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Could blackbird mortality from avicide DRC-1339 contribute to avian botulism outbreaks in North Dakota?

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Abstract Blackbird (family Icteridae) depredation on sunflower (*Helianthus annuus*) crops in the prairie states of the United States has motivated the proposed use of an avicide, DRC-1339 (3-chloro-4-methylaniline), to decrease their numbers. The resulting mortality of blackbirds at wetland roosts could increase the potential of avian botulism occurring in affected marshes. To assess this possibility, we seeded (artificially placed) blackbird carcasses in selected wetlands in Stutsman County, North Dakota, during August–September 2000 and July–September 2001 to evaluate their rate of decomposition and role in initiating avian botulism outbreaks. We monitored carcasses to determine their persistence, the frequency and amount of maggots produced, and the presence of type C botulinum toxin. In 10 of our 12 study wetlands, blackbird carcasses were not rapidly removed by scavengers, thus providing substrate for maggot growth and potential production of *Clostridium botulinum* toxin. Decomposition of carcasses occurred rapidly, and maggot production averaged 4–5 g per carcass within 9 days. We were unable to detect *C. botulinum* type C toxin in any of the 377 blackbird carcasses or the 112 samples of maggots we collected in 2000 or 2001. None of the 25 blackbird carcasses we tested contained botulinum spores, the most probable explanation for the absence of botulinum toxin production. Our results indicate that the likelihood of DRC-1339-poisoned blackbirds causing botulism outbreaks would be minimal in North Dakota wetlands during late summer and early autumn.

Key words avian botulism, blackbird carcasses, *Clostridium botulinum*, DRC-1339, Icteridae, North Dakota, wetland ecosystems

Blackbirds (family Icteridae) are responsible for extensive damage to ripening sunflower (*Helianthus annuus*) crops in North Dakota, South Dakota, and Minnesota, ranging from 5.1–7.9 million dollars annually (Hothem et al. 1988). The compound 3-chloro-4-methylaniline (also known as 3-chloro-p-toluidine hydrochloride or DRC-1339) has been tested for efficacy as an avicide in North Dakota and South Dakota to reduce local blackbird abundance (Linz and Bergman 1996, Linz et al.

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2003). The goal of applying DRC-1339 is to reduce depredation on ripening sunflower crops, particularly by red-winged blackbirds (*Agelaius phoniceus*). Sunflower fields nearest to large roost sites tend to experience greatest depredation (Otis and Kilburn 1988) and thus are commonly chosen for baiting. In 1998 DRC-1339-treated brown rice was applied to over 450 individual bait sites throughout the Prairie Pothole Region of North Dakota. These sites were 0.1–0.4 ha in size and baited, respectively, with 5,000–11,000 treated rice grains. Each treated rice grain contained enough DRC-1339 to kill a blackbird (K. Johnson, United States Fish and Wildlife Service [USFWS], personal communication). The application may also expose nontarget birds to DRC-1339 (Custer et al. 2003, Pipas et al. 2003). Blackbirds typically die within 24–72 hours subsequent to ingesting treated bait (Besser et al. 1967). Affected birds frequently seek water and often die in or near wetlands where they are roosting. During baiting operations in 1998, over 75% of bait sites were located within 5 km of at least one Waterfowl Production Area (WPA) (K. Johnson, USFWS, personal communication). Mortality of blackbirds occurring at wetland roost sites in WPAs could put these areas at an increased risk for avian botulism outbreaks.

Avian botulism is a lethal disease of waterfowl and other waterbirds caused by *Clostridium botulinum* type C, an anaerobic spore-forming bacterium that produces a potent neurotoxin. *Clostridium botulinum* spores are widely distributed in wetland ecosystems including sediments, invertebrates, fish, and other vertebrates that use wetlands (Rocke and Friend 1999). Botulism outbreaks have been linked to decomposing vertebrate carcasses. Tissues of wetland birds not killed by botulism also have been found to contain *C. botulinum* spores (Gunderson 1933, Reed and Rocke 1992), and many animals in wetland environments frequently ingest botulism spores. After these animals die, regardless of the cause of mortality, their carcasses can provide ample substrate in which botulinum spores can germinate and produce toxin (Duncan and Jensen 1976, Wobeser and Galmut 1984). These decomposing carcasses may contain thousands of maggots, and even a few maggots may have a sufficient amount of toxin to kill other birds (Wobeser 1997). Wobeser (1997) described many of the factors that could influence the probability of the occurrence of an avian botulism outbreak caused by vertebrate carcasses. These factors include the portion of carcasses that contain spores, the rate of carcass removal by scavengers, and the proportion of carcasses that become infested with toxin-laden maggots.

A second pathway for initiating botulism outbreaks may occur when suitable wetland environmental factors favor the production of toxin that is ingested by susceptible birds, resulting in a mortality event. In these situations, botulism outbreaks may occur within wetland ecosystems in the absence of carcasses. Both types of outbreaks are more likely under favorable environmental conditions associated with water temperature, pH, and redox conditions (Rocke and Samuel 1999). Although the relative importance and interaction of these 2 pathways is not understood, it is clear that conditions such as warm temperatures, a large amount of suitable substrate, and abundance of susceptible birds are likely to increase the probability and magnitude of outbreaks. In general, the likelihood of avian botulism typically increases during mid to late summer (August–September), when high ambient and wetland temperatures, fly activity, and maggot production can increase substantially. Maggots accumulate toxin from these carcasses, and consumption of toxin-laden maggots causes mortality in waterfowl and other wetland species. An avian botulism carcass–maggot cycle can result, leading to potentially explosive outbreaks of avian botulism in wetland systems. Botulism outbreaks have occurred sporadically in the prairies of North Dakota and South Dakota since the early 1900s and have become much more frequent in recent decades (Rocke and Friend 1999). Although there is annual variation in botulism cycles in prairie wetlands, these outbreaks pose a considerable risk to migrating waterfowl, shorebirds, and other wetland species.

The purpose of our study was to investigate whether blackbird carcasses, which could result from the application of DRC-1339, provide a suitable substrate for maggot growth and type C botulinum toxin production in wetlands. To accomplish this we seeded (artificially placed) freshly dead and frozen blackbird carcasses in a number of wetlands and monitored them to determine how long carcasses persisted in wetlands, the percentage that became maggot-infested, the amount of maggots produced, the duration of maggot infestation, and the presence of *C. botulinum* toxin and spores in these carcasses.

Methods

Wetland seeding trials

During spring and summer 2000 and spring 2001, we collected carcasses of blackbirds in North Dakota and South Dakota. We placed carcasses in plastic bags and froze them for subsequent use in field trials. We identified wetlands for field trials based on characteristics that indicated potential for use as blackbird roosts (shallow water, abundance of cattails [*Typha* spp.], and submerged trees) and presence of blackbirds at sunrise and sunset, indicating active roost sites. All study wetlands were located in Stutsman County, North Dakota. We seeded carcasses in 3 wetlands during August–September 2000: Soupir (47°11′41″N, 98°34′26″W), Edmunds (47°15′19″N, 98°56′40″W), and Eldridge (46°50′51″N, 98°51′59″W). We seeded carcasses in 9 wetlands from July–September 2001: Soupir, Edmunds, Legge (47°00′46″N, 98°21′15″W), Ladish (46°55′16″N, 98°28′45″W), Huber (46°56′31″N, 98°28′49”W), Homer (46°50′58”N, 98°39′53″W), O'Meara (46°54′32″N, 98°25′03″W), Miedema (46°40′41″N, 98°18′48″W), and Wendel (46°50′49″N, 98°19′54″W).

To begin each trial, we placed up to 60 blackbird carcasses in each study wetland. We placed 30 wooden stakes (5 cm × 5 cm × 2.5 m) >15 m apart in or near cattail vegetation throughout each test wetland. We numbered, painted, and flagged each stake with ribbon to assist in relocation. One or 2 blackbirds were attached securely to each stake at water level with monofilament fishing line (0.33 mm diameter). Each carcass was designated "A" or "B" by affixing a piece of marked cloth tape to each of the monofilament lines. We obtained georeferenced locations at all stakes using a Garmin 12 Global Position System unit (Garmin International, Olathe, Kans.). We estimated density of carcasses seeded into each wetland by determining the area of a minimum convex polygon that contained all carcasses and divided number of carcasses by polygon area.

Trials lasted 9–15 days, depending on rates of carcass disappearance and decomposition that occurred during the trial. We visited stakes approximately every 3 days to monitor the status and condition of blackbird carcasses and to obtain carcasses for type C botulinum testing. On day 3 of each seeding trial, we monitored stakes labeled #1–10 (or alternative combination totaling 10 stakes). We used a fishnet to collect 1 carcass from each of the 10 stakes and drain excess water. We stored collected carcasses in Zip-loc® bags (S. C. Johnson & Son, Inc., Racine, Wisc.) and labeled them with the date, wetland, stake number, and carcass letter. We chilled these carcasses in an insulated ice chest within 1 hour, and they were frozen (–20°C) within 6 hours. We repeated monitoring on days 6 and 9 of the seeding trial until all 30 stakes had been visited. Carcasses were periodically monitored for up to 6 additional days during cooler weather to allow continued decomposition and potential production of *C. botulinum* toxin for the Soupir and Eldridge wetlands in 2000 and the Homer, O'Meara, and Miedema wetlands in 2001. We collected remaining carcasses on the last day we checked the wetland (9–15 days after seeding).

In 2000 we recorded both the status (floating, sunken, or missing) and condition (degree of decomposition—fresh, maggots present, maggot infested, or decomposed) of the carcasses at each monitored stake and opportunistically at other

Blackbird dissection and maggot collection were performed at the National Wildlife Health Center, Madison, Wisconsin.

stakes visited during monitoring activities in each wetland. In 2001 we recorded the status of each carcass as above, but we divided carcass condition into 2 subcategories: 1) decomposition (fresh, partially decomposed, and very decomposed), and 2) presence of maggots (present, absent, or infested). For comparability between years, the 2000 data were converted to this format as follows: birds with maggots were considered partially decomposed; those classified as decomposed were considered very decomposed; and the presence of maggots for the decomposed group was determined from samples collected from the carcasses during laboratory dissection (see below).

We collected environmental and water-quality measurements (depth, temperature, conductivity, dissolved oxygen, pH, and redox potential) at 5 of the 30 stakes, at points distributed throughout each wetland. We collected measurements one morning during the trial between 0600 and 1000 using a Yellow Springs Instruments, Inc., 600 XL meter (Yellow Springs, Oh.). We calculated average measurements for each environmental parameter from the 5 locations in each wetland (Table 1). We calculated the relative risk of avian botulism outbreaks based on environmental conditions in each wetland using the risk model developed by Rocke and Samuel (1999). They found that water temperature, pH, and redox potential were associated with probability of avian botulism outbreaks occurring in wetland ecosystems. Higher levels of water temperature, lower ranges of redox potential, and neutral pH values were associated with increased probability of botulism outbreaks.

Analysis of carcass persistence

We used nonparametric methods for the analysis of survival data to estimate the disappearance rate of blackbird carcasses seeded in the study wetlands. The periodic monitoring and removal of carcasses required that the survival analysis consider both right- and interval-censored data. A carcass removed for *C. botulinum* toxin testing provided a minimum estimate of survival time, or right-censored data. Carcasses that disappeared from the wetland between monitoring periods provided interval-censored data. Although the exact day of loss was unknown, a lower limit for survival was based on the last day a carcass was observed and the upper limit was the day when the carcass was first determined to be missing. Carcasses that disappeared were assumed to have been removed by an unknown scavenger or predator. We estimated and plotted survival curves for carcasses in each wetland using the Kaplan-Meier product-limit survival methods (Lee 1980). We used correlation

Table 1. Average values of selected environmental parameters, carcass disposition, and maggot growth in 3 North Dakota wetlands, August–September 2000 and 9 North Dakota wetlands, July–September 2001.

									Carcasses		
Wetland	Date seeded	Depth (cm)	Conductivity Temp (ºC)	(uS/cm)	DO(mg/L)	pH	Redox (mV)	Botulism risk[a]	Density[b]	Disappearance[c]	% with maggots[d]
Soupir	15 August 2000	44.4	17.90	2,166.4	0.322	7.65	–251.90	0.71	5.02	2.5	1.7
Edmunds	25 August 2000	56.3	15.31	1,125.0	0.392	7.20	–266.94	0.44	17.9	14.0	32.6
Eldridge	19 September 2000	35.7	11.64	465.6	4.794	8.46	–87.14	0.31	7.11	43.7	0
Soupir	27 July 2001	41.1	20.61	2,041.2	ND[e]	7.25	–139.64	0.24	5.14	2.6	84.7
Edmunds	1 August 2001	55.0	22.97	1,445.2	ND	7.24	–118.50	0.21	13.8	0	56.7
Legge	14 August 2001	31.1	18.13	499.8	0.72	7.76	–45.02	0.31	1.98	0	41.7
Ladish	15 August 2001	38.2	17.58	1,725.8	1.17	8.56	–51.44	0.39	2.00	0	58.3
Huber	19 August 2001	52.5	22.24	1,125.0	0.32	8.04	–37.10	0.47	27.4	3.3	34.4
Homer	29 August 2001	53.1	17.38	512.8	0.23	7.75	–41.30	0.29	3.44	0	26.7
O'Meara	30 August 2001	20.6	18.04	555.8	0.36	7.85	–37.20	0.33	3.66	0	53.3
Miedema	3 September 2001	31.8	18.74	1,041.2	0.10	7.82	–85.00	0.43	3.34	6.7	61.4
Wendel	4 September 2001	29.0	16.71	551.4	0.55	8.10	–18.40	0.32	2.01	71.9	3.6

[a] Relative risk (0.0–1.0) of avian botulism outbreak based on environmental values of redox, pH, and temperature (Rocke and Samuel 1999).

[b] Carcass density estimated as # blackbirds per hectare.

[c] Disappearance (100 – persistence) measured as percent of carcasses present on day 9.

[d] Percent of observed carcasses supporting maggot growth on or before day 9.

[e] Level not detectable.

analysis to test for covariates that influenced the estimated disappearance rate of carcasses at 9 days post-seeding.

Analysis of maggot abundance

We determined maggot presence, absence, and infestation on carcasses for each wetland by using necropsy findings (2000) or field observations (2001) at days 3, 6, and 9 post-seeding. For each collection period (including days 12 and 15 in 2001), we determined percentage of collected carcasses from each wetland that had maggots present. We also determined the cumulative percentage of carcasses with maggots at day 9 for each wetland from observations and collections for all periods up to day 9; we considered a carcass positive if maggots were present on day 3, 6, or 9. We excluded birds that disappeared from the wetland and were not observed during any of the collection periods from the number of total birds checked for that collection period. We determined the average mass of maggots for carcasses only for blackbirds that had maggots during the collection period.

Blackbird carcass testing

To assess levels of type C botulinum toxin produced in the blackbird carcasses, we thawed each bird at 8ºC for approximately 48 hours. We then dissected each bird using sterile procedures to remove all maggots and separated soft tissues (including organs) from feathers and bones. We weighed maggots and tissues separately to the nearest 0.01 g and stored them in heavy-duty plastic bags. Tissues from each blackbird were diluted 1:3 with sterile saline solution and pulverized separately in a stainless-steel industrial-strength blender (Waring Commercial blender with Model MC1 attachment, New Hartford, Conn.). Maggot samples were diluted 1:10 with sterile saline solution and pulverized with a mortar-and-pestle grinder. We stored samples in 15- or 50-ml polypropylene centrifuge tubes (Corning, Inc., Corning, N.Y.) at 8ºC until tested (within 2–4 hours) for presence of toxin. We centrifuged blended samples at 3,000 RPM for 30 minutes at 5–8ºC. In 2001 only half of the blackbird carcasses from each collection period at a wetland were analyzed, each individually. Maggots collected from all birds during 2001 were pooled by wetland and collection date, and the combined samples were diluted 1:10 in saline for testing. Pooled maggot samples contained maggots from 3 to 10 carcasses, all collected on the same date, with an equal weight of maggots from each carcass.

We conducted a screening test of blackbird tissue and maggot samples for presence of type C botulinum toxin using an indirect antigen-capture enzyme-linked immunosorbent assay (ELISA). This assay followed the method of Rocke et al. (1998), utilizing polystyrene immunosticks as the solid substrate, with 3 modifications: 1) the immunosticks were refrigerated for 36 hours (rather than overnight) before the blocking step; 2) samples were incubated for 48 hours (rather than overnight) before processing; and 3) Sigma-Aldrich reagents (St. Louis, Mo.) were substituted for the Kirkegaard and Perry reagents in 2000. Little or no color change on the bottom half of the immunostick indicated a negative result, moderate uneven color change was classified as nonspecific reactivity, moderate solid color change was considered a suspect positive, and strong solid color change was ELISA positive.

We placed half of each sample supernatant (1.6–7 ml) into either a 2-ml cryovial (Nunc, Inc., Naperville, Ill.) or 50-ml polypropylene centrifuge tube (Corning) to use for the ELISA test. We used MaxiSorp immunosticks (Nunc, Inc., Naperville, Ill.), which were previously coated with affinity-purified chicken antibody as described in Rocke et al. (1998). Pooled maggot samples that were positive by ELISA were retested using 1.6-ml maggot sample from each individual carcass in the pool, if sufficient sample remained. Blackbird tissue or individual maggot samples that were ELISA-positive or suspect-positive were tested by a mouse toxin neutralization bioassay, following the general methods described by Quortrup and Sudheimer (1943), with modifications reported in Rocke et al. (1998), to confirm presence of type C botulinum toxin. We thawed the frozen sample at 4ºC and mixed it 7:3 with antibiotic media (Rocke et al. 1998). For each sample tested, 2 white mice (female, Institute of Cancer Research strain, 15–20 g), 1 nonprotected and 1 control, were then inoculated intraperitoneally (IP) with 0.5 ml of the mixture. We protected the control mouse with an IP inoculation of 0.2 ml type C botulinum antitoxin, previously produced in rabbits at the National Wildlife Health Center (NWHC), 30 minutes prior to injection with the sample. We observed mice for signs of botulism toxicity (hind-limb paralysis, wasp waist, labored breathing) or death for 4 days post-inoculation. A positive test was indicated if the nonprotected

mouse died or showed signs of botulism and the control mouse remained healthy. A negative test was indicated if both mice survived. If both mice died, we considered the test inconclusive. For a few tissue and maggot samples, there was not adequate material remaining to test at the dilutions described, so these samples were diluted further (in the range of 1:2 to 1:10) before testing. We also tested a portion of the tissue and all the maggot samples showing a nonspecific ELISA reaction by the mouse toxin neutralization assay to determine whether these samples contained botulinum toxin.

Samples from 9 seeded carcasses and 16 maggot samples collected in 2001 that had enough sample remaining were cultured in cooked meat carbohydrate media (Gibco/BRL, Gaithersburg, Md., with added trypticase peptone, potassium phosphate, yeast extract, L-cysteine hydrochloride, glucose, and vitamin K) for 2 days to determine whether dormant C. botulinum spores were present. This procedure causes dormant spores to germinate and produce toxin. We tested these samples to determine whether blackbirds (and maggots) used in the seeding trials had ingested spores that had not germinated while carcasses were in the wetland. For each sample, we inoculated 0.1 ml of the blended mixture into cooked meat media and incubated for 5 days at 32ºC. We then filtered each sample through a 0.45-micron filter and tested the filtrate for botulinum toxin using the ELISA, as described previously. We conducted a mouse bioassay for any ELISA-positive or suspect-positive sample, using the same procedures as done previously.

Results

Environmental conditions (temperature, conductivity, dissolved oxygen, pH, and redox potential) varied among wetlands and years sampled (Table 1). Wetland temperature was typically higher in midsummer (August) and declined toward autumn (September). Conductivity in the 12 wetlands ranged from approximately 500–2,000 uS/cm, and dissolved oxygen varied from undetectable levels to a high reading of 4.8 mg/L, but was typically less than 1 mg/L. Measurements of pH were between 7.00 and 8.56, and redox potential ranged from approximately –20 to –270. Except for Soupir during 2000, environmental conditions at the 12 wetlands were not strongly favorable for occurrence of avian botulism outbreaks (Rocke and Samuel 1999). In addition, we observed no avian botulism epizootics on these wetlands during experimental trials.

Carcass persistence

In 2000 each wetland had different persistence rates for seeded blackbird carcasses (Figure 1*a*). Few blackbird carcasses disappeared from Soupir (3.3%) and Edmunds (14%) during the study period; however, an estimated 15% of blackbird carcasses disappeared within 3 days at Eldridge, and an additional 30% disappeared by day 9. In 2001 the persistence rate was high (>85%) for 8 of the 9 wetlands seeded (Figure 1*b*). However, at Wendel, 55% of the carcasses were missing by day 3, and an additional 17% had disappeared by day 6. We found that disappearance rates of carcasses were positively correlated ($P=0.045$) with date that wetlands were seeded, with carcasses disappearing more rapidly in wetlands that were seeded later in the summer. Carcass disappearance was not correlated with wetland depth ($P=0.99$), density of blackbird carcasses ($P=0.49$), predicted risk of a botulism outbreak ($P=0.43$), or related to differences between years in the study ($P=0.62$).

Carcass decomposition occurred rapidly in most

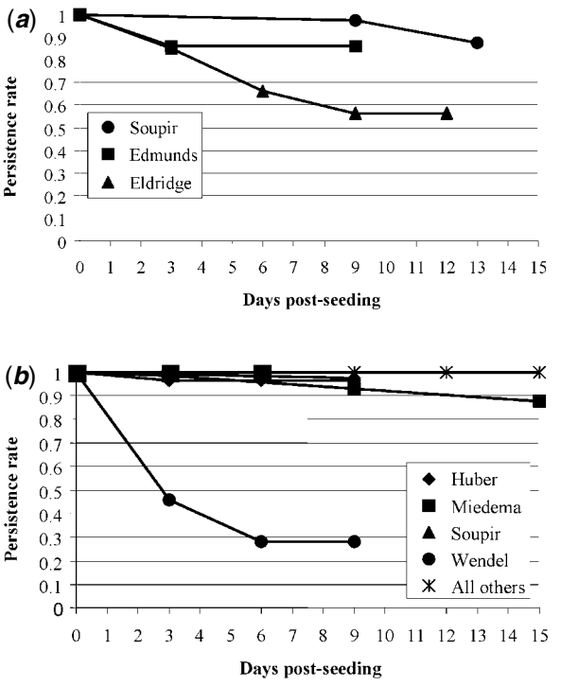

Figure 1. Persistence of blackbird carcasses seeded in 12 North Dakota wetlands during August–September 2000 (*a*) and during July–September 2001 (*b*). Persistence estimated using Kaplan-Meier survival methods (see text).

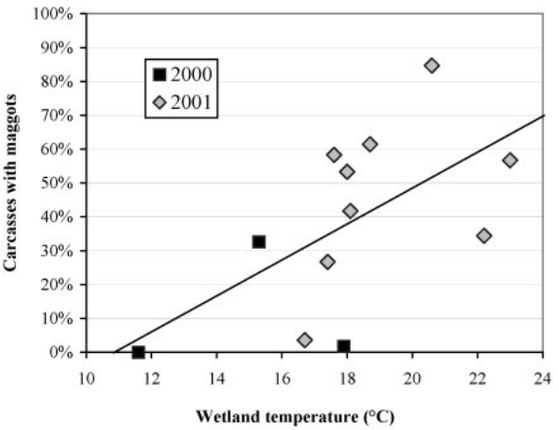

Figure 2. Percent of seeded blackbird carcasses with maggots at 12 wetlands in North Dakota in 2000 and 2001 by wetland water temperatures. Cumulative percent of carcasses with maggots measured at day 9 post-seeding.

wetlands. Blackbirds in all wetlands were very decomposed by day 13, except in Eldridge (2000) and Wendel (2001), both of which were seeded late in the season. Carcasses in Edmunds and Huber (2001) showed the fastest decomposition, with almost all birds being very decomposed by day 6. The percentage of carcasses that sank to the bottom increased with the level of decomposition.

Maggot production

In 2000 the percentage of carcasses with maggots at 9 days post-seeding was greatest in Edmunds wetland (32.6%), which was seeded 25 August. There was no significant maggot production in the 2 other wetlands during 2000 (Table 1). In 2001 the percentage of carcasses with maggots was greatest for Soupir wetland (84.7% on day 9), which was seeded on 27 July. For both years combined, there was a reduction in the percentage of carcasses with maggots as the summer progressed ($r = 0.62$, $P = 0.03$, $n = 12$). A trend was also observed between wetland water temperature and percentage of carcasses with maggots (Figure 2). This association showed a positive correlation when both years were combined ($r = 0.59$, $P = 0.04$, $n = 12$).

For the 3 wetlands in 2000, the percentage of collected carcasses with maggots (23.1%) and average weight of maggots per carcass with maggots (2.1 g ± 1.14 SE) were greatest 3 days post-seeding, and subsequently declined. No maggots were present ≥12 days post-seeding. In 2001 the percentage of carcasses with maggots (77.5%) and average weight of maggots (5.9 g ± 0.74 SE) was highest at 6 days post-seeding. Some maggots were still present after 15 days post-seeding in 5.3% of the carcasses, but the average weight declined to 0.8 g (±0.13 SE) per carcass. For both years combined, the percentage of carcasses with maggots by day 9 was 34.6% (Table 1), with an average maggot weight of 3.5 g (±0.34 SE) per blackbird.

C. botulinum toxin assays

In 2000 we screened 125 carcasses using the ELISA method. Of these, 74 (59.2%) were negative, 42 (33.6%) exhibited nonspecific reactivity, and 9 samples (7.2%) were suspect-positive (Table 2). Of the 15 maggot samples we tested, 3 (20%) were negative, 10 (66.7%) displayed nonspecific reactivity, and 2 (13.3%) were suspect-positive (Table 3). All 11 carcasses and maggot samples that were suspect-positive by ELISA were found to be negative for mouse toxicity. Of the 52 samples with nonspecific reactivity, 25 of these samples (15 blackbirds, 10 maggots) were determined negative by mouse toxin neutralization assay.

In 2001 we screened 252 blackbird carcasses by ELISA. Of these, 221 (87.7%) were negative for the presence of botulinum toxin, 12 (4.8%) exhibited nonspecific reactivity, 8 samples (3.2%) were suspect-positive, and 11 samples (4.4%) tested positive (Table 2). Of the 93 individual maggot samples we screened by ELISA, 40 samples (43%) were negative, 8 samples (8.6%) had nonspecific reactivity, 23 (24.7%) were suspect-positive, and 22 samples (23.7%) showed a positive reaction (Table 3). Four of the initial pooled maggot samples, representing 31 individual carcass samples, were negative by ELISA screening, and therefore maggot samples from individual carcasses were not retested. All 65 carcass and maggot samples that were suspect-positive or positive by ELISA, and 17 samples with nonspecific ELISA reactivity (9 blackbird, 8 maggot) were negative by mouse toxin neutralization test. The 25 maggot and blackbird samples cultured for spores were all negative for active toxin by mouse test, but 3 of the maggot and 3 of the blackbird samples had positive reactivity by ELISA.

Discussion

We found that the rate of carcass disappearance generally was low ($\bar{x} = 12\%$) in the 12 wetlands seeded with blackbird carcasses during 2000 and 2001. There were no carcasses missing (0% disappearance) in 5 of the wetlands, and disappearance was

Table 2. Seeded blackbird carcasses collected from 12 marshes in Stutsman County, North Dakota, August–October 2000 and July–September 2001, tested for *Clostridium botulinum,* type C toxin by an enzyme-linked immunosorbent assay (ELISA) and mouse toxin neutralization test.

	ELISA test					Mouse test	
Year Wetland	*n*	Negative	Nonspecific reactivity	Suspect positive	Positive	*n*[a]	Positive
2000							
Soupir	55	34	20	1	0	8	0
Edmunds	40	21	14	5	0	12	0
Eldridge	30	19	8	3	0	4	0
2001							
Soupir	34	31	1	2	0	3	0
Edmunds	30	26	2	1	1	2	0
Legge	33	25	5	0	3	7	0
Ladish	16	15	0	1	0	1	0
Huber	30	28	1	0	1	2	0
Homer	32	24	2	3	3	8	0
O'Meara	33	28	1	1	3	5	0
Miedema	34	34	0	0	0	None tested	
Wendel	10	10	0	0	0	None tested	
Total	377	295	54	17	11	52	0

[a]All samples that were suspect positive and a portion of those that had nonspecific reactivity by ELISA test were tested for toxicity in mice.

<20% in 5 additional wetlands. Linz et al. (1991) reported that a higher rate (32% in 7 days) of blackbird carcasses disappeared in wetlands with water depths comparable to those used in our study (>30 cm). However, Cliplef and Wobeser (1993) reported that only 2% of waterfowl carcasses were removed from wetlands in prairie Canada. Linz et al. (1991) found an inverse relationship between water depth and rate of carcass disappearance. We were unable to detect any relationship between carcass persistence and water depth in the wetlands seeded in this study, although wetland depth in our study sites was usually >30 cm. In addition to wetland depth, the rate of scavenging also may be influenced by seasonal or local food availability and location of carcasses relative to wetland edge. We observed that carcass disappearance was highest in wetlands seeded late in the season, with greatest losses at Eldridge and Wendel, which were seeded in September. Carcasses in these wetlands were situated closer to the edges than in other wetlands and may have been more accessible to scavengers. Linz et al. (1991) reported that carcass disappearance was greater in blackbirds placed in a high-density pattern in the wetland at depths <30 cm. We did not observe a correlation between carcass disappearance rate and carcass density in our wetlands; however, it is difficult to determine how the range of carcass densities used in our study compared with those used by Linz et al. (1991).

For all wetlands we found maggots on an average of 35% of carcasses collected for laboratory necropsy; however, results were highly variable in each of the wetlands. The proportion of carcasses observed in the field with maggots by day 9 averaged 8.6% overall in 2000 but increased to 46.1% in 2001. This relationship most likely occurred because maggot growth is increased by warm conditions (Reed and Rocke 1992). Maggot production and water temperature were positively correlated in our 12 wetlands. Other environmental conditions such as rainfall and wind speed also may have been responsible for these patterns by hindering flies from landing and laying eggs on carcasses (Reed and Rocke 1992); however, we did not assess these factors during our seeding trials.

Blackbird carcasses provided a suitable substrate for growth of maggots, which could accumulate botulinum toxin produced in the carcasses. We observed that decomposition of carcasses and maggot production occurred rapidly; typically blackbirds were substantially decomposed and infested with maggots within 3–9 days, with an average of 3.5 g of maggots produced for each carcass collected. We estimated a potential mass of approximately 1,200 g of maggots per 1,000 blackbird carcasses (1,000 birds × 34.6% birds with maggots × 3.5 g /bird). Rocke et al. (2000) reported that ingestion of <1 g of maggots collected during the peak of a botulism outbreak could cause mortality in green-winged teal (*Anas crecca*), pintail (*A. acuta*), and mallard (*A. platyrhynchos*) ducks. Given optimal conditions, blackbird carcasses provide a suitable protein substrate with sufficient maggot production to pose a potential risk for initiating a botulism outbreak.

Table 3. Maggot samples collected and tested for type C botulinum toxin by an enzyme-linked immunosorbent assay (ELISA) and mouse toxin neutralization test from blackbird carcasses from 3 wetlands in Stutsman County, North Dakota, seeded August–October 2000 and 9 wetlands seeded July–September 2001.

	ELISA test					Mouse test	
Year Wetland	*n*	Negative	Nonspecific reactivity	Suspect positive	Positive	*n*[a]	Positive
2000							
Soupir	1	1	0	0	0	None tested	
Edmunds	14	2	10	2	0	12	0
Eldridge	No maggots collected						
2001							
Soupir	18	9	0	4	5	9	0
Edmunds	14	6	4	3	1	8	0
Legge	18	5	0	5	8	14[c]	0
Ladish	3 pools[b]	3 pools	0	0	0	None tested	
Huber	1 pool	1 pool	0	0	0	None tested	
Homer	7	1	2	2	2	6	0
O'Meara	17	5	1	7	4	12	0
Miedema	17	13	0	2	2	4	0
Wendel	2	1	1	0	0	1	0
Total	108 + 4 pools	43 + 4 pools	18	25	22	66	0

[a] All samples that were suspect positive and those that had nonspecific reactivity by ELISA test were tested for toxicity in mice.

[b] In 2001 all maggots were first tested as pooled samples (by marsh and date). If the pool was negative, no further testing was done. If positive, maggots were tested individually and data presented separately.

[c] One additional maggot sample was tested by mouse toxin neutralization, but there was insufficient material for an ELISA.

Development of type C botulinum toxin in wetland environments is dependent on the presence of *C. botulinum* spores, a suitable substrate for cell growth, and environmental conditions that are conducive to spore germination, bacterial growth, bacteriophage activation, and toxin production (Rocke and Friend 1999). In our field study we were unable to detect type C botulinum toxin (using the combination of ELISA screening with mouse toxin neutralization) in any of the 377 blackbird carcasses and 112 maggot samples collected from the 12 wetlands during the seeding trials. There are several possible reasons for the difference between ELISA and mouse neutralization tests results for toxin detection. First, ELISA positives could result from the binding with non- or low-functional toxins caused by point mutations in the toxin gene (Oguma et al. 1984, Gregory et al. 1996) or by botulinum strains with low toxicity (Lee and Riemann 1970) that were not detectable by mouse bioassay. Second, low quantities of toxin might have been present in the carcasses but were denatured by carcass enzymes and rendered inactive so that they could not be detected by the mouse test. Finally, it is possible that the ELISA provided false positive results from a cross-reaction to proteins other than toxin that were present in our tissue and maggot samples. For the types of samples we processed in this study, ELISA tests were most useful for screening large numbers of tissues to identify those that were toxin-negative. Verification of active toxin from positive ELISA tests required mouse test neutralization, although we failed to detect any toxin in our samples.

We also were unable to detect the presence of spores in the 16 maggot and 9 carcass samples tested. In contrast, Reed and Rocke (1992) detected spores in 50% of healthy mallards sampled in California by culture and mouse toxin assay. The low recovery of type C botulinum toxin in our study indicates that blackbird carcasses generally were not favorable for production of toxin. Although these birds may provide a suitable protein source for maggot growth, the production of *C. botulinum* toxin is unlikely to occur if no spores are present within the carcass prior to death (Wobeser 1997). Since these birds feed primarily on terrestrial insects and seeds (Martin et al. 1951) where type C botulinum spores are scarce, the potential for spore ingestion and toxin production in these carcasses may be minimal.

In addition to a suitable substrate provided by animal carcasses, favorable environmental conditions also may play a role in determining the relative risk of a botulism outbreak within a wetland (Rocke and Samuel 1999). The environmental conditions measured at the 12 wetlands we sampled

Large numbers of blackbirds typically roost in wetland environments near sunflower fields in the central prairies of the United States.

indicated that conditions were most favorable at Soupir wetland in 2000. Based on environmental conditions in our study wetlands, it appeared the risk of botulism outbreaks occurring in the absence of animal carcasses generally was low, and no botulism mortality was observed in our study wetlands. As a result, it is difficult to determine whether type C botulinum toxin and bird mortality would have been produced in these wetlands under more favorable environmental conditions. If botulism mortality had occurred during our study, it may have been difficult for us to establish whether blackbird carcasses or favorable environmental conditions were responsible for initiating the outbreak.

Management implications

Our findings indicate that application of DRC-1339, and subsequent blackbird mortality, during late summer and early autumn is unlikely to increase the occurrence of avian botulism outbreaks in North Dakota wetlands. Although our blackbird carcasses were not actively removed by scavengers and provided a suitable substrate for maggot production, none of the carcasses or maggots we tested had detectable levels of type C botulinum toxin or had evidence of *C. botulinum* spores. Absence of toxin was likely related to the lack of *C. botulinum* spores ingested by blackbirds. To minimize the risk of outbreaks, we recommend that DRC-1339 applications occur in early autumn when maggot production is reduced. Assuming that blackbirds are not ingesting spores, the probability of botulism also would be low during the cool spring months, when maggot growth would be reduced and toxin production could potentially occur. Although there are a few reports of outbreaks of botulism in the winter and spring, these are typically in diving ducks that ingest sediments containing residual toxin produced in the wetland the previous summer (Rocke and Friend 1999).

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