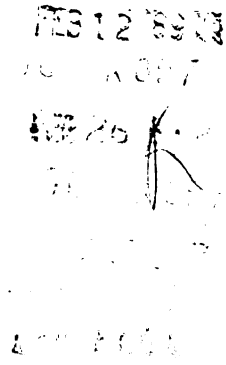

ABSTRACT

THE EFFECT OF ORAL INGESTION OF *CLOSTRIDIUM BOTULINUM* TYPE E ON CAPTIVE GULLS

by Richard H. Monheimer

Twenty-six juvenile Ring-billed Gulls and six juvenile Herring Gulls were successfully raised in captivity. Culture lots of *C. botulinum* type E were produced to force feed to the gulls: the maximum titer of toxin obtained was 15,900 mouse LD50 doses per milliliter. Seventy-six percent of the Ring-billed Gulls fed culture died. Only one of five Herring Gulls died and it drowned when placed in water while sick with botulism. Blood samples were taken from the gulls following the forced feeding. These samples were titered to determine the amount of toxin in the circulatory system at intervals after feeding. The possibility of the growth of *C. botulinum* type E in Lake Michigan and its relationship to the waterbird mortalities is discussed.

THE EFFECT OF ORAL INGESTION OF *CLOSTRIDIUM BOTULINUM* TYPE E ON CAPTIVE GULLS

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX TABLES

INTRODUCTION

Clostridium botulinum type E is a rod-shaped, anaerobic bacterium whose optimum growth temperature is between 25 and 30 C (Bergey's Manual for Determinative Bacteriology, 1957). As this culture grows, it produces a very potent exotoxin which, upon oral ingestion by selected vertebrates, is extremely lethal (Lamanna, 1959). A review of the literature (Dolman, 1960) shows that cases of type E botulism in humans have usually been associated with marine foodstuffs, such as pickled herring and salmon eggs; Pedersen (1955) concluded that *C. botulinum* type E is very prevalent on the sea bottom. Thus, the organism has been generally associated with a marine environment and not until recently has it been found in bodies of fresh water in North America.

The first evidence that *C. botulinum* type E was present in the Great Lakes came in 1960, when an outbreak of botulism occurred in Minneapolis, Minnesota. Smoked "ciscos" from the Great Lakes served as the vehicle for the food poisoning (Kautter, 1964). In September, 1963, an outbreak of botulism occurred in Tennessee, Alabama, and Kentucky which was attributed to smoked "chubs" taken from Lake Michigan (Scholtens et al., 1965). Later that same fall there occurred a high mortality of gulls (*Larus argentatus*; *L. delawarensis*), loons

(*Gavia immer*) and several other species of waterbird in southern Lake Michigan. Examination of blood and tissues from a random sampling of these dead birds showed the presence of varying amounts of *C. botulinum* type E toxin (Kaufmann and Fay, 1964). A similar mortality occurred in northern Lake Michigan in the summer and fall of 1964, and an examination of the carcasses also showed type E toxin to be present in the birds (Fay, 1965).

While there is considerable documentation of *C. botulinum* type C as the causative agent of waterbird mortalities in the North American prairies (Kalmbach and Gunderson, 1934), nothing is known of the association between such mortalities and *C. botulinum* type E. To ascertain whether the Lake Michigan waterbird mortalities could be caused by *C. botulinum* type E, it must be determined whether or not these birds are susceptible to the type E toxin. It is this question which this investigation attempted to answer.

MATERIALS AND METHODS

Rearing gulls for experimental purposes. Early in July, 1964, six Herring Gulls (Larus argentatus) and 30 Ring-billed Gulls (L. delawarensis), whose ages were estimated to be between one and five weeks, were captured at the gull nesting colony located near Rogers City, Michigan. These gulls were transported to the Rose Lake Wildlife Research Center, East Lansing, Michigan, where they were raised and kept until used in the feeding experiments, which were done at Michigan State University.

Upon arrival at Rose Lake, the gulls were sorted and placed into five outdoor cages; the six Herring Gulls were placed in one cage and the Ring-billed Gulls, in groups of seven, in four other cages. Two of the Ring-billed Gulls were much smaller than the others (about one week of age) so these were placed in a small cage indoors.

The outdoor cages used for the first three weeks were constructed of wood and chicken wire and measured four by eight by three feet. Later, the gulls were transferred to cages measuring ten by ten by six feet. The roofs of these cages were covered with burlap to provide the birds with some protective shade. The gulls were held in the larger cages until used for experimental purposes.

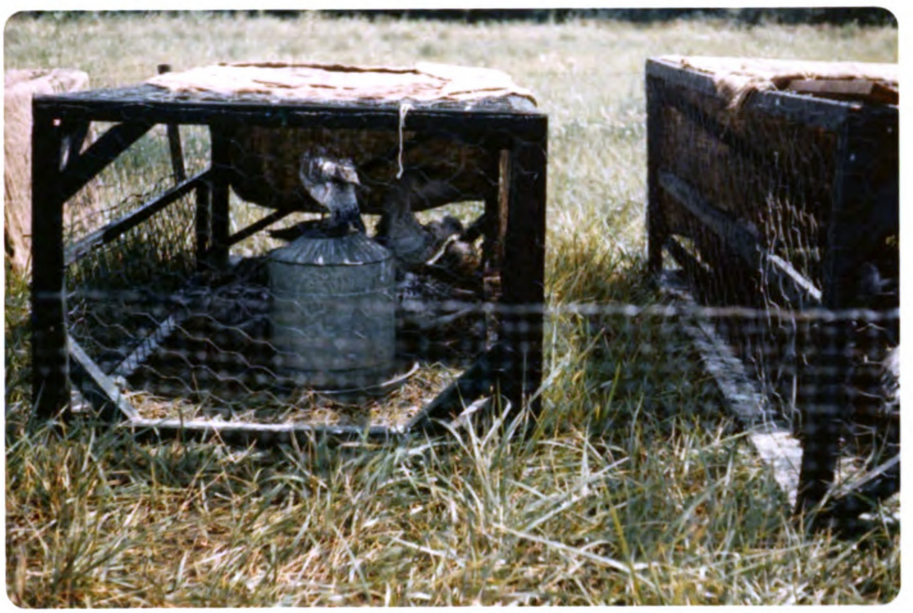

Figure 1. The smaller outdoor cages used for holding the gulls. The fence in the foreground was to keep out predators.

Figure 2. The larger outdoor cages used for holding the gulls.

During their first three days in captivity, the gulls were fed live minnows, fresh frozen smelts, and other commercially packaged frozen fish. After this, they were fed venison (supplied by the Michigan Department of Conservation) and chopped frozen "whiting" of Atlantic Ocean origin (obtained from National Foods Sales, Inc., Fond du Lac, Wisconsin). The gulls ate, without hesitation, all of these foods when dropped on the floor of the cage. The amount of food given to a cage of gulls during one feeding was slightly more than that which the gulls would eat immediately. During the first two weeks the gulls were fed four times a day while during the third week they were fed only twice a day, and after the third week they were fed only once a day.

While the gulls were in the smaller cages, water was supplied to them in inverted-can water dispensers. Although this left much to be desired, it appeared to be sufficient for the gulls' needs. When the larger cages were used, a system of continually running water was constructed. A pipe three quarters of an inch in diameter and 50 feet long was placed through the row of cages, two feet above the ground and one foot from the rear of the cages. This pipe was attached to a hose which carried fresh water. From a hole in the bottom of the pipe, water flowed, in each cage, into a water-tight galvanized chicken feeder which measured eight inches by six feet by eight inches deep. This provided ample space for the gulls to bathe as well as drink. To prevent flooding, the

overflow water passed through a hole near the top of the feeder into rubber hosing which carried the water outside and away from the cages.

Sanitation of the smaller cages was accomplished by moving them whenever necessary to fresh ground. The larger cages, which were too heavy to be moved, had cedar posts anchored upright in them. The gulls preferred to sit on these posts rather than the ground and the fecal material thus tended to accumulate. Sand was used periodically to cover this soiled area, and sand also was spread occasionally over the entire floor of the cage.

The cages in which the gulls were held indoors measured 20 by 30 by 14 inches high and were constructed of wire and sheet metal. Food and water were served in clay or glass crocks. Sanitation was accomplished by placing the cages over pans containing disinfectant (Medic, Michigan State Industries, or Roccal, Sterwin Chemicals Incorporated) and water at a ratio of about two ounces per gallon. This disinfectant solution was changed daily.

Preparation of cultures of C. botulinum type E for toxin. Production of C. botulinum type E toxin suitable for use in feeding experiments necessitated using a strain that produced a high titer of toxin under suitable culturing conditions. The organism chosen was a strain isolated by Mr. Ralph Johnston (Food and Drug Administration, Detroit, Michigan) from fresh "whitefish chubs" (Coregonus spp.) taken from Lake Michigan.

This strain is identified by the Food and Drug Administration as 026-080 X and will hereafter be referred to, in this paper, as C. botulinum type E - J.

The medium selected for culturing C. botulinum type E - J contained 5.0% Trypticase (B.B.L.), 0.5% peptone, and 0.4% glucose (T.P.G. medium) with 0.004% methylene blue added as an indicator of the oxidation-reduction potential. Anaerobic conditions were produced by filling the culture bottles completely immediately after autoclaving while the medium was still hot; 165-ml prescription bottles were filled so as to contain 160 ml of medium, 500-ml containers were filled with 475 ml of medium, and 625-ml prescription bottles were filled with 600 ml of medium. The bottles were inoculated after cooling to approximately 30 C. Five different lots of culture were produced and these lots were designated 'a' through 'e'.

When viewing isolated colonies of C. botulinum type E - J using light reflected from a concave mirror passing up through the plate, two different colonial types were seen: a light and a dark type. Light or dark colonies, or a mixture of the two types, were inoculated into tubes containing ten milliliters fluid thioglycollate medium (Difco). Incubation for 48 hours at 25 C produced an actively growing starter culture, of which two milliliters served as the inoculum for each bottle of T.P.G. medium in a culture lot. The colony type used to inoculate each lot was designated as 'd' for dark, 'l' for light, and 'm' for a mixture of light and dark colonies.

A summary of the conditions under which the various lots of toxin were cultured is shown below:

Culture code	Incubation time (days)	Incubation temperature (C)	Volume of medium (ml)
Lot 'a'	1.5	25	160
Lot 'b'	5	25	475
Lot 'c'	5	30	475
Lot 'd'	5	30	475
Lot 'e'	5	30	600

Following appropriate incubation, the broth from each container comprising a culture lot was pooled into a large sterile flask and dispensed in 80-ml quantities into 100-ml screw-cap bottles; these bottles were stored at -15 C. A sample of the unfrozen culture was used immediately to test the toxicity of the culture. A titration was also undertaken whenever a bottle was thawed for use in a feeding experiment. The cultures were thawed by placing the bottle in a 400-ml beaker of cold tap water and gently shaking every ten minutes; approximately one hour was required to thaw the bottle of culture.

The titer of each lot of culture was determined by injecting white mice with various dilutions of the culture broth. Appropriate dilutions were made with a buffer containing 6.8%

potassium dihydrogen phosphate and 0.2% gelatin. White mice weighing 18 to 27 grams were injected with 0.2 ml of the diluted culture. The mice were observed for three days although when a lethal dose of toxin was present, death usually occurred within 24 hours. The LD50 dose of toxin for each lot of culture was calculated by the cumulative method of Reed and Muench (1938) as described by Carpenter (1956); an example is given with Table 1. The cumulative survivor totals were calculated by totaling the number of mice that lived, beginning with the smallest dose of toxin injected; the cumulative death totals were calculated by totaling the number of mice that died, beginning with the largest dose of toxin injected. The mortality rate was determined from these cumulative totals, and the LD50 determined by using a simple proportion.

A specific toxin neutralization test was employed to ascertain that the lethal agent in the broth was C. botulinum type E toxin. A sample of culture was mixed with monovalent C. botulinum type E antiserum (obtained from the Communicable Disease Center, Atlanta, Georgia), incubated for 30 minutes, and injected intraperitoneally into white mice. Survival of the protected mice and deaths of the unprotected mice identified the lethal agent as C. botulinum type E toxin. Lots 'a', 'b', 'c', and 'e' were tested using this procedure.

Experimentation with birds. Prior to feeding C. botulinum type E - J cultures to the gulls, experience in handling birds

was gained by experimenting with chickens. Twenty-one White Leghorn Chickens weighing approximately five pounds each were fed toxic culture at levels containing from 345 to 2,760 mouse LD50 doses: 12 chickens received lot 'a'-d diluted to contain 675, 1,350, 2,025, and 2,700 LD50 doses in five milliliters of solution; nine chickens received various volumes of undiluted culture from lot 'a'-1.

The feeding was accomplished by placing a plastic tube, which was attached to a syringe, down the bird's esophagus so that the end of the tube was in the proventriculus. This tube-syringe system contained the appropriate amount of culture so that the desired volume was injected directly into the gastrointestinal tract. During the 24 hours following forced feeding, water was withheld from the birds. Each chicken was fed culture once only.

Most of the gulls were fed by the method described for feeding chickens. A second method, however, was used also; a pipette containing the desired amount of culture was placed into the bird's esophagus and the culture, which was released slowly, flowed directly into the proventriculus.

All lots of C. botulinum type E-J culture except 'a' were fed to the gulls. Ring-billed Gulls were fed volumes of up to 48 ml while the Herring Gulls received up to 60 ml. Because of the large volumes used, the feedings often consisted of two or three smaller doses given over a period of about 15 minutes rather than in one large dose. Six Ring-billed

Gulls received doses of partially purified C. botulinum type E toxin (obtained from the United States Army Biological Laboratories, Fort Detrick, Fredrick, Maryland). To prevent possible regurgitation of forced-fed materials, food was withheld from the gulls for 24 hours prior to feeding and for approximately 24 hours after feeding. Water was withheld during the 12 hours following feeding.

Eighteen of the gulls received a second feeding of culture while nine received a third feeding. Following their third feeding four Ring-billed Gulls were injected intraperitoneally with culture which was passed through a Seitz filter.

Two Herring Gulls were fed 30 ml of culture containing 477,000 LD50 doses in three feedings (159,000 LD50 doses per feeding) over a period of four hours. After these gulls became sick, but appeared to be recovering, they were placed in water to determine their ability to swim.

One Ring-billed Gull and one Herring Gull, after being forced-fed four and five times respectively, were fed culture containing 131,000 LD50 doses in their daily diet of fish for two five day periods five days apart (the normal consumption of food for Ring-billed Gulls was about three quarters of a pound per gull per day and for Herring Gulls was about one pound of food per gull per day).

To determine the amount of toxin entering the circulatory system of the gull and the length of time the toxin remains

in the bird, blood samples were taken from the jugular vein, as described by McClure et al. (1955), and titered for toxin by mouse injection.

Four Ring-billed Gulls, three of which were never fed toxin and one which was fed once with ten milliliters of culture containing 131,000 LD50 doses, were used as controls to ascertain that some other agent in the culture, rather than type E botulinum toxin, was not the specific lethal factor. These gulls were injected intravenously with 0.75 ml C. botulinum type E antiserum (obtained from Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada) containing 225,000 anti-LD50 doses. Five hours after this injection the gulls were fed ten milliliters of culture (lot 'e'-m), which contained 131,000 LD50 doses of toxin. One week later these gulls again were fed the same amount of culture, but they were not injected with antiserum.

Each gull that died while on test was examined for gross mechanical injuries and pathologic abnormalities. Samples of fat, breast muscle, and brain were removed and are being held frozen for future pesticide analysis.

RESULTS

Rearing Gulls

All except four of the Ring-billed Gulls were raised successfully. One gull died while being bled from the brachial vein but death was apparently due to improper handling. One gull died from injuries obtained from the pecking of other gulls. The cause of death of the other two gulls is not known; one bird, immobilized due to a leg injury, died following an all-night rain storm, and the other gull died after being moved indoors prior to experimentation.

All Herring Gulls were raised successfully. However, one was not used in the feeding experiments because it was in poor health due to a broken leg.

Production of C. botulinum Type E-J Cultures

Data used in calculating the toxicities of each lot of culture are shown in Tables 1 through 6. Lot 'b'-d, at a dilution of 1:500, was lethal for mice; no higher dilutions were tested. The toxicity is, therefore, considered as greater than 2,500 LD50 doses/ml of broth. All of the control tests indicated that the lethal agent of each lot of culture was C. botulinum type E toxin.

The effect of freezing and storage upon the toxicity of a culture is shown in Table 7. The toxicity of the undiluted

culture remained constant over the 31 day freezing period. Because of this, the level of toxin fed was computed on the basis of the data obtained from all of the titrations made on each lot of culture (Table 1 through 6).

Experimentation with Birds

Feeding experiments. The data obtained from the studies in which chickens were fed culture (Table 8) indicate that a dose of C. botulinum type E - J toxin lethal to White Leghorn Chickens is approximately 1300 to 1400 LD50 doses. Three chickens survived feedings of greater than 2000 LD50 doses, but two of them (2273 and 2879) exhibited severe paralysis from which they recovered. No symptom of sickness was observed in any other chickens which survived.

The first symptom of botulism that was observed in the chickens was inactivity. Following this, their eyes closed part way and the birds lost the ability to stand or control the wings. Finally, the neck was limp, and the bird appeared lifeless for several hours before death. These symptoms are similar to those described by Biester and Schwarte (1948) for type C botulism in chickens.

The feeding of Ring-billed Gulls with C. botulinum type E - J culture resulted in the death of 16 of 21 gulls, a mortality rate of 76% (Table 9). Eight of the gulls died following the first feeding, seven of the 13 receiving a second feeding died, while one of the two receiving a third

Table 1. Summary of deaths of mice injected with C. botulinum type E - J lot 'a'-1.

Dilution of culture	Dose of culture* (ml)	Results in 3 days		Cumulative total		Percent mortality
		Lived	Died	Lived	Died	
1:40	0.00500	2	4	2	10	83
1:60	0.00332	0	4	2	6	75
1:80	0.00250	4	2	6	2	25
1:100	0.00200	4	0	10	0	0

*Total volume of diluted culture injected was 0.2 ml

LD50 = 0.00332 - (75-50/75-25) (0.00332-0.00250)
= 0.00332 - (0.5) (0.00082)
= 0.00332 - 0.00041
= 0.00291 ml

Toxicity = 1/0.00291 = 345 LD50 doses/ml

LD50 = that amount of toxin that will kill 50% of the mice injected

Table 2. Summary of deaths of mice injected with C. botulinum type E - J lot 'a'-d.

Dilution of culture	Dose of culture* (ml)	Results in 3 days		Cumulative total		Percent mortality
		Lived	Died	Lived	Died	
1:80	0.00250	0	4	0	13	100
1:100	0.00200	0	4	0	9	100
1:120	0.00166	0	4	0	5	100
1:140	0.00142	2	1	2	1	33

*Total volume of diluted culture injected was 0.2 ml
Calculated toxicity = 675 LD50 doses/ml

Table 3. Summary of deaths of mice injected with C. botulinum type E - J lot 'c'-m.

Dilution of culture	Dose of culture* (ml)	Results in 3 days		Cumulative total		Percent mortality
		Lived	Died	Lived	Died	
1:400	0.00050	0	8	0	43	100
1:500	0.00040	2	6	2	35	95
1:600	0.00034	0	8	2	29	94
1:800	0.00025	1	8	3	21	88
1:1000	0.00020	0	7	3	13	81
1:2000	0.00016	1	4	4	6	60
1:4000	0.00014	3	2	7	2	22

*Total volume of diluted culture injected was 0.2 ml
Calculated toxicity = 6,300 LD50 doses/ml

Table 4. Summary of deaths of mice injected with C. botulinum type E - J lot 'd'-m.

Dilution of culture	Dose of culture* (ml)	Results in 3 days		Cumulative total		Percent mortality
		Lived	Died	Lived	Died	
1:600	0.00034	0	4	0	22	100
1:800	0.00025	4	6	4	18	82
1:1000	0.00020	7	5	11	12	52
1:1200	0.00016	4	4	15	7	32
1:1400	0.00014	5	3	20	3	13
1:1600	0.00013	2	0	22	0	0

*Total volume of diluted culture injected was 0.2 ml
Calculated toxicity = 5,100 LD50 doses/ml

Table 5. Summary of deaths of mice injected with C. botulinum type E - J lot 'e'-d.

Dilution of culture	Dose of culture* (ml)	Results in 3 days		Cumulative total		Percent mortality
		Lived	Died	Lived	Died	
1:2000	0.000100	0	8	0	34	100
1:2400	0.000082	2	12	2	26	93
1:2800	0.000072	1	5	3	14	82
1:3200	0.000063	6	8	9	9	50
1:4000	0.000050	13	1	22	1	4
1:4800	0.000041	2	0	24	0	0

*Total volume of diluted culture injected was 0.2 ml
Calculated toxicity = 15,900 LD50 doses/ml

Table 6. Summary of deaths of mice injected with C. botulinum type E - J lot 'e'-m.

Dilution of culture	Dose of culture* (ml)	Results in 3 days		Cumulative total		Percent mortality
		Lived	Died	Lived	Died	
1:2000	0.000100	0	2	0	20	100
1:2400	0.000082	6	10	6	18	75
1:3200	0.000063	11	5	17	8	32
1:4000	0.000050	6	2	23	3	12
1:4800	0.000041	6	1	29	1	3
1:5600	0.000036	3	0	32	0	0

*Total volume of diluted culture injected was 0.2 ml
Calculated toxidity = 13,100 LD50 doses/ml

Table 7. Effect of freezing and storage upon the toxicity of C. botulinum type E - J lot 'e'-d.

Days held at -15 C.	Dilutions of culture injected			
	1:1600	1:2400	1:3200	1:4000
0	2/2*			
1	2/2	2/2	1/2	0/2
2	2/2	1/2	1/2	0/1
8	2/2	2/2	2/2	0/2
20		2/2	1/2	0/2
31	4/4	3/4	3/4	0/4

*Number of mice dead per number of mice injected

Table 8. Summary of feeding of chickens with C. botulinum type E - J.

Chicken number	Culture lot fed	Volume of broth fed (ml)	Total LD50 doses fed	Results
2204	'a'-1	1	345	Lived
2319	'a'-1	1	345	Lived
2289	'a'-d	5	675	Lived
2344	'a'-d	5	675	Lived
2525	'a'-d	5	675	Lived
2221	'a'-1	2	690	Lived
2254	'a'-1	2	690	Lived
2243	'a'-d	5	1350	Died
2255	'a'-d	5	1350	Died
2385	'a'-d	5	1350	Lived
2227	'a'-1	4	1380	Lived
2275	'a'-1	4	1380	Died
2201	'a'-d	5	2025	Died
2273	'a'-d	5	2025	Lived*
2879	'a'-d	5	2025	Lived*
2248	'a'-1	6	2070	Died
2506	'a'-1	6	2070	Died
2832	'a'-d	5	2700	Died
2549	'a'-d	5	2700	Died
2215	'a'-d	5	2700	Died
2828	'a'-1	8	2760	Lived

*Recovered from severe paralysis

Table 9. Summary of feeding of Ring-billed Gulls with C. botulinum type E-J.

Gull number	Culture lot fed	Volume of culture fed (ml)	LD50 doses fed	Results
138*		20	0	Lived
138(1)**	'c'	10	63,000	Lived
138(2)	'c'	40	252,000	Died
134(1)	'b'	10	25,000	Lived
134(2)	'e'-m	10	131,000	Died
143(1)	'b'	10	25,000	Lived
143(2)	'e'-m	10	131,000	Lived
143(3)	'e'-m	30	393,000	Lived
230(1)	'c'	10	63,000	Died
231(1)	'c'	10	63,000	Lived
231(2)	'c'	40	252,000	Died
139(1)	'b'	20	50,000	Died
137(1)	'b'	35	87,000	Died
243(1)	'e'-m	10	131,000	Died
244(1)	'e'-m	10	131,000	Died
291(1)	'd'	30	153,000	Lived
291(2)	'd'	48	244,800	Died
293(1)	'd'	30	153,000	Lived
293(2)	'd'	48	244,800	Died
295(1)	'd'	30	153,000	Died
297(1)	'd'	30	153,000	Died
290(1)	'd'	48	244,800	Lived
290(2)	'd'	30	153,000	Died
294(1)	'd'	48	244,800	Lived
294(2)	'd'	30	153,000	Lived
296(1)	'd'	48	244,800	Lived
296(2)	'd'	30	153,000	Lived
298(1)	'd'	48	244,800	Lived
298(2)	'd'	30	153,000	Lived
232(1)	'c'	40	252,000	Lived
232(2)	'c'	40	252,000	Lived
135(1)	'c'	40	252,000	Lived
135(2)	'c'	40	252,000	Died
234(1)	'c'	40	252,000	Died
150(2)	'e'-m	10	131,000	Lived
150(3)	'e'-m	30	393,000	Died
242(1)	'e'-m	10	131,000	Lived
242(2)	'e'-m	10	131,000***	Lived
242(3)	'e'-m	10	131,000	Lived
289(1)	'e'-m	10	131,000***	Lived
289(2)	'e'-m	10	131,000	Lived
299(1)	'e'-m	10	131,000***	Lived
299(2)	'e'-m	10	131,000	Lived
300(1)	'e'-m	10	131,000***	Lived
300(2)	'e'-m	10	131,000	Lived

*Control fed uninoculated T.P.G. medium; **(1) - first culture feeding; (2) - second culture feeding; ***Injected I.V. with 225,000 anti-LD50 doses antiserum five hours prior to feeding of culture.

feeding died. Gull 290 broke its wing after the first feeding and was in poor health when it was fed the second time.

Only one of the four gulls injected intraperitoneally with Seitz filtered culture died, and this was apparently due to the accidental injection of culture into an abdominal air sac, because immediately after treatment the bird emitted a gurgling sound as it breathed and fluid ran from its mouth upon turning the bird upside down. One of the gulls fed purified toxin died, but the symptoms which it exhibited indicated that it may have died from some type of respiratory ailment and not from botulism. There was no evidence that the gulls fed toxic culture on their food were affected, although during the last three days of the feeding trial they ate nothing, refusing even culture-free food offered to them.

The first symptom of botulism usually became apparent in the Ring-billed Gulls about five hours after feeding. The symptoms were similar to those seen in the chickens; first the eyes were closed slightly and the feathers became ruffled, giving the bird a 'rough' appearance. The wings of the bird soon drooped considerably and the inability of the bird to stand followed quickly. In some instances there followed a short period in which the gull's head bobbed up and down, moving from a 'normal' position to resting the head on the cage floor; this 'bobbing' was in synchrony with the breathing movements. All gulls that reached this stage died, usually within five hours (15 hours after feeding), although

some died without showing this particular symptom. The final stage in the symptoms was complete limpness and a lifeless appearance of the bird.

Only two of the five Herring Gulls fed C. botulinum type E - J showed any symptoms of botulism, and only one of these died. The two that received 477,000 LD50 doses in three feedings became sick (Table 10). One of the birds which exhibited symptoms of botulinum poisoning drowned within 25 minutes after being placed in water: the other sick Herring Gull was able to swim when placed in water. The condition of the drowned gull, prior to being placed in water, indicated that it otherwise might have survived the attack of botulism.

The symptoms seen in the two sick Herring Gulls were similar to those observed in the Ring-billed Gulls except that the Herring Gulls seemed to be stronger and never lost the ability to stand.

Titration of blood samples. The data obtained from the blood samples taken from Ring-billed Gulls indicate that following a single feeding of culture, the maximum concentration of toxin occurred in the blood two to three hours after the feeding (Figure 5). Blood samples were taken from 21 birds prior to feeding; in every instance, no toxic agent for white mice was present in 0.2 ml of blood. In general, gulls that showed a titer of less than 500 mouse MLD at the end of six hours survived; one of the gulls, however, that had a titer of more than 1000 mouse MLD for six hours lived. In most instances

Figure 3. A ring-billed Gull unable to stand due to botulism. Note the puffy appearance of the eyes and ruffled feathers.

Figure 4. A Ring-billed Gull showing complete limpness at the terminal stage of botulism.

Table 10. Summary of feeding of Herring Gulls with C. botulinum type E - J lot 'e'-d.

Gull number	Volume of culture fed (ml)	LD50 doses fed	Results
283 (1)	35	556,500	Lived
283 (2)	35	556,500	Lived
283 (3)	60	954,000	Lived
284 (1)	35	556,500	Lived
284 (2)	35	556,500	Lived
284 (3)	60	954,000	Lived
285 (1)	35	556,500	Lived
285 (2)	35	556,500	Lived
285 (3)	60	954,000	Lived
286 (1)	10	159,000	Lived
286 (2)	30*	477,000	Lived
288 (1)	10	159,000	Lived
288 (2)	30*	477,000	Died (from drowning)

*This volume was fed in three doses during a four-hour period.

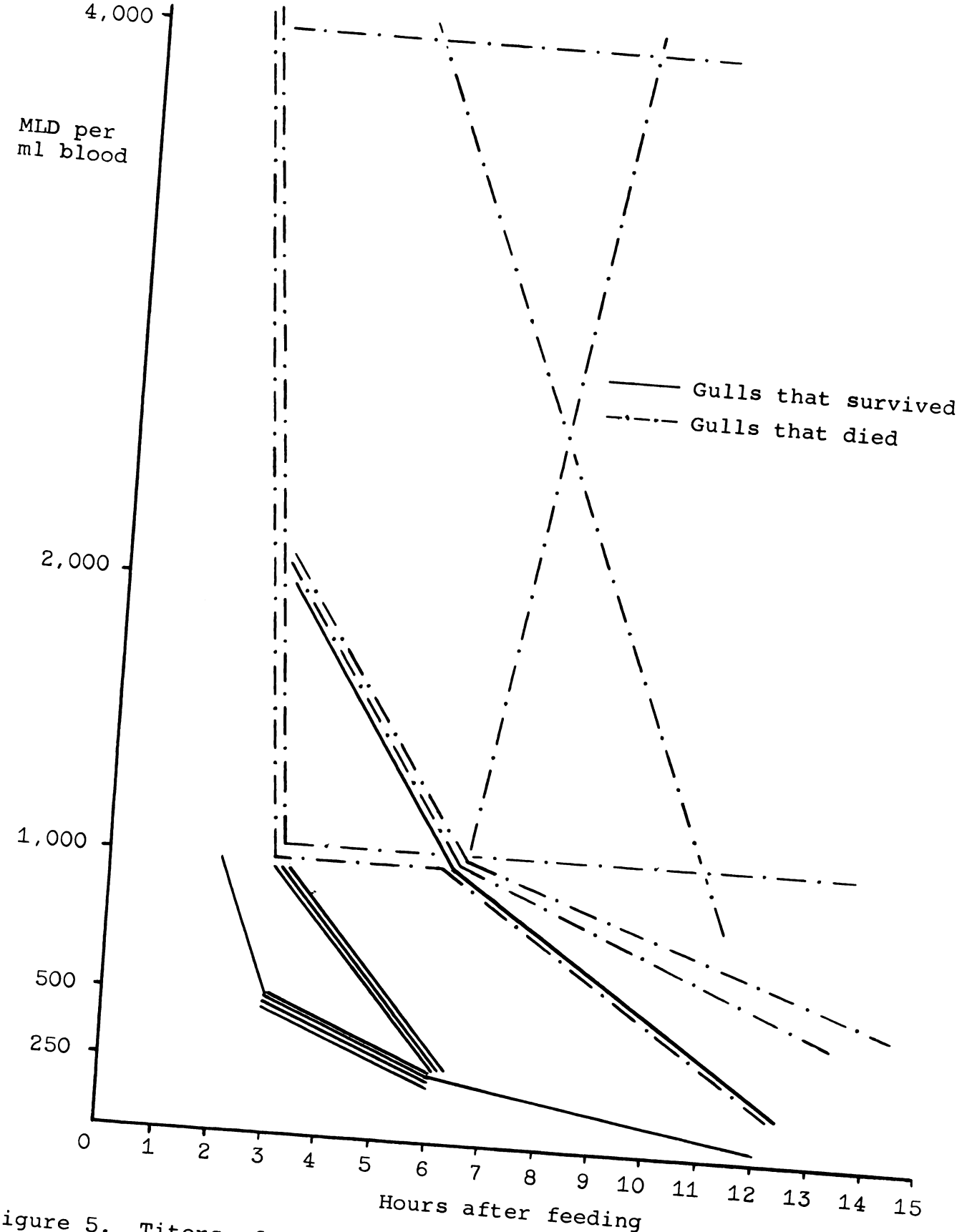

Figure 5. Titers of toxin in the blood of Ring-billed Gulls following feeding of C. botulinum type E - J.

the toxin titer of the blood dropped to less than 1000 mouse MLD prior to death.

Results from titrating blood samples taken from Herring Gulls indicate that the maximum toxin concentration following a single feeding of culture occurred in the blood about three hours after feeding (Figure 6). The toxin concentration in the blood following a schedule of feeding at zero, two, and four hours is higher than after a single feeding. The Herring Gulls were fed higher titers of toxin and, therefore, show higher titers in their blood. Because only two Herring Gulls displayed any symptoms of botulism (both would probably have survived had they not been placed in water), plus the fact that up to 16 times as much toxin was found in their blood as in the surviving Ring-billed Gulls, it is evident that the Herring Gulls have a greater resistance to the toxin than do the Ring-billed Gulls.

Control testing. All four of the Ring-billed Gulls used to ascertain that C. botulinum type E was the lethal agent in the culture survived the first feeding without exhibiting any symptoms of botulism. Following the second feeding, two of the gulls appeared to get very slightly sick, but this sickness was so slight that it was detectable only because of previous experience in working with gulls. The other two gulls did not show any symptoms following the second feeding.

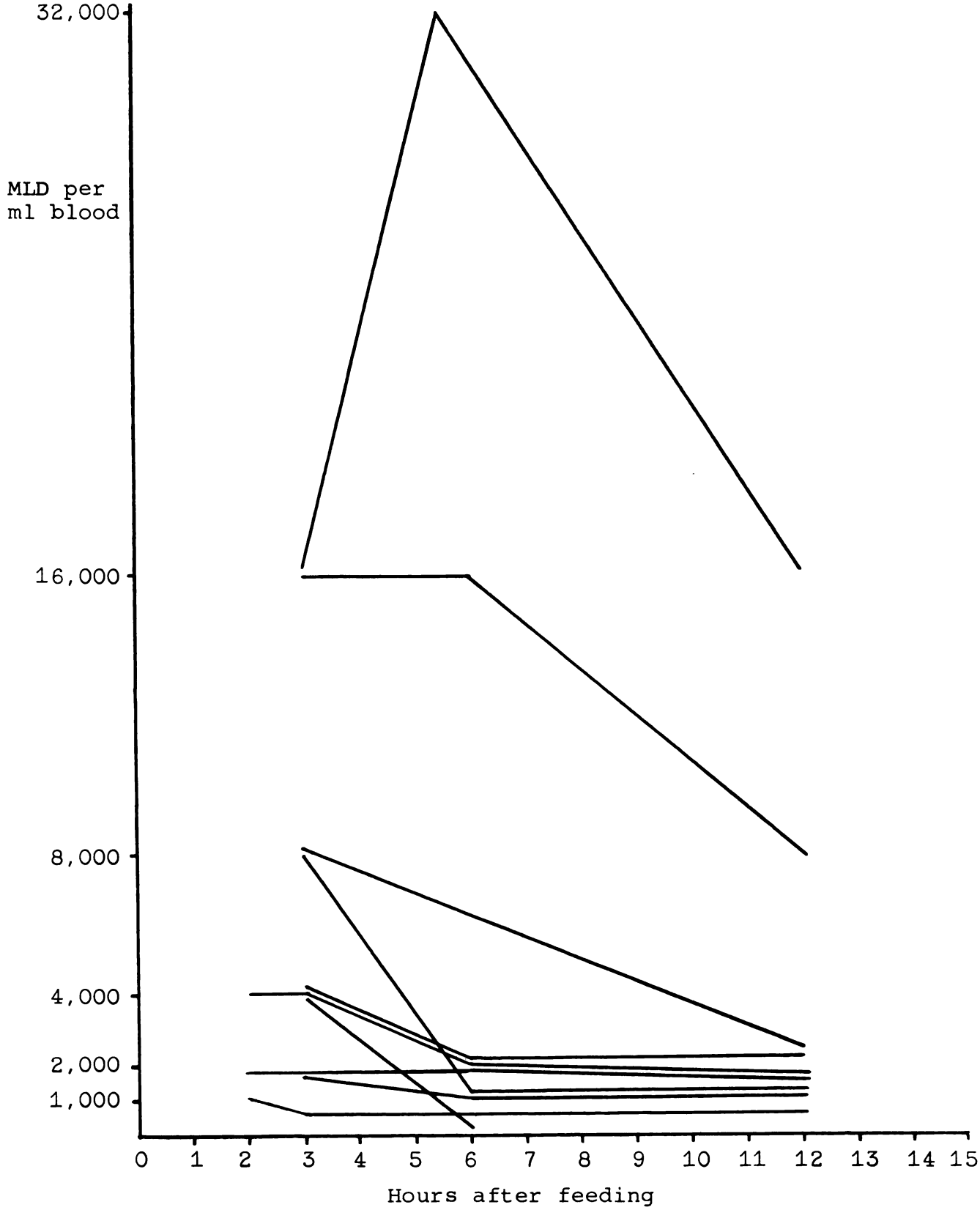

Figure 6. Titers of toxin in the blood of Herring Gulls following feeding of C. botulinum type E - J. The two highest curves are the gulls fed 477,000 LD50 doses in three feedings during a four hour period.

Post mortem examination. The results of the post mortem examinations indicate that the death of the gulls was not due to gross mechanical injuries or pathologic abnormalities.

DISCUSSION

Although 76% of the Ring-billed Gulls died after being forced fed C. botulinum type E - J culture, the overall mortality rate did not increase in proportion to the increase of toxin fed. Table 11 indicates that the death rate was about the same for each dosage level. However, deaths resulting from the first feeding decreased at the higher levels of toxin while at the same levels following the second feeding, the mortality rate increased. The initial decrease in mortality may be due to the large volumes of culture which were forced-fed, while the increased mortality following the second feeding may be a result of the botulinum toxin from the first feeding.

Table 12 shows the results of the Ring-billed Gull feeding trials, but lists them by volume of culture fed rather than by (and regardless of) the LD50 doses fed. These results are almost identical with those listing the deaths by toxicity (Table 11). This similarity is to be expected because the toxic dose is directly related to the volume. However, these tables do show that there was a decreased mortality at the higher doses following the first feeding and that this decrease may be associated with the larger volumes fed rather than the higher level of toxin.

Table 11. Summary of deaths of Ring-billed Gulls fed *C. botulinum* type E - J listed by LD50 dose fed.

LD50 doses fed	Results	Feeding trial	Total % dead	% dead of 1st feeding trial	% dead of 2nd feeding trial
63,000	Died	1			
63,000	Lived	1	33.3	33.3	----
63,000	Lived	1			
131,000	Lived	1			
131,000	Died	1			
131,000	Died	1			
131,000	Died	2			
131,000	Lived	2			
131,000	Lived	2			
153,000	Lived	1	42.9	57.1	28.5
153,000	Lived	1			
153,000	Died	1			
153,000	Died	1			
153,000	Died	2			
153,000	Lived	2			
153,000	Lived	2			
153,000	Lived	2			
244,800	Lived	1			
244,800	Lived	1			
244,800	Lived	1			
244,800	Lived	1			
244,800	Died	2			
244,800	Died	2			
252,000	Lived	1	46.2	14.3	83.3
252,000	Lived	1			
252,000	Died	1			
252,000	Died	2			
252,000	Died	2			
252,000	Lived	2			
252,000	Died	2			
393,000	Lived	2	50.0	----	50.0
393,000	Died	2			

Table 12. Summary of deaths of Ring-billed Gulls fed *C. botulinum* type E - J listed by volume fed.

Volume of culture fed (ml)	Results	Feeding trial	Total % dead	% dead of 1st feeding trial	% dead of 2nd feeding trial
10	Lived	1			
10	Lived	1			
10	Lived	1			
10	Died	1			
10	Lived	1			
10	Lived	1	36.4	37.5	33.3
10	Died	1			
10	Died	1			
10	Died	2			
10	Lived	2			
10	Lived	2			
30	Lived	1			
30	Lived	1			
30	Died	1			
30	Died	1			
30	Died	2	40.0	50.0	33.3
30	Lived	2			
30	Lived	2			
30	Lived	2			
30	Lived	2			
30	Died	2			
40	Lived	1			
40	Lived	1			
40	Died	1			
40	Died	2			
40	Died	2			
40	Lived	2			
40	Died	2	46.2	14.3	83.3
48	Lived	1			
48	Lived	1			
48	Lived	1			
48	Lived	1			
48	Died	2			
48	Died	2			

Dr. Robert Ringer, M.S.U. Avian Physiologist, expressed agreement that higher volumes could decrease the mortality rate and he also pointed out that the constituents of the gastrointestinal tract are believed to act according to Starling's "law of the heart" (Starling, 1918). This law states that the more a muscle (the heart) is stretched, the harder that muscle contracts. If this is correct for the gastrointestinal tract, then the larger the volume of culture forced into it, the more the muscles stretched so the harder these muscles contracted. Subsequently, the culture was defecated more quickly resulting in less toxin absorption and a decreased mortality.

Some evidence to demonstrate the effect of forced feeding with large volumes of culture appears in the data obtained in the Herring Gull feeding trials; the two gulls which received 477,000 LD50 doses in three feedings of ten milliliters each at two-hour intervals showed symptoms of botulinal poisoning; the three gulls which received greater than 500,000 LD50 doses in one or two feedings of at least 30 ml per feeding showed no symptoms. Chicken 2828 may also have survived due to the larger volume of culture fed. Limited evidence following forced feeding of Ring-billed Gulls with various volumes of water containing dye also supports the above.

As previously suggested, the high mortality rate following the second feeding with high doses of culture may be due to the effect of the residual botulinal toxin from the first

feeding. Brooks (1964), in discussing the investigations on pharmacological action of botulinal toxin (type A), showed that the autonomic nervous system is affected by the toxin at the cholinergic synapses. Since all of the synapses of the parasympathetic system are cholinergic and the action of the parasympathetic system increases intestinal contractions and tone (Guyton, 1961), botulinal toxin could slow or inhibit the movement of materials through the gastrointestinal tract. Thus, following a previous feeding of toxin, the larger volumes of culture would not be removed as quickly and more toxin would be absorbed, resulting in a mortality rate which would tend to increase in proportion to the toxin level fed.

The role that immunity played in the degree of susceptibility of the gulls to the C. botulinum type E toxin is uncertain. It is recognized that possibly the gulls may have had some level of immunity to the type E toxin prior to the feeding trials and that it may take several weeks following exposure for immunity to develop. However, it seems that immunity had little, if any, effect because the percent mortality was higher as a result of the second feeding.

Another factor which may have influenced the fate of the gulls is lack of acclimatization to environmental conditions and human handling. The gulls quickly adapted to the outdoor cages but when placed in the small indoor cages, most of the gulls became quite restless. Handling caused the

gulls to become very excited, usually resulting in reguritation of undigested foods. For this reason, liquid cultures were fed. Defecation usually occurred immediately upon replacing the bird in its cage.

A problem that remains unanswered is the survival of the gulls fed partially purified toxin. It may be that the molecule(s) of purified toxin and those of the crude culture are in some way different and that the former is somehow affected by our techniques or perhaps by the gulls' digestive processes.

It has been shown that a lethal amount of C. botulinum type E toxin can be present in the blood of gulls which appear to be normal but later, when death occurs, a much smaller amount of toxin will be found upon examination of the carcasses. If the Lake Michigan mortalities are being caused by the ingestion of botulinum type E toxin by the birds, the above observation is important because it aids in explaining the generally low level (less than 100 MLD) of toxin found in the carcasses by Kaufmann and Fay (1964). However, most gulls probably would not need to consume large doses of toxin for it to be lethal, since environmental 'stresses' which a bird may encounter while sick with botulism can be enough additional strain to kill. An example of such stress is the drowning of the Herring Gull which, had it not been placed in water, probably would have survived.

A problem of primary concern is whether the necessary conditions exist in Lake Michigan to enable C. botulinum type E to grow and produce toxin in sufficient quantities to be of significance in the waterbird mortalities. Schmidt et al. (1961) found toxin produced from a pure culture at 3.3 C after 31 days incubation. Danish health officials found that fish, lethal to a man who consumed a portion of it, contained 100,000 mouse I.P. fatal doses of toxin; they indicated that this toxin was produced in three days at a temperature of 4 C (Dahlerup and Wilhjelm, 1964). Sakaguchi and Tohyama (1955), Dolman (1964), Duff et al. (1956) and others have indicated that cultures of C. botulinum type E growing in association with certain other bacteria produce higher titers of toxin than does a pure culture, and it is very likely that C. botulinum type E growing in Lake Michigan would be growing in a mixed culture. The nutrient requirements could be supplied, in Lake Michigan, by some source of pollution or by the bodies of invertebrates (Jensen and Allen, 1960) or fish (Kautter, 1964). Kaufmann and Marshall (1965) showed that C. botulinum type 62A would produce toxin in a trypticase medium autoclaved in the presence of lactose without special precautions to eliminate atmospheric oxygen. Kautter (op. cit.) showed that C. botulinum type E would grow in fish incubated under aerobic or anaerobic conditions. Therefore, theoretically, all of the necessary conditions can exist in Lake Michigan for a substantial amount of C. botulinum type E toxin to be produced, although the presence of such toxin has not yet been demonstrated.

The presence of the toxin in the carcasses of the birds from the Lake Michigan mortalities indicates that the toxin probably played some role in the die-offs, but it cannot be stated categorically that C. botulinum type E was the sole cause of the deaths. It is possible, however, that the mortalities were caused by botulinum toxin acting synergistically with some other factor(s), such as chlorinated hydrocarbons or organic phosphates. Until some of the other suspect agents have been investigated or until a reasonably large source of C. botulinum type E is discovered in nature, the cause of the recent Lake Michigan waterbird mortalities will remain unknown.

SUMMARY

Twenty-one Ring-billed Gulls and five Herring Gulls were raised successfully in captivity for use in studies on botulinal poisoning. Clostridium botulinum type E was cultured to obtain toxin for feeding trials. Under various conditions, the culture produced toxin titers which varied from 345 to 15,900 LD50 doses per milliliter.

Ring-billed Gulls and Herring Gulls were fed varying levels of the culture. Sixteen of 21 Ring-billed Gulls fed the culture died. Only one of five Herring Gulls fed the culture died and it drowned when placed in water while sick with botulism. The Herring Gulls appear to be more resistant to the toxin than the Ring-billed Gulls. Control tests indicated that the lethal agent in the culture was C. botulinum type E toxin.

Titration of blood samples taken from gulls following feeding of toxin indicated that a normal appearing gull may have a lethal amount of toxin in its blood, but there may be little toxin detectable in its blood when the gull is sick or dead. In nature, most gulls probably would not need to consume large doses of toxin because environmental 'stresses' which a bird may encounter while sick with botulism may be enough additional strain to cause death.

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APPENDIX

Table I. Conditions under which gulls were forced-fed C. botulinum type E - J.

Gull number	Feeding method	Number of aliquots fed	Time interval between aliquots (minutes)	Volume per aliquot (ml)
134(1)	t-s*	1	--	10
134(2)	pipette	1	--	10
135(1)	t-s	2	35	20
135(2)	t-s	2	42	20
137(1)	t-s	1	--	35
138(1)	t-s	1	--	10
138(2)	t-s	2	38	20
139(1)	t-s	1	--	20
143(1)	t-s	1	--	10
143(2)	pipette	1	--	10
143(3)	pipette	3	120	10
143(4)	pipette	3	120	10
150(1)	pipette	1	--	10
150(2)	pipette	3	120	10
230(1)	t-s	1	--	10
231(1)	t-s	1	--	10
231(2)	t-s	2	41	20
232(1)	t-s	2	35	20
232(2)	t-s	2	38	20
234(1)	t-s	2	33	20
242(1)	pipette	1	--	10
242(2)**	pipette	1	--	10
242(3)	pipette	1	--	10
243(1)	pipette	1	--	10
244(1)	pipette	1	--	10
283(1)	t-s	2	12	15
283(2)	t-s	1	--	35
283(3)	t-s	2	13	30
284(1)	t-s	2	11	15
284(2)	t-s	1	--	35
284(3)	t-s	2	15	30
285(1)	t-s	2	13	15
285(2)	t-s	1	--	35
285(3)	t-s	2	13	30

continued

*Feeding was by tube-syringe system.
**Injected I.V. with 225,000 anti-LD50 doses antiserum five hours prior to feeding of culture.

Table I - Continued

Gull number	Feeding method	Number of aliquots fed	Time interval between aliquots (minutes)	Volume per aliquot (ml)
286(1)	pipette	1	--	10
286(2)	pipette	3	120	10
286(3)	pipette	4	120	10
286(4)	pipette	4	120	10
288(1)	pipette	1	--	10
288(2)	pipette	3	120	10
289(1)**	pipette	1	--	10
289(2)	pipette	1	--	10
290(1)	t-s	2	20	24
290(2)	t-s	2	14	15
291(1)	t-s	2	22	15
291(2)	t-s	2	13	24
293(1)	t-s	2	20	15
293(2)	t-s	2	14	24
295(1)	t-s	2	11	15
296(1)	t-s	2	10	24
296(2)	t-s	2	11	15
297(1)	t-s	2	13	15
298(1)	t-s	2	11	24
298(2)	t-s	2	6	15
299(1)**	pipette	1	--	10
299(2)	pipette	1	--	10
300(1)**	pipette	1	--	10
300(2)	pipette	1	--	10

Table II. Summary of data obtained by post mortem examination of the gulls.*

Gull number	Sex	Weight (gm)	External condition	Amount of internal fat	Parasites observed
134	female	375	good	good	none
135	male	275	poor	poor	none
138	male	345	good	poor	few tapeworms
139	male	430	good	fair	none
149	?	320	good	fair	one tapeworm
150	female	345	poor	poor	two tapeworms
230	female	360	good	good	none
231	male	275	good	fair	none
232	female	375	fair	good	few tapeworms
234	male	325	fair	fair	few tapeworms
243	male	410	fair	poor	many tapeworms
244	female	320	good	fair	few tapeworms
283	female	550	fair	poor	none
284	male	730	good	fair	none
285	?	850	fair	good	none
288	male	950	good	good	none
290	female	270	poor	poor	few tapeworms
291	?	250	good	poor	one tapeworm
293	male	270	fair	poor	few tapeworms
294	female	365	good	poor	none
295	female	310	good	poor	few tapeworms
296	male	400	good	good	none
297	male	390	good	poor	one tapeworm
298	female	350	good	good	one tapeworm

*Autopsy reports are available at Rose Lake Research Center.

Table III. Summary of data obtained from titration of gull blood samples following feeding of C. botulinum type E - J.

Gull number	Time of bleeding*	MLD of toxin found in blood
134(1)	0	0
134(2)	3	2,000
	6	1,000
	13	250
135(1)	0	0
135(2)	6	1,280
	9	6,000
137(1)	0	0
	17	10
138(2)	0	0
138(3)	6	2,560
139(1)	0	0
143(1)	0	0
	24	0
143(2)	3	250
150(2)	3	250
230(1)	0	0
	12	10
231(1)	0	0
231(2)	0	0
	6	2,560
232(1)	0	0
232(2)	0	0
	6	160
234(1)	0	0
	53	10
242(1)	3	250
243(1)	3	2,000
	6	1,000
	14	250
244(1)	3	4,000
	9	4,000
283(1)	0	0
	2	1,000
	3	500
	6	500
	11	250
283(2)	0	0
	3	4,000
	6	2,000
	12	2,000
284(1)	0	0
	2	2,000
	3	2,000
	6	2,000
	11	1,000
284(2)	0	0
	3	4,000
	6	250
285(1)	0	0
	2	4,000
	3	4,000
	6	2,000
	11	1,000
285(2)	0	0
	3	8,000
	6	6,000
	12	2,000
286(1)	3	8,000
	6	1,000
	12	250
286(2)	3	16,000
	6	16,000
	12	8,000
288(1)	3	2,000
	6	1,000
	12	250
288(2)	3	16,000
	6	32,000
	12	16,000
290(1)	0	0
	3	500
	6	160

continued

*Hours after feeding of culture.

Table III - Continued

Gull number	Time of bleeding*	MLD of toxin found in blood	Gull number	Time of bleeding*	MLD of toxin found in blood
290(2)	0	0	293(2)	0	0
	2	4,000		2	4,000
	3	1,000		3	1,000
	6	1,000		6	1,000
	12	1,000		12	200
291(1)	0	0	295(1)	0	0
	3	2,000		3	2,000
	6	1,000		6	1,750
	12	160	296(1)	0	0
291(2)	0	0		3	500
	2	8,000		6	250
	3	12,000	296(2)	3	500
	6	4,000		6	250
	12	500	297(1)	0	0
	30	500		3	20
293(1)	0	0	298(1)	0	0
	3	1,000	298(2)	0	0
	6	160		3	1,000
	24	0		6	250

Table IV. Summary of bolulism symptoms observed in gulls following forced feeding with C. botulinum type E - J.

Gull number	Hours after feeding	Symptoms*	Gull number	Hours after feeding	Symptoms*
134(1)	---	1	230(1)	12.0	5
134(2)	5.5	5		14.0	6
	8.5	6		23.5	8
	10.5	7	231(1)	18.0	4
	12.5	8		23.0	1
135(1)	12.0	1	231(2)	5.5	3
	16.0	3		8.5	6
	18.0	4		17.0	9
	22.0	1	232(1)	12.0	1
135(2)	4.0	4		18.0	2
	5.5	7		23.0	1
	8.5	8	232(2)	5.5	2
137(1)	---	1		8.5	1
	17.0	8	232(3)	---	1
138(1)	---	1	234(1)	11.5	5
138(2)	6.0	4		13.5	6
	9.0	6		37.0	7
	17.5	9		42.0	8
139(1)	---	1	242(1)	2.0	2
	72.0	9		4.0	1
143(1)	---	1	242(2)	---	1
143(2)	7.0	2	242(3)	---	1
	12.0	4	243(1)	2.0	2
	24.0	1		6.5	4
143(3)	3.0	2		7.0	5
	8.0	3		8.0	7
	24.0	1		14.0	8
143(4)	7.5	2	244(1)	4.0	2
	9.0	2		5.0	4
	19.0	1		6.0	6
150(1)	5.0	2		9.0	8
	12.0	3	283(1)	---	1
	17.0	1	283(2)	---	1
150(2)	3.0	2	283(3)	---	1
	4.0	3	284(1)	8.0	2
	6.0	5		9.0	1
	8.0	6	284(2)	---	1
	9.0	7	284(3)	---	1
	10.0	8			

continued

*Key to botulism symptoms: 1 = No symptoms observed; 2 = eyes partly closed, feathers becoming ruffled; 3 = eyes appear puffy, feathers ruffled; 4 = as above, wings drooping; 5 = as above, can no longer stand; 6 = head bobbing; 7 = complete limpness; 8 = death occurred; 9 = found dead.

Table IV - Continued

Gull number	Hours after feeding	Symptoms*	Gull number	Hours after feeding	Symptoms*
285(1)	---	1	291(1)	---	1
285(2)	---	1	291(2)	5.0	2
285(3)	---	1		7.5	4
286(1)	---	1		9.5	5
286(2)	6.0	2		16.0	7
	7.5	3		36.0	9
	9.0	4	293(1)	7.0	2
	24.0	3		13.0	5
	48.0	1		48.0	1
286(3)	---	1	293(2)	5.0	2
288(1)	---	1		9.5	5
288(2)	3.0	2		16.0	7
	7.5	3		48.0	9
	11.0	4	294(1)	---	1
	24.0	4	294(2)	---	1
	28.0	8**	295(1)	7.0	2
289(1)	---	1		10.0	6
289(2)	---	1		22.5	9
290(1)	5.0	2	296(1)	---	1
	8.0	3	296(2)	---	1
	9.0	5	297(1)	7.0	2
	10.0	7		8.0	3
	48.0	1		10.0	4
290(2)	5.0	3		23.0	9
	6.0	5	298(1)	---	1
	7.0	7	298(2)	---	1
	10.0	8	299(1)	---	1
			299(2)	---	1
			300(1)	---	1
			300(2)	---	1

** Death occurred by drowning.