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PERSISTENCE OF *CLOSTRIDIUM BOTULINUM* TYPE C TOXIN IN BLOW FLY (CALLIPHORIDAE) LARVAE AS A POSSIBLE CAUSE OF AVIAN BOTULISM IN SPRING

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ABSTRACT: Diverse samples were examined at a site of water-bird mortality, caused by *Clostridium botulinum* type C toxin in southern Moravia (Czechoslovakia). The toxin was detected in high concentrations in mute swan (*Cygnus olor*) carcasses ($\leq 1 \times 10^6$ LD_{50}/g) as well as in necrophagous larvae and pupae of the blow flies *Lucilia sericata* and *Calliphora vomitoria* ($\leq 1 \times 10^5$ LD_{50}/g) collected from them. It was detected in lower concentrations ($\leq 1 \times 10^3$ LD_{50}/g) in other invertebrates (ptychopterid fly larvae, leeches, sow-bugs) associated with these carcasses, and occasionally in water samples (8 LD_{50}/ml) close to the carrion. The toxin was not detected in the samples of water, mud or invertebrates collected at a distance ≥5 m from the carcasses. The toxin-bearing larvae of *L. sericata* and *C. vomitoria*, containing 80,000 LD_{50}/g of type C toxin, were exposed in the mud at the study site for 131 days from November to March. Although the toxin activity decreased 25-fold and 40-fold in the two samples of maggots exposed during this period, it remained very high (≤3,200 LD_{50}/g). Birds ingesting a relatively low number of these toxic larvae (or pupae) in the spring could receive a lethal dose of the toxin.

Key words: *Clostridium botulinum*, botulism, birds, *Cygnus olor*, flies, *Lucilia sericata*, *Calliphora vomitoria*, experimental study.

INTRODUCTION

An outbreak of type C avian botulism occurred on the water reservoirs at Nové Mlýny (48°52′ to 48°55′N, 16°31′ to 16°44′E) in southern Moravia (Czechoslovakia) in 1988 and early spring 1989, killing >3,000 birds including 68 mute swans *Cygnus olor* (Hubálek et al., 1990). Moreover, three mute swans died in August and September 1988 on the "Hlohovecký" fishpond (48°47′N, 16°46′E) near Lednice, where botulism epornitics are notoriously known to occur (Hudec and Pellantová, 1985). An examination of diverse samples from the fishpond ecosystem at the site of avian mortality revealed a very high level of toxicity in sarcophagous fly larvae and pupae collected from the carrion. The purpose of this study was to test whether or not botulinum toxin could "overwinter" in these fly larvae when exposed under natural conditions. Although a long-term stability of *Clostridium botulinum* type C toxin stored at lower temperatures has been described (Boroff and DasGupta, 1971; Graham et al., 1978; Hubálek and Halouzka, 1988), specific sources of the toxin have not been identified in the spring outbreaks of botulism among waterfowl (Graham et al., 1978; Wobeser et al., 1983; Rachač, 1986; Hubálek et al., 1990). Mud, water, plankton, or invertebrates are suspected reservoirs. The results of this study could contribute to a better understanding of the mechanism of avian botulism occurring in early spring.

MATERIALS AND METHODS

Materials examined

All samples were collected on the "Hlohovecký" fishpond during three visits. These are described in the following paragraphs.

On 12 September 1988, three carcasses (1- to 2-wk-old) of mute swans and one ill swan were present at the site. The samples collected were liver fragments from the two swan carcasses and two pools (56 and 67 specimens) of larval (2nd and 3rd instar) *Lucilia sericata* and *Calliphora vomitoria* from these carcasses; a sample of water (pH 8.1) about 1 m from one of the carcasses; and a number of invertebrates: 100 water boatmen (*Notonecta glauca*), 32 may fly (Ephemeridae) larvae, one leech (*Erpobdella octoculata*), and four snails (*Lymnaea stagnalis*) caught in water at a distance of 5 to 30 m from the carcasses. Zooplankton was absent at the site during that time period.

On 18 October 1988, several dead pikepearches (*Stizostedion lucioperca*) and pike (*Esox lucius*) were observed in addition to the three swan carcasses. Materials examined were organs (liver, heart, brain, gonads, muscles and intestine) of one dead pikepearch, and invertebrates collected from the original swan carcasses including 15 fly larvae (*L. sericata, C. vomitoria, Fannia canicularis*), 17 fly pupae (mostly *L. sericata,* several *C. vomitoria*), one ptychopterid larva, four sow-bugs (*Asellus aquaticus*) and three leeches (Hirudinidae). Additional samples were collected apart from the carrion and included 300 chironomid larvae (benthos), 10 *Asellus aquaticus* (benthos), five *Lymnaea stagnalis,* one sample of mud and two samples of water. Zooplankton was absent.

On 4 November 1988, the swan carcasses were already decomposed except for skeletal, plumage and skin remnants. Only two maggots (*L. sericata*) were found in them. Other fly larvae (about 30) and three pupae (*F. canicularis*) were collected from the soil under the carrion.

Experimental persistence of botulinum toxin in maggots

Two screw-capped and perforated (but impermeable to fly larvae) polyethylene 100-ml bottles were filled with the local pond mud and supplied each with 22 larvae of *L. sericata* (75%) and *C. vomitoria* (25%) that were collected from the swan carrion, stored at −60 C, and found to contain 80,000 LD_{50}/g of *C. botulinum* type C toxin. These two bottles (A, B) were then placed on the shore of "Hlohovecký" fishpond, in the soil at a depth of 10 cm. Sample A was located in a less humid place (never overflooded with the pond water), while the sample B was placed in the pond, at a water-level of 10 to 20 cm. Both samples were exposed on 4 November 1988 and excavated for examination on 15 March 1989, 131 days later. The larvae were then eluted from the content of the bottles, counted and assayed for the toxin.

Weather variables at the study site are described as the overall mean (or sum) and the range of monthly averages (in parentheses) in the period from 1 November 1988 to 15 March 1989. Air temperature: daily mean 2.0 C (0.1 to 5.0 C), mean daily maximum 5.5 C (2.9 to 9.7 C), mean daily minimum −1.0 C (−3.3 to 1.0 C), absolute maximum 15.5 C (8.0 to 15.5 C), absolute minimum −15.3 C (−3.1 to −15.3 C). Soil temperature (in a 10-cm depth): daily mean 2.3 C (0.6 to 4.6 C), mean daily maximum 2.5 C (0.7 to 5.1 C), mean daily minimum 2.0 C (0.6 to 3.9 C). Total precipitation was 101 mm (9 to 42 mm), and there were 12 days (0 to 6 days) with snow cover ≥1 cm. The fishpond was frozen from 23 November 1988 to 6 March 1989, but open for several days during December and February.

Toxin assays

All the specimens were stored at −60 C until assay for botulinum toxin. Invertebrates were washed thoroughly using tap water and sterile distilled water, homogenized (mortar) in physiological saline supplemented with gentamicin (200 μg/ml) to prepare a 10% (w/v) suspension, left for 30 min at 20 C in the dark, and centrifuged for 15 min at 3,000 × *g*. The supernatant fluids were tested for toxicity by injecting 0.4-ml volumes intraperitoneally into two to four 5- to 6-wk-old specific-pathogen free mice. Ten percent (w/v) homogenates in saline with gentamicin were also prepared from the liver of swan carcasses and from various organs of the fish. Aliquots of the organ homogenates from the fish were additionally treated with 0.1% trypsin for 30 min at 37 C before centrifugation and inoculation into mice. The samples of water and 10% mud suspensions (in saline with gentamicin) were centrifuged and inoculated into mice as above.

The suspensions that caused death of mice within 5 days post inoculation were subjected to mouse neutralization tests with the *C. botulinum* type A, B, C, D and E toxin antisera purchased from Imuna (082 22 Šarišské Michalany, Czechoslovakia). Positive (toxin-containing) suspensions were then titrated in mice by using decimal or four-fold dilutions, and the titres were expressed as LD_{50}/g.

RESULTS

Toxin distribution in the samples

Clostridium botulinum type C toxin was detected in a number of samples (the titer given in parentheses).

In the collection on 12 September 1988 it was found in the liver fragments from two mute swan carcasses (800,000 and 250,000 LD_{50}/g), two pools of calliphorid larvae from these carcasses (80,000 LD_{50}/g), and the sample of water (8 LD_{50}/ml). Toxin was not demonstrated in the other samples. In the collection on 18 October 1988 toxin was found in calliphorid larvae (250 LD_{50}/g) and pupae (80,000 LD_{50}/g), a ptychopterid larva (800 LD_{50}/g), three leeches (800 LD_{50}/g), and four sow-bugs (160 LD_{50}/g). All these toxin-bearing invertebrates were scavenging and were collected directly from the two swan car-

casses. Toxin was not demonstrated in the other samples of invertebrates (collected outside the carrion), the fish, mud or water. In the collection on 4 November 1988 toxin was found in two calliphorid larvae (6,300 LD_{50}/g) collected from swan carrion and two larvae (2.5 LD_{50}/g) and three pupae (2.5 LD_{50}/g) of *F. canicularis* collected from the soil below the carcass.

Overwintering of botulinum toxin in fly larvae

The toxin concentration in the pools of larval *L. sericata* and *C. vomitoria* was originally 80,000 LD_{50}/g. Of the 22 larvae installed in each sample A and B, 14 almost intact and three partially decomposed specimens were found in the drier sample A, while 9 nearly intact and one decomposed specimen were found in the humid sample B after 131 days. The toxin titer was estimated as 3,200 and 2,000 LD_{50}/g in the pools of the nearly intact larvae of the samples A and B, respectively, after the exposure. Although the reduction of larval toxicity was 25-fold and 40-fold, respectively, the toxin concentrations still remained very high in both samples. Moreover, botulinum toxin was detected in the water eluate of the soil in sample B (5 LD_{50}/g) whereas not in that of the drier sample A with a lower proportion of decomposed maggots.

DISCUSSION

During the epornitic of botulism, type C toxin was detected in the affected bird carcasses and in the living invertebrates associated with the avian carcasses. There were high levels (80,000 LD_{50}/g) of *C. botulinum* type C toxin in pupae of sarcophagous Calliphoridae (*L. sericata, C. vomitoria*). Potential toxicity of the pupae has not been reported previously. Botulinum toxin obviously persists through the metamorphosis of a calliphorid larva to the pupal stage, contrary to the results of Häagsma et al. (1972) or Duncan and Jensen (1976). The considerable acidity with pH values 2.8 to 3.3 (Greenberg, 1973) of central parts of the blow fly larval midgut even during metamorphosis does not seem to affect the activity of botulinum type C toxin. This toxin is very stable at a wide range of pH values from at least 2.7 to 10.2, and only very acid (pH 1.8) or alkaline (pH 12.0) conditions destroy it (J. Halouzka and Z. Hubálek, unpubl. data).

Various scavenging invertebrates other than calliphorid larvae, collected directly on the carrion, also revealed detectable levels (160 to 800 LD_{50}/g) of botulinum toxin. Alternatively, similar invertebrates collected on the bottom or in water from 5 to 30 m distance from the avian carcasses were free of detectable level of toxin. Fish also were free of toxin; they probably died of an acute anoxia. However, a single water sample collected at a distance of 1 m from a dead swan contained 8 LD_{50}/g of the toxin.

Our results confirm the importance of avian carcasses as the principal vehicle (reservoir) of intoxication for other birds during botulism epornitics (Duncan and Jensen, 1976). Scavenging invertebrates might serve as vectors of the toxin (and bacterium) and the source of intoxication for insectivorous birds. Considerable concentrations (about 1×10^5 LD_{50}/g, 1×10^4 LD_{50}/larva) of botulinum toxin were detected in the larvae of *L. sericata* and *C. vomitoria*. This is in agreement with the observations of other authors (Lee et al., 1962; Richardson et al., 1965; Häagsma et al., 1972; Duncan and Jensen, 1976; Shayegani et al., 1984) who all report up to 1×10^5 to 1×10^6 LD_{50}/g of toxin in larvae. Experimental peroral applications of type C botulinum toxin showed that, on average, 5×10^4 (from 5×10^3 to 5×10^5) mouse LD_{50} will kill an adult duck (Richardson et al., 1965; Häagsma et al., 1972; Duncan and Jensen, 1976; Smith, 1977; Wobeser, 1988). This means that one very toxic maggot of the 3rd instar might contain a dose of the toxin lethal for a mallard duck.

The ability of botulinum type C toxin to "overwinter" in the blow fly larvae or

pupae is probably very important to development of avian botulism in the early spring. Large numbers of the 3rd stage instar larvae emigrate from each carcass before pupation in summer or autumn. When the carrion is situated in water, a majority of these larvae will sink to the bottom and die. Interestingly, *L. sericata* and *C. vomitoria* are able to overwinter as diapause prepupal larvae under appropriate conditions at temperatures above −9 C to −11 C (Davies, 1929; Ring, 1972; Block et al., 1988). Our experiment revealed that (1) the toxin-bearing larvae in the mud remained largely intact (though dead) over 131 days in winter; and (2) the toxin content decreased by only 25 to 40 times over the winter season, to the values of about 1×10^2 to 1×10^3 LD_{50} per larva. Such a toxin concentration could mean that about 10 of these toxic dead larvae (or pupae) would be sufficient to kill a duck when ingested from the bottom in spring.

The toxin inactivation rate in the larvae over the winter season seemed to follow the thermal inactivation curve of *C. botulinum* type C toxin obtained in the laboratory (Hubálek and Halouzka, 1988). The regression equations of the time (in $\log_{10}$ hr) necessary for the in vitro inactivation of type C toxin by 90% (Y_{90}) or 99% (Y_{99}) on temperature X (C) have been $Y_{90} = 3.275 - 0.0595\ X$ and $Y_{99} = 4.053 - 0.0678\ X$, respectively (Hubálek and Halouzka, 1988). Since the mean temperature of the soil at the study site was 2.3 C during the period, this means that a tenfold and hundredfold decrease of toxin activity should be achieved in 57 and 329 days, respectively. The actual data of the study seem to correspond to this model, in that a 25-fold to 40-fold decrease of the larval toxicity was reached within 131 days.

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