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## A Clinico-Pathologic Study of Botulism in Ring-Necked Pheasants

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A CLINICO-PATHOLOGIC STUDY OF BOTULISM  
IN RING-NECKED PHEASANTS

BY  
HAZEL J. SHAVE

A thesis submitted  
in partial fulfillment of the requirements for the  
degree Master of Science, Major in  
Wildlife Biology, South Dakota  
State University

1971

A CLINICO-PATHOLOGIC STUDY OF BOTULISM  
IN RING-NECKED PHEASANTS

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

/ Thesis Adviser U Date

Sciences Département U

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A CLINICO-PATHOLOGIC STUDY OF BOTULISM  
IN RING-NECKED PHEASANTS

Abstract

HAZEL J. SHAVE

Toxins were produced from 6 strains of Cl. botulinum, 2 of which killed pheasants. Inoculated birds were observed and a description of the progression of signs of the disease is given. Juvenile pheasants were inoculated with Types A and C<sub>x</sub> toxin intramuscularly and were given the toxin orally. These birds were observed and their clinical signs noted. Adult pheasants were inoculated with toxoid prepared from type C<sub>x</sub> toxin and commercial toxoid; injections were repeated in 21 days. Fourteen days following the final injection, the birds were challenged with toxin. Those protected with toxoid survived, while those receiving saline succumbed. Gross necropsy findings, bacteriologic and histopathologic examinations on all birds were negative.

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## INTRODUCTION

In 1793 an outbreak of an unknown kind of poisoning in southern Germany was attributed to the consumption of blood sausage. Because this became the type case, the name botulism was suggested, referring to sausage (Lewis and Cassell, 1964). Although botulism was undoubtedly present for centuries, the practice of medicine had not expanded sufficiently to recognize the specific syndrome. Until the 1920's, only types A and B Clostridium botulinum were recognized, although many isolates did not fall into these groups. During this decade, types C (1922) and D (1926) and E (1928) were identified in wild birds and animals. Type F was isolated in the early 1960's. It has been recognized that although there is a geographical predominance of one type over another, the organism is ubiquitous.

Cl. botulinum type C was found to be the cause of extensive outbreaks of botulism in wild waterfowl. An excellent history of botulism is given by Kalmbach (1968). He listed the earliest recorded occurrence in the United States as in 1910. At this time the disease was called "Western Duck Sickness," since the etiologic agent was unknown. The most striking feature of the disease was high morbidity. He stated that in 1912, groups of 30,000 and 44,000 dead birds were found in two areas of Utah. This high mortality stimulated first public, then subsequently research interest. While there were many ideas as to the specific etiology of the disease, it was generally accepted that alkalinity of water was somehow important. In 1917 botulism was first diagnosed in domestic birds

in the United States. However, it was not until 1930 that "Western Duck Sickness" was recognized as botulism.

From this short history it is evident that diagnosis of botulism has been elusive. More than 130 years elapsed from the first recorded outbreak in humans until the disease was found in wild birds.

In 1955 botulism was recognized as a disease causing great losses in game farm pheasants (Rosen, 1955). Presumably, pheasants in the wild are dispersed sufficiently to avoid the close contact necessary for the spreading of the toxin. Birds are raised indoors on game farms to a certain age, at which time they are transferred to open-top out-door pens, about 600 birds to a pen. Vegetation in the pens is allowed to grow high to provide a protective cover against flying scavengers. Sick pheasants usually seek dense cover and remain there until death. Cl. botulinum may be either in the intestinal tract of the bird, or may be introduced into the carcass by flies laying eggs. On a warm, early fall day, a pheasant carcass decomposes rapidly, creating anaerobic habitat necessary for growth of the organism. As the organism multiplies and dies, the toxin is released. Healthy pheasants eat the flesh or fly larvae, which seem capable of concentrating the toxin, and a botulism outbreak is in progress. Lee et al. (1962) found that ingestion of eight blow-fly larvae was fatal to a 10-week-old pheasant. Diagnosis of the disease is dependent on demonstration of toxin in the serum of the bird. The toxin is present only transiently in the blood stream in quantities large enough to detect by mouse inoculation.

This study was undertaken to demonstrate the clinical signs of botulism in ring-necked pheasants. Since gross necropsy findings are negative, one must rely on clinical signs to be alert to the possibility of botulism. Losses on a game farm can be high, and preventative measures must be taken early to avert an economic disaster.

## REVIEW OF LITERATURE

The exact mode of action of botulinum toxin is unknown. The toxin acts specifically at cholinergic neuromuscular junctions, somehow blocking acetylcholine formation or release. The nerve impulse to the muscles is blocked, resulting in a flaccid paralysis, as opposed to tetanus toxin where inhibition of muscular tension is suppressed (van Heyningen, 1968). Burgen et al. (1949) found that when toxin was added to a muscle-nerve preparation, there was a latent period during which the toxin was attached but non-reactive. However, once the latent period had started, onset of paralysis was irreversible. Neither the acetylation of choline nor the action of cholinesterase was affected by toxin. Burgen felt that the effect of the toxin was on nerves in the end-plate where the medullary sheath is absent, preventing transmission of the impulse. Ambache (1951) believed that the action of botulinum toxin was to produce a defect in acetylcholine secretion affecting post-ganglionic cholinergic junctions in both the sympathetic and parasympathetic systems. He reported that transmission across a preganglionic junction in the ciliary ganglion could be blocked and that the action of the toxin does not pertain to the presence or absence of the medullary sheath. Brooks (1954) found that excised, paralyzed motor nerve filaments were able to release acetylcholine by current pulses passing through the muscle bath. He concurred with Burgen that conduction was blocked anterior to the pre-synaptic part of

the end-plate, probably by causing the nerve filaments to be locally impermeable or by hyperpolarizing their membranes.

The sequence of clinical signs of botulism intoxication varies among animals. Peripheral nerves are primarily affected. In humans, symptoms start anteriorly with blurred vision and dryness of the mouth, followed by difficulty in breathing, speaking, and swallowing. Paralysis progresses posteriorly affecting the arms and then the legs (Rogers et al., 1964).

Cattle and horses show the typical flaccid paralysis, beginning posteriorly. Sheep exhibit stiffness, incoordination and excitability. Paralysis occurs in the final stages of the disease in sheep (Blood and Henderson, 1968). Experiments have shown that mink (Mustela vison), ferrets (Mustela nicripes), and muskrats (Ondatra zibethica) are susceptible to types A or C toxin and coyotes (Canis latrans), wildcats (Lynx rufus) and foxes (Vulpes fulva) are resistant to botulinum toxins (Quortrup and Gorham, 1949).

The most common sign of botulism in birds is paralysis. In chickens and turkeys, although paralysis begins with the legs and progresses anteriorly, the most marked sign is paralysis of the neck muscles, so-called "limberneck." Frequently this is the only paralysis noticed (Biester and Schwarte, 1965).

The importance of botulism in wild birds is indicated by a study made at the Bear River Wildlife Disease Research Station in Utah where 3,000 wild birds were necropsied with a 47.9 percent incidence of botulism (Quortrup et al., 1941). This morbidity is

higher than would be expected in other localities since botulism is prevalent in Utah. The only consistent lesions found were petechiation of the myocardium and congestion of the smaller blood vessels. Capillaries in the brain were frequently ruptured. Their experiments showed that when high or low doses of toxin were given, petechiation of the myocardium did not occur. If death came in 36 to 50 hours after the toxin was administered, petechiation was present. Bloating and excess mucoid secretion of the cloaca were found in some birds.

A study at Tulare Lake Basin, California, indicated that botulism produced a weakening and progressive paralysis in the birds (Mays, 1941). Pain did not appear to be manifested. The first clinical sign in ducks is difficulty in rising from water, followed by an inability to fly. At this time the bird can still swim, dive and run rapidly over the ground. Apparently the wing muscles are paralyzed before the leg muscles. This is followed by progressive paralysis until the neck is affected. If the bird is in water, it cannot keep its head up and will drown.

Since botulism is not contagious, the apparent spread of the disease from Utah, where it was first noted, to areas such as South Dakota has been studied. Kalmbach (1934) suggested that migrating birds may carry the organism in mud or vegetation adhering to their feet. He felt that birds can fly many miles before becoming paralyzed after ingestion of the toxin. Decaying carcasses with proliferation of organisms would provide new foci of infection.

Coburn and Quortrup (1938) tested many natural duck foods for the presence of preformed toxin. They found organic material from animal origin necessary for the production of toxin. In the fall, as shore lines change due to drought or heavy rains, the feather edge of shallow water contains much animal matter in the form of annelids, crustaceans and insect larvae, which provide culture medium for Clostridium botulinum.

In 1955, Rosen's report on new diseases in California game farm pheasants included botulism. In the same year, an outbreak occurred at a game farm in New York (Cheatum et al., 1957). Leg weakness was the first sign noticed. When flushed, birds would start flying from a sitting position. This was followed by wing and neck paralysis. Fly larvae were found to be the primary source of toxin. Wisconsin was the next state in which botulism was diagnosed on a pheasant game farm (Vadlamudi et al., 1959). Lee (1962), also working in Wisconsin, observed large amounts of mucus in the intestinal tract of affected birds. Suspensions of liver, spleen and intestines were toxic to mice. He also found that maggots from dead birds, ground in saline, were toxic to mice and healthy pheasants. An outbreak of botulism among previously vaccinated birds in Ontario stimulated typing of the organism isolated from fly larvae containing toxin (Fish et al., 1967). The toxoid used in vaccination was type C<sub>β</sub>, while the organism isolated was type C<sub>α</sub>. Type C<sub>α</sub> contains antigenic sites not found in C<sub>β</sub>, while all those found in C<sub>β</sub> are included in C<sub>α</sub>. Protection given by C<sub>β</sub> toxoid is incomplete against C<sub>α</sub> toxin (Burrows, 1963).

Much research has been done in the use of toxoid immunization of pheasants against botulism. Boroff and Reilly (1959) found that two injections at 3-4 week intervals resulted in better immunity than a single injection. Active immunity lasted at least eight months. They questioned whether this immunity would be sufficient to protect a bird against a natural outbreak. Rosen (1959) conducted field studies on the efficacy of toxoid immunization. He found among immunized birds an average mortality of 6.5 percent, while control birds had a mortality of 17 percent in a natural outbreak of botulism. Another experiment under field conditions that was done by Reilly and Boroff (1961) involved thirty unimmunized and six immunized birds which died in a brief epizootic. They felt that immunization was of limited value.

At Jasper-Pulaski State Fish and Game area in Indiana large scale tests were made on toxoid immunized pheasants (Demaree, 1968). They felt that a low level of protection was produced by immunization, but proper management and strict sanitation were more important to pheasant raising success.



## MATERIALS AND METHODS

Cultures of Clostridium botulinum used in this study were obtained from the American Type Culture Collection and the National Communicable Disease Center. The cultures used were:

## American Type Culture Collection

Type A - #19397

Type C<sub>α</sub> - #17850

Type C<sub>β</sub> - #17784

Type E - #17786

## National Communicable Disease Center

Type C<sub>α</sub> - NCDC #4496

Type D - NCDC #KA-9

The cultures were grown in chopped meat broth from 24 hours at 37 C in a Torbal jar which had been evacuated and filled three times with a gas mixture containing 80 percent nitrogen, 10 percent hydrogen, and 10 percent carbon dioxide (Dowell and Hawkins, 1968). The broth was used to inoculate flasks for toxin production. The flasks contained 500 ml. of Brain Heart Infusion Broth (Difco) containing 0.5 percent glucose and 0.1 percent calcium chloride. Dialysis tubing (Union Carbide Corporation) was intussuscepted to form a sack with a large surface area (Sterne and Wentzel, 1950). A short piece of glass tubing was placed in the open end of the sack and secured with a rubber band. The external opening of the glass tubing was plugged loosely with cotton to prevent contamination. The opening of the flask was sealed with cotton which also

held the sack suspended in the broth. The top half of the flask was covered with paper during autoclaving at 121 C for 15 minutes. After the flask had cooled, 25 ml. of sterile saline was introduced into the sack through the glass tubing, followed by 1 ml. of inoculum. The flask was placed in an anaerobic incubator (Thelco) which was also evacuated and flushed three times with gas. Cultures were incubated 10 days at 35 C. Toxin was recovered from the dialysis tubing sack in 2 ml. aliquots, frozen and stored at -70 C.

For the production of toxoid, 20 ml. of type C<sub>2</sub> toxin was mixed with 0.1 ml. concentrated formalin and incubated at 37 C for 30 days (Broff and Reilly, 1959). To test for attenuation of the toxin, each of two mice was inoculated intraperitoneally with 0.1 ml. of this mixture. If the mice showed no ill effects, equal volumes of toxoid and complete Freund's adjuvant (Difco) were stirred in a blender to a thick consistency.

Eight-week-old pheasants used in this study were donated by Mr. David Suter of the South Dakota Pheasant Company, Canton, South Dakota. Adult birds were obtained from the Wildlife and Fisheries Sciences Department, South Dakota State University. The birds were kept in individual cages and fed commercial pheasant ration (Zip Feed Mills, Inc.).

White Swiss mice averaging 30 grams body weight were used for testing toxins and toxoids. The intraperitoneal route of inoculation at a dosage of 0.1 ml. was used. To determine the titer of toxins, 6 mice were used for each toxin dilution. The 50 percent endpoint was determined by the method of Reed and Muench (1938).

Histopathologic examination of liver, kidney, spleen, lung, heart, brain and spinal cord was done on all experimental birds. Spinal cords were removed using the method of Levine (1965). Tissues were fixed in 10 percent neutral formalin, processed and embedded in paraffin. Sections were cut at 6 microns and stained with hematoxylin and eosin.

Bacteriologic examination was done on bone marrow, lung, liver, kidney, spleen, heart, and intestine of each bird. These tissues were cultured on Blood Agar Base (Difco) containing 5 percent defibrinated sheep blood. The plates were incubated in an atmosphere containing 5 percent carbon dioxide at 37 C.

## RESULTS AND CONCLUSIONS

Toxin Verification

It has been found by other workers that individual isolates of Clostridium botulinum vary in their ability to produce toxin (Appleton and White, 1957). Mice were used to determine the toxin producing capacity of the organisms used in this study. Each of three mice was inoculated with 0.1 ml. of toxin intraperitoneally. Surviving mice were observed for 5 days. Type C<sub>β</sub> -ATCC #17784 had been subcultured many times and apparently had lost its toxin producing capabilities. All other toxins were fatal to mice within 24 hours (Table 1).

Neutralization tests were done to verify identity of the toxins. Toxin (0.1 ml.) was mixed with 0.1 ml. of specific antitoxin in a one ml. syringe and incubated at 37 C for 30 minutes. A sample (0.1 ml.) of this mixture was inoculated into each of two mice. Two mice received 0.1 ml. each of the toxin alone. Type C<sub>β</sub> was omitted from this and future tests. Except for those receiving type A toxin, all mice receiving antitoxin survived, while those receiving toxin alone died. All mice receiving type A toxin or toxin-antitoxin mixture died. Type A toxin was heated to 50 C for 30 minutes and 0.1 ml. was inoculated into each of two mice which later died. At 1:5 and a 1:10 dilution of the toxin was prepared with sterile saline. 0.1 ml. of these dilutions was incubated with 0.1 ml. of type A antitoxin, and mice were inoculated as before. 0.1 ml. of the diluted toxins was also inoculated into two mice each.

Table 1. Effects of various botulinum toxins in mice.

Toxin	Number of mice inoculated	Number of mice dead	Time of death
Type A - ATCC #19397	3	3	1 hr.
Type C <sub>α</sub> - ATCC #17849	3	3	24 hr.
Type C <sub>β</sub> - ATCC #17784	3	0	- -
Type C <sub>α</sub> - NCDC #4496	3	3	24 hr.
Type D - NCDC #KA-9	3	3	24 hr.
Type E - ATCC #17786	3	3	24 hr.

Only those mice receiving the 1:10 dilution with antitoxin survived. This dilution was heated to 60 C for 30 minutes. All mice inoculated with this heated toxin survived while control mice receiving the unheated toxin died. Biochemical tests were done to further identify type A culture (Table 2). Results of tests proved it to be a type A, and it was much more potent than any other toxin.

#### Response of Pheasants to Toxins

It was necessary to determine the toxicity of the toxins to pheasants. For these tests, adult birds weighing 800 to 1200 grams were used. Four pheasants were given 0.1 ml. of toxin, type A (ATCC #19397) intramuscularly; two were given 0.5 ml. of toxin orally. Those receiving intramuscular injection were found dead

Table 2. Biochemical reactions of Clostridium botulinum type A.

Glucose - acid	Milk - acid and digestion
Mannitol - no change	Gelatin - digestion
Lactose - no change	Nitrate - negative
Sucrose - no change	Indole - negative
Maltose - acid	Urea - negative
Salicin - acid	Lecithinase - negative
Glycerol - slightly acid	Lipase - positive
Starch - no change	Hemolysis - positive
Cooked meat - digestion	

in 20 hours, while those receiving the oral dose of toxin showed no signs of paralysis or illness. The latter were observed for 22 days, then each given 0.5 ml. of toxin intramuscularly. Both birds were dead in 18 hours.

One pheasant was given 0.1 ml. of toxin, type C<sub>α</sub> (NCDC #4496) intramuscularly and another received 0.1 ml. of toxin orally. The former was dead in 16 hours while the other bird showed paralysis of the legs and wings within 16 hours. This condition lasted 36 hours. Twelve hours later it was able to take a few steps and 24 hours later a complete recovery was observed. The bird was then given 0.3 ml. of toxin orally. Paralysis of leg and wing musculature occurred in 8 to 12 hours respectively followed by death in 24 hours.

Two pheasants were injected with 0.1 ml. of toxin, type C<sub>x</sub> (ATCC #17850) intramuscularly. These birds showed no signs of paralysis. Nine days following infection, both received 0.4 ml. of toxin intramuscularly. Paralysis did not occur. The birds were observed for 12 days following the second injection with no change in clinical condition.

Four pheasants were given type D toxin (NCDC #KA-9). Two birds were injected intramuscularly, one receiving 0.2 ml. and the other 0.4 ml. Similar doses were given to two birds orally. The birds showed no signs of botulism for 28 days. At that time the pheasant that had been injected with 0.2 ml. was given 1 ml. of toxin intramuscularly. The bird that had received 0.4 ml. orally was injected with 2 ml. intramuscularly. All four birds remained clinically normal.

Eight, 9-week-old pheasants were injected intramuscularly with 0.1 ml. of toxin, type E (ATCC #17786) while five, 9-week-old pheasants were given 0.3 ml. of the toxin orally. These birds were observed for 30 days and no signs of paralysis were noted.

Observations of inoculated birds were made at varying intervals and a description of the clinical progression of the disease is given (Table 3). The first signs noted were ruffling of the feathers and general uneasiness. These signs are common in many disease conditions in birds. Following this stage the bird ran a few steps then sank back on its hocks, apparently as if unable to stay on its legs for any length of time and soon after could only walk on its hocks. Although this appeared to be awkward, birds in

Table 3. Progressive clinical signs of botulism in pheasants.

Stage of Disease	Signs
I	Bird is uneasy with ruffled feathers
II	Bird runs a few steps, then sinks back on its hocks
III	Bird walks on its hocks
IV	Bird is unable to move legs but moves wings
V	Wings are paralyzed
VI	Eyes tend to close but open if bird is startled
VII	Neck is paralyzed
VIII	Bird is prostrate and unable to raise head; breathing is deep and slow
IX	Breathing is shallow
X	Bird is unresponsive, but breathing continues



this stage were able to move around easily. Next the legs were completely paralyzed and the bird pushed itself around by its wings. The tremendous fortitude of pheasants was demonstrated by these determined efforts of movement. Locomotion ceased with wing paralysis, the next stage, during which the bird remained alert and protected itself from intruders with its beak. This alertness was gradually lost, and the bird sat with its head resting against its breast and its eyes closed. When startled, it would raise its head and open its eyes, only to assume the previous position with eyes closed. Loss of neck control, "limberneck," followed and the bird fell over, unable to raise its head. Breathing was deep and slow, but then became very shallow, almost undiscernible. This stage preceded death, but some birds in this condition for three days made a complete recovery. All infected birds did not show each stage, and the duration of clinical signs varied greatly. Since the response to oral doses of toxin was generally unpredictable, greater emphasis was placed on the intramuscular route of administration, which gave more reproducible results.

Type C<sub>α</sub> (NCDC #4496) and type A toxins were used in succeeding tests, since these were the only strains which affected pheasants. Mouse inoculations were used to determine the strength of the toxins. Six mice were used for each toxin dilution. Six dilutions were used for type C<sub>α</sub>, ranging from 10<sup>-1</sup> to 10<sup>-6</sup>; nine dilutions were used for type A, 10<sup>-1</sup> to 10<sup>-9</sup>. With type C<sub>α</sub>, all mice died within 48 hours through dilution 10<sup>-4</sup>, while those in greater dilutions survived.

With type A, all the mice died through  $10^{-6}$ , and the other mice survived. This gave an  $LD^{50}$  of  $10^{-4.5}$  for C and  $10^{-6.5}$  for A toxin.

Twelve, 8-week-old birds were acquired from the South Dakota Pheasant Company and maintained for four days to allow for acclimation to new surroundings. At that time six birds were given 0.1 ml. of type  $C_{\alpha}$  toxin intramuscularly and six 0.2 ml. of toxin orally. Results of this experiment are shown in Tables 4 and 5.

Five, 11-week-old pheasants were inoculated intramuscularly with 0.1 ml. each of type  $C_{\alpha}$  toxin. One of these birds sustained a broken leg. Three, 11-week-old birds were given 0.1 ml. of toxin orally. The reactions are given in Tables 6 and 7. Nine days after the initial dose, the two normal birds were given 0.3 ml. of toxin orally. Twenty-four hours later both birds were dead.

Seven, 13-week-old pheasants were inoculated intramuscularly with 0.1 ml. each of type  $C_{\alpha}$  toxin (Table 8).

Four sets of two adult pheasants were inoculated intramuscularly with varying amounts of type  $C_{\alpha}$  toxin to determine time of death and dose relationships in larger birds. Both birds receiving 0.4 ml. of toxin were dead in 3 days as were both birds receiving 0.2 ml. One bird receiving 0.1 ml. was dead in 3 days; the other died in five days. One bird receiving 0.05 ml. died in 5 days, while the other became paralyzed, and then recovered.

Table 4. Stages of clinical disease of pheasants inoculated with 0.1 ml. of type C botulinum toxin intramuscularly.

Hours Post Inoculation	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6
6	1	1	1	1	1	1
8	1	2	2	2	2	2
10	3	3	3	3	3	3
14	3	3	4	4	4	4
18	4	4	4	4	7	7
20	5	5	5	5	7	7
22	6	6	6	6	8	9
30	8	9	9	9	9	Dead
44	9	9	Dead	Dead	Dead	
49	10	10				
68	10	Dead				
164	Dead					

Table 5. Stages of clinical disease of pheasants inoculated with 0.2 ml. of type C botulinum toxin orally.

Hours Post Inoculation	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6
4	Normal	Normal	Normal	Normal	Normal	2
6	1	1	1	1	1	8
8	2	2	2	2	6	Dead
10	2	6	6	6	Dead	
14	3	7	7	7		
18	4	9	9	9		
20	4	9	Dead	Dead		
22	4	10				
30	3	10				
44	2	10				
49	Normal	6				
53		5				
68		4				
92		3				
116		Normal				

Table 6. Stages of clinical disease of pheasants inoculated with 0.1 ml. of type C<sub>α</sub> botulinum toxin intramuscularly.

Hours Post Inoculation	Bird 1*	Bird 2	Bird 3	Bird 4	Bird 5
4	Normal	Normal	Normal	2	2
8	3	3	3	3	3
14	3	3	3	5	5
21	3	4	4	7	7
27	3	9	9	10	Dead
45	4	10	Dead	Dead	
67	Normal	Dead			

\* Bird sustaining a broken leg

Table 7. Stages of clinical disease of pheasants inoculated with 0.1 ml. of type C botulinum toxin orally.

Hours Post Inoculation	Bird 1	Bird 2	Bird 3
14	Normal	Normal	3
17	Normal	Normal	5
21	Normal	1	7
27	Normal	1	Dead
33	Normal	Normal	

Table 8. Stages of clinical disease of pheasants inoculated with 0.1 ml. of type C botulinum toxin intramuscularly.

Hours Post Inoculation	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6	Bird 7
18	2	2	2	2	2	2	4
21	2	4	4	4	4	4	4
30	4	4	4	7	7	7	Dead
45	5	5	5	9	9	9	
54	7	7	7	9	10	Dead	
60	7	8	8	9	10		
70	9	9	9	9	Dead		
80	9	9	9	Dead			
94	9	Dead	Dead				
142	6						
166	4						
190	Normal						

Six, 11-week-old pheasants were inoculated intramuscularly with 0.1 ml. of type A toxin, and three were given 0.1 ml. orally. Results are shown in Tables 9 and 10. Nine days after the initial dose the two clinically normal birds were given 0.3 ml. of toxin orally. They remained clinically normal for eight days. At that time they were given 0.5 ml. of toxin orally. Within 24 hours both birds were dead.

Six, 8-week-old pheasants were inoculated intramuscularly with 0.1 ml. of type A toxin. Six birds were given 0.3 ml. of the toxin orally. The results are given in Tables 11 and 12. Three days later the three remaining birds were given 0.5 ml. of type A toxin orally. One bird was dead within 18 hours, while the other two showed no signs of illness. Fifteen days later these two birds were given 1 ml. of toxin orally and no abnormal signs were observed. To determine if they had developed immunity, they were injected intramuscularly with 0.5 ml. of type A toxin. Both birds were dead in 18 hours.

#### Immunization Study

Twelve birds were used for an immunization study. Four birds were injected with 2 ml. each of toxoid prepared from type C<sub>2</sub> toxin, four birds were administered 1 ml. each of commercial toxoid (Clostridium botulinum, type C Bacterin-Toxoid, Salsbury Laboratories), and each of four birds was injected with 1 ml. of sterile saline. In each case two birds were inoculated intramuscularly and two intraperitoneally. These birds ranged in weight from 1050 gms to



Table 9. Stages of clinical disease of pheasants inoculated with 0.1 ml. of type A betulinum toxin intramuscularly.

Hours Post Inoculation	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6
2	Normal	Normal	Normal	2	2	4
4	4	4	Dead	Dead	Dead	Dead
5	Dead	Dead				

Table 10. Stages of clinical disease of pheasants inoculated with 0.1 ml. of type A betulinum toxin orally.

Hours Post Inoculation	Bird 1	Bird 2	Bird 3
21	Normal	Normal	3
25	Normal	Normal	4
30	Normal	Normal	7
33	Normal	4	9
45	Normal	Normal	9
67	Normal	Normal	Dead

Table 11. Stages of clinical disease of pheasants inoculated with 0.1 ml. of type A botulinum toxin intramuscularly.

Hours Post Inoculation	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6
3	Normal	3	3	3	4	7
5	2	4	4	4	4	Dead
8	3	8	Dead	Dead	Dead	
13	8	Dead				
18	Dead					

Table 12. Stages of clinical disease of pheasants inoculated with 0.3 ml. of type A botulinum toxin orally.

Hours Post Inoculation	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6
3	Normal	Normal	2	Normal	Normal	Normal
5	Normal	Normal	4	Normal	Normal	Normal
13	Normal	Normal	Dead	Normal	Normal	Normal
21	Normal	7		Normal	Normal	Normal
25	Normal	Dead		Normal	Normal	Normal
34	5			Normal	Normal	Normal
41	9			Normal	Normal	Normal
45	Dead			Normal	Normal	Normal

1775 gms. The injections were repeated in 21 days. Two weeks following the last injection, challenge doses of toxin were given. The route of administration of toxoid, saline, and the challenge dose of toxin are shown in Table 13.

Within 48 hours both saline-treated control birds receiving 0.5 ml. of toxin intramuscularly and one saline control receiving 1 ml. of toxin orally were dead. The remaining saline control was in stage 4 and continued in this stage for 48 hours and then made a complete recovery. The toxoid-protected birds were observed for two weeks during which time they showed no signs of disease. From this it was postulated that while the challenge dose was not large enough to kill all the unprotected birds, the toxoids seemed to protect birds from the disease. No difference was seen between the commercial toxoid and that prepared from the C<sub>2</sub> toxin.

#### Pathology Studies

From a total of 76 pheasants necropsied in this study, gross lesions were found in twelve birds. Seven of these had received type C<sub>2</sub> toxin intramuscularly, four had been injected with type A toxin intramuscularly, and one type A toxin orally. The most frequent lesion seen (hydropericardium) occurred in seven birds. The amounts of fluid varied from 0.25 ml. to 2 ml. Four birds had enlarged spleens; three had slightly impacted cloacae. Two birds had fatty livers. Necropsy findings in single birds were crop mycosis, hemorrhage on the surface of the pancreas, distended

Table 13. Route of administration of toxoid, saline, and challenge dose of toxin.

Initial Treatment		Challenge Treatment	
Toxoid	Route of Inoculation	Toxin	Route of Inoculation
Non-Commercial	Intraperitoneal	1.0 ml.	Oral
Commercial	Intraperitoneal	1.0 ml.	Oral
Non-Commercial	Intraperitoneal	0.5 ml.	Intramuscular
Commercial	Intraperitoneal	0.5 ml.	Intramuscular
Saline	Intraperitoneal	1.0 ml.	Oral
Saline	Intraperitoneal	0.5 ml.	Intramuscular
Non-Commercial	Intramuscular	1.0 ml.	Oral
Commercial	Intramuscular	1.0 ml.	Oral
Non-Commercial	Intramuscular	0.5 ml.	Intramuscular
Commercial	Intramuscular	0.5 ml.	Intramuscular
Saline	Intramuscular	1.0 ml.	Oral
Saline	Intramuscular	0.5 ml.	Intramuscular

gall bladder, petechiation of serous membranes, splenic hemorrhages and leg abscesses. (Table 14)

Bacteriologic examination of tissues taken at necropsy failed to reveal the presence of pathogens. Staphylococcus epidermidis with 105 isolations was the most frequent organism found. Escherichia coli, with 94 isolations, and alpha hemolytic Streptococcus, with 79 isolations, were the next most often encountered. The following organisms were found in the following order of frequency: Micrococcus sp., Staphylococcus aureus, Pseudomonas aeruginosa, Flavobacterium sp., Corynebacterium pseudodiphtheriticum, and Enterobacter aerogenes.

Culture of bone marrows revealed the least number of isolates-- 3 alpha hemolytic Streptococci, one Staphylococcus epidermidis and one Staphylococcus aureus. Lungs showed the greatest variety of organisms, with eight species. Intestines contained seven species; kidneys, spleen and liver, six each; and heart cultures had five. (Table 15). All of these organisms are normally present in the environment and could be expected to be found in any bird. Since most of the pheasants were necropsied several hours after death, organisms in the digestive tract could be found throughout the carcass.

There was essentially no difference between organisms isolated from birds receiving type C<sub>4</sub> and those receiving type A toxin. Pseudomonas aeruginosa, Enterobacter aerogenes, and Corynebacterium pseudodiphtheriticum were found only in birds receiving type C<sub>4</sub> toxin, but the other species were common to both groups. Of the 76

Table 14. Lesions found on necropsy of birds with various doses of botulinum toxin and routes of administration.

Toxin and Dose	Necrotic Lesions
0.1 ml. type C, intramuscular	hydropericardium, 2 ml. of fluid friable liver mild crop mycosis
0.2 ml. type C, intramuscular	cloacal impaction
0.3 ml. type C, orally	hemorrhage on pancreas distended gall bladder
0.5 ml. type C, intramuscular	hydropericardium, 1 ml. of fluid
0.5 ml. type C, intramuscular	hydropericardium, 1 ml. of fluid enlarged spleen fatty liver
4 ml. type C, intramuscular	cloacal impaction
4 ml. type C, intramuscular	petechial hemorrhages on serosa enlarged spleen cloacal impaction
0.1 ml. type A, intramuscular	hemorrhagic spleen hydropericardium, 0.5 ml. of fluid
0.1 ml. type A, intramuscular	enlarged spleen hydropericardium, 1 ml. of fluid
0.1 ml. type A, intramuscular	hydropericardium, 0.1 ml. of fluid
0.1 ml. type A, intramuscular	hydropericardium, 0.25 ml. of fluid
0.3 ml. type A, orally	leg abscess enlarged spleen

Table 15. Distribution of organisms isolated from pheasants necropsied.

Organism	Kidney	Spleen	Liver	Heart	Lung	Bone marrow	Intestine
<u>Staphylococcus epidermidis</u>	17	10	15	16	20	1	23
<u>Escherichia coli</u>	19	6	12	16	15		23
Alpha hemolytic <u>Streptococcus</u>	11	8	8	12	14	3	11
<u>Micrococcus sp.</u>	4	4	5	6	8		6
<u>Staphylococcus aureus</u>	5	5	2	4	5	1	5
<u>Pseudomonas aeruginosa</u>	1		1		1		1
<u>Flavobacterium sp.</u>		1			1		
<u>Corynebacterium pseudodiphtheriticum</u>					2		
<u>Enterobacter aerogenes</u>							1



birds cultured, 49 had received type C<sub>α</sub> toxin, and 27 had received type A. The predominance of type C<sub>α</sub> birds cultured could account for the greater diversity of isolates of these birds.

Histopathologic lesions were few and randomly distributed. The most prevalent lesions were liver congestion of varying degrees, followed by crystals in the renal tubules. Heart and spleen inflammations were next frequent, with one instance each of cerebral inflammation, a small area of inflammation in the spinal cord, and numerous leukocytes found in the liver, kidney and heart. There was no correlation between histopathologic, bacteriologic and gross necropsy findings.

#### Conclusions

It is concluded that pheasants are susceptible to botulism through either oral or intramuscular route of administration. A summary of all toxin inoculation experiments done in this study is given in Table 16. Of the strains of Cl. botulinum used in this study, types A and C<sub>α</sub> were toxic to pheasants. Literature reports of toxicity of type C<sub>β</sub> were not verified because this strain became nontoxic.

Great individual variations in response to intramuscular injection of type C<sub>α</sub> were found. Birds inoculated with 0.1 ml. of toxin intramuscularly survived from 30 to 164 hours. Of 18 birds inoculated, 2 survived, including one with a broken leg. The other survivor remained in stage 9 for three days before recovery. Pheasants receiving type C<sub>α</sub> orally died within 27 hours or recovered.

Table 16. Summary of inoculation studies.

Toxin Toxicity Studies									
Toxin type A		Toxin type C <sub>α</sub> (NCDC #4496)		Toxin type C <sub>α</sub> (ATCC #17850)		Toxin type D		Toxin type E	
I.M. <sup>1</sup>	Oral	I.M.	Oral	I.M.	I.M.	Oral	I.M.		
4 birds 0.1 ml.	2 birds 0.5 ml.	2 birds 0.05 ml.	1 bird 0.4 ml.	2 birds 0.5 ml.	1 bird 1.2 ml.	1 bird 0.2 ml.	8 birds 0.4 ml.		
		3 birds 0.1 ml.			1 bird 0.4 ml.	1 bird 2.4 ml.			
		2 birds 0.2 ml.							
		2 birds 0.4 ml.							
Experiments to Study Clinical Signs of Botulism									
Toxin type C <sub>α</sub>				Toxin type A					
Intramuscular		Oral		Intramuscular		Oral			
18 birds 0.1 ml.		3 birds 0.1 ml.		12 birds 0.1 ml.		3 birds 0.1 ml.			
		6 birds 0.2 ml.				6 birds 0.3 ml.			
Immunization Studies									
Toxoid I.P. <sup>2</sup>		Saline I.P.		Toxoid I.M.		Saline I.M.			
Toxin oral	Toxin I.M.	Toxin oral	Toxin I.M.	Toxin Oral	Toxin I.M.	Toxin Oral	Toxin I.M.		
2 birds 1.0 ml.	2 birds 0.5 ml.	1 bird 1.0 ml.	1 bird 0.5 ml.	2 birds 1.0 ml.	2 birds 0.5 ml.	1 bird 1.0 ml.	1 bird 0.5 ml.		

<sup>1</sup>I.M. - Intramuscular

<sup>2</sup>I.P. - Intraperitoneal

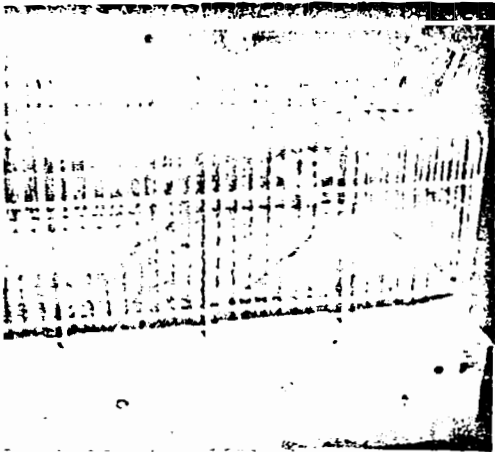
The majority of those recovering had showed few signs of intoxication, however one bird remained in stage 10 for two days before recovery. Oral doses of toxin must be larger than intramuscular inoculations to elicit signs of toxicosis. Oral administration of 0.1 ml. of toxin killed one bird, while 2 others remained normal. 0.2 ml. of oral administered toxin killed 4 birds of 6, while 0.3 ml. of toxin was lethal to all birds.

Although type A toxin given intramuscularly reacted more rapidly than type C<sub>α</sub>, pheasants were more resistant to oral doses of type A. It may be that type A toxin is more readily detoxified before absorption into the nervous system. Type A toxin (0.1 ml.) when administered intramuscularly killed all pheasants within 18 hours. When given orally 0.1 ml. killed one bird of three, 0.3 ml. killed three birds of six, 0.5 ml. killed three birds of five; while the remaining two birds were refractive to 1.0 ml.

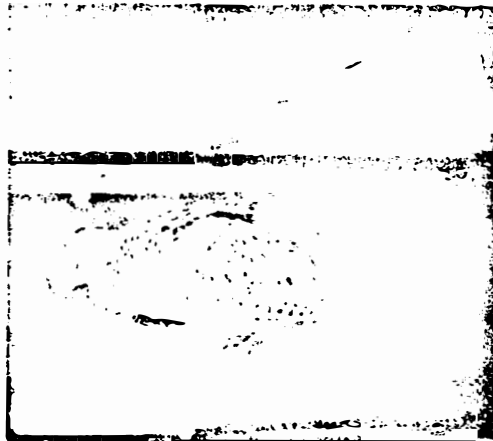
Immunization studies indicated that toxoid given either intramuscularly or intraperitoneally will protect pheasants against lethal doses of toxin. There is danger in extrapolating laboratory findings to natural conditions, because the amount of toxin a pheasant may ingest in the wild could be much greater than the amount administered as a challenge.

Clinical signs of botulism, although not pathognomonic, are typical enough to alert an observer to the probability of the presence of botulism. In early stages of the disease, loss of use of the legs is seen, followed by wing and neck paralysis. Due to

great individual variances in resistance, a specific time-table of signs cannot be made. Within limits, duration of signs depends on the size of the dose. Adult birds require larger doses of toxin to elicit a response than do juveniles. Gross necropsy, bacteriologic and histopathologic findings do not confirm the diagnosis of botulism in pheasants, since no common lesions are found. These examinations must be done, however, to exclude other diseases which may mimic botulism.



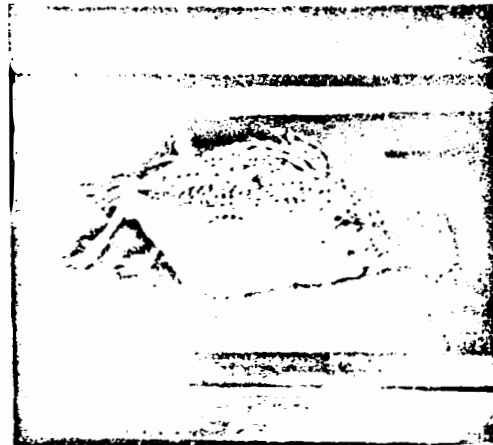
A.



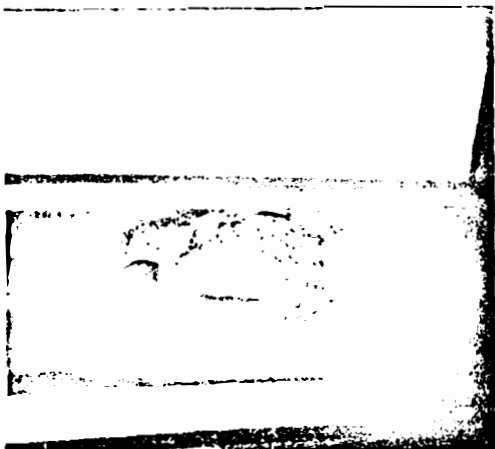
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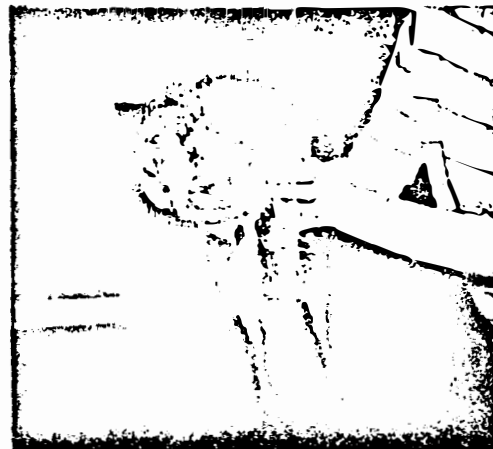
C.



D.



E.



F.

Pheasants in various stages of botulism. A. Normal; B. Stage 4; C. Stage 5; D. Stage 6; E. Stage 8; F. Stage 10.

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