposed to this toxin, but are not bioaccumulating it. Consequently, it is not surprising that histopathology showed little to no damage in the livers.

ELISA vs LC-MS/MS

All samples contained concentrations of microcystin within the limit of detection of the ELISA, but no samples, even those with highest concentrations of microcystin (127.0 ppb), were within the detectable limit of the LC-MS/MS, which had an average limit of detection of 149.8 ppb. This discrepancy is likely due to the fact that only one congener, MC-LR, was measured with LC-MS/MS, whereas ELISA measures all congeners of microcystin (MC-eq). MC-LR may be present in these samples, but not in high enough concentrations to be detected with the LC-MS/MS method. It is likely that another congener or congeners constitute the majority of microcystin present in these samples.

Are *M. lucifugus* bioaccumulating microcystin?

Because there was no significant difference between the concentrations of MC-eq in the livers collected from *M. lucifugus* and the negative control livers, we can conclude that there is likely little to no MC-eq in the *M. lucifugus* livers. Rather, the non-zero values measured by ELISA are likely a result of matrix effects from the liver tissue itself. Conversely, the feces collected from individual *M. lucifugus* and from underneath the roosting colony contained significantly higher concentrations of MC-eq than the negative control feces. The low positive results from the negative control indicate the presence of matrix effects with this sample type, but the significant differences between the negative control feces and the feces from *M. lucifugus* show that there are relatively high concentrations of MC-eq in *M. lucifugus* feces. The presence of high concentrations of MC-eq in *M. lucifugus* feces confirms that these bats were exposed to this toxin. However, the higher concentrations of MC-eq present in the *M. lucifugus* feces compared to the livers indicate that *M. lucifugus* are excreting more MC-eq than they are accumulating. Furthermore, the nonspecific nature of the liver damage found in the histopathology of the livers indicates that while this population of *M. lucifugus* was exposed to MC, it is likely that they are not accumulating high concentrations of the toxin in the liver and are therefore impacted only minimally, if at all.

Route of microcystin exposure

Because Jones et al. [20] showed that *Hexagenia* are a common prey item of *M. lucifugus* during this insect's emergence and Woller-Skar et al. [22] confirmed that volant *Hexagenia* contain high concentrations of MC-eq, we can infer that *Hexagenia* are a likely source of MC-eq in *M. lucifugus*. Thus, we can conclude that *M. lucifugus* were exposed to MC through trophic transfer. The concentrations of MC-eq found in

Hexagenia and *M. lucifugus* feces in this study are similar to, but lower than those found by Woller-Skar et al. [22]. This apparent decrease in MC-eq concentrations in these two sample types may be attributable to different extraction methods used, or natural annual variation in MC production.

However, *Hexagenia* may only be one route of MC exposure for *M. lucifugus*. We also detected MC-eq in fecal samples of *M. lucifugus* in which no *Hexagenia* were detected. This could be attributable to false negatives in the dietary analysis (*i.e.,* these bats were consuming *Hexagenia*, but this prey item was not detected during the dietary analysis). The presence of MC-eq without *Hexagenia* could also indicate that *M. lucifugus* were consuming other prey items that contain MC-eq. Woller-Skar [6] showed that chironomid nymphs (Order Diptera) can also bioaccumulate MC-eq, although to a much lesser extent than *Hexagenia* mayflies. Although MC has not yet been confirmed in adult chironomids, we found that *M. lucifugus* were consuming two species of these non-biting midges. Furthermore, over half of the diet of *M. lucifugus* consisted of aquatic insects. However, because we sampled a maternity colony next to Little Traverse Lake, it is more likely that these bats were eating toxic aquatic insects as opposed to *M. lucifugus* that may be roosting further from our sampling site. Not all aquatic insects have been tested for MC, but benthic insects are more likely to contain MC since *M. aeruginosa* overwinters in lake sediments [15].

Jones et al. [20] found that *Hexagenia* were the most frequently occurring prey item in the diet of *M. lucifugus* overall, but no *Hexagenia* were detected in feces collected on the second sampling date (27 June). This absence could be a false negative, it could be due to decreasing numbers of *Hexagenia* in the environment causing bats to find

alternative MC-contaminated prey items, or it could be due to conditioned taste aversion of *M. lucifugus* to *Hexagenia.* If consuming toxic *Hexagenia* mayflies makes *M. lucifugus* exhibit symptoms of illness, as microcystin does in other mammals, it is possible that these bats may avoid or decrease consumption of microcystin-laden insects. Conditioned taste aversion has been documented in one other species of bat, *Carollia perspicillata* [29]. However, Jones et al. [20] did not collect available insects to test for prey selectivity for or against *Hexagenia* relative to other insects, and we consider that the lack of *Hexagenia* in the feces of *M. lucifugus* on 27 June is more likely due to the decrease in *Hexagenia* in the environment or to false negatives.

Although we did not test the water column for the presence of MC-eq, it is unlikely that *M. lucifugus* are ingesting this toxin through drinking water. Woller-Skar [6] showed that concentrations of MC-eq at the surface of Little Traverse Lake (where bats would be skimming water to drink) had little to no MC-eq before blooms of *M. aeruginosa*.

How toxic are *Hexagenia* to *M. lucifugus?*

During a 13-week study using mice, Fawell et al. [30] determined that daily oral dosages of 40 µg MC-LR per kg of bodyweight (=40 ppb) was the No Observed Adverse Effect Level (NOAEL; where there are clearly no signs of pathological changes in the liver). This study resulted in the WHO guideline for a tolerable daily intake (TDI) of 0.04 µg MC-LR per kg of bodyweight (= 0.04 ppb) for humans. Almost all *Hexagenia* individuals contained concentrations of MC that exceed the NOAEL in mice. Moreover, average measurements of MC (= 152 ppb) in *Hexagenia* mayfly not only exceeded this NOAEL, but more than tripled this guideline. However, techniques used by Jones *et al.*

[20] do not provide quantitative data on the number of individual *Hexagenia* consumed by each bat; therefore, it is not possible to approximate the quantity of MC-eq to which these bats were exposed.

Fawell et al. [30] also showed that exposure to 200 ppb over the same time period resulted in minor observed effects in the liver and only in a few animals. This minimal-effect level more closely mimics the exposure that *M. lucifugus* would have by ingesting one individual *Hexagenia* per night that contains average concentrations of MC-eq. However, *M. lucifugus* are likely consuming many *Hexagenia* over a shorter time period than the mice tested by Fawell et al. [30].

It has been estimated that lactating *M. lucifugus* may eat more than their body mass in insects each night to cope with the metabolic demand of reproduction. Thus, during this time of increased energy demands, it is reasonable that the *M. lucifugus* in this study were likely consuming large quantities of *Hexagenia*. However, *Hexagenia* were not found in all fecal samples, suggesting that levels of MC exposure may vary considerably among bats and across time. It is also important to note that we did not do an emergence count and, therefore, we cannot estimate the proportion of the colony sampled. Additionally, we were only able to make observations on individual bats that were alive and roosting in this maternity colony. If individuals that feed primarily or exclusively on *Hexagenia* or other toxic aquatic insects became ill or died away from the roost, they would not have been sampled for this study, which may bias assessments of the extent to which *M. lucifugus* are impacted by microcystin.

Impact of microcystin on bats

The degree of susceptibility, or even resistance, to MC varies highly among species as well as by the MC congener [31]. Our data show that bats are being exposed to MC, but that they are excreting more microcystin in their feces than they are accumulating in the liver. It is possible that bats have a physiological mechanism for detoxification that prevents or slows the rates of absorption or accumulation of microcystin, or that bats aren't ingesting enough microcystin to cause liver damage. It is also possible that the low hydrophobicity of MC-LR [32] makes this congener more likely to be excreted rather than bioaccumulated [33]. Although mass mortalities of bats from microcystin have not been recorded, this toxin may have some physiological impact on bats that may increase vulnerability of bats to predators, parasites, or disease [34].

Currently, North American bats are experiencing several threats of mortality. The spread of white-nose syndrome and an increase in wind turbine facilities has led to population declines in many bat species [35,36]. In addition, insectivorous bats are more susceptible to ingesting environmental toxins than other organisms due to their longevity relative to mammals of similar size, and higher metabolic rates associated with the ability of flight [37]. It has been shown that bats are at risk of ingesting environmental pollutants such as mercury and polychlorinated biphenyls (PCBs) through the consumption of aquatic insects [38]. Although there are several studies that have analyzed the bioaccumulation of environmental contaminants such as polybrominated diphenyl ethers (PBDEs) and the insecticide DDT in bats [39–41], to date, only one study has assessed whether or not bats bioaccumulate naturally occurring toxins, particularly the secondary metabolites of cyanobacteria that may bioaccumulate in many aquatic organisms such as those that serve as prey items for bats. The single documented case of mass mortality of

bats due to a cyanotoxin was attributed to Anatoxin-a, a neurotoxin produced by *Anabaena flos-aquae* [42], although it is unclear whether the toxin was ingested while drinking contaminated water or from consuming insects. Even though mass mortalities due to algal toxins may be rare, water quality can impact the health of bats and should therefore be monitored for both inorganic and organic toxins.

Future work

More in depth dietary analyses, such as those using next generation sequencing (NGS) techniques, would be useful in providing more data on which species of insects the bats in this area are consuming. Because NGS yields millions of sequence reads, it reduces the likelihood of false negatives in dietary analyses, a potential limiting factor in this study.

In order to monitor the movement of microcystin through an ecosystem, other emerging aquatic insects, particularly those with benthic life stages, should be tested for microcystin, as they may provide alternative routes for this toxin to be transferred to terrestrial organisms. For bats in particular, feces from nearby roosting colonies should be tested over a longer time period. In this study, feces were collected before a bloom event, but microcystin concentrations in lakes have been shown to be the highest during and right after blooms. It is important to test if the bats are ingesting more microcystin over time or if major insect emergences, such as those of *Hexagenia,* are the only relatively short time periods during which bats are exposed to microcystin. Because North American bats are presently facing so many threats, it is essential that we understand all possible threats to their health.

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References

1. Paerl HW, Huisman J. Blooms like it hot. Science 2008; 320: 57–58.
2. Huisman J, Matthijs H, Visser P. Harmful cyanobacteria. Dordrecht: Springer; 2005.
3. Paerl HW, Fulton RS. Ecology of harmful cyanobacteria. In: Granelí E, Turner JT, editors. Ecology of harmful algae. Berlin: Springer; 2006.
4. Vanderploeg H a., Liebig JR, Carmichael WW, Agy M a., Johengen TH, Fahnenstiel GL, et al. Zebra mussel (*Dreissena polymorpha*) selective filtration promoted toxic *Microcystis* blooms in Saginaw Bay (Lake Huron) and Lake Erie. Can J Fish Aquat Sci. 2001; 58: 1208–1221.
5. Kuiper-Goodman T, Falconer I, Fitzgerald J. Human health aspects. In: Chorus I, Bartram J, editors. Toxic cyanobacteria in water, a guide to their health public health consequences, monitoring and management. London: Routledge Press; 1999.
6. Woller-Skar MM (2009) Zebra mussel (*Dreissena polymorpha*) promotion of cyanobacteria in low-nutrient lakes and the subsequent production and fate of microcystin. Ph.D. dissertation, Bowling Green State University. Available: http://scholarworks.bgsu.edu/bio_diss/33/
7. Raikow DF, Sarnelle O, Wilson AE, Hamilton SK. Dominance of the noxious cyanobacterium *Microcystis aeruginosa* in low-nutrient lakes is associated with exotic zebra mussels. Limnol Oceanogr. 2004; 49: 482–487.
8. Fahnenstiel GL, Lang, Gregory A, Nalepa TF, Johengen TH. Effects of zebra mussel (*Dreissena polymorpha*) colonization on water quality parameters in

Saginaw Bay, Lake Huron. J Great Lakes Res. 1995; 21: 435–448.

9. Sivonen K, Jones G (1999) Cyanobacterial Toxins. In: Chorus I, Bartram J, editors. Toxic cyanobacteria in water, a guide to their health public health consequences, monitoring and management. London: Routledge Press. 1999.

10. Falconer I, Dornbusch M, Moran G, Yeung S (1992) Effect of the cyanobacterial (blue-green algal) toxins from *Microcystis aeruginosa* on the isolated enterocytes from the chicken intestine. Toxicon. 1992; 30: 790–793.

11. Vasconcelos V. Uptake and depuration of the peptide toxin microcystin-LR in the mussel *Mytilus galloprovincialis*. Aquat Toxicol. 1995; 32: 227–237.

12. Kotak BG, Zurawell RW, Prepas EE, Holmes CFB. Microcystin-LR concentration in aquatic food web compartments from lakes of varying trophic status. Can J Fish Aquat Sci. 1996; 53: 1974–1985.

13. Mohamed Z. Accumulations of cyanobacterial hepatotoxins by *Daphnia* in some Egyptian irrigation canals. Ecotox Environ Safe. 2001; 50: 4–8.

14. Hunt B. The life history and economic importance of a burrowing mayfly, *Hexagenia limbata*, in southern Michigan lakes. B I Fish Res. 1953; 4: 1-151.

15. Reynolds C, Jaworski G, Cmiech H, Leedale G. On the annual cycle of the blue-green alga *Microcystis aeruginos*a Kütz. Emend. Elenkin. Philos Trans R Soc B Biol Sci. 1981; 293: 419–477.

16. Edsall TA. Burrowing mayflies (*Hexagenia*) as indicators of ecosystem health. Aquat Ecosyst Heal Manag. 2001; 4: 283–292.

17. Belwood J, Fenton M. Variation in the diet of *Myotis lucifugus* (Chiroptera: Vespertilionidae). Can J Zool. 1976; 54: 1674–1678.

18. Anthony ELP, Kunz TH. Feeding strategies of the little brown bat, *Myotis lucifugus*, in southern New Hampshire. Ecology. 1977; 58: 775–786.

19. Clare EL, Barber BR, Sweeney BW, Hebert PDN, Fenton MB. Eating local: Influences of habitat on the diet of little brown bats (*Myotis lucifugus*). Mol Ecol. 2011; 20: 1772–1780.

20. Jones D, Woller-Skar M, Russell A. Rapid shift in the diet of *Myotis lucifugus* during mid maternity season. Acta Chiropterologica. In prep.

21. WHO. Cyanobacterial toxins: Microcystin-LR in drinking-water. Background document for development of WHO Guidelines for Drinking-water Quality. Guidelines for drinking-water quality. 2003. http://www.who.int/water_sanitation_health/dwq/chemicals/cyanobactoxins.pdf. Downloaded 1 September 2015.

22. Woller-Skar MM, Jones DN, Russell AL, Luttenton MR. Microcystin detected in little brown bats (*Myotis lucifugus*). Am Midl Nat. 2015; 174: 331–334.

23. Henry M, Thomas DW, Vaudry R, Carrier M. Foraging distances and home ranges of pregnant and lactating little brown bats (*Myotis lucifugus*). J Mammal. 2002; 83: 767–774.

24. Southwood T. Ecological methods: with particular reference to the study of insect populations. London: Methuen; 1966.

25. Mirura G, Robinson N, Geisbert T, Bostian K, White J, Pace J. Comparison of *in vivo* and *in vitro* toxic effects of microcystin-LR in fasted rats. Toxicon. 1989; 27: 1229–1240.

26. Moreno I, Herrador M, Atencio L, Puerto M, González A, Camean A.

Differentiation between microcystin contaminated and uncontaminated fish by determination of unconjugated MCs using an ELISA anti-ADDA test based on receiver-operating characteristic curves threshold values: Application to *Tinca tinca* from natural ponds. Environ Toxicol. 2009; 26: 45–56.

27. Geis-Asteggiante L, Lehotay S, Fortis L, Paoli G, Wijey C, Heinzen H. Development and validation of a rapid method for microcystins in fish and comparing LC-MS/MS results with ELISA. Anal Bioanal Chem. 2011; 401: 2617–2630.
28. Luna L. Manual of histological staining methods of the Armed Forces Institute of Pathology. 3rd ed. New York: McGraw Hill; 1968.
29. Terk M, Green L. Taste aversion learning in the bat, *Carollia perspicillata*. Behav Neural Biol. 1980; 28: 236–242.
30. Fawell JK, Mitchell RE, Everett DJ, Hill RE. The toxicity of cyanobacterial toxins in the mouse: I microcystin-LR. Hum Exp Toxicol. 1999; 18: 162–167.
31. Schmidt JR, Wilhelm SW, Boyer GL. The fate of microcystins in the environment and challenges for monitoring. Toxins. 2014; 6: 3354–3387.
32. Ward C, Codd G. Comparative toxicity of four microcystins of different hydrophobicities to the protozoan, *Tetrahymena pyriformis*. J Appl Microbiol. 1999; 68: 874–882.
33. Kozlowsky-Suzuki B, Wilson AE, Ferra S. Biomagnification or biodilution of microcystins in aquatic foodwebs? Meta-analyses of laboratory and field studies. Harmful Algae. 2012; 18: 47–55.
34. Smith JL, Boyer GL, Zimba PV. A review of cyanobacterial odorous and bioactive

metabolites: Impacts and management alternatives in aquaculture. Aquaculture. 2008; 280: 5–20.

35. Arnett E, Baerwald E. Impacts of wind energy development on bats: Implications for conservation. In: Adams R, Pedersen S, editors. Bat evolution, ecology, and conservation. New York: Springer; 2013. pp. 435–456.

36. Coleman JTH, Reichard JD. Bat white-nose syndrome in 2014: A brief assessment seven years after discovery of a virulent fungal pathogen in North America. Outlooks Pest Manag. 2014; 25: 374–377.

37. Clark D, Shore R. Chiroptera. In: Shore R, Rattner B, editors. Ecotoxicology of wild mammals. London: Wiley; 2001. pp. 159–214.

38. Baron LA, Sample BE, Suter GW. Ecological risk assessment in a large river-reservoir: 5. Aerial insectivorous wildlife. Environ Toxicol Chem. 1999; 18: 621–627.

39. Secord AL, Patnode KA, Carter C, Redman E, Gefell DJ, Major AR, et al. Contaminants of emerging concern in bats from the northeastern United States. Arch Environ Contam Toxicol. 2015; 69: 411-421.

40. Kannan K, Yun SH, Rudd RJ, Behr M. High concentrations of persistent organic pollutants including PCBs , DDT , PBDEs and PFOS in little brown bats with white-nose syndrome in New York , USA. Chemosphere. 2010; 80: 613–618.

41. O'Shea T, Johnston J. Environmental contaminants and bats: Investigating exposure and effects. In: Kunz T, Parsons S, editors. Ecological and behavioral methods for the study of bats. 2nd ed. Baltimore: The Johns Hopkins University Press; 2009. pp. 500–528.

42. Pybus M, Hobson D, Onderka D. Mass mortality of bats due to probable blue-green algal toxicity. J Wildl Dis. 1986; 22: 449–450.

Figure Legends

Fig 1: Map of Little Traverse Lake in Leelanau County, Michigan from Jones et al. [20].The sampled *Myotis lucifugus* roost is indicated with a star next to Little Traverse Lake (44.923580, -85.821265).

Fig 2: Boxplot showing the median concentrations of MC-eq in each sample type as measured with ELISA, including interquartile range. Outliers are shown with open circles. Significant differences in mean concentrations of MC-eq are denoted by letters above boxes. Red dashed line represents the NOAEL for mice (40 ppb, [21]) and the dashed blue line represents the TDI for humans (0.04 ppb, [29]). Samples from *M. lucifugus* are denoted "Mylu."

Table 1: P-values from Tukey's HSD pairwise comparisons of mean concentrations of MC-eq.

	Feces (Roost)	Feces (Individuals)	*M. lucifugus* Livers	Livers (Negative Control)	Feces (Negative Control)
Hexagenia	4.2×10^{-3}	1.0	$< 1.0 \times 10^{-7}$	$< 1.0 \times 10^{-7}$	$< 1.0 \times 10^{-7}$
Feces (Roost)	-	0.53	1.4×10^{-6}	7.2×10^{-4}	$< 1.0 \times 10^{-7}$
Feces (Individuals)		-	5.6×10^{-6}	2.9×10^{-4}	1.0×10^{-7}
M. lucifugus Livers			-	0.87	0.45
Livers (Negative Control)				-	0.077

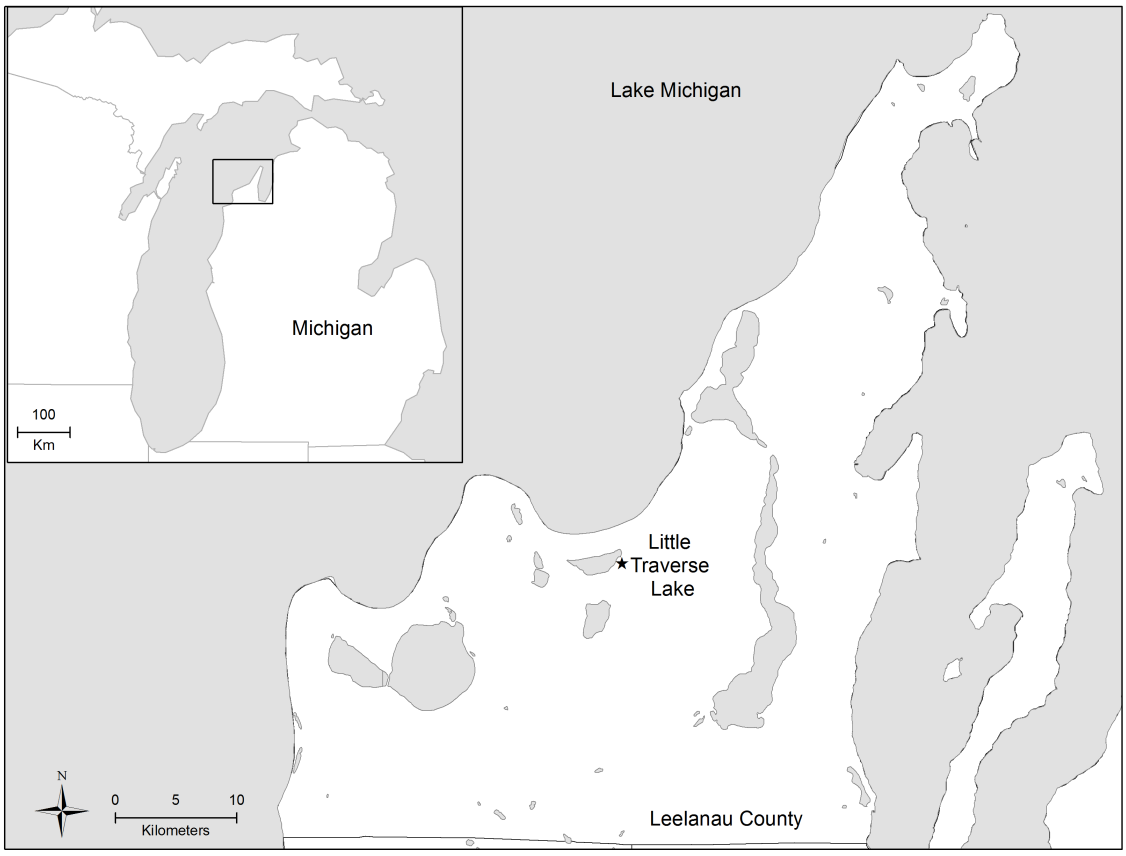
Lake Michigan
Michigan
100
Km
Little
Traverse
Lake
N
0
5
10
Kilometers
Leelanau County

Fig. 1

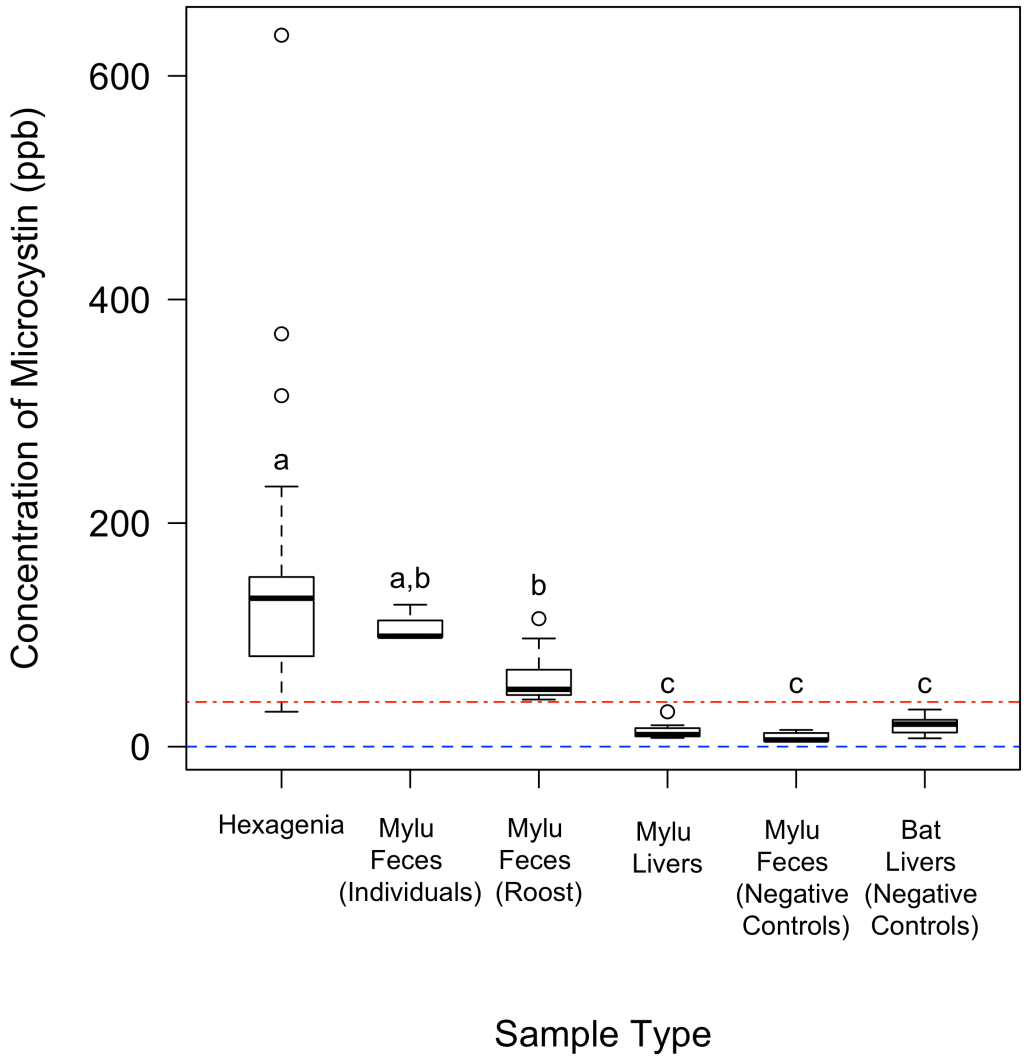
Concentration of Microcystin (ppb)
600
400
200
0
a
a,b
b
c
c
c
Hexagenia
Mylu Feces (Individuals)
Mylu Feces (Roost)
Mylu Livers
Mylu Feces (Negative Controls)
Bat Livers (Negative Controls)
Sample Type

Fig. 2.

Chapter 4

Extended Literature Review

INTRODUCTION

Trophic interactions are the transfer of energy between trophic levels, but at the aquatic-terrestrial interface, these interactions may result in more than the movement of energy and nutrients between ecosystems. Emerging aquatic insects are important prey items for riparian predators (Baxter *et al.* 2005), but they can also facilitate the export of contaminants to terrestrial ecosystems (Menzie 1980; Runck 2007; Cristol *et al.* 2008). Although many studies have focused on the movement of both organic (*e.g.*, pesticides) and inorganic contaminants (*e.g.*, metals and other toxic elements) from aquatic to terrestrial ecosystems, few have assessed the transfer of naturally occurring algal toxins into terrestrial ecosystems. As harmful algal blooms (HABs) increase in frequency and intensity due to human alterations such as climate change (Paerl and Huisman 2008), eutrophication (Huisman *et al.* 2005; Paerl and Fulton 2006), and invasive species (*e.g.* zebra mussels; (Vanderploeg *et al.* 2001), it is increasingly important that we understand the impact they may have on ecosystems.

Microcystis aeruginosa is a species of cyanobacteria responsible for many HABs since it produces a hepatotoxin, microcystin (MC, Chorus and Bartram 1999). This toxin can bioaccumulate in emerging aquatic insects that may then become toxic prey for riparian predators such as bats. Little brown bats, *Myotis lucifugus,* are a common bat species in North America that consumes aquatic insects and may take advantage of emergences of Ephemeroptera. Mass emerging *Hexagenia* mayflies (Ephemeroptera) have been shown to contain high concentrations of microcystin (Woller-Skar *et al.* 2015).

Thus, predation of *M. lucifugus* on *Hexagenia* may result in the trophic transfer of an algal toxin into the terrestrial ecosystem. This literature review gives detailed background information on methods of dietary analyses in insectivorous bats with special focus on the diet of *M. lucifugus*, current knowledge of how HABs affect bats, production and toxicity of microcystin, and the examined route of exposure (*Hexagenia* mayflies) of microcystin to *M. lucifugus*.

DIETARY ANALYSIS OF INSECTIVOROUS BATS

Because bats are volant and forage at night, direct observation of prey consumption is difficult or impossible. Therefore, examination of gut contents or feces has become the standard approach to determining the diets of insectivorous bats. There are a variety of techniques that can be used to identify prey taxa in the gut and fecal content of these species. There are three main methods that have been used to identify prey taxa, each of which has strengths and weaknesses. Traditional methods of dietary analysis involve visual identification of hard insect parts in feces or stomach contents. This type of analysis allows us to broadly understand which types of insects are consumed by bats, but does not identify the exact species that are consumed. Modern molecular techniques enable identification of prey items to species level by targeting insect DNA present in the bat feces. These molecular techniques use the polymerase chain reaction (PCR) to amplify insect DNA, which is then sequenced at a specific "barcoding" region and compared to voucher sequences of known species in online databases (Hebert *et al.* 2003, 2004). Unlike visual identification of insects, the use of barcoding can distinguish among morphologically cryptic taxa or degraded specimens.

However, not all insects are represented in online databases and these techniques can be costly. There are also studies that use a combination of both traditional and molecular techniques, which have some of the strengths and weaknesses of each type of analysis. Here I will provide a brief review of visual and molecular analyses of the diet of insectivorous bats, using *Myotis lucifugus* as the primary example, and discuss the strengths and weaknesses of various approaches.

DIET OF *M. LUCIFUGUS* BASED ON VISUAL ANALYSIS OF GUT CONTENT OR FECES

Traditionally, gut and fecal analyses have been conducted by visual identification of hard, undigested insect parts under a dissecting scope. Due to incomplete digestion, analyses of stomach contents allow for greater levels of confidence by identifying more intact ingested prey items compared with fecal samples (Pine 1969; Easterla and Whitaker 1972; Whitaker 1972; Kunz 1974; Buchler 1976). However, this technique requires the animals to be euthanized, and is therefore not preferable, particularly for threatened or endangered species. By comparing the stomach contents of the bats to the available insect fauna, Buchler (1976) was able to determine that *M. lucifugus* exhibit selectivity towards locally available aquatic insects. For example, during mayfly emergences, *M. lucifugus* shifted their diets from primarily Diptera (true flies) to almost exclusively Ephemeroptera (>80% of insects detected in the stomach contents), even though mayflies made up only 4% of available insects.

Unlike analyses of gut content, fecal analyses can be completely non-invasive (Rabinowitz and Tuttle 1982; Kunz and Whitaker 1983), as the samples can be collected

from underneath bat roosts or by holding bats in bags temporarily. One drawback, however, is that prey items in feces are less intact after mastication and digestion; thus, prey items may be more difficult to identify, and soft-bodied prey such as Ephemeroptera may be underrepresented (Kunz and Whitaker 1983). Due to the ease of collection of guano, several studies have focused on fecal pellets of *M. lucifugus* to determine this species' diet. By comparing insects found in *M. lucifugus* feces to insects in malaise traps, Belwood and Fenton (1976) showed that *M. lucifugus* are opportunistic feeders that feed heavily on swarms of readily available aquatic insects, primarily chironomid midges (Diptera). Likewise, Anthony and Kunz (1977) found a high diversity of insects consumed by *M. lucifugus* including Diptera, Coleoptera, Lepidoptera, Trichoptera, Ephemeroptera, Neuroptera, Homoptera, and Hymenoptera, many of which are aquatic. However, *M. lucifugus* were more likely to be selective when a higher diversity of insects were available, shown by a shift in diet from primarily Diptera to Ephemeroptera and Coleoptera (Anthony and Kunz 1977).

One drawback to these types of analyses is that is it often difficult for non-entomologists to find and identify insect body parts correctly, and the effects of digestion can result in biases and incomplete characterizations of a bat's diet. Even in the best circumstances, the parts of prey left behind after digestion are typically identifiable only to order or family (Clare *et al.* 2009).

MOLECULAR TECHNIQUES FOR DIETARY ANALYSIS USING BARCODING

Molecular techniques present an alternative approach for characterizing the diet of insectivorous bats when coupled with mitochondrial barcoding. DNA barcoding is based

on the idea that every species can be identified from one particular region of their DNA. Mitochondrial DNA, as opposed to nuclear DNA, is used for barcoding in animals because of its unimodal inheritance and lack of recombination (Saccone et al. 1999). In addition, mtDNA is ideal for fecal analyses in particular because of both its circular shape and abundance relative to nuclear DNA (approximately 100 times more numerous; Avise 1994), which makes it more likely for copies of mtDNA to survive mastication and digestion. DNA barcoding in animals uses an approximately 650 bp region of the cytochrome oxidase I (COI) gene. COI is used because it is a highly conserved (Lynch and Jarrell 1993) protein coding region (Brown 1985) with robust universal primers (Folmer *et al*. 1994) and strong phylogenetic signal (Hebert *et al.* 2003). The COI region currently has the largest database of sequences available and thus has become the locus of choice for animal barcoding. The region of COI targeted by the universal LCO1490/ HCO2198 primers (Folmer *et al.* 1994) has been shown to be effective in making species-level identifications (Hebert *et al.* 2003). Once sequenced, DNA amplified with these primers can be entered into an online database such as BOLD (Barcode of Life Database, Ratnasingham and Hebert 2007) or GenBank and compared to sequences from vouchered specimens.

Mitochondrial DNA fragments larger than 300 bp can be difficult to recover in highly degraded samples such as feces and stomach contents (Deagle *et al.* 2006); thus, many studies have utilized "mini-barcodes" which are mtDNA fragments typically 100-200 bp in length (*e.g.,* Hebert *et al*. 2004; Meusnier *et al*. 2008; Zeale *et al*. 2011). Hajibabaei *et al.* (2006) showed that mini-barcodes can accurately differentiate interspecific variation, and consequently, are sufficient to distinguish among arthropod

species present in bat feces, for example. As with all methods, there are some limitations. Short sequences have an increased likelihood of showing high similarity to other sequences, thereby increasing the chance of false positives; it is therefore best to use conservative identification criteria when identifying species using mini-barcodes. The length and location of mini-barcodes can impact their ability to discriminate among species (Hajibabaei *et al*. 2006). Within the last five years, the most commonly used mini-barcode to identify insect prey in bat feces is delimited by the ZBJ-Art primers (Zeale *et al.* 2011). These primers do not amplify non-target DNA such as bacterial, fungal, or bat DNA, and have been shown to have good coverage of prey items of insectivorous bats in Africa, Europe, and North America (Bohmann *et al.* 2011; Clare *et al*. 2011, 2013, 2014; Krüger *et al*. 2014, Razgour *et al*. 2011; Vesterinen *et al*. 2013, 2016).

COMBINING TRADITIONAL AND MODERN DIETARY ANALYSIS TECHNIQUES

Since fragments of many different insects can be present in one fecal sample, the DNA from each prey item must be separated before it can be sequenced. This can be done mechanically by using traditional methods of pulling out insect parts or molecularly after DNA has been amplified with barcoding primers. Some studies have used a combination of morphological and molecular techniques to identify prey items in bat feces. Clare *et al.* (2009) was one of the first to combine the two techniques. They used a dissecting microscope to pick out insect fragments from the feces of eastern red bats (*Lasiurus borealis*), then extracted DNA from these fragments, and identified the prey items to species by comparing COI mini-barcoding sequences to BOLD (Clare *et al*.

2009). Clare *et al*. (2011) used the same technique to identify arthropod prey in the diet of *M. lucifugus*. These authors were able to get identifiable sequence data from approximately 66 prey taxa and of these, 39 were confidently identified to species level. Clare *et al*. (2011) showed that aquatic insects constituted a large proportion of the *M. lucifugus* diet, confirming what previous traditional and molecular studies found (Belwood and Fenton 1976; Buchler 1976; Anthony and Kunz 1977). Notably, this study detected a temporal shift in *M. lucifugus* food habits from primarily Diptera in the early maternity season to mainly Ephemeroptera in mid-maternity season, corresponding with ephemeropteran emergences. Overall, the *M. lucifugus* diet comprised about 30% Ephemeroptera. The authors also detected spatial differences in diet where *M. lucifugus* in forest roosts had higher dietary richness than those in agricultural roosts. This study is valuable in discovering what *M. lucifugus* consume during a critical time period in their life history.

Although this hybrid approach is cheaper than strictly molecular methods, it is more time consuming and requires expertise in being able to find arthropod fragments in feces. The number and condition of insect fragments that may be extracted from the fecal pellets also limits these analyses. Like strictly visual methods, this technique is also susceptible to false negatives since soft-bodied insects may have been more thoroughly digested and, therefore, distinguishable parts from those insects may be missed during dissection (Kunz and Whitaker 1983).

PURELY MOLECULAR TECHNIQUES FOR DIETARY ANALYSES

Unlike the methods of Clare *et al*. (2009, 2011), there are two completely molecular techniques that have been used to isolate the DNA of different insects after extraction and PCR: cloning and next-generation sequencing (NGS). These techniques do not require insect fragments to be picked out of feces visually. Cloning exploits the bacteria *Escherichia coli*, each cell of which is transformed with a plasmid containing one fragment of the amplified insect DNA. Because each colony contains a different fragment of insect DNA, the proportion of clones has been shown to roughly correspond with dietary proportions (Deagle *et al*. 2005). Therefore, dietary analyses from cloning may be semi-quantifiable in that relative proportions of insect prey in the diet may be estimated. In the same way that visual analyses of fecal pellets are limited by the number of insect fragments selected, cloning is also constrained by the number of clones selected, and may fail to detect rare species (Alberdi *et al*. 2012).

Zeale *et al*. (2011) assessed the dietary breadth of three species of insectivorous bats that exploit different dietary niches. The authors compared the results of morphological identification of prey from bat feces to the results from cloning (selecting 16 clones/sample) and found that there was general agreement between the morphological and molecular techniques, but that cloning allowed higher taxonomic resolution of the bats' diets. Similarly, Alberdi *et al.* (2012) used cloning to assess the foraging ecology of the European insectivorous bat *Plecotus macrobullaris*. These authors selected 20 colonies per sample and used a rarefaction analysis to show that the number of clones/sample was sufficient to detect all prey present. Although this method detected 54 operational taxonomic units (OTUs), it is still possible that not all prey items were detected, particularly those that may be rare or less abundant.

DIETARY ANALYSES USING NEXT GENERATION SEQUENCING

Next generation sequencing (NGS) is not limited by the selection of hard body parts for visual inspection or of clones for sequencing, but rather yields millions of individual sequence reads in just one run. Consequently, NGS platforms are rapidly replacing cloning techniques as they become more cost effective and faster than both cloning and traditional analyses. Recent dietary studies using NGS have increased our understanding of resource partitioning (Bohmann *et al*. 2011; Razgour *et al.* 2011; Emrich *et al*. 2014), niche differentiation (Krüger *et al*. 2014), and predator-prey dynamics (Vesterinen *et al*. 2013). Studies using NGS to assess the composition of bat diets have yielded datasets of approximately 36,000 (Bohmann *et al.* 2011) to over 90,000 (Razgour *et al*. 2011) high quality sequence reads and identified approximately 100 (Razgour *et al*. 2011; Krüger *et al*. 2014) to over 200 (Bohmann *et al*. 2011; Clare *et al*. 2014) molecular OTUs (MOTUs). Although Coutts *et al*. (1973) found, using traditional visual analyses, that one fecal pellet typically contains either one large insect or several smaller ones, Bohmann *et al*. (2011) found DNA from as many as 52 unique haplotypes in a single fecal pellet using NGS.

Recently, Clare *et al.* (2014) used NGS to measure temporal and spatial variation in the diet of *M. lucifugus* across Canada during the reproductive season. Their analyses identified 211 MOTUs in the diet of *M. lucifugus* constituting approximately 45% Lepidoptera, 34% Diptera, 11% Ephemeroptera, 6% Trichoptera, 4% Coleoptera, as well as 5 minimally represented orders with only 1 or 2 species found. Some of the most commonly consumed prey items were mayflies in the genus *Caenis,* which were found in

about 50% of the fecal samples. With all locations pooled, Clare *et al*. (2014) found that there was a temporal shift in the diet of *M. lucifugus* from primarily Diptera in the early season (during pregnancy) to Lepidoptera as the summer progressed, while levels of Ephemeroptera, Coleoptera, and Trichoptera remained stable throughout the summer. Although Clare *et al*. (2011) saw a shift from Diptera to Ephemeroptera using hybrid techniques, that pattern was not seen by Clare *et al*. (2014) in any location, even at sites that were sampled for both studies, although this may be due to differences in methodology. Clare *et al.* (2014) also found a significant decrease in dietary richness through the progression of summer, unlike Anthony and Kunz (1977) who saw no temporal change in diversity. Furthermore, Clare *et al*. (2014) found significant spatial variation in diet. The finding of both temporal and spatial variation in the diet of *M. lucifugus* confirms that this species takes advantage of aquatic insects that are locally abundant. Consequently, population declines of *M. lucifugus* due to white-nose syndrome (WNS, Frick *et al.* 2010) may have regionally specific consequences on insect populations.

QUANTIFICATION OF DIET

In visually-based dietary analyses where hard insect parts are used to characterize and quantify diet, the proportional representation of each insect taxon has been based on a reconstruction of parts using the minimum number possible (*e.g.*, four antennae found could belong to four individuals, but is presumed to represent two individuals (Buchler 1976). Kunz and Whitaker (1983) tested the efficacy of this quantification method by using *M. lucifugus* in feeding trials. Based on both volume (portion of feces that contains

a particular prey item) and frequency (proportion of animals in which a prey item was detected), the four most common prey taxa were identified in order of importance (*i.e.,* taxa that constitute the largest proportions of the diet), demonstrating that fecal analyses can yield "reasonable estimates" of food habits by *M. lucifugus*. Nevertheless, there are still some sources of error that may prevent extrapolation of food habits between age, sex, or reproductive classes. Therefore, Kunz and Whitaker (1983) suggested that quantified results should be interpreted with caution. For example, some bats may cull parts of their insect prey before ingestion. Therefore, if a bat consumes the body of an insect, but few or no legs or antennae, the representation of that prey item may be underestimated. Likewise, soft-bodied insects may be underrepresented as they are not fully sclerotized and may be more easily digested, leaving fewer parts behind to be included in fecal analyses.

In molecular analyses using cloning, the number of clones that contain DNA of a certain prey item has been shown to roughly correspond to the mass of the prey item consumed (Deagle *et al*. 2005). Thus, the number of clones can be used to provide semi-quantitative data on prey items at least in relative proportion to other prey consumed. However, due to biases in DNA survival during digestion (Deagle and Tollit 2007), quantification of prey items in feces is limited.

In their NGS-based analyses, Clare *et al*. (2014) determined that species level and ordinal level analyses reveal different patterns, thus it is important to identify MOTUs to species level whenever possible. These authors quantified dietary proportions by looking at the presence or absence of a MOTU in a sample (*i.e.,* frequency of occurrence) and defining the proportions as the frequency of occurrences of an order out of the total

number of occurrences. Unlike morphological sampling, NGS does not allow for the assumption of independence of each haplotype, but does maximize the diversity of prey recovered (Clare *et al*. 2014). Independence cannot be assumed because multiplex identifier (MID) tags, primers, adaptors, and sequencing direction can influence sequence yield. Even using the same PCR product in different runs can yield different results (Pompanon *et al*. 2012; Deagle e*t al*. 2013). Bohmann *et al*. (2011) suggested that it is not possible to truly quantify the number of insects eaten by individual bats or to determine proportions of insect prey because dietary proportions are quantified using the presence or absence of MOTUs. Clare *et al.* (2014) also found that molecular methods may overrepresent rare items and underestimate the importance of common items, since both are quantified as presence or absence in a sample. Furthermore, richness in an order is not always related to abundance. For example, mayflies have mass emergences and are highly abundant, but not particularly diverse and thus can be underrepresented in molecular analyses (Clare *et al*. 2014).

Dietary analyses produced through NGS may provide some quantitative measurements on the abundance or mass of prey items consumed by using the number of times a sequence is produced. However, there are no studies to date that have compared the number of sequences produced by NGS techniques to the mass of prey items consumed. In order to compensate for this lack of clarity, some studies have compared NGS results to morphological analyses. Razgour *et al*. (2011) found that there were no significant differences in prey proportions between morphological and molecular diet analysis although the morphological analysis showed some orders of prey not present in the molecular analysis and vice versa. Similarly, Krüger *et al.* (2014) found high overlap

between morphological and NGS methods in determining dietary proportions, but found minor differences between the two techniques. Prey-specific biases in both hard body part and DNA survival (Deagle and Tollit 2007) may result in soft bodied insects being underrepresented in both morphological and molecular techniques of identifying prey, making quantification difficult. However, differential survival of DNA does not necessarily mean that there will be quantification biases using presence/absence data (Deagle and Tollit 2007), provided that a sufficient number of DNA fragments are examined.

Understanding the diet of a species can answer many ecological questions such as resource partitioning and niche overlap in sympatric or cryptic species, interspecific competition, the impact of land use on foodwebs, and ecosystem responses to disturbance. Like Clare *et al*. (2011, 2014) used the diet of *M. lucifugus* to make inferences about habitat quality, we can also use information on the diet of bats to further our knowledge of the trophic transfer of environmental toxins.

IMPACT OF PREY ON BATS

Bats have high metabolic rates due to their size and flight habits, and require higher levels of food intake; thus, bats may be more exposed to environmental toxins via trophic transfer (Secord *et al*. 2015). Moreover, females must increase food consumption due to increase in energy demand during reproduction (Anthony and Kunz 1977; Kurta *et al.* 1989) and may be at additional risk for consuming these toxins. Globally, many bat species have been exposed to organic contaminants such as pesticides and industrial compounds, as well as inorganic contaminants like heavy metals and other toxic elements

(O'Shea and Johnston 2009). Studies have demonstrated the bioaccumulation of mercury (Baron *et al.* 1999; Little *et al*. 2015) in *M. lucifugus* as well as pesticides (Clark 1988) via trophic transfer from aquatic insects. Clark (2001) showed that the use of DDT (dichlorodiphenyltrichloroethane) was responsible for major population declines of *Tadarida brasiliensis* at Carlsbad Cavern, New Mexico between 1955 and 1971. The local population of *T. brasiliensis* plummeted from over 8 million in 1936 to under 40,000 by 1967 most likely due to DDT (Constantine 1967, Clark 2001).

Recently, several studies have focused on the bioaccumulation of toxins and contaminants that may affect immunological or other physiological functions and ultimately the survival of bats that are susceptible to white-nose syndrome (WNS). Kannan *et al.* (2010) found that all bat carcasses contained PBBs (polybrominated biphenyl) and many samples contained high levels of PCBs (polychlorinated biphenyl), PBDEs (polybrominated diphenyl ether), DDT, and chlordanes. PCBs have been shown to cause changes in biochemistry and behavior and can reduce the survival of young (Clark and Stafford 1981). Specimens collected for this study had some of the highest levels of PBDEs reported in wildlife (Kannan et al. 2010). Many of the chemicals detected in these bats can have synergistic and/or additive harmful effects (Kannan *et al*. 2010).

Similarly, Secord *et al*. (2015) analyzed carcasses of a few species of common bats, including *M. lucifugus*, in the northeastern U.S. for contaminants of emerging concern (CECs). Although the use and concentration of many "legacy contaminants" such as PCBs and organochloride pesticides have decreased since the 1970s (Bayat *et al*. 2014), there has been an increase in new types of contaminants being introduced into the

environment such as pharmaceuticals, antibiotics, detergents, plasticizers, PBDEs, and many others. Park and Cristinacce (2006)found that sewage-treatment filter beds are important foraging sites for insectivorous bats. Aerial invertebrates caught in these locations contained relatively high concentrations of estrogenic compounds (Park and Cristinacce 2006). Secord *et al.* (2015) found 25 types of CECs in the carcasses. Almost all bat carcasses had bioaccumulated at least one type of antibiotic. Recent work (Cornelison *et al*. 2014; Hoyt *et al*. 2015) has shown probiotic inhibition of *Pseudogymnoascus destructans* (the causative fungal agent of WNS); thus, antibiotics may hinder a bat's natural defenses to the fungus. They also found that the highest average concentrations of CECs found were bisphenol A and PBDEs, both of which have been shown to inhibit immune function in mammals (Thuvander and Darnerud 1999; Vandenberg *et al.* 2013). Many of the CECs detected in this study can affect hibernation and immune function in bats, which may also make them more susceptible to WNS.

BATS AND ALGAL TOXINS

Though there has been extensive research on inorganic and organic CECs, there is only one record of bats being affected by naturally occurring toxins. Pybus *et al.* (1986) documented a mass mortality event of bats due to an algal toxin. In Alberta, Canada, over a thousand bats were found dead floating in a lake with green slime. This slime was determined to be *Anabaena flos-aquae*, which is capable of producing a neurotoxin called anatoxin-a. This toxin was found in high concentration in the bat carcasses. However, it is not clear if the bats ingested this toxin through contaminated drinking water or contaminated prey.

To date, only one published study has analyzed whether or not bats are exposed to microcystin. Feces from little brown bats (*Myotis lucifugus*) were shown to contain concentrations of MC similar to those found in volant *Hexagenia* from the same area (Woller-Skar *et al.* 2015). On average, both *Hexagenia* mayflies and *M. lucifugus* feces contained over 200 ppb (dry weight). This suggests that these bats are ingesting MC through trophic interactions. However, it remains to be seen whether or not bats exposed to MC are bioaccumulating this algal toxin in the liver.

WHAT IS MICROCYSTIN?

Microcystin (MC) is a secondary metabolite produced by cyanobacteria (Carmichael 1992). This naturally occurring hepatotoxin is produced by several genera of cyanobacteria including *Microcystis*, *Planktothrix, Anabaena*, *Hapalosiphon* (terrestrial), *Nostoc*, and *Anabaenopsis* (Carmichael *et al*. 1988), but was named for the first species in which it was found (*Microcystis aeruginosa*, Chorus and Bartram 1999). Microcystin occurs globally (Sivonen and Jones 1999, Zurawell *et al*. 2005) most commonly in fresh water, but has also been found in marine environments (Chorus and Bartram 1999).

Microcystin is a cyclic heptapeptide with the structure cyclo (D-Ala-L-X-D-MeAsp-L-Z-Adda-D-Glu-Mdha), where MeAsp is D-erythro-b-methylaspartic acid, Mdha is N-methyldehydroalanine, Adda is 2S, 3S, 8S, 9S-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4E, 6E-dienoic acid, and X and Z are variable L-amino acids (Carmichael *et al.* 1988). The Adda and Mdha groups bind to and inhibit protein phosphatases, making microcystin toxic to animals (MacKintosh *et al.* 1995). The structural variation in the X and Z amino acid groups results in different congeners of

microcystin. To date, over 80 congeners of microcystin have been found (Hoeger *et al*. 2005). The type of congener determines how toxic the molecule is, as the polarity influences the ease with which the toxin can pass through the bile acid transport system (Gorham and Carmichael 1988). Microcystin-LR (MC-LR; leucine in the X position and arginine in the Z position) is the most common and most toxic congener (Mirura *et al*. 1989; Sivonen and Jones 1999). Other common congeners with similar toxicological effects are MC-RR, MC-LA, and MC-YR (Yoshizawa *et al*. 1990). High chemical stability and water solubility allow MC to persist in the environment, thus increasing potential exposure to many organisms (Sivonen and Jones 1999).

TOXICOLOGICAL EFFECTS OF MICROCYSTIN

After an organism ingests MC, this toxin cannot penetrate cell membranes (Zurawell *et al*. 2005), therefore the toxin targets the liver through the bile acid transport system which actively transports peptides and biliary acids into hepatocytes (Gorham and Carmichael 1988). Once MC penetrates liver cells, the cells undergo apoptosis (Hooser 2000), which leads to bleeding, degredation of sinusoidal structure and architecture, (Falconer *et al.* 1981; Runnegar and Falconer 1982), and oxidative stress in the liver (Smith e*t al*. 2008). This architectural breakdown in the liver, and hepatocellular necrosis results in increased blood flow and enlargement of the liver which in turn decreases systemic arterial blood flow, ultimately resulting in death from hypovolemic shock in vertebrates (Zurawell *et al*. 2005). In addition to liver damage, Ueno *et al*. (1996) found a significant correlation between MC intake and high cancer rates in humans in China. However, there is not enough evidence to consider MC to be carcinogenic (Butler *et al.*

2009). Although the rate of metabolism of and the degree of resistance to MC depends on the species that is exposed to this toxin (Schmidt *et al.* 2014), MC has been shown to be more toxic to terrestrial mammals than to aquatic organisms (Sivonen and Jones 1999).

MICROCYSTIN POISONING

In a short-term study using mice, Fawell *et al.* (1999) found that oral doses of 2,000 μg MC-LR per kg of body weight (ppb) resulted in maternal toxicity that included both macroscopic changes in the liver and death of several individuals. Fetuses did exhibit lower weight and delayed ossification, but there was no evidence of fetal lethality, teratogenicity, or treatment-related physical abnormalities. With regards to developmental toxicity, a clear no observed adverse effect level (NOAEL) was determined at 600 ppb. In their long-term study spanning 13 weeks, doses of 200 ppb resulted in minimal effects with few changes in the livers of few animals. A clear NOAEL of MC-LR for pathological changes in mouse liver was determined at 40 ppb. This study confirmed that the World Health Organization's recommendation for MC-LR levels in safe drinking water (1 ppb) was adequate. This long term, clear NOAEL was used to create a tolerable daily intake (TDI) for humans over a lifetime at 0.04 ppb (Sivonen and Jones 1999).

Although ingestion of MC typically results in minor symptoms such as nausea, vomiting, and diarrhea (WHO 2003), there has been one documented case involving multiple human fatalities due to MC (Jochimsen *et al.* 1998; Carmichael *et al.* 2001; Azevedo *et al*. 2002). In 1996, over one hundred patients were exposed to MC while receiving dialysis in Brazil. The use of untreated water led to the death of over 50

patients. Contaminated water can be a health threat to humans and many other organisms, but it is not the only route of exposure of wildlife to MC.

TROPHIC TRANSFER OF MC

Intoxication due to MC has been documented in many animals such as livestock, dogs, deer, and aquatic organisms such as freshwater turtles, waterfowl, and fish (Roegner *et al*. 2014). However, not all poisoning occurs directly from drinking contaminated water. There has been much debate as to whether or not MC bioaccumulates or biodilutes in food webs. Kozlowsky-Suzuki *et al*. (2012) used a meta-analysis of 42 studies and found that, in general, MC tends to biodilute in food webs rather than bioaccumulate, but this trend is species-dependent. Zooplankton and zooplanktivorous fish show potential for biomagnification of MC. Kozlowski-Suzuki *et al.* (2012) also found that several factors influence the bioaccumulation of MC. For example, the availability of alternative nontoxic prey items may influence the accumulation of MC, possibly decreasing the intake of toxic prey. Similarly, the length of exposure impacts the accumulation of MC.

To date, few studies have focused on the trophic transfer of MC between ecosystems. Livestock and pets have been reported most frequently with MC poisoning from drinking contaminated water, but not through the ingestion of contaminated food sources. Fewer examples are known from natural systems. Miller *et al*. (2010) found that MC moved from freshwater lakes into Monterey Bay, California, resulting in the death of several sea otters. Once in marine water, high concentrations of MC bioaccumulated in invertebrates such as snails, crabs, and several species of bivalves, which are food

sources for sea otters. The affected sea otters showed symptoms of hepatocellular vacuolation, apoptosis, necrosis and hemorrhage consistent with MC intoxication, whereas two captive sea otter livers did not. Furthermore, chemical testing using liquid-chromatography with tandem mass spectrometry (LC-MS/MS) showed that the sea otter livers contained concentrations of MC ranging from 1.36-104.46 ppb wet weight. Although these sea otters were impacted by MC found in a marine environment, Miller *et al.* (2010) were able to trace the toxin back to a freshwater lake that frequently has large blooms of *M. aeruginosa.*

MICROCYSTIS AERUGINOSA

Microcystin can impact wildlife both in the water column and in the sediments of lakes. How and when this toxin is bioaccumulated by other organisms depends on the life cycle of *M. aeruginosa*, one of the principal producers of MC. The annual life cycle of *M. aeruginosa* is divided into four main stages (Ihle *et al*. 2005). In the spring, *M. aeruginosa* cells move from the benthic zone to the pelagic zone. During the summer, these cells then experience population growth that results in bloom formation. The length and timing of these blooms depends on a combination of biological, chemical, and physical factors such as lake depth, stratification, and temperature, resulting in large annual fluctuations in levels of this cyanobacteria and its toxins (Sivonen and Jones 1999). In natural settings, many strains of *M. aeruginosa* may produce different combinations of MC congeners (Sivonen and Jones 1999).

Blooms of *M. aeruginosa* may be exacerbated by human alterations including eutrophication, climate change (Paerl and Huisman 2008), and invasive species such as

zebra mussels. Established populations of zebra mussels change aquatic communities via filter feeding (Bastviken *et al.* 1998). Zebra mussels consume and remove algal species that compete with *M. aeruginosa* (Fahnenstiel *et al*. 1995; Vanderploeg *et al*. 2001), but selectively reject *M. aeruginosa* (Vanderploeg *et al*. 2001). There is high spatial and temporal variation in MC production. MC production is regulated at the genetic, cellular, and population level (Zurawell *et al.* 2005).

In late summer and early autumn, living *M. aeruginosa* cells sink to the benthic zone and return to the sediment. Typically, MC is contained within the cells of *M. aeruginosa* and is released when a cell lyses (Sivonen and Jones 1999). Vegetative cells of *M. aeruginosa* then overwinter in lake sediments where they may be ingested by other organisms overwintering in sediments. One benthic invertebrate that is particularly susceptible to ingesting overwintering cells of *M. aeruginosa* is the burrowing mayfly, *Hexagenia.*

LIFE CYCLE OF *HEXAGENIA*

Hexagenia mayflies are widely distributed throughout North and South America (Edmunds *et al.* 1976). In the Great Lakes region, the most commonly occurring species are *H. limbata, H. rigida* (Corkum *et al*. 1997)*,* and *H. bilineata* (Cochran 1992; Cochran and Kinziger 1997). The species in this genus are characterized by large, synchronous emergences of adults in spring and summer (Hunt 1953). *Hexagenia* spend the majority of their lives (1-2 years), as nymphs that burrow into the sediments of lakes. In the Great Lakes region, populations of *Hexagenia* spend two years as nymphs and populations consist of two overlapping cohorts. As the nymphs overwinter in sediment, they feed on

detritus and algae (Hunt 1953) from which they may ingest toxic *M. aeruginosa*. They may also be exposed to microcystin as they move water through their burrows with their gills.

Once mature, nymphs come to the surface of the lake where they molt into subimagos (subadults, Hunt 1953). Within a day or two, subimagos molt again into sexually mature imagos (adults) which swarm to mate (Hunt 1953). Females deposit their eggs on the surface of the water, and spent imagos die within a few hours of mating (Hunt 1953). The eggs sink to the lake sediment and hatch after several weeks (Edsall 2001). As volant subimagos and imagos, *Hexagenia* may be a potential food source for terrestrial animals that did not have access to them in their aquatic life stages.

Their unique life history make this aquatic insect highly susceptible to bioaccumulating MC. Individual *Hexagenia* mayflies have been shown to contain high concentrations of MC at the nymph (Woller-Skar 2009) and volant stages (Woller-Skar *et al.* 2015) of their life cycle. In Little Traverse Lake (Leelanau County, Michigan, USA), *Hexagenia* nymphs have been shown to contain over 80,000 ppb MC-eq while volant (both subadult and adult) *Hexagenia* contain an average of 200 ppb MC-eq (Woller-Skar *et al*. 2015). Thus, these emerging aquatic insects can potentially move high concentrations of MC from freshwater lakes to terrestrial ecosystems when they are consumed by terrestrial predators.

Conclusions and Future Directions

This study is the first to show that *M. lucifugus* are ingesting microcystin through trophic transfer and that *M. lucifugus* are excreting most of this toxin, rather than

bioaccumulating it in the liver. Consequently, this study shows that *Hexagenia* mayflies are one route of transfer of this naturally occurring algal toxin from a freshwater ecosystem into a terrestrial ecosystem. It is interesting that *M. lucifugus* do not seem to bioaccumulate microcystin in their livers. From these results, it appears that *M. lucifugus* populations are only minimally affected by microcystin if at all. Future research should focus on depuration rates of MC in bats to determine if bats are unique in their ability to excrete rather than bioaccumulate this hepatotoxin. To monitor the health of bat populations, future work should focus on quantifying MC in bat feces throughout the year. There may be temporal shifts in MC accumulation in their feces that may indicate when bats are at the greatest risk for MC poisoning. It would also be interesting to monitor insect diversity in tandem with dietary analyses of bat feces to determine if they are avoiding toxic prey. Furthermore, future work should also focus on measuring MC concentrations in other emerging aquatic insects that may also be prey for riparian predators like *M. lucifugus* and, therefore, potential routes of trophic transfer of microcystin. Additionally, researchers should determine other potential terrestrial receptors of this toxin such as birds, reptiles, and amphibians. Research documenting the movement of this algal toxin through ecosystems may provide insight into the interconnectedness of ecosystems and may help predict ecosystem responses to disturbance such as trophic cascades.

LITERATURE CITED

ALBERDI A., GARIN I., AIZPURUA O., AND AIHARTZA J. 2012. The foraging ecology of the mountain long-eared bat *Plecotus macrobullaris* revealed with DNA mini-barcodes. PLoS ONE, 7: e35692.

ANTHONY E. L. P., AND KUNZ T. H. 1977. Feeding strategies of the little brown bat, *Myotis lucifugus*, in southern New Hampshire. Ecology, 58: 775–786.

AVISE J. 1994. Molecular markers, Natural History and Evolution. Chapman & Hall, New York.

AZEVEDO S., CARMICHAEL W.W., JOCHIMSEN E., RINEHART K., LAU S., SHAW G., AND EAGLESHAM G. K. 2002. Human intoxication by microcystins during renal dialysis treatment in Caruaru Brazil. Toxicology, 181-182: 441–446.

BARON L., SAMPLE B.E., AND SUTER G.W. 1999. Ecological risk assessment in a large river-reservoir: 5. Aerial insectivorous wildlife. Environmental Toxicology and Chemistry, 18: 621–627.

BASTVIKEN D., CARACO N., AND COLE J. 1998. Experimental measurements of zebra mussel (*Dreissena polymorpha*) impacts on phytoplankton community composition. Freshwater Biology, 39: 375–386.

BAXTER C., FAUSCH K., AND CAUNDERS W. 2005. Tangled webs: reciprocal flows of invertebrate prey link streams and riparian zones. Freshwater Biology, 50: 201–220.

BAYAT S., GEISER F., KRISTIANSEN P., AND WILSON S. 2014. Organic contaminants in bats: trends and new issues. Environment International, 63: 40–52.

BELWOOD J., AND FENTON M. 1976. Variation in the diet of *Myotis lucifugus* (Chiroptera: Vespertilionidae). Canadian Journal of Zoology, 54: 1674–1678.

BOHMANN K., MONADJEM A., NOER C. L., RASMUSSEN M., ZEALE M. R. K., CLARE E., JONES G., WILLERSLEV E., AND GILBERT M. T. P. 2011. Molecular diet analysis of two African free-tailed bats (Molossidae) using high throughput sequencing. PLoS ONE, 6: e21441.

BROWN W. 1985. The mitochondrial genome of animals. Pp. 95–130, *in* Molecular Evolutionary Genetics (R. MACINTYRE, ed.). Plenum Press, New York.

BUCHLER E. R. 1976. Prey selection by *Myotis lucifugus* (Chiroptera: Vespertilionidae). The American Naturalist, 110: 619–628.

BUTLER N., CARLISLE J. C., LINVILLE R., AND WASHBURN B. 2009. Microcystins: A brief overview of their toxicity and effects, with special reference to fish, wildlife and livestock. Program, Ecotoxicology Risk, Integrated Branch, Assessment Environmental, California Agency, Protection.

CARMICHAEL W. W. 1992. Cyanobacteria secondary metabolites— the cyanotoxins. A review. Journal of Applied Bacteriology, 72: 445–459.

CARMICHAEL W. W. , AZEVEDO S. M. F. O., AN J. S., MOLICA R. J. R., JOCHIMSEN E. M., LAU S., RINEHART K. L., SHAW G. R., AND EAGLESHAM G. K. 2001. Human fatalities from cyanobacteria: Chemical and biological evidence for cyanotoxins. Environmental Health Perspectives, 201: 663–668.

CARMICHAEL W.W., BEASLEY V., BUNNER D. L., ELOFF J. N., FALCONER I., GORHAM P., HARADA K.-I., KRISHNAMURTHY T., MIN-JUAN Y., MOORE R. E., RINEHART K., RUNNEGAR M., SKULBERG O. M., AND WATANABE M.1988. Naming of cyclic heptapeptide toxins of cyanobacteria (blue-green algae) (Letter). Toxicon, 26: 971–973.

CHORUS I. AND BARTRAM J. 1999. Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management. Routledge Press, London.

CLARE E. L., BARBER B. R., SWEENEY B. W., HEBERT P. D. N. AND FENTON M. B. 2011. Eating local: Influences of habitat on the diet of little brown bats (*Myotis lucifugus*). Molecular Ecology, 20: 1772–1780.

CLARE E. L., FRASER E. E., BRAID H. E., FENTON M. B., AND HEBERT P. D. N. 2009. Species on the menu of a generalist predator, the eastern red bat (*Lasiurus borealis*): Using a molecular approach to detect arthropod prey. Molecular Ecology, 18: 2532–2542.

CLARE E. L., SYMONDSON W. O. C., BRODERS H., FABIANEK F., FRASER E. E., MACKENZIE A., BOUGHEN A., HAMILTON R., WILLIS C. K. R., MARTINEZ-NUÑEZ F., MENZIES A. K., NORQUAY K. J. O., BRIGHAM M., POISSANT J., RINTOUL J., BARCLAY R. M. R., AND REIMER J. P. 2014. The diet of *Myotis lucifugus* across Canada: Assessing foraging quality and diet variability. Molecular Ecology, 23: 3618–3632.

CLARE E. L., SYMONDSON W. O. C., AND FENTON M. B. 2013. An inordinate fondness for beetles? Variation in seasonal dietary preferences of night-roosting big brown bats (*Eptesicus fuscus*). Molecular Ecology, 23: 3633-3647.

CLARK D. R. 1988. How sensitive are bats to insecticides? Wildlife Society Bulletin, 16: 399–403.

CLARK D. R. 2001. DDT and the decline of free-tailed bats (*Tadarida brasiliensis*) at Carlsbad Cavern, New Mexico. Archives of Environmental Contamination and Toxicology, 40: 537–543.

CLARK D. AND STAFFORD C. 1981. Effects of DDE and PCB (Aroclor 1260) on

experimentally poisoned female little brown bats (*Myotis lucifugus*): Lethal brain concentrations. Journal of Toxicology and Environmental Health, 7: 925–934.

COCHRAN P. 1992. The return of *Hexagenia* (Ephemeroptera: Ephemeridae) to the lower Fox River, Wisconsin. Great Lakes Entomology, 25: 79–81.

COCHRAN P., AND KINZIGER A. 1997. *Hexagenia limbata* (Ephemeroptera: Ephemeridae) persists at low levels of abundance in the lower Fox River, Wisconsin. Great Lakes Entomology, 30: 89–92.

CONSTANTINE D. G. 1967. Activity patterns of the Mexican free-tailed bat. University of New Mexico Publications in Biology, 7: 1-79.

CORKUM L., CIBOROWSKI J., AND POULIN R. 1997. Effects of emergence date and maternal size on egg development and sizes of eggs and first-instar nymphs of a semelparous aquatic insect. Oecologia, 111: 60–75.

CORNELISON C., KEEL M., GABRIEL K., BARLAMENT C., TUCKER T., PIERCE G., AND CROW S. A. 2014. A preliminary report on the contact-independent antagonism of *Pseudogymnoascus destructans* by *Rhodococcus rhodochrous* strain DAP96253. BMC Microbiology, 14: e246.

COUTTS R., FENTON M., AND GLEN E. 1973. Food intake by captive *Myotis lucifugus* and *Eptesicus fuscus* (Chiroptera: Vespertilionidae). Journal of Mammalogy, 54: 985–990.

CRISTOL D. A., BRASSO R. L., CONDON A. M., FOVARGE R. E., FRIEDMAN S. L., HALLINGER K. K., MONROE A. P., AND WHITE A. E. 2008. The movement of aquatic mercury through terrestrial food webs. Science, 320: 335.

DEAGLE B. E., EVESON J. P., AND JARMAN S. N. 2006. Quantification of damage in DNA

recovered from highly degraded samples- a case study on DNA in faeces. Frontiers in Zoology, 3: e11.

DEAGLE B. E., THOMAS A. C., SHAFFER A. K., TRITES A. W., AND JARMAN S. N. 2013. Quantifying sequence proportions in a DNA-based diet study using Ion Torrent amplicon sequencing: Which counts count? Molecular Ecology Resources, 13: 620–633.

DEAGLE B. E., AND TOLLIT D. J. 2007. Quantitative analysis of prey DNA in pinniped faeces: Potential to estimate diet composition?. Conservation Genetics, 8: 742-747.

DEAGLE B. E., TOLLIT D. J., JARMAN S. N., HINDELL M. A., TRITES A. W., AND GALES N. J. 2005. Molecular scatology as a tool to study diet: Analysis of prey DNA in scats from captive Steller sea lions. Molecular Ecology, 14: 1831–1842.

EASTERLA D., AND WHITAKER J. 1972. Food habits of some bats from Big Bend National Park, Texas. Journal of Mammalogy, 53: 887–890.

EDSALL T. A. 2001. Burrowing mayflies (*Hexagenia*) as indicators of ecosystem health. Aquatic Ecosystem Health and Management, 4: 283–292.

EMRICH M., CLARE E. L., SYMONDSON W. O. C., KOENIG S. E., AND FENTON M. B. 2014. Resource partitioning by insectivorous bats in Jamaica. Molecular Ecology, 23: 3648–3656.

FAHNENSTIEL G. L., LANG A. G., NALEPA T. F., AND JOHENGEN T. H. 1995. Effects of zebra mussel (*Dreissena polymorpha*) colonization on water quality parameters in Saginaw Bay, Lake Huron. Journal of Great Lakes Research, 21: 435–448.

FALCONER I., JACKSON A., LANGLEY J., AND RUNNEGAR M. 1981. Liver pathology in mice poisoning by the blue-green alga *Microcystis aeruginosa*. Australian Journal of

Biological Science, 24: 179–187.

FAWELL J. K., MITCHELL R. E., EVERETT D. J., AND HILL R. E. 1999. The toxicity of cyanobacterial toxins in the mouse: I microcystin-LR. Human & Experimental Toxicology, 18: 162–167.

FOLMER O., BLACK M., HOEH W., LUTZ R., AND VRIJENHOEK R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3: 294–299.

FRICK W. F., POLLOCK J. F., HICKS A. C., LANGWIG K. E., REYNOLDS D. S., TURNER G. G., BUTCHKOSKI C. M., AND KUNZ T. H. 2010. An emerging disease causes regional population collapse of a common North American bat species. Science, 329: 679–682.

GORHAM P., AND CARMICHAEL W.W. 1988. Hazards of freshwater blue-green algae (cyanobacteria). *in* Algae and Human Affairs (LEMBI C., AND WAALAND J., eds.). Cambridge University Press, Cambridge, UK, 403–431 pp.

HAJIBABAEI M., SMITH M. A., JANZEN D. H., RODRIGUEZ J. J., WHITFIELD J. B., AND HEBERT P. D. N. 2006. A minimalist barcode can identify a specimen whose DNA is degraded. Molecular Ecology Notes, 6: 959–964.

HEBERT P. D. N., CYWINSKA A., BALL S. L., AND DEWAARD J. R. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society B Biological Sciences, 270: 313–321.

HEBERT P.D.N., PENTON E.H., BURNS J.M., JANZEN D.H. AND HALLWACHS W. 2004. Ten species in one: DNA barcoding reveals cryptic species in the Neotropical skipper butterfly *Astraptes fulgerator*. Proceedings of the National Academy of Sciences of

the United States of America, 101: 14812–14817.

HOEGER S., HITZFELD B., AND DIETRICH D. 2005. Occurrence and elimination of cyanobacterial toxins in drinking water treatment plants. Toxicology and Applied Pharmacology, 203: 231–242.

HOOSER S. 2000. Fulminant hepatocyte apoptosis *in vivo* following microcystin-LR administration to rats. Toxicologic Pathology, 28: 726–733.

HOYT J., CHENG T., LANGWIG K., HEE M., FRICK W., AND KILPATRICK A. 2015. Bacteria isolated from bats inhibit the growth of *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome. PLoS ONE, 10: e0121329.

HUISMAN J., MATTHIJS H., AND VISSER P. 2005. Harmful Cyanobacteria. Springer, Dorcrecht, Netherlands, 240 pp.

HUNT B. 1953. The life history and economic importance of a burrowing mayfly, *Hexagenia limbata*, in southern Michigan lakes. Bulletin of the Institute for Fisheries Research. 151 pp.

IHLE T., JÄHNICHEN S., AND BENNDORF J. 2005. Wax and wane of *Microcystis* (Cyanophyceae) and microcystins in lake sediments: A case study in Quitzdorf Reservoir (Germany). Journal of Phycology, 41: 479–488.

JOCHIMSEN E., CARMICHAEL W. W., AN J., CARDO D. M., COOKSON S. T., HOLMES C. E. M., ANTUNES M. B. DE C., FIHLO D. A. DE M., LYRA T. M., BARRETO V. S. T., AZEVEDO S. M. F. O., AND JARVIS W. R. 1998. Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. The New England Journal of Medicine, 338: 873–878.

KANNAN K., YUN S. H., RUDD R. J., AND BEHR M. 2010. High concentrations of persistent

organic pollutants including PCBs, DDT, PBDEs and PFOS in little brown bats with white-nose syndrome in New York, USA. Chemosphere, 80: 613–618.

KOZLOWSKY-SUZUKI B., WILSON A. E. AND FERRA S. 2012. Biomagnification or biodilution of microcystins in aquatic foodwebs? Meta-analyses of laboratory and field studies. Harmful Algae, 18: 47–55.

KRUGER F., CLARE E. L., GREIF S., SIEMERS B. M., SYMONDSON W. O. C., AND SOMMER R. S. 2014. An integrative approach to detect subtle niche differentiation in the sympatric trawling bat species *Myotis dasycneme* and *Myotis daubentonii*. Molecular Ecology, 23: 3657-3671.

KUNZ T. 1974. Feeding ecology of a temperate insectivorous bat (*Myotis velifer*). Ecology, 55: 693–711.

KUNZ T. H. AND WHITAKER J. O. 1983. An evaluation of fecal analysis for determining food habits of insectivorous bats. Canadian Journal of Zoology, 61: 1317-1321.

KURTA A., BELL G. P., NAGY K., AND KUNZ T. H. 1989. Energetics of pregnancy and lactation in free-ranging little brown bats (*Myotis lucifugus*). Physiological Zoology, 62: 804–818.

LITTLE M. E., BURGESS N. M., BRODERS H. G., AND CAMPBELL L. M. 2015. Mercury in little brown bat (*Myotis lucifugus*) maternity colonies and its correlation with freshwater acidity in Nova Scotia, Canada. Environmental Science and Technology, 49: 2059-2065.

LYNCH M., AND JARRELL P. 1993. A method for calibrating molecular clocks and its application to animal mitochondrial DNA. Genetics, 135: 1197–1208.

MACKINTOSH R., DALBY K., CAMPBELL D., COHEN P., COHEN P. AND MACKINTOSH C.

1995. The cyanobacterial toxin microcystin binds covalently to cysteine-273 on protein phosphatase 1. FEBS Letters, 371: 236–240.

MENZIE C. A. 1980. Potential significance of insects in the removal of contaminants from aquatic ecosystems. Water, Air, and Soil Pollution, 13: 473–479.

MEUSNIER I., SINGER G. A. C., LANDRY J.-F., HICKEY D. A., HEBERT P. D. N. AND HAJIBABAEI M. 2008. A universal DNA mini-barcode for biodiversity analysis. BMC Genomics, 9: e214.

MILLER M. A., KUDELA R. M., MEKEBRI A., CRANE D., OATES S. C., TINKER M. T., STAEDLER M., MILLER W. A., TOY-CHOUTKA S., DOMINIK C., HARDIN D., LANGLOIS G., MURRAY M., WARD K., AND JESSUP D. A. 2010. Evidence for a novel marine harmful algal bloom: Cyanotoxin (microcystin) transfer from land to sea otters. PLoS ONE, 5: e12576.

MIRURA G., ROBINSON N., GEISBERT T., BOSTIAN K., WHITE J., AND PACE J. 1989. Comparison of *in vivo* and *in vitro* toxic effects of microcystin-LR in fasted rats. Toxicon, 27: 1229–1240.

O'SHEA T., AND JOHNSTON J. 2009. Environmental contaminants and bats: Investigating exposure and effects. Pp. 500–528 in Ecological and Behavioral Methods for the Study of Bats (KUNZ T., AND PARSONS S., eds.). 2nd edition. The Johns Hopkins University Press, Baltimore, 901 pp.

PAERL H. W., AND FULTON R. 2006. Ecology of harmful cyanobacteria. *in* Ecology of Harmful Algae (GRANELI E. AND TURNER J. T. eds.). Springer: Berlin, 95-109 pp.

PAERL H. W., AND HUISMAN J. 2008. Blooms like it hot. Science, 320: 57–58.

PARK K., AND CRISTINACCE A. 2006. Use of sewage treatment works as foraging sites by

insectivorous bats. Animal Conservation, 9: 259–268.

PINE R. 1969. Stomach contents of a free-tailed bat, *Molossus ater*. Journal of Mammalogy, 50: 162.

POMPANON F., DEAGLE B. E., SYMONDSON W. O. C., BROWN D. S., JARMAN S. N., AND TABERLET P. 2012. Who is eating what: Diet assessment using next generation sequencing. Molecular Ecology, 21: 1931–1950.

PYBUS M., HOBSON D., AND ONDERKA D. 1986. Mass mortality of bats due to probable blue-green algal toxicity. Journal of Wildlife Diseases, 22: 449–450.

RABINOWITZ A. R., AND TUTTLE M. D. 1982. A test of the validity of two currently used methods of determining bat prey preferences. Acta Theriologica, 27: 283–293.

RATNASINGHAM S., AND HEBERT P. 2007. BOLD: the barcode of life data system (http://www.barcodinglife.org). Molecular Ecology Notes, 7: 355–364.

RAZGOUR O., CLARE E. L., ZEALE M. R. K., HANMER J., SCHNELL I. B., RASMUSSEN M., GILBERT T. P., AND JONES G. 2011. High-throughput sequencing offers insight into mechanisms of resource partitioning in cryptic bat species. Ecology and Evolution, 1 (4): 556–570.

ROEGNER A. F., BRENA B., GONZÁLEZ-SAPIENZA G., AND PUSCHNER B. 2014. Microcystins in potable surface waters: Toxic effects and removal strategies. Journal of Applied Toxicology, 34: 441–457.

RUNCK C. 2007. Macroinvertebrate production and food web energetics in an industrially contaminated stream. Ecological Applications, 17: 740–753.

RUNNEGAR M., AND FALCONER I. 1982. The *in vivo* and *in vitro* biological effects of the peptide hepatotoxin from the blue-green alga *Microcystis aeruginosa*. South African

Journal of Science, 78: 363–366.

SACCONE C., DECARLA G., GISSI C., PESOLE G., AND REYNES A. 1999. Evolutionary genomics in the Metazoa: the mitochondrial DNA as a model system. Gene, 238: 195–210.

SCHMIDT J. R., WILHELM S. W., AND BOYER G. L. 2014. The fate of microcystins in the environment and challenges for monitoring. Toxins, 6: 3354–3387.

SECORD A. L., PATNODE K. A., CARTER C., REDMAN E., GEFELL D. J., MAJOR A. R., AND SPARKS D. W. 2015. Contaminants of emerging concern in bats from the Northeastern United States. Archives of Environmental Contamination and Toxicology, 69: 411-421.

SIVONEN K. AND JONES G. 1999. Cyanobacterial toxins. *in* Toxic cyanobacteria in water, a guide to their health public health consequences, monitoring (CHORUS I. AND BARTRAM J., eds.). Routledge Press, London. 41–111 pp.

SMITH J. L., BOYER G. L., AND ZIMBA P. V. 2008. A review of cyanobacterial odorous and bioactive metabolites: Impacts and management alternatives in aquaculture. Aquaculture, 280: 5–20.

THUVANDER A., AND DARNERUD P. 1999. Effects of polybrominated diphenyl ether (PBDE) and polychlorinated biphenyl (PCB) on some immunological parameters after oral exposure in rats and mice. Toxicological & Environmental Chemistry, 70: 229–242.

UENO Y., NAGATA S., TSUTSUMI T., HASEGAWA A., WATANABE M., PARK H.-D., CHEN G. C., CHEN G., AND YU S. Z. 1996. Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of

primary liver cancer in China, by highly sensitive immunoassay. Carcinogenesis, 17: 1317–1321.

VANDENBERG L., EHRLICH S., BELCHER S., BEN-JONATHAN N., DOLINOY D., HUGO E., HUNT, P. A., NEWBOLD R. R., RUBIN B. S., SAILI K.S., SOTO, A. M., WANG, H. S., AND VOM SAAL F. S. 2013. Low dose effects of bisphenol A: An integrated review of *in vitro*, laboratory animal, and epidemiology studies. Endocrine Disruptors, 1: e25078.

VANDERPLOEG H. A., LIEBIG J. R., CARMICHAEL W. W., AGY M. A., JOHENGEN T. H., FAHNENSTIEL G. L., AND NALEPA T. F. 2001. Zebra mussel (*Dreissena polymorpha*) selective filtration promoted toxic *Microcystis* blooms in Saginaw Bay (Lake Huron) and Lake Erie. Canadian Journal of Fisheries and Aquatic Sciences, 58: 1208–1221.

VESTERINEN E. J., LILLEY T., LAINE V. N., AND WAHLBERG N. 2013. Next generation sequencing of fecal DNA reveals the dietary diversity of the widespread insectivorous predator Daubenton's Bat (*Myotis daubentonii*) in southwestern Finland. PLoS ONE, 8(11): e82168.

VESTERINEN E. J., RUOKOLAINEN L., WAHLBERG N., PENA C., ROSLIN T., LAINE V. N., VASKO V., SAAKSJARVI I. E., NORRDAHL K., AND LILLEY T. M. 2016. What you need is what you eat? Prey selection by the bat *Myotis daubentonii*. Molecular Ecology, 25: 1581–1594.

WHITAKER J. 1972. Food habits of bats from Indiana. Canadian Journal of Zoology, 50: 877–883.

WHO. 2003. Cyanobacterial toxins: Microcystin-LR in drinking-water. Background document for development of WHO Guidelines for Drinking-water Quality. in

Guidelines for drinking-water quality.

WOLLER-SKAR M. M. 2009. Zebra mussel (*Dreissena polymorpha*) promotion of cyanobacteria in low-nutrient lakes and the subsequent production and fate of microcystin. Ph.D. Dissertation, Bowling Green State University.

WOLLER-SKAR M. M., JONES D. N., RUSSELL A. L., AND LUTTENTON M. R. 2015. Microcystin detected in little brown bats (*Myotis lucifugus*). American Midland Naturalist, 174: 331–334.

YOSHIZAWA S., MATSUSHIMA R., WATANABE M., HARADA K-I., ICHIHARA A., CARMICHAEL W.W., AND FUJIKI H. 1990. Inhibition of protein phosphatases by microcystins and nodularin associated with hepatoxicity. Journal of Cancer Research and Clinical Oncology, 116: 609–614.

VANDER ZANDEN M. J., AND SANZONE D. M. 2004. Food web subsidies at the land-water ecotone. *in* Food webs at the landscape level (POLIS G. A., POWER M. E. AND HUXEL G. R., eds.). The University of Chicago Press, Chicago, Illinois. 185–188 pp.

ZEALE M. R. K., BUTLIN R. K., BARKER G. L. A., LEES D. C., AND JONES G. 2011. Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. Molecular Ecology Resources, 11: 236–244.

ZURAWELL R. W., CHEN H., BURKE J. M., AND PREPAS E. E. 2005. Hepatotoxic cyanobacteria: A review of the biological importance of microcystins in freshwater environments. Journal of Toxicology and Environmental Health, Part B 8:1–37.