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Authors: Spieker, John O., Yuill, Thomas M., and Burgess, Elizabeth C.

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VIRULENCE OF SIX STRAINS OF DUCK PLAGUE VIRUS IN EIGHT WATERFOWL SPECIES

John O. Spieker,[1] Thomas M. Yuill,[1,2,4] Elizabeth C. Burgess[1,3]

[1] Department of Veterinary Science, University of Wisconsin, Madison, Wisconsin 53706, USA
[2] School of Veterinary Medicine, Department of Pathobiological Sciences, University of Wisconsin, Madison, Wisconsin 53706, USA
[3] Department of Medical Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin, 53706, USA
[4] Author for reprints

ABSTRACT: Susceptibility of New World waterfowl to the Lake Andes strain of duck plague virus (DPV) was assessed by intramuscular inoculation of adult muscovies (*Cairina moschata*), mallards (*Anas platyrhynchos*), Canada geese (*Branta canadensis*), wood ducks (*Aix sponsa*), redheads (*Aythya americana*), gadwalls (*Anas strepera*), blue-winged teal (*Anas discors*), and pintails (*Anas acuta*). The relative virulence of DPV strains isolated from five United States and one Canadian location was established in muscovies, mallards, and Canada geese. Differences in DPV strain virulence were detected by formation of plaques in cell culture. Two strains that consistently formed plaques killed adult mallards while non-plaque forming strains killed hatchling but not adult mallards. Based on mortality after exposure to the Lake Andes strain, blue-winged teal, then wood ducks and redheads were highly susceptible, muscovies and gadwalls moderately susceptible, mallards and Canada geese less susceptible, and pintails the least susceptible. Mean death times were significantly ($P < 0.01$) different between adult muscovies (4.5 days) versus mallards and Canada geese (5.8 days each). Mean death time of the virulent Lake Andes and Minnesota strains were shorter ($P < 0.05$) than for the other four, less virulent DPV strains. Four of the less virulent strains killed hatchling but not adult mallards. Susceptibility to mortality was dependent upon age and route of inoculation. The intramuscular route of inoculation required the least amount of virus to kill mallard and muscovy ducks, the intranasal and conjunctival routes required more virus, and the oral route the most virus. This study was conducted from 1974 to 1977 between the months of September and April, with the exception of two titrations conducted in early May at the University of Wisconsin Department of Veterinary Science and the Charmany research facility of the University of Wisconsin-Madison.

Key words: Duck plague virus, duck virus enteritis, susceptibility, virulence, strains, waterfowl.

INTRODUCTION

Duck plague (DP) (duck virus enteritis) is caused by a herpesvirus that infects ducks, geese and swans (Leibovitz, 1984). Since its first appearance in Long Island, New York (USA) in 1967 (Leibovitz, 1970), scattered epizootics have occurred across the United States and Canada (Brand and Docherty, 1988).

In January 1973, an epornitic of DP in South Dakota (USA) killed approximately 43% of an estimated 100,000 wild waterfowl, mostly mallard ducks (*Anas platyrhynchos*) and Canada geese (*Branta canadensis*) (Friend and Pearson, 1973). A focal DP epizootic occurred among captive black ducks (*Anas rubripes*) in Wisconsin (USA), but spared Canada geese, mallards, and other species in adjacent pens (Jacobsen et al., 1976). A DP epizootic in the Washington, D.C. (USA) Zoological Park caused mortality among eight duck species, but did not affect geese or swans (Montali et al., 1976). Based on these observations, we believe that duck plague virus (DPV) strains may vary in virulence, as occurs with Newcastle disease (Beard and Hanson, 1984), and herpesviruses including Marek's disease (Purchase and Biggs, 1967) and infectious laryngotracheitis (Pulsford, 1961).

Mortality among some species, but not others, could also be due to differences in species susceptibility to DPV. Differences occur in susceptibility of domesticated ducks and wild European waterfowl to a Dutch strain of DPV (Van Dorssen and Kunst, 1955). A Long Island, New York, DPV strain killed all Canada geese, muscovy ducks (*Cairina moschata*), and a

TABLE 1. Origin of duck plague virus strains used, year isolated, and source species. All strains were passed once in Muscovy duck liver prior to use for inoculation trials.

Strain	Strain origin	Year isolated	Source species
LA-SD-73	Lake Andes, South Dakota, USA	1973	Mallard duck
CO-WI-73	Coloma, Wisconsin, USA	1973	Black duck
SP-MN-73	St. Paul, Minnesota, USA	1973	Mute swan
WA-NY-73	Wantagh, New York, USA	1973	Muscovy duck
SA-CA-74	Sacramento, California, USA	1974	Muscovy duck
HE-PQ-CAN-76	Hemmingford, Quebec, Canada	1976	Muscovy duck

mute swan (*Cygnus olor*), as well as lesser scaup (*Aythya affinis*), inoculated with high doses of the virus (Dardiri and Butterfield, 1969). Differential mortality occurred in an epizootic of DP in a captive flock of mallards, black ducks, and Canada geese housed together. Because mortality and morbidity were confined to the black ducks and Canada geese, we propose there are differences in species susceptibility (Montgomery et al., 1981). However, the wider range of relative susceptibility of North American waterfowl to infection and death from different DPV strains has not been studied extensively.

We had four objectives: to determine if six North American DPV strains differed in virulence; if eight waterfowl species differed in their susceptibility to DP-induced mortality; if there was a difference in susceptibility due to age; and if susceptibility varied by route and exposure to DPV.

MATERIALS AND METHODS

This study was conducted from 1974 to 1977 between the months of September and April, with the exception of two titrations conducted in early May at the University of Wisconsin Department of Veterinary Science research facility of the University of Wisconsin, Madison, Wisconsin. The six tested strains of DPV came from six sites and four species (Table 1). The strains were obtained for the study as follows: the HE-PQ-CAN-76 strain as 20% Muscovy duck liver suspension from the original epizootic; the CO-WI-73 and LA-SD-73 strains as fourth passages on muscovy embryo fibroblast (MEF) cells; the SP-MN-73 strain as fourth passage on duck chorioallantoic membrane (DCAM); the WA-NY-73 strain as first passage in DCAM; and the SA-CA-74 strain as a second passage on MEF cells. Following receipt, each strain was separately passed once by inoculation of four 12- to 18-mo-old male muscovy ducks except for the HE-PQ-CAN-76 strain. All strains produced death 3 to 8 days postinoculation (DPI). Livers were aseptically removed as soon as possible after death and frozen at −70 C or immediately chilled at 4 C and processed. No two strains were handled the same day.

Twenty per cent liver suspensions were prepared in chilled minimum essential medium (MEM) (International Scientific Industries, Inc. Crystal Lake, Illinois, USA) with 10% fetal bovine serum diluent and triturated in a 60 ml Sorvall mixer vessel (DuPont Co., Wilmington, Delaware, USA) at maximum speed for 5 min. After dispensing into vials, inocula were stored at −70 C or immediately titrated unfrozen (plaque-forming strains).

Muscovy ducks, mallard ducks and Canada geese were used most frequently as they were relatively available throughout the year. A flock of breeder muscovies with no known history of DP was purchased from an area farm and kept in pens loose on the floor as were flocks of mallard ducks. Growing and egg-laying birds were fed chicken laying mash, whole corn, oyster shell, and granite grit. Some birds were kept indoors on a photoperiod of 18 hr light per day to promote egg production. Hatchlings were kept in heated brooders and fed the same laying mash given to adults.

Mallards came from a Beloit, Wisconsin, park (42°31′N, 88°55′W) and also from the Northern Prairie Waterfowl Research Center (NPWRC) of the U.S. Fish and Wildlife Service at Jamestown, North Dakota USA. The Canada geese were picked up as gunshot cripples at the Horicon National Wildlife Refuge at Horicon, Wisconsin (43°33′N, 88°38′W), and held until they had recovered from their wounds. The geese and many of the mallards were maintained year-round in top-enclosed outdoor pens with shelters and were given chicken mash, whole corn, grit, and water. The wood ducks were captured by cannon-net at

the Mead Wildlife Area near Stevens Point, Wisconsin (44°32′N, 89°32′W). The redheads, gadwalls, blue-winged teal, and most of the pintails were received as downy young from the NPWRC and were raised to adulthood. Additional pintails were donated by the Horicon State Wildlife Refuge. Additional blue-winged teal were netted at a farm pond near Baldwin, Wisconsin USA (44°58′N, 92°23′W), and the remaining species were donated by the NPWRC. Other than occasional feeding of lettuce, no special attempt was made to simulate the natural foods of the noncommercial species.

Muscovy embryo fibroblast (MEF) primary cells were prepared fresh as needed from 14- to 17-day embryonated eggs by the method described by Revozzo and Burke (1973). The cells were grown in Linbro (Flow Laboratories, Rockeville, Maryland, USA) plastic 6- and 24-well cell culture plates for DPV titration, identification, and serum neutralization tests. Cell monolayers were grown at 37 C in a 5% CO_2 atmosphere at a pH of approximately 7.2.

Heat-treated (56 C, 30 min) pre-inoculation serum samples were tested for neutralizing antibody by adding 1 undiluted ml from each pool of 10 sera to 1 ml diluent containing 1.2×10^3 plaque-forming units (PFU) of the LA-SD-73 DPV strain and incubating for 1 hr at 37 C. An antiserum-free control inoculum was prepared substituting diluent for antiserum. Control antiserum was obtained from sheep inoculated with the DPV vaccine (Holland B) (Brand and Docherty, 1984). One-tenth ml from each sample was inoculated onto each of three MEF monolayers in 24-well plates and incubated at 37 C for 30 min. After overlaying each well with 2 ml of MEM with 3% fetal bovine serum, plates were incubated at 37 C for 3 to 4 days. Due to the cell-associated nature of DPV, no viscous overlay was required to produce plaques. Monolayers were then fixed, stained and disinfected by dipping the plates in 1% crystal violet in 10% formalin for 10 min. The approximately 0.5 mm plaques were counted using a dissecting microscope. A 50% or greater reduction in plaque count of experimental versus control wells was regarded as positive for DPV antibody.

Birds free of DP antibody were used in titrations of each DPV strain. They were housed in individual cages in separate rooms of a high efficiency particulate air (HEPA)-filtered isolation room. Two-day-old hatchlings were housed in heated cages in groups of four to eight birds. Food and water were available at all times. Ten-fold serial dilutions of each DPV strain were made in MEM medium after thawing, titrated in MEF cells (plaque-forming strains) and inoculated into birds. Adult birds received 0.5 ml of virus suspension in the pectoral muscle and hatchlings were given 0.2 ml in the thigh muscles. It was necessary to employ fewer individual gadwalls, redheads, and blue-winged teal per dilution because of their limited availability. Control birds were inoculated with diluent only (titration controls), or were uninoculated (room controls housed in same room in individual cages). Birds were observed at least once and usually twice a day for 14 days and the time of death was recorded. Birds not dying were euthanized with CO_2 and cervical dislocation. All birds were necropsied; only those birds from which DPV was isolated or that had gross lesions consistent with DP were included in the LD_{50} calculations (Reed and Muench, 1938). The presence of LA-SD-73 and SP-MN-73 DPV strains in the livers of dead birds was confirmed in MEF cultures by plaque assay followed by neutralization with specific DPV antiserum as described. Mean death times were calculated using a Student's *t* test, (Snedecor and Cochran, 1980) only for that subset of birds receiving 1 to 10 LD_{50} of virus.

TABLE 2. Relative susceptibility of adults of eight waterfowl species to mortality from intramuscular inoculation with the LA-SD-73 strain of duck plague virus (titrations from 0.01 10^3 plaque-forming units).

Host species	LD_{50}[a]	Mean days to mortality
Blue-winged teal	<0.01	4.4
Wood duck	0.1	4.0
Redheads	0.1	4.9
Muscovy	0.5	4.5
Gadwals	0.5	5.6
Canada goose	1.0	5.8
Mallard	1.0	5.8
Pintail	$>3 \times 10^3$	NC[b]

[a] Number of plaque-forming units (in muscovy embryo fibroblast cells) to produce 1 LD_{50}.
[b] NC = Not calculated, no deaths resulted.

Tests of species susceptibility to mortality by DPV and of virus strain virulence were tested by intramuscular (IM) titration of the LA-SD-73 virus strain in all eight species (Table 2). All six DPV strains were titered by IM inoculation in adult muscovy ducks, adult Canada geese, and adult and hatchling mallards; four to six birds per dilution were used for each species. Four to six birds per species of titration control birds for each species also were inoculated IM with diluent. An additional three to four birds were used as room controls. Age-related changes in susceptibility were determined by

TABLE 3. Virus strain LD_{50} and mean days to mortality in waterfowl inoculated intramuscularly with three plaque-forming strains of duck plague virus.

Species	Virus strain		
	LA-SD-73	SP-MN-73	CO-WI-73
Muscovy, adult	0.5[a]	0.7	0.1
Days (*n*)	4.5 (8)[b]	4.5 (6)	6.7 (11)
Canada goose, adult	1.0	0.8	0.1
Days (*n*)	5.8 (6)	5.4 (5)	7.4 (5)
Mallard, 2-day old	0.1	0.4	12.0
Days (*n*)	5.2 (12)	4.2 (7)	6.1 (10)
Mallard, adult	1.0	0.7	NC[c]
Days (*n*)	5.8 (6)	6.4 (7)	NC (0)

[a] Number of plaque forming units in muscovy embryo fibroblast cells = 1 LD_{50}. Calculated for birds receiving <10 LD_{50} of virus.
[b] Mean days to mortality (number of those birds dying following inoculation with 1–10 LD_{50}).
[c] Not calculated. No birds died.

TABLE 4. Virus strain LD_{50} and mean days to mortality in waterfowl inoculated intramuscularly with three non-plaque forming strains of duck plague virus.

Species	Virus strain		
	SA-CA-74	WA-NY-73	HE-PQ-CAN-76
Muscovy adult	6.8[a]	6.7	3.8
Days (*n*)	6.5 (10)[b]	5.8 (9)	5.6 (9)
Canada goose adult	6.0	5.5	2.6
Days (*n*)	8 (6)	10.8 (5)	7.3 (3)
Mallard 2-day-old	2.2	5.0	3.0
Days (*n*)	7.3 (1)	8.4 (5)	4.5 (4)
Mallard adult	NC[c]	NC	NC
Days (*n*)	NC (0)	NC (0)	NC (0)

[a] Reciprocal of the $\log_{10}$ dilution of virus that is one LD_{50}.
[b] Mean days to mortality (number of those birds dying following inoculation with 1-10 LD_{50}).
[c] Not calculated. No birds died.

IM titration of the six virus strains in adult and hatchling mallard and muscovy ducks.

The effect of route of inoculation on LA-SD-73 strain DPV mortality was determined in adult and hatchling mallard and muscovy ducks. Approximately 0.008 ml of MEM containing virus was put onto the surface of each eye and worked manually into the conjunctival sac. The birds were inoculated intranasally by introduction of 0.05 ml of virus inocula for adult ducks and 0.025 for each hatchling, into each external nares. Ducks were orally inoculated by dropping the virus suspension (0.5 ml for adult ducks, 0.1 ml for hatchlings) onto the maxillary palate with the duck's head tilted back.

RESULTS

The LA-SD-73 strain was virulent in all host species except pintails, which survived exposure to $>10^{3.5}$ PFU (Table 2). The SP-MN-73 strain was as virulent for muscovy and mallard ducks, and Canada geese, as was the LA-SD-73 strain (Table 3). The mean death times for muscovies and Canada geese (combined) for the LA-SD-73 strain were significantly ($P < 0.01$) shorter than for CO-WI-73 and the three nonplaque forming DPV strains. The CO-WI-73 was virulent in muscovies and Canada geese but did not produce mortality in adult mallards inoculated IM with up to $10^{4.5}$ PFU. However, one LD_{50} for 2-day-old mallards was 12 PFU. Four pintails became infected following inoculation of $10^{3.5}$ PFU of the virus and developed antibody but did not die. These three virus strains all produced plaques in MEF cells.

The non-plaque-forming DPV strains proved more difficult to evaluate because no in vitro system of virus quantification was available. High dilutions of SA-CA-74 killed muscovies (titering $10^{-6.8}$ = 1 LD_{50}) and Canada geese (10^{-6} = 1 LD_{50}) although the LD_{50} titer was 10,000-fold lower in hatchling mallards ($10^{-2.2}$). Adult mallards survived injections of undiluted SA-CA-74 inoculum containing more than $10^{6.8}$ muscovy LD_{50}. (Table 4).

The remaining two non-plaque forming virus strains also failed to kill adult mallards but did produce hatchling mortality. The WA-NY-73 strain proved virulent in muscovies (titering $10^{-6.7}$ = 1 LD_{50}), Canada geese ($10^{-5.7}$), and hatchling mallards (10^{-5}). The HE-PQ-CAN-76 strain killed muscovies, Canada geese, and hatchling mallards at LD_{50} titers of $10^{-3.8}$, $10^{-2.6}$, and 10^{-3}, respectively (Table 4).

Blue winged teal appeared to be the most susceptible to the LA-SD-73 DPV strain (<0.01 plaque-forming unit = 1 LD_{50}) of the species tested, but the number of birds available was small (Table 2).

Wood ducks and redheads were also very susceptible (0.1 PFU = 1 LD_{50}) to mortality. Muscovies, gadwalls, Canada geese and mallards appeared to be somewhat less susceptible to death from this DPV strain (0.5 to 1.0 PFU = 1 LD_{50})(Table 2). The small number of pintails tested were resistant to DP mortality and survived inoculation with up to $10^{3.5}$ PFU of this virus strain. None of the room control or media inoculated control birds died; when euthanized no DPV was isolated from their livers.

Susceptibility of muscovy ducks to mortality by all six DPV strains was equal to or greater than that of mallards or Canada geese (Tables 3 and 4). The difference in species susceptibility appeared to be greatest for the non-plaquing virus strains (SA-CA-73, WA-NY-73, and HE-PQ-CAN-76). Adult mallards were at least $10^{3.8}$ to $10^{6.8}$ times more resistant than muscovies to death from four (CO-WI-73, SA-CA-74, WA-NY-73, and HE-PQ-CAN-76) of the six virus strains tested. The mean death times for muscovy ducks was significantly ($P < 0.01$) shorter than for Canada geese, which died more quickly than mallards ($P < 0.01$). No control birds died; when euthanized no DPV was isolated from their livers.

Susceptibility to DPV mortality of adult and 2-day-old muscovy ducks was similar for all six DPV strains; differences between titers in the adults and the hatchlings were 10-fold or less. Titers of LA-SD-73 and in adult vs hatchling mallards inoculated by any of four routes varied 15-fold or less (Table 5). However, hatchling mallards died following IM inoculation with four DPV strains, whereas adults did not die (Tables 3 and 4). None of the control birds died and no DPV was isolated from the livers.

Adult muscovy and mallard ducks died following exposure to DPV strain LA-SD-73, by all four routes (Table 5). Birds were 30- to 180,000-fold more susceptible to death from the virus by the IM route than for conjunctival, intranasal, or oral routes.

TABLE 5. Mortality in two waterfowl species to the LA-SD-73 strain of DP virus when administered by four different routes.

Inoculation route	Muscovy		Mallard	
	Adult	2-day[a]	Adult	2-day
Intramuscular	0.4[b]	0.1	1.0	0.1
Conjunctival	2.2	NT[c]	2.2	2.5
Intranasal	3.5	NT	3.4	2.2
Oral	3.7	4.3	3.4	3.7

[a] 2-day-old hatchling duck.
[b] Titer as $\log_{10}$ plaque-forming units in muscovy embryo cell.
[c] Not tested.

DISCUSSION

There is a significant difference in virulence among the North American strains of DPV tested. These strains fell into two groups according to their ability to kill adult mallards. Strains LA-SD-73 and SP-MN-73 were the most virulent and killed muscovy ducks, Canada geese, and adult and hatchling mallards at very low doses. The other four strains did not kill adult mallards, although mortality did result following inoculation of Canada geese, muscovy ducks, and hatchling mallards. However, the mean death times for these four strains in muscovy ducks and Canada geese were significantly longer than for the two highly virulent strains. Although differences in DPV strain virulence have not been quantified previously, Liebovitz (1984) suggested that field strains may differ and reported that some DPV strains produced no mortality in commercial Pekin duck (*Anas platyrhynchos*) flocks, although egg production declined 95%.

Based on our results there are differences in susceptibility to mortality by DPV in several species of North American waterfowl, similar to differences in susceptibility of European waterfowl inoculated with a Dutch strain of DPV (Jansen, 1968). In our study, blue-winged teal were more sensitive for virus detection than was our MEF cell plaquing system: <0.01 PFU of virulent virus equaled 1 LD_{50}. Wood ducks, redheads, muscovies, gadwalls, Canada geese, and mallards were, in order,

increasingly resistant to mortality from virulent virus. However, adult mallards, but not Canada geese, survived inoculation with large doses of the less virulent DPV strains. Pintails were resistant to mortality but not infection with relatively large doses of LA-SD-73, a virulent strain of DPV.

These findings are similar to the findings in a previous experimental inoculation study where blue-winged teal and Canada geese were highly susceptible to mortality from DPV (Wobeser, 1987). Mean death times also reflected species differences; muscovies died significantly more quickly than Canada geese and mallards.

Differences in species susceptibility could be used to establish DPV virulence groups. Avirulent strains would be those that killed neither adult nor hatchling muscovy ducks. Avirulence could be further tested by inoculation of blue-winged teal, the most susceptible species we tested. The DPV strains of low virulence would be those that killed hatchling mallards and adult muscovies but not adult mallards. Virulent strains would kill adult mallards, but not pintails. Highly virulent strains would kill pintails. Mean death times in muscovy ducks might also be a useful parameter of assessing relative virulence of DPV. In this study, it varied from 4.5 days for virulent strains to over 6 days for strains of low virulence. Mean death time is one of the standard measures of virulence for Newcastle disease in chickens (Beard and Hanson, 1984). The production of plaques by DPV in muscovy embryo fibroblast cells may also prove to be a useful tool for assessing virulence, and warrants further study. The two most virulent strains that we tested produced clear plaques well, whereas the low virulence strains produced no or poorly defined plaques. Virus plaque morphology has been associated with virulence with other viruses such as Venezuelan equine encephalitis virus (Frank and Johnson, 1971).

The greater susceptibility of young birds to mortality from some of the DPV strains is likely to be more important in captive birds than in free-flying waterfowl populations. Captive birds are usually kept at high population densities, facilitating transmission. In contrast, broods of most wild ducks and geese are small in number and relatively isolated until the young are fairly large and able to fly.

The route of inoculation had an important effect on mortality from DPV. The IM route required the least amount of virus to kill. Conjunctival and intranasal exposure required 100- to 1000- fold more virus to produce mortality. Although oral exposure required the most virus to cause mortality, we have found that acutely infected birds shed over $10^{3.7}$ PFU of DPV per ml of feces before they die (Spieker, 1970)—quantities we have shown sufficient to produce mortality following oral exposure. However, the likelihood of birds ingesting sufficient amounts of virus to cause infection and death would depend on a variety of factors such as virus stability in the environment and dilution in water. The virulence of the virus strain would probably be an important factor too. Oral inoculation of high doses of a Long Island, New York, strain killed all seven Canada geese exposed, but only some of the adult and 3-wk-old mallards tested (Dardiri and Butterfield, 1969). Given the drinking and feeding habits of waterfowl, conjunctival and intranasal DPV exposure in nature would be likely, and would require somewhat smaller amounts of virus.

The effects of host age and sex, and of the route of exposure, have been better studied for other avian viral diseases than for DP. As we found with four DPV strains, the severity of disease and death usually decreases with age. For example, susceptibility to duck hepatitis diminishes by 1 mo of age in mallards and in domestic ducks (Friend and Trainer, 1972). The severity of Newcastle disease (Beard and Hanson 1984) and Mareks disease (Calnek, 1973) in chickens usually decreases from hatching to maturity. Differences in sex-related susceptibility to DPV mortality

have not been shown as they have been in some other avian viral diseases. Marek's disease was found to be more virulent in female chickens (Purchase and Biggs, 1967) and Japanese quail (Khare et al., 1975). It is possible that the reproductive state of the hen may influence susceptibility and could vary seasonally as has been shown with shedding of DPV by persistently infected birds (Burgess and Yuill, 1983). Although DP has occurred at all times of year, there is evidence of a trend for focal epizootics in the spring. This route of exposure can have a major effect on infection and the development of disease. More natural routes of exposure (intranasal, oral, and ocular) to Newcastle disease virus in chickens led to respiratory signs, while intramuscular, intravenous, and intracerebral inoculation of the same strains increased its neurovirulence (Beard and Easterday, 1967). Marek's disease virus infection produces disease only when inoculated via the intraperitoneal and intranasal routes, but not when given orally (Witter and Burmester, 1967).

Thus, it is clear that DPV pathogenesis and epizootiology is complex. Duck plague appears to be a relatively new disease to the North American continent, having been first described in Long Island, New York, in 1967 (Leibovitz, 1970). Already, strains with a considerable range of virulence have appeared. It seems likely that continued evolution will occur as these strains evolve in waterfowl populations comprised of species of widely different habits, susceptibilities, population distributions and densities.

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