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TOXICITY OF *CLOSTRIDIUM BOTULINUM* TYPE E NEUROTOXIN TO GREAT LAKES FISH: IMPLICATIONS FOR AVIAN BOTULISM

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ABSTRACT: Since 1999, large-scale mortalities of fish-eating birds have been observed on the Great Lakes, and more specifically on Lake Erie. Type E botulism has been established as the primary cause of death. The mechanism of type E botulism exposure in fish-eating birds is unclear. Given that these birds are thought to eat live fish exclusively, it seems likely that their prey play a key role in the process, but the role of fish as potential transport vectors of botulinum neurotoxin type E (BoNT/E) to birds has not been adequately investigated. Between June 2003 and April 2004 a methodological model for exposing fish to *Clostridium botulinum* was developed and used to compare the sensitivity of rainbow trout (*Oncorhynchus mykiss*), round goby (*Neogobius melanostomas*), walleye (*Stizostedion vitreum*), and yellow perch (*Perca flavescens*) to four doses (0, 800, 1,500, and 4,000 Mouse Lethal Doses) of *Clostridium botulinum* type E neurotoxin. Each fish species expressed unique changes in both behavior and skin pigmentation prior to death. Yellow perch survived significantly longer ($P<0.05$) than the three other species at all toxin treatments. Results of this study suggest that live fish can represent a significant vector for transfer of BoNT/E to birds.

Key words: Avian botulism, *Clostridium botulinum*, fish, Lake Erie, perch, rainbow trout, round goby, type E, walleye.

INTRODUCTION

Clostridium botulinum is a Gram-positive, anaerobic bacterium that produces botulinum neurotoxin, the most toxic substance known, which is the causative agent of botulism. Spores are found in freshwater, marine, and terrestrial environments throughout the world. *Clostridium botulinum* is divided into seven serotypes, denoted A through G, based on the serologic specificity of the toxin produced (Austin, 2001). Fish farmers historically referred to botulism caused by *C. botulinum* neurotoxin type E (BoNT/E) as 'bankrupt disease' in response to the significant mortalities and staggering economic loss that it caused (Huss and Eskildsen, 1974; Eklund et al., 1984). Huss and Eskildsen (1974) first reported the occurrence of type E botulism in farmed fish in rainbow trout (*Oncorhynchus mykiss*) in Denmark. Subsequently outbreaks of botulism in farmed fish have been well documented (Huss and Eskildsen, 1974; Cann and Taylor, 1982), but very little research on the toxicology of BoNT/E in fish has been conducted.

Recent outbreaks of type E botulism in water birds that are thought to eat live fish exclusively once again have highlighted the need for research regarding the toxicology of BoNT/E in fish. The first case of type E botulism causing large bird mortalities in the Great Lakes occurred in 1963 (Fay et al., 1965). Three outbreaks on Lake Michigan occurred between 1963 and 1964, killing an estimated 12,000 birds (Fay et al., 1965). Since 1999 large-scale mortalities of fish-eating birds due to BoNT/E have been observed on the Great Lakes, especially on Lake Erie (Domske, 2003). Although smaller sporadic outbreaks of type E botulism occurred in the 1970s and 1980s (Brand et al., 1983,

1988), recent epizootics have become annually recurrent over the past seven years and involve Lakes Huron, Erie, and Ontario (Canadian Cooperative Wildlife Health Centre database, 2005).

The mechanism of BoNT/E exposure in fish-eating birds is unknown. Given that these birds are thought to eat live fish exclusively, it seems likely that their prey species play a key role in the process. To date, the role of fish as potential transport vectors of BoNT/E to birds has not been adequately investigated. Since the first experimental oral exposure of fish (rainbow trout) to BoNT/E (Skulberg and Grande, 1967), only two other fish species have been subjected to oral exposure: carp (*Cyprinus carpo*) (Haagsma, 1975) and coho salmon (*Oncorhynchus kisutch*) (Eklund et al., 1982, 1984, 2004). There currently is no generally accepted protocol to test the relative sensitivity of various fish species to this toxin.

In an 11-month study (June 2003–April 2004), our objective was to develop a fish botulism exposure model and utilize it to test the sensitivities of selected freshwater fish species to BoNT/E. This allowed insight into the toxicity of BoNT/E in fish and helped to elucidate the role that fish play in epizootics of avian botulism in fish-eating birds.

MATERIALS AND METHODS

Fish acquisition and holding

This project was carried out under the University of Guelph Animal Care Committee Animal Use Protocol 02R161. The four species utilized were juvenile domesticated rainbow trout (214.5±SE 3.4 g) acquired from the Alma Aquaculture Research Station (Alma, Ontario, Canada); round gobies (*Neogobius melanostomas*) (75.7±SE 2.3 g) caught in Lake Erie by the Ontario Ministry of Natural Resources trawler (Erie Explorer, Port Dover, Ontario, Canada); pond-raised walleye (*Stizostedion vitreum*) (61.2±SE 3.0 g) acquired from Leonard Walleye Culture (Kingston, Ontario, Canada); and pond-raised yellow perch (*Perca flavescens*) (88.0±SE 3.5 g) from Cooper Bay Fisheries (Selkirk, Ontario, Canada).

Each species was held in individual 350 l (1.1 m×1.1 m×0.29 m) tanks supplied with continuously flowing (30 l/min) fresh water with excellent water chemistry characteristics (in accordance with University of Guelph, Animal Care Committee Guidelines). A photoperiod of 12D:12L was provided with an automated, shielded fluorescent lighting system. Prior to experimentation, fish were fed a size six-point regular sinking pellet feed (Martin Mills, Elmira, Ontario, Canada). All fish were quarantined in the laboratory for a minimum of two months prior to experimentation.

BoNT/E production

Clostridium botulinum type E (strain Russ) was grown for four days in broth medium containing special peptone, peptone, glucose, and yeast extract (5% special peptone; Oxoid, Basingstoke, Hampshire, UK) in an anaerobic chamber (Coy Laboratory Products, Grass Lake, Michigan, USA) at 30 C. After four days the culture was centrifuged at 20,000 × G with a RC5 Sorvall centrifuge (Dupont Instruments, Wilmington, Delaware, USA) for 30 min at 4 C and the supernatant harvested. One lot of culture supernatant was utilized for the duration of the study. The supernatants then were trypsinized by addition of 10% w/vol trypsin solution (1.4%) at 37 C for 1 hr (Austin and Blanchfield, 1996). The culture supernatant was then two-fold serially diluted, and each dilution was tested for toxicity by mouse bioassay (two mice × five dilutions = 10 mice total) by intraperitoneal injection of 0.5 ml. The highest dilution that was lethal to both mice was defined as one mouse lethal dose (MLD) per 0.5 ml^{-1} (because 0.5 ml was injected into the mouse). The reciprocal of the dilution factor was corrected to 1 µl and was used to describe the concentration of toxin present in the original supernatant (MLD/µl).

For the purposes of dosing, the volume of supernatant that fit conveniently into a No. 5 gelatin capsule (Gaines Nutrition, Santee, Ca, USA) was 100 µl. The original supernatant (400 MLD per 100 µl), was then concentrated 2× to achieve 800 MLD per 100 µl, 3.75× to achieve 1,500 MLD per 100 µl, and 10× to achieve 4,000 MLD per 100 µl, using centrifugal filters (Whatman, Clifton, New Jersey, USA) with a molecular weight cutoff of 30 kDa. Negative control (0 MLD) capsules consisted of the same concentrated culture supernatants neutralized with specific *C. botulinum* type E monovalent antiserum (Aventis, Toronto, Ontario, Canada). No fish treated with these capsules showed clinical signs or died.

No. 5 gelatin capsules were 75% filled with gelatinized corn starch (National 1215, Brampton, Ontario, Canada), and 100 µl of the trypsinized supernatant were added. Capsules were frozen immediately in a conventional freezer and maintained at a temperature of −20 C until the time of intubation.

Experimental design

Four 10-day trials were conducted for each species, with four replicates, each consisting of 12 fish, three fish each intubated with each of 0 (neutralized controls), 800, 1,500, and 4,000 MLD of BoNT/E, respectively, such that 48 fish were utilized (four treatments × three fish per treatment × four replicates of experiment). Fish surviving at the end of the tenth day were considered survivors and were humanely killed by tricaine methanesulfate (MS-222) overdose (3.0 gl^{-1} for 10 min).

Fish acclimation

Fish were held individually at 19.3 C, SE 0.1 C, in 12 90-l aerated aquaria. Fish were fasted for six days prior to experimentation to empty the digestive system and were allowed to acclimate in the experimental aquaria for the final three days of fasting. Experimental fish were not fed for the duration of the experiment.

Fish intubation

Fish were individually placed into a 0.42 gl^{-1} MS-222 anesthetic bath. Once anaesthetized to stage 4 (Summerfelt and Smith, 1990), fish were removed and weighed (Mettler BB2440 Delta Range, Mettler-Toledo, Greifensee, Switzerland). A gelatin capsule was placed into the distal end of a 6-mm-diameter rigid tygon tube, which was slowly passed down the esophagus into the stomach of the fish. Once the end of the tube was inside the stomach, a tube of smaller diameter was advanced down the outer tube, forcing the capsule into the stomach. Then fish were immediately returned to the experimental tanks for recovery. Fish were observed continuously for one hour to ensure that they did not regurgitate the capsule and that they recovered fully from anesthesia.

Capsule degradation

To determine the time after insertion that toxin was released (time 0 for the experiments), placebo capsules were produced using No. 5 gelatin capsules that were 75% filled with corn starch but no toxin was added. Six rainbow trout, round goby, walleye, and 12 yellow perch were fasted and acclimated as described previously and were intubated with the placebo capsules. Beginning 30 minutes later, one fish of each species was killed humanely at half-hour intervals by MS-222 overdose, and their stomachs were dissected to visually determine capsule integrity.

Behavioral assessment and observations

Following capsule administration fish were continuously recorded by video camera, and behavioral changes in the fish were observed for 10 days or until prior death.

Statistical methods

Statistical Application Software (version 8e, Statistical Analysis Software Institute) was used for all statistical analysis; significance level was $P<0.05$. A Levene's test was used to test for homogeneous variances among the three treatment groups. To test for significant differences in the mean time to death at each BoNT/E dose, the data were subjected to a Tukey analysis. A linear regression was performed to test for a significant relationship between toxin dosage and individual times to death. A logistic regression (Proc Cat Mod procedure) accounting for species (4), dose (3), and their interactions was performed to analyze the proportion of fish of each species that died versus the dosages tested.

RESULTS

Capsule degradation

In the rainbow trout, round goby, and walleye, capsule degradation began approximately one hour postintubation and lasted up to two hours. Therefore, two hours postintubation was used as the standardized time of capsule degradation and of toxin release. In yellow perch, capsule degradation began approximately three hours postintubation and lasted up to five hours. Five hours postintubation was used as the standardized time of capsule degradation and of toxin release. The times taken for capsule degradation to occur were subtracted from the time to each clinical sign (including death).

Clinical signs in rainbow trout

No capsule regurgitation was observed. Following intubation, fish exhibited nor-

TABLE 1. Number of rainbow trout showing signs and time (hours) to onset of clinical signs, loss of equilibrium, loss of motion, and death, following exposure to 800, 1,500, and 4,000 mouse lethal dose (MLD) of *Clostridium botulinum* neurotoxin type E at 19.3 C.

Dose (MLD)	Loss of equilibrium	Loss of motion	Death
800	44.2±13.4[a] (5/12)[b]	47.4±14.1 (5/12)	51.8±14.6 (5/12)
1,500	23.5±6.9 (5/12)	24.6±6.5 (5/12)	28.7±8.7 (5/12)
4,000	23.5±6.9 (11/12)	31.1±3.9 (11/12)	31.9±3.8 (11/12)

[a] Time (hr) to onset of clinical sign (mean±SE).

[b] Number of fish with clinical sign/total number of fish.

mal behavior, actively maintaining themselves off the bottom and swimming occasionally around the aquarium, but mainly staying stationary. Their behavior was comparable to that of the control fish.

The first behavioral abnormality observed was an increase in swimming bursts. This swimming behavior was often intermittent; in between bursts fish would settle to the bottom and remain immobile for a period lasting a few seconds and sometimes minutes. As time progressed, loss of equilibrium and increased respiratory rates were observed (as determined subjectively). Fish would settle on the bottom, exhibit loss of equilibrium (usually tilting to one side), and swim erratically for a period. Loss of equilibrium ultimately became more severe, and fish would spend an increasing amount of time resting on the bottom. Fin coordination and righting ability were gradually lost, and the fish would become inverted for short periods. At this stage fish would undergo swimming bursts with no coordination. Loss of equilibrium in 10/21 (48%) fish that exhibited clinical signs resulted in a change in vertical orientation termed "head up/tail down orientation." When these fish attempted to swim, they often breached the surface of the water. Loss of motor function then progressed to total loss of equilibrium. Fish would remain motionless, ventral surface up, resting on the bottom with an increased respiratory rate until loss of respiratory reflexes and death occurred. Dead fish remained in dorsal recumbency, resting on the tank bottom exhibiting opercular abduction.

The mean times following intubation to reach complete loss of equilibrium and loss of motion were 23±6.9 to 44±13.4 hr and 25±6.5 to 47±14.1 hr, respectively. There were no significant differences (all P values >0.05) in the overall time to death between doses (Table 1).

Clinical signs in round goby

No regurgitation of capsules was observed in any test fish. Following intubation, fish exhibited normal behavior, spending the majority of their time resting on the bottom, periodically swimming upwards. The first clinical sign was the development of a faint 1–2-cm-wide black band around the girth of the fish just posterior the pectoral fins. With time, the band of pigment darkened and extended toward the tail. This continued until the entire fish posterior to the pectoral fins was darkened. Later the anterior end also darkened, resulting in the entire body being very dark, almost black.

Despite the drastic hyperpigmentation, behavior of gobies remained otherwise unremarkable until the onset of the second clinical sign, loss of equilibrium, which coincided closely with the later stages of hyperpigmentation. Once observed, loss of equilibrium progressed very rapidly and was complete (fish lying on its back) within a few hours.

TABLE 2. Number of round gobies showing signs and time (hours) of onset of clinical signs, start of hyperpigmentation, complete hyperpigmenation, loss of motion, and death, following exposure to 800, 1,500, and 4,000 mouse lethal dose (MLD) of *Clostridium botulinum* neurotoxin type E at 19.3 C.

Dose (MLD)	Start of hyperpigmentation	Complete hyperpigmentation	Loss of motion	Death
800	13.5±1.2[a] (11/12)[b]	18.4±1.2 (11/12)	19.2±1.1 (11/12)	20.9±1.1 (11/12)
1,500	15.6±1.7 (12/12)	21.8±2.7 (12/12)	23.4±3.0 (12/12)	25.2±3.0 (12/12)
4,000	14.1±1.8 (12/12)	16.7±2.1 (12/12)	18.6±2.2 (12/12)	18.9±2.3 (12/12)

[a] Time (hr) to onset of clinical sign (mean±SE).

[b] Number of fish with clinical sign/total number of fish.

As loss of equilibrium progressed, fish would undergo swimming bursts with no coordination. This often would result in the fish resting on its side or in dorsal recumbency for a period lasting from several seconds to several minutes. This intermittent swimming continued until all voluntary motor function was lost. The time taken to loss of voluntary motor function ranged from 18.6±2.2 to 23.4±3.0 hr and coincided very closely with the time of loss of respiratory reflexes and death (Table 2). There were no significant differences (all P values >0.05) in the overall time to death between doses (Table 2). Dead, completely hyperpigmented fish were observed on the bottom orientated on their side, abdomen, or in dorsal recumbency.

Clinical signs in walleye

No regurgitation of capsules was observed. Following intubation, fish exhibited normal behavior, generally maintaining their bodies slightly off the aquarium bottom with very few swimming episodes. For the duration of the experiment, fish spent the majority of the time at or near the bottom of the aquaria.

The first clinical sign was a slight abduction of the opercular plates. As the condition progressed, opercular abduction became more severe until, ultimately, maximum opercular abduction occurred, impairing respiration. The result was progressively more shallow beating of the operculum with time.

The second clinical sign was progressive loss of equilibrium. This coincided with advanced opercular abduction. Fish would briefly tilt to one side, or their tail would drop slightly. As this progressed, loss of equilibrium resulted in 10/20 (50%) of fish that exhibited clinical signs adopting a "head up/tail down orientation" at some point. When such fish attempted to swim, they often breached the surface of the water. Ultimately, complete loss of equilibrium was observed, and the fish turned in dorsal recumbency. The mean time taken to complete loss of equilibrium decreased as dose increased, from 49±7.5 to 39±4.1 hr (Table 3). This coincided very closely with loss of respiratory activity and death (Table 3). There were no significant differences (all P values >0.05) in the overall time to death between doses (Table 3). Following death the fish were orientated in dorsal recumbency with their opercular plates abducted and bodies arched ventrally.

Clinical signs in yellow perch

No regurgitation of capsules was observed. Following intubation, fish exhibited normal behavior, actively maintaining themselves off the bottom and swimming regularly around the aquarium, staying in one spot only for a few minutes at time.

The first clinical sign was a darkening of pigmented areas that coincided with a slight abduction of the opercular plates.

TABLE 3. Number of walleye showing clinical signs and time (hours) of onset of clinical signs, start of opercular abduction, maximum opercular abduction, and death, following exposure to 800, 1,500, and 4,000 mouse lethal dose (MLD) of *Clostridium botulinum* neurotoxin type E at 19.3 C.

Dose (MLD)	Start of opercular abduction	Maximum opercular abduction	Death
800	38.4±5.6[a] (7/12)[b]	49.1±7.5 (7/12)	50.5±7.5 (10/12)
1,500	31.7±3.5 (10/12)	40.6±4.1 (10/12)	41.4±4.1 (11/12)
4,000	30.7±2.7 (9/12)	38.9±4.0 (9/12)	39.4±4.1 (11/12)

[a] Time (hr) to onset of clinical sign (mean±SE).

[b] Number of fish with clinical sign/total number of fish.

The time taken to the onset of hyperpigmentation decreased as dose increased (Table 4). Opercular abduction was very subtle and progressed only slightly over the duration of the experiment. In contrast, the increase in extent and degree of hyperpigmentation became extreme, and the majority of the fish that exhibited clinical signs ultimately darkened over the entire body surface. As hyperpigmentation progressed, fish showed reduced swimming behavior and intermittently rested on the aquarium bottom for several minutes at a time.

The next clinical sign was loss of equilibrium. Fish first exhibited a slight loss of righting ability, which coincided with the fish spending an increasing amount of time near the surface of the water. Loss of equilibrium progressed, and 17/32 (53%) of fish that exhibited clinical signs adopted a "head up/tail down orientation" at some time. When these fish attempted to swim they often breached the surface of the water. Loss of equilibrium continued until fish rested on the aquarium bottom in dorsal recumbency or on their side (Table 4). The time taken for this to occur decreased with increasing BoNT/E dose. Intermittently, such fish attempted to swim, but they showed no fin coordination or righting ability. Signs then progressed to total loss of voluntary motor function and death. The mean time to death ranged from 140±34.6 to 67±14.9 hr (Table 4) and was significantly longer at the 800 MLD dose than at the 1,500 and 4,000 MLD doses, which did not differ significantly. Dead fish remained on the bottom with darkened pigmentation and slight opercular abduction.

TABLE 4. Number of yellow perch showing clinical signs and time (hours) of onset of clinical signs, start of hyperpigmentation, loss of equilibrium, and death, following exposure to 800, 1,500, and 4,000 mouse lethal dose (MLD) of *Clostridium botulinum* neurotoxin type E at 19.3 C.

Dose	Start of hyperpigmentation	Loss of equilibrium	Death
800	62.7±6.3[a] (3/12)[b]	107.9±27.2 (2/12)	140.3±34.6 (3/12)
1,500	41.5±13.5 (4/12)	NA[c]	79.7±20.9 (8/12)
4,000	36.3±15.4 (4/12)	64.5±15.2 (7/12)	67.7±14.9 (8/12)

[a] Time (hr) to onset of clinical sign (mean±SE).

[b] Number of fish with clinical sign/total number of fish.

[c] NA = sample size too small.

Mortality patterns

At the 800 MLD dose, both mortality and time taken to reach maximum mortality were species dependent. Mortality was highest in the round goby (92%) and was lower in the walleye (83%), rainbow trout (42%), and yellow perch (25%) (Fig. 1). The round goby reached its maximum mortality in the shortest time (24 hr). Despite having different cumulative mortalities, the walleye and rainbow trout arrived at their maximums at the same time (102 hr). Yellow perch took the longest (204 hr) to reach its maximum mortality (Fig. 1).

At the 1,500 MLD dose, the mortality and time taken to reach maximum mortality were also species dependent. The highest mortality occurred in the round goby (100%). Mortality then decreased in the walleye (92%), yellow perch (67%), and rainbow trout (42%). Mortality rate in the goby was 50% per day. Time taken to reach maximum mortality then increased in the rainbow trout (66 hr), walleye (72 hr), and yellow perch (186 hr) (Fig. 1).

At the 4,000 MLD dose the round goby had the highest mortality (100%). Mortality then decreased in the rainbow trout and walleye (92%) and was the lowest in the yellow perch (67%). Overall the round goby reached its maximum mortality in the shortest time (30 hr). Time taken to reach maximum mortality then increased in the rainbow trout (54 hr) and walleye (60 hr) and was the longest in the yellow perch (138 hr) (Fig. 1).

Overall, mortality in each species stayed the same or increased as a function of increasing toxin dose. Mortality ranged from as low as 25% in the yellow perch administered 800 MLD to as high as 100% in the round goby in both the 1,500 and 4,000 MLD treatment groups. The logistic regression showed that only the rainbow trout had a near-significant ($P=0.058$) linear trend when analyzing the number of fish dying in each species over log dose.

A small proportion of fish showed clinical signs during the experiment, mainly slight loss of equilibrium (tilting to one side or the other) and increased numbers of swimming bursts, but subsequently recovered fully prior to the end of the 10-day period.

Dose response

At the 800 MLD dose, significant differences in mean time to death were observed between species. Mean time to death (hr) was significantly longer in the yellow perch (140.2 ± 34.6, $n=3$) than in the rainbow trout (51.8 ± 14.6, $n=5$, $P=0.0006$), walleye (50.8 ± 7.5, $n=10$, $P<0.0001$), and round goby (20.9 ± 1.1, $n=11$, $P<0.0001$) (Fig. 2). The mean time to death of the rainbow trout, round goby, and walleye did not differ significantly (all P values >0.05) from each other.

At the 1,500 MLD dose, the mean time to death was again longest in the yellow perch (79.7 ± 20.9, $n=8$), then decreased in the walleye (41.4 ± 4.2, $n=11$), rainbow trout (28.7 ± 8.7, $n=5$), and round goby (25.2 ± 3.1, $n=11$). The mean times to death for the rainbow trout, walleye, and round goby did not differ significantly (all P values >0.05) from each other, while the mean time to death of the yellow perch was significantly longer than the rainbow trout ($P=0.037$) and round goby ($P=001$) but not walleye ($P=0.0804$).

At the 4,000 MLD dose, the mean time to death was still highest in the yellow perch (67.6 ± 14.9, $n=8$), then decreased in the walleye (39.4 ± 4.2, $n=11$), rainbow trout (31.9 ± 3.8, $n=5$), and round goby (18.9 ± 2.3, $n=11$) (Fig. 2). The mean times to death for the rainbow trout, walleye, and round goby did not differ significantly (all P values >0.05) from each other, while the mean time to death of the yellow perch was significantly longer than the round goby ($P=0.0057$), but not the rainbow trout ($P=0.1348$) and walleye ($P=0.4521$).

To account for the variation in fish weight between species, the individual

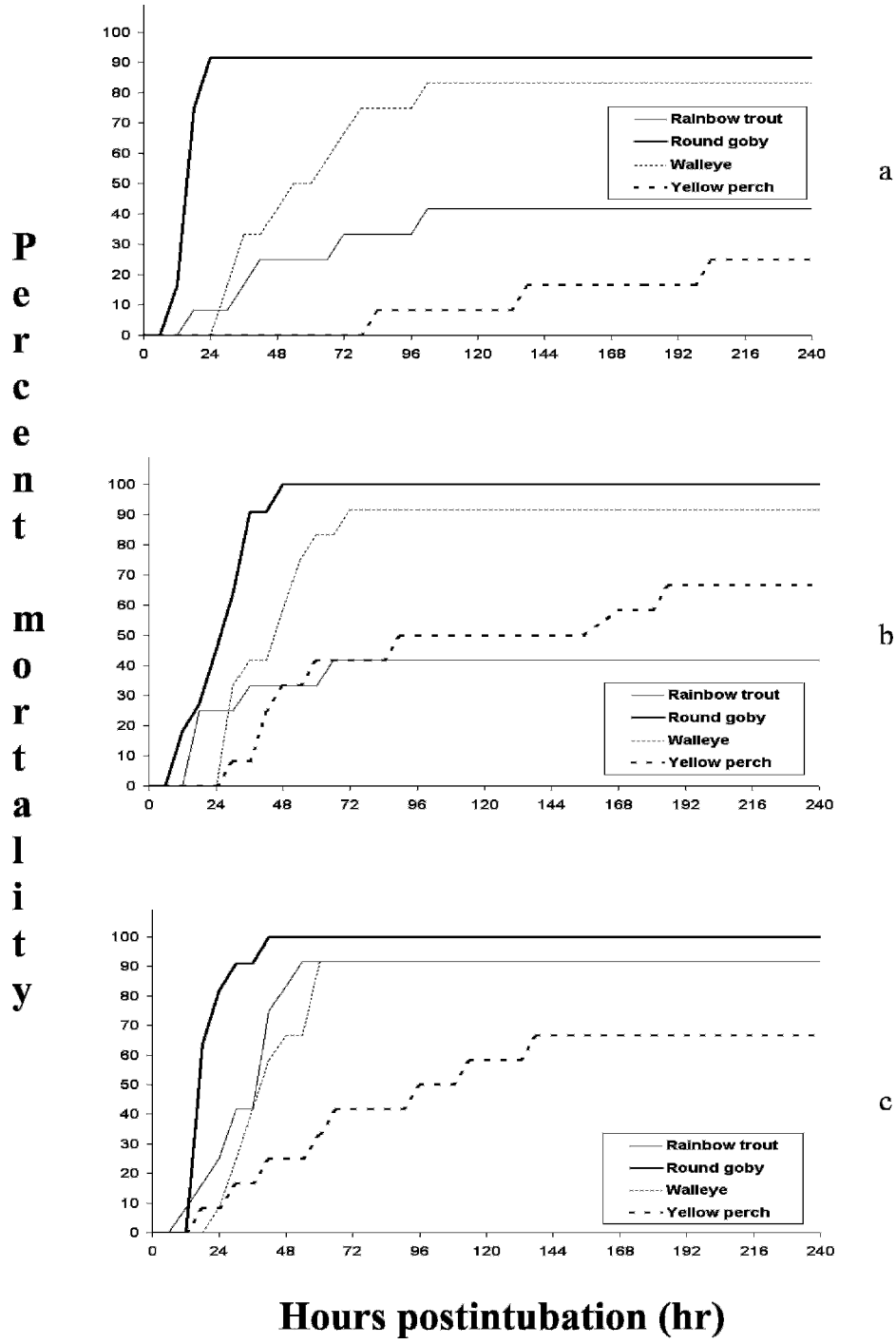

FIGURE 1. Cumulative percent mortality in rainbow trout, round goby, walleye and yellow perch following exposure to 800 (a), 1,500 (b) and 4,000 (c) Mouse Lethal Doses of *Clostridium botulinum* neurotoxin Type-E at 19.3 C (n=12 in each species).

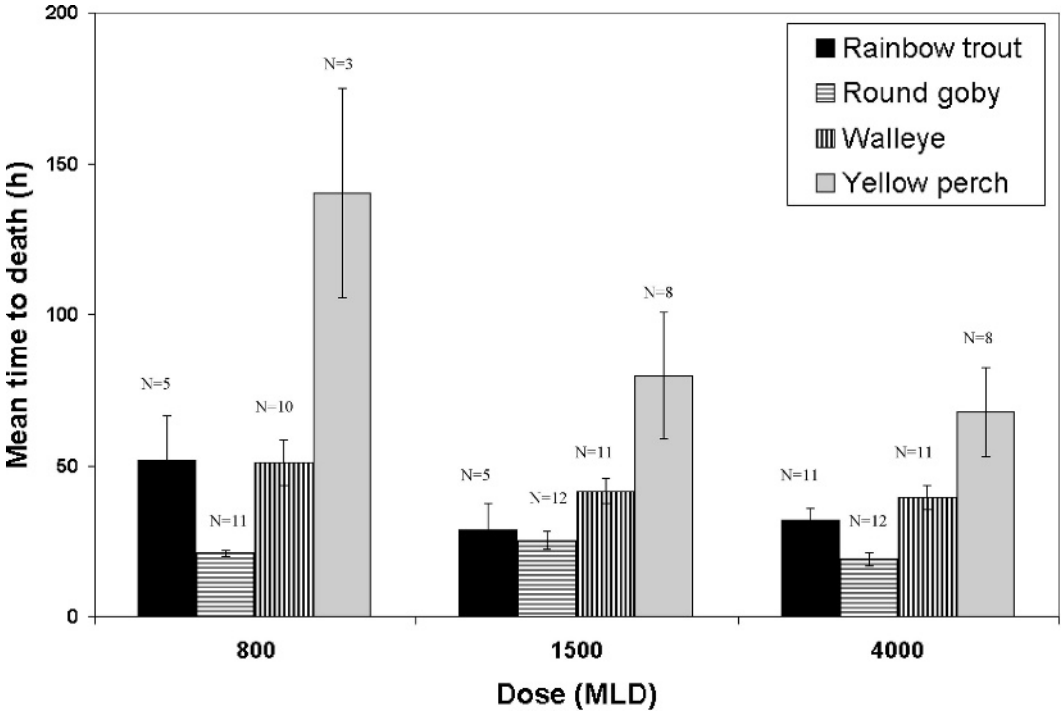

FIGURE 2. Time to death (hr) of rainbow trout, round gobies, walleye and yellow perch exposed to 800, 1,500 and 4,000 Mouse Lethal Doses (MLD) of *Clostridium botulinum* neurotoxin Type-E at 19.3 C (mean ± S.E.). At the 800 and 1,500 MLD doses the time to death of yellow perch was significantly longer than the three other species. At the 4,000 MLD the time to death of yellow perch was significantly longer than the round goby, but not the rainbow trout and walleye. The time to death of rainbow trout, round goby and walleye did not differ significantly at any dose.

times to death were combined and expressed as a function of toxin dosage (Fig. 3). Time to death as a function of $MLDg^{-1}$ showed a negative linear trend (slope$=-0.1238$); however, the slope of the line was not significantly different from zero ($R^2 = 0.0066$) (Fig. 3). Individuals receiving more toxin per body weight did not die faster.

DISCUSSION

Experimental exposure of freshwater fish to BoNT/E has been reported previously, but the doses administered, techniques of toxin administration, and experimental methodology varied greatly among studies (Skulberg and Grande, 1967; Cann and Taylor, 1982; Eklund et al., 2004). Thus, an easily replicated, standard methodological model for testing BoNT/E toxicity in fish such as reported here was needed to compare the sensitivities of various fish species to BoNT/E accurately.

Mortality due to BoNT/E exposure was observed in all four species of fish and occurred at each experimental dose, but never in negative control fish. The mortality pattern in rainbow trout is consistent with observations by Skulberg and Grande (1967), but is contrary to the findings of Cann and Taylor (1982), who observed no mortality in five rainbow trout orally administered 10,000 MLD of BoNT/E. Whether the fish regurgitated toxin after they were returned to the water was unknown (Cann and Taylor, 1982), but regurgitation could explain lack of mortality.

The clinical signs observed in the current study varied among species, al-

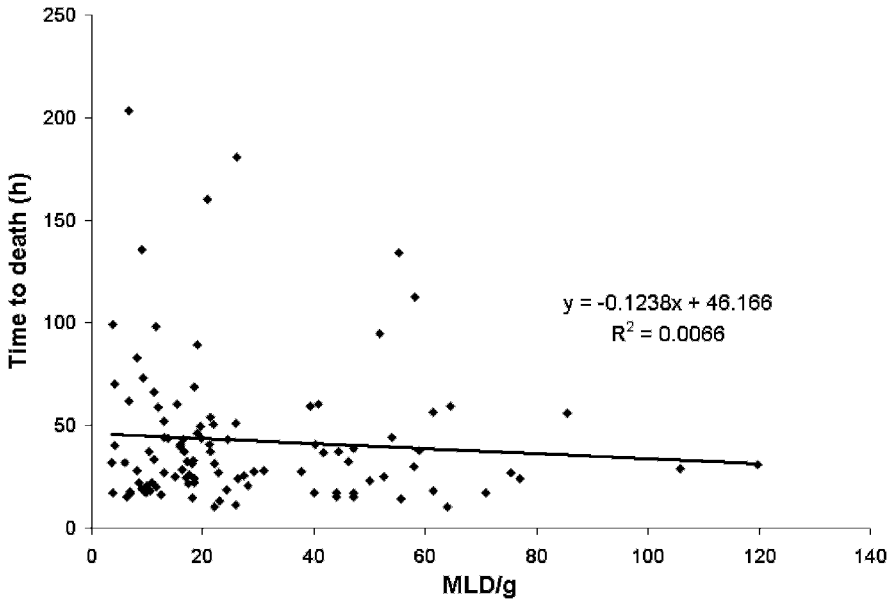

FIGURE 3. Time to death (hr) of rainbow trout, round gobies, walleye and yellow perch as a function of *Clostridium botulinum* neurotoxin Type-E dosage (Mouse Lethal Dose g^{-1}) administered at 19.3 C. The slope of the regression line was not significantly different from zero.

though some signs, such as loss of equilibrium and loss of motion, were seen in all. Cann and Taylor (1982) also described signs similar to those seen here in their rainbow trout, while Skulberg and Grande (1967) reported that following death, rainbow trout exhibited abducted operculums and open mouths. Opercular abduction following death also was observed in the walleye and to a lesser degree the yellow perch in the current study.

Following exposure to BoNT/E, some of the rainbow trout, walleye, and yellow perch exhibited a head up/tail down orientation, and attempts to swim resulted in breaching of the surface of the water. This orientation has been reported in tilapia exposed to BoNT/E (Lalitha and Gopakumar, 2001). Similarly, coho salmon exposed to BoNT/E moved toward the surface of the water, following which they would sink to the bottom as though they were "tail heavy" (Eklund et al., 1982, 2004). *Clostridum botulinum* neurotoxins act by inhibiting the release of the acetylcholine from synaptic vesicles at the peripheral motor nerve terminal (Austin, 2001). The head up/tail down orientation in the affected fish species may be due to inhibition of cholinergic mechanisms controlling the swim bladder, or the motor control of fins that stabilize the fish and maintain its postural balance. Failure of round gobies to display this sign may be because they lack a swim bladder (Jude, 1997).

In this study hyperpigmentation was observed in the round goby, walleye, and yellow perch, a sign also reported in carp administered BoNT/E (Haagsma, 1975). In contrast, a reduction of color intensity has been reported in BoNT/E-intoxicated tilapia (Lalitha and Gopakumar, 2001) and rainbow trout (Cann and Taylor, 1982) but was not observed in the rainbow trout intoxicated in this study.

Melanophores are probably affected, since pigmentation darkens. In bony fish nervous control of chromatophores has been demonstrated (Fujii and Navales, 1972; Fujii, 1993), and acetylcholine

causes either melanophore dispersion (resulting in darkening) or aggregation (resulting in lightening) depending on the species of fish (Fujii, 1969). Our results suggest that in the round goby, walleye, and yellow perch, acetylcholine induces pigment aggregation in chromatophores, as the effect of inhibition of cholinergic nervous mechanisms is pigment dispersion and hyperpigmentation.

The progression of hyperpigmentation toward the tail, then the head, observed in the round goby was unique. However, a head-to-tail progression has been observed in the onset of paralysis of fin muscle control in BoNT/E-intoxicated coho salmon (Eklund et al., 1984, 2004). Similarly, in humans, signs of botulism progress in a descending (head to feet) and symmetrical pattern (Smith and Sugiyama, 1988; Tacket and Rogawski, 1989). However, the onset of clinical signs in avian botulism occurs in the opposite direction, with loss of leg function occurring first with a gradual progression to complete flaccid paralysis (Wobeser, 1997).

Fish exhibiting loss of equilibrium, erratic swimming, hyperpigmentation, and in extreme cases breaching behavior will stand out from normal fish. Consequently, birds that selectively prey on live fish could easily target intoxicated fish. Preferential prey selection by birds that feed on live fish is known to occur. Loons are very opportunistic and tend to select prey whose swimming behavior makes capture easier (Barr, 1973). Prior to feeding, loons lower their heads into the water and look around, in a gesture called "peering" (McIntyre, 1989). Loons peer while swimming over a school of fish and tend to dive and chase any fish that swims erratically, or at a distance from the school. Furthermore, loons show a preference for disabled fish over fish behaving normally if given the choice (Barr, 1973). Free toxin is present in the fillet and visceral fraction of fish following exposure to BoNT/E (Yule et al., in press). Thus, fish-eating birds may ingest BoNT/E because they selectively prey on the intoxicated fish among the population.

Numerous reports have identified BoNT/E in birds after death (Kaufman and Fay, 1964; Fay, 1966; Jensen and Gritman, 1966; Brand et al., 1983, 1988), but experimental estimates of species sensitivities to BoNT/E have been conducted for only a small number of birds. Monheimer (1968) tested the sensitivity of ring-billed gulls (*Larus delawarensis*) to BoNT/E, and the LD50 value was less than 20,000 MLD. The level needed to induce obvious illness in 50% of the gulls was approximately 12,000 MLD. Monheimer also reported herring gulls, great blue heron, common loon, and horned grebe to be susceptible to type E toxin. Eklund et al. (1982) reported that a toxin titer of 400 MLD was detected in the stomach and intestines of 10-g Coho salmon that had been fed 2,000 MLD of BoNT/E. Although sensitivity to BoNT/E of the majority of avian species has not been determined, according to these results only 30 10-g salmon would have to be consumed to cause obvious illness in ring-billed gulls. For more sensitive avian species, this number would be lower.

The sensitivity of fish to the effects of BoNT/E varies between species. The low mortality observed in the yellow perch may be significant, because in the natural setting more tolerant fish could harbor a higher toxin level before clinical signs and mortality occurs, effectively reducing the number of fish required to induce type E botulism in fish-eating birds. However, more susceptible species, such as the round goby and walleye, can still represent a potentially significant vector for toxin transfer. In the natural setting, higher susceptibility would result in a higher proportion of fish exhibiting clinical signs, making them attractive to selective fish-eating birds.

With the exception of the yellow perch, in which the mean time to death at the 800 MLD dose was significantly longer,

toxin dose did not affect this parameter. Cann and Taylor (1982) also reported inconsistent dose responses to experimental BoNT/E exposure in freshwater fish. They had no explanation for the highest mortality occurring in rainbow trout administered the lowest BoNT/E dose. Furthermore, Skulberg and Grande (1967) reported that rainbow trout administered 160,000 and 120,000 MLD of BoNT/E took longer to die than ones administered 80,000 and 40,000 MLD.

Time to death following toxin administration contributes to the potential role that each species plays in the intoxication of fish-eating birds. The longer a fish can survive while harboring BoNT/E, the higher the probability that in a natural setting these fish will be preyed upon by fish-eating birds.

Yellow perch survive significantly longer than the other species following exposure to BoNT/E at all three doses tested. Yellow perch are an abundant species in the Great Lakes (MacDougall et al., 1998), and loons breeding in Ontario, Minnesota, Wisconsin, and Michigan have been reported to feed predominantly on this species (Olson and Marshall, 1952; Barr, 1973). Yellow perch follow the 20 C isotherm, within the upper 5 m of the lake (McIntyre, 1989), and therefore are in a stratum with good visibility and are in the normal feeding zone of fish-eating birds.

The round goby, a benthic species, was not highly tolerant of BoNT/E; the mean time to death of the rainbow trout, round goby, and walleye did not differ significantly at any dose. Although these species exhibited shorter times to death in comparison with yellow perch, their potential role in mortalities of fish-eating birds should not be overlooked. The round goby may be more likely to be consumed by fish-eating birds in Lake Erie than rainbow trout and walleye. The species is very abundant in the areas where bird mortalities occur, and their population density has been reported to exceed 20 fish per square meter of lake bottom (Marsden and Jude, 1995). Gobies also inhabit near shore rocky areas and have been reported to be most predominant in depths of three to five meters. In contrast, walleye and rainbow trout are less abundant and prefer deeper, cooler water.

Round gobies spend the majority of their time near or on the bottom (Jude, 1997), where BoNT/E is more likely to be present, and so one might have expected this species to evolve a higher tolerance to the toxin. However, this appears not to be the case. Round gobies are also very sensitive to low-level chronic BoNT/E exposure (Yule et al., 2006). Haagsma (1975) reported that carp, another benthic species, were also very sensitive to BoNT/E.

The sensitivity of freshwater fish to BoNT/E is to some extent independent of body weight. The time to death of individual fish across species was not related to the dosage of toxin administered to the fish. These results suggest that a threshold dose of toxin is required to induce clinical signs and mortality.

Fish can express prolonged clinical signs of intoxication, and in rare cases fish recovered fully and returned to normal behavior. Recovery from type E botulism has been reported in horses (Cooper, 1964), waterfowl (Klein, 1985; Locke and Friend, 1989), and carp (Haagsma, 1975). The recovery of fish in our study is unique in that it occurred within 10 days of exposure. Once BoNT/E has bound to its receptor the reaction is irreversible and persistent (Raciborska and Charlton, 1999), and recovery occurs by complete regeneration of axons and nerve terminals (Santafe et al., 2000). In most other animals, this process starts two to three weeks postinhibition and can take months to complete (Duchen, 1972).

It is not known if fish can acquire resistance to BoNT/E. Hiroki (1970) reported that carp responded to botulinum toxoid in the same manner as warm-blooded animals and that active immunity can be produced. Such a response has not been confirmed in any other fish species,

nor has an immune response to small doses of toxin been demonstrated.

This study simulated ingestion-preformed toxin. In the natural setting fish may ingest *C. botulinum* spores that germinate in their digestive tract. The conditions within the digestive system of fish, however, may not provide a suitable environment for the growth and proliferation of *C. botulinum*. Fish fed pellets containing 500,000 type E spores daily for five days showed no signs of toxin production as evidenced by clinical signs (Eklund et al., 1984).

If BoNT/E is not produced in the gut, there must be a source of BoNT/E in the environment. In the Great Lakes this source may be linked to the zebra and quagga mussels (*Dreissena* spp.), which filter feed on suspended particles including algae, organic detritus, and bacteria. As zebra mussels feed, they accumulate certain particles and chemicals (DeKock et al., 1993), and this may include *C. botulinum* spores. *Clostridium botulinum* spores may be filtered from the water and concentrated within the feeding mussel. When mussels die and decompose, conditions suitable for spore germination and toxin production may be met. Since the diet of the round goby consists mainly of zebra mussels (Jude, 1997), they might become intoxicated from feeding on, or around, mussel beds.

Dead fish may also contribute to the germination of BoNT/E. As fish decay, conditions are ideal for spore germination, bacterial proliferation, and subsequent neurotoxin production. Dead and decaying fish can contain high levels of BoNT/E, regardless of the initial cause of death (Cann and Taylor, 1982; Eklund et al., 1984). These decaying fish are often on the lake bottom and could be a potentially significant source of BoNT/E. Trout and coho salmon, for example, are voracious scavengers and feed on the soft tissues of dead fish, where toxin often is concentrated (Huss et al., 1974; Eklund et al., 1984).

Invertebrates also might represent a significant transport vector for toxin. The BoNT/E has been demonstrated in snails, earthworms, and small nematodes (Huss et al., 1974). Invertebrates are unaffected by BoNT/E and can contain very high levels within their bodies (Duncan and Jensen, 1976).

Scavenging fish and those that feed on toxin-laden invertebrates may become intoxicated, and fish-eating birds may prey selectively upon these moribund fish, being exposed to lethal BoNT/E doses. The result is amplification of the cycle, similar to what occurs with type C botulism in ducks and gulls (Duncan and Jensen, 1976).

Epizootics of botulism in fish may occur frequently but are not evident until migrating fish-eating birds are present as the top trophic level species to indicate such events. Without dead and moribund birds to act as sentinels, drawing attention to these episodes, dead fish may settle to the bottom and go unnoticed.

The recent type E avian botulism epizootics in the Great Lakes have raised many questions about the mechanisms of BoNT/E transfer to fish-eating birds. This work has demonstrated that fish exposed to BoNT/E show prolonged moribund states and express changes in behavior and pigmentation, both of which in a natural setting could increase the likelihood of consumption by fish-eating birds. Thus, results of this study suggest that live fish can represent a significant transport vector for BoNT/E from its point of origin in the ecosystem to fish-eating birds.

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