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REPEATED LOW-LEVEL EXPOSURE OF THE ROUND GOBY (*NEOGOBIUS MELANOSTOMAS*) TO *CLOSTRIDIUM BOTULINUM* TYPE E NEUROTOXIN

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ABSTRACT: In a 4-mo study (June 2004–September 2004), round gobies (*Neogobius melanostomas*) were dosed orally every 72 hr for up to 21 days with *Clostridium botulinum* neurotoxin type E (BoNT/E) at one of four doses: 0, 50, 250, and 500 mouse lethal doses (MLD). Fish were observed for changes in pigmentation and behavior for the duration of the experiment. Mortality was observed with all treatments, with the exception of the 0 MLD control. Clinical signs observed were consistent with prior research and appeared to occur in a threshold manner. The mean times to death and percent mortalities were dose dependant. Hazard ratios were determined to have a significant positive (parameter estimate=0.03) linear relationship with dose. The hazard ratio showed that per one unit dose increase, the instantaneous probability of a fish dying increased 1.02%. Postmortem analysis of experimental fish demonstrated that 11% (3/27) of fish contained detectable BoNT/E in their visceral fraction. The other 89% tested negative for BoNT/E, despite the fact that all fish died as a result of BoNT/E exposure. Therefore, botulism should not necessarily be ruled out as the cause of a fish kill, even if the fish test negative for BoNT/E.

Key words: Botulism, *Clostridium botulinum*, fish, Lake Erie, *Neogobius melanostomas*, repeated low-level exposure, round goby, type E.

INTRODUCTION

Since 1999, outbreaks of type E avian botulism have caused thousands of mortalities in live fish-eating birds on the lower Great Lakes, especially Lake Erie (McLaughlin, 2003). These outbreaks have raised many questions about the role that live fish may play in epizootics of avian botulism. Live fish may represent a significant vector for the transfer of BoNT/E to live fish-eating birds (Yule et al., 2006b). Most research has focused on one scenario in which fish become acutely intoxicated from BoNT/E because of a single ingestion of preformed toxin. It is also plausible, however, that some fish may ingest sublethal (very low-level) doses repeatedly over much longer time periods. Repeated low-level exposure may be the more likely pattern of uptake and toxin transport into recipient fish.

To date, little research has been conducted on repeated low-level BoNT/E exposure in fish. Eklund et al. (1984) reported high mortality in 12–15-g coho salmon, *Oncorhynchus kisutch*, administered 10 or 20 mouse lethal doses (MLD) BoNT/E per day for 10–20 days.

In this study, round gobies (*Neogobius melanostomas*) were exposed to repeated low-level doses of BoNT/E. Round gobies were introduced inadvertently into Lake Erie prior to 1990 and the species has become prolific and widespread in the lake. Population density of gobies has been reported to exceed 20 fish per square meter of lake bottom (Marsden and Jude, 1995; Jude, 1997). Analysis of stomach contents of dead birds during botulism events on both Lakes Erie and Huron between 1999 and 2000 found gobies to be a prominent food item, with 38% of the birds' stomachs containing goby remains (Campbell, 2002). The goby may be a key factor in the recent avian botulism epizootics. The mortalities of fish-eating birds on Lake Erie have coincided with popu-

lation peaks of the round goby as the expansion of the species progressed from west to east in the lake (Romblee, 2003).

The round goby is not highly resistant to acute intoxication with BoNT/E (Yule et al., 2006b); however, nothing is known about the species' response and sensitivity to repeated low-level exposure. If, in the natural environment, round gobies ingest low doses of BoNT/E on a repeated basis, the species may have an evolved tolerance to low-level toxin exposures.

In a 4-mo study (June 2004–September 2004), our objective was to determine the sensitivity and clinical responses of the round goby to repeated low-level BoNT/E exposure. Furthermore our goal was to determine if BoNT/E is detectable postmortem in round gobies following repeated exposures to known BoNT/E doses. Such a determination will provide further insight into the pathogenesis of BoNT/E in fresh water fish, and help to further elucidate the role that round gobies may play in recent avian botulism epizootics.

MATERIALS AND METHODS

Fish acquisition and holding

This project was carried out under the University of Guelph Animal Care Committee Animal Use Protocol: 02R161. Round gobies (54.6±2.7g SE) caught in Lake Erie by the Ontario Ministry of Natural Resources trawler (*Erie Explorer*, Port Dover, Ontario, Canada) were utilized for the study.

The fish were held in 350-l (1.1×1.1×0.29 m) tanks supplied with continuously flowing freshwater at flow rates of 30 l/min. A photoperiod of 12 hr dark:12 hr light was provided with an automated shielded fluorescent lighting system. Prior to experimentation, fish were fed a 6-point regular sinking pellet feed (Martin Mills, Inc., Elmira, Ontario, Canada). All fish were quarantined in the laboratory for a minimum of 2 mo prior to experimentation.

BoNT/E production and encapsulation

Clostridium botulinum type E (strain Russ) was grown for 4 days in broth medium containing special peptone, peptone, glucose, and yeast extract (5% special peptone-Oxoid, Basingstoke, Hampshire, England) in an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, Michigan, USA) at 30 C. After 4 days the culture was centrifuged at 20,000 × G with an 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 were then 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 twofold 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 MLD per 0.5 ml (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 Number 5 gelatin capsule (Gaines Nutrition, Santee, California, USA) was 100 µl. The original was then concentrated using centrifugal filters (Whatman, Clifton, New Jersey, USA) with a molecular weight cutoff of 30 kDa to achieve the desired doses (50, 250, and 500 MLD). 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.

Number 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 immediately frozen in a conventional freezer and maintained at a temperature of −20 C until the time of intubation.

Fish acclimation

During the course of the experiment fish were held at (±SE) 19.3±0.1 C in 12 individual 90-l aerated aquaria. Fish were fasted for 6 days prior to experimentation to empty their digestive system, and allowed to acclimate in the experimental aquaria for the final 3 days of fasting. Experimental fish were not fed for the duration of the experiment.

Experimental design

Three 21-day trials were conducted, with each trial consisting of 12 fish. A different dose was tested in each trial. In each trial, three fish each were intubated into the stomach with 0 MLD (neutralized controls) capsules, and the other nine fish were intubated with the treatment dose (either 50, 250, or 500 MLD of BoNT/E). Every 3 days, the intubation procedure was repeated on surviving fish, until the conclusion of the experiment. Fish surviving at the end of the 21st day were considered survivors and were killed humanely by tricaine methanesulfate (MS-222) overdose. Intubation of fish was conducted as described previously (Yule et al., 2006b). A capsule degradation time of 2 hr postintubation (Yule et al., 2006b) was subtracted from all time observations to account for the time needed for the gelatine capsule to degrade in the fishes' stomachs and the toxin to be released.

Behavioral assessment and observations

Following capsule administration fish were continuously recorded by video camera (24 hr/day) for the duration of the experiment. Clinical signs including pigmentation changes and equilibrium loss were observed over the 21-day period or until the fish died. The first sign of equilibrium loss was defined as the first observation of the fish turning upside down. Complete equilibrium loss was defined as the point at which the fish remained upside down, lacking the ability to right itself.

Free-toxin analysis

After death (as a result of intoxication, or in the case of survivors to 21 days and MS-222 overdose), experimental fish were immediately removed from their observation aquaria. Individual fish were then placed into a double bag and frozen at −20 C in a conventional freezer. At the time of analysis, fish were thawed and axial muscle and nonmuscle samples were prepared and tested for the presence of BoNT/E as described previously (Yule et al., 2006a).

Statistical analysis

A Cox proportional hazard regression model was utilized to test for a significance relationship between survival curves and dose. This analysis accounted for fish that survived the duration of the experiment by treating them as censored values. Statistical Application Software ('proc phreg' procedure) and a significance level of $P<0.05$ were used for all statistical analysis's (Version 8e, Statistical Analysis Software Inc.).

RESULTS

Clinical signs

Control fish that received capsules containing antiserum-neutralized toxin demonstrated no clinical signs of disease or distress throughout the duration of the experiment. There were no mortalities in the control group.

Prior to death, intoxicated round gobies expressed total loss of voluntary motor function (body and fin movement) and hyperpigmentation (resulting in a progressive head-to-tail darkening of pigmentation as described by Yule et al., 2006b). There was a marked difference in time to onset of these clinical signs across the three treatments. The times to initial loss of equilibrium, complete loss of equilibrium, and total hyperpigmentation were longest in the 50 MLD treatment, and then decreased progressively in the 250 MLD and 500 MLD treatments (Table 1). Time to death was longest in the 50 MLD treatment (180±31.2 hr), decreased in the 250 MLD treatment (78±21.2 hr) and was shortest in the 500 MLD treatment (25.6±1.1 hr) (Fig. 1).

Mortality patterns

Fish treated with 50 MLD of toxin took the longest time to reach maximum mortality (14.3 days) and had the lowest percentage of mortality (89%) of the three treatments (Fig. 2). The 250 MLD and 500 MLD treatments had the same percentage of mortalities (100%), but the time taken to reach this point differed (9.5 days at 250 MLD and 1.3 days at 500 MLD).

Multiple Cox proportional hazard regression models were investigated using all dose levels (0, 50, 250, and 500 MLD). The first model evaluated was a full model including linear, cubic, and quadratic effects. Analysis of reduced models

TABLE 1. Number of round gobies showing clinical signs, and time (hr) to onset of clinical signs (mean±SE): Initial loss of equilibrium, complete loss of equilibrium, and total hyperpigmentation following low-level, repeated exposures to either 0, 50, 250, and 500 mouse lethal doses (MLD) of botulinum neurotoxin Type-E for up to 504 hr (21 days).

Dose (MLD)	Initial loss of equilibrium (hr)	Complete loss of equilibrium (hr)	Total hyperpigmentation (hr)
0	NA[1] (0/9)	NA (0/9)	NA (0/9)
50	180.3±31.2 (8/9)	180.6±31.2 (8/9)	180.8±31.2 (8/9)
250	77.4±21.3 (9/9)	77.9±21.2 (9/9)	78.0±21.2 (9/9)
500	24.5±1.6 (9/9)	25.3±1.14 (9/9)	25.5±1.13 (9/9)

[1] NA = not applicable: fish did not express clinical signs.

showed a significant linear effect ($P<0.001$), but cubic and quadratic effects were not significant. The hazard ratios (sometimes referred to as relative risk 'RR') were determined to have a significant positive (parameter estimate=0.03) relationship with dose. The hazard ratio showed that per one unit dose increase, the instantaneous probability of a fish dying increased (HR=1.02).

Free-toxin analysis

The presence of BoNT/E in postmortem fish was both sample and treatment dependent. No filet samples tested positive for the presence of BoNT/E. Three viscera samples tested positive, one from the 250 MLD treatment and two from the 500 MLD treatment.

DISCUSSION

These results suggest that round gobies are sensitive to repeated low-level BoNT/E exposure. Clinical signs observed were consistent with those described previously (Yule et al., 2006b), and high mortality rates occurred at all treatments. At

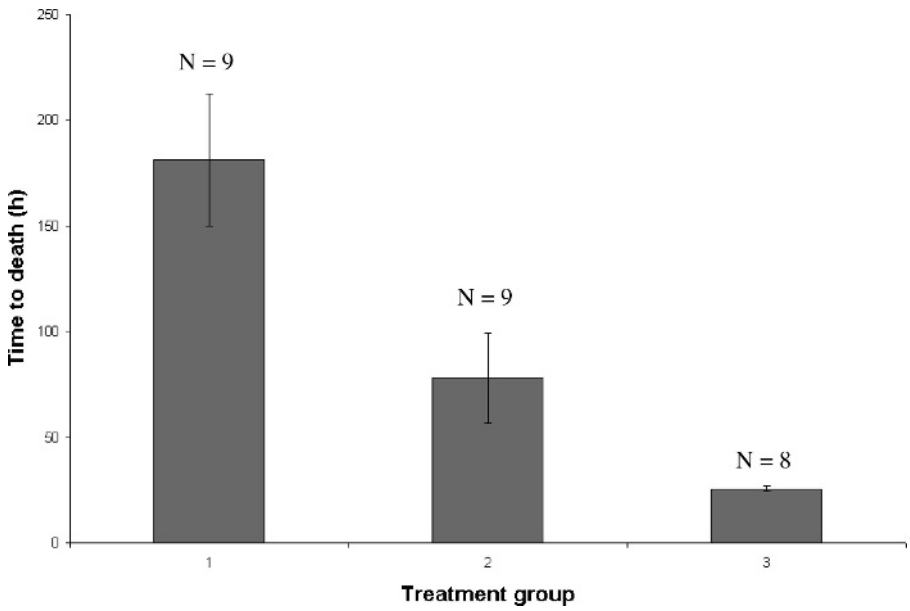

FIGURE 1. Mean time to death (hr±SE) of round gobies exposed to repeated oral treatments of *Clostridium botulinum* neurotoxin type E every 72 hr, for a maximum of 504 hr (21 days) at one of the following doses: 1=50, 2=250 and 3=500 Mouse Lethal Doses ($n=9$ in each treatment). The control group ($n=3$) for each treatment showed no mortality.

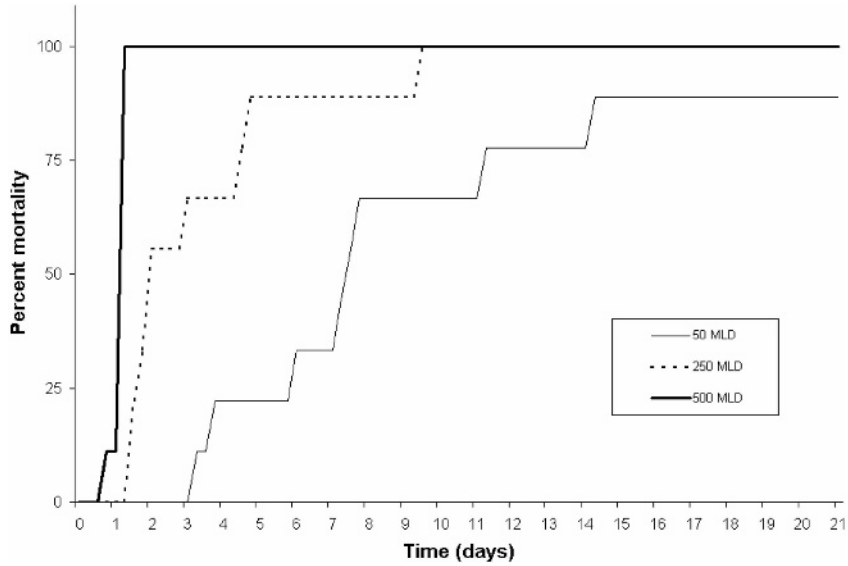

FIGURE 2. Cumulative percent mortality in round gobies following repeated oral treatment with 50, 250, or 500 Mouse Lethal Doses of *Clostridium botulinum* neurotoxin type E every 72 hr for up to 504 hr (21 days) ($n=9$ in each treatment). The control group ($n=3$) for each treatment showed no mortality.

500 MLD the response was essentially acute; no fish survived to receive a second dose of toxin. Times to death for the 500 MLD treatment were similar to round gobies exposed to 800–4,000 MLD BoNT/E (Yule et al., 2006b). However, in fish treated with 250 MLD and 50 MLD BoNT/E, mean times of death were longer, and some fish survived to receive multiple doses.

These data suggest that there is an additive effect of low-level toxin exposures in the round goby, with clinical signs occurring beyond a threshold dose of toxin. This suggests there are a minimal number of key neuromuscular junctions that need to be inhibited to cause clinical signs. At lower doses, it appears that repeated toxin administration is needed to block neurotransmitter release. Once blocked, the reaction is irreversible and persistent (Raciborska and Charlton, 1999). In most animals, once neuromuscular junctions are inhibited by BONT/E, recovery occurs by complete regeneration of nerve terminals (Santafe et al., 2000), which starts 2 to 3 wk postinhibition and can take months to fully regenerate (Duchen, 1972). Postintubation, all fish behaved normally for most of the time prior to death. During this period, each sequential exposure may inhibit further movement of neuromuscular junctions, until the critical threshold for induction of clinical signs occurs. This may explain why the time taken for clinical signs to occur is longer at lower toxin doses, as evidenced by the 50 MLD treatment results. The critical threshold for initiation of clinical signs in these fish appeared to be in the range of 100 MLD of toxin.

Postmortem analysis revealed that a small percentage (11%, 3/27) of intoxicated round gobies had free BoNT/E. In contrast, over 75% of gobies receiving higher single doses of toxin (500, 1,500, and 4,000 MLD) had toxin in their tissues (Yule et al., 2006a), and the mean times to death were shorter than in the present study (Yule et al., 2006b). Eklund et al. (1984) reported that the long time period between toxin ingestion and the onset of botulism signs in coho salmon made it difficult to demonstrate type E toxin in the fish postmortem. In our study, 89% of fish tested negative for BoNT/E, despite the

fact that all fish died as a result of BoNT/E exposure. Once BoNT/E is bound to the neuron, it is no longer available to cause toxicity in the mouse bioassay. This raises an interesting problem in determining the cause of fish kills. Under typical diagnostic procedures a small number of fish are tested to determine the cause of death. If the fish are negative for BoNT/E, botulism cannot necessarily be ruled out as the cause of death, because false negative conclusions could be reached in fish dying from botulism at doses near the lower threshold for intoxication.

Although the percentage of round gobies with detectable BoNT/E postmortem was low, toxin was present in some fish. In nature, if such fish are preyed upon (by birds or other fish) they could become a vector for BoNT/E in the ecosystem. Furthermore, a very small amount of BoNT/E could kill numerous round gobies. It has been hypothesized that dead fish decaying on a lake bottom has the potential to initiate a large-scale botulism epizootic (Yule et al., 2006b).

At the lower dose exposures, round gobies survived for multiple days, but only one of the 27 fish tested did not show clinical signs and die. If the same sensitivity exists in nature, the round goby might be useful as a sentinel species for BoNT/E. The clinical signs the species expresses (dark banding, for example) are very easy visible to the naked eye and could be used to signal the presence BoNT/E, assuming they are unique to botulism.

The present study reveals that round gobies are sensitive to repeated low-level BoNT/E exposure and express clinical signs similar to gobies exposed to higher BoNT/E doses acutely. Exposed round gobies show an additive effect of repeated toxin exposures, with clinical signs occurring in a threshold manner. The high mortality observed at repeated low-level doses suggests that BoNT/E could have significant effects on wild round goby populations.

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