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STOMACH CONTENT ANALYSIS OF BOTULISM-AFFECTED BIRDS IN LAKE MICHIGAN

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STOMACH CONTENT ANALYSIS OF BOTULISM-AFFECTED BIRDS IN LAKE
MICHIGAN

By

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ABSTRACT

STOMACH CONTENT ANALYSIS OF BOTULISM-AFFECTED BIRDS IN LAKE

MICHIGAN

By

David A. Essian

Avian botulism type E has caused large-scale bird and fish die-offs on the Great Lakes

annually since 1998, and continues to threaten breeding and migratory waterbirds in the

region. From 2010 to 2012, several northern Lake Michigan beaches were monitored for

avian mortalities by National Park Service, Common Coast Research and Conservation,

and U.S. Geological Survey personnel, partners, and volunteers. Bird carcasses were

collected and tested for the presence of botulinum toxin type E (BoNT/E) at the USGS

National Wildlife Health Center. I compare the prey composition and prey sizes

consumed by the birds that were collected. Additionally, I examine the diets of culled

double-crested cormorants (*Phalacrocorax auritus*) from three breeding colonies on

northern Lake Michigan during the 2012 breeding season to use as BoNT/E-free

references. Round gobies were found in 86% of the BoNT/E-positive birds, 76% of the

BoNT/E-negative birds, and 94% of the BoNT/E-free cormorants examined in this study.

The grand mean total length of gobies consumed by bird species ranged from 70.82 ±

3.42 mm in horned grebes (*Podiceps auritus*) to 120.60 ± 11.83 mm in ring-billed gulls.

There were significant intraspecies differences in the stomach content of ring-billed gulls

that were compared across collection period (June – July versus August – October) and of

BoNT/E-free double-crested cormorants that were compared across location and age

i

group. These results provide further evidence linking round gobies to BoNT/E outbreaks in fish-eating waterbirds. The stomach contents found in BoNT/E-affected birds suggests multiple possible pathways for BoNT/E transmission in different groups of birds (e.g.transfer through benthic macroinvertebrates to mussel-eating ducks vs. transfer through live fish to top-level fish-eating birds).

ACKNOWLEDGMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	…………………….	Adult
BELL	…………………….	Bellow Island, Lake Michigan
BoNT	………………..........	*botulinum* neurotoxin
BoNT/E	…………………….	Type E *botulinum* neurotoxin
F	…………………….	Female
FRET	…………………….	Förster Resonance Energy Transfer
GI	…………………….	Gastrointestinal
GULL	…………………….	Little and Big Gull Islands, Lake Michigan
J	…………………….	Juvenile
LPSB	…………………….	Ludington Pump Storage Breakwall
M	…………………….	Male
n	…………………….	Sample size
NMU	…………………….	Northern Michigan University
NWHC	…………………….	National Wildlife Health Center
S.E.	…………………….	Standard error
TL	…………………….	Total Length
USGS	…………………….	United States Geological Survey

CHAPTER ONE: BACKGROUND

The earliest recorded avian botulism die-offs in Lake Michigan occurred throughout the 1960's (Fay et al., 1965), in 1976, and in the early 1980's (Brand et al., 1988). Outbreaks were not recorded again until the late 1990's. However, since the late 1990's there have been regular die-offs in the Great Lakes, and they have occurred annually in Lake Michigan since 2006. Fish-eating and mussel-eating birds generally account for the most mortalities. Over 110,000 bird deaths in the Great Lakes have been attributed to avian botulism since 1963, most of which occurred since 1998 (Environment Canada and US EPA, 2014). Outbreaks contributed to the extirpation of breeding great black-backed gulls (*Larus marinus*) on Lake Ontario (Shutt et al., 2014), and affected federally endangered piping plover (*Charadrius melodus*) (Roche et al., 2010). Die-offs are often conspicuous, leaving hundreds of bird carcasses stranded on Great Lakes beaches. Avian botulism has generated public concern in the Great Lakes region, not only because of the ecological impacts, but because of perceived public health risks. This has spurred an effort to determine the environmental and biological factors that contribute to these outbreaks.

Clostridium botulinum is a globally distributed anaerobic bacteria that produces spores which can persist in freshwater and marine sediments for up to 30 years. *Botulinum* neurotoxin (BoNT) is a single polypeptide chain that is released from lysed, vegetative *C. botulinum* cells. There are seven *C. botulinum* serotypes (A-G) which are classified based on the structure and function of the BoNT. *Botulinum* neurotoxins

interfere with vertebrate neurotransmission by disabling binding proteins on the synaptic

vesicle or the presynaptic membrane, resulting in paralysis of the extremities (Wilson et

al., 2002). Type E *botulinum* toxin (BoNT/E), which inactivates the SNAP-25 protein, is

the serotype responsible for recent bird die-offs in the Great Lakes. Waterbirds that ingest

lethal doses of the toxin can die from drowning when paralysis of the neck muscles

prevent them from holding their heads above water (Rocke and Friend, 1999).

Several methods of BoNT/E detection in wildlife have been used in the Great

Lakes (Getchell et al., 2006; Piazza et al., 2011; Skerratt et al., 2005). Since only

vegetative *C. botulinum* cells produce the toxin, birds could ingest *C. botulinum* spores

without exhibiting symptoms of BoNT intoxication. Diagnosis of BoNT/E intoxication

must be carried out before decomposition occurs to reliably diagnose BoNT/E as the

cause of death, because decomposing plant and animal tissues create anaerobic

environments that promote *C. botulinum* growth. In the past, mouse bioassays have been

used to diagnose wildlife suspected of carrying BoNT/E (Skerratt et al., 2005). A recently

developed *in vitro* method of BoNT/E detection using Förster resonance energy transfer

(FRET) is less expensive, requires less care, and presents fewer ethical issues (Piazza et

al., 2011).

Warmer water temperatures are known to facilitate type C botulism outbreaks in

shallow wetlands (Rocke and Samuel, 1999), and Lafrancois et al. (2011) showed a

correlation between botulism outbreaks, lake levels, and surface temperatures in Lake

Michigan. Low lake levels may promote *C. botulinum* growth by stranding nearshore

macroinvertebrates and vegetation which would create a favorable microhabitat for *C.*

botulinum when stranded organisms begin to decompose (Lafrancois et al., 2011).

Moreover, higher temperatures would improve growing conditions for *C. botulinum* by reducing levels of dissolved oxygen (Lafrancois et al., 2011). There were higher rates of BoNT/E detection in sediments with low dissolved oxygen, pH, and redox potentials (Perez-Fuentetaja et al., 2006), and annual variation in these values corresponded with water temperatures (Perez-Fuentetaja et al., 2011). These parameters are also likely important drivers of type C botulism outbreaks (Barras and Kadlec, 2000; Rocke and Samuel, 1999).

Recent type E avian botulism die-offs have also coincided with major ecological changes in the Great Lakes. Nutrients have been redirected to the benthos (Dayton et al., 2014) and light penetration has increased in the littoral zone as a result of the introduction of dreissenid mussels (*Dreissina polymorpha* and *Dreissina bugensis*), which filter zooplankton and fine particles from the water column (Malkin et al., 2008). Increased availability of hard substrate (i.e., empty mussel shells) and increased light penetration have facilitated large-scale blooms of *Cladophora glomerata* (Malkin et al., 2008). *Cladophora glomerata* (hereafter referred to as *Cladophora*) grows until mid-summer when it begins to senesce and slough to the bottom of the lake (Higgins et al., 2005). *Cladophora* deposition sites likely provide protein-rich anoxic microhabitats, important for the production of *C. botulinum*. Furthermore, large mats of *Cladophora* that wash ashore may provide an anoxic habitat for *C. botulinum* as they begin to decay. Detection of the BoNT/E gene was higher in mats of *Cladophora* collected from beaches where avian botulism outbreaks occurred compared with samples collected from beaches where they did not occur (Byappanahalli and Whitman, 2009). In 2011, BoNT/E produced by vegetative *C. botulinum* cells was detected in 73% of beached mats of *Cladophora* on the

3

Sleeping Bear Dunes National Lakeshore shoreline, and 74% of mats sampled elsewhere in the Great Lakes in 2011 (Chun et al., 2013).

While the physicochemical requirements and distribution of type E botulinum in the environment have been a major focus of research related to avian botulism outbreaks, few studies have examined how the feeding strategies of BoNT/E-affected birds may help facilitate BoNT/E outbreaks. A carcass-maggot pathway, in which waterfowl consume live maggots that have concentrated the toxin off of the carcasses of other botulism-affected birds, has been implicated in type C *botulinum* die-offs in shallow wetlands (Reed and Rocke, 1992). However, many of the species affected by type E botulism on the Great Lakes consume almost exclusively live fish or live benthic macroinvertebrates. Therefore, pathways that include live fish and live macroinvertebrates are likely important in the transfer of BoNT/E to birds (Byappanahalli and Whitman, 2009).

The species that have been most often implicated in the transfer of BoNT/E are dreissenids and round gobies (*Neogobius melanostomus*). A few studies have examined the diets of BoNT/E-affected birds (Campbell, 2003; Hannett et al., 2011; Hebert et al., 2014), but none have examined the size class distribution of fish consumed. My research objective was to compare the species composition and size classes of prey found in the stomach contents of seven species of BoNT/E-affected birds to infer important trophic pathways for BoNT/E. To strengthen my comparisons between fish-eating birds in Lake Michigan, I examined the stomach contents of BoNT/E-free double-crested cormorants (*Phalacrocorax auritus*) that were collected as part of Michigan's cormorant control program. I hypothesized that abundant benthic prey, particularly dreissenid mussels and round gobies, would comprise a large proportion of the stomach contents of botulism-

4

affected waterbirds, which would suggest that gobies play an important role in the transfer of BoNT/E in the Great Lakes.

CHAPTER TWO: STOMACH CONTENT ANALYSIS OF BOTULISM-AFFECTED BIRDS IN LAKE MICHIGAN

Abstract

Large-scale waterbird die-offs caused by *Clostridium botulinum* neurotoxin type E (BoNT/E) have occurred annually in the Great Lakes since the late 1990's. Invasive round gobies (*Neogobius melanostomus*) have been implicated as important vectors for the transfer of toxin to fish-eating birds because they are an abundant benthic prey fish and their establishment in the Great Lakes coincides with recent bird die-offs. I examined the stomach contents of waterbirds collected from northern Lake Michigan and tested for BoNT/E during summer and fall, 2010 – 2012. I also examined the diets of presumably BoNT/E-free cormorants (*Phalacrocorax auritus*) collected from three locations in Lake Michigan as references. Round gobies were consumed by all groups of birds examined in this study: 86% of the BoNT/E-positive individuals, 76% of the BoNT/E-negative birds, and 94% of the BoNT/E-free cormorants examined consumed round gobies. Double-crested cormorants, ring-billed gulls (*Larus delewarensis*), and common loons (*Gavia immer*) consumed gobies that averaged > 100 mm total length, whereas horned and red-necked grebes (*Podiceps auritus and P. grisegena*), white-winged scoters (*Melanitta fusca deglandi*), and long-tailed ducks (*Clangula hymealis*) consumed gobies that averaged < 80 mm total length. Other common prey items in the diets of waterbirds examined included dreissenid mussels, terrestrial insects, and alewives (*Alosa pseudoharengus*). The importance of round gobies in diets of Lake Michigan waterbirds suggests they could play a role in the transfer of BoNT/E to fish-eating birds; however,

6

gobies were found in most individuals of all categories (BoNT/E-positive, -negative, and
–free) of birds. Variability in botulism impact on bird species in the region may be linked
to trophic interactions based on goby size.

Keywords: Round goby, type E botulism, piscivorous birds, size class distribution,
stomach contents.

Introduction

Wild birds acquire *Clostridium botulinum* neurotoxin (BoNT) by ingesting
contaminated prey (Reed and Rocke, 1992; Smith and Sugiyama, 1988). Type C botulism
outbreaks often kill large numbers of waterbirds on warm, shallow bodies of water
(Newman et al., 2007). Type C botulism is usually transferred to waterbirds through
contaminated invertebrates, such as maggots consumed from decaying carcasses (Reed
and Rocke, 1992). In contrast, type E avian botulism die-offs more commonly affect
piscivorous birds that have presumably ingested live, intoxicated fish, suggesting
alternative toxin transfer pathways for BoNT/C (carcass-maggot pathway) and BoNT/E
affected birds (live-fish pathway).

Plant and animal tissues act as reservoirs for *C. botulinum* spores (Espelund and
Klaveness, 2014). After organisms harboring *C. botulinum* spores die, their decomposing
tissues create anaerobic conditions that support vegetative cell growth and BoNT
production (Espelund and Klaveness, 2014). Mass deposition and decomposition of
Cladophora glomerata (hereafter *Cladophora*), a common green alga in the Great Lakes,

7

is seen as a potential driver of BoNT/E production. BoNT/E has been detected in *Cladophora* mats (Byappanahalli and Whitman, 2009; Chun et al., 2013), fish (Getchell et al., 2006) and invertebrates (Perez-Fuentetaja et al., 2011) in the Great Lakes. For avian botulism outbreaks to occur, BoNT/E must either be transferred through food webs from substrates containing BoNT/E to live prey, and from live prey to birds, or inadvertently consumed in pursuit of live prey (Espelund and Klaveness, 2014; Getchell et al., 2006). Also, transmission of BoNT/E through food webs must coincide with the presence of foraging waterbirds, many of which are migratory. Therefore, type E avian botulism outbreaks are driven by spatial and temporal patterns of *C. botulinum*, prey items that that can act as vectors for the transfer of BoNT/E, and the birds that are affected.

Recent avian botulism outbreaks in Lake Michigan began in 2006, about the same time that round gobies (*Neogobius melanostomus*) appeared in annual surveys of prey fish abundance in Lake Michigan (Madenjian et al., 2014). Unlike most Lake Michigan prey fish, round gobies, particularly larger individuals, consume dreissenid mussels (Barton et al., 2005; Bunnell et al., 2005; Campbell et al., 2009). Thus, round gobies provide a link between mussel-bound nutrients (and toxins) and higher trophic levels (Hebert et al., 2014; Johnson et al., 2010; Poste and Ozersky, 2013), including piscivorous waterbirds in the Great Lakes. Based on stomach content examinations, round gobies were an important prey item in birds that succumbed to BoNT/E in Lake Erie (Campbell, 2003; Hannett et al. 2011).

In their non-native ranges, round gobies have become important prey for some piscivorous birds and have caused shifts in the foraging behavior of birds (Jakubas, 2004;

Johnson et al., 2010; Tucker and Seefelt, 2014). After round gobies were introduced to Lake Ontario, they became the dominant prey of breeding double-crested cormorants at Pigeon and Snake Island. Moreover, seasonal shifts in cormorant diets that corresponded with prey abundance before the introduction, were no longer observed (Johnson et al., 2010). Similarly, round gobies became the dominant prey of double-crested cormorants at the Beaver Archipelago in Lake Michigan, and other prey items became less important than previously observed (Van Guilder and Seefelt, 2013). The response of other waterbird species to the round goby invasion has not been investigated.

Round gobies in the Great Lakes consume a variety of prey species, including chironomids, mayflies, *Mysis* shrimp, *Gammarus* spp., zooplankton, and dreissenid mussels. Stomach content analysis of round gobies in the Great Lakes reveal that gobies in all size classes (range 30 – > 150 mm total length) ate dreissenid mussels, but the contribution of dreissenid mussels to the diets of gobies generally increases with fish size class (Campbell et al., 2009; Walsh et al., 2007). Smaller gobies (< 85 mm total length) did not consume larger dreissenid mussels (> 10 mm shell length), whereas larger gobies (> 85 mm total length) consumed dreissenid mussels ≤ 12.9 mm shell length (Ray and Corkum, 1997). Gobies in all size classes preferred smaller dreissenids. A stable isotope analysis of gobies in Lake Erie showed that round gobies display a switchpoint where the dreissenid mussels comprised a larger proportion of the diets of round gobies that were ≥ 112 mm TL (Campbell et al., 2009). Barton et al. (2005) reported that predation by round gobies in Lake Erie caused a decline in dreissenid mussels < 14 mm in length, which subsequently led to increased consumption of chironomids and amphipods by gobies. After smaller dreissenid mussels are depleted, free-living macroinvertebrates, like

9

chironomids and amphipods, may be the only prey available to smaller gobies because they are limited by gape size (Campbell et al., 2009; Ray and Corkum, 1997).

Avian botulism events offer an opportunity to examine the diets of waterbirds staging on the Great Lakes in autumn, an understudied aspect of the life history of these birds, and it is key to understanding the transfer of type E botulinum toxin in food webs. Adams et al. (2003) examined shorebirds during type C botulism outbreaks in Saskatchewan and found that certain feeding guilds experienced higher mortality rates, and attributed the difference to the distribution of BoNT/C in habitats where birds were feeding. A comparison of the stomach contents of BoNT/E-affected birds across feeding groups in Lake Michigan may be useful in generating hypotheses about the location of BoNT/E in the environment, as well as suggesting alternate pathways for the transfer of the toxin in food webs. Since BoNT/E affects birds acutely (Smith and Sugiyama, 1981) knowledge of what BoNT/E-affected birds were eating before they succumbed to the toxin could provide clues about which prey items are important in the transfer of the toxin.

A comparison of the stomach contents of BoNT/E-positive, -negative and -free birds in Lake Michigan might also be useful in determining important pathways for BoNT/E transfer. For most birds that use Lake Michigan, diet studies would be difficult because birds are often dispersed and would require lethal capture methods. However, due to perceived conflicts between cormorants and fisheries, there is an ongoing cormorant control program in Lake Michigan implemented by the U.S. Department of Agriculture. By examining the diets of cormorants culled as part of this program, I was

10

able to make useful comparisons between the diets of BoNT/E-affected birds and presumably BoNT/E-free birds.

Previous studies have identified round gobies as an important prey item for top-level predators in the Great Lakes (Johnson et al., 2010), but their importance in the diets of BoNT/E-affected birds in Lake Michigan is not known. An examination of the importance (percent abundance and frequency of occurrence) and size class distribution of prey consumed by BoNT/E-affected birds on the Great Lakes was conducted to help elucidate important microhabitats and trophic pathways of the toxin (e.g. carcass-maggot, live fish, benthic macroinvertebrates), and improve our understanding of the ecology of *C. botulinum*. In this study, I examined the stomach content of BoNT/E-affected birds collected from northern Lake Michigan and determined the size classes of their prey using allometric relationships.

Methods

BoNT/E-affected birds

Between the months June and November, 2010 – 2012, sick or dead birds were collected from fixed beach transects around northern Lake Michigan (Figure 1) by National Park Service , Common Coast Research and Conservation, and United States Geological Survey (USGS) personnel, partners, and volunteers (Chipault, USGS, personal communication). Due to increased local awareness of avian botulism related mortality, some bird carcasses were also reported and collected from nearby areas off-transect. To ensure the toxin was present in the bird before death, only birds that were fresh and showed no signs of decomposition were collected. Bird carcasses in suitable

condition were sent to the USGS National Wildlife Health Center (NWHC) in Madison, Wisconsin to be tested for BoNT/E. Mouse bioassays were used to detect BoNT/E in birds (Skerratt et al., 2005); in 2010, a subset were also tested at the NWHC with an *in vitro* method of detecting BoNT/E using a Förster resonance energy transfer (FRET) assay (Piazza et al., 2011). Birds that tested positive for BoNT/E by either mouse bioassay or FRET are hereafter referred to as BoNT/E-positive. Birds are referred to as BoNT/E-negative if the assay(s) used to test the bird for BoNT/E were unanimously negative. Gastrointestinal (GI) tracts were removed from carcasses at NWHC, botulinum toxin was inactivated with 10% formalin, and GI tracts were preserved in 95% ethanol.

BoNT/E-free cormorants

To strengthen comparisons between the diets of sick and dead birds found in northern Lake Michigan, I incorporated seemingly healthy cormorants that were culled as part of cormorant control efforts by the United States Department of Agriculture and the Band of Ottawa and Chippewa Indians Department of Natural Resources. These cormorants were obtained following United States Fish and Wildlife Service regulations (CFR 21.13). Culled cormorants were considered BoNT/E-free because they were not visibly injured or sick before they were shot, and there were no reports of botulism-related deaths of double-crested cormorants in the area (Figure 1) during the time frame of collection. Furthermore, most of the birds investigated by the NWHC were collected post-mortem, which can lead to biases in the estimation of prey composition. For example, prey items with hard parts may be over-represented in birds that were not collected and frozen immediately upon death because soft prey items decompose rapidly.

12

At Bellow Island (BELL), Grand Traverse Bay, Michigan, cormorants were culled in the morning (10:00 – 13:00) 12 June 2012. At Gull Island and Little Gull Island (GULL), Michigan, and the Ludington Pump Storage Breakwall (LPSB), Michigan, cormorants were culled in the evening (17:00 – 21:00) 28 June 2012 and 24 July 2012, respectively. Culled birds were immediately placed on ice and then frozen (-20ºC) to be processed later. Placing the birds on ice likely slowed post-mortem digestion and freezing birds halted it. The amount of time the cormorants were on ice before they were transferred to the freezer was approximately 5.5, 6.5, and 2.0 hours for BELL, LPSB, and GULL birds, respectively. The GI tracts were removed and preserved in 95% ethanol.

Stomach content analysis for all birds

Sex (male, female), age (adult, juvenile), and mass (g) were recorded for all groups of birds, although these data are missing for some individuals within each group. The wet mass of entire GI tract, and the separate wet masses of esophagus, proventriculus, gizzard, and intestine were measured. Contents of the esophagus, proventriculus, and gizzard were removed separately, and the prey items were washed through nested sieves (2 mm, 1 mm, and 0.59 mm). To reduce bias against soft-bodied prey, many diet studies examine only the esophageal content of birds (Anderson et al., 2008); however, most of the BoNT/E-affected birds in this study were collected 1-2 days post-mortem, and often had no prey items left in their esophagus. Since sample sizes were already limited, we pooled esophagus and gizzard contents for each bird examined.

The prey items were sorted into the following categories: diagnostic fish bones (cleithra, otolith, dentary, premaxillary, dentigirous plate, pharyngeal branchial plate, and

13

operculum), Michiganscellaneous bones (vertebrae, ribs, fin rays, etc.), intact fish, insect, mussel, crayfish, parasites, and other (stones, sand, feathers, etc.). Items in each category were identified and enumerated to calculate the minimum number of individuals from each species consumed.

Diagnostic bones were measured and used to estimate the total length (TL) of fish using published regression coefficients (Burnett et al., 2002; Dietrich et al., 2006; Scharf et al., 1998). There were rarely intact fish in the stomach contents of birds that were collected post-mortem. To select the most effective diagnostic bone for estimating TL of round gobies, the numerical abundance, frequency of occurrence (refers to the proportion of birds containing the bone), and relative condition (intact vs. damaged), of each bone was assessed (Table 3). I examined the size class distribution of each fish species consumed by birds. When possible, I reported the grand mean TL of fish consumed by calculating the average TL of each fish species consumed by each bird, and averaging those values.

Numerical abundance of fish was determined by summing the number of each diagnostic bone found in a bird and dividing by the number of bones that belong in one fish. The same principle was applied to calculate the minimum number of macroinvertebrate species consumed. For instance, since each dreissenid mussel has two umbos, the number of umbos in each stomach was counted and divided by two to calculate the minimum number of dreissenids consumed.

I described the diets of birds sampled in this study by calculating the frequency of occurrence (%) of each prey item in the stomach contents of birds, as well as the relative

contribution of each prey item by percent abundance and estimated wet mass (g) to the stomach content of birds.

The mass of macroinvertebrates (insects and mussels) in the diets of birds could not be estimated because invertebrate samples were generally too degraded. I attempted to compare the percent numerical abundance of prey items in the diets of culled cormorants across location, sex, and age class; however, data were best represented by proportions and therefore were subject to unit sum constraint, lack of normality, and variance equality. Therefore, I used the percent abundance of each prey item to compare the distribution of prey items in different groups of birds using Pearson's Chi square analysis. The use of percent numerical abundance instead of percent biomass will result in an overestimation of the importance of insects in the diets of birds. However, it was important to include invertebrates in this analysis, since they could be important in the transfer of BoNT/E.

For each species examined, I used modified Costello graphs (Marshall and Elliot 1997) to graphically analyze the importance of each prey item with mean percent abundance on the y axis instead of mean percent biomass to allow inclusion of invertebrates. Limited sample sizes precluded meaningful comparisons between groups of most BoNT/E-affected species. However, sample sizes of common loons were large enough to compare using Chi square contingency tables; the size class distributions of round gobies consumed by BoNT/E-positive and BoNT/E-negative loons were defined as 30 – 60 mm, 60 – 90 mm, 90 – 120 mm, 120 – 150 mm, and 150 mm. The Mann-Whitney U test was used to compare the mean length of gobies consumed by BoNT/E-

15

positive and BoNT/E-negative loons. I also compared the diets of ring-billed gulls collected before and after 1 August using Chi square contingency tables because these intervals correspond to the nesting and post-nesting periods (Pollet et al., 2012). For all analyses, only prey items that were consumed by birds in both comparison groups were considered in the Chi square contingency tables.

Results

Eighty-nine birds (seven species) observed sick or dead and collected from Lake Michigan were tested for BoNT/E and included in this study. The BoNT/E testing yielded positive intoxication results for 58 birds and negative results for 30 birds (Table 1). The mouse assay for one red-necked grebe was equivocal and that specimen was not included in the subset of samples that received FRET testing so results remain equivocal. One of the BoNT/E-negative loons exhibited signs of lead poisoning (there was a lead fishing sinker in the gizzard and the lining of the GI tract was black). In addition, we examined the stomach content of 77 BoNT/E-free double-crested cormorants that were culled at breeding colonies on northern Lake Michigan (Table 2).

The dentary structure was used to back-calculate the lengths of round gobies consumed because it was the most frequently occurring (found in 90% of stomachs that contained goby bones) and abundant bone (mean 4.03 ± 0.70 gobies per stomach) found in good condition in BoNT/E-affected birds (Table 3).

There were too many differences in the diets of birds between species to analyze them as groups. Nevertheless, the birds examined in this study comprised four general feeding groups which are reported separately for conceptual purposes. I refer to white-

winged scoters and log-tailed ducks as mussel-eating ducks, although they are not limited to bivalve prey (Anderson et al., 2008; Anderson and Lovvorn, 2012; and Ross et al., 2005). Grebes are aquatic generalists (Piersma, 1988), and ring-billed gulls are aquatic-terrestrial generalists (Pollet et al., 2012). I refer to loons and cormorants are top-level piscivores (Barr, 1996; Doucette et al., 2011), although they will also consume benthic crustaceans

Mussel-eating ducks

All of the long-tailed ducks and white-winged scoters examined in this study were BoNT/E-positive (Table 1). Dreissenid mussel shells were found in all long-tailed ducks and white-winged scoters, and round gobies occurred in 50% and 83% of long-tailed ducks and white-winged scoters, respectively (Figure 2c-d). The stomach contents of long-tailed ducks and white-winged scoters were usually degraded and most of the hard parts (mussel shells and fish bones) were fragmented. Only four intact round goby dentary bones were found in white-winged scoters and one was found in a long-tailed duck. The estimated lengths of round gobies ranged from 60.42 – 82.09 mm (grand mean 72.86 ± 3.21 mm) in white-winged scoters. The estimated length of the round goby found in the long-tailed duck was 55.60 mm (Table 4). As expected, dreissenid mussels were more important in the diets of mussel-eating ducks than other species. Unlike previous diet studies (Brown and Fredrickson, 1986; Ross et al., 2005), amphipods were not present in the stomach content of white-winged scoters or long-tailed ducks.

Grebes

All of the grebes examined in this study tested positive for BoNT/E except for one red-necked grebe which was equivocal (Table 1). The stomach contents of horned grebes included round gobies, alewives, dreissenid sp., chironomid larvae, and land insects (Hemiptera, Hymenoptera, and Coleoptera). All of the grebes that were examined consumed terrestrial insects. Terrestrial insects were important in the diets of grebes compared to other bird feeding groups. Round gobies comprised the largest proportion of prey items in horned grebe stomachs by abundance (Figure 2a-b). Individual, fragmented dreissenid mussel shells were found in one horned and one red-necked grebe. Both birds also consumed round gobies, so it is possible that the dreissenids were indirectly consumed by the birds. Only one horned grebe consumed an alewife, and its estimated length was 46.36 mm. The estimated lengths of round gobies ranged from 46.81 – 108.58 mm (grand mean, 70.81 ± 3.42 mm) in horned grebes (Figure 3a) and 47.17 – 121.82 mm (mean =72.74 ± 15.32 mm) in the single red-necked grebe that contained intact round goby bones (Figure 3b, Table 4). Horned and red-necked grebes appeared to primarily be limited to gobies that were < 100 mm TL (Figure 3a-b).

Ring-billed gull

There were 19 ring-billed gulls from which GI tracts were collected during this study period; 78.9% (15/19) tested positive for BoNT/E (Table 1). Prey items that occurred most frequently in the stomach content of ring-billed gulls were round gobies (73.6% frequency of occurrence), terrestrial insects (68% frequency of occurrence), and

alewives (37% frequency of occurrence), respectively (Figure 2e). Chironomids were also found in four ring-billed gulls and comprised a large proportion of ring-billed gull diets (by number). However, 119 of 144 chironomid larvae in ring-billed gull stomachs were found in one bird. Similarly, 108 of 151 terrestrial insects observed in ring-billed gull stomachs were found in one bird, suggesting opportunistic feeding on patches of prey.

A small sample size for BoNT/E-negative gulls precluded meaningful statistical comparisons of proportions of prey items between BoNT/E-positive and BoNT/E-negative ring-billed gulls. Estimated alewife TL ranged from 49.91 – 121.80 mm (grand mean = 73.13 ± 6.82 mm) in BoNT/E ring-billed gulls; one alewife (TL = 67.70 mm) was found in BoNT/E-negative gulls. Goby length ranged from 34.53 – 195.26 mm (grand mean = 120.10 mm ± 17.00 mm) in BoNT/E-positive ring-billed gulls and 55.60 – 145.90 mm (grand mean = 120.14 . ± 13.40 mm) in BoNT/E-negative ring-billed gulls (Table 4). Visual inspection of graphs suggested that there was little difference between the size class distribution of round gobies in BoNT/E-positive and BoNT/E-negative ring-billed gulls, so samples were pooled to calculate mean estimated length.

A relatively large sample size of ring-billed gulls allowed comparison of the stomach contents of gulls in the nesting period (June 27 – July 30, $n = 10$) with gulls in the post-breeding period (August 1 – September 30, $n = 9$). The mean proportion of prey items consumed by ring-billed gulls in the nesting and post-breeding period differed (χ^2 [df = 2] = 324.93, p < 0.01). Alewives comprised a higher proportion of the stomach content of gulls collected in the nesting period (nesting period mean, 23.0 ± 11.2%; post-breeding mean, 4.7 ± 3.7%), whereas round gobies comprised a larger proportion of the

stomach content of gulls collected in the post-breeding period (nesting period mean, 11.7

± 6.8%; post-breeding mean, 52.7 ± 6.50 %). Chironomid larvae were only found in

nesting period birds, and dreissenid mussels were only observed in post-breeding birds.

Ring-billed gull stomach contents contained benthic and pelagic fish, as well as aquatic

and terrestrial invertebrates, which suggests that ring-billed gulls have adaptable diets.

Although they consume a variety of prey types, they may specialize on certain prey based

on seasonal availability, or energetic demands (i.e. chick rearing demands).

Top-level piscivores

Of the 39 common loon GI tracts examined, 31 contained prey items. Seven of

eight (87.5%) common loons with empty GI tracts tested negative for BoNT/E. Round

gobies were present in 100% of common loon GI tracts that contained prey items (13

BoNT/E-negative loons and 18 BoNT/E-positive loons; Figure 2). Other frequently

occurring prey items, dreissenid mussels and crayfish, were each present in 29.0% of

loons that contained prey items. Round gobies comprised the highest mean proportion

(85.8%) of loon stomach content by abundance (Figure 2f). Round goby size class

distribution of BoNT/E-positive and negative common loons did not differ (χ^2 (df = 4) =

8.792, p = 0.07) (Figure 3). The mean estimated length of gobies consumed by loons did

not differ between BoNT/E-positive and negative loons (Mann-Whitney, U (248) = 7324,

z = 0.02, p = 0.98) (Table 4). The estimated TL of round gobies in all loons examined

ranged from 47.17 – 207.30 mm (mean 109.73 ± 6.12 mm) (Figure 3d, Table 4).

Dreissenid mussels were more abundant, whereas crayfish were less abundant in

BoNT/E-positive birds (χ^2 [df = 2] = 199.51, p < 0.01); however, both prey items were

relatively unimportant in the diets of Lake Michigan loons (Figure 2f). Round gobies were, by far, the most important prey item consumed by common loons.

The most frequently occurring prey items in double-crested cormorant were round gobies (100%), followed by dreissenid mussels (71.4%), crayfish (28.5%), alewives (14.3%), and yellow perch (14.3%) (Figure 2g). Round gobies comprised the largest mean proportion of prey consumed by cormorants (70.4%) (Figure 2g). The estimated length of gobies consumed by double-crested cormorants ranged from 54.40 – 188.04 mm (grand mean 114.41 ± 5.80 mm) (Figure 3e, Table 4).

Despite differences in collection methods used, the prey composition of BoNT/E-positive and BoNT/E-free double-crested cormorants appear to be comparable (Figure 2g-h). Many of the cormorants at BELL had empty stomachs (45.5%, 5/11), whereas few of the birds at LPSB (3.7%, 1/27) and GULL (20.5%, 8/39) had empty stomachs. Round gobies were found more frequently than all other prey items at each location (Figure 2f-h). Comparisons of the abundance of prey items across locations showed that round gobies comprised the highest mean proportion of stomach content at LPSB (mean =61.6 ± 4.4%) and BELL (mean = 48.8 ± 14.7%), and the second highest proportion of stomach content at GULL (mean = 36.3 ± 6.4%). Alewives comprised the highest mean proportion of stomach content at GULL (mean = 41.3 ± 7.2%) (Figure 2f-h). Comparisons of fish biomass in the stomach contents at each location showed that round gobies comprised the highest proportion of biomass at LPSB (mean = 88.7 ± 4.9%), BELL (mean = 80.7 ± 12.6%), and GULL (mean = 48.9 ± 8.0%) (Figure 4). When double-crested cormorants were pooled across sites, round gobies comprised a larger mean proportion and biomass (g) of the stomach contents of juvenile cormorants than

adult cormorants; conversely, alewives comprised a larger proportion and biomass (g) of the stomach contents of adult cormorants than juvenile cormorants (χ^2 [df = 3] = 548.37, p < 0.01) (Figures 4 and 5).

The estimated size ranges of gobies consumed by BoNT/E-free cormorants at BELL, GULL, and LPSB were 39.50 – 225.50 mm (grand mean, 118.73 ± 18.66 mm), 32.72 – 218.14 mm (grand mean 91.31 ± 6.10 mm), and 37.10 – 171.90 mm (grand mean 105.10 ± 3.52 mm), respectively. The size class distribution of gobies consumed differed among locations (χ^2 [df = 8] = 226.94, p < 0.01) (Figure 5). Round gobies that were > 90 mm comprised a larger portion of the diets of LPSB cormorants than of GULL and BELL cormorants. The mean length of gobies consumed by culled cormorants differed between sites (Kruskal-Wallis, H = 198.81, $p < 0.01$). The estimated size ranges of alewives consumed by BELL, GULL, and LPSB double-crested cormorants were 98.30 – 141.70 mm (grand mean, 117.20 ± 6.62 mm), 20.24 – 162.33 mm (grand mean 103.63 ± 5.44 mm), and 39.24 – 147.53 mm (grand mean 114.13 ± 12.90 mm), respectively (Figure 3f-h). Yellow perch comprised < 3% of the stomach content at all locations. The grand mean lengths of yellow perch consumed at GULL and LPSB were 95.10 ± 8.67 mm and 111.93 ± 6.60 mm, respectively. Yellow perch were not found in the stomach content of BELL double-crested cormorants.

The diets of BoNT/E-free cormorants differed by location (χ^2 [df = 8] = 1267.84, p < 0.01). Dreissenid mussels comprised a higher proportion of the stomach contents at LPSB than at GULL and BELL (Figure 2f-h). The abundance of dreissenid mussels and round gobies in cormorants was positively correlated when sites were pooled together (Pearson correlation, $r^2 = 0.70$, $p < 0.01$). Dreissenid mussels comprised a higher

proportion of gizzard contents than esophageal contents in culled cormorants, suggesting that retention time for dreissenid shells in cormorant gizzards is high compared to other prey items. Therefore, dreissenid mussels may be overrepresented in the diets of double-crested cormorants.

Discussion

Round gobies are clearly the dominant prey fish of Lake Michigan waterbirds examined in this study. At least one individual bird from all of the BoNT/E-affected species included in this study contained round gobies, and the frequency of occurrence of gobies within a species ranged from 40%-100% (mean 78.1 ± 9.4%). Although round gobies were the most important prey consumed by most of the species examined, there were differences in prey composition and round goby size class distribution among species. For instance, alewives were important prey for both of the species that are reeding residents in Lake Michigan (double-crested cormorants and ring-billed gulls). However, they were nearly absent from the diets of fall migrants (common loons, horned grebes, red-necked grebes, white-winged scoters, and long-tailed ducks). Dreissenid mussels were the most important prey item in the stomach contents of mussel-eating ducks, but they were relatively unimportant in the stomach contents of other species (except double-crested cormorants). The analyses suggests that round gobies are the main prey fish involved in a live-fish pathway for the transfer of the botulinum toxin to fish-eating bird species, but that alternative pathways are possible. For example, mussel-eating ducks are likely exposed to the toxin through live benthic macroinvertebrates, rather than live-fish. Furthermore, waterbirds found sick or dead but BoNT/E-negative

had consumed round gobies, as had apparently healthy cormorants that were culled, consumption of gobies alone cannot account for botulinum intoxication.

Differences in the stomach content of BoNT/E-free double-crested cormorants collected at three locations on separate dates suggest that the diets of fish-eating birds in Lake Michigan vary spatially and temporally. Alewives were far more important in the stomach content of double-crested cormorants from GULL. At GULL, alewives were found less frequently than round gobies, but comprised nearly the same mean proportion of biomass. Several GULL cormorants contained well over 50 alewives, and one contained 166. Double-crested cormorants may focus their foraging efforts on patches of abundant prey. The mean length of round gobies found in the stomach contents of BoNT/E-free double-crested cormorants seemed to decrease based on the dates that birds were collected. Huo et al. (2014) reported high rates of mortality in Lake Michigan round goby populations. Round gobies may experience increased predation pressure in areas near double-crested cormorant breeding colonies. If double-crested cormorants are selecting larger size classes of gobies, the mean length of round gobies that are available in these areas may decrease throughout the year because of predation on larger size classes.

The prevalence and quantity of BoNT/E in different prey species, let alone different size classes within prey species, is not well understood. Nor is the sensitivity of bird species to the toxin. BoNT/E has been detected in fish and invertebrate species that birds in this study consumed (Getchell et al., 2006; Perez-Fuentetaja et al., 2006). Die-offs of several fish species, including round gobies, have been attributed to BoNT/E (Getchell et al., 2006). BoNT/E intoxicated fish may also exhibit symptoms that increase

their susceptibility to predation by fish-eating birds (Yule et al., 2006a). For instance, round gobies may become hyperpigmented and exhibit erratic swimming behavior, which could attract the attention of predators (Yule et al., 2006a, 2006b). Ontogenetic shifts in goby diets (Ray and Corkum, 1997; Campbell et al., 2009) could result in increased prevalence of BoNT/E intoxication in gobies of a certain size class. If this occurs in the wild, round goby size selection by birds could influence the likelihood of BoNT/E impact.

The size class distribution of round gobies consumed differed between bird species. Male gobies have higher growth rates than female gobies in Lake Michigan (Huo et al., 2014). Gobies prefer to spawn in shallow water where males aggressively guard their nests (Corkum et al., 1998; Meunier et al., 2009). The relatively high representation of larger gobies in ring-billed gull diets may reflect a foraging strategy in which ring-billed gulls preferentially depredate male gobies guarding their nests in shallow water. Horned grebes, on the other hand, consumed almost exclusively gobies that were < 100 mm TL, likely because they are limited by gape size (Piersma, 1988).

Top-level piscivores consumed all size classes of round gobies. The diets of double-crested cormorants appeared skewed toward larger gobies compared to common loons, which consumed gobies evenly among size classes. These differences may reflect spatial and temporal differences in round goby distribution throughout the lake. Double-crested cormorants feed in shallower, nearshore habitats during the round goby spawning season (Kornis et al., 2012), so it may be more profitable for double-crested cormorants to selectively prey on larger gobies when they are concentrated. However, common loons

are fall migrants that forage deeper and further offshore (Kenow, USGS, personal communication). Round gobies may be more dispersed in the fall as they begin to move away from spawning sites. If so, size selectivity may not be as profitable for common loons.

Round goby diets can differ based on gape size, depth, and habitat (Ray and Corkum, 1997; Walsh et al., 2007). Dreissenid mussels comprise a higher proportion of the diets of gobies exceeding ~ 100 mm (Campbell et al., 2009). Where goby abundance is high, this pattern may be especially pronounced because round gobies can alter size structure in dreissenid populations by consuming smaller mussels (Barton et al., 2005). Therefore, the prey species involved in the transfer of BoNT/E to piscivorous birds may vary depending on the date and location that the birds are feeding, as well as the size of gobies upon which the birds are feeding. Horned and red-necked grebes appear to prefer smaller gobies because of a gape size limitation (Piersma, 1988; this study), suggesting that the dreissenid – goby transfer pathway may not be as important for these bird species. Conversely, larger gobies are more important in the diets of common loons, double-crested cormorants, and ring-billed gulls which are commonly collected in die-offs. These differences suggest that while the initial carriers of BoNT/E could vary, round gobies are likely an important final vector of BoNT/E to piscivorous birds.

Small sample sizes precluded a statistical comparison of diets across years in this study. However, round gobies are consistently a dominant component of piscivore diets in the Great Lakes (Johnson et al., 2010; Van Guilder and Seefelt, 2013; Figures 3 and 4). Although the dominant role of round gobies in the diets of fish-eating waterbirds may not

vary annually, the impact of BoNT/E on individual bird species may be variable (Chipault, USGS, personal communication). While changes in Great Lakes foodwebs likely play an important role, there are other important ecological factors involved in the die-offs. For large-scale die-offs to occur, the conditions that promote BoNT/E production and transfer through foodwebs must coincide with the presence of large numbers of waterbirds in the Lake Michigan ecosystem (e.g., during autumn migration). In years when large-scale outbreaks occur, low water levels, high surface temperatures, and mass blooms of *Cladophora* may extend the period and area suitable for BoNT/E production, and increase the likelihood that birds will encounter the toxin (Lafrancois et al., 2011).

Prey availability in the habitats where BoNT/E is produced could also affect the likelihood that a bird will encounter the toxin. For example, larger round gobies may displace smaller round gobies from rocky habitats, which are optimal for round gobies, to sandy habitats (Ray and Corkum, 2001). Predators that focus their foraging efforts on smaller gobies that have been displaced to peripheral habitats may be exposed to different trophic pathways than avian predators that focus their foraging efforts on rocky areas. These differences could affect the likelihood that a predator will encounter the toxin. To predict the impact of botulism outbreaks on Great Lakes waterbirds, it will be critical to understand the interplay between spatial and temporal trends of *C. botulinum* in the Great Lakes as it relates to the foraging strategies and habitat use of Great Lakes waterbirds and their prey items.

Many studies have described the biases associated with stomach content analyses in waterbirds, including studies on some of the species examined in this study (Brown

and Ewins, 1996; Seefelt and Gillingham, 2006; and Anderson et al., 2008). Since the BoNT/E-affected birds in this study were collected post-mortem, their stomach contents were highly degraded (there was little or no tissue attached to the bones of vertebrates, and soft-bodied prey would have been completely digested), and most of the identifiable prey items were bones from fish and invertebrate exoskeletons. As a result the data from stomach contents of BoNT/E-affected birds surely over represents species that were indirectly consumed by prey fish or birds (such as dreissenid mussels and chironomids), and underrepresents soft-bodied prey in these birds (such as amphipods). Amphipods were conspicuously absent from the stomach contents of the mussel-eating ducks in this study. Amphipods are a key prey item of nesting white-winged scoters, and previous studies have suggested that they may be important prey for long-tailed ducks on the Great Lakes (Brown and Fredrickson, 1986; Peterson and Ellarson, 1977; Ross et al., 2005), and North Atlantic (White et al. 2009). Any bias against soft-bodied prey in this study is likely most extreme for long-tailed duck and white-winged scoter diets and less important for known piscivores such as loons and double-crested cormorants (Barr et al., 1996; Seefelt and Gillingham, 2006).

Conclusion

Predation of round gobies by BoNT/E-affected waterbirds in the Great Lakes occurred across species and across large spatial scales suggesting that round gobies are likely important vectors for BoNT/E to waterbirds. However, birds found not to have BoNT/E also consumed gobies, indicating that consumption of gobies cannot alone explain why some birds are intoxicated and others are not. Although round gobies dominated the diets of most species examined, there were spatial and temporal

differences in the stomach content of waterbirds within and among bird species. The likelihood of a waterbird ingesting BoNT/E contaminated prey items can be influenced by the foraging strategies of birds that are affected. Further research is needed to discover how dietary differences affect the susceptibility of birds to BoNT/E ingestion. Information about the specific locations and habitats where birds forage and the distribution and diet of round gobies throughout the Great Lakes will also be necessary to better evaluate the risk of exposure to BoNT/E in each bird species.

Table 1. Sample sizes of birds collected on northern Lake Michigan beaches from 2010 – 2012. Samples are grouped based on whether they tested positive (+) or negative (-) for type E botulinum toxin (BoNT/E) using either a mouse bioassay or the FRET assay. The asterisk next to the red-necked grebe collected in 2010 indicates that the assay result was equivocal.

Species	2010		2011		2012		Total	
	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)
Common loon (*Gavia immer*)	3	2	5	3	12	14	20	19
Double-crested cormorant (*Phalacrocorax auritus*)	3	4	3	3	0	1	6	7
Horned grebe (*Podiceps auritus*)	0	0	0	1	0	4	0	5
Long-tailed duck (*Clangula hyemalis*)	0	2	0	0	0	0	0	2
Red-necked grebe (*Podiceps grisegena*)	1*	3	0	1	0	3	0	4
Ring-billed gull (*Larus delewarensis*)	0	3	1	6	3	6	4	15
White-winged scoter (*Melanitta deglandi*)	0	0	0	1	0	2	0	6
Total	6	14	9	14	15	29	30	58

Table 2. The collection dates, approximate locations, and sample sizes (n) of cormorants culled at three locations in northern Lake Michigan. The sex (M, male; F, female) and age class (J, juvenile; A, adult) of most of the birds at each site were also determined.

					Sex		Age class	
	Collection Date	Latitude	Longitude	n	M	F	J	A
Bellow Island (BELL)	12 June 2012	45.10004	85.56673	11	9	2	2	8
Ludington Pump Storage Breakwall (LPSB)	28 June 2012	43.89298	86.45562	27	14	5	10	11
Little and Big Gull Island (GULL)	24 July 2012	45.51412	86.71902	39	21	12	6	27

31

Table 3. The percent occurrence (proportion of birds in which round goby bones were detected) and mean number of round goby diagnostic bone structures in the stomach contents of all botulism-affected birds that contained goby bones.

Bone Structure	% Occurrence	Mean # of bones per bird
Cleithra	0.43	2.38 ± 0.74
Dentary	0.90	4.03 ± 0.70
Dentigerous plate	0.80	4.62 ± 0.74
Operculum	0.24	0.80 ± 0.30
Otolith	0.43	1.70 ± 0.50
Pharyngeal branchial plate	0.70	2.71 ± 0.80
Premaxillary	0.70	2.63 ± 0.60

Table 4. Grand mean estimated lengths (GMEL ± S.E.) of fish consumed by birds collected on northern Lake Michigan shorelines. The sample sizes (n) of botulism-positive (+) and botulism negative (-) birds that contained each fish are included. The mean lengths of gobies consumed by BoNT/E-positive and BoNT/E-negative common loons did not differ (Mann-Whitney, U (248) = 7,324, z = 0.02, p = 0.98), so samples were pooled. Ring-billed gull sample-sizes were too small for such an analysis, so botulism-positive and negative samples are displayed separately*. The dash marks (--) indicate an absence of the corresponding prey item in GI tracts.

| | n | Round Goby | n | Alewife | n | Yellow Perch |
	+/-	GMEL (mm)	+/-	GMEL (mm)	+/-	GMEL
Common Loon (*Gavia immer*)	17/12	109.73 ± 6.12	1/0	48.20	--	--
Double-crested cormorant (*Phalacrocorax auritus*)	7/0	114.41 ± 5.80	1/0	99.46	1/0	138.44
Horned grebe (*Podiceps auritus*)	5/0	70.81 ± 3.42	1/0	46.36	--	--
Long-tailed duck (*Clangula hyemalis*)	2/0	55.60	1/0	69.12	--	--
Ring-billed gull (*Larus delewarensis*)	7/3	120.10 ± 17.00 / 120.34 ± 19.85*	5/1	73.13 ± 6.82 / 67.70*	--	--
Red-necked grebe (*Podiceps grisegena*)	1/0	72.74	--	--	--	--
White-winged scoter (*Melanitta fusca*)	2/0	72.90 ± 3.21	--	--	--	--
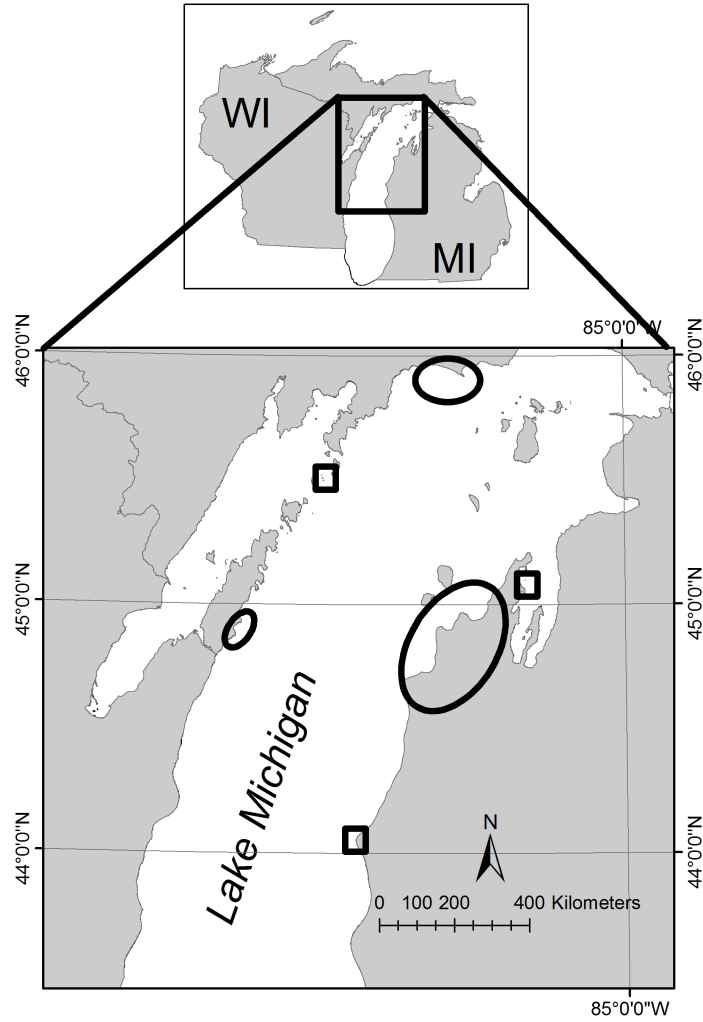
Figure 1. Sample locations where BoNT/E affected waterbirds (circled) and culled double-crested cormorants (squares) were collected.
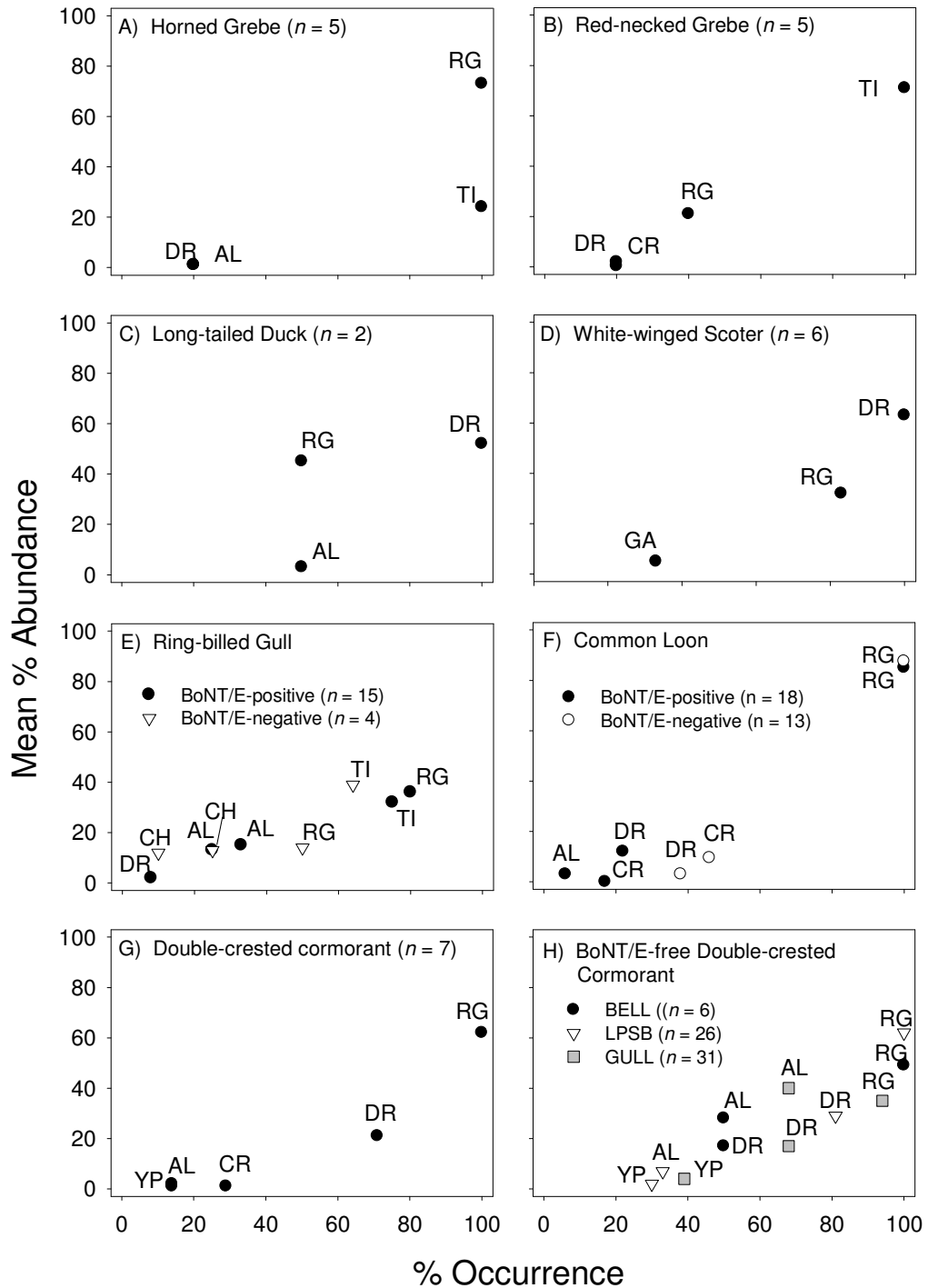
Figure 2. Modified Costello graphs which show the importance of different prey items in the stomach contents of birds based on mean proportion of diet (percent abundance) and frequency of occurrence (proportion of birds containing the prey item). Prey items depicted in the graphs include round gobies (*Neogobius melanostomus*) (RG), alewives (*Alosa pseudoharengus*) (AW), yellow perch (*Perca flavescens*) (YP), crayfish (CF), chironomids (CH), terrestrial insects (TI), and gastropods (GA). Prey that comprised < 1% of the total prey consumed by a species, or that were considered incidentally consumed, were excluded. Since percent abundance is used instead of percent mass these graphs overemphasize the importance of macroinvertebrates. When BoNT/E-negative birds were examined they were represented separately.
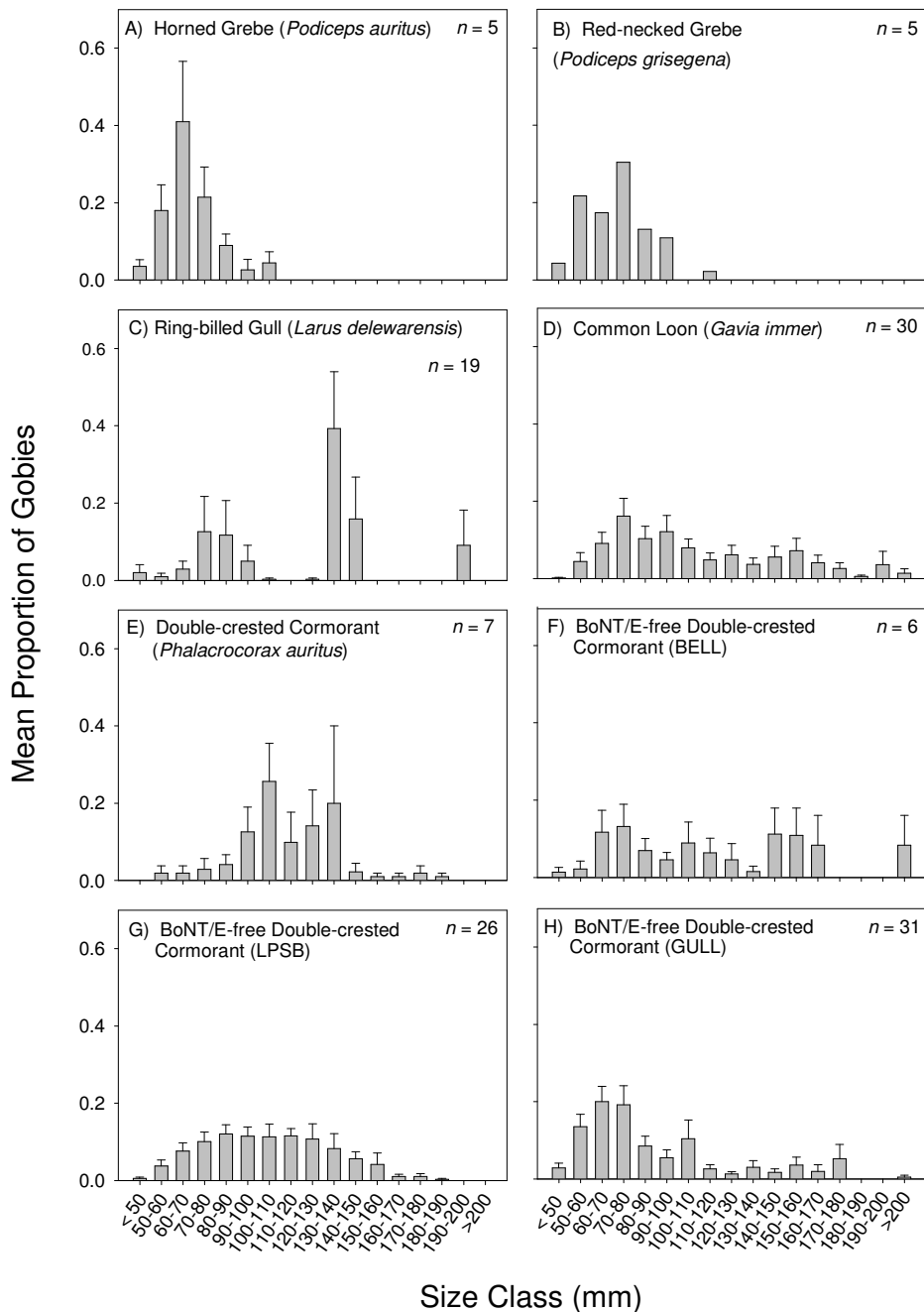
Figure 3. A comparison of the size class distribution of round gobies (Neogobius melanostomus) consumed by waterbirds collected in Lake Michigan from 2010 to 2012. The bars show the mean proportion (± S. E.) of gobies in each size class. Intact round goby dentaries were found in one red-necked grebe, so standard errors were not calculated. Except for 3/10 ring-billed gulls and 12/30 common loons, all of the birds represented tested positive for botulinum neurotoxin type E (BoNT/E). There was no difference between the size class distributions found in BoNT/E-positive and –negative common loons (χ^2 (df = 4) = 8.792, p = 0.07). Small sample sizes precluded statistical comparisons, but visual inspection of the data suggested that there was little difference between the size class distribution of round gobies in BoNT/E-positive and –negative ring-billed gulls, so samples were pooled.
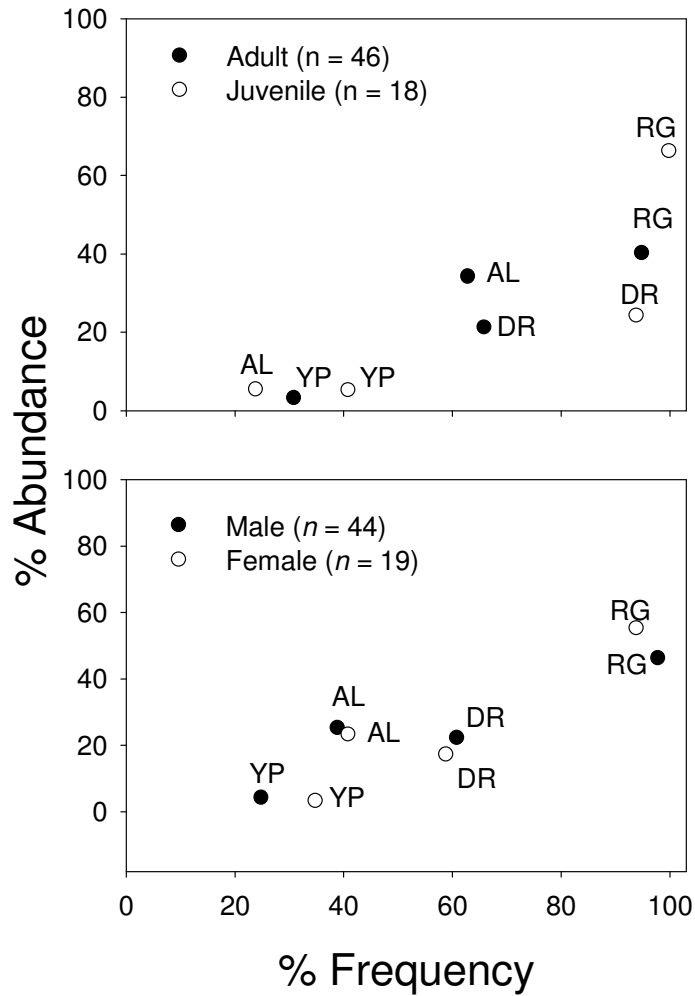
Figure 4. Modified Costello graphs (left) that show the importance of round gobies (RG), alewives (AL), yellow perch (YP), and dreissenid mussels (DR) consumed by botulism-free double-crested cormorants (*Phalacrocorax auritus*) grouped by age class (Adults and Juveniles) and sex (Male and Female). Sample sizes (*n*) are displayed. All birds were collected from breeding colonies in Lake Michigan during summer 2012, and the importance of each prey item is based on mean proportion of stomach contents (percent abundance) and frequency of occurrence (proportion of birds that consumed the prey item). It is likely that the importance of dreissenid mussels are over-represented in these graphs, since they are much smaller than fish. The diets of adult and juvenile double-crested cormorants differed significantly (χ^2 [df = 3] = 548.37, p < 0.01).
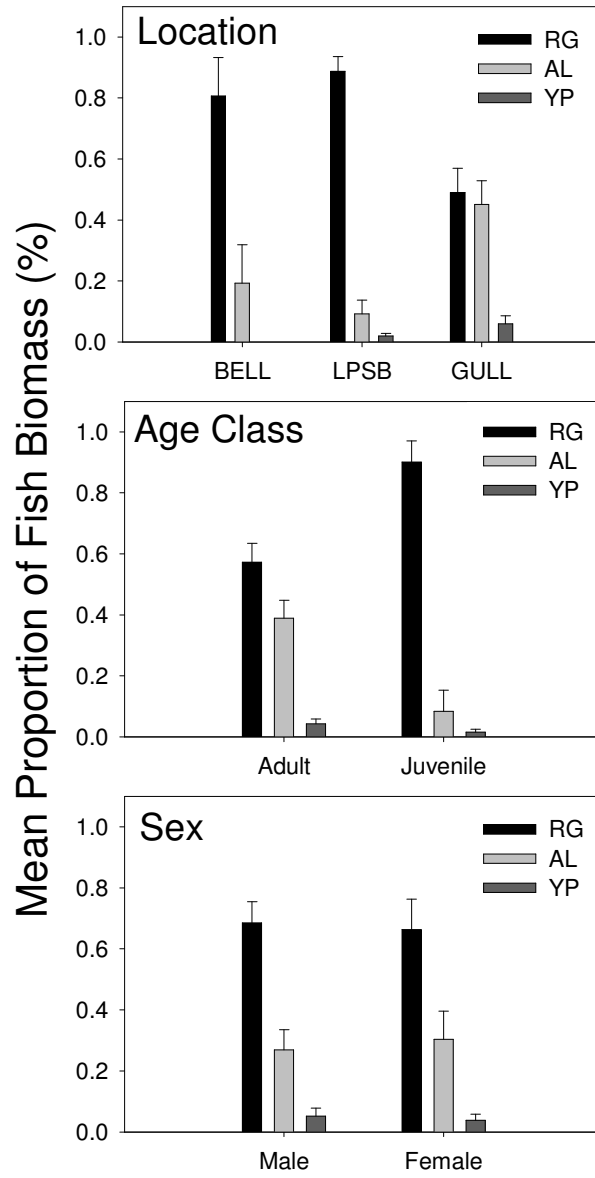
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