# NEWCASTLE DISEASE AND OTHER CAUSES OF MORTALITY IN DOUBLE-CRESTED CORMORANTS (PHALACROCORAX AURITUS)

A thesis submitted to the
College of Graduate Studies and Research in
partial fulfillment of the requirements for the
degree of Doctor of Philosophy in the
Department of Veterinary Pathology
University of Saskatchewan
Saskatoon

by

Thijs Kuiken

May 1998

**UNIVERSITY OF SASKATCHEWAN**

College of Graduate Studies and Research

**SUMMARY OF DISSERTATION**

Submitted in partial fulfillment

of the requirements for the

**DEGREE OF DOCTOR OF PHILOSOPHY**

by

**THIJS KUIKEN**

Department of Veterinary Pathology
Western College of Veterinary Medicine
University of Saskatchewan

Fall, 1998

Examining Committee:

| | |
|---|---|
| Dr. L.W. Oliphant | Dean/Associate Dean/Dean's Designate, Chair, College of Graduate Studies and Research |
| Dr. C. Riddell | Chair of Advisory Committee, Department of Veterinary Pathology |
| Dr. F.A. Leighton | Co-supervisor, Department of Veterinary Pathology |
| Dr. G. Wobeser | Co-supervisor, Department of Veterinary Pathology |
| Dr. T.K. Bollinger | Department of Veterinary Pathology |
| Dr. K.A. Hobson | Canadian Wildlife Service, Environment Canada |
| Dr. V. Misra | Department of Veterinary Microbiology |
| External Examiner: | |
| Dr. D.E. Stallknecht | Southeastern Cooperative Wildlife Disease Study<br>College of Veterinary Medicine<br>University of Georgia<br>Athens GA 30606 |

**Newcastle disease and other causes of mortality in double-crested cormorants (*Phalacrocorax auritus*)**

Since 1990, Newcastle disease (ND) caused repeated epidemics in juvenile double-crested cormorants (*Phalacrocorax auritus*; DCC). This disease is important because it may: (1) spread to poultry, (2) spread to wild birds cohabiting with DCC, and (3) modify population dynamics of DCC. This study had three parts:

1. Mortality of DCC in a breeding colony on Doré Lake (Saskatchewan, Canada) was monitored from 1994 to 1996. Birds were observed every third day from inside a tunnel-and-blind system, causing minimal investigator disturbance. The most important causes of mortality, not induced by humans, were ND (21% of hatched chicks in 1995), starvation (4 to 12% per year), and coyote predation (2% in 1994). Newcastle disease only affected juvenile DCC, which had wing and leg paralysis. Affected DCC examined histologically ($n = 25$) had non-suppurative encephalomyelitis, with significantly more ($P < 0.001$) neuronal necrosis, gliosis, perivascular infiltration with mononuclear cells, and endothelial hypertrophy than in control DCC ($n = 18$). Immunohistochemically, Newcastle disease virus (NDV) antigen was limited to central nervous system and kidney, and velogenic NDV was isolated most frequently and in the highest concentration from the kidney. The predicted amino acid sequence of the fusion protein cleavage site was identical to those from previous ND epidemics in DCC, indicating that the same virus has been circulating in DCC since 1990.

2. Twelve 16-week-old captive-raised DCC were infected experimentally with NDV. No birds died, possiby due to age-related resistance. Duration of NDV excretion from the cloaca was $15 \pm 6.2$ days post infection, with a maximum of 28 days post infection, suggesting that DCC populations may maintain velogenic NDV year-round through transmission between susceptible individuals.

3. A reverse transcriptase-polymerase chain reaction test (RT-PCR) was developed to detect NDV in diagnostic samples, as an alternative to virus isolation. The RT-PCR test was performed on allantoic fluid samples containing different strains of hemagglutinating viruses, and on tissues of chickens and DCC infected with NDV. The test cross-reacted with other paramyxovirus strains and with avian influenzavirus strains, and its detection limit was higher than virus isolation, so that it was not considered a suitable alternative.

## BIOGRAPHICAL

| | |
|---|---|
| 1963 | Born in Victoria, Hong Kong |
| 1988 | Doctor of Veterinary Medicine, University of Utrecht, The Netherlands |
| 1989-1990 | Research associate, Central Veterinary Institute, Lelystad, The Netherlands |
| 1990-1993 | U.K. coordinator for marine mammal strandings, Zoological Society of London |

## PUBLICATIONS


Birkun, Jr., A., Kuiken, T., Krivokhizhin, S., Haines, D.M., Osterhaus, A.D.M.E., van de Bildt, M.W.G., Joiris, C.R., Siebert, U. (1998) An epizootic of morbilliviral disease in common dolphins (*Delphimus delphis ponticus*) from the Black Sea. *Veterinary Record*: in press.

Gortel, K., Houston, D.M., Kuiken, T., Fries, C.L., Boisvert, B. (1996) Inherited myopathy in a litter of Labrador retrievers. *Canadian Veterinary Journal* **37**: 108-110.

Jepson, P.D., Brew, S., MacMillan, A.P., Baker, J.R., Barnett, J., Kirkwood, J.K., Kuiken, T., Robinson, I.R., Simpson, V.R. (1997) Antibodies to *Brucella* in marine mammals around the coast of England and Wales. *Veterinary Record* **141**: 513-515.

Kennedy, S., Kuiken, T., Ross, H.M., McAliskey, M., Moffett, D., McNiven, C.M., Carole, M. (1992) Morbillivirus infection in two common porpoises (*Phocoena phocoena*) from the coasts of England and Scotland. *Veterinary Record* **131**: 286-290.

Kirkwood, J.K., Bennett, P.M., Jepson, P.D., Kuiken, T., Simpson, V.R., Baker, J.R. (1997) Entanglement in fishing gear and other causes of death in cetaceans stranded on the coasts of England and Wales. *Veterinary Record* **141**: 94-98.

Kuiken, T. (1985) Influences of diet, gestation and age on haematology and plasma chemistry of the harbour seal, *Phoca vitulina*. *Aquatic Mammals* **11**: 40.

Kuiken, T. (1988) Anaesthesia in the European otter (*Lutra lutra*). *Veterinary Record* **123**: 59.

Kuiken, T. (editor) (1996) Diagnosis of by-catch in cetaceans: proceedings of the second European Cetacean Society workshop on cetacean pathology, Montpellier, France, 2 March 1994. *European Cetacean Society Newsletter* **26**, 43 pp.

...the Double-crested Cormorant..., though unfortunate in some respects, is by no means as unpleasant as it has often been painted, but is actually a reputable avian citizen, not without intelligence, amiability, and interest.

H. F. Lewis, 1929

## PERMISSION TO USE

In presenting this thesis in partial fulfilment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by Dr. F.A. Leighton, Dr. G.A. Wobeser, or, in their absence, by the Head of the Department of Veterinary Pathology or the Dean of the Western College of Veterinary Medicine. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or in part should be addressed to:

Head of the Department of Veterinary Pathology
University of Saskatchewan
52 Campus Drive
Saskatoon, Saskatchewan S7N 5B4

## ABSTRACT

Since 1990, Newcastle disease (ND) caused repeated epidemics in juvenile double-crested cormorants (Phalacrocorax auritus; DCC). This disease is important because it may: (1) spread to poultry, (2) spread to wild birds cohabiting with DCC, and (3) modify population dynamics of DCC. This study had three parts:

1. Mortality of DCC in a breeding colony on Doré Lake (Saskatchewan, Canada) was monitored from 1994 to 1996. Birds were observed every third day from inside a tunnel-and-blind system, causing minimal investigator disturbance. The most important causes of mortality, not induced by humans, were ND (21% of hatched chicks in 1995), starvation (4 to 12% per year), and coyote predation (2% in 1994). Newcastle disease only affected juvenile DCC, which had wing and leg paralysis. Affected DCC examined histologically ($n = 25$) had non-suppurative encephalomyelitis, with significantly more ($P < 0.001$) neuronal necrosis, gliosis, perivascular infiltration with mononuclear cells, and endothelial hypertrophy than in control DCC ($n = 18$). Immunohistochemically, Newcastle disease virus (NDV) antigen was limited to central nervous system and kidney, and velogenic NDV was isolated most frequently and in the highest concentration from the kidney. The predicted amino acid sequence of the fusion protein cleavage site was identical to those from previous ND epidemics in DCC, indicating that the same virus has been circulating in DCC since 1990.

2. Twelve 16-week-old captive-raised DCC were infected experimentally with NDV. No birds died, possiby due to age-related resistance, and duration of NDV excretion from the cloaca was $15 \pm 6.2$ days post infection, with a maximum of 28 days post infection, suggesting that DCC populations may maintain velogenic NDV year-round through transmission between susceptible individuals.

3. A reverse transcriptase-polymerase chain reaction test (RT-PCR) was developed to detect NDV in diagnostic samples, as an alternative to virus isolation. The RT-PCR test was performed on allantoic fluid samples containing different strains of hemagglutinating viruses, and on tissues of chickens and DCC infected with NDV. The test cross-reacted with other paramyxovirus strains and with avian influenza virus strains, and its detection limit was higher than virus isolation, so that it was not considered a suitable alternative.

## ACKNOWLEDGMENTS

Ted Leighton and Gary Wobeser were the two main reasons I came to Saskatchewan. I could not have wished for better supervisors. Annemie, my wife, interrupted her career to come with me and let me do this project. Sandra Honour was a great support, especially in my first years here. Barbara Hanbidge, who had studied cormorants on Doré Lake before me, gave me invaluable advice. Ferdie Edquist and Rod Laliberté shared freely their intimate knowledge of Doré Lake and her treacherous reefs. My advisory committee, Drs Trent Bollinger, Keith Hobson, Vikram Misra, and Craig Riddell, helped me whenever I requested it.

The research for this thesis was done mainly in Saskatoon, at the Western College of Veterinary Medicine, in Hull, at the Virology Section of the Animal Diseases Research Institute, and on DoréLake, where I was assisted by the local office of Saskatchewan Environment and Resource Management (SERM). Many people in those places helped me with my work, and I am grateful to them. In particular, I would like to thank: Ian Shirley, Kathie Caspell, Lois Ridgway, and Jan Diederichs in the Department of Veterinary Pathology, Debbie Haines, Brian Chelack, Lori Hassard, Bob Norman, Jaret Bogdan, Brent Wagner and Lydden Polley in the Department of Veterinary Microbiology, Juliane Deubner in the Department of Veterinary Anatomy, Colette Wheler in the Department of Veterinary Internal Medicine, Ed Cowal and Glenn Honing of SERM, and Ahmad Afshar, Rob Heckert, Martha Harding, Seeven Vydelingum, José Riva, and André Laflamme in the Virology Section of the Animal Diseases Research Institute.

This project was supported by the Canadian Cooperative Wildlife Health Center, Canadian Wildlife Service (Environment Canada), Alberta Sports, Recreation, Parks and Wildlife Foundation, University of Saskatchewan, Western College of Veterinary Medicine Wildlife Health Fund, SERM, Canadian Food Inspection Agency, and Animal Health Trust of Canada.

TO HEIT AND MEM

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AIV | Avian influenza virus |
| BHI | Bovine heart infusion |
| DCC | Double-crested cormorant |
| $EID_{50}$ | Embryo infectious dose 50%: virus concentration, expressed as the negative exponent of the dilution at which 50% of the embryos were infected |
| $ELD_{50}$ | Embryo lethal dose 50%: virus concentration, expressed as the negative exponent of the dilution at which 50% of the embryos died |
| F | Fusion |
| H & E | Hematoxylin and eosin |
| HA | Hemagglutination |
| HI | Hemagglutination inhibition |
| ICPI | Intracerebral pathogenicity index |
| IPA | Immunoperoxidase assay |
| IVPI | Intravenous pathogenicity index |
| MEM | Earle's minimum essential medium supplemented with 20% fetal bovine serum and 50 µg/ml gentamycin |
| ND | Newcastle disease |
| NDE | Newcastle disease epidemic |
| NDV | Newcastle disease virus |
| p.i. | Post infection or post exposure |
| PBS | 0.01 M phospate-buffered saline solution (pH 7.4) |
| PBST | PBS containing 0.05% Tween |
| PCR | Polymerase chain reaction |
| PMV-1 | Paramyxovirus serotype 1 |
| RPA | Rapid plate agglutination test |
| RT | Reverse transcriptase |
| SPF | Specific-pathogen-free |
| TAB | Tunnel-and-blind |

# 1. GENERAL INTRODUCTION[1]

## 1.1 Introduction

Newcastle disease (ND) caused high mortality of juvenile double-crested cormorants (Phalacrocorax auritus; DCC) in multiple breeding colonies across western Canada in 1990 (Wobeser et al., 1993). In 1992, a more wide-spread ND epidemic in DCC occurred, encompassing breeding colonies in western Canada, the Great Lakes area, and north-central USA (Roffe, 1992; Heckert, 1993). These occurrences were the first time that this disease was reported to have caused high mortality in wild birds, although infections with mildly pathogenic strains of the causative virus are common (Spalatin and Hanson, 1975). The source, epidemiology, and pathogenesis of the virus in DCC were unknown.

As the global human population and its impact on the environment increases, there is more interest in studying factors affecting natural ecosystems (Vitousek, 1992), including infectious diseases of wild animal populations (Gulland, 1995). Concerns about wildlife diseases include: (1) threat of extinction of the host species, e.g. canine distemper in black-footed ferrets (Mustela nigripes; Thorne and Williams, 1988), (2) spread of disease from wildlife to humans, e.g. rabies in raccoons (Procyon lotor; Coyne et al., 1989), (3) spread of disease from wildlife to domestic animals, e.g. brucellosis in bison (Bison bison; Meyer and Meagher, 1995), and (4) disease as an indication of environmental pollution, e.g. phocine distemper in harbour seals (Phoca vitulina; Ross et al., 1995).

The presence of ND in DCC has generated considerable interest because of its potential to: (1) spread to domestic poultry in the USA and Canada, which currently are

---

[1]A version of this chapter, combined with a version of chapter 9, was submitted for publication as Kuiken (1998).

free of the highly virulent form of the disease (Mixson and Pearson, 1992; Office International des Épizooties, 1997), (2) spread to wild birds cohabiting with DCC, including relatively uncommon species such as American white pelicans (Pelecanus erythrorhynchos) (Wobeser et al., 1993), and (3) modify population dynamics of DCC (Nisbet, 1995).

This study was initiated to increase knowledge of pathogenesis and epidemiology of ND in DCC, and to improve diagnosis of ND in this species. This information should help to assess, and possibly reduce, the risk of transmission of ND from DCC to other species, and to estimate the effect of ND on population dynamics of DCC.

## 1.2 Newcastle disease

This section is based mainly on the review of ND in domestic chickens by Alexander (1997), unless indicated otherwise. Newcastle disease virus (NDV) is infectious for virtually all bird species, including domestic poultry (Kaleta and Baldauf, 1988). Newcastle disease, caused by this virus, is an important disease for the poultry industry because of devastating epizootics it causes and because of far-reaching effects on trade in poultry products. For example, the cost of eradication of ND from California in the early 1970s was over 20 million U.S. dollars (Walker et al., 1973). Currently, the domestic poultry of Canada and the USA are free of highly virulent ND (Office International des Épizooties, 1997).

Newcastle disease virus, also called avian paramyxovirus serotype 1 (PMV-1), is one of nine serotypes of avian paramyxoviruses which constitute the genus Rubulavirus of the family Paramyxoviridae. The genome of NDV is a single strand of RNA of negative sense, i.e. complementary to the messenger RNA which codes for virus proteins. This RNA genome codes for six proteins: (1) large protein, which is a RNA-directed RNA polymerase, (2) hemagglutinin-neuraminidase protein, responsible for hemagglutination and for cleavage of neuraminic acid residues from glycoproteins and lipids, (3) fusion (F) protein, responsible for fusion of virus and host membranes, (4) nucleocapsid protein, a major structural component of the nucleocapsid, (5)

phosphorylated, nucleocapsid-associated protein, which has a role in transcription and replication of the virus, and (6) matrix protein, which organizes virus assembly and moderates transcription. Virions of NDV are more or less spherical, and consist of a long helical nucleocapsid structure surrounded by an envelope covered by spike glyco-proteins (hemagglutinin-neuraminidase and F proteins; Samson, 1988).

Avian paramyxoviruses are grouped into nine so-called serotypes on the basis of their antigenic relatedness in hemagglutination inhibition (HI) tests using polyclonal antisera specific for the prototype strain of a given serotype. Newcastle disease virus, belonging to serotype 1, cross-reacts with several other avian paramyxovirus serotypes, particularly PMV-3 isolates from psittacines. The possibility of cross-reaction can be eliminated by the use of monoclonal antisera. Of the nine serotypes, NDV is the most important pathogen for poultry, but PMV-2 and PMV-3 can also cause severe disease. Except for PMV-5 and PMV-9, all serotypes have been found in wild ducks and geese (Stallknecht et al., 1991).

Newcastle disease virus isolates may be classified according to their pathogenicity as highly virulent (velogenic), moderately virulent (mesogenic), mildly virulent (lentogenic), or asymptomatic, based on the severity of disease caused in chickens and chicken embryos. The most widely used tests to determine pathogenicity are those to determine the intracerebral pathogenicity index (ICPI) in 1-day-old chicks and the intravenous pathogenicity index (IVPI) in 6-week-old chickens: (1) asymptomatic isolates have $0.0 \leq \text{ICPI} \leq 0.2$ and $\text{IVPI} = 0.0$, (2) lentogenic isolates have $0.2 \leq \text{ICPI} \leq 0.5$ and $\text{IVPI} = 0.0$, (3) mesogenic isolates have $1.0 \leq \text{ICPI} \leq 1.5$ and $0.0 \leq \text{IVPI} \leq 0.5$, and (4) velogenic isolates have $1.5 \leq \text{ICPI} \leq 2.0$ and $2.0 \leq \text{IVPI} \leq 3.0$ (Alexander, 1989).

The pathogenicity of a NDV isolate is determined largely by the amino acid structure of the F protein. Before a NDV virion can be infective, the precursor ($F_0$) protein has to be split into two smaller segments, the $F_1$ and $F_2$ proteins, by enzymes of the host cell. The location on the $F_0$ protein where this occurs is called the cleavage site. Lentogenic isolates have $F_0$ proteins with only one basic amino acid, arginine, at the cleavage site, and can be cleaved only by trypsin-like enzymes. The distribution of these enzymes in the host organism is restricted, so that infection with these isolates remains

localized to areas such as the intestinal tract. In contrast, mesogenic and velogenic isolates have $F_0$ proteins with two basic amino acids at the cleavage site and can be cleaved by an enzyme or enzymes with a much wider distribution in the host organism. Therefore, such isolates can produce systemic infection and lead to death.

Transmission of NDV may take place by inhalation of infected aerosol or by ingestion of infected excreta; the latter is probably the main method in the pigeon variant of NDV and in avirulent enteric NDV, neither of which normally produces respiratory signs. The length of time that NDV remains infective outside the host depends on a number of environmental factors including nature of suspending medium, temperature, exposure to light, and humidity. Temperature is an important factor; NDV loses its infectivity within 5 minutes at 60 C, but may remain infective up to 10 years if kept at -20 C (Lancaster, 1966, p. 43). Olesiuk (1951) tested the infectivity of NDV when seeded on various materials and under a variety of temperature regimes. She found that the duration of infectivity decreased at temperatures of 20 C and higher, with a marked decrease above 37 C. Newcastle disease virus remained infective for 255 days when left in a hen house, where the temperature varied from -11 to 36 C. She thought it likely that NDV could survive in the natural environment from one season to the next.

The age of the bird is an important variable in the morbidity and mortality caused by NDV infection. In general, the younger the bird, the more severe the disease. Another important factor is immune status. In general, antibody will prevent the virus from growing in the visceral organs and will keep the bird clinically healthy. However, it will not prevent virus replication at mucosal surfaces and therefore the virus can still be excreted by immune but infected birds (McFerran and McCracken, 1988). The most commonly used method to detect antibody to NDV in serum or yolk is the hemagglutination inhibition (HI) test. Hemagglutination inhibiting antibodies are usually detectable in the serum within 6 to 10 days after infection, and generally peak at about 3 to 4 weeks. Hemagglutination inhibiting antibodies may remain detectable for up to 1 year after infection.

The average time between entrance of NDV and appearance of clinical symptoms is 5 to 6 days, and fecal excretion of mesogenic strains of NDV lasts from 15 to 21 days

(Lancaster, 1966). After review of experimental and field data, Lancaster (1966) concluded that a permanent carrier state in ND was rare in chickens. However, in other species, chronic excretion has been found, with a maximum of more than 1 year in yellow-headed Amazon parrots (Amazona ochrocephala oratrix) infected with velogenic NDV (Erickson et al., 1977). Macpherson (1956) isolated NDV from fecal samples of two juvenile great cormorants (Phalacrocorax carbo) 1 month after infection.

The clinical signs, gross lesions, and histological lesions produced by virulent strains of NDV are highly variable, and there are no pathognomonic lesions associated with any form of ND. Clinical signs may include respiratory distress, diarrhea, and signs of nervous disease. Gross lesions may be absent or include edema around the eyes, and hemorrhage in the trachea and gastro-intestinal tract. Histological lesions may include non-suppurative encephalomyelitis, hemorrhages and necrotic foci in multiple tissues, and lymphocyte depletion in lymphoid organs.

Currently, the only unequivocal method of ND diagnosis is virus isolation. Because live vaccines, based on lentogenic and mesogenic strains, are commonly used in the poultry industry, and migratory birds in North America are regularly infected by lentogenic strains (Spalatin and Hanson, 1975), mere identification of NDV infection without determination of the pathogenicity of the isolate is not sufficient for control measures to be imposed. Only infections with the more virulent strains of NDV are reportable diseases in Canada (Heckert et al., 1996) and the USA (United States Department of Agriculture, 1996). For Canada, these are NDV isolates with an ICPI > 0.7 (Commission of the European Communities, 1993); for the USA, these are NDV isolates with an ICPI of 1.5 to 2.0 and an IVPI > 2.0 (Alexander, 1989).Virus isolation is usually done by inoculation of diagnostic specimens into embryonated chicken eggs, a labour-intensive method which may take several weeks to complete.

In recent years, more rapid techniques, based on the polymerase chain reaction (PCR), have been developed for the diagnosis of viral infections. The PCR is a method used for the in vitro amplificaton of selected targeted DNA molecules, resulting in a several-million-fold amplification of the target sequence within a few hours.These large amounts can then easily be detected and identified. In the case of RNA viruses, such as

NDV, the viral RNA has to be purified and transcribed to complementary DNA by use of the enzyme reverse transcriptase (RT) before starting the PCR. The specificity of the test, i.e., its characteristic of only reacting positively with the desired DNA sequence, depends on the design of the primers. The sensitivity of the test, i.e., the lowest concentration of agent in the sample it can detect, depends on how many times the target sequence has been amplified. A variety of parameters can affect the reaction kinetics and the success of amplification. The advantages of PCR-based methods to diagnose viral infections are that it is sensitive, specific, rapid and cheap in comparison to conventional virus isolation. The main reason that it is not used more widely in routine diagnosis of viral diseases is the high risk of contamination of samples with amplified DNA from previous experiments, leading to false-positive results (Bélak and Ballagi-Pordány, 1993; Pfeffer et al., 1995). Reverse transcriptase-PCR tests have been developed for the detection of NDV in allantoic fluid (Jestin and Jestin, 1991) and in vaccines (Stäuber et al., 1995), but their performance with diagnostic specimens has not been tested.

The epidemiology of ND in DCC is unknown, but in some ways it may be similar to the epidemiology of ND in wild waterfowl and village chickens. In a study of wild ducks wintering in Louisiana, USA, Stallknecht et al. (1991) observed a seasonal decrease in the prevalence of paramyxovirus infection, possibly related to increased flock immunity. This was supported by the observed differences between prevalence in juveniles (4%) and adults (<1%). They could not determine whether viruses persisted in the population through a cycle of transmission between susceptible individuals or through persistent infections in individual birds. A computer model of the interaction between velogenic NDV and a flock of village chickens–which resembles a population of wild birds in many respects–indicated that the virus would only persist in a population of 500 birds or more, and that discernible waves of infection with high mortality occurred every 1.5 to 2 years due to an increased proportion of young, susceptible birds. In between outbreaks, the mortality level was very low and difficult to detect (Spradbrow, 1993/1994).

## 1.3 The double-crested cormorant

This section is based on a review by Johnsgard (1993), except where indicated otherwise. The DCC is a fish-eating bird, which breeds in colonies of a few to many thousands of pairs. Adults have black feathers, maxilla, and legs, yellow to orange mandible and gular pouch, and emerald-green eyes. The common and scientific name is derived from the white to black tufts of feathers on the side of the head in the prebreeding plumage. The DCC is the most wide-spread of six species of cormorant present in North America, and the only one that breeds inland. It belongs to the order Pelecaniformes, which traditionally includes cormorants and shags (Phalacrocoridae), darters (Anhingidae), pelicans (Pelecanidae), gannets and boobies (Sulidae), frigatebirds (Fregatidae), and tropicbirds (Phaethontidae), although there is currently debate about this classification.

Double-crested cormorants breed in 40 of the 50 states of the United States, all 10 Canadian provinces, and in Mexico, Cuba, and the Bahamas. There are six more-or-less distinct populations: (1) the Atlantic population on the northeast coast (96,000 breeding pairs), (2) the Interior population centred in the Canadian Prairie Provinces and Great Lakes (220,000 breeding pairs), (3) the Florida and Caribbean population (14,000 breeding pairs), (4) the San Salvador population (212 breeding pairs), (5) the Alaska population (3,000 breeding pairs), and (6) the West Coast population (31,000 breeding pairs). There is little intermixing between populations east and west of the Rocky Mountains, although some birds banded in Alberta have been recovered west of the Rocky Mountains. The migratory Interior and Atlantic populations overlap extensively with each other in their winter range in Florida and the Gulf of Mexico, and with the resident Florida and Caribbean populations (Hatch, 1995).

Double-crested cormorants often nest together with a variety of other birds: alcids (Alcidae) including common murre (Uria aalge), black-legged kittiwake (Rissa tridactyla), anhinga (Anhinga anhinga), great cormorant, Brandt's cormorant (P. penicillatus), pelagic cormorant (P. pelagicus), red-faced cormorant (P. urile), American white pelican, great blue heron (Ardea herodias), great egret (Ardea alba), cattle egret

(Bubulcus ibis), American crow (Corvus brachyrhynchos), black-crowned night-heron (Nycticorax nycticorax), common grackle (Quiscalus quiscula), common eider (Somateria mollissima), gulls (Larus spp.), and terns (Sterna spp.; Lewis, 1929; Mendall, 1936; Hanbidge, 1989; Hatch, 1995). They also have close contact with bird species that scavenge and prey on their breeding colony sites: ring-billed (Larus delawarensis), California (L. californicus), herring (L. argentatus), and great black-backed gulls (L. marinus), American crow, fish crow (C. ossifragus), northwestern crow (C. caurinus), common raven (C. corax), and bald eagle (Haliaeetus leucocephalus).

Nesting sites consist of sandy or rocky areas along islands, cliffs, reefs, or other water-lined sites, or of trees or towers standing in or near water, such as swamps, coastal woodlands, or tree-lined lakes. The breeding season of the DCC is highly variable across its geographic range, with egg laying beginning in March in Florida and Baja California and delayed seasonally northward, as late as June in Alaska. The normal clutch size ranges from three to seven eggs, which are incubated for an average of 28 days. Young are hatched naked and blind, and are fed by regurgitation of fish remains from the stomach of the parents. The eyes of the nestlings open after 3 to 5 days, and the body is mostly covered with black woolly down by about 2 weeks. The young begin to leave their nests at 3 to 4 weeks, and are able to fly at 5 to 6 weeks. Independence from the parents occurs when the young are about 9 to 10 weeks of age.

For the past 20 years, numbers of breeding birds have been increasing rapidly, particularly in the Interior and Atlantic populations. This increase is attributed to a combination of factors in the breeding range: reduced human persecution, declining levels of organochlorine contaminants, and abundance of prey fish following decimation of predatory fish. In the wintering range, the replacement of natural wetlands by catfish culture ponds in the southern USA may have improved winter survival by increased food availability (Weseloh and Ewins, 1994; Weseloh et al., 1995).

### 1.4 Newcastle disease in cormorants from 1897 to 1992

From 1897 to 1898 there was extensive mortality of domestic poultry in the

Western Isles of Scotland, recorded in the Gaelic poem "Call nan cearc" (The loss of the hens). Although no reference was made to ND in any reports of this die-off, Macpherson (1956) argued that it was caused by ND contracted from great cormorants and/or European shags (Phalacrocorax aristotelis). Macpherson made little distinction between these two species in his article and referred to both as "cormorants." His conclusions were based on: (1) the similarity of the geographical distribution of affected poultry flocks between the 1897-98 die-off and the 1949-51 ND epidemic (see below), (2) the symptoms of respiratory and nervous disease in affected poultry suggestive of ND, and (3) the lack of mortality in ducks, which are more resistant to ND than chickens. Three years previously, in the winter of 1893-94, there was high mortality of European shags in the Orkneys. Many birds lost all fear of humans and even entered poultry- and cow-houses, where some were so weak that they were eaten alive by rats (Rattus norvegicus). The cause of this mortality was not determined (Robinson, 1913), but it has some similarities to clinical signs seen in DCC with ND (Wobeser et al., 1993). Potts (1969) attributed this and later mortality events–which he called eruptions–in European shags to food shortage.

From 1949 to 1951 there was an epidemic of ND in domestic poultry in Scotland, mainly along the coast of the mainland, and on the Hebridean and Orkney Isles (Macpherson, 1956). This epidemic spread to Northern Ireland in 1949 and to the south-west of the Republic of Ireland in 1950 (Anonymous, 1949). These outbreaks were linked to feeding offal from European shags and great cormorants, shot for human consumption, to poultry (Macpherson, 1956). NDV was isolated from the bone marrow of some of the European shags shot by the owner of the poultry in one of the outbreaks in Scotland in 1949, and from six apparently healthy European shags shot off the coast of two Hebridean islands in November and December 1949. Pathogenicity indices of these strains were not determined, but all six killed 4- to 5-day-old chicks 3 to 5 days after infection, indicating high pathogenicity. Newcastle disease virus was not isolated from great cormorants, although at least one apparently healthy bird shot at the same time and place had an HI titre to NDV of 1:320, indicating recent infection (Blaxland, 1951). Newcastle disease virus also was isolated from a northern gannet (Morus bassanus),

which was found on a beach on one of the Orkney Isles, and which had a severe nephritis and subcapsular renal petechiae. Pathogenicity indices were not determined, but the isolate caused hemorrhagic laryngitis and tracheitis, proventricular hemorrhage, and death of chickens 6 to 9 days after infection (Wilson, 1950). Gannets also were shot by local people for human consumption (Macpherson, 1956).

Except for the lesions in the one northern gannet, there was no evidence that NDV caused morbidity or mortality in free-living seabirds. However, there was only one reported visit to a seabird breeding colony to look for signs of disease, and this visit probably took place outside the breeding season. A veterinarian and an ornithologist visited colonies of 2.5 million seabirds on the Saltee Islands, which were considered a possible source of NDV infection for two poultry flocks on the nearby coast of south-west Ireland, but found no evidence for the presence of infection (Anonymous, 1949).

There was no agreement about how great cormorants or European shags had become infected with NDV. According to Blaxland (1951), the most likely explanation was that they were infected by contact with gulls that had fed on NDV-infected poultry carcasses and viscera thrown overboard by passing ships, or that great cormorants and European shags might have mistaken floating intestines for fish and become infected by ingesting them. In contrast, Macpherson (1956) suggested that great cormorants or European shags might be the primary reservoir of NDV, because the low mortality and comparative mildness of the disease indicated a long-established biological adaptation of NDV to these birds. He considered Blaxland's explanations unlikely, because there was no import of poultry or poultry carcasses at some of the islands where ND had occurred, and because his experience with feeding captive great cormorants indicated that they were purely fish-eaters.

In June 1974, NDV was isolated from 3 of 56 tissue pools collected from 111 great cormorant nestlings on two islands in the Volga delta (Astrakhan region, Russia). No clinical signs of ND were seen in great cormorants or cohabiting species, nor was the pathogenicity of the isolates determined (Lvov et al., 1975).

In 1975, several DCC with ocular opacity, partial paralysis, and extreme weakness were observed on Île Gros Pèlerin, St Lawrence Estuary (Quebec, Canada). Newcastle

disease virus was isolated from 14 of 27 (52%) birds collected before August (Cleary, 1977). At the same time, NDV was isolated from juvenile DCC–also from this island–that had been transported to Quebec City Zoo and had developed signs of ND while in quarantine. (E. Broughton, pers. comm.; Reeker, 1975). The virus isolates were characterized as velogenic (Reeker, 1975; Heckert et al., 1996).

In 1976, sera of three of 27 adult DCC and zero of eight juvenile DCC from Île Gros Pèlerin had HI antibody to NDV, but NDV was not isolated (Cleary, 1977). From 1976 to 1979, five to six nests on each of four DCC colony sites in the St Lawrence Estuary (Île Gros Pèlerin, Île Verte, and Grand and Petite Île aux Pommes) were marked. The colonies were visited four times each breeding season to check for clinical signs of ND in DCC, blood samples from the chicks in the marked nests were collected to test for HI antibody to NDV, and one chick per marked nest was killed and submitted for virus isolation. One serum sample from 1976 had a low level of HI antibody to NDV. No DCC with clinical signs of ND were observed, nor was NDV isolated from any of the chicks. (E. Broughton, pers. comm.).

In 1990, extensive mortality of DCC, American white pelicans and gulls (Larus spp.) was observed on at least ten different lakes in Alberta, Saskatchewan and Manitoba (Canada). The major clinical sign in DCC was inability to fly, often with unilateral wing or leg paralysis. Focal non-suppurative inflammation was present in the brain and spinal cord of DCC and American white pelicans. Newcastle disease virus was isolated from DCC, an American white pelican, and a ring-billed gull from Saskatchewan, and DCC from Alberta were positive for NDV in an immunofluorescence test. The virus isolates were classified as velogenic or mesogenic. The overall extent of mortality and the source of the virus were not determined. It was speculated that the affected species might have been exposed to NDV during the winter and brought it north during migration, or that NDV infection was endemic among waterbirds (Wobeser et al., 1993).

In 1992, ND again caused mortality in DCC. The epidemic was more widespread than in 1990, and involved western Canada, the Great Lakes area, and the north-central states of the USA (Roffe, 1992; Heckert, 1993). The disease affected only juvenile DCC, with similar clinical signs and lesions as in 1990 and an estimated mortality of 10 to 90%

(Meteyer et al., 1997). The epidemic was associated with unusually high mortality in pelicans and gulls, but attempts to isolate NDV from these species failed (Roffe, 1992). Newcastle disease was diagnosed in a flock of domestic range turkeys located near a DCC colony affected by ND on Devils Lake, North Dakota (Mixson and Pearson 1992). Velogenic NDV was isolated from both turkeys and DCC and the isolates were considered identical by monoclonal antibody typing and nucleotide sequence analysis, supporting the belief that the virus was transmitted from the DCC to the turkeys (Heckert et al., 1996).

The NDV isolates from different geographical areas of the ND outbreaks in DCC in 1990 and 1992 were similar by the predicted amino acid sequence of parts of the fusion gene and matrix gene, suggesting that DCC from widely distant breeding colonies were infected by viruses from a similar, very stable NDV population, possibly in the common wintering range (Seal et al., 1995; Heckert et al., 1996). On the basis of the predicted amino acid sequence of the fusion protein cleavage site and the matrix protein, Seal et al. (1995) and Seal (1996) considered NDV isolates from turkeys and DCC in the USA collected in 1992 to be most closely related to NDV isolates from psittacines and domestic poultry in the USA collected in the early 1970s, and suggested that these virus types from 1992 were introduced from psittacine sources outside the USA or were circulating in wild and pet bird populations in North America.

### 1.5 Objectives

Based on the above literature review, the objectives of this study were to: (1) describe aspects of the pathogenesis, epidemiology and immunology of ND in DCC, (2) characterize the histological lesions of ND in DCC, (3) develop a PCR method to identify NDV in the tissues of DCC and other birds, (4) determine the occurrence of NDV infection in wild birds sharing habitat with DCC, and (5) estimate the relative importance of ND as a mortality factor for DCC.

This study can be divided into three parts. The first part was a 3-year-long field study of a DCC breeding colony. A tunnel-and-blind system was developed to be able to

observe and sample the birds on the colony site with minimal disturbance (Chapter 2). In the second year of the field study a ND epidemic occurred. The clinical signs and lesions of DCC with ND were described, and the level of HI antibody to NDV in yolk of DCC eggs and serum of juvenile DCC was compared between the year when ND occurred and the years preceding and following the epidemic. Other wild birds that died during the ND outbreak were tested for the presence of NDV infection (Chapters 3 and 4). Other causes of DCC mortality during the breeding season were characterized (Chapter 7), and their effect on DCC reproductive success was compared with that of ND (Chapter 8).

The second part was an experimental infection of DCC with NDV (Chapter 5). Recently-hatched DCC were raised in captivity and infected with a NDV isolate from the above epidemic at 4 months of age. Morbidity, mortality, immune response and virus excretion were monitored for 10 weeks after infection.

The third part was the development of a PCR test to detect NDV in tissues of DCC and other birds (Chapter 6). A reverse transcriptase-PCR test, with primers spanning the nucleotide sequence coding for the fusion protein cleavage site, was tested on allantoic fluid samples containing different embryo-lethal hemagglutinating viruses, and on tissues from DCC and chickens infected with NDV.

# 2. A TUNNEL-AND-BLIND SYSTEM TO REDUCE INVESTIGATOR DISTURBANCE OF COLONIAL WATERBIRDS[1]

## 2.1 Introduction

Traditionally, investigators have employed blinds for observing colonial waterbirds (e.g., Lewis, 1929; Mendall, 1936) and tunnels have been used to reduce the disturbance caused by entering and leaving blinds. Munro and Bédard (1977) used three cabins connected by 70 m of tarpaulin-covered tunnel to study common eiders (Somateria mollissima); Shugart et al. (1981) used a 90-m-long plastic-covered tunnel to reach two blinds, one adjacent to a Caspian tern (Sterna caspia) colony, the other to a herring gull (Larus argentatus) colony; and Cairns et al. (1987) used a Fabrene-covered tunnel to reach a blind in a northern gannet (Morus bassanus) colony.

I describe the construction and use of an above-ground tunnel to reach multiple blinds in a colony of double-crested cormorants (Phalacrocorax auritus; DCC) and American white pelicans (Pelecanus erythrorhynchos), and of a light-weight telescopic rod with attachments to secure specimens without having to leave the tunnel or blinds.

## 2.2 Materials and methods

The study site (Fig. 2.1) was a 300 x 100 m island (Island A) in Bazill Bay, Doré Lake (Saskatchewan, Canada; 54°46'N 107°17'W). During 1994-96, about 3000 pairs of DCC and 500 pairs of American white pelicans nested on the island. Most DCC and all pelicans nested on the ground.

---

[1]A version of this chapter was published as Kuiken et al. (1997).

**Figure 2.1 Aerial view of the tunnel-and-blind system on Island A at Doré Lake, June 1996.**

The tunnel design was modified from that of Cairns et al. (1987). I used a combination of steel and wood for the tunnel frames and divided the tunnel into 3.5-m-long modules, which could be attached to each other or to a blind as required, while Cairns et al. (1987) used wooden frames and built the tunnel in one piece.

Tunnel modules consisted of a sheet of 200 g $m^{-2}$ woven polyethylene fabric (Inland Plastics, Saskatoon, Canada), black on one side and silver on the reverse, stretched between two frames. The polyethylene was cut into a 3.5 x 2.82 m sheet, with a 5-cm-wide fold along each long side. Each fold was sewn into a channel, through which a 6-mm-diameter polypropylene rope was threaded. The rope exited the channel through a copper grommet near each end of the sheet. Frames were made of a 284 x 3.2 x 0.3 cm steel band bent into the appropriate shape and fastened with screws to a 4.5 x 4.5 x 104 cm wooden bar (Fig. 2.2). One short side of the polyethylene sheet was wrapped around the steel band and pressed between the band and the side and top bars, which were then screwed to the band (Fig. 2.2). The same procedure was followed for the other short side of the polyethylene sheet. The sheet was placed with the black side to the exterior of the tunnel.

Blinds were 0.95-cm-thick plywood boxes measuring 1.5 x 1.2 x 0.9 m. Blinds were painted (white) to increase weather resistance. One side panel in each blind had a 80 x 60 cm hinged door for access onto the colony site. Both side panels had a 10 x 40 cm hinged window. The front and back panel each had an opening, shaped like the cross-section of the tunnel, but slightly smaller.

A tunnel module was connected to a blind by placing one tunnel frame inside the opening of a front or back panel of a blind and pulling the tunnel module tight, so that the frame jammed against the inside of the opening (Fig. 2.3). Tunnel modules were attached one to another with nuts and bolts through the top and bottom corners of adjacent tunnel frames (Figs. 2.2 and 2.3). Once the tunnel-and-blind (TAB) system was in place, the ropes sewn into the bottom edges of the tunnel modules were pulled taut and tied to the bottom of the tunnel frames (Fig. 2.3).

The TAB system was anchored by 6-mm-diameter polypropylene ropes, attached from the top of the tunnel frames and the top corners of the blinds to large standing and

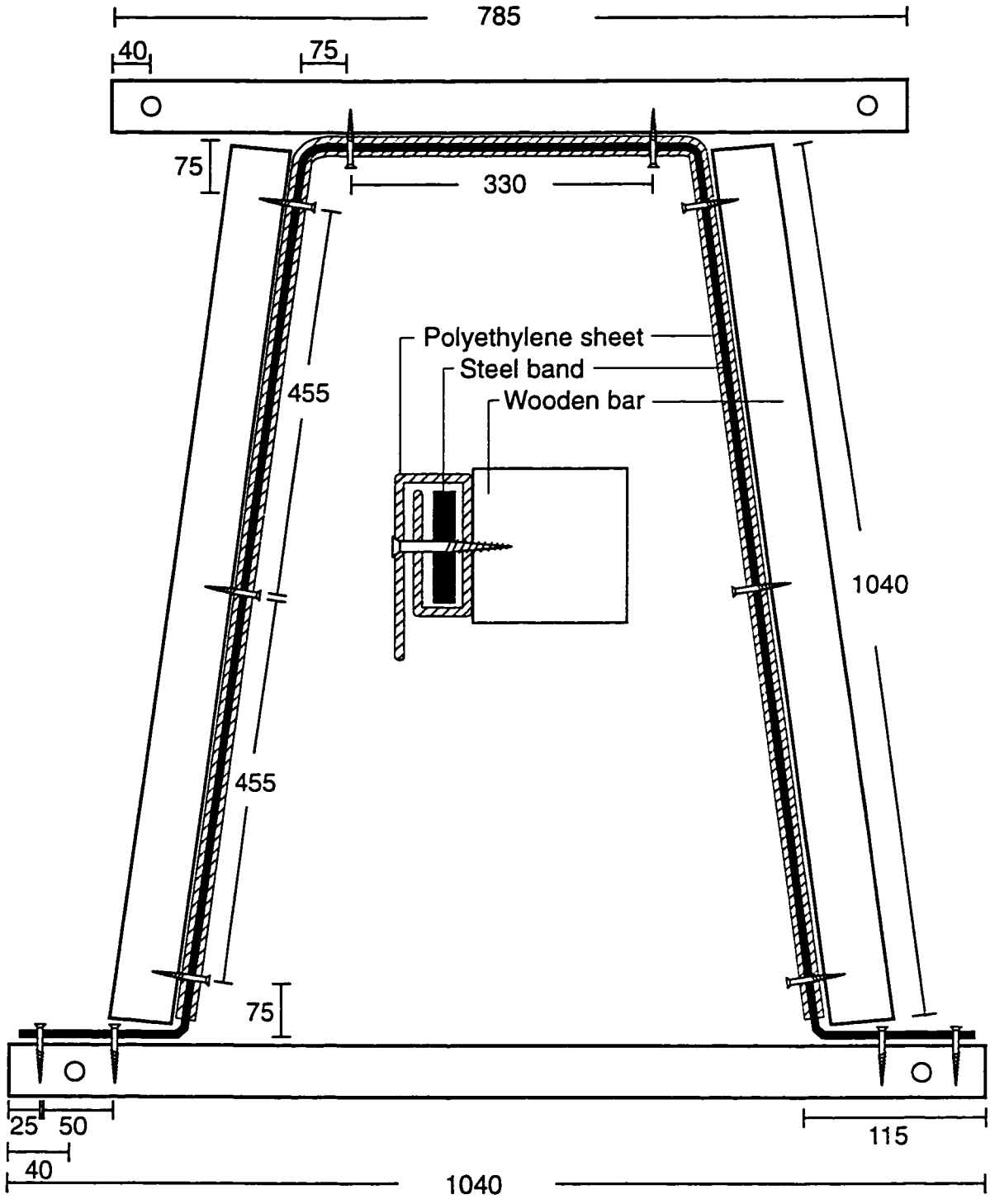



**Figure 2.2 Front view of the frame for the tunnel module. The inset is a cross-section of the frame to show how the polyethylene sheet was wrapped around the steel band and pressed between the band and the wooden bar by use of screws. All dimensions are in mm.**

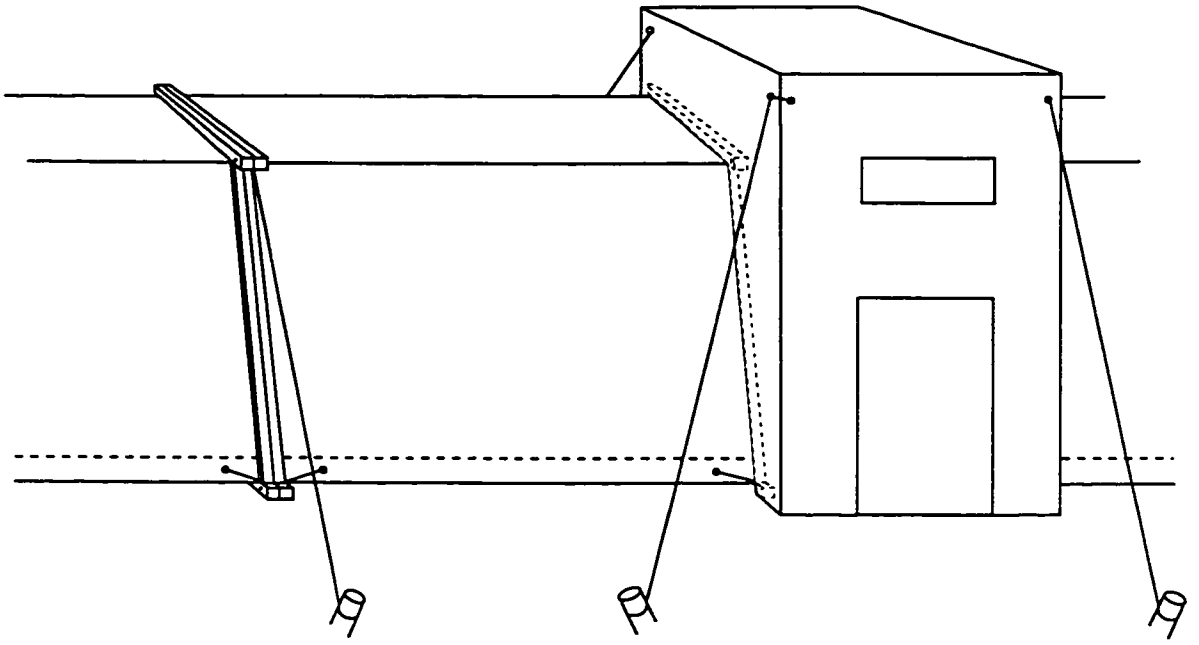


**Figure 2.3 Oblique view of the tunnel-and-blind system, showing how tunnel modules were attached to each other and to a blind.**

fallen trees, to stakes made from 1 x 25 cm steel bars, or to boulders. In addition, large stones were placed on the roof of some blinds.

The tunnel entrance was at the water's edge, the subsequent 10 m of tunnel was on a gentle sandy slope, and the remainder of the TAB system was on level ground with a peat substrate. The main TAB system (Fig. 2.1), about 72 m long, consisted of seven blinds, with six tunnel modules between the tunnel entrance and the first blind, and two or three tunnel modules between blinds. There was an 8-m-long side branch that consisted of two tunnel modules with a terminal blind from the side door of a blind in the main TAB system.

The TAB system was erected each spring from 1994-96, before DCC or pelicans were present on the colony site. About 3.5 hours of work by each of seven people was required to establish the TAB system; of this, 1 hour was spent clearing snow from the site and 2.5 hours was needed to erect the system. The TAB system was taken down each autumn, after most DCC and pelicans had left the colony site, and stored on Island A covered with plastic sheets.

From about 1 June to 1 September each year, I canoed to the tunnel entrance every third day and remained in the TAB system for about 6 hours per visit. Eggs and birds, both live and dead, were collected without leaving the TAB system during these visits. Specimens within about 50 cm of the TAB system were taken by hand; for specimens further away, a telescopic rod with attachments was used. The 500-g telescopic carbon-fibre fishing rod (Stippy Gildehengel, Sneek, The Netherlands) had five sections and could be elongated from 115 to 600 cm. The rod was extended through a narrow space in a door or window of a blind, or under the bottom edge of the tunnel. Depending on the specimen to be retrieved, one of three attachments was fastened with pipe clamps to the tip of the fishing rod: eggs were retrieved with a 10-cm-diameter aquarium net; bird carcasses were impaled on a 5-cm-long barbed fishing hook; and live DCC were caught around the tarsometarsus by use of a 12-cm-long blunt hook with a 1.25 cm inside diameter, made from 3-mm-diameter aluminum rod. In this way, specimens up to about 6 m from the TAB system could be collected.

## 2.3 Results

Few adjustments to the TAB system were necessary after it was set up. Wind blew down the first tunnel module once; this was solved by tying it to large stones dug into the sand instead of to stakes. A dead tree fell on a tunnel frame and ripped the polyethylene; this was repaired from inside the tunnel using duct tape. The blinds were repainted once, but no other maintenance or repair was required in three years of use.

Cormorants and pelicans within about 20 m of the tunnel entrance left the area for a few minutes after each arrival and departure of the investigator by canoe. Eggs and chicks in the temporarily abandoned nests were killed by herring, California (L. californicus), and ring-billed (L. delawarensis) gulls and by ravens (Corvus corax). Within a few weeks of beginning the observation period, all nests within about 20 m of the tunnel entrance, approximately five pelican nests and 25 DCC nests, were permanently abandoned. Cormorants and pelicans breeding around the rest of the TAB system did not appear to be disturbed by the investigator's arrival or departure, nor by his presence in the TAB system. Birds did not appear to notice human voices speaking at low conversational level, but turned their heads towards the source of sudden noises, such as coughing. Fledged juvenile DCC regularly sat on or entered the TAB system.

I collected 16 DCC and 8 pelican eggs, 67 pelican and 61 DCC carcasses (all juvenile), and caught 74 fledged juvenile and 3 adult DCC from inside the TAB system in the period 1994-96. The disturbance caused by specimen collection depended on the type of specimen, its location, the stage of reproduction, and the investigator's skill in remaining hidden from the birds. Collection of an egg or a bird carcass from beside a nest generally did not cause birds to leave their nests. If an egg or carcass was taken from inside a nest, the adult bird on the nest usually left it temporarily. When trapping a live DCC, I had to widen the opening through which the rod had been extended to pull the struggling bird inside. This sometimes exposed part of the investigator's body to the other birds on the colony site, and they temporarily abandoned an area within 5-10 m of the trapping location.

## 2.4 Discussion

Use of the TAB system, in combination with the telescopic rod with attachments, allowed me to approach within a few metres of several hundred DCC and pelican nests and to collect specimens every third day during the breeding season without being seen, thus decreasing my disturbance of the colony. The assemblage of the TAB system was carried out before the breeding season and therefore did not disturb the birds.

The arrival and departure of the investigator by canoe caused the most severe disturbance, but this was limited to an area within about 20 m of the entrance to the TAB system. Gull predation and nest abandonment as a result of such investigator disturbance have been reported before in colonies of DCC (Kury and Gochfeld, 1975; Ellison and Cleary, 1978) and American white pelicans (Johnson and Sloan, 1976). The disturbance could have been reduced, had it been possible to place the entrance in an area of tall vegetation, or to extend the tunnel for about 25 m into the water.

The other main source of disturbance was visual exposure of the investigator when a live DCC was pulled into the TAB system. This might have been solved by extending the rod through a special opening in the blind, instead of through a door or window or under the edge of the tunnel. For example, a 50-cm-diameter hole in the side of the blind, covered by a loose-fitting cloth sleeve, would allow the rod to be manipulated freely without the investigator being seen by the birds on the colony site.

The 6-m-long carbon-fibre fishing rod used to collect specimens was three times lighter than the 8-m-long aluminum pole used by Hines and Custer (1995) to collect heron eggs, and was therefore easier to manipulate in the confinement of the TAB system.

In retrospect, the blinds should have been taller, so that an investigator could stand upright, which would have increased visibility from the blinds and investigator comfort.

The tunnel design of Cairns et al. (1987) was an improvement over that by Shugart et al. (1981), because the covering material was stronger, it was easy to set up on irregular terrain, and birds were unaware of traffic through the tunnel to and from the blind. In comparison with the tunnel of Cairns et al. (1987), which was built in one piece,

our modular design allowed flexibility in the number of tunnel modules and blinds used and the position of the blinds within the system. The TAB system was also easy to adjust during assemblage to topographic features of the colony site and nest distribution. I believe this system could be useful in other studies of ground-nesting colonial waterbirds, especially where repeated access to a large number of nests and collection of specimens is required.

# 3. AN EPIDEMIC OF NEWCASTLE DISEASE IN DOUBLE-CRESTED CORMORANTS IN 1995[1]

## 3.1 Introduction

Newcastle disease (ND) caused high mortality in double-crested cormorants (Phalacrocorax auritus; DCC) several times since 1975 (Cleary, 1977; Wobeser et al., 1993; Roffe, 1992; Heckert, 1993), but affected breeding colonies have never been observed in any detail during the course of an epidemic. As part of a disease study of a cormorant breeding colony in Saskatchewan from 1994 to 1996, I had the rare opportunity to observe the start of a ND epidemic in 1995 and to follow closely its course for 1 month. Herein, I (1) describe the epidemic, including clinical signs, contact with other bird species, mortality rate, virus isolation and serology, and (2) compare results among the three consecutive breeding seasons.

## 3.2 Materials and methods

### 3.2.1 Study area

The study area was Doré Lake (Saskatchewan, Canada; 54°46'N, 107°17'W); the lake has three islands, designated as Island A, Island B, and Rock Island, each with ground-nesting colonies of DCC (Fig. 3.1). Besides DCC, American white pelicans (Pelecanus erythrorhynchos) breed on Island A, herring (Larus argentatus) and California gulls (L. californicus) breed on Island B, and Caspian terns (Sterna caspia), ring-billed (L.

[1]A version of this chapter was accepted for publication as Kuiken et al. (1998c).

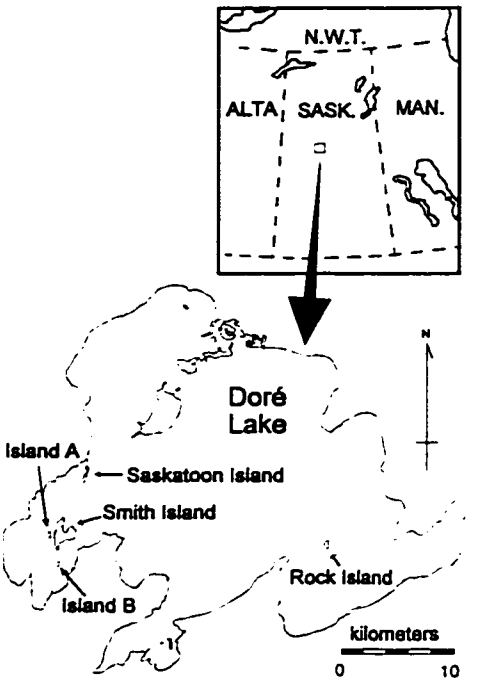



**Figure 3.1 Map of Doré Lake. Island A, Island B, and Rock Island have breeding colonies of double-crested cormorants.**

delawarensis), herring and California gulls breed on Rock Island. The main study site was Island A, a 300 x 100 m island in the south-west part of Doré Lake (Fig. 3.2).

### 3.2.2 Monitoring of reproduction, morbidity, and mortality

Observations were made on Island A from within an 88-m-long tunnel-and-blind (TAB) system (Fig. 3.2; Chapter 2) every third day from 1 June to 1 September 1994 to 1996. About 10% of the total nesting area of DCC was visible from within this system. All cormorant and pelican nests within about 6 m of the TAB system were marked with numbered stakes at the beginning of each breeding season. At each visit, I recorded the number of eggs and chicks in each marked nest, presence of sick or dead birds, and presence of birds other than DCC or pelicans. The location of carcasses was recorded in relation to the numbered stakes or TAB system to prevent counting them more than once per visit. Whenever possible, carcasses were classified as fresh or old. Criteria for designating carcasses as fresh were convex shiny eyes, clean plumage, red musculature, and glistening viscera. Criteria for designating carcasses as old were desiccation, flattening, and soiled plumage. In some cases, only skin, bones, esophagus and stomach were present when carcasses were first observed. I assumed that fresh carcasses were of birds that had died $< 3$ days previously, and old carcasses were of birds dead for $\geq 3$ days. In some cases it was not possible to determine the state of decomposition of carcasses because they were either too far away from the TAB system or partly hidden from view; these carcasses were classified as unknown. At the end of each breeding season, I counted carcasses and nests on Island A. I corrected for the disappearance of nests after fledging by determining the proportion of marked nests that were still visible, and dividing the total number of nests counted on Island A by this proportion.

During a ND epidemic in 1995 (23 July to 24 August), I also visited the cormorant colonies on Island B (28 July and 18 August) and on Rock Island (14 August), and walked along the shores of the adjacent Smith Island and Saskatoon Island (20 to 24 August) to look for sick and dead birds (Fig. 3.1).

I estimated the mortality rate of juvenile DCC from Island A in the period of the

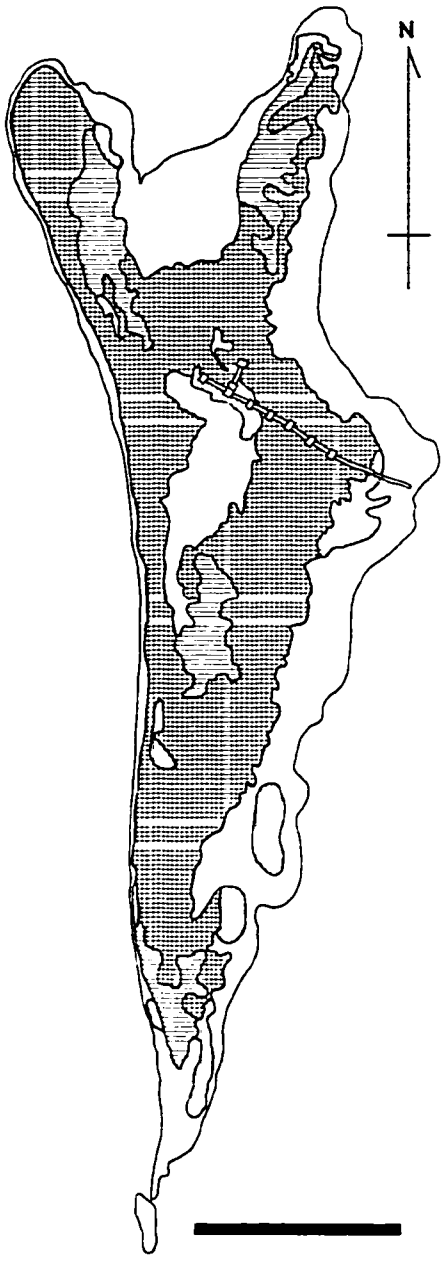



**Figure 3.2 Location of tunnel-and-blind system and nesting areas of double-crested cormorants (horizontal lines), American white pelicans (vertical lines), and both species (cross-hatching) on Island A at Doré Lake. This map was modified from an aerial photograph taken in June 1996. Bar = 50 m.**

ND epidemic as the number of deaths during the period divided by the number alive at the beginning of the period. The number of deaths during a period, or cumulative mortality, is not the same as the number of carcasses observed at the end of that period, because of carcass disappearance due to scavenging and decomposition (Wobeser, 1994). Because I only counted the total number of cormorant carcasses on Island A at the end of the epidemic, I used a correction factor $\alpha$ for carcass disappearance. To determine $\alpha$, I divided the number of juvenile cormorant carcasses found in the area visible from the TAB system at the end of the epidemic by the cumulative mortality in that area during the epidemic. The sum of the number of fresh carcasses seen at each visit during the epidemic provided a minimum estimate of the cumulative mortality; the sum of the number of fresh carcasses and carcasses of unknown stage of decomposition provided a maximum estimate. I used the average of these two values. Including this correction factor, the formula was: mortality rate = number of carcasses observed at end of period/($\alpha$ x number alive at beginning of period).

### 3.2.3 Virological examination

In 1994, samples for virus isolation were collected from 13 juvenile and one adult cormorant found moribund or dead on Island A (7 July to 1 September). In 1995, samples were collected from five DCC with clinical signs of ND collected on Island A (23 and 24 July), and from the following individuals of other species found dead or moribund on Island A, Island B, Rock Island, or Smith Island (16 July to 24 August): 10 juvenile pelicans, two adult ring-billed gulls, two juvenile herring gulls, one juvenile dabbling duck, probably a mallard (Anas platyrhynchos), one juvenile Caspian tern, one red-necked grebe (Podiceps grisegena) of unknown age, one adult common merganser (Mergus merganser), and one juvenile great blue heron (Ardea herodias). In 1996, samples were collected from one adult pelican found moribund on Island A on 16 June, two juvenile DCC found moribund on Island A (29 July and 19 August), and one juvenile ring-billed gull found dead on Rock Island (23 August).

Samples for virus isolation were stored in liquid nitrogen until processing. Virus

isolation was attempted by the Diagnostic Virology Laboratory, Western College of Veterinary Medicine, University of Saskatchewan, on pooled samples of brain, trachea, lung, liver, kidney, spleen, jejunum, and femoral bone marrow from each bird according to the method described by Wobeser et al. (1993) for samples from Saskatchewan. Confirmation of virus identity and assessment of pathogenicity (Alexander, 1988) was done by the Virology Section, Animal Diseases Research Institute, Canadian Food Inspection Agency, on a pool of Newcastle disease virus (NDV)-positive first-passage allantoic fluid samples from embryonating chicken eggs inoculated with tissue suspensions from the five DCC from 1995.

### 3.2.4 Examination for antibodies to Newcastle disease virus

Examination for hemagglutination inhibiting (HI) antibodies to NDV was carried out on yolk and serum samples. I arbitrarily collected 28 to 147 cormorant eggs per year from marked nests around the TAB system between 25 and 29 May from 1994 to 1996. Only one egg was collected per nest. Eggs were opened at the blunt end by use of scissors and length of incubation was estimated as: (1) marginal vein not yet visible grossly at 0-1 day (Romanoff, 1960), (2) marginal vein clearly visible as a red ring in the yolk sac and crown-rump length of embryo $\leq$ 10 mm at 2-8 days (Van Scheik, 1985), (3) crown-rump length of embryo > 10 mm and $\leq$ 20 mm at 9-13 days (Van Scheik, 1985), and (4) crown-rump length of embryo > 20 mm and $\leq$ 50 mm at 14-25 days (Van Scheik, 1985). The yolk was separated from the white by use of a kitchen egg separator. One ml of yolk was collected by use of a 1-ml insulin syringe without a needle and was diluted 1:10 in 0.01 M phosphate-buffered saline (pH 7.4).

I bled and banded 25 to 81 DCC from Island A per year between 1994 and 1996. Juvenile DCC that had not yet left their nests were caught by hand. Juvenile DCC that had left their nests and adult DCC were trapped from within the TAB system by hand or by use of a telescopic rod with a blunt hook at the end (Chapter 2). Birds were bled from the brachial vein, banded with a size 8 U.S. Fish and Wildlife Service aluminum band (Laurel, Maryland, USA), and released. In case of moribund birds, free-flowing blood

was collected from the jugular vein after cervical dislocation. Serous fluid from the heart lumen or celomic cavity was collected from 10 cormorant carcasses from Island A. I also bled four DCC from Rock Island (14 August 1995) and one cormorant from Island B (18 August 1995); all were juveniles with clinical signs of ND.

Blood samples were collected from other bird species as well. In 1994, I bled nine pelicans and one ring-billed gull, all juveniles. Before the ND epidemic in 1995, I bled one duckling, probably a mallard, and two juvenile pelicans. During the ND epidemic in 1995, I bled one great blue heron, two herring gulls, one ring-billed gull, five pelicans, and one Caspian tern, all juveniles. In 1996, I bled three juvenile and one adult pelican. All birds were from Island A, except for one herring gull from Island B, the great blue heron, duckling, and one ring-billed gull from Smith Island, and the Caspian tern from Rock Island.

Yolk and serum samples were frozen in liquid nitrogen until further processing. They were held at 56 C for 30 minutes, adsorbed once (serum) or twice (yolk) with guinea pig erythrocytes to remove nonspecific hemagglutinins, and tested for HI antibodies to NDV (Beard, 1989b) using guinea pig erythrocytes by the Diagnostic Virology Laboratory, Western College of Veterinary Medicine, University of Saskatchewan. Eight hemagglutination units of NDV B1 strain were used as antigen. An HI titer of $\geq$ 1:20 was considered positive (Brown et al., 1990).

Twenty-eight newly-hatched DCC from Island A (19 and 27 June 1995) were captured and raised in captivity so that the HI antibody titer to NDV in their serum could be measured over time without repeated disturbance of the breeding colony. These DCC were taken from nests from which eggs had been collected previously that season for measurement of HI antibody titer to NDV in yolk, and choice of nests was biased towards those containing eggs with high antibody titers. Twenty-two of 28 eggs (79%) in nests from which newly-hatched DCC were taken were seropositive for NDV, with titers ranging from < 1:20 to 1:160 (geometric mean titer: 51). Birds were bled when captured and weekly thereafter for at least 5 weeks. Newly-hatched birds were bled from the jugular vein; older birds were bled from the medial metatarsal vein or brachial vein.

### 3.2.5 Statistical analysis

Statistical analysis was done with Statistix software (Analytical Software, 1996). I tested the hypothesis that presence of antibody in egg yolk was independent of length of incubation and collection year by use of a log-linear model for multidimensional contingency tables (Zar, 1996). Following this, I tested the conditional independence of each of the three variables from the other two. I also tested the hypothesis that presence of antibody in yolk from eggs incubated < 2 days was independent of collection year by use of chi-square analysis (Zar, 1996).

## 3.3 Results

### 3.3.1 Clinical signs in double-crested cormorants

Juvenile DCC with clinical signs of ND were first seen on Island A on 23 July 1995 and were seen at each visit thereafter until the end of the observation period on 24 August 1995. Clinical signs of ND were not seen in adult DCC nor in any other bird species in 1995, nor in any birds in 1994 or 1996.

The most obvious clinical signs of ND were partial paralysis (paresis) or complete paralysis of one or more limbs. Signs varied with severity of paralysis and number and combination of limbs affected. All possible variations were observed.

Cormorants with mild unilateral leg paralysis limped, occasionally stumbled, and occasionally stood on the affected leg with toes curled (knuckling). Cormorants with severe unilateral leg paralysis held the affected leg tucked up against the body or partly or completely stretched out forwards, backwards or sideways, with toes held curled or straight (spastic paralysis). They hopped on the unaffected leg and maintained balance by flapping their wings in the air or leaning on the ground with wings, tail, or both. They were capable of flying and swimming, but were unable to dive. They often rested in sternal recumbency, whereas normal DCC usually rest standing. Cormorants with bilateral leg paralysis sometimes showed knuckling or sat on their intertarsal joints, but

were usually seen in sternal recumbency. They tried to move forwards by use of beak and wings pivoted against the ground (Fig. 3.3A), but made very slow progress.

Birds with unilateral wing paralysis let the affected wing hang loose by the side of the body or held it tucked against the body in the same way as an unaffected wing in resting position. In such cases, the abnormality only became visible when the birds carried out behavior in which they normally would use both wings, such as drying their wings, flying, or hopping onto a log or other object. Then, they spread the normal wing fully and the other partially or not at all (Fig. 3.3B). They could not fly and, in cases in which the lame wing hung loose, they had difficulty swimming and diving. A less apparent consequence of unilateral wing paralysis was that birds falling on their back had difficulty in righting themselves (Fig. 3.3C). Birds lying on their back paddled vigorously with their legs, swung their tail and head back and forth, pushed off the ground with the functioning wing, and in this way were eventually able to right themselves. Cormorants with bilateral wing paralysis were unable to right themselves if they fell on their back.

Cormorants with unilateral leg paralysis and unilateral (either ipsi- or contralateral) wing paralysis were able to hop on one leg and maintain balance by flapping the functioning wing in the air or leaning on the ground with it, and by leaning on the ground with beak and tail. They were able to swim slowly but were unable to dive, and, if they fell on their back, could right themselves only with great difficulty. Cormorants with more than two paralyzed limbs were only able to move short distances and were usually found lying in sternal recumbency.

Paralysis of the head and neck was rarely seen. One bird walked with its head and anterior half of the neck hanging vertically, as if the anterior neck muscles were paralyzed. When it stood still, it rested the tip of the beak on the ground beside its right foot. Another bird was apparently unable to tuck its head between its shoulders, as DCC normally do when resting, and instead held its head to one side of its breast.

The head of many affected birds trembled constantly. Such birds were often unable to peck accurately, for example at an approaching hand. One bird with head tremors repeatedly bobbed its head up and down. Two birds with unilateral leg paralysis had trembling of the whole body: one constantly, the other for about 30 sec after it had

**Figure 3.3A Double-crested cormorant with bilateral leg paralysis due to Newcastle disease. It is trying to move forward by use of its wings pivoted against the ground, and is leaning on the ground with its tail.**

**Figure 3.3B Double-crested cormorant with unilateral wing paralysis due to Newcastle disease. The normal wing is spread and the affected wing is held close to the body.**

**Figure 3.3C Double-crested cormorant with unilateral wing paralysis due to Newcastle disease. It is lying on its back and has difficulty in righting itself. Note the scuff marks on the ground caused by the tail movements.**

been fed.

Other signs of neurologic disease included circling, excessive elevation of the legs during walking (goose stepping), and an unsteady gait. Some affected birds apparently lost their fear of humans and avian predators such as ravens (Corvus corax). Others were found in unusual places, such as around human dwellings. Several birds walked from my camp site on Smith Island along a path through the forest towards the middle of the island.

Ocular involvement was common in affected birds. Clinical signs included closure of the eyelids, watery exudate, a plaque of yellow-white friable material in the conjunctival sac, and reddened and opaque cornea, sclera, and third eyelid. Two birds had bilateral pupillary dilatation and were apparently blind: they walked without coordination, constantly bumping into objects, and did not react to an approaching hand.

Other common, non-specific, clinical signs included depression, soiled and damaged plumage, cloacal area matted with grey-green droppings, outside of beak caked with dirt, laborious breathing—in one case with beak opened at each inspiration, emaciation—more apparent in the last 2 weeks of observations, many lice on the plumage and in the oral cavity, and leeches in the oral cavity, nasal cavity, or conjunctival sac.

There was abundant contact between affected juvenile DCC and other DCC and pelicans on Island A. Normal juvenile DCC pecked at and clutched various body parts of affected DCC, which often lacked defensive behavior. Adult DCC continued to feed affected juveniles as long as they were able to beg. Immediately before and after being fed, juvenile pelicans pecked at any birds in their proximity, including DCC with signs of ND. Juvenile pelicans also tried to steal food from juvenile DCC being fed by adult DCC. Both juvenile DCC and juvenile pelicans regularly pecked at and extensively mouthed carcasses lying on the ground, and in one case a juvenile pelican ate the intestine of a juvenile cormorant carcass. Large shallow pools of water in the center of Island A formed as a result of heavy rains between 29 July and 1 August. These pools and the shallow water along the shore of Island A were used extensively by both juvenile DCC and juvenile pelicans. Many partly paralyzed DCC remained on the shoreline and died there because they were unable to get out of the water after swimming.

### 3.3.2 Course of morbidity and mortality in double-crested cormorants

The number of juvenile DCC with clinical signs of ND observed from the TAB system reached a peak about 2 weeks after the start of the epidemic, and the number of freshly dead carcasses reached a peak about 10 days later (Fig. 3.4). Between 20 and 24 August 1995, I found 1,005 juvenile cormorant carcasses: 972 on Island A, 32 on the shore of Smith Island, and one on the shore of Saskatoon Island (Fig. 3.1). I assumed that they were from the Island A colony, and had died of ND. On 24 August 1995, 3,219 cormorant nests were counted on Island A, and 166 of 232 (72%) nests marked in May were visible. Therefore, the estimated number of cormorant nests on Island A in 1995 was 3,219 ÷ 0.72 = 4,499. The mean number of juveniles surviving to 3 weeks of age per nest, excluding those nests from which eggs and juveniles were collected, was 1.38 (n = 101, SD = 1.00, range 0 to 3.5), so that the estimated number of juveniles surviving to 3 weeks on Island A was 4,499 x 1.38 = 6,208. The median hatching date in 1995 on Island A was 16 June (n = 191, first quartile 13 June, third quartile 23 June, range: 10 June to 8 July), so that the median date on which the surviving juveniles were 3 weeks old was 7 July. No mortality was observed from the TAB system between this date and 23 July, when the ND epidemic was first noted, so that the number of juveniles surviving to 3 weeks of age is a reasonable estimate of the number of juveniles alive at the beginning of the epidemic. During the course of the epidemic, the total number of juvenile cormorant carcasses observed from the TAB system at each visit progressively underestimated the calculated cumulative mortality (Table 3.1). At the end of the epidemic, the number of cormorant carcasses present around the TAB system was 50% of the cumulative mortality, so $\alpha = 0.5$ (Table 3.1). Therefore, the mortality rate of juvenile DCC of Island A from ND from 23 July to 24 August 1995 was approximately: number of deaths observed at end of period/($\alpha$ x number alive at beginning of period) = 1,005/(0.5 x 6,208) = 0.32.

The behavior and development of unaffected juvenile DCC on Island A is summarized here to put the epidemic in the context of the cormorant's reproductive cycle. Juvenile DCC started wandering among the nests and mingling with birds from other

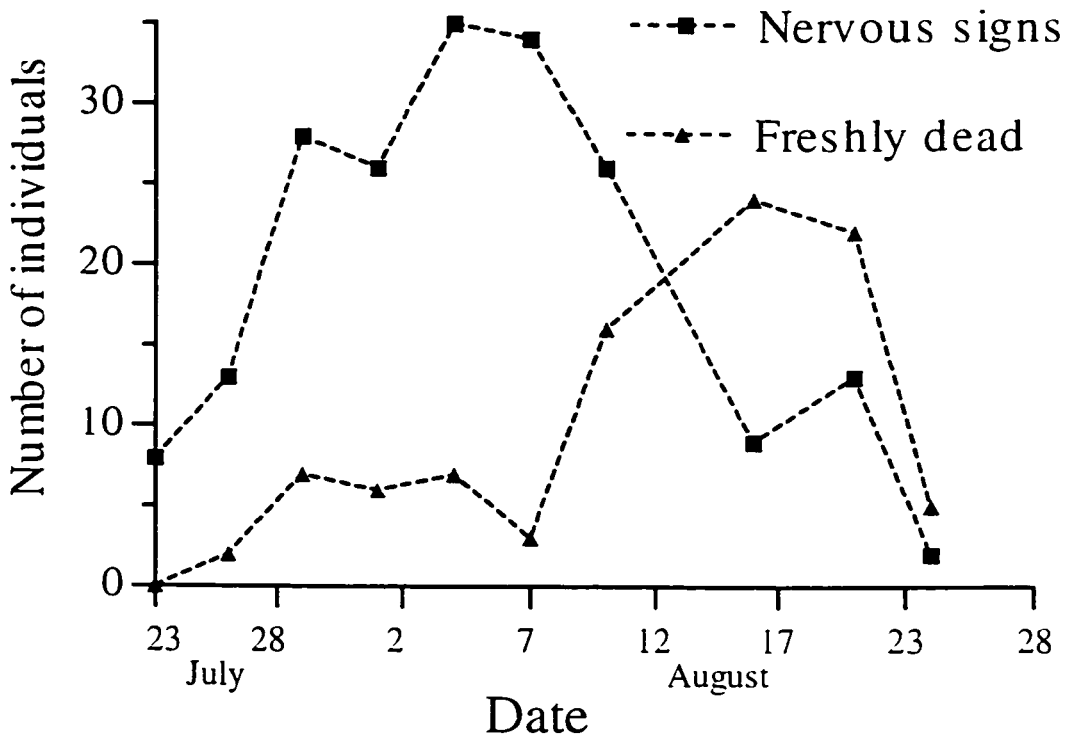



**Figure 3.4 Morbidity and mortality of juvenile double-crested cormorants during the Newcastle disease epidemic in 1995 in the area visible from the tunnel-and-blind system on Island A at Doré Lake.**

**Table 3.1 Mortality of juvenile double-crested cormorants during the Newcastle disease epidemic of 1995 in the area visible from the tunnel-and-blind system on Island A at Doré Lake.**

| Date | | Number of carcasses observed | | | | Cumulative mortality | | Total number of carcasses observed/mean cumulative mortality |
|---|---|---|---|---|---|---|---|---|
| | | State of decomposition | | | Total | | | |
| | | Fresh | Old | Unknown | | | | |
| July | 23 | 0 | 0 | 0 | 0 | 0 | (0-0)[a] | - |
| | 26 | 2 | 0 | 0 | 2 | 2 | (2-2) | 1 |
| | 29 | 7 | 0 | 5 | 12 | 12 | (9-14) | 1 |
| August | 1 | 6 | 1 | 12 | 19 | 24 | (15-32) | 0.8 |
| | 4 | 7 | 13 | 3 | 23 | 32 | (22-42) | 0.7 |
| | 7 | 3 | 10 | 18 | 31 | 44 | (25-63) | 0.7 |
| | 10 | 16 | 7 | 4 | 27 | 62 | (41-83) | 0.4 |
| | 16 | 24 | 9 | 9 | 42 | 91 | (65-116) | 0.5 |

[a]Mean (minimum-maximum) where the minimum was calculated as the sum of fresh carcasses seen at each visit and the maximum was calculated as the sum of fresh carcasses and carcasses of unknown state of decomposition seen at each visit.

nests on 14 July 1995 (median age = 4 weeks), and by 17 July, most of them were no longer on their nests. They were very curious, pecking at sticks, feathers, and other objects on the ground. On 20 July, they were first seen at the water's edge, and on 23 July, some were swimming and diving in the shallows next to Island A. On 26 July, juvenile DCC were first seen flying a few meters in the air, and by 3 August, many were flying around Island A and swimming and diving in the water around the island. They also started catching fish from about this date onwards, although adult DCC were still seen feeding juveniles until the end of the observation period. On 16 August there appeared to be fewer juvenile DCC on Island A than on the previous visit, and their number decreased progressively until 24 August, when < 100 juveniles were seen on Island A out of several thousand juveniles fledged.

Morbidity and mortality also were noted on the other two cormorant colony sites on Doré Lake. On Island B, which had 49 cormorant nests, I found one juvenile cormorant carcass on 28 July 1995, and two juvenile DCC with unilateral wing paralysis and 17 juvenile cormorant carcasses on 18 August 1995. On Rock Island, which had 1,120 cormorant nests, I found 27 juvenile DCC with signs of lameness and 169 juvenile cormorant carcasses on 14 August 1995.

### 3.3.3 Observations of other bird species

Several bird species, besides DCC and pelicans, were present on or around Island A during the epidemic, and had contact with affected DCC. Up to 200 juvenile and adult herring, California, and ring-billed gulls were present on Island A at each visit. Most roosted at the water's edge or in the open area in the center of Island A (Fig. 3.2). They often swam in the shallows and in the stagnant pools in the central open area, walked in the nesting areas, and fed on cormorant and pelican carcasses. One to three ravens were present at each visit, usually feeding on a cormorant or pelican carcass in the central open area, or sitting in a tree. Once, several American crows (Corvus brachyrhynchos) were seen feeding on a cormorant carcass. An immature bald eagle (Halilaeetus leucocephalus) was seen at Island A twice during the epidemic, once soaring over the island, the other

time sitting in a tree. Occasionally, but not during the epidemic, an immature bald eagle was seen scavenging on cormorant and pelican carcasses on Island A and drinking in the shallows. A pair of American coots (Fulica americana) with their chicks, four pairs of red-necked grebes with their chicks, and up to 20 yellow-headed blackbirds (Xanthocephalus xanthocephalus) were seen regularly among the bulrushes (Scirpus lacustris) around Island A, and had presumably nested there. Up to 22 mallards were regularly seen swimming in the shallows and feeding in the mud at the water's edge. Occasionally, a common goldeneye (Bucephala clangula), a green-winged teal (Anas crecca), and several American wigeon (Anas americana) were seen swimming in the shallows. Three semi-palmated plovers (Charadrius semipalmatus) and five sandpipers (Calidris sp.) were seen once at the water's edge.

Morbidity and mortality were noted in pelicans, gulls, and terns during the epidemic. On Island A, I found one adult and 638 juvenile pelican carcasses, 18 moribund juvenile pelicans, one adult herring gull carcass, two adult California gull carcasses, three juvenile herring or California gull carcasses, and one adult and one juvenile ring-billed gull carcass on 24 August 1995. On Island B, I found one adult herring gull carcass on 28 July 1995, and one juvenile and one adult herring gull carcass on 18 August 1995. On Rock Island, I found one juvenile Caspian tern with a broken humerus, 11 ring-billed gull carcasses, 11 herring or California gull carcasses, and one adult Caspian tern carcass on 14 August 1995.

### 3.3.4 Virological examination

Newcastle disease virus was isolated from the five DCC collected from Island A on 23 and 24 July 1995. The virus (designated PMV-1/cormorant/Saskatchewan-Canada/2035/95) had an intracerebral pathogenicity index of 1.61 and an intravenous pathogenicity index of 1.23, and was classified as pathogenic on the basis of an intracerebral pathogenicity index > 0.7 (Commission of the European Communities, 1993). Newcastle disease virus was not isolated from any birds sampled in 1994 or 1996, nor from birds other than the DCC sampled in 1995.

### 3.3.5 Examination for antibodies to Newcastle disease virus

Yolk samples were more difficult to collect from incubated eggs (≥ 2 days of incubation) than from freshly laid eggs (< 2 days of incubation) because embryonic development and higher fluidity and volume of the yolk increased the risk of rupturing the yolk sac. The antibody status of cormorant eggs, their length of incubation and year of collection (Table 3.2) were not all mutually independent (Pearson's $X^2 = 151.80$, 17 df, n = 225, $P < 0.00001$). Antibody status was dependent on length of incubation, holding collection year constant (Pearson's $X^2 = 43.41$, 9 df, n = 225, $P < 0.0001$), but independent of collection year, holding length of incubation constant (Pearson's $X^2 = 11.98$, 8 df, n = 225, $P = 0.15$). Length of incubation was dependent on collection year, holding antibody status constant (Pearson's $X^2 = 116.21$, 12 df, n = 225, $P < 0.00001$). If only eggs of < 2 days of incubation were considered, there was a significant difference in antibody status between years ($X^2 = 6.20$, 2 df, n = 86, $P = 0.045$), due to the high prevalence (93%) in 1995 (Table 3.2).

During the ND epidemic, 37 of 63 (59%) of cormorant sera tested positive for antibodies to NDV, with titers of 1:20 or 1:40 (Table 3.3); all positive sera were from 5- to 9-week-old birds (Table 3.4). The highest prevalence and the highest geometric mean titers were found during the first 2 weeks of the epidemic (Table 3.3). However, bird capture was strongly biased towards birds with clinical signs of ND, which were easier to catch, so that these samples do not accurately represent the juvenile DCC of the Island A colony. There was no clear relationship between the presence of clinical signs of disease and titer (Table 3.5). Sera from three of four DCC from Rock Island collected during the ND epidemic tested positive for antibodies to NDV, one with a titer of 1:40, two with a titer of 1:20; the serum from the cormorant from Island B tested negative.

The only cormorant sera that tested positive for antibodies to NDV before or after the ND epidemic were from two 2-day-old birds taken into captivity in 1995 (Table 3.4). Both had a titer of 1:20. The titer of the sibling egg of one of these chicks was 1:40, that of the other was < 1:20. One of the seropositive chicks tested negative 1 week later, and the other died a few days after sampling. It was dehydrated and emaciated; histological

**Table 3.2 Hemagglutination inhibiting antibody titer to Newcastle disease virus in yolks of double-crested cormorant eggs from Island A at Doré Lake.**

| Year | Length of incubation (days)[a] | Prevalence | Geometric mean titer | Titer | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | < 1:20 | 1:20 | 1:40 | 1:80 | 1:160 |
| 1994 | 0-1 | 70 (10)[b] | 44 | 3 | 2 | 3 | 1 | 1 |
| | 2-8 | 40 (10) | 34 | 6 | 1 | 3 | 0 | 0 |
| | 9-13 | 40 (5) | 20 | 3 | 2 | 0 | 0 | 0 |
| | 14-25 | 67 (3) | 20 | 1 | 2 | 0 | 0 | 0 |
| | Total | 54 (28) | 33 | 13 | 7 | 6 | 1 | 1 |
| 1995 | 0-1 | 93 (27) | 41 | 2 | 6 | 13 | 5 | 1 |
| | 2-8 | 60 (60) | 39 | 24 | 13 | 13 | 8 | 2 |
| | 9-13 | 23 (35) | 22 | 27 | 7 | 1 | 0 | 0 |
| | 14-25 | 24 (25) | 32 | 19 | 3 | 2 | 1 | 0 |
| | Total | 51 (147) | 37 | 72 | 29 | 29 | 14 | 3 |
| 1996 | 0-1 | 68 (49) | 26 | 16 | 22 | 10 | 1 | 0 |
| | 2-8 | 0 (1) | < 20 | 1 | 0 | 0 | 0 | 0 |
| | Total | 66 (50) | 26 | 17 | 22 | 10 | 1 | 0 |

[a]Length of incubation was estimated as: (1) marginal vein not yet visible at 0-1 day, (2) marginal vein visible as red ring on yolk sac and crown-rump length of embryo ≤ 10 mm at 2-8 days, (3) crown-rump length of embryo > 10 mm and ≤ 20 mm at 9-13 days, and (4) crown-rump length of embryo > 20 mm and ≤ 50 mm at 14-25 days.
[b]Percent positive (number of yolks tested).

**Table 3. 3 Relationship between sampling date and hemagglutination inhibiting antibody titer to Newcastle disease virus in sera of double-crested cormorants from Island A at Doré Lake during the Newcastle disease epidemic in 1995.**

| Sampling date | Prevalence | Geometric mean titer | Titer | | |
|---|---|---|---|---|---|
| | | | < 1:20 | 1:20 | 1:40 |
| 23 to 29 July | 63 (19)[a] | 28 | 7 | 6 | 6 |
| 30 July to 5 August | 84 (19) | 27 | 3 | 9 | 7 |
| 6 to 12 August | 57 (7) | 24 | 3 | 3 | 1 |
| 13 to 19 August | 29 (18) | 20 | 13 | 5 | 0 |
| Total | 59 (63[b]) | 26 | 26 | 23 | 14 |

[a]Percent positive (number of sera tested).
[b]This includes four sera which were collected from two cormorants which had been bled at least once before where one was bled twice and the other four times.

**Table 3.4 Relationship between age and prevalence of hemagglutination inhibiting antibody titer to Newcastle disease virus in double-crested cormorants from Island A at Doré Lake.**

| Age class | Age[a] (wk) | Prevalence | | |
|---|---|---|---|---|
| | | 1994 | 1995 | 1996 |
| Juvenile | 0 | 0 (6)[b] | 10 (20[c]) | nd[d] |
| | 1 | 0 (6) | 0 (11[e]) | 0 (17) |
| | 2 | 0 (17) | 0 (9) | 0 (3) |
| | 3 | 0 (8) | 0 (6) | nd |
| | 4 | 0 (13) | 0 (4) | 0 (4) |
| | 5 | 0 (4) | 57 (14) | nd |
| | 6 | 0 (11) | 84 (19) | 0 (1) |
| | 7 | 0 (8) | 67 (12) | nd |
| | 8 | 0 (12) | 38 (8) | nd |
| | 9 | 0 (2) | 33 (15) | nd |
| | 10 | 0 (1) | nd | nd |
| Adult | | 0 (2) | 0 (3) | nd |
| Total | | 0 (90[f]) | 34 (121[g]) | 0 (25) |

[a]The age of juvenile cormorants captured in their nest was based on the estimated hatching date for that nest. The age of juvenile cormorants captured outside their nest was based on the median hatching date of the marked cormorant nests (20 June 1994, 16 June 1995, 29 June 1996).
[b]Percent positive (number of sera tested).
[c]All 20 were the first sera taken from 20 juvenile cormorants captured on Island A and taken into captivity.
[d]No data
[e]Eight of the 11 were the first sera taken from eight juvenile cormorants captured on Island A and taken into captivity.
[f]This includes 23 sera taken from free-living cormorants which had been bled at least once before where 10 were bled twice, two were bled five times, and one was bled six times.
[g]This includes four sera taken from free-living cormorants which had been bled at least once before where one bird was bled twice and the other was bled four times, each at intervals of 1 week. One of the paired sera had a titer of 1:20 and the titers of the others was < 1:20.

**Table 3.5 Relationship between clinical signs and hemagglutination inhibiting antibody titer to Newcastle disease virus in sera of double-crested cormorants from Island A at Doré Lake during the Newcastle disease epidemic in 1995.**

| Clinical signs | Prevalence | Geometric mean titer | Titer | | |
|---|---|---|---|---|---|
| | | | < 1:20 | 1:20 | 1:40 |
| Nervous disease | 66 (41)[a] | 33 | 14 | 15 | 12 |
| Emaciated/moribund/dead | 36 (14) | 20 | 9 | 5 | 0 |
| None observed | 63 (8) | 26 | 3 | 3 | 2 |
| Total | 59 (63[b]) | 26 | 26 | 23 | 14 |

[a]Percent positive (number of sera tested).
[b]This includes four sera which were collected from two cormorants which had been bled at least once before where one was bled twice and the other four times.

lesions of ND, such as non-suppurative encephalitis and neuronal necrosis, were absent (Kuiken et al., unpublished data).

Sera from birds other than DCC all tested negative for antibodies to NDV.

## 3.4 Discussion

The clinical signs of ND described here correspond to those reported in previous ND epidemics in DCC (Cleary, 1977; Wobeser et al., 1993; Banerjee et al., 1994; Meteyer et al., 1997). Most of the nervous signs may be attributed to cerebellar lesions (Clippinger et al., 1996). However, some suggest lesions in other parts of the nervous system. Knuckling suggests damage to sensory or motor pathways of the peripheral nerve, spinal cord, brain stem, or cerebral cortex. Loss of fear and walking into the forest may be interpreted as changes in learned behavior, which suggest cerebral dysfunction. Blindness, in the absence of structural changes within the eye itself, suggests damage to the optic nerve or the cerebral pathways. Fixed dilated pupils also may be a result of damage to these structures or to the oculomotor nerve (Clippinger et al., 1996).

Placing the ND epidemic in the context of the cormorant's breeding cycle may help to identify risk factors. The traditional "mass action" formulation for the transmission of directly transmitted diseases states that "the rate of appearance of new infections is proportional to the product of susceptibles, X, times infectious cases, Y, thus $dY/dt = \beta XY$, the $\beta$ being the rate of effective contact between individuals." (Fine et al., 1982). Assuming that NDV was carried to Island A by returning DCC (Meteyer et al. 1997), infectious cases (Y) were present from the beginning of the breeding season. Juveniles were present from around 16 June, the median hatching date. Passive HI antibodies to NDV, which correlates with protection of the host against NDV (Alexander, 1991), was not detectable in juveniles > 1-week-old (Table 3.4). This indicates that large numbers of susceptibles (X) were present from about 23 June. Until 17 July, most juveniles remained in their nests and only had direct contact with parents and siblings. After that date, the number of intra-and inter-species contacts increased greatly as the juveniles left their nests and wandered about the island. This greatly increased the rate of

effective contact among individuals ($\beta$). The epidemic started on 23 July, 6 days later, which is the approximate incubation period for ND in chickens (Alexander, 1991). This sequence of events suggests that departure of juvenile DCC from their nests, with the associated increase in contact rate among birds, was a critical initiating factor for the epidemic. If this also is true for other ND epidemics in cormorant breeding colonies, monitoring activities can be focused on times when the mean age of juveniles exceeds 5 to 6 weeks.

Because juveniles leave nests on the ground at 4 weeks of age, while they leave nests in trees or on cliffs at 6 weeks of age (Lewis, 1929), colonies with ground nests might have earlier and more severe ND epidemics than colonies with tree nests or nests on cliffs. This is consistent with the higher mortality in juveniles from ground nests than from tree nests during the ND epidemic in Quebec (Canada) in 1975 (Cleary, 1977). In more southerly colonies, where egg laying is less synchronized and age variation in juvenile DCC is greater (Palmer, 1962), ND might occur in DCC much younger than 4 weeks of age due to contact with juveniles that hatched earlier and already have left their nests.

Spread of NDV between juvenile DCC by direct and indirect contact may have been increased by their investigative behavior, including pecking at affected birds and at inanimate objects contaminated with droppings. Their intensive use of shallow water on and around Island A, and the tendency of affected birds to die on the shoreline, may have increased transfer of NDV by water.

Besides ND in juvenile DCC on Island A, which was confirmed by virus isolation, the occurrence of this disease in juvenile DCC on Island B and Rock Island may be inferred from the presence of birds with clinical signs consistent with ND on these islands and from positive sera of birds from Rock Island. This was later confirmed by histological and virological examination of those individuals (Chapter 4). Because I did not study the interaction between birds from breeding colonies on Island A, Island B, and Rock Island, I can only speculate about the possible mechanisms of NDV transfer among these colonies. Island A is about 2 km from Island B, and these two islands are about 25 km from Rock Island (Fig. 3.1). First, adult DCC from different colonies may have

infected each other at common foraging areas, which may be up to 20 km from nesting sites (Hobson et al., 1989). Second, fledged juvenile DCC with ND may have swum or flown between Island A and Island B, and may have flown between these two islands and Rock Island, and so transferred NDV between colonies. Third, other bird species moving between colonies, such as gulls, may have transferred NDV. Finally, spread of NDV between Islands A and B may have occurred by air or water (Alexander, 1991).

The importance of ND as a cause of mortality for DCC depends on several factors, including (1) age of bird affected, (2) mortality rate, and (3) whether mortality is additive or compensatory. Life history studies of six species of cormorants suggest that "a key to successful long-term survival in these birds is the maintenance of a considerable proportion of older and reproductively experienced birds in the breeding flock..." (Johnsgard, 1993). Because mortality in this and previous ND epidemics in DCC was limited to juveniles (Cleary, 1997; Meteyer et al., 1997), ND may not be as important per bird lost as causes of mortality that also affect older birds, such as oil spills and pesticide poisoning (Johnsgard, 1993).

I calculated that the ND epidemic on Island A in 1995 had a mortality rate of 32% of juveniles. This is a minimum estimate that assumes that all carcasses present on the colony site on 24 August 1995 were found when searched for. Such success in finding carcasses is most unlikely. Given the brown-black color of the cormorant carcasses and the speed with which they were hidden by bird droppings and debris, it might be realistic to assume that I found only about 50% of cormorant carcasses present. In that case, the calculated mortality rate would be twice as high as the minimum rate, or 64%. This is higher than the overall first-year mortality rate for DCC from Mandarte Island (British Columbia, Canada) which was 59%, calculated by subsequent-year sightings of birds banded as nestlings (van de Veen, 1973). It is impossible to speculate whether mortality from ND in juvenile DCC is additive or compensatory, because little is known about other causes of first-year mortality in DCC, particularly after fledging (Johnsgard, 1993; Erwin, 1995).

Newcastle disease did not appear to be an important cause of morbidity or mortality in other wild bird species cohabiting with DCC on Doré Lake. I found no

clinical signs, or serological or virological evidence of ND in species other than the cormorant, despite potential transfer of NDV by direct contact with affected live and dead DCC and indirect contact with their droppings on land and in water. During the ND epidemic in DCC, unusual mortality in other species was seen only in juvenile pelicans. However, NDV was not isolated and necropsy findings suggested that they died of starvation of undetermined cause (Kuiken et al., unpublished data). Mortality in juvenile pelicans and gulls was recorded in association with the 1990 and 1992 epidemics in DCC (Roffe, 1992; Wobeser et al., 1993). Pathogenic NDV was isolated from one pelican, which also had focal encephalitis, and one ring-billed gull from 1990 (Wobeser et al., 1993), but this was the only evidence that the virus caused mortality in those species. Virus was not isolated from any gulls or pelicans in 1992 (Roffe, 1992). There were no reports of ND outbreaks in poultry in Canada, the USA, or Mexico in 1995 (Welte, 1997), suggesting that NDV from the epidemic on Doré Lake in 1995 did not spread to poultry during or after the breeding season.

Statistical analysis of antibody titers to NDV in cormorant eggs indicates that the antibody status of the eggs was dependent on the incubation stage, and, once corrected for this variable, did not differ between years. The most likely explanation for the variation in antibody titers to NDV at different stages of incubation (Table 3.2) is the change in yolk concentration due to absorption of water from the albumen in the first half of incubation and loss of water from the yolk in the second half (Romanoff, 1967). Transfer of antibodies from the yolk to the embryo and enzymatic breakdown of antibodies may also cause fluctuation in yolk antibody titer (Kramer and Cho, 1970). For comparison of antibody titers in eggs among years, examination should be limited to unincubated eggs. These have the additional advantage that yolk samples are easier to collect than from partly incubated eggs.

In chickens, egg yolk antibody titers to NDV correspond with serum titers of the dam (Heller et al., 1977). Thus, the significantly higher prevalence of antibody titer to NDV in eggs incubated < 2 days in 1995 compared to 1994 and 1996 (Table 3.2) suggests that a higher percentage of adult DCC had been in contact with NDV in that year. In chickens, HI antibodies to NDV usually become undetectable within a year after infection

(Hanson, 1980). By extrapolation, the DCC that had antibodies to NDV in May 1995 probably had contact with NDV some time after the 1994 breeding season. This is consistent with the assumption that in 1995 NDV was carried to Island A by returning DCC infected during winter or migration.

Antibodies to NDV were present in sera of juvenile DCC in July and August 1995, when there was a ND epidemic on Island A, but not in 1994 and 1996, when there was no epidemic (Tables 3.3 and 3.4); this corresponds with the findings of Wobeser et al. (1993) and Meteyer et al. (1997). It confirms the suggestion of Meteyer et al. (1997), that serology can be used as a non-lethal way of determining exposure to NDV in a cormorant population > 1 to 2 weeks of age, when passive antibodies become undetectable.

# 4. PATHOLOGY OF NEWCASTLE DISEASE IN DOUBLE-CRESTED CORMORANTS, WITH COMPARISON OF DIAGNOSTIC METHODS[1]

## 4.1 Introduction

Newcastle disease (ND) caused high mortality in juvenile double-crested cormorants (Phalacrocorax auritus; DCC) several times since 1975 (Cleary, 1977; Wobeser et al., 1993; Roffe, 1992; Heckert, 1993; Chapter 3). Despite the importance of ND both for wild birds and domestic poultry, there are few studies on its pathology and diagnosis in DCC.

In past studies, DCC suspected of ND were examined by necropsy, histology, virus isolation, immunofluorescence, and serology. Characteristic histological lesions were found in the brain and spinal cord, and included Purkinje cell necrosis, lymphoplasmacytic vasculitis, perivascular cuffs, and gliosis. Brain and intestine appeared to be the best tissues for virus isolation (Wobeser et al., 1993; Banerjee et al., 1994; Meteyer et al., 1997).

In July and August 1995, a ND epidemic in DCC on Doré Lake (Saskatchewan, Canada) was monitored. Epidemiological aspects of this epidemic have been described previously (Chapter 3). Herein, the pathological findings in 25 DCC from this epidemic are compared with those in 18 DCC without ND. The main objectives were (1) to describe the distribution of Newcastle disease virus (NDV) and associated lesions, and (2) to evaluate different methods of ND diagnosis in DCC.

---

[1]A version of this chapter was submitted for publication as Kuiken et al. (1998e).

## 4.2 Materials and methods

### 4.2.1 Birds

Twenty-five DCC with clinical signs of nervous disease, hereafter referred to as ND epidemic (NDE) birds, were collected alive during a ND epidemic on Doré Lake (Saskatchewan, Canada; 54°46'N, 107°17'W). They were found between 23 July and 18 August 1995 on one of three colony sites on Doré Lake: Island A (n = 19), Island B (n = 1) and Rock Island (n = 5) (Chapter 3).

Eighteen DCC, hereafter referred to as control birds, were collected dead (n = 12) or alive (n = 6) from Island A, Doré Lake. They were found between July and September 1994 to 1996, except during the ND epidemic. The primary diagnoses were severe trauma, caused by coyote (Canis latrans) predation (n = 12), a fallen branch (n = 1), or an unknown cause (n = 2), starvation (n = 1), beak malformation (n = 1), and bilateral rotation of the carpal joint (n = 1).

### 4.2.2 Necropsy

Necropsies were done on all birds. Live birds were monitored for about 5 minutes to observe their clinical signs, then killed by cervical dislocation. Necropsies were carried out by one person using a standard protocol. The birds were divided into three categories of body condition: birds in good body condition had well-developed musculature and visible fat in the coronary groove, mesentery and abdominal subcutis; birds in moderate body condition had moderately developed musculature, visible fat in the coronary groove but not in the mesentery or abdominal subcutis; and birds in poor body condition had atrophied musculature and no visible fat in the coronary groove, mesentery, or abdominal subcutis. Samples for virological, bacteriological and serological examination were stored in liquid nitrogen, and samples for histological examination were stored in 10% neutral-buffered formalin.

### 4.2.3 Serology

Sera from 20 NDE and eight control birds were held at 56 C for 30 minutes, adsorbed once with guinea pig erythrocytes to remove nonspecific hemagglutinins, and tested for hemagglutination inhibiting antibodies to NDV using guinea pig erythrocytes (Beard, 1989b) by the Diagnostic Virology Laboratory, Western College of Veterinary Medicine, University of Saskatchewan. Eight hemagglutination units of NDV B1 strain were used as antigen. A titer of $\geq$ 1:20 was considered positive (Brown et al., 1990).

### 4.2.4 Histology

Formalin-fixed samples from all birds were embedded in paraffin, sectioned at 5 $\mu$m, and stained with hematoxylin and eosin (H & E) for examination by light microscopy. Selected heart sections also were stained with hematoxylin-basic fuchsin-picric acid to detect muscle degeneration or oil red-O to detect fat (Luna, 1968). The following tissues were examined: brain (sagittal sections taken a few mm from the median plane), spinal cord (transverse sections at the level of the seventh cervical vertebra, third thoracic vertebra, and middle of the synsacrum), left and right brachial plexus (transverse sections), left and right lumbosacral plexus (transverse sections), left and right eye (sections including optic nerve), eyelid, bursa of Fabricius, thymus, spleen, femoral bone marrow, esophagus, stomach, duodenum, pancreas, liver, jejunum, ileum and ceca, colon, trachea, left and right lung, heart, thoracic aorta, pectoral muscle, quadriceps muscle, proximal tibia, left and right kidney, left and right thyroid and parathyroid, left and right adrenal gland, uropygial gland, ovary and oviduct or left and right testis.

Sections of cerebrum, cerebellum, brain stem, cervical, thoracic, and lumbar spinal cord, and brachial and lumbosacral plexus of all NDE birds were examined and a list of all lesions seen was prepared. Then, the identification numbers of sections of all birds were masked, the sections were re-examined in arbitrary order, and scored for the presence or absence of listed lesions without knowledge of the bird's origin.

Other organ systems of all NDE birds also were examined by light microscopy. Any tissue with lesions found in more than six NDE birds also was examined for the presence of similar lesions in all control birds.

### 4.2.5 Virus isolation

Virus isolation was attempted on pooled tissues of all birds by the Diagnostic Virology Laboratory, Western College of Veterinary Medicine, University of Saskatchewan. Samples of brain, trachea, lung, liver, kidney, spleen, jejunum, and femoral bone marrow were pooled and homogenised with 0.01 M phosphate-buffered saline solution (pH 7.4; PBS) in a Stomacher (Seward Medical, London, U.K.) to obtain a 1:10 dilution. After freezing at -80 C, samples were thawed in a 37 C water bath, centrifuged at 2600 G at 4 C for 10 minutes, and the supernatant was filtered, the final filter having a pore size of 0.22 $\mu$m. A 0.15 ml volume of filtrate was injected into each of 5 embryonated chicken eggs (Alexander, 1989) and three passages without 100% embryo mortality were carried out before a case was considered negative.

Allantoic fluid from eggs with dead embryos was tested for the presence of hemagglutinating agents with a hemagglutination (HA) test (Beard, 1989a). Hemagglutination-positive samples were tested for the presence of NDV with a hemagglutination inhibition test (Beard, 1989b), using polyclonal chicken antiserum to NDV. Because many of the allantoic fluid samples from dead embryos were negative in the HA test, an indirect immunoperoxidase assay (IPA), which does not depend on the hemagglutination activity of NDV, was used to determine whether NDV was present (see below).

Virus isolation was attempted on individual tissues of five NDE birds (cases 1 to 5), found on Island A, Doré Lake, on 23 and 24 July, 1995, at the beginning of the ND epidemic. A sample of each tissue (brain, trachea, lung, liver, kidney, spleen, jejunum, and femoral bone marrow) was homogenized with bovine heart infusion (BHI) broth in a Ten Broeck tissue grinder to obtain a 1:10 dilution. The BHI broth consisted of 33.3 mg/ml BHI powder (BDH, Toronto, Ontario, Canada), 10% (vol/vol) bovine serum, 10 x

$10^3$ U/ml penicillin and 10 mg/ml streptomycin in distilled water. After centrifugation at 2000 G at 4 C for 20 minutes, the supernatant was harvested and diluted 9:10 with PBS containing gentamycin (5 mg/ml). The diluted supernatant was left for 1 hr at room temperature, centrifuged again, and the supernatant was used for virus isolation procedures as described above. Allantoic fluid samples from eggs with dead embryos were tested for the presence of NDV only with IPA (see below). Samples were considered negative if no embryo mortality occurred during two passages and allantoic fluid harvested at the end of the second passage tested negative in the IPA.

4.2.6 Indirect immunoperoxidase assay (IPA)

This method, adapted from Afshar et al. (1989), was carried out by the Virology Section, Animal Diseases Research Institute, Canadian Food Inspection Agency. One ml of allantoic fluid sample was filtered through a 0.45 $\mu$m filter, and then diluted 1:10 in Earle's minimum essential medium supplemented with 20% fetal bovine serum and 50 $\mu$g/ml gentamycin (MEM). Of this mixture, 25 $\mu$l was added to a well of a 96-well flat-bottom tissue culture microtiter plate (Nunc, Roskilde, Denmark), containing about 5 x $10^4$ confluent Vero cells in 75 $\mu$l MEM per well. The plate was incubated in a humidified atmosphere of 5% $CO_2$ and 95% air for 72 hr at 37 C, by which time an infected cell monolayer was formed. The plate was voided of its medium by gentle inversion. The monolayers were rinsed briefly with PBS, drained, and fixed for 10 minutes at room temperature with 20% acetone in PBS containing 0.02% bovine serum albumin. The plate was voided of acetone by gentle inversion and dried with a blow dryer. The monolayers were rehydrated by rinsing with PBS. Fifty $\mu$l of a 1:800 dilution of polyclonal chicken antiserum against NDV was added to each well and the plate was incubated at 25 to 30 C. After 30 minutes, the wells were drained of serum and rinsed with PBS containing 0.05% Tween (PBST) three times for 2 minutes each at room temperature. The wells were filled with 50 $\mu$l of a 1:1000 dilution of horseradish peroxidase-labelled rabbit anti-chicken IgG (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA) and incubated for 30 minutes at 25-30 C. The wells were again emptied, rinsed in PBST, and filled with

50 $\mu$l of substrate solution containing $H_2O_2$ (Afshar et al., 1989). The enzymatic reaction was stopped after 12 minutes by emptying the wells and rinsing them with PBST and then with distilled water. Cells were examined with an inverted light microscope. Positive wells had red-brown staining of Vero cells. Wells containing Vero cells infected with NDV (B1 vaccine strain) were run concurrently as positive controls. Wells containing Vero cells, allantoic fluid samples, and serum from NDV-free chickens were also run as negative controls.

### 4.2.7 Virus titration

The virus concentration of NDV-positive tissue samples was determined by making serial ten-fold dilutions of each sample in PBS, injecting 0.1 ml into the allantoic cavity of each of five chicken eggs containing 9 to 10-day-old embryos, and monitoring embryo death daily for 7 days post inoculation. The virus concentration was expressed as the negative exponent of the dilution at which 50% of the embryos died ($ELD_{50}$; Villegas and Purchase, 1989).

### 4.2.8 Immunohistochemistry

Immunohistochemical staining was performed on the tissues of five NDE birds (cases 1 to 5) by the Diagnostic Immunology Laboratory, Western College of Veterinary Medicine, University of Saskatchewan. The same tissues were examined as for histological examination. Immunohistochemical staining was conducted with formalin-fixed, paraffin-embedded tissue sections using an avidin-biotin complex immunoperoxidase method (Haines and Chelack, 1991) adapted for a robotic stainer (Brigati et al., 1988). A chicken polyclonal antiserum to NDV (Spafas, Preston, Connecticut, USA) was used as the primary antibody. Briefly, duplicate sections of each tissue block were stained with NDV antiserum diluted 1:2000. The enzyme reactions were developed in diaminobenzidine to produce a dark brown precipitate and the sections were counterstained with hematoxylin. The amount of staining was scored from negative

to 3+ relative to that in the positive control tissue. Sections of cerebellum from a DCC from which NDV had been isolated were used as a positive control. Tissue sections of a captive-raised DCC, which was serologically and virologically negative for NDV, were used as a negative control. Tissue sections of a DCC from which NDV had been isolated were processed as above, but omitting the primary antibody, as an omission control.

#### 4.2.9 Reverse transcriptase-polymerase chain reaction (RT-PCR) test and nucleotide sequencing

A RT-PCR test (Chapter 6) was used to determine the presence of NDV in kidney and jejunum of a NDE bird (case 2). The identity of RT-PCR products was confirmed by nucleotide sequencing (Chapter 6).

#### 4.2.10 Bacteriology

Samples of brain, trachea, lung, liver, spleen, kidney, jejunum, and femoral bone marrow from a NDE bird with multisystemic fibrino-suppurative inflammation were cultured on blood agar (Prepared Media Laboratory, Richmond, British Columbia, Canada) by the Diagnostic Bacteriology Laboratory, Western College of Veterinary Medicine, University of Saskatchewan. The inoculated media were incubated at 37 C in an aerobic atmosphere and examined daily for 2 days for bacterial growth. An isolated colony representative of each bacterial variant detected visually was selected and identified according to methods of Carter and Cole (1990).

#### 4.2.11 Statistical analysis

The Fisher exact test was used to test the null hypothesis that the proportion of birds with a given lesion was the same in the NDE and control groups (Zar, 1996). Because this test was used 12 times to compare the two groups, a Bonferroni-corrected P-value of 0.05/12 = 0.004 was employed to maintain a study-wise P-value of 0.05 (Howell,

1992). The Cochran's Q test was used to test the null hypothesis that the probability of a ND-positive result in NDE birds was the same for histological, virological, and serological examination (Zar, 1996).

## 4.3 Results

### 4.3.1 Clinical signs

All 25 NDE birds showed clinical evidence of central nervous system dysfunction. Twenty-two of the 25 had partial or complete paralysis of one or more limbs: one wing (one bird), both wings (four birds), one leg (two birds), both legs (six birds), one wing and one leg (two birds), one wing and both legs (one bird), both wings and one leg (one bird), and both wings and both legs (five birds). Other nervous signs included head tremors (seven birds), ataxia (four birds), standing on a foot with toes curled (two birds), walking in circles (one bird), and apparent blindness (one bird).

### 4.3.2 Necropsy

All 25 NDE birds were young-of-the-year in complete or nearly complete juvenal plumage. Ten were male and 15 were female. Twelve birds were in good, five in moderate, and eight in poor body condition.

Externally, one bird had a breast wound with surrounding hemorrhage at the level of the thoracic inlet, which perforated the body wall and trachea about 1 cm anterior to the bifurcation. Another bird had a featherless area of 2 cm diameter on the rump, with hemorrhage in the subcutis. These wounds were probably caused by gulls (Larus spp.). In two birds with leg paralysis, the legs were held tucked against the body with curled toes, and the knee, tarsometatarsal and digital joints were stiff and could only be extended with considerable force. In five birds with wing paralysis, the wings were held against the body and the elbow and carpal joints were stiff and could not be extended fully. In two birds with leg paralysis, the skin and feathers of the leading edges of both carpal joints were

abraded, due to leaning on the ground with the wings. In one bird with paralysis of all four limbs, the skin and feathers on the dorsal aspect of the right elbow joint were abraded due to wing movements while the bird was lying on its back.

Eye lesions were found in 11 birds. Excess watery fluid, wetting the surrounding plumage, exuded from the eye in six birds. Plaques of yellow-white friable material, which were easily dislodged, were present in the conjunctival sac of five birds. The cornea of one bird had a circumscribed white spot 2 mm in diameter, and the cornea of another bird was slightly opaque. The conjunctiva of the sclera (two birds), nictating membrane (one bird) and eyelid (one bird) were reddened. The nictating membrane in the bird with the reddened nictating membrane also was thickened and opaque. Two birds had bilateral pupil dilation.

Internally, the brain parenchyma was edematous in two birds, and there was excess watery fluid in the subdural space of the cranium in three birds. One bird had generalized venous congestion. There was pallor of the middle layer of the myocardium of the left ventricle in three birds. The spleen appeared reduced in size in one bird, enlarged in three birds, and had miliary subcapsular hemorrhages in one bird. One of the enlarged spleens had white nodules of 1-2 mm diameter. The pancreas had a white nodule of 1 mm diameter in the parenchyma in one bird. The liver of six birds had multiple white contorted cords about 0.5 mm in diameter and up to 30 mm long, multiple white miliary nodules, or both, in the parenchyma. The liver was reduced in size with sharp edges in five birds, and had a hemorrhage of 7 mm diameter on the surface in one bird. Nematodes were present in the stomach of 25 birds, either loose in the lumen or attached to the wall. The stomach of 24 birds contained no food, and the intestine of all 25 birds was virtually empty.

### 4.3.3 Histology

#### 4.3.3.1 Nervous system

Lesions in the brain and spinal cord of NDE birds included neuronal necrosis,

spongy change, gliosis, perivascular infiltration with mononuclear cells, endothelial hypertrophy, and hemorrhage (Tables 4.1 and 4.2). These lesions often were found together in discrete foci. However, perivascular infiltration with mononuclear cells and endothelial hypertrophy were found regularly in the absence of other lesions.

Neuronal necrosis was most often seen as shrunken angular neurons with eosinophilic cytoplasm and no nucleus (Fig. 4.1A). Less common lesions included cell swelling, central chromatolysis, peripheral displacement of the nucleus, and pyknosis. Necrotic neurons were most often seen in the cerebellar nuclei and in the brain stem. Neuronal necrosis also was recognized by the decreased number or absence of neurons in the Purkinje cell layer of the cerebellum and in the ventral horn of the grey matter of the spinal cord.

Spongy change consisted of round, optically empty spaces in the parenchyma, and probably was caused by necrosis and loss of neurons and axons. It was found most often as discrete foci in the cerebellar nuclei, in the brain stem, and in the ventral horn of the grey matter of the lumbar spinal cord (Fig. 4.1B).

Gliosis consisted of a multifocal to diffuse increase of small cells in the parenchyma, usually around necrotic neurons. These gliotic foci were most easily visible in the molecular layer of the cerebellum, because it normally had few visible small cells (Fig. 4.1C).

Perivascular infiltration with mononuclear cells in the parenchyma of brain and spinal cord usually consisted of a compact cuff, one to four cells thick (Fig. 4.1D), but in some cases up to 15 cells thick. The cells were mainly medium-sized lymphocytes; plasma cells were regularly present, and heterophils were rarely found. The meninges usually had a more diffuse infiltration of mononuclear cells.

Endothelial hypertrophy usually was found in small blood vessels, and was characterized by large endothelial nuclei with vesiculated chromatin protruding into the vascular lumen (Fig. 4.1D). Hemorrhages, when present, were small and perivascular.

Lesions in the brachial and lumbosacral plexus of NDE birds consisted of axonal degeneration, endothelial hypertrophy, and perivascular infiltration with mononuclear cells. Axonal degeneration consisted of swelling and fragmentation of axons, and the

**Table 4.1 Distribution of neurological lesions in double-crested cormorants with clinical signs consistent with Newcastle disease. Newcastle disease virus was isolated from 21 of 25 birds.**

| | | | Number of cormorants with each lesion | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Neuronal necrosis | Spongy change | Gliosis | Perivascular infiltration with mononuclear cells | | Endothelial hypertrophy | Hemorrhage | Axonal degeneration |
| Tissue | | n | | | | Parenchyma | Meninges | | | |
| Cerebrum | | 25 | 8 | 4 | 13 | 20 | 9 | 12 | 7 | NE[a] |
| Cerebellum | | 25 | 17 | 11 | 18 | 18 | 15 | 19 | 2 | NE |
| Brain stem | | 25 | 13 | 11 | 15 | 21 | 11 | 21 | 1 | NE |
| Spinal cord: | Cervical | 23 | 2 | 3 | 2 | 8 | 7 | 8 | 1 | NE |
| | Thoracic | 24 | 3 | 6 | 6 | 9 | 3 | 7 | 1 | NE |
| | Lumbar | 24 | 10 | 8 | 8 | 6 | 5[b] | 7 | 0 | NE |
| Plexus: | Brachial | 22 | NE | NE | NE | 3 | NE | 5 | 0 | 7 |
| | Lumbosacral | 25 | NE | NE | NE | 3 | NE | 12 | 0 | 14 |

[a]Not examined or not applicable.
[b]Only 21 of 24 sections of lumbar spinal cord had meninges present.

**Table 4.2 Prevalence of lesions in the central nervous system[a] of double-crested cormorants with clinical signs consistent with Newcastle disease, compared to negative control cormorants.**

| | | Number of birds with each lesion (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Neuronal necrosis | Spongy change | Gliosis | Perivascular infiltration with mononuclear cells | | Endothelial hypertrophy | Hemorrhage |
| Cormorant group | n | | | | Parenchyma | Meninges | | |
| Newcastle disease epidemic | 25[b] | 19 (76) | 17 (68) | 22 (88) | 24 (96) | 20 (80) | 23 (92) | 8 (32) |
| Negative control | 17[c] | 2 (12) | 9 (53) | 0 (0) | 1 (6) | 0 (0) | 3 (8) | 7 (41) |
| P-value[d] | | < 0.001 | 0.35 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.78 |

[a]Cerebrum, cerebellum, brain stem, and/or cervical spinal cord.
[b]Cervical spinal cord was available from only 23 cormorants.
[c]Brain stem was available from only 13 cormorants and cervical spinal cord from only 14 cormorants.
[d]Statistic of the Fisher exact test. A value of < 0.004 indicates a statistically significant difference between the two groups.

**Figure 4.1A** Neuronal necrosis in the brain stem of a double-crested cormorant with Newcastle disease. The necrotic neuron (arrowhead) is shrunken and has no nucleus. Adjacent neurons (arrows) are normal. H & E. Bar = 40 $\mu$m.

**Figure 4.1B** Spongy change in the brain stem of a double-crested cormorant with Newcastle disease. H & E. Bar = 160 $\mu$m.

**Figure 4.1C** Gliosis in the cerebellum of a double-crested cormorant with Newcastle disease. The molecular layer (M) has a focal increase of small cells, and Purkinje cells in the subjacent Purkinje cell layer (P) are missing. G = granular layer. ME = meninges. H & E. Bar = 100 $\mu$m.

**Figure 4.1D** Perivascular infiltration with mononuclear cells in the cerebrum of a double-crested cormorant with Newcastle disease. The blood vessel has a compact cuff of mononuclear cells. Inset: The endothelial cells have large nuclei (arrowheads) protruding into the vascular lumen. H & E. Bar = 50 $\mu$m.


A
B
C
D
E

formation of ellipsoids (Fig. 4.2A).

Negative control birds also had some of the above lesions in the nervous system (Table 4.2). A necrotic neuron was found in a cerebellar nucleus of a bird killed by a coyote. Several necrotic Purkinje cells were found in a bird with a bacterial meningo-encephalitis associated with puncture wounds of the skull. Spongy change was found in nine birds killed by coyotes, and was usually more diffuse than in NDE birds. A one to four-cell-thick cuff of mononuclear cells was found around three blood vessels in the white matter of the cerebellum of a bird killed by a coyote. Endothelial hypertrophy was found in three birds which died of starvation, coyote predation, and head trauma of unknown cause, respectively. Hemorrhage was found in seven birds which died of trauma, six of which were killed by coyotes and one by a falling branch. The hemorrhage was usually more extensive than in NDE birds.

There was a significant difference ($P < 0.001$) between NDE and control birds in the prevalence of neuronal necrosis, gliosis, perivascular infiltration with mononuclear cells, and endothelial hypertrophy in the central nervous system (Table 4.2).

#### 4.3.3.2 Lymphoid system

Newcastle disease epidemic birds had lymphocyte necrosis and depletion in the thymus (25/25 birds), bursa of Fabricius (23/23 birds), and spleen (11/25 birds). Lymphocyte necrosis was seen as small cells with pyknosis or karyorrhexis, often within optically empty spaces; lymphocyte depletion was characterized by fewer lymphocytes than normal, or none. In one bird, the parenchyma of a thymic lobule was largely replaced by serous fluid admixed with erythrocytes and heterophils; in two birds, the spleen had pools of serous fluid and fibrin around necrotic sheathed capillaries.

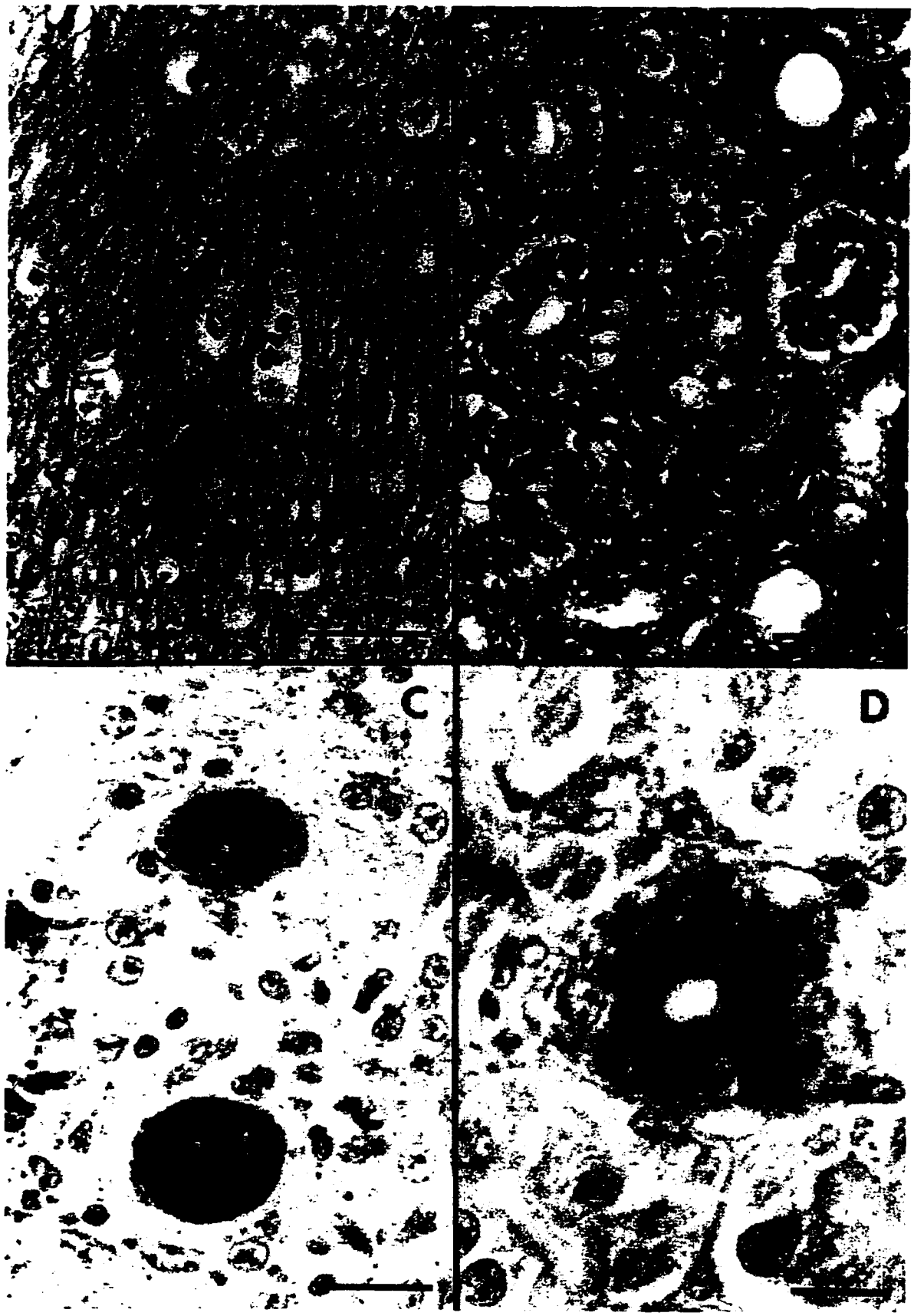
The spleen had hyperplasia, probably of reticulum cells, in 3 of 25 birds; it was characterized by fields of large, round to oval cells around hypertrophic sheathed capillaries. These cells had large nuclei with finely stippled chromatin, a moderate amount of light blue vacuolated cytoplasm, and $\leq 1$ mitotic figure per high power (400X) field. The three birds were all from the first week of the ND epidemic and had enlarged

**Figure 4.2A Axonal degeneration in the lumbosacral plexus of a double-crested cormorant with Newcastle disease. One axon is swollen and fragmented, and has formed an ellipsoid (arrowhead). H & E. Bar = 40 $\mu$m.**

**Figure 4.2B Tubular epithelial necrosis in the kidney of a double-crested cormorant with Newcastle disease. Necrotic tubular epithelial cells (arrowheads) are small and have pyknotic nuclei and dark cytoplasm. H & E. Bar = 30 $\mu$m.**

**Figure 4.2C Immunohistochemical staining of Newcastle disease virus in the cerebellum of a double-crested cormorant. Positive staining is visible as variably-sized granules in the cytoplasm of Purkinje cells. Hematoxylin counterstain. Bar = 20 $\mu$m.**

**Figure 4.2D Immunohistochemical staining of Newcastle disease virus in the kidney of a double-crested cormorant. Positive staining is visible as variably-sized granules in the cytoplasm of tubular epithelial cells, and is strongest on the apical side. Hematoxylin counterstain. Bar = 10 $\mu$m.**


C
D

spleens.

#### 4.3.3.3 Other systems

The prevalence of lesions consistent with ND and found in more than six NDE birds is compared to that in control birds in Table 4.3. In NDE birds, foci of mononuclear cells were found in the following tissues: lung, not associated with bronchi (6/25 birds); lamina propria, submucosa and serosa of proventriculus (5/25 birds); submucosa of gall bladder (3/21 birds); pancreas (12/25 birds); myocardium (2/24 birds); kidney (21/25 birds); submucosa of ureter (2/25 birds); wall of oviduct (1/14 birds); adrenal gland (2/25 birds); and uropygial gland (1/25 birds). There was necrosis of a few tubular epithelial cells adjacent to the foci in the kidneys in 10 of the above 21 birds (Fig. 4.2B).

There was suppurative conjunctivitis in 23 of 25 birds, varying from mild (nine birds) to moderate (11 birds) to severe (three birds), and characterized by diffuse heterophil infiltration in the epithelium and lamina propria; the lamina propria also had a variable number of lymphocytes and plasma cells. All 11 birds with gross eye lesions had a moderate to severe conjunctivitis histologically. The friable material found in the conjunctival sac at necropsy consisted of plaques of fibrin, heterophils, sloughed epithelial cells and bacterial colonies. There was mild to moderate suppurative keratitis in five of 25 birds, all of which had gross eye lesions. The keratitis was characterized by diffuse heterophil infiltration of the corneal stroma. In addition, the cornea had multiple erosions (one bird), a corneal ulcer and neovascularisation (one bird), or epithelial hyperplasia (one bird). Three of the above birds also had mild to moderate suppurative anterior uveitis, characterized by diffuse heterophil infiltration in the filtration angle of the anterior chamber and the iris. The skin of the eyelid had multifocal epidermal erosions or ulcers in 13 of 25 birds. These lesions were covered by crusts of necrotic epithelial cells, heterophils, erythrocytes, fibrin, bacterial colonies, and debris. The underlying subcutis was infiltrated by heterophils.

There was mild to moderate superficial granulomatous proventriculitis, associated with the presence of nematodes in 22 of 25 birds. The liver (16/25 birds) and pancreas

**Table 4.3 Prevalence of lesions in lung, pancreas, kidney and eyelid of double-crested cormorants with clinical signs consistent with Newcastle disease, compared to negative control cormorants.**

| Cormorant group | Number of cormorants with each lesion/number of cormorants tested (%) | | | | | |
|---|---|---|---|---|---|---|
| | Foci of mononuclear cells in the: | | | Renal tubular necrosis | Suppurative conjunctivitis | Erosions or ulcers in the skin of the eyelid |
| | Lung | Pancreas | Kidney | | | |
| Newcastle disease epidemic | 6/25 (24) | 12/25 (48) | 21/25 (84) | 10/21 (48) | 23/25 (92) | 13/25 (52) |
| Negative control | 5/18 (28) | 7/11 (64) | 11/17 (65) | 2/11 (18) | 9/10 (90) | 4/12 (33) |
| P-value[a] | 0.94 | 0.62 | 0.28 | 0.14 | 0.63 | 0.48 |

[a]Statistic of the Fisher exact test. A value of $< 0.004$ indicates a statistically significant difference between the two groups.

(1/25 birds) had mild to moderate multifocal granulomatous inflammation, associated with the presence of trematodes. The myocardium had locally extensive fatty change in three of 24 birds. These birds had pallor of the left ventricle on necropsy. The areas consisted of myocytes with variably sized small intracytoplasmic vacuoles, which stained positive for fat with oil red O. There was no evidence of myocyte degeneration in H & E stained sections nor in sections stained with hematoxylin-basic fuchsin-picric acid. The interrenal (cortical) cells of the adrenal gland were small and eosinophilic due to loss of lipid vacuoles in 14 of 25 birds. The chromaffin (medullary) cells of the adrenal gland were infiltrated by a few to moderate numbers of mature granulocytes in three of 25 birds. There was serous atrophy of fat, in which the fat was replaced by a network of fine eosinophilic fibrils, in six of 18 birds. One bird, which had generalized venous congestion on necropsy, had a fibrino-suppurative inflammation of the meninges, pericardium, myocardium, pleura, pectoral muscle, arteries, veins, ovary liver, and uvea, associated with a generalized Escherichia coli infection.

There was no statistically significant difference between NDE and control birds in the prevalence of lesions in the lung, pancreas, kidney or eyelid (Table 4.3).

### 4.3.4 Virus isolation

Newcastle disease virus was isolated from pooled tissues of 21 of 25 NDE birds. The allantoic fluid had a titre in the HA test of <1:2 in 19 cases, 1:32 in one case, and 1:64 in one case. The two HA test-positive allantoic fluid samples had titres of 1:240 in the hemagglutination inhibition test. The allantoic fluid of all 21 cases was positive in the IPA. The four NDV-negative NDE birds were found in the last two weeks of the ND epidemic. Newcastle disease virus was not isolated from pooled tissues of any of 18 control birds.

Newcastle disease virus was isolated from eight individual tissues in the five NDV-positive birds thus examined. Kidney was the tissue most frequently positive and had the highest virus concentration (Table 4.4). All eight samples were positive in the IPA.

**Table 4.4 Virus isolation and immunohistochemical detection of Newcastle disease virus in individual tissues of double-crested cormorants.**

| Cormorant number | Brain[a] | | Spinal cord[b] | | Kidney | | Jejunum | |
|---|---|---|---|---|---|---|---|---|
| | VI[c] | IHC[d] | VI | IHC | VI | IHC | VI | IHC |
| 1 | - | + | NE[e] | - | 2.6 | + | - | - |
| 2 | 3.3 | +++ | NE | ++ | 4.7 | +++ | 2.2 | - |
| 3 | - | ++ | NE | - | 4.8 | ++ | - | - |
| 4 | - | ++ | NE | + | < 2.0 | ++ | - | - |
| 5 | < 2.0 | + | NE | + | - | - | - | - |

[a]Cerebrum, cerebellum, and brain stem.
[b]Cervical, thoracic, and lumbar spinal cord. Cervical spinal cord of only four cormorants was examined.
[c]Virus isolation in embryonated chicken eggs. The result is expressed as negative (-) or. in positive tissues, as log Egg Lethal Dose 50% per g tissue.
[d] Immunohistochemical examination. The amount of staining scored from negative (-) to strongly positive (+++) relative to that in the positive control tissue.
[e]Not examined.

### 4.3.5 Immunohistochemistry

Specific staining for NDV was limited to the nervous system and the kidney (Table 4.4), and consisted of variably-sized distinct dark-brown granules in the cytoplasm. Specific staining in the nervous system was found in the cerebrum, cerebellum, and brain stem (5/5 cases), thoracic spinal cord (1/5 cases) and lumbar spinal cord (3/5 cases). The strongest staining was found consistently in the cerebellum. Specifically stained cells were neurons (including Purkinje cells and granule cells in the cerebellum; Fig. 4.2C), glial cells, and, rarely, endothelial cells of small blood vessels. Areas of specific staining usually corresponded with foci of neuronal necrosis and inflammation. However, many marked histological lesions in the nervous system had no or weak specific staining. No specific staining was found in the cervical spinal cord (0/4 cases), brachial plexus (0/3 cases), or lumbosacral plexus (0/5 cases). Specific staining in the kidney was found in four of five cases. Specifically stained cells were tubular epithelial cells in cortex and medulla (Fig. 4.2D), and usually corresponded with tubular epithelial necrosis adjacent to interstitial foci of mononuclear cells.

Non-specific staining consisted of diffuse light-brown staining of epithelial cells, most marked in the intestine, and dark-brown staining of granules of heterophils. This non-specific staining did not interfere with interpretation of the tissue sections, except in intestine and tissues with many heterophils, such as spleen, thymus, and eyelid.

### 4.3.6 Reverse transcriptase-polymerase chain reaction test and nucleotide sequencing

Kidney and jejunum samples from the one NDE bird tested (case 2) were positive for NDV by RT-PCR test. The nucleotide sequence of part of the RT-PCR products, which were about 500 base pairs long, was $^{370}$ATCTAGAGGAAGGAGACAGAAACGTTTTGTAGGTGCT$^{406}$, with the predicted amino acid sequence $^{109}$SRGRRQKRFVGA$^{120}$, which corresponds to the fusion protein cleavage site of NDV (Seal et al., 1995).

### 4.3.7 Serology

Fourteen of 20 NDE sera tested positive for hemagglutination inhibiting antibodies to NDV, with titres of 1:20 (n = 9) or 1:40 (n = 5). All eight control sera tested negative.

### 4.3.8 Comparison of methods for Newcastle disease diagnosis

There was no significant difference in the probability of a ND-positive result between histological, virological, and serological examination ($Q = 4.22$, $P > 0.05$; Table 4.5).

## 4.4 Discussion

Newcastle disease virus was isolated from 21 of 25 NDE birds, confirming that they had ND. A virus isolate from the same group of birds (PMV-1/cormorant/Saskatchewan-Canada/2035/95) was previously confirmed as pathogenic, with an intravenous pathogenicity index of 1.23 and an intracerebral pathogenicity index of 1.61 (Chapter 3). Pathogenic NDV is here defined as a NDV isolate with an intracerebral pathogenicity index > 0.7 (Commission of the European Communities, 1993). The virus isolate had the same predicted amino acid sequence of the fusion protein cleavage site as those from ND epidemics in DCC in 1990 and 1992 (Seal et al., 1995; Heckert et al., 1996). This sequence included the R-for-G substitution at position 110, which appears to be unique for NDV isolates obtained from DCC and associated species since 1990, and suggests that this virus has been circulating in the DCC population for several years.

The four NDE birds that were negative for NDV by virological examination had brain lesions characteristic for ND (Meteyer et al., 1997; this study); three of them also had hemagglutination inhibiting antibodies to NDV (Table 4.5). Together, these findings indicate that these four birds also had ND. One NDE bird, from which NDV was isolated, had no visible ND lesions in the brain. Wobeser et al. (1993) also found no microscopic

**Table 4.5 Comparison of three methods used for the diagnosis of Newcastle disease in 25 double-crested cormorants with clinical signs consistent with this disease.**

| Diagnostic method | Number positive (%) | Test result combinations[a] | | | | | |
|---|---|---|---|---|---|---|---|
| Histology[b] | 24 (96) | + | + | + | + | + | - |
| Virus isolation | 21 (84) | + | + | + | - | - | + |
| Serology | 14 (70) | + | NE | - | + | - | - |
| Number of birds per combination | | 11 | 5 | 4 | 3 | 1 | 1 |

[a] + = positive, - = negative, NE = not examined.
[b] Histology was considered positive if cerebrum, cerebellum, brain stem, and/or cervical spinal cord had neuronal necrosis, perivascular infiltration with mononuclear cells, and/or gliosis.

lesions in the brain of one of three DCC from which NDV was isolated. However, Meteyer et al. (1997) found that all 16 NDV-positive DCC had brain lesions.

Nineteen of the 21 virus isolates from the NDE birds were negative in the HA test, which is traditionally used as a screening test for NDV (Alexander, 1989). In a laboratory where only the HA test is used, therefore, ND would not have been diagnosed in these birds. Hemagglution may not have occurred because these isolates were highly pathogenic to chicken embryos and killed them before reaching sufficient concentration in the allantoic fluid to cause hemagglutination. Alternatively, the virus isolates may have had no or little ability to cause hemagglutination. Although hemagglutination is considered a characteristic property of NDV (Alexander, 1997), exceptions are known; for example, Karzon and Bang (1951) isolated a velogenic NDV strain (CG 179) from an outbreak in California which did not consistently agglutinate erythrocytes. Whatever the reason, a test which did not depend on hemagglutination was necessary to identify reliably the isolates from these birds. In this study an IPA was used, which makes use of the specific binding between NDV and anti-NDV antibody. This assay was straightforward to use, inexpensive, and allowed the testing of many samples concurrently.

The distribution of NDV in five NDE birds was limited to brain, kidney, and jejunum (Table 4.4). Of these, the kidney had both the highest prevalence of infection and the highest concentration of NDV. This is comparable to chickens infected with neurotropic NDV, in which the virus concentration in the kidney was about $10^3$ greater than in other tissues during clinical disease (Asdell and Hanson, 1960). Therefore, kidney should be included in the tissues collected for virus isolation in cases of suspected ND in DCC. In previous studies (Wobeser et al., 1993; Banerjee et al., 1994; Meteyer et al., 1997) this was not done, probably because kidney is not on the standard list of tissues for NDV isolation (Alexander, 1989) and because kidney lesions in DCC with ND are not conspicuous (Banerjee et al., 1994; this study).

The brain of NDE birds had NDV antigen in neurons, glial cells, and endothelial cells, which corresponds with the localization of neurotropic NDV in chickens (Wilczynki et al., 1977). The presence of NDV antigen usually was associated with

neuronal necrosis, gliosis, endothelial hypertrophy, and perivascular infiltration with mononuclear cells (Figs. 4.1A to D). These lesions correspond with those found previously in DCC with ND (Wobeser et al., 1993; Banerjee et al., 1994; Meteyer et al., 1997) and are considered characteristic for this disease in other bird species also (Kaleta and Baldauf, 1988; Alexander, 1997). However, although these lesions are useful to help establish a diagnosis of ND in DCC, they are not pathognomonic. Other viral diseases, e.g. avian influenza and avian encephalomyelitis, although not reported in DCC, cause similar lesions (Swayne, 1996). Also, in this study neuronal necrosis, endothelial hypertrophy, and perivascular infiltration with mononuclear cells were found in a few DCC with no clinical or virological evidence of ND (Table 4.2).

The kidney of NDE birds had NDV antigen in tubular epithelial cells, in both the cortex and medulla, which corresponds to its location in chickens with ND (Kölbl, 1978). The presence of NDV antigen was often associated with small foci of necrotic tubular epithelial cells and interstitial infiltration with mononuclear cells (Fig. 4.2B); similar kidney lesions were found in ND in DCC (Banerjee et al., 1994), pigeons (Barton et al., 1992), turkeys (Al-Sheikhy and Carson, 1975), chickens (Jungherr et al., 1946), and house sparrows (Passer domesticus) (Gustafson and Moses, 1952). The lesions in the kidney were mild in relation to the high concentration of NDV in that tissue, in contrast to the situation in the brain. The high prevalence of NDV in the kidney suggests that it may be an important source of excreted virus.

The lymphocyte depletion and necrosis in the lymphoid organs of the NDE birds are consistent with lesions found previously in DCC (Wobeser et al., 1993; Banerjee et al., 1994; Meteyer et al., 1997) and domestic poultry (Alexander, 1997). Such lesions may have a variety of causes (Riddell, 1987), and therefore have little diagnostic value.

Comparison of histology, virus isolation, and serology to diagnose ND showed that each method was useful, but that none of them were positive in all 25 NDE cases (Table 4.5). In my opinion, diagnosis of ND in DCC is best attempted with a combination of methods. Exposure to NDV may be ascertained by serology, both in live and dead birds. Double-crested cormorant carcasses may be screened for ND by histological examination of the central nervous system and kidneys; multiple locations should be

examined, because lesions may have a restricted distribution. Lesions caused by ND may be differentiated from those caused by other diseases with immunohistochemistry. However, cross-reaction with other avian paramyxoviruses, particularly serotype 3, is possible in both immunohistochemistry—if a polyclonal antiserum is used—and serology (Alexander, 1997). Furthermore, it is not possible to determine the pathogenicity of the virus with these methods. Currently, the only unequivocal method of ND diagnosis, which also allows characterization of the infecting strain, is virus isolation (Alexander, 1997). Tissues for virus isolation should include kidney, brain and intestine. Identification of isolated viruses should not depend only on the HA test, because NDV isolates from DCC may be HA-negative. Because NDV virulence is related to the predicted amino acid sequence of the fusion protein cleavage site, the RT-PCR test, combined with nucleotide sequencing of the fusion gene coding for the cleavage site, may eventually result in a partial reduction in traditional live-animal testing for assessment of pathogenicity of NDV isolates (Seal et al., 1995). In addition, this test is useful to determine the epidemiological relationship between isolates. The RT-PCR test usually is done on viruses amplified in embryonated chicken eggs (e.g., Seal et al., 1995) , but it can also be performed directly on tissues of diseased DCC, as demonstrated in this study and in Chapter 6.

# 5. EXPERIMENTAL INFECTION OF DOUBLE-CRESTED CORMORANTS WITH PATHOGENIC NEWCASTLE DISEASE VIRUS[1]

## 5.1 Introduction

Pathogenic Newcastle disease virus (NDV) has caused epidemics in double-crested cormorants (Phalacrocorax auritus; DCC) in Canada and the USA several times in recent years (Cleary, 1977; Wobeser et al., 1993; Meteyer et al., 1997; Chapter 3). Pathogenic NDV is here defined as a NDV isolate with an intracerebral pathogenicity index in chickens of > 0.7 (Commission of the European Communities, 1993). Many aspects of Newcastle disease (ND) in DCC are unknown, and experimental infection of this species with NDV has not been reported. Here an experimental infection of DCC with a pathogenic NDV isolate is described. The objective was to monitor morbidity, mortality, immune response and virus excretion as a result of the infection.

## 5.2 Materials and methods

### 5.2.1 Birds and housing

Thirteen DCC (nos. 1 to 13), hatched between 9 and 23 June 1995, on Island A, Doré Lake, Saskatchewan, Canada, were captured on 19 or 27 June 1995, and raised in captivity at the Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada (Chapter 3). On 18 September 1995, the birds were moved to a biosafety level 3 containment area at the Animal Diseases Research Institute, Canadian Food Inspection Agency, Hull, Quebec, Canada.

---

[1]A version of this chapter was accepted for publication as Kuiken et al. (1998b).

### 5.2.2 Virus

A pathogenic NDV strain (PMV-1/cormorant/Saskatchewan-Canada/2035/95) was isolated from five DCC found during a ND epidemic on Doré Lake, Saskatchewan, Canada, in 1995. It had an intracerebral pathogenicity index of 1.61 and an intravenous pathogenicity index of 1.23 (Chapter 3). In total, the virus was passed three times through specific pathogen-free (SPF) chicken eggs containing a 9- to 10-day-old embryo before it was used to infect DCC in this experiment. A stock of virus was made by harvesting allantoic fluid from embryonated eggs, inoculated with the virus. This virus stock, which had a virus titer of $10^{9.5}$ $ELD_{50}$/ml, was divided into aliquots which were stored frozen at -70 C. To confirm that the virus in the stock had maintained its pathogenicity, 0.1 ml of a 1:10 dilution of the stock was injected into the brachial vein of each of 10 6-week-old SPF chickens. Within 10 days, 7 of the 10 chickens died and the remaining three chickens had nervous signs consistent with ND.

### 5.2.3 Vaccination

A 0.5 ml dose of an inactivated NDV vaccine (Newcavac, Intervet, Millsboro, Delaware, USA) was injected into the quadriceps muscle of one bird (no. 3) on 4 July and 8 August 1995.

### 5.2.4 Experimental design

At the beginning of the experiment, all 13 birds, 14 to 16 weeks old, were held in the same pen. On 26 September 1995, two birds (no. 1 and 2) were placed in a separate pen, hereafter referred to as the infected pen. They were each inoculated in the conjunctival sac and oral cavity with 0.05 ml of undiluted virus stock, and in the cloaca with a cotton swab dipped in undiluted virus stock.

On 5 October 1995, nine birds were moved to the infected pen. Seven of these (nos. 3 to 9) were inoculated as above; the remaining two (nos. 10 and 11) were not

inoculated.

On 12 October 1995, none of the birds had yet developed clinical signs consistent with ND, suggesting that the route of infection was incorrect or the dose was too low to cause ND. Therefore, one of the remaining uninfected birds (no. 12) was placed in the infected pen and 0.5 ml of a 1:10 dilution of the virus stock was injected into a brachial vein.

The remaining bird (no. 13) was kept separate as a negative control.

### 5.2.5 Clinical examination

From 26 September to 14 October 1995, birds were observed for about 15 minutes twice daily, usually around the time of feeding. Posture and ability to walk, fly, wash and feed were recorded on a clinical examination sheet, and any abnormal posture or behaviour was described. From 14 October onwards, the birds were observed once daily.

### 5.2.6 Serology

Serum samples were collected weekly from the time of capture until a bird was infected or placed in contact with infected birds. Thereafter, serum samples were collected at 4, 7, 11, 14, 21, 28, 49, and 70 days post infection or exposure (p.i). Serum samples were tested for the presence of hemagglutination inhibiting (HI) antibodies to NDV by use of the hemagglutination inhibition test (Beard, 1989b), starting at a serum dilution of 1:2, by the Virology Section, Animal Diseases Research Institute, Canadian Food Inspection Agency. Four to six hemagglutination units of NDV B1 strain were used as the antigen.

### 5.2.7 Virus isolation

Pharyngeal and cloacal swabs were collected just before each bird was infected or placed in contact with infected birds, and at 2, 4, 7, 11, 14, 21, 28 and 42 days p.i. After

collection, swabs were placed in tubes containing 2 ml of brain heart infusion (BHI) broth. The BHI broth consisted of 33.3 g/l BHI powder (BDH, Toronto, Ontario), 10% bovine serum, 10 x $10^6$ U/l penicillin and 10 g/l streptomycin in distilled water. After mixing, the swabs were squeezed out and removed from the tubes. Cloacal samples were centrifuged at 2000 G for 15 minutes at 4 C and the supernatants were harvested. The cloacal and pharyngeal samples were each mixed with 0.2 ml of an antibiotic solution, containing 1 mg/ml streptomycin, 1 mg/ml vancomycin, 500 $\mu$g/ml nystatin, and 500 $\mu$g/ml gentamycin in sterile phosphate-buffered saline (pH 7.2), left at room temperature for 1 hour, and centrifuged at 2000 G for 15 minutes at 4 C.

The supernatants were used for virus isolation procedures in embryonated SPF chicken eggs (Alexander, 1989). A 0.2 ml volume of supernatant was used per egg; 5 eggs were used per passage. When embryos died, harvested allantoic fluid was examined for the presence of hemagglutinating agents by use of the rapid plate agglutination (RPA) test (Beard, 1989a). The test was considered positive if hemagglutination was seen within 5 minutes. If a test was negative, it was repeated after a second passage through embryonated chicken eggs, as described above. One RPA test-positive cloacal sample per bird was tested for the presence of NDV by use of an indirect immunoperoxidase test (see below). Samples were considered negative if no embryos died during two passages and allantoic fluid harvested at the end of the second passage tested negative in the RPA test.

Virus isolation procedures also were carried out on individual tissues, collected from each bird at necropsy. A sample of each tissue (brain, trachea, lung, liver, kidney, spleen, jejunum, and femoral bone marrow) was homogenized with BHI broth in a Ten Broeck tissue grinder to obtain a 1:10 dilution. After centrifugation at 2000 G at 4 C for 20 minutes, the supernatant was harvested and diluted 9:10 with gentamycin (5 mg/ml) in sterile phosphate-buffered saline (pH 7.2). The diluted supernatant was held for 1 hour at room temperature, centrifuged at 2000 G at 4 C for 20 minutes and the supernatant was used for virus isolation and identification procedures as described above.

### 5.2.8 Indirect immunoperoxidase assay (IPA)

This assay was performed as described in Chapter 4.2.6.

### 5.2.9 Pathology

Eight birds (nos. 1, 3-5, 7, 8, 10, and 13) were killed by exposure to $CO_2$ on 27 October 1995, and the remaining five (2, 6, 9, 11, and 12) on 14 December 1995. Full necropsies were carried out, using a separate set of sterilized forceps, scissors, and scalpel for each bird and taking care to prevent cross-contamination between carcasses.

Samples of brain (cerebrum, cerebellum, and brain stem) from birds killed on 27 October were taken for histological examination. These were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5 $\mu$m, and stained with hematoxylin and eosin for examination by light microscopy (Luna, 1968). Selected sections were examined for the presence of NDV antigen by use of an avidin-biotin complex immunoperoxidase method (Chapter 4).

## 5.3 Results

Four birds developed transient clinical signs consistent with ND: bird no. 2 between 23 and 27 days p.i., bird no. 4 at 12 to 16 days p.i., bird no. 5 at 17 days p.i., and bird no. 10 at 14 days p.i. All four birds had ataxia, characterized by walking with irregular steps, falling down, and using the tail to support part of the body weight. Birds no. 2 and 4 also had hypermetria, evident as lifting the feet higher than normal when walking, so-called "goose-stepping", and hopping past, instead of onto, a branch. Bird no. 2 had head tremors.

None of the non-vaccinated birds had a detectable HI antibody titer to NDV until 7 days p.i., when 7 of 11 birds tested positive. By 11 days p.i., all 11 birds tested positive and remained so until they were killed. The mean HI antibody titer to NDV in non-vaccinated birds reached a maximum of 1:630 (range: 1:128 to 1:2048) at 21 days p.i. and

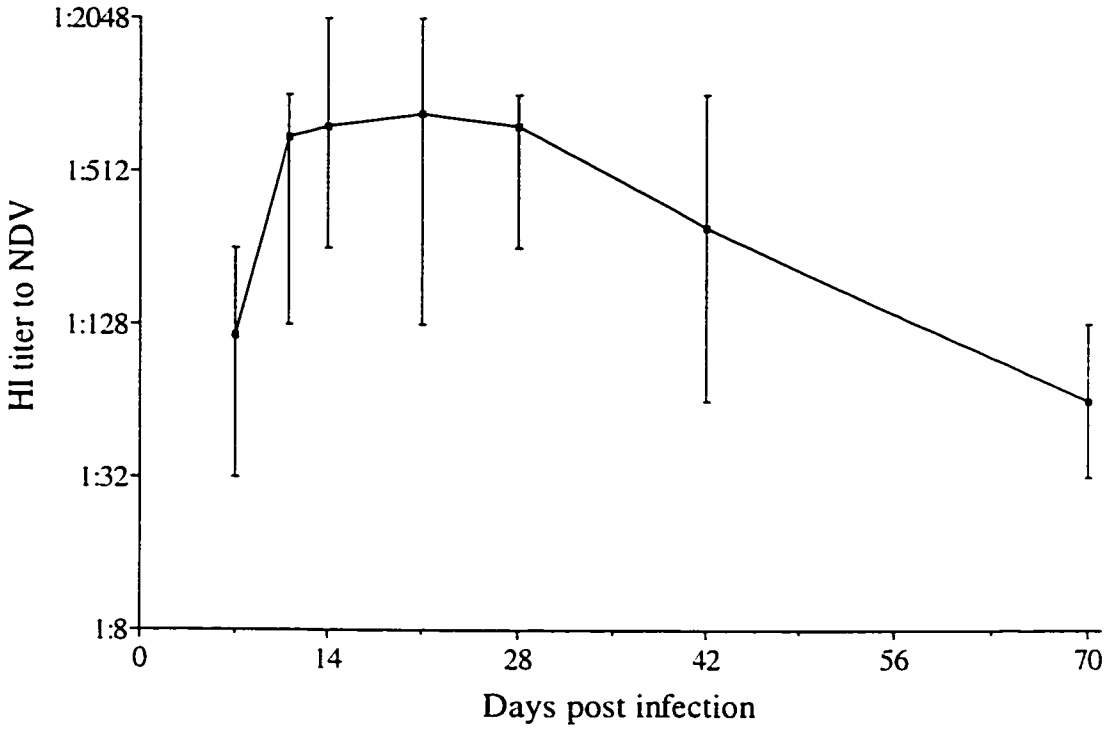



**Figure 5.1** Hemagglutination inhibiting antibody titers to Newcastle disease virus (mean and range) in double-crested cormorants infected with pathogenic Newcastle disease virus. n = 11 from 0 to 21 days post infection; n = 5 from 28 to 70 days post infection.

gradually declined thereafter to 1:56 (range: 1:32 to 1:128) at 70 days p.i. (Fig. 5.1). There was no apparent difference in immune response among birds infected by different methods, including by contact with inoculated birds. The vaccinated bird had a titer of 1:128 at the time of infection, which rose to 1:512 at 7 days p.i., reached a maximum of 1:1024 at 14 days p.i. and remained at this level until the bird was killed. The negative control bird tested negative for HI antibody to NDV throughout the study.

Newcastle disease virus was detected in pharyngeal and cloacal swabs of all non-vaccinated birds (Fig. 5.2). The duration of virus excretion by non-vaccinated birds was 7 ± 2.6 days p.i. (mean ± standard deviation) from the pharynx, and 15 ± 6.2 days p.i. from the cloaca. Newcastle disease virus was last detected at 14 days p.i. in a pharyngeal swab from bird no. 6, and at 28 days p.i. in a cloacal swab from bird no. 9. There was no apparent difference in the pattern of virus excretion among birds infected by different methods, including by contact with inoculated birds. Newcastle disease virus was not isolated from swabs of the vaccinated bird except from a pharyngeal swab collected at 4 days p.i. Newcastle disease virus was not isolated from any swabs of the negative control bird, nor from any tissues collected at necropsy from the 13 birds 22 to 79 days p.i.

A cerebral section from bird no. 5 had multiple foci of gliosis, perivascular infiltration with mononuclear cells, and endothelial hypertrophy. A few neurons in these areas had eosinophilic cytoplasm and no nucleus. On immunohistochemical examination, these areas did not have specific staining for NDV.

## 5.4 Discussion

No mortality occurred in the experimentally infected DCC. This is surprising because the isolate was obtained from an epidemic which killed approximately 32 to 64% of juvenile DCC at the breeding colony from which the experimental birds originated (Chapter 3). Even the intravenously inoculated DCC did not die, although it was inoculated with a dose that was 5 x higher than the dose which killed 7 of 10 chickens and caused disease in all 10. An important difference between the juvenile DCC that were infected naturally on Doré Lake and those in the laboratory was age of infection; most

DCC on Doré Lake were about 6 weeks old when they were infected, compared to about 16 weeks old for the experimentally-infected DCC. In general, the pathogenicity of NDV decreases with the age of the bird (Alexander, 1997). Age-related resistance to NDV infection may also explain why ND has never been diagnosed in adult DCC, despite close contact between affected juveniles and adult birds (e.g., Meteyer et al., 1997; Chapter 3).

Morbidity was limited to transient ataxia observed in four DCC. These nervous signs correspond with those seen in free-living DCC with ND and may be attributed mainly to cerebellar lesions (Clippinger et al., 1996). The ataxia probably would have had more serious consequences in free-living birds, through predation and decreased ability to feed (Meteyer et al., 1997; Chapter 3).

The immune response to NDV infection in these DCC corresponds to that seen in chickens (Alexander, 1997). If one extrapolates, HI antibody titers to NDV would become undetectable (< 1:2) by about 18 weeks p.i. (Fig. 5.1). This suggests that DCC infected with NDV during the breeding season in which they hatched would not have detectable HI antibody to NDV in the following breeding season. The usual age of initial breeding in DCC is probably 3 years, although successful breeding has occurred among 2-year-olds (Palmer, 1962). Thus, any HI antibody to NDV found in serum of breeding DCC or in yolk of their eggs (Wobeser et al., 1993; Chapter 3) indicates exposure to NDV or a related avian paramyxovirus after the year of hatching.

The maximum length of NDV excretion in these birds was 28 days. In theory, it is possible that this bird, which was infected on 5 October, was reinfected on 12 October by bird no. 12, so that the maximum length of excretion was 21 instead of 28 days. These results correspond to those of MacPherson (1956), who isolated NDV from two juvenile great cormorants (P. carbo) 32 days after they had been infected intranasally with 1 ml of allantoic fluid containing pathogenic NDV (Herts strain).

The neuronal necrosis and non-suppurative encephalitis found in the cerebrum of one DCC were probably caused by the experimental NDV infection, because their appearance was characteristic for ND in DCC (Meteyer et al., 1997; Chapter 4).This tissue may have been negative for NDV by immunohistochemistry and virus isolation because virus was no longer present at 22 days p.i. In chickens with neurotropic NDV

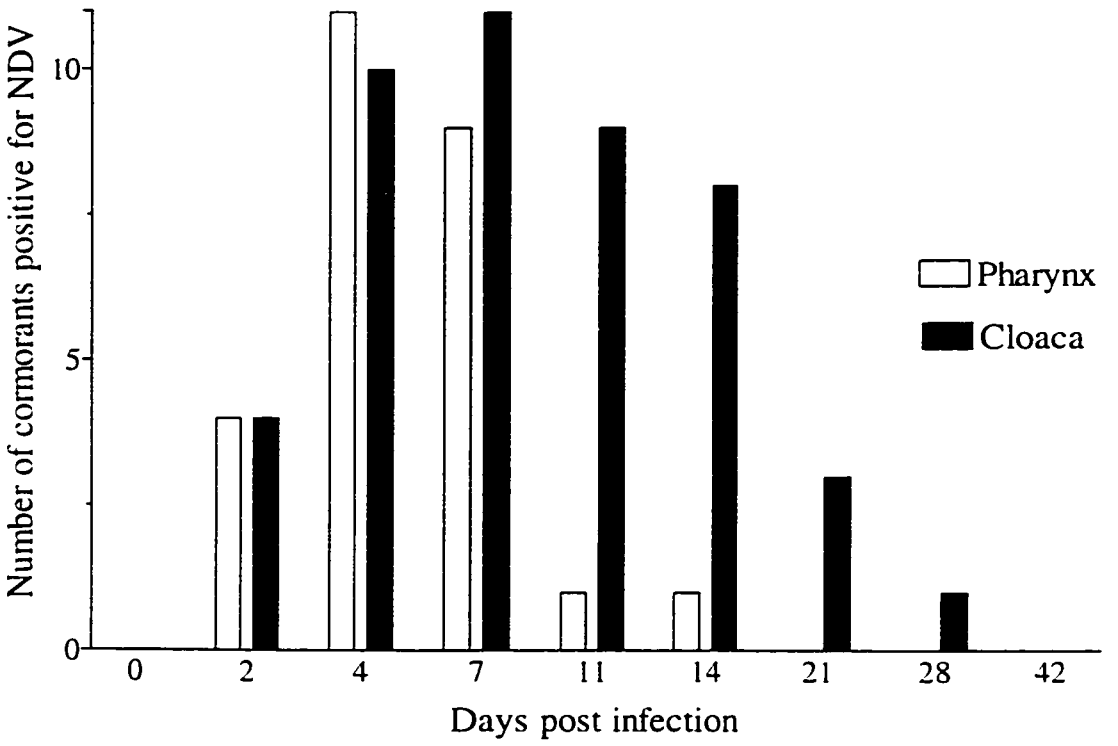



**Figure 5.2** Newcastle disease virus excretion by double-crested cormorants infected with pathogenic Newcastle disease virus. n = 11 from 0 to 21 days post infection; n = 5 at 28 and 42 days post infection.

infection, NDV was not detectable in the brain by immunofluorescence later than 8 days p.i., nor by virus isolation 1 month p.i. (Wilczynski et al., 1977). Alternatively, the lesions, which are not pathognomonic for ND (Meteyer et al., 1997; Chapter 4), had another cause.

It is unknown how NDV is maintained between epidemics, which have been recognized in DCC every 2 or 3 years since 1990. It has been speculated that DCC are infected by psittacines, or that the virus is maintained in the migrating DCC population (Seal et al., 1995; Meteyer et al., 1997). The results of this study show that DCC can shed pathogenic NDV for several weeks without clinical signs of ND; therefore, the DCC population could be a maintenance reservoir for NDV through serial infection of susceptible birds. This is consistent with the work of Heckert et al. (1996) on NDV isolates from DCC, in which they showed that isolates from different geographical locations in 1990 and 1992 were identical by nucleotide sequencing.

The greatest risk of spread of NDV from DCC to poultry is likely at the end of the breeding season. At this time, some DCC (mostly young) disperse in any direction from their colony site, and loiter in bays and estuaries for 2 to 6 weeks before starting southward migration (Palmer, 1962). The Interior population of DCC, which is centred on the Canadian prairie provinces and the Great Lakes area and in which most recorded ND epidemics have occurred, winters mainly in the Gulf of Mexico, from Texas to North Carolina. Large numbers of DCC can be seen from late August to early November at intermediate points (Hatch, 1995). This post-breeding dispersal and migration would allow infected juveniles to spread the virus over a large area surrounding breeding sites and along migration routes to the wintering grounds.

Poultry kept outside, such as range turkeys, are especially at risk of coming into contact with DCC or with their excreta. This was thought to be the cause of an outbreak of ND in a flock of domestic range turkeys located near a DCC colony in North Dakota in 1992 (Mixson & Pearson, 1992; Heckert et al., 1996). In situations where poultry farms use water from lakes or rivers on which DCC reside, virus might be introduced by water. Contamination of water with waterfowl excreta was considered the most likely origin of NDV in 1990 in a turkey flock from Northern Ireland (Graham et al., 1996).

# 6. DETECTION OF NEWCASTLE DISEASE VIRUS BY REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION TEST

## 6.1 Introduction

Newcastle disease (ND) is an infectious disease of birds, caused by Newcastle disease virus (NDV), also called avian paramyxovirus serotype 1 (PMV-1). Currently, the only unequivocal method for diagnosis of ND is virus isolation in combination with assessment of the pathogenicity of the infecting strain. Virus isolation usually is done in the allantoic cavity of embryonated chicken eggs, and may take several weeks (Alexander, 1997). This method requires fresh specimens, is slow, expensive, and labour-intensive. A potential alternative method is based on the polymerase chain reaction (PCR), which is being used increasingly in human and veterinary medicine for the diagnosis of infectious diseases. For RNA viruses such as NDV, a reverse transcriptase (RT) step to convert RNA to a DNA copy precedes the PCR. In comparison with traditional virus isolation techniques, PCR-based methods can be rapid, sensitive, specific, cheap, and used on partly degraded specimens (Belák and Ballagi-Pordány, 1993; Pfeffer et al., 1995).

Although RT-PCR has been used extensively to determine the nucleotide sequence of parts of the NDV genome (e.g. Collins et al., 1993; Seal et al., 1995; Heckert et al., 1996), there are few reports of its use to detect NDV in diagnostic samples from birds suspected of ND. As a preliminary step to using RT-PCR as a diagnostic method in organs, Jestin and Jestin (1991) applied a RT-PCR test, using primers spanning the nucleotide sequence coding for the cleavage site of the fusion glycoprotein precursor, to allantoic fluid samples infected with different serotypes of PMV. The test was considered specific (i.e., it only reacted positively with NDV), although the only other viruses tested

were strains of PMV-2, -3, and -4. The detection limit of the test was not determined. Stäuber et al. (1995) used a RT-PCR test, again with primers spanning the cleavage site of the fusion gene, to detect NDV in NDV vaccines. The test also was used successfully on clinical samples of NDV-infected birds, but results were not shown. The specificity was tested by use of vaccines against other poultry diseases, but it was not stated which ones, nor were results of these tests shown. The detection limit of the RT-PCR test in live vaccines was reported as $5 \times 10^2$ mean embryo infective dose ($EID_{50}$) per test on 2.5 µl of sample, i.e., $2 \times 10^5$ $EID_{50}$ per ml sample. However, this figure was based on the vaccine manufacturer's statement that "live vaccine preparations contained at least $10^6$ $EID_{50}$ NDV per dose", so the actual detection limit may have been higher.

In this study, a RT-PCR test was used to detect NDV in allantoic fluid, organs, and cloacal swabs. The main objective was to develop a test that distinguished NDV from other embryo-lethal hemagglutinating viruses such as avian influenza virus (AIV), could be used on various diagnostic samples, and had a detection limit at least as low as virus isolation.

## 6.2 Materials and methods

### 6.2.1 Samples

Allantoic fluid samples containing > 8 hemagglutinating units per ml of one of the following embryo-lethal hemagglutinating viruses were provided by the Virology Section, Animal Diseases Research Institute, Agriculture Canada, Hull, Quebec: eight reference strains and 16 field isolates of PMV, 14 reference strains of AIV, a strain of turkey rhinotracheitis virus, and a strain of avian adenovirus (Tables 6.1 and 6.2). These samples were obtained by harvesting allantoic fluid from embryonated, specific pathogen-free (SPF) chicken eggs infected with a given virus strain (Alexander, 1989). Negative control allantoic fluid samples were harvested from non-infected embryonated SPF chickens; attempts to isolate virus from these samples failed (Chapter 5.2.7).

Chicken tissues containing PMV-1/GB Texas were obtained from SPF chickens

**Table 6.1 Results of the reverse transcriptase-polymerase chain reaction (RT-PCR) test at different annealing temperatures with reference strains of avian paramyxovirus (PMV), avian influenza virus (AIV), turkey rhinotracheitis virus (TRV) and avian adenovirus (AAV).**

| No. | Virus | Name of strain | RT-PCR result per annealing temp. (C) | | | |
|---|---|---|---|---|---|---|
| | | | 53 | 56 | 59 | 62 |
| PMV serotype (pathogenicity) | | | | | | |
| a | 1 (velogenic) | GB Texas | +[a] | + | + | + |
| b | 1 (mesogenic) | Roakin | + | + | + | + |
| c | 1 (lentogenic) | B1 | + | + | + | + |
| d | 2 | chicken/California/Yucaipa/56 | + | + | -[b] | [c] |
| e | 3 | parakeet/Netherlands/449/75 | + | + | + | + |
| f | 4 | duck/HongKong/03/75 | + | - | | |
| g | 6 | duck/HongKong/199/77 | - | | | |
| h | 8 | goose/Delaware/1058/76 | - | | | |
| AIV subtype | | | | | | |
| i | H1N1 | A/swine/Iowa/31 | - | | | |
| j | H2N3 | A/mallard/Alabama/77/77 | - | | | |
| k | H3N8 | A/duck/Ukraine/1/63 | - | | | |
| l | H4N6 | A/duck/Czechoslovakia/56 | - | | | |
| m | H5N2 | A/quail/Oregon/20719/86 | + | + | + | + |
| n | H6N | A/willet/Ontario/3724/63 | - | | | |
| o | H7N3 | A/turkey/Minnesota/29206/83 | - | | | |
| p | H8N4 | A/turkey/Ontario/6118/167 | - | | | |
| q | H9N2 | A/turkey/Minnesota/12877/1285/81 | - | | | |
| r | H10N7 | A/chicken/Germany/N/49 | - | | | |
| s | H11N9 | A/duck/Memphis/546/74 | - | | | |
| t | H12N5 | A/duck/Alberta/60/76 | + | + | - | |
| u | H13N6 | A/gull/Maryland/704/77 | - | | | |
| v | H14N5 | A/mallard/Gurjev/263/82 | - | | | |
| w | TRV | B1994 | - | | | |
| x | AAV | chicken/127/82 | - | | | |

[a]Positive.
[b]Negative.
[c]Not tested.

**Table 6.2 Results of the reverse transcriptase-polymerase chain reaction (RT-PCR) test at an annealing temperature of 53 C with field isolates of avian paramyxovirus (PMV).**

| Serotype (pathogenicity) | Host species | Location from where submitted | Laboratory number | RT-PCR result |
|---|---|---|---|---|
| PMV-1 (velogenic) | pelican | Saskatchewan | 90-DC-1478 | +[a] |
| PMV-1 (velogenic) | cormorant | Ontario | 95-DC-2345 | + |
| PMV-1 (velogenic) | parrot | Quebec | 90-X-988 | + |
| PMV-1 (mesogenic) | duck | Ontario | 93-DC-1348 | + |
| PMV-1 (mesogenic) | peregrine falcon | Canada | 94-DC-3306 | + |
| PMV-1 (lentogenic) | duck | Ontario | 93-DC-1647 | + |
| PMV-1 (lentogenic) | turkey | Ontario | 94-DC-0691 | + |
| PMV-1 lentogenic | pigeon | Ontario | 94-DC-1435 | + |
| PMV-1 (lentogenic) | chicken | Ontario | 95-DC-0085 | + |
| PMV-1 (lentogenic) | rock dove | Canada | 95-DC-0655 | + |
| PMV-2 | cordon bleu | Canada (import) | 92-IM-2925 | -[b] |
| PMV-2 | pigeon | Ontario | 93-DC-1347 | - |
| PMV-2 | finch | Quebec (import) | 95-IM-1373 | - |
| PMV-3 | budgerigar | Ontario | 83-X-4542 | + |
| PMV-3 | cockatiel | Ontario | 94-DC-3028 | + |
| PMV-3 | finch | Alberta | 95-DC-0227A | - |
| PMV-3 | parakeet | Quebec (import) | 95-IM-1047 | - |

[a]Positive.
[b]Negative.

that had died of ND after inoculation in the nares and conjunctival sacs with $10^4$ Egg Lethal $Dose_{50}$ ($ELD_{50}$) of the above virus. The concentration of the virus in the brain ($ELD_{50} = 10^{3.5}$/g), lung ($ELD_{50} = 10^{6.2}$/g), liver ($ELD_{50} = 10^{4.2}$/g), and spleen ($ELD_{50} < 10^2$/g) was determined by virus titration in embryonated chicken eggs (Chapter 4.2.7). Negative control tissues were obtained from non-infected SPF chickens; attempts to isolate virus from these samples failed.

Double-crested cormorant (Phalacrocorax auritus; DCC) tissues containing PMV-1/cormorant/Saskatchewan-Canada/2035/95 were obtained from a free-living DCC that had died of ND (Chapters 3 and 4). The concentration of the virus in the brain ($ELD_{50} = 10^{3.3}$/g), kidney ($ELD_{50} = 10^{4.7}$/g), and jejunum ($ELD_{50} = 10^{2.2}$/g) was determined as above. Negative control tissues were obtained from a captive-raised DCC without detectable hemagglutination inhibiting (HI) antibody to NDV (Beard, 1989b) in its serum; attempts to isolate virus from these tissues failed.

Cloacal swabs containing PMV-1/cormorant/Saskatchewan-Canada/2035/95 were obtained from three captive-raised DCC sampled 0 to 48 days after inoculation in the conjunctival sac and oral cavity with $2 \times 10^8$ $ELD_{50}$ of the above virus (Table 6.3). The presence of NDV in these swabs was determined by virus isolation in combination with an indirect immunoperoxidase test (Chapter 5).

In all RT-PCR tests, allantoic fluid containing PMV-1/GB Texas or PMV-1/cormorant/Saskatchewan-Canada/1480/90 was used as a positive control.

### 6.2.2 Reverse transcriptase-polymerase chain reaction test

Samples were diluted 1:10 in bovine heart infusion (BHI) broth and incubated with 0.02 mg/ml of proteinase K and 1% sodium dodecyl sulfate (final concentrations) for 2 hours at 56 C to extract the nucleic acid. The BHI broth consisted of 33.3 mg/ml BHI powder (BDH, Toronto, Ontario, Canada), 10% bovine serum, $10 \times 10^3$ U/ml penicillin and 10 mg/ml streptomycin in distilled water. After RNA extraction with phenol-chloroform-isoamyl alcohol and concentration with ethanol, the nucleic acid sample was centrifuged in vacuum and the resulting pellet was resuspended in 40 µl of

**Table 6.3 Comparison of results of virus isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) test on cloacal swabs of three double-crested cormorants infected with Newcastle disease virus.**

| Cormorant | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| A | | | B | | | C | | |
| Days after infection | Virus isolation result | RT-PCR result | Days after infection | Virus isolation result | RT-PCR result | Days after infection | Virus isolation result | RT-PCR result |
| 0 | -[a] | - | 0 | - | - | 0 | - | - |
| 3 | - | - | 2 | - | - | 2 | +[b] | + |
| 6 | + | + | 4 | + | - | 4 | + | + |
| 9 | + | + | 7 | + | + | 7 | + | + |
| 11 | + | + | 11 | + | - | 11 | + | - |
| 13 | + | - | 14 | + | - | 14 | + | - |
| 20 | + | - | 21 | - | - | 21 | - | - |
| 27 | - | - | 28 | - | - | 35 | - | - |
| 34 | - | - | 42 | - | - | | | |
| 48 | - | - | | | | | | |

[a]Negative.
[b]Positive.

distilled water per ml of 10% tissue suspension. Of this, 5 µl was used per RT-PCR test.

For the RT step, a mixture of the following reagents was added to each 5 µl sample of nucleic acid suspension: 1 µl of 40 U/µl recombinant RNAsin ribonuclease inhibitor (Promega, Madison, Wisconsin, USA), 0.33 µl of 3 µg/µl random primers (GibcoBRL, Burlington, Ontario, Canada), 2 µl of 0.1 M dithiothreitol (GibcoBRL, Burlington, Ontario, Canada), 4 µl of 5X First Strand Buffer (GibcoBRL, Burlington, Ontario, Canada), 1 µl each of 10 mM deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate (Perkin Elmer, Branchburg, New Jersey, USA), 1 µl of 200 U/µl Moloney murine leukemia virus reverse transcriptase (GibcoBRL, Burlington, Ontario, Canada), and 2.66 µl distilled water. This mixture was covered with a layer of 50 µl mineral oil and placed in a Thermolyne (Dubuque, Iowa, USA) or Coy (Ann Arbor, Michigan, USA) thermal cycler. The mixture was incubated for 60 minutes at 37 C, the enzyme was denatured by heating for 5 minutes at 95 C, and the mixture was maintained for maximally 60 minutes at 80 C until the PCR step.

For the PCR step, a mixture of the following reagents was added to the above sample: 3 µl of 10X PCR buffer II (Perkin Elmer, Branchburg, New Jersey, USA), 3.6 µl of 25 mM $MgCl_2$ (Perkin Elmer, Branchburg, New Jersey, USA), 1 µl each of 10 mM deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate (Perkin Elmer, Branchburg, New Jersey, USA), 5 µl of 10 pmol/µl primer 2 (5'-AGTCGGAGGATGTTGGCAGC-3'), 5 µl of 10 pmol/µl primer 7 (5'-TTAGAAAAAACACGGGTAGAA-3'), both synthesized in a Oligo 1000 DNA synthesizer (Beckman Instruments, Palo Alto, California, USA), 0.25 µl of 5 U/µl Amplitaq DNA polymerase (Perkin Elmer, Branchburg, New Jersey, USA), and 9.15 µl distilled water. The primers were chosen to span the first part of the fusion gene, including the nucleotide sequence coding for the cleavage site of the precursor fusion glycoprotein. The PCR protocol consisted of 40 cycles of denaturation for 1 minute at 94 C, primer annealing for 1 minute at 53 C, and elongation for 2 minutes at 72 C. Ten µl of the resulting RT-PCR products were loaded onto a 1% agarose (BRL, Bethesda, Maryland, USA) gel containing 0.5 µg/ml ethidium bromide. After electrophoresis of the

gel in Tris-acetate-EDTA buffer for 2 hours at 50 V, the bands were visualized in ultraviolet light and compared with a λ DNA/HindIII marker (GibcoBRL, Burlington, Ontario, Canada).

To confirm their identity, RT-PCR products generated from the brain and jejunum of the free-living DCC with ND were purified by electrophoresis in LMP agarose (GibcoBRL, Burlington, Ontario, Canada), followed by DNA extraction using the Magic PCR-Prep system (Promega, Madison, Wisconsin, USA). Nucleotide sequencing was carried out by use of Amplitaq DNA polymerase (Perkin Elmer, Branchburg, New Jersey, USA), primer 7 (5'-TTAGAAAAAACACGGGTAGAA-3'), and an automated nucleic acid sequencer by the Virology Section, Animal Diseases Research Institute, Canadian Food Inspection Agency. Nucleotide sequence editing, analysis, prediction of amino acid sequences, and alignments were carried out with GeneWorks 2.5.1 software (Oxford Molecular Ltd., Oxford, UK).

The optimal concentration of $MgCl_2$ in the reaction mix was determined by carrying out the RT-PCR test on allantoic fluid containing PMV-1/pelican/Saskatchewan-Canada/1478/90 at different $MgCl_2$ concentrations (0.5, 1, 2, 3, and 4 mM). The specificity (i.e., the characteristic of only reacting positively with PMV-1) was determined by carrying out the RT-PCR test on reference strains of avian paramyxovirus and AIV (Table 6.1) at different annealing temperatures. Initially, the RT-PCR test was carried out at an annealing temperature of 53 C. In subsequent tests, the annealing temperature was increased by 3 C steps (up to 62 C) to increase the specificity. The detection limit was determined by carrying out the RT-PCR test on serial ten-fold dilutions of allantoic fluid containing PMV-1/cormorant/Saskatchewan-Canada/2035/95 ($ELD_{50} = 10^{9.5}$ per ml fluid) or chicken lung containing PMV-1/chicken/GB Texas/48 ($ELD_{50} = 10^{6.2}$ per g tissue) in 0.01 M phosphate-buffered saline solution (pH 7.4). The performance on different tissues was determined by carrying out the RT-PCR test on brain, lung, liver, spleen, kidney, jejunum, and cloacal material of DCC or chickens infected with NDV.

Because the RT-PCR test reacted positively with AIV A/quail/Oregon/20719/86 (H5N2) and AIV A/duck/Alberta/60/76 (H12N5), the nucleotide sequence of both

primers was compared with nucleotide sequences in the database of the National Center for Biotechnology Information (GenBank), including nucleotide sequences of part of the hemagglutinin gene of various H5N2 subtypes (but not of AIV A/quail/Oregon/20719/86), and of part of the hemagglutinin gene of AIV A/duck/Alberta/60/76, by use of a protein database search program (BLAST version 2.0: Altschul et al., 1997).

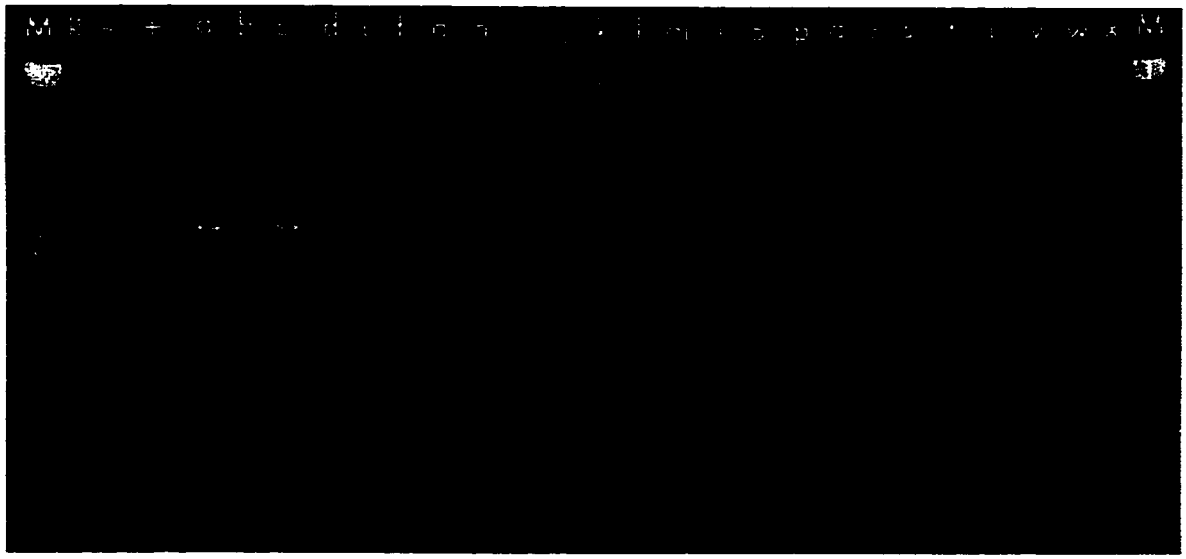
## 6.3 Results

The RT-PCR test on PMV-1 isolates generated a product of approximately 564 base pairs (Fig. 6.1), which matches the predicted size based on the location of the primers (Collins et al., 1996). Part of the nucleotide sequence was $^{370}$ATCTAGAGGAAGGAGACAGAAACGTTTTGTAGGTGCT$^{406}$, with the predicted amino acid sequence $^{109}$SRGRRQKRFVGA$^{120}$, which corresponds to the fusion protein cleavage site of NDV (Seal et al., 1995), and confirms that the RT-PCR products were generated from PMV-1 genome in the predicted region.

The RT-PCR tests with reaction mixes containing 1 and 2 mM $MgCl_2$ generated the most product. Therefore, the rest of the RT-PCR tests were carried out with reaction mixes containing 1.8 mM $MgCl_2$, which was the standard concentration in the laboratory where this study was done.

All reference strains and field isolates of PMV-1 reacted positively in the RT-PCR test at an annealing temperature of 53 C (Tables 6.1 and 6.2; Fig. 6.1). In addition, the reference strains of PMV-2, PMV-3, and PMV-4, and two reference strains of AIV reacted positively. At higher annealing temperatures, progressively fewer non-PMV-1 strains reacted positively, but two still reacted positively at an annealing temperature of 62 C. The negative control allantoic fluid samples had a weakly positive reaction in the RT-PCR tests at annealing temperatures of 59 and 62 C. The three reference strains of PMV-1 continued to react positively at annealing temperatures > 53 C, but the staining intensity of the bands decreased. Therefore, the rest of the RT-PCR tests were carried out at an annealing temperature of 53 C.

**Figure 6.1 Results of the reverse transcriptase-polymerase chain reaction test at an annealing temperature of 53 C on reference strains of avian paramyxovirus and avian influenza virus, a turkey rhinotracheitis virus strain, and an avian adenovirus strain: lane M = marker, lane R = reagent control, lane - = negative control, lane + = positive control, lanes a-x correspond to the letters of the virus strains in Table 6.1. Arrowhead = 564 bp fragment.**



The $10^{-3}$ dilution of NDV-infected chicken lung, corresponding to a virus concentration of $10^{3.2}$ $ELD_{50}$ per g tissue, was the lowest concentration that reacted positively in the RT-PCR test (Fig. 6.2A). The negative control lung sample and the $10^{-10}$ dilution of infected chicken lung had a weakly positive reaction. The $10^{-5}$ dilution of NDV-infected allantoic fluid, corresponding to a virus concentration of $10^{4.5}$ $ELD_{50}$ per ml fluid, was the lowest concentration that reacted positively in the RT-PCR test. The negative control allantoic fluid sample had a weakly positive reaction.

All NDV-infected chicken and DCC tissues reacted positively in the RT-PCR test, and all negative control tissues reacted negatively (Figs. 6.2B and 6.2C). However, the band generated from the NDV-infected DCC kidney was weak relative to the virus concentration in the sample, and there was a large amount of DNA < 564 bp long in the negative control DCC kidney and jejunum samples.

Seven of 14 (50%) cloacal swabs, from which NDV was isolated, reacted positively in the RT-PCR test, and 13/13 (100%) cloacal swabs, from which NDV was not isolated, reacted negatively (Table 6.3).

There were no significant alignments (score > 26 bits, corresponding with 13/20 or 13/21 matching nucleotides) with sequences of AIV for either primer in the BLAST search of GenBank, indicating that hybridization of these primers with nucleotide sequences of AIV was unlikely.

## 6.4 Discussion

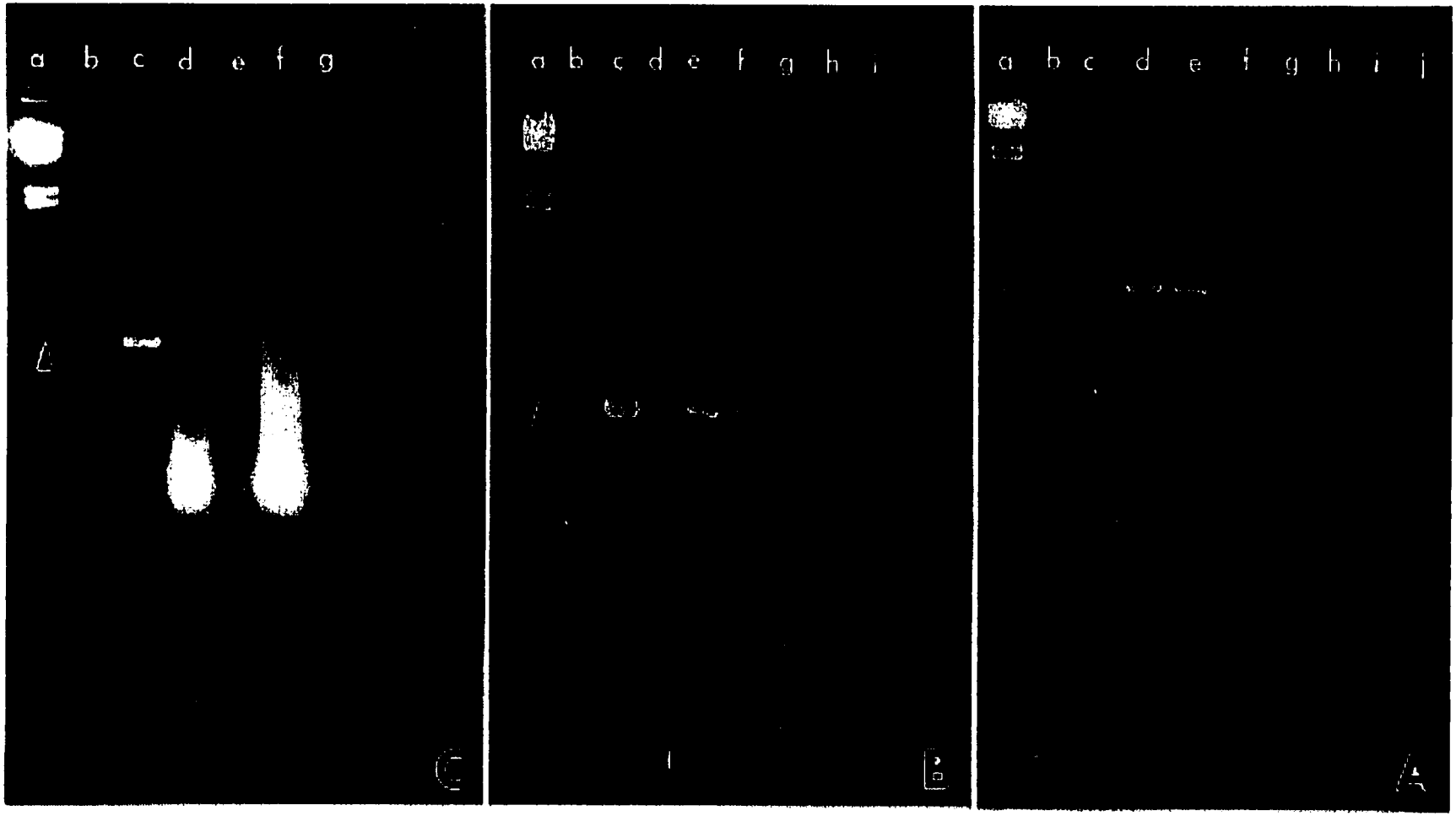
The development of a NDV-specific RT-PCR test for use with diagnostic specimens and with a detection limit at least as low as virus isolation was not successful. Although the RT-PCR test detected NDV in all types of samples tested, there was cross-reaction with other PMV serotypes and with AIV, and the test had a higher detection limit than virus isolation.

The cross-reaction of the RT-PCR test with PMV-2, -3, and -4 (Table 6.1) is not unexpected, given the phylogenic relationships between these serotypes (Alexander, 1997). The cross-reaction with two subtypes of AIV is more surprising, because

**Figure 6.2A Results of the reverse transcriptase-polymerase chain reaction test at an annealing temperature of 53 C on serial ten-fold dilutions of chicken lung infected with Newcastle disease virus (NDV): lane a = marker, lane b = reagent control, lane c = NDV-negative chicken lung, lane d = positive control, lanes e-j = $10^{-2}$ to $10^{-7}$ dilutions of NDV-infected chicken lung. Arrowhead = 564 bp fragment.**

**Figure 6.2B Results of the RT-PCR test at an annealing temperature of 53 C on chicken tissues infected with Newcastle disease virus (NDV): lane a = marker, lane b = reagent control, lane c = positive control, lane d = NDV-negative (-) chicken lung, lane e = NDV-positive (+) chicken lung, lane f = - chicken liver, lane g = + chicken liver, lane h = - chicken spleen, lane i = + chicken spleen. Arrowhead = 564 bp fragment.**

**Figure 6.2C Results of the RT-PCR test at an annealing temperature of 53 C on double-crested cormorant tissues infected with Newcastle disease virus (NDV): lane a = marker, lane b = NDV-negative (-) cormorant brain, lane c =NDV-positive (+) cormorant brain, lane d = - cormorant kidney, lane e = + cormorant kidney, lane f = - cormorant jejunum, lane g = + cormorant jejunum. Arrowhead = 564 bp fragment.**


a b c d e f g
a b c d e f g h i
a b c d e f g h i j

comparison of the nucleotide sequences of the primers with published nucleotide sequences of AIV provided no evidence of homology. It is more likely that the positive results with these AIV subtypes occurred because the samples contained PMV RNA due to cross-contamination, either before or during the RT-PCR procedure. Nucleotide sequencing of the RT-PCR products from the tests on AIV would have confirmed whether this was the case.

The main problem in this study was false-positive results, demonstrated by positive results with negative control samples in some of the experiments; the results of these experiments were invalid. False-positive results were probably due to cross-contamination with amplified DNA from one experiment to another. This carry-over has been termed the Achilles heel of the PCR, and is the main reason why it is routinely performed as a clinical service in only a few laboratories (Belák and Ballagi-Pordány, 1993). Despite applying most of the guidelines suggested to avoid false positives (Kwok and Higuchi, 1989), they still occurred in this study.

The RT-PCR test had the lowest detection limit with NDV-infected DCC spleen ($< 10^2$ $ELD_{50}$ per g tissue). This was at least 2 x $10^3$ times lower than the RT-PCR test of Stäuber et al. (1995) on live NDV vaccines, but up to 10 times higher than virus isolation. The detection limit of the RT-PCR test in this study was higher still with NDV-infected chicken lung and allantoic fluid. In principle, virus isolation allows detection of 10 $ELD_{50}$ NDV per g, given the inoculation of each of 5 embryonated chicken eggs with 0.2 ml of a 10% suspension of diagnostic sample. An important reason for the lower detection limit of virus isolation probably is the 200 times larger sample size: 100 mg per test for virus isolation compared to 0.5 mg for RT-PCR. The detection limit of the RT-PCR test may be decreased by using a protein-less buffer instead of BHI broth to dilute samples. In this study, BHI broth was used because it was the standard diluent for virus isolation (Chapter 5.2.7).

Despite the high detection limit of the RT-PCR test for NDV relative to virus isolation, and cross-reaction with other PMV serotypes, this test may be useful in some research applications, for example the rapid and inexpensive screening of a large number of samples for the presence of NDV. However, positive results from such a survey would

need to be verified by virus isolation in combination with serotyping to distinguish between NDV and other PMV serotypes (Alexander, 1997).

# 7. PATHOLOGY OF CAUSES OF MORTALITY AND DISEASES, OTHER THAN NEWCASTLE DISEASE, IN DOUBLE-CRESTED CORMORANTS [1]

## 7.1 Introduction

Little is known about the pathology of causes of mortality and diseases, other than Newcastle disease, in double-crested cormorants (Phalacrocorax auritus; DCC). In a recent review of the published literature on DCC, Erwin (1995) pointed out that research efforts had been skewed largely toward nesting and feeding ecology during the breeding season, and that disease and parasites had received very little attention. Because of the recent publilcation of studies on pathology of Newcastle disease in DCC (e.g., Wobeser et al., 1993; Meteyer et al., 1997; Chapter 4), the lack of such studies on pathology of other diseases in this species has only been accentuated.

The most extensive pathological study in DCC, excluding Newcastle disease, is of embryonal abnormalities, including subcutaneous edema, ascites, hemorrhage, gastroschisis, and bill defects (Ludwig et al., 1996). Bill defects in DCC nestlings also have been illustrated (Fox et al., 1991). The pathology of infections with the bacterium Pasteurella multocida (Mutalib and Hanson, 1989), and the trematode Amphimerus elongatus (Pense and Childs, 1972), in adult DCC have been described. Lewis (1929) mentioned an ovoid tumor "about the size of a pullet's egg" in the thoracic cavity of a DCC.

As part of a study of the reproductive success and disease of a DCC breeding colony in Saskatchewan from 1994 to 1996, DCC were observed throughout the breeding

---

[1]A version of this chapter, combined with a version of chapter 8, was submitted for publication as Kuiken et al. (1998d).

season for morbidity and death. The objective of this study was to categorize different forms of mortality and disease conditions, describe their clinical signs and pathology, and, where possible, determine their cause. Various aspects of Newcastle disease in DCC from this colony have been described in Chapters 3 and 4. The effect of these causes of mortality on the reproductive success of the DCC will be discussed in Chapter 8.

## 7.2 Materials and methods

### 7.2.1. Study site

The study site was Island A, a 300 x 100 m island in the south-west part of Doré Lake (Saskatchewan, Canada; 54°46'N, 107°17'W) with ground-nesting colonies of DCC and American white pelicans (Pelecanus erythrorhynchos). Island A is separated by a 250-m-wide shallow water channel from Smith Island, which is 2 x 1.5 km (Chapter 3).

### 7.2.2 Monitoring of reproduction, morbidity, and mortality

Observations were made on Island A from within an 88-m-long tunnel-and-blind (TAB) system every third day from 1 June to 1 September from 1994 to 1996 (Chapter 2). About 10% of the total nesting area of DCC was visible from within this system. All DCC and pelican nests within about 6 m of the TAB system were marked with numbered stakes at the beginning of each breeding season. At each visit, the number of eggs and chicks in each marked nest, presence of broken eggs, eggs displaced from the nest, and sick or dead birds were recorded. The location of broken and displaced eggs and carcasses was recorded in relation to the numbered stakes or TAB system to prevent counting them more than once. Whenever possible, displaced eggs, and sick or dead birds were collected by hand or by use of a telescopic rod with attachments, without leaving the TAB system (Chapter 2).

In addition to observations made from inside the TAB system, observations were

made and samples were collected each year from outside the TAB system during the incubation period when collecting eggs, during the nestling period when bleeding DCC chicks, and at the end of breeding season when surveying Island A for the presence of carcasses.

### 7.2.3 Identification of birds with color bands

Fourteen to twenty-six DCC nestlings were banded per year with one or two colored bands to allow identification from a distance. To make these bands, 95 x 1 x 0.5 mm strips of colored plastic (Darvic Plastics, Middlesex, U.K.) were wrapped double, held in shape with pliers, and heated in boiling water for 5 s. After cooling in cold water for 5 s, the plastic bands retained their rolled-up shape.

### 7.2.4 Necropsy

Necropsies were carried out according to a standard protocol. Carcasses were divided into three categories of state of decomposition: fresh, moderately decomposed, and very decomposed. Fresh carcasses had no visible signs of autolysis; moderately decomposed carcasses were visibly autolyzed, but their internal organs were intact and recognizable and could be assessed for the presence of lesions; and very decomposed carcasses had internal organs which were discolored, misshapen or ruptured, and were difficult to recognize. Carcasses also were divided into three categories of body condition: good, moderate, and poor. Birds in good body condition had well-developed musculature and visible fat in the coronary groove, intestinal mesentery and abdominal subcutis; birds in moderate body condition had moderately developed musculature, visible fat in the coronary groove but not in the intestinal mesentery or abdominal subcutis; and birds in poor body condition had atrophied musculature and no visible fat in the coronary groove, intestinal mesentery, or abdominal subcutis. Samples for virological, bacteriological and serological examination were stored in liquid nitrogen, samples for

histological examination were stored in 10% neutral-buffered formalin, and samples for parasitological identification were stored in 70% ethanol until further use. In the case of parasites in the liver, small pieces of liver were fixed in 10% neutral-buffered formalin and parasites were dissected free of the tissue for identification.

### 7.2.5 Histology

Formalin-fixed samples were embedded in paraffin, sectioned at 5 $\mu$m, and stained with hematoxylin and eosin (H & E) for examination by light microscopy (Luna, 1968). To quantify liver infection with the trematode Amphimerus elongatus, which has been reported before in DCC (Pense and Childs, 1972) but was not always detectable grossly in this study, liver from all carcasses was examined histologically to detect A. elongatus infection. Other tissues from 22 5 to 9-week-old DCC chicks with Newcastle disease also were examined for A. elongatus infection and are included in this study. Tissues examined were brain, spinal cord, brachial plexus, lumbosacral plexus, eye, eyelid, bursa of Fabricius, thymus, spleen, femoral bone marrow, esophagus, ventriculus, duodenum, pancreas, liver, jejunum, ileum, cecum, colon, trachea, lung, heart, aorta, pectoral and quadriceps muscle, kidney, thyroid and parathyroid, adrenal gland, uropygial gland, and testis or ovary and oviduct. Tissues were considered positive if sections of adult trematodes, their eggs, or both were detected. The stomach of 20 of these 22 DCC was examined histologically for lesions associated with Contracaecum sp. infection, which is common in DCC (Huizinga, 1971). Tissues from selected gross lesions also were examined histologically.

### 7.2.6 Bacteriology

Samples from selected cases were cultured on blood agar (Prepared Media Laboratory, Richmond, British Columbia, Canada) by the Diagnostic Bacteriology Laboratory, Western College of Veterinary Medicine, University of Saskatchewan. The

inoculated media were incubated at 37 C in an aerobic atmosphere and examined daily for 2 days for bacterial growth. An isolated colony representative of each bacterial variant detected visually was selected and identified according to methods of Carter and Cole (1990).

### 7.2.7 Identification of parasites

Parasites were identified by the Diagnostic Parasitology Laboratory, Western College of Veterinary Medicine, University of Saskatchewan, according to Yamaguti (1961) for nematodes, McDonald (1981) for trematodes, Emerson (1972a,b,c) for lice, Holland (1949) for fleas, and Davies (1991) for leeches.

### 7.2.8 Determination of length of incubation, age of embryos, and age of chicks

The length of incubation of eggs was estimated on the basis of the median date of start of incubation. This was calculated as the median hatching date minus 28 days, which is the mean incubation period for DCC (Johnsgard, 1993). Hatching dates of marked nests were estimated by taking the average of the date when a chick was first seen in a nest and the preceding observation date. In those cases where a chick was seen pipping, that date was taken as the hatching date.

Age of embryos incubated up to 8 days was estimated as follows: (1) marginal vein not yet visible grossly at 0 to 1 day (Romanoff, 1960), and (2) marginal vein clearly visible as a red ring in the yolk sac, and crown-rump length of embryo $\leq$ 10 mm at 2 to 8 days (Van Scheik, 1985). Age of embryos incubated more than 8 days was estimated according to the criteria provided by Hanbidge and Fox (1996).

Age of chicks was estimated on the basis of the median hatching date for that year. Nestlings were defined as chicks no more than 4 weeks old, because DCC chicks begin to leave their nests when 3 to 4 weeks old (Johnsgard, 1993). Post-nestling chicks were defined as chicks at least 5 weeks old.

## 7.3 Results

The median hatching dates were 20 June 1994 (n = 159, first quartile 14 June, third quartile 23 June, range 8 June to 5 July), 16 June 1995 (n = 191, first quartile 13 June, third quartile 23 June, range 10 June to 8 July), and 29 June 1996 (n = 116, first quartile 29 June, third quartile 2 July, range 18 June to 17 July).

### 7.3.1 Infertility and embryonal mortality

Eggs failed to hatch because of infertility or pre-hatching mortality due to embryonal death, avian predation, and displacement from the nest. Infertility is not a cause of death but was included in this list because it was not possible to distinguish infertility from early embryonal death. In 33 of 79 (42%) cases, avian predation or displacement from the nest was associated temporally and spacially with human disturbance (Table 7.1).

Eggs were considered infertile or the embryo was considered to have died during incubation if they had been incubated at least 5 weeks when found. Fourteen of these eggs were examined by necropsy. All eggs had brown-white to brown-grey egg shells. Ten had no visible embryo; one of these was fresh, the other nine were decomposed with ruptured yolk sac and foul-smelling contents. Two had embryos, incubated about 13 days and 26 days, with no visible abnormalities. Two had deformed embryos. One of these, incubated about 21 days, had an encephalocele and spina bifida. The dorsal part of the skull and cervical vertebral column were absent, leaving the dorsal part of cerebrum, cerebellum, and cervical spinal cord exposed (Fig. 7.1A). The maxilla (12 mm long) was 6 mm shorter than the mandible and deviated 4 mm to the left, and no eye development was visible (Fig. 7.1B). The other, incubated about 19 days, had eyes fused along the medial edges, and the bill was a 2-mm-long stump (Figs. 7.1C and 7.1D).

All eggs predated in the nest or displaced from the nest were found at 0 to 4 weeks of incubation. Avian predators taking an egg directly from the nest either broke it and ate

its contents in the nest, or flew away with the egg in their bill.

One adult DCC in its nest was observed attempting to roll a displaced egg back into the nest bowl by placing its closed bill beyond the egg and then drawing the head back toward the nest, but this manoeuvre was not successful. Nine displaced eggs were examined by necropsy. One had a brown-white egg shell, ruptured yolk sac, no visible embryo and foul-smelling contents, suggesting that it was infertile or that the embryo had died in an early stage of incubation. Six had blue-white egg shells, an intact yolk sac, and fresh contents. Three had been incubated about 0-1 day and two had been incubated about 2-8 days; the yolk sac of the sixth was broken on opening the egg, so that embryonal development could not be determined. Two eggs had embryos with multiple hemorrhages; one, incubated about 16 days, had about five petechiae in the skin of the rump. The other, incubated about 21 days had petechiae in the skin of head, neck, rump, wings, and legs.

### 7.3.2 Nestling mortality

Main causes of mortality in nestlings were avian predation, displacement from the nest, and sibling competition. In 76 of 136 (56%) cases, avian predation or displacement from the nest was associated temporally and spatially with human disturbance (Table 7.1).

Gulls taking live nestlings pecked them on the back of the rump and head, picked them up by the skin on the back of the rump and dropped them onto the ground, in some cases after flying up into the air. They gained access to and ate internal organs through a hole pecked in the back of the rump adjacent to the vertebral column. In some cases, herring gulls (Larus argentatus) ate nestlings up to about 500 g body weight whole. When the nestlings were 3 to 4 weeks old, adult DCC were no longer constantly present at their nest, and nestlings were seen themselves warding off herring gulls.

Necropsies were done on eight nestlings killed by an unidentified gull (n = 7) or herring gull (n = 1). Seven nestlings had wounds on the back of the rump, consisting of

**Table 7.1 Causes of mortality in double-crested cormorants on Island A at Doré Lake from 1994 to 1996.**

| Cause of mortality | Eggs (n = 105) | Nestlings (n = 178) | Post-nestling chicks (n = 1393) | Adults (n = 10) |
|---|---|---|---|---|
| Infertility or embryonal death | 26[a] | -[b] | - | - |
| Avian predation | 6[c] | 29[d] | 0 | 0 |
| Displacement from nest | 73[e] | 107[f] | - | - |
| Sibling competition | - | 39 | 0 | - |
| Newcastle disease | 0 | 0 | 1041[g] | 0 |
| Coyote predation | 0 | 0 | 241 | 5 |
| Suffocation | - | 0 | 61 | 0 |
| Entrapment | - | 1 | 6 | 2 |
| Other[h] | 0 | 2 | 2 | 0 |
| Undetermined | 0 | 0 | 42 | 3 |

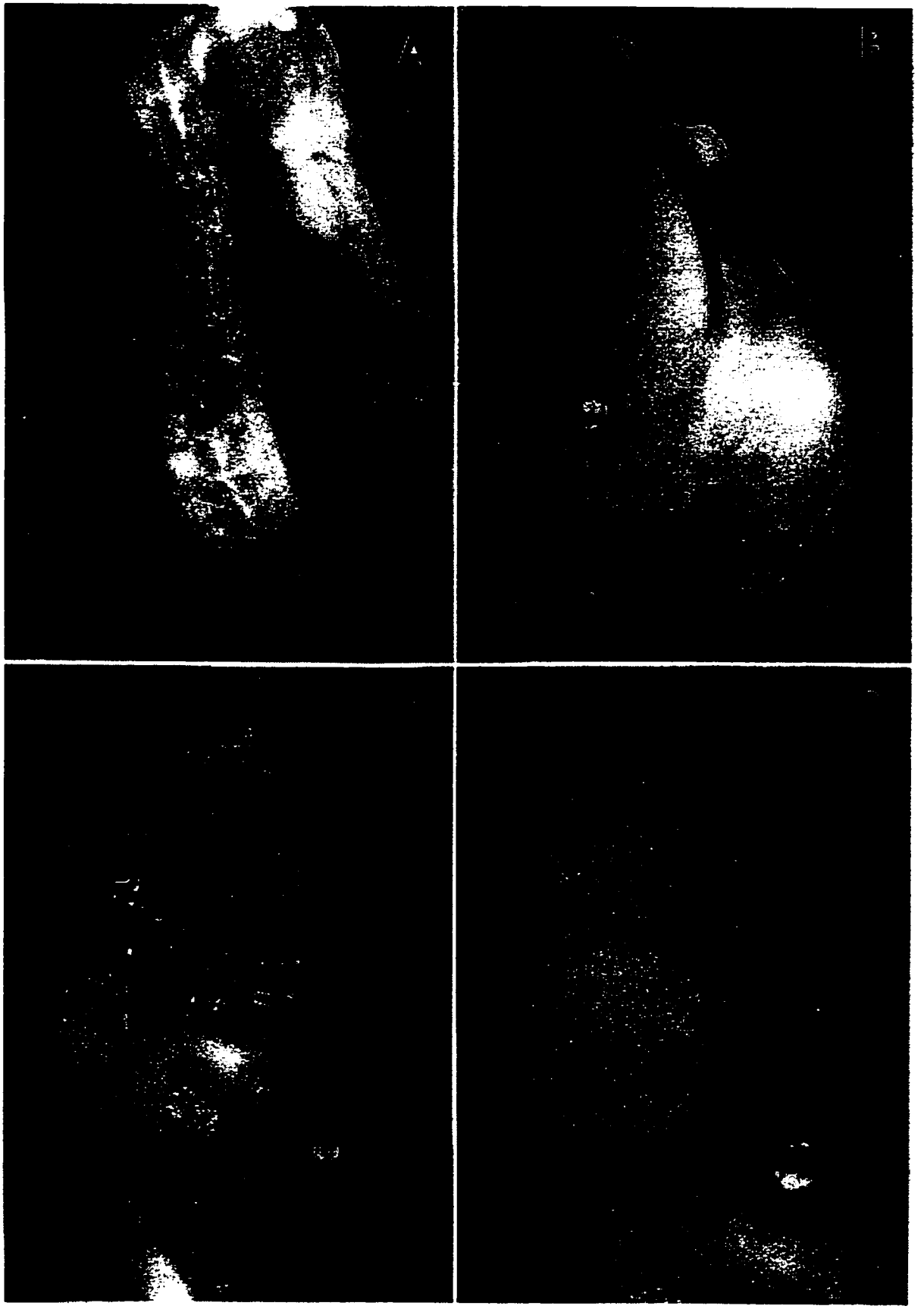
[a]Twenty-two eggs had been displaced from the nest.
[b]Not relevant.
[c]The eggs were taken directly from the nest by a common raven (n = 1), California gulls (n = 2), and gulls of undetermined species (n = 3), after the nest had been temporarily abandoned by the adult cormorant due to arrival of a human at the entrance of the TAB system by canoe.
[d]Twenty-six nestlings were killed by herring gulls (n = 22) or gulls of undetermined species (n = 4), after the nest was temporarily abandoned by the adult cormorant due to human disturbance, either bleeding of cormorant chicks (n = 19), disturbance from inside the TAB system (n = 6), or arrival at the entrance of the TAB system by canoe (n = 1). Three nestlings were killed or scavenged by a bald eagle.
[e]In 27 cases, displacement was associated temporally and spatially with human disturbance, either arrival at the entrance of the TAB system by canoe (n = 13), presence in the nesting area to collect eggs (n = 6) or to extend the TAB system (n = 6), or presence in the TAB system (n = 2). Fifteen displaced eggs were subsequently killed by herring gulls (n = 3), California gulls (n = 8), or gulls of undetermined species (n = 4).
[f]In 50 cases, displacement was associated temporally and spatially with human disturbance, either arrival at the entrance of the TAB system by canoe (n = 13), bleeding of cormorant chicks in the nesting area (n = 9), disturbance from inside the TAB system (n = 22), or extension or repair of the TAB system (n = 6). Twenty-seven displaced nestlings were subsequently killed by herring gulls (n = 15), a California gull (n = 1), a ring-billed gull (n = 1), gulls of undetermined species (n = 9), or a common raven (n = 1).
[g]Two of these also had bacterial disease, one caused by Escherichia coli, the other by Salmonella typhimurium.
[h]Abrasion wounds of the head (n = 2), back broken by falling branch (n = 1), and emaciation of unknown cause (n = 1).

**Figure 7.1A Spina bifida in a double-crested cormorant embryo incubated about 21 days.**

**Figure 7.1B Deformed bill and encephalocele in a double-crested cormorant embryo incubated about 21 days.**

**Figure 7.1C Fused eyes in a double-crested cormorant embryo incubated about 19 days.**

**Figure 7.1D Hypoplasia of the bill in a double-crested cormorant embryo incubated about 19 days.**


A
B

subcutaneous hemorrhage of 3 cm diameter (n = 1), a shallow skin wound of 2.5 cm diameter and extensive subcutaneous hemorrhage (n = 1), or a hole of 1.5 to 4 cm diameter penetrating the celomic cavity (n = 5). One nestling had subcutaneous hemorrhage of 0.5 cm diameter on the back of the head, and another had subdural hemorrhage at the level of the cerebellum. In the five carcasses with penetrating wounds into the celomic cavity, most internal organs were absent except for the stomach. This protruded from the wound, attached internally by the esophagus. The three nestlings without penetrating wounds into the celomic cavity were in good body condition and had fish remains in the stomach. They had a transverse rupture of the liver adjacent to the edge of the sternum, presumably caused by a gull either pecking at the nestling or dropping it onto the ground. There was extensive hemorrhage around the liver, and very pale musculature, indicating that the nestlings exsanguinated from the liver rupture (Fig. 7.2A).

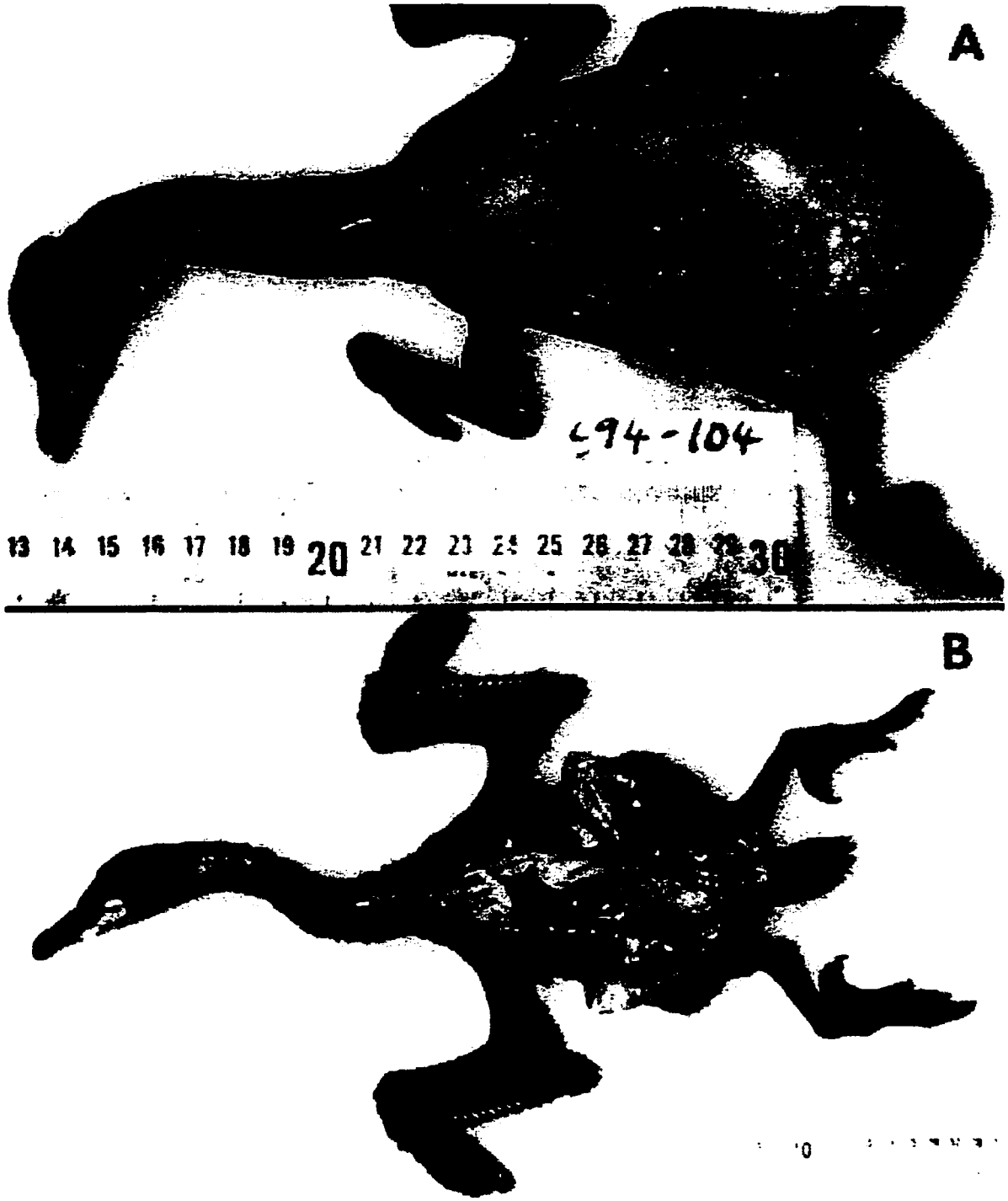
An immature bald eagle (Halilaeetus leucocephalus) in a tree on the colony site was observed eating a 2-week-old, freshly dead nestling on three consecutive days. It could not be determined whether this represented predation or scavenging. One or two juvenile or adult bald eagles were seen on the colony site on 4 to 12 days each year, either in a tree or on the ground. Movement of the eagles across the colony site caused DCC below the flight path to fly up from their nests.

Adult DCC made no attempt to return displaced nestlings into the nest; nor were these nestlings brooded or fed outside the nest. Necropsies were done on six displaced nestlings found alive (n = 3) or dead (n = 3) inside the TAB system. They were in good body condition; four had fish remains in the stomach. No abnormalities were detected on gross necropsy, except congestion of lungs and kidneys in one case.

Nestlings that died from sibling competition came from nests with two (n = 4), three (n = 18), or four (n = 11) chicks. The nest of origin was not determined for six of 39 nestlings. The average size difference between the affected nestlings and their siblings was estimated as four times smaller (range 1 to 10, n = 22). Fifteen nestlings which died of sibling competition were necropsied. They were in poor body condition and

**Figure 7.2A Traumatic lesions in a 2-week-old double-crested cormorant killed by a gull. Note the hemorrhage around the liver, the pale heart, the stomach greatly distended with fish, and presence of fat in the mesentery.**

**Figure 7.2B Emaciation due to sibling competition in a 3-week-old double-crested cormorant. Note the skeletal muscle atrophy, liver atrophy, distended gall bladder, shrunken stomach, and absence of fat in the mesentery.**


A
94-104
13 14 15 16 17 18 19 20 21 22 23 25 26 27 30
B

dehydrated (Fig. 7.2B). The stomach was empty except for nematodes, pebbles, feathers, and plant material, and the intestine had green-brown to red-brown opaque viscous contents. One nestling had a transverse closed fracture of the right humerus, and another had a complex fracture, 1 cm in diameter, on the left dorsal side of the skull, with a piece of fractured skull penetrating the cerebrum. The carcasses retrieved from inside the nest (n = 5) were flattened, the down was worn off the dorsal surface, and the part of the carcass which had lain under the siblings was more decomposed than the rest.

Two 4-week-old nestlings died with wounds on the head. One was found moribund at the water's edge, the other dead inside the TAB system. Both had multiple abrasion wounds of the skin on top of the head, with dry yellow-brown crusts, subcutaneous hemorrhage and edema, and loss of down. One had two 1-cm-long incision wounds in the skin on the left side of the head, about 2 cm from each other. Subjacent to one of these wounds was a skull fracture, with a 1-cm-long bone fragment penetrating the left cerebral hemisphere. The brain tissue around this fragment was stained red, yellow, and green. Histologically, both birds had multifocal suppurative dermatitis and fasciitis, with extensive hemorrhage and multiple foci of rod-shaped and coccoid bacteria. The bird with skull fracture had multifocal suppurative encephalitis, with extensive hemorrhage, multiple foci of rod-shaped bacteria, edema and neuronal necrosis.

One 3-week-old nestling died from entrapment in a monofilament nylon fishing line. The line was entangled around its neck and around the branch of a nearby tree.

### 7.3.3 Post-nestling chick mortality

Main causes of mortality in post-nestling chicks were Newcastle disease, coyote (Canis latrans) predation, and suffocation (Table 7.1).

An outbreak of Newcastle disease occurred between 23 July and 24 August 1995, and caused mortality in 5 to 10-week-old chicks (Chapters 3 and 4). Two of these birds also had bacterial disease: colibacillosis in one (Chapter 4), salmonellosis in the other. The heart, liver, and spleen of the latter bird were enlarged and congested with blood.

Histologically, there were foci of rod-shaped bacteria and thrombi in blood vessels throughout the body, including cerebrum, lungs, myocardium, spleen, liver, and kidney, from which tissues Salmonella typhimurium was isolated.

Coyote predation was detected from 20 August to 14 September 1994. This was the only year when coyotes were heard and their tracks seen on adjacent Smith Island. Most DCC killed were 9 to 12 weeks old. Carcasses were found distributed across Island A, without any apparent pattern, except an area of 2 $m^2$, strewn with dead tree trunks and branches, where five carcasses lay together, and the border of stinging nettle (Urtica dioica) and common great bulrush (Scirpus lacustris) lining the shore, where 48 carcasses were found in piles of up to 20 carcasses. There was a maze of paths with clearly visible coyote tracks in this vegetation. There was a 0.5-m-wide path through the bulrushes on Island A opposite the closest point on Smith Island, where there were many fresh coyote tracks, including some that led into the water. Necropsies were carried out on 14 chicks that died from coyote predation. They were in full juvenal plumage and in good body condition. Seven birds had puncture wounds in the thorax (n = 7), back (n = 4), head (n = 2), or legs (n = 1), with hemorrhage in the surrounding subcutis and muscle. The wounds were 3 to 5 mm in diameter and about 10 mm deep. Three birds had three to four wounds 1 to 3 cm apart in a row. All 14 birds had hemorrhage in subcutis of head, neck, and/or thorax (n = 13), lung (n = 6; two of these were associated with blood in the oral cavity), kidney (n = 4), air sac (n = 3), subdural space (n = 3), liver (n = 1), or pericardial sac (n = 1). Ten birds had skeletal fractures in the skull (n = 5), cervical vertebrae (n = 3), thoracic vertebrae (n = 3), or ribs (n = 2). One bird had a ruptured liver, and another had a ruptured trachea. Six birds had been partly eaten. The pectoral musculature and overlying skin was missing in four birds, and three birds had a 6 to 10-cm-wide hole in the back of the rump adjacent to the vertebral column with part of the internal organs missing.

Suffocation following human disturbance caused death in 5-week-old chicks, which had been herded together for blood sampling. Ten people herded several hundred chicks into a corner made by the main part and a side-branch of the TAB system. The chicks in the corner climbed on top of each other, and within a few minutes chicks on the

bottom had died. The side-branch of the TAB system was dismantled immediately and the live birds in the pile were placed in the shade, where they recovered and walked away after a few minutes. Necropsies were done on four of the suffocated chicks. They were in good body condition and were partly in juvenal plumage. They had multiple hemorrhages in pectoral musculature (n = 3), quadriceps muscle (n = 1), spleen (n = 3), or myocardium (n = 1), and diffuse acute congestion of the lungs (n = 3) or liver (n = 1).

Entrapment caused death in six 5 to 10-week-old chicks in full juvenal plumage. They were found hanging by the neck in a Y-shaped fork of a branch (n = 1), by the mandible from a 5-cm-long vertical side branch of a larger horizontal branch (n = 1), by the cloaca on a vertical branch (n = 1), by the prepatagial ligament from a branch (n = 1), by the forearm in between two vertical branches about 5 cm away from each other (n = 1), and by the neck in between the supports of two adjacent tunnel modules of the TAB system (n = 1). A necropsy was done on the last-mentioned bird. Severe dehydration was the only lesion.

The vertebral column of one 7-week-old chick was broken by a 3-m-long, 10-cm-thick branch falling on its back from about 6 m high. It was found alive on 19 August 1996 with paralysis of the tail and both legs, and a necropsy was carried out. The vertebral column was dislocated at the junctions between the synsacrum and adjacent thoracic and caudal vertebrae, there were fractures of left and right pubis and a 2-cm-long fissure on the right side of the synsacrum, with extensive hemorrhage of the surrounding tissue. The spinal cord at both sites of dislocation, the liver and both kidneys had ruptures and hemorrhage.

A 5-week-old chick was found dead in the water. At necropsy, it was in poor body condition and had pulmonary congestion.

### 7.3.4 Adult mortality

Causes of mortality in adults were coyote predation and entrapment (Table 7.1). Five adults were found dead among post-nestling chicks killed by coyotes (see above),

and were also presumed to have died from this cause.

Two adults were found impaled on tree branches. Both were necropsied. One was a male in nuptial plumage, which was found hanging by the gular pouch from a branch. The gular pouch had a 2-cm-wide perforation at the base, through which the tip of the branch protruded. The bird was in good body condition, had fish remains in the stomach, and was dehydrated. The other was impaled by the rump on a vertical branch of a fallen tree. The branch entered the body near the cloaca and left the dorsal part of the rump near the posterior edge of the synsacrum.

### 7.3.5 Parasitic diseases

Nematodes (Contracaecum spiciligerum), trematodes (Amphimerus elongatus), biting lice (Eidmanniella pellucida, Pectinopygus faralloni, Piagetiella incomposita), fleas (Ceratophyllus sp., probably C. niger), and leeches (Theromyzon sp.) were found in DCC examined (Table 7.2). Other parasites were found in the intestine but were not identified. During observation of the DCC colony from the TAB system, one breeding adult was observed regurgitating fish remains admixed with live nematodes for its newly-hatched nestlings.

The nematode Contracaecum spiculigerum was found by gross necropsy mainly in the proventriculus, either attached to the mucosa or loose in the lumen. The prevalence of C. spiculigerum in nestlings appeared to increase with age: 2/4 (50%) at 1 week, 15/21 (71%) at 2 weeks, 5/6 (83%) at 3 weeks, and 4/5 (80%) at 4 weeks of age. Prevalence in post-nestling chicks and adults was 100% (43/43). At least one of the 2-week-old nestlings was infected with mature C. spiculigerum. Grossly, six 5 to 10-week-old chicks had 4 to 10 ulcers, 0.1 to 15 mm in diameter and 1 to 2 mm deep, in the proventricular mucosa; one other 8-week-old chick had multiple hemorrhages, 5 mm in diameter, in the ventricular mucosa. Histologically, 19 of 20 chicks had a superficial gastritis, characterized by multifocal infiltration of granulocytes, lymphocytes, plasma cells, and macrophages in variable proportions into the lamina propria. The inflammation was

**Table 7.2 Prevalence of parasite infections in different age classes of double-crested cormorants on Island A at Doré Lake from 1994 to 1996.**

| Parasite | Nestlings | | Post-nestling chicks | | Adults | |
|---|---|---|---|---|---|---|
| Contracaecum spiculigerum | 72 | (26/36)[a] | 100 | (40/40) | 100 | (3/3) |
| Amphimerus elongatus[b] | 35 | (8/23) | 45 | (18/40) | 0 | (0/2) |
| Eidmanniella pellucida and/or Pectinopygus farallonii[c] | 6 | (1/17) | 51 | (19/37) | 0 | (0/1) |
| Piagetiella incomposita[c] | 6 | (1/17) | 30 | (11/37) | 0 | (0/1) |
| Ceratophyllus sp.[c] | 6 | (1/17) | 0 | (0/37) | 0 | (0/1) |
| Theromyzon sp.[c] | 0 | (0/17) | 5 | (2/37) | 0 | (0/1) |

[a]Percent infected (number of birds infected/number of birds examined).
[b]Presence of trematodes was determined by detection of adult trematodes and/or their eggs in the liver by light microscopy. Small pieces of liver were fixed in 10% neutral-buffered formalin and adult trematodes were dissected free of the tissue for identification of species.
[c]Because few moderately or very decomposed carcasses had ectoparasites: 2/22 (9%) for feather lice, 0/12 (0%) for pouch lice, 1/3 (33%) for fleas, and 0/2 (0%) for leeches, the prevalence of ectoparasites was calculated on the basis of freshly dead carcasses only.

closely associated with the presence of nematodes, which penetrated to the bottom of the crypts of the mucus-secreting epithelium and into the lumen of the proventricular glands. Other changes associated with the presence of C. spiculigerum in the superficial epithelium included ulceration, hemorrhage into the lamina propria and stomach lumen, and foci of rod-shaped and coccoid bacteria in the damaged epithelium.

Trematodes Amphimerus elongatus and its eggs were found by histology mainly in the liver. However, trematode eggs, probably from A. elongatus, also were found in the kidney (n = 1), pancreas (n = 1), and gall bladder (n = 1). Prevalence of A. elongatus infection in nestlings appeared to increase with age: 0/3 (0%) at 1 week, 3/13 (23%) at 2 weeks, 3/4 (75%) at 3 weeks, and 2/3 (67%) at 4 weeks of age. In some cases, it was not clear where in the liver trematodes and their eggs were located; in other cases, they were clearly loose in the liver parenchyma. The inflammatory response around trematodes and foci of eggs varied from a few granulocytes in the adjacent liver parenchyma to a thick wall of macrophages and fibroblasts, admixed with variable numbers of granulocytes, lymphocytes, and multinucleated cells. Some foci included hemorrhage in the adjacent liver parenchyma. In general, the inflammatory response around trematode eggs was more marked than around trematodes. The liver parenchyma had spaces filled loosely with dissociated hepatocytes, erythrocytes and variable numbers of heterophils and macrophages; these were probably migration paths of trematodes. Some bile ducts were hyperplastic, contained a few trematode eggs, or both. Four 5 to 7-week-old chicks with histological evidence of A. elongatus infection had gross liver lesions; these varied from miliary white nodules to contorted tubular white structures, 0.5 mm in diameter and up to 30 mm long in the liver parenchyma.

The lice Eidmanniella pellucida and Pectinopygus farallonii were collected from the plumage, while Piagetiella incomposita was collected from inside the gular pouch. Between 1 and 10 P. incomposita (mean ± SD = 4 ± 3.3, n = 12) were found per bird. Grossly, the gular pouch mucosa was mildly thickened and roughened. Histologically, the mucosa of one of these cases had multiple areas of orthokeratotic hyperkeratosis and ulceration. The ulcers had crusts of necrotic cells and coc-shaped bacteria, and many

granulocytes and lymphocytes in the subjacent connective tissue. A cross-section of a louse was present in one ulcer.

Two chicks with Newcastle disease, one found in the water and the other at the water's edge, had about 10 leeches of the genus Theromyzon attached to the mucosa of the nasal and oral cavity.

### 7.3.6 Other disease conditions

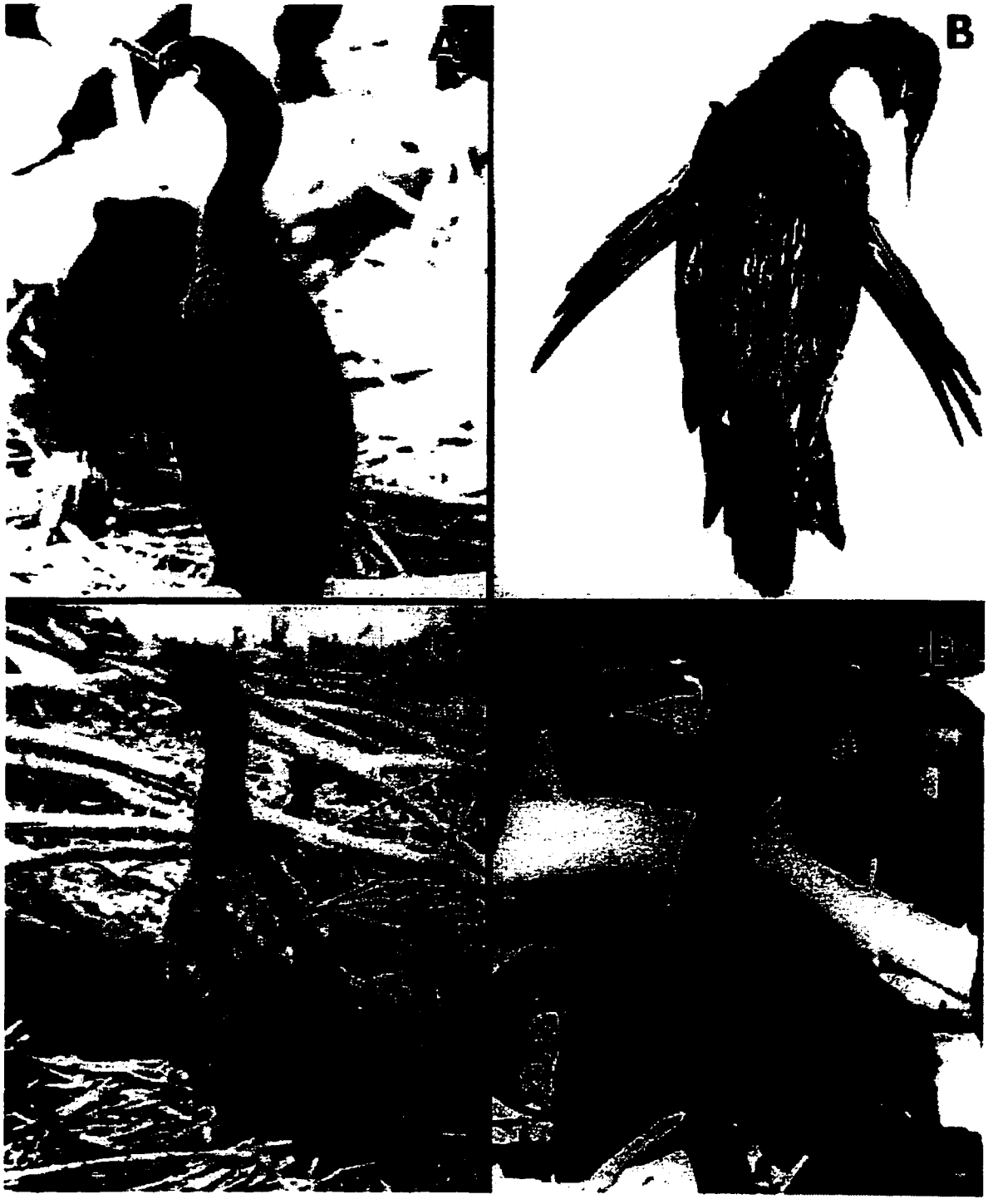
Deformed bills were observed in five chicks. The first chick was tagged at 1 week of age, at which time the bill appeared normal. At 2 weeks, the maxilla deviated slightly downwards and was curved to the left, describing an arc of about 180°. The mandible appeared normal. The deformation became progressively more severe, until, by 8 weeks, the maxilla described a full circle and the tip was pointing forwards. The maxilla touched the left side of the mandible about 1 cm below the left eye, and there was blood on the mandible and gular pouch. The mandible was rotated 10° to the left along the longitudinal axis. At this age, the bird could fly and swim and appeared healthy except for the deformed bill (Fig. 7.3A). The second chick was captured alive at 3 weeks of age. The maxilla was curved about 80° to the right and about 160° downwards. The mandible was straight and shaped normally, except for a rotation of about 10° to the right along the longitudinal axis, which fitted against the deformed maxilla. Except for the deformed bill and a wheezing call, which was different from other nestlings, no abnormalities were detected on clinical examination or gross necropsy of this bird. In the third chick, observed alive at 7 weeks of age, the maxilla was curved downwards and formed a full circle, and the mandible was curved about 90° to the right. In the fourth chick, observed alive at 7 weeks of age, the maxilla was 0.5 cm shorter than the mandible, and the hook on the maxilla crossed the mandible on the left side. The fifth chick was found dead, in poor body condition, and very decomposed at 10 weeks after the median hatching date. About halfway along the maxilla, there was a deviation of about 5° to the left, so that the tip of the maxilla was about 0.5 cm to the left of the median line.

**Figure 7.3A Deformed bill in an 8-week-old double-crested cormorant. The maxilla is curved to the left, forming a full circle, and the tip of the maxilla rubs against the side of the mandible.**

**Figure 7.3B Bilateral rotation of 90° of the carpal joint in a 10-week-old double-crested cormorant. The primary feathers are held at a 30 to 45° angle to the median plane, with the undersurface facing up.**

**Figure 7.3C Hypopigmentation in a 4-week-old double-crested cormorant. It has light gray-brown down, feathers, legs and maxilla.**

**Figure 7.3D Normal pigmentation in a 4-week-old double-crested cormorant. It has black down, feathers, legs and maxilla.**


A
B

Abnormal rotation of the carpal joint was observed in at least five post-nestling chicks from 6 to 12 weeks of age. The abnormality affected the left (n = 2), the right (n = 2), or both wings (n = 1). All affected birds were in good body condition. In resting position, the primary feathers of the affected wing were held horizontal at a 30 to 45° angle to the median plane, with the undersurface of the primary feathers facing upward (Fig. 7.3B). The elbow of the affected wing was held slightly higher than normal and on the contralateral side of the median plane. When the wing was spread, the carpal joint could not be extended more than about 90 to 135°. In the water, the primary feathers of the affected wing dragged through the water and slowed the bird's progress down. The bilaterally affected bird was observed diving, but it was slower than normal, water weeds caught in its wings, and did not stay under water for longer than about 5 s. A necropsy was carried out on this bird, a male in good body condition. In resting position of the wing, the right carpal joint was rotated 90° in a clock-wise direction, and the left carpal joint was rotated 90° in a counter-clock-wise direction. Other joints appeared normal.

Abnormal coloration of the plumage occurred in one chick, observed several times from 2 to 8 weeks of age. As a nestling, it had light gray-brown down, legs and maxilla (Fig. 7.3C). In juvenal plumage, throat, neck and breast were whitish, head, wings, and tail were light gray-brown, gular pouch was yellow, and maxilla and legs were brown. In contrast, other nestlings had black down, legs, and maxilla (Fig. 7.3D), and other chicks in juvenal plumage had whitish to light-brown breast, and dark-brown head, wings and tail, dark brown maxilla and black legs. The sibling and parents of this abnormally colored chick had normal coloration.

Absence of one eye occurred in at least three 5 to 10-week-old chicks. The eye was replaced by pink to brown-black material over which the nictating membrane could move freely, and the affected birds showed no evident discomfort or change in behavior.

Constriction by a leg band occurred in one chick, observed several times from 8 to 10 weeks of age. It was in full juvenal plumage, could fly, appeared in good body condition and was being fed by its parents. One of the two plastic color leg bands used to mark this bird had slipped above the intertarsal joint, causing obstruction of venous blood

flow and swelling of the leg distal to the band. It did not use the affected leg.

## 7.4 Discussion

### 7.4.1 Causes of mortality

Embryonal deformities and hemorrhages, such as those found in this study, may be produced by a large variety of causes, including mutations, heat or cold, chemical compounds, nutritional deficiencies, and infectious agents (Romanoff, 1972). Ludwig et al. (1996) ascribed similar lesions in DCC embryos from the Great Lakes to exposure to planar halogenated compounds. However, Doré Lake is remote from direct industrial discharges, and the concentration of these compounds, measured as 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents, in DCC eggs from a nearby lake in Saskatchewan was one-sixth of that in DCC eggs from a Great Lakes colony (Sanderson et al., 1994).

Eggs and nestlings are easily displaced from the nest when adult birds leave hurriedly, because DCC incubate eggs and brood nestlings up to 1 week of age on top of their feet (Snow, 1960; Johnsgard, 1993). Human disturbance was the only observed cause of hurried departure and subsequent egg or nestling displacement in this study. However, the occasional presence of bald eagles on the colony site probably had the same effect. Drent et al. (1964) considered disturbance by bald eagles to cause most natural egg loss of DCC on Mandarte Island, British Columbia, and Hobson et al. (1989) saw bald eagles feeding on DCC chicks on Lake Winnipegosis, Manitoba.

Displaced eggs or nestlings were not retrieved by their parents and had no chance of survival. Those not taken by a gull or raven probably died from exposure or starvation. Embryos inside displaced eggs and DCC nestlings up to about 2 weeks of age are very susceptible to hyperthermia, and probably died from exposure to the sun (van Scheik, 1985). Double-crested cormorant nestlings older than 2 weeks can effectively maintain homeothermy in the natural environment (Dunn, 1976). However, displaced nestlings

from 2 to 4 weeks of age may have died of starvation, because they were incapable of climbing into the nest and were not fed by their parents outside the nest.

Sibling competition and avian predation are important causes of nestling mortality (Johnsgard, 1993; Hunt and Evans, 1996), but the associated lesions have not been described previously. Because sibling competition caused substantially different lesions in nestlings from those caused by avian predation (Figs. 7.2A and 7.2B), pathological examination may be used to distinguish between them.

Coyote predation has been recorded previously in colonial waterbirds, e.g., California gulls (Larus californicus; Vermeer, 1967) and American white pelicans (Dunbar, 1984), but not in DCC. The coyotes on Smith Island in summer 1994 probably had reached the island over the ice of the frozen lake in winter and were unable to swim to the nearest mainland, which was about 1 km away. Therefore, the DCC and pelican colony on Island A was likely an important source of food for them. The puncture wounds, bone fractures, and associated hemorrhage in DCC killed by coyotes were caused by coyotes gripping the live birds with their teeth. Carcasses partly eaten by coyotes were characterized by loss of pectoral musculature and overlying skin; this was different from carcasses fed upon by gulls, in which only the internal organs were removed through a small hole in the body wall.

Suffocation of DCC chicks in large creches caused by human disturbance has been recorded previously (Brechtel, 1983). It also occurs in domestic poultry, due to birds crowding or piling up in a corner, in which the main lesion is tracheal and pulmonary congestion (Riddell, 1997), similar to findings in DCC from this study. After this accident, post-nestling chicks were not herded into a corner of the TAB system for blood sampling. Instead, post-nestling chicks were trapped by leg-pole from within the TAB system, with no mortality of trapped birds and minimal disturbance of surrounding birds (Chapter 2).

Most cases of entrapment on a branch or other object involved birds at 5 to 10 weeks of age, when the chicks were learning to climb trees and to fly (Johnsgard, 1993). In this period they often were seen slipping off their intended perch, flapping their wings

vigorously, and holding on with the hook on the maxilla or with the entire head before falling to the ground. These activities probably increased the risk of entrapment.

### 7.4.2 Parasitic diseases

Contracaecum spiculigerum (Huizinga, 1971), Amphimerus elongatus (Pense and Childs, 1972), Eidmanniella pellucida, Pectinopygus faralloni, Piagetiella incomposita (Threlfall, 1982), and Ceratophyllus niger (Hubbard, 1947) have been found in DCC before. Theromyzon sp. is common in waterfowl (Wobeser, 1997), but has not been reported in DCC. Infections with Contracaecum spiculigerum (Obendorf and McColl, 1980), A. elongatus (Kingston, 1984), and Theromyzon sp. (Wobeser, 1997) cause mortality in other species. The parasitic infections of DCC in this study were not considered to be the primary cause of death, although they may have caused some degree of debilitation.

The prepatent period of the nematode C. spiculigerum is not known, but is at least 28 days in other members of the same superfamily (Soulsby, 1982). Therefore, cormorant chicks less than 4 weeks old in this study probably did not acquire mature C. spiculigerum by eating fish containing encapsulated larvae, which is how fish-eating birds normally become infected with this parasite (Huizinga, 1971). The observation in this study of an adult DCC feeding fish admixed with live nematodes to newly-hatched chicks indicates that DCC nestlings acquire mature C. spiculigerum in this way, as suggested by Huizinga (1971). Infections with the trematode Amphimerus elongatus are obtained by eating fish containing encysted metacercariae. After ingestion, young flukes (postmetacercariae) move quickly up the bile ducts, pancreatic ducts, or both (Kingston, 1984). In contrast to an adult DCC from Louisiana, which had A. elongatus only in the bile ducts (Pense and Childs, 1972), DCC in this study also had A. elongatus loose in the liver parenchyma. In addition, trematode eggs were found in the kidney and pancreas, suggesting that A. elongatus in the DCC migrates via the bile duct, pancreatic duct, and ureter to reach these organs. This is supported by the detection of A. elongatus-like

trematodes in the pancreas of a juvenile DCC from another breeding colony on Doré Lake and in the kidney of a juvenile DCC from Florida (Kuiken, unpublished data). The lesions caused by the louse Piagetiella incomposita, which have not been described before, were similar in character, but much less severe, than those found in a juvenile American white pelican infected with the closely related P. peralis (Wobeser et al., 1974). Leeches of the genus Theromyzon were found only in DCC with Newcastle disease, a disease of the central nervous system which probably impaired their ability to remove these parasites by preening.

### 7.4.3 Other disease conditions

The bill deformities in DCC in this study were similar to those found in DCC from the Great Lakes (Fox et al., 1991). These were ascribed to exposure to chemicals such as polyhalogenated aromatic hydrocarbons, an unlikely cause for the condition in DCC in this study, as discussed above for embryonal abnormalities. Bill deformity due to vitamin D deficiency has been reported in DCC chicks in captivity (Nichols et al., 1983). However, the captive birds also had deformed limbs, soft bones, and difficulty in staying upright, in contrast to the affected birds in this study, which appeared otherwise healthy. Whatever the cause of the bill deformities in this study, once affected chicks reached independence from their parents at 9 to 10 weeks of age (Johnsgard, 1993), it is questionable whether they were able to obtain enough food to survive.

The abnormal rotation of the carpal joint in some of the DCC in this study is similar to a condition with a variety of names, including angel wing, slipped wing, and airplane wing. Angel wing occurs in young waterfowl when the weight of the rapidly growing flight feathers on the manus is insufficiently supported by the non-rigid bones of the wing, the manus twists outwards, and remains in that position after the bones are ossified. It has been reported in domestic waterfowl and wild waterfowl fed by humans, and has been associated with overfeeding and an unbalanced diet, including an excessive level of protein, and deficiencies in calcium, manganese, and vitamin D. It is found in

rapidly growing birds, and occurs more frequently in male birds, in the larger individuals of a brood, and in warm weather conditions, when energy otherwise used to maintain body temperature may be used for growth (Kear, 1973; Kreeger and Walser, 1984; Ritchie et al., 1994). The only report of an angel-wing-like condition in wild birds not fed by humans is in American white pelicans (Drew and Kreeger, 1986). A possible explanation for this condition in DCC in the wild is very rapid growth. Such growth is most likely to occur in older chicks in the brood, in broods with parents superior in fishing skill and reproductive performance, and in years of mild weather and abundant food supply (Dunn, 1976; DesGranges, 1982).

The abnormal coloration in the DCC in this study was probably hypopigmentation or hypomelanism, an abnormally diminished pigmentation resulting from decreased melanin production (Taylor, 1988). Bancroft (1993) reported an albino DCC in North Carolina, and Ross (1963) reported one sight record of a white DCC and another of a partial albino. In chickens, several mutations occur which dilute eumelanin, the pigment of black or blue feathers. Black Australorp chickens have an autosomal recessive mutation which dilutes the black pigment to a light to dark brownish-grey (Smyth, 1990), similar in appearance to the hypopigmented DCC in this study.

Absence of one eye was seen at 5 to 10 weeks of age, when chicks are wandering around the island and have a high rate of interaction with other chicks. During such interactions the birds often make pecking motions at each other and occasionally strike each other. Chicks trying to climb into strange nests are repelled by jabs from juvenile and adult birds in the nest (Lewis, 1929; Mendall, 1936). Possibly eye loss occurred as a result of strikes with the hooked bill during such interactions.

Even if the leg band causing constriction in one of the DCC had been lost soon after the bird was last seen, the leg may have been permanently damaged, and decreased the bird's chance of survival. Crippling due to displacement of non-metal bands has been reported previously. To prevent the recurrence of such damage, it may be better to use more rigid colored bands, for example made from plexiglass (Nietfeld et al., 1994).

### 7.4.4 Conclusions

Colonial waterbirds are considered to be more susceptible to infectious diseases than their non-colonial counterparts, due to the high density of birds on colony sites, and there are many examples of substantial mortality of colonial waterbirds from outbreaks of viral, bacterial, and parasitic infections (Wittenberger and Hunt, 1985; Parnell et al., 1988; Bourne, 1989).

Surprisingly, no outbreaks of infectious disease were detected in DCC from this study except for a Newcastle disease epidemic in 1995. Spread of infectious disease on the colony site may have been inhibited by gulls and other scavengers, which removed carcasses and food remains (Chapter 3), and by DCC excreta, which contain 22% phosphoric acid (dry weight basis) and may act as a disinfectant (Lewis, 1929).

The description of disease conditions in DCC from this study allows more precise differentiation between different causes of mortality and provides baseline data to estimate the relative importance of various causes of mortality for the life history of the DCC. A number of these conditions, e.g. bill deformities, require further studies to determine their cause and pathogenesis.

# 8. RELATIVE IMPORTANCE OF DIFFERENT CAUSES OF MORTALITY FOR REPRODUCTIVE SUCCESS OF DOUBLE-CRESTED CORMORANTS[1]

## 8.1 Introduction

Little is known about age-specific survival rates and effects of diseases and parasites on survival of double-crested cormorants (Phalacrocorax auritus; DCC). These parameters are important to develop population models for the management of the species (Erwin, 1995). The potential effect of disease on DCC populations became apparent when Newcastle disease (ND) was diagnosed as a cause of apparently high mortality in DCC chicks every 2 or 3 years since 1990 (Wobeser et al., 1993; Meteyer et al., 1997; Chapter 3). However, without estimates of mortality rates from other causes, it was not possible to assess the relative importance of ND.

According to a recent review by Johnsgard (1993), DCC lay 3.0 to 4.3 eggs on average, which have a mean hatching success of 49 to 80%. Of the chicks hatched, between 77 and 95% survive to fledging, resulting in a breeding success of 1.8 to 2.4 young fledged per nest. Predation by gulls (Larus spp.) and corvids (Corvus spp.) is considered an important cause of mortality of eggs and nestlings. Hunt and Evans (1997) found that last-hatched chicks in broods of three to four chicks commonly die of starvation due to non-aggressive sibling competition. Mortality from ND outbreaks, which generally occurs after the chicks have left their nests, was estimated at 10 to 90% of young of the year (Meteyer et al., 1997; Chapter 3). Van de Veen (1973) estimated a first-year mortality rate of 52%, a second-year mortality rate of 26%, and a subsequent year mortality rate of 15%, based on subsequent-year sightings of DCC from Mandarte

[1]A version of this chapter, combined with a version of chapter 7, was submitted for publication as Kuiken et al. (1998d).

Island, British Columbia, banded as juveniles.

As part of a field study of a DCC colony in Saskatchewan from 1994 to 1996, reproductive success was monitored and, where possible, causes of mortality were determined (Chapters 3, 4 and 7). Reproductive success was estimated directly rather than by use of the Mayfield method (1975). The latter method performs poorly in situations such as those found on the DCC colony site, with high visibility of nests, synchrony of nesting, and hatch rates, and catastrophic mortality (Johnson and Shaffer, 1990). The main objectives of this study were to estimate the weekly mortality rates of juvenile DCC from different causes, and to compare the importance of different causes for overall DCC survival.

## 8.2 Materials and methods

### 8.2.1 Study area

The study site was Island A, a 300 x 100 m island in Doré Lake (Saskatchewan, Canada; 54°46'N, 107°17'W) with ground-nesting colonies of DCC and American white pelicans (Pelecanus erythrorhynchos; Chapter 3).

### 8.2.2 Monitoring of reproduction and mortality

Observations were made on Island A from within an 88-m-long tunnel-and-blind (TAB) system every third day from 1 June to 1 September from 1994 to 1996 (Chapter 2). About 10% of the total nesting area used by DCC was visible from within this system. All DCC and pelican nests within about 6 m of the TAB system were marked with numbered stakes at the beginning of each breeding season. At each visit, number of eggs and chicks per marked nest were recorded, and samples of eggs displaced from the nest and moribund or dead chicks were collected for necropsy (Chapter 7). At the end of each breeding season, all nests and carcasses on Island A were counted. Correction for the disappearance of nests after fledging was made by determining the proportion of marked

nests that were still visible, and dividing the total number of nests counted on Island A by this proportion. The distance of marked nests to the entrance of the TAB system was measured to assess the effect of arrival at and departure from the entrance of the TAB system on nest abandonment.

Human activities outside the TAB system during the breeding season consisted of marking DCC and pelican nests and collecting DCC eggs from the marked area (25 May 1994, 29 May 1995, and 29 May 1996), bleeding DCC chicks in the marked area (25 June, 4 and 7 July 1994, 8 July 1995, 9 and 10 July 1996), collecting DCC nestlings from the marked area (19 and 27 June 1995), surveying Island A for carcasses (25 July 1994), and repair of the TAB system (20 June 1996).

### 8.2.3 Calculation of reproductive success parameters

Hatching date was estimated as the mid-point between the date when a chick was first seen in a nest and the preceding observation date, except where a chick was seen pipping, in which cases that date was taken as hatching date. Clutch size was the maximum number of eggs present in the nest within the average incubation period, which is 28 days (Johnsgard, 1993). Brood size was the maximum number of chicks in the nest within 4 days after the hatching date. Nestling mortality between two observations was considered to have occurred at the mid-point of the interval. The number of nestlings per nest was determined up to 3 weeks after hatching, the point at which nestlings start leaving the nest (Johnsgard, 1993). The number of young fledged per nest was defined as the number of 3-week-old chicks per nest, although DCC chicks only learn to fly when they are 5 to 6 weeks old (Johnsgard, 1993). Hatching success was calculated as brood size divided by clutch size. Nests with eggs or chicks on previous visits were considered abandoned up to 3 weeks after hatching if contents were lost and no adult birds were present. Reproductive success parameters were calculated only for nests from which no eggs or chicks had been collected, and for which clutch size, hatching date, brood size, and number of chicks surviving to 1, 2, and 3 weeks of age were known.

### 8.2.4 Necropsy

Diagnosis of cause of death and other diseases was carried out as described in Chapters 3, 4 and 7. In those cases where the nest of origin of DCC examined was not known, the median hatching date for the colony was used to estimate their age.

### 8.2.5 Calculation of mortality rates

Weekly mortality rates for the DCC chicks from the marked area of the colony were calculated as the number of birds that died or disappeared during a given week divided by the number of birds alive at the beginning of that week. For the period up to 3 weeks after hatching, mortality was estimated both by calculation and by observation. By calculation, the average loss of chicks in a given week was multiplied by the total number of marked nests. By observation, the total number of nestlings of corresponding age observed moribund or dead in the marked area was summed.

For the period between 3 weeks after hatching and the end of the breeding season, weekly mortality rates from suffocation, coyote (Canis latrans) predation, and ND were calculated as the number of juvenile DCC carcasses in the whole colony attributable to a given cause divided by the total number of fledged chicks and the number of weeks over which the carcasses were found. Carcass counts from ND and coyote predation were multiplied by two to account for carcass disappearance between death and detection (Chapter 3). The mortality rate from suffocation was calculated on the basis of the number of fledged chicks in the marked area, because only these chicks were considered to have been at risk; mortality rates from ND and coyote predation were calculated on the basis of the number of fledged chicks in the whole colony. The number of fledged chicks in the marked area was calculated using the mean number of fledged chicks per nest. The mean number of fledged chicks per nest outside the marked area was calculated by extrapolation from data for the marked area, after removing the abandoned nests from the data set (Table 8.1); nest abandonment in the marked area was mainly caused by human disturbance (see below) and human disturbance was largely limited to the marked area.

**Table 8.1 Comparison of reproductive parameters at nests of double-crested cormorants in the marked area on Island A at Doré Lake from 1994 to 1996.**

| Year | Total nests | Hatching date | Clutch size | | Hatching success | Brood size | | Nests abandoned | Young fledged per nest | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | including abandoned nests | | excluding abandoned nests | |
| 1994 | 53 | 20/6[a] (14/6-23/6) | 3.2 ± 0.7[b] | A[c] | 78[d] | 2.5 ± 1.3[b] | A | 30[e] | 1.6 ± 1.3[b] | A | 2.3 ± 0.9 | A |
| 1995 | 63 | 16/6 (13/6-23/6) | 2.8 ± 1.0 | B | 51 | 1.4 ± 1.1 | B | 35 | 1.1 ± 0.9 | B | 1.6 ± 0.6 | B |
| 1996 | 53 | 29/6 (29/6-2/7) | 2.4 ± 0.8 | C | 60 | 1.4 ± 1.1 | B | 40 | 1.1 ± 1.0 | B | 1.75 ± 0.6 | B |
| Mean | 56 | | 2.8 ± 0.9 | | 63 | 1.8 ± 1.3 | | 35 | 1.2 ± 1.1 | | 1.9 ± 0.8 | |

[a]Median date, recorded as day/month (first quartile - third quartile).
[b]Mean ± standard deviation.
[c]Tukey multiple comparison test; values with the same letter do not differ significantly, $P > 0.05$.
[d]Percentage of eggs that hatched.
[e]Percentage of nests with eggs or chicks on previous visits.

Overall mortality rates were calculated as the cumulative calculated mortality from a specific cause divided by the number of chicks hatched.

### 8.2.6 Statistics

The null hypothesis that clutch size and number of young fledged per nest in DCC nests in the marked area were the same between years was tested by one-way ANOVA, followed by the Tukey test (Zar, 1996). The Wilcoxon-Mann-Whitney test was used to test the null hypothesis that the distribution of the distance of DCC nests to the entrance of the TAB system was the same for abandoned and non-abandoned nests in the marked area (Zar, 1996). Because we used this test three times to compare the two groups from 1994 to 1996, we employed a Bonferroni-corrected P-value of 0.05/3 = 0.02 to maintain a study-wise P-value of 0.05 (Howell, 1992).

## 8.3 Results

The number of DCC nests on Island A declined nearly three-fold from 1994 to 1996 (Table 8.2). Clutch size and number of young fledged per nest was significantly higher in 1994 than in the subsequent 2 years (Table 8.1).

Nest abandonment ranged from 30 to 40% (Table 8.1). Abandonment was associated with human disturbance due to human activities in the marked area (Fig. 8.1) and to arrival at and departure from the TAB system by canoe. In 1994, the mean distance to the entrance of the TAB system was significantly less ($t = 3.90$; $P = 0.0002$) for abandoned nests (mean ± standard deviation = 26 ± 11 m, $n = 40$) than for nests not abandoned (36 ± 13 m, $n = 99$). Of 49 marked DCC nests closer than 20 m to the TAB system entrance in 1994, 17 (43%) were abandoned. Four nests in 1995 and zero nests in 1996 closer than 20 m to the entrance of the TAB system were marked. The difference in mean distance to the entrance between abandoned and non-abandoned nests was not significant ($P > 0.02$) in 1995 ($t = 0.73$, $n_1 = 21$, $n_2 = 68$) and 1996 ($t = 2.33$, $n_1 = 34$, $n_2 = 76$).

**Table 8.2 Number of double-crested cormorant nests on Island A at Doré Lake from 1994 to 1996.**

| Year | Marked area | | Island A | |
|---|---|---|---|---|
| | Number marked in May | Proportion visible in August | Number visible in August | Number corrected for disappearance |
| 1994 | 232 | 0.51 | 3551 | 6963 |
| 1995 | 244 | 0.72 | 3219 | 4496 |
| 1996 | 189 | 0.65 | 1689 | 2619 |

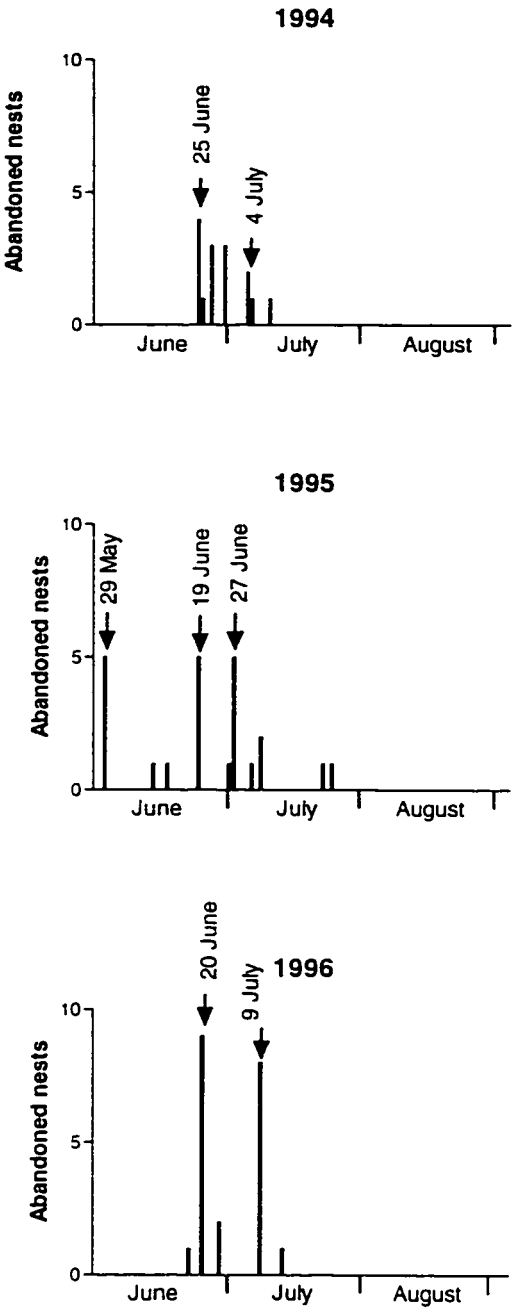



**Figure 8.1 Relationship between abandonment of double-crested cormorant nests and human disturbance from 1994 to 1996. Peaks of abandonment of marked nests on Island A at Doré Lake correspond with dates on which human activities in the marked area occurred (arrows).**

Mortality in the nestling period in all 3 years was caused by displacement of chicks from the nest, predation by gulls, and starvation due to sibling competition (Fig. 8.2). The calculated and observed weekly mortality rates were the same except for the second week after hatching in 1994, when observed mortality was six less than the number calculated, and for the third week after hatching in 1995, when observed mortality was seven less than the number calculated. The agreement between these two methods indicates that most nestling mortality in the marked area was observed during visits at 3-day intervals.

Mortality in the post-nestling period had different causes each year (Fig. 8.2). Mortality from suffocation, caused by birds climbing on top of each other due to human disturbance, was limited to DCC in the marked area. Mortality from coyote predation and ND occurred across the colony site.

Between 26 and 34% of overall mortality of juvenile birds from 1994 to 1996 was caused in the nestling period by displacement from the nest, avian predation, and sibling competition (Fig. 8.3). The most important single cause of mortality of juvenile birds was ND, which killed 21% of hatched chicks in 1995, two to ten times more than coyote predation (2% in 1994) or sibling competition (12% in 1994). In 1996, when there was no measurable mortality in the post-nestling period, the overall survival was 20% better than in the previous 2 years. Other causes of mortality in the post-nestling period were classified as entrapment, emaciation of unknown cause, abrasion wounds of the head, back broken by a fallen branch, and not determined. These causes together resulted in $\leq$ 1% mortality of juvenile birds per year.

## 8.4 Discussion

Newcastle disease was important not only because it caused the highest mortality of juvenile DCC during the study period, but also because it killed juveniles that had survived the vulnerable nestling period. Newcastle disease was first diagnosed in juvenile DCC from Island A in 1990, and has been recorded there in 1992, 1995, and 1997 (Wobeser et al., 1993; F. A. Leighton, pers. comm.; Chapter 3; Kuiken et al., 1998a).

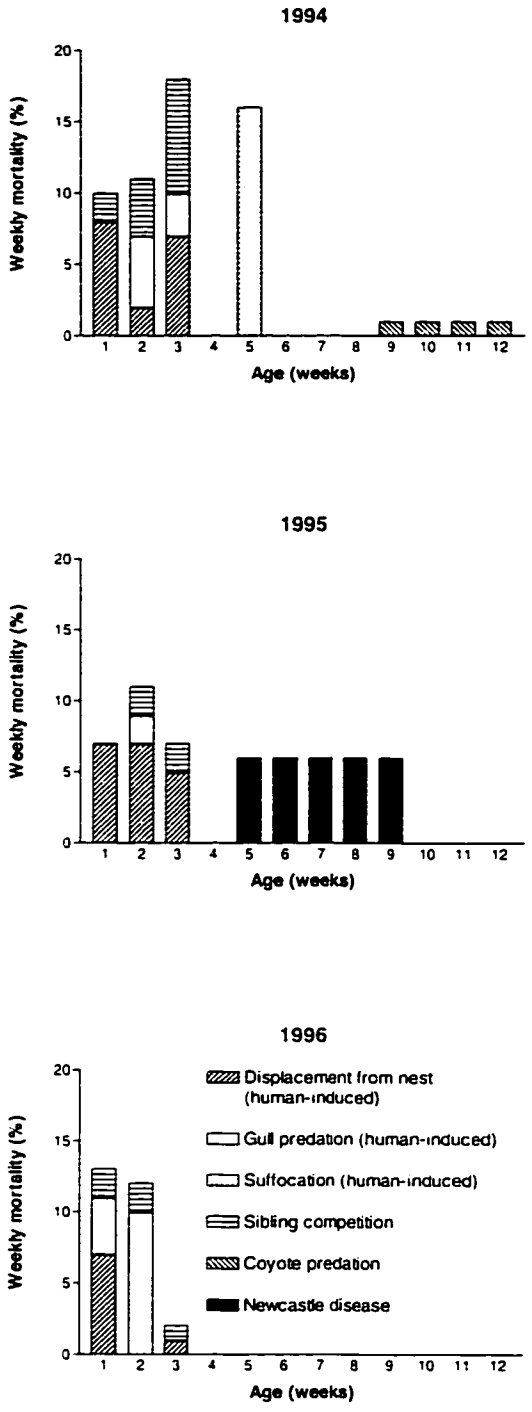



**Figure 8.2 Weekly mortality rates in double-crested cormorants in the marked area of Island A at Doré Lake from 1994 to 1996.**

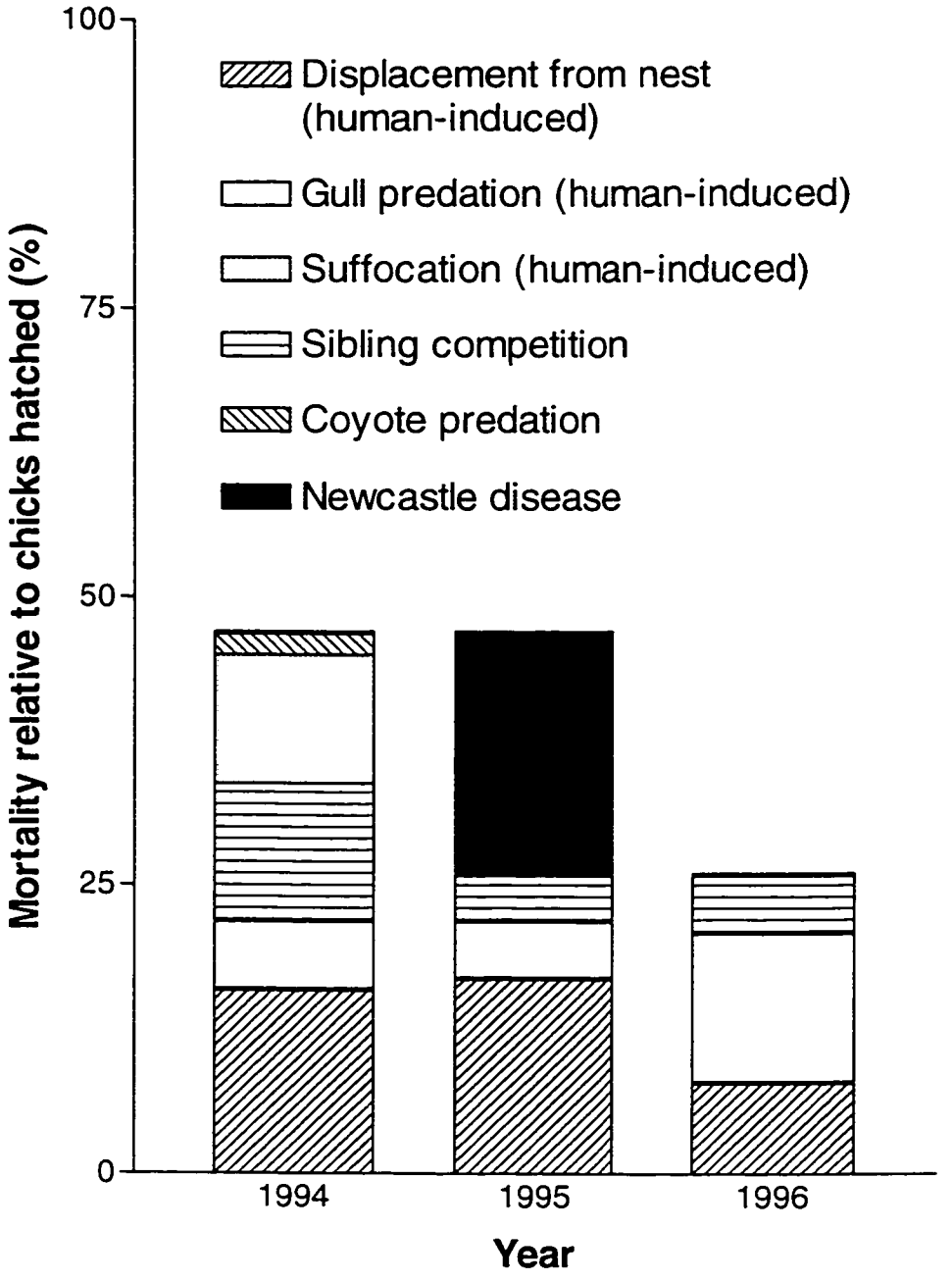



**Figure 8.3 Overall mortality in double-crested cormorants from hatching until the end of the breeding season in the marked area of Island A at Doré Lake from 1994 to 1996.**

Comparison of survival of juvenile birds in 1995, when ND occurred, and in 1996, when there was no measurable mortality in the post-nestling period, suggests that 20% fewer juvenile DCC were recruited into the DCC population of Island A due to mortality from ND (Fig. 8.3). Based on initial breeding at 3 years of age (Johnsgard, 1993), lower recruitment due to a ND outbreak would start to affect the breeding population 3 years later. However, it is not known whether higher mortality from ND in a given year-class of DCC is compensated by lower mortality later in life. A long-term study is needed to understand the effects of ND on population trends of DCC. Such a study should include analysis of population data by use of computer models to formulate hypotheses, and field experiments to test these hypotheses (Potts and Robertson, 1994). Long-term research on other species (e.g. Potts and Aebischer, 1991; Woiwod and Hanski, 1992; Pelton and van Manen, 1996) suggests that at least 20 years of continuous data are required to discover causes of population trends. The long-term study of the closely-related European shag (Phalacrocorax aristotelis; Potts et al., 1980) should provide a useful model for similar work on DCC.

Coyote predation was probably a chance event linked to coyotes being trapped on nearby Smith Island when the ice on Doré Lake melted in spring 1994 (Chapter 7), and there was no evidence of coyotes on Island A nor on Smith Island in 1995 or 1996. Vermeer (1969) suggested that the preference of DCC for nesting on islands may be a mechanism directed against mammalian predation.

Suffocation of DCC chicks disturbed by humans caused substantial mortality of birds from the marked area in 1994 (Fig. 8.3). However, it was of limited importance for the reproductive success on a colony-wide basis, because it did not occur elsewhere on the colony site and would not have occurred in the absence of humans.

Displacement of chicks from the nest and avian predation occurred each year during the nestling period (Fig. 8.2). In general, these causes affected all siblings in a nest and led to permanent nest abandonment. More nest abandonment occurred close to the entrance of the TAB system and on days when humans were present in the marked area, suggesting a link between human disturbance and these causes of nestling mortality (Fig. 8.1). Gull predation and nest abandonment as a result of human disturbance have been

reported before in DCC colonies (Kury and Gochfeld, 1975; Ellison and Cleary, 1978). The level of human activity on the colony site was a compromise between the requirements of different parts of this field study. For example, the collection of blood samples and chicks during the nestling period, to study immunology of ND (Chapter 3), caused substantial disturbance and affected the data on reproductive success shown here.

Starvation from sibling competition also occurred each year during the nestling period (Fig. 8.2) and resulted in 1 to 2% mortality per week, except in the second and third weeks of 1994, when it was 4 to 8%. The higher rate in 1994 probably was related to the significantly larger mean brood size in that year. Mortality from sibling competition occurs at higher rates in nests with larger broods (Hunt and Evans, 1997).

The decrease in clutch size and number of young fledged per nest from 1994 to 1995 (Table 8.1) may reflect a colony-wide change or a human-induced redistribution of breeding pairs on the colony site. Due to the human disturbance in the marked area, which first occurred in 1994, that part of Island A may have been considered a less desirable nesting area in 1995 and 1996, and may have been used by reproductively less successful birds. In other cormorant species, poor nest sites are occupied later in the breeding season by younger, less experienced birds, which have a lower clutch size and fledging success (e.g. Potts et al., 1980; Boekelheide and Ainley, 1989).

Double-crested cormorants first colonized Island A in 1983 (Hanbidge, 1989). Their number increased until 1994, and then declined nearly three-fold in the next 2 years (Fig. 8.4). Unfortunately, only one nest count was carried out between 1986 and 1994, during which period outbreaks of ND occurred in 1990 and 1992 (Wobeser et al., 1993; F. A. Leighton, pers. comm.).

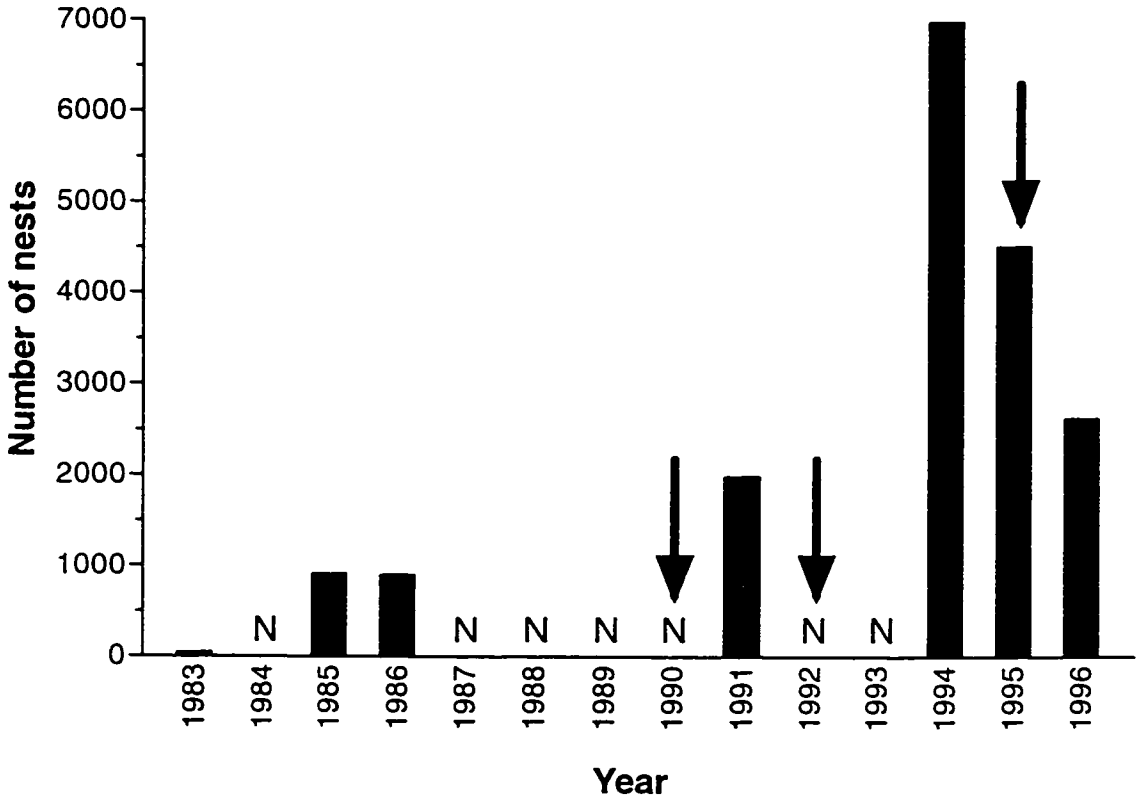



**Figure 8.4** Number of double-crested cormorant nests and recorded Newcastle disease epidemics (arrows) on Island A at Doré Lake from 1983 to 1996. Nest count data from 1983 to 1986 from Hanbidge (1989). Nest count datum from 1991 from K. Roney (pers. comm.). N = no nest count done.

# 9. GENERAL DISCUSSION[1]

## 9.1 Incidents of Newcastle disease in double-crested cormorants from 1993 to 1997

In this section, Newcastle disease (ND) incidents in cormorants from the start of this study in 1993 to winter 1997-98 are summarized, supplementing the review from 1897 to 1992 (Chapter 1.4). In 1995, Newcastle disease virus (NDV) was isolated from double-crested cormorants (Phalacrocorax auritus; DCC) in Alberta, Saskatchewan, Ontario, and Prince Edward Island. In addition, a DCC with an HI antibody titre to NDV of 1: 128, indicating recent exposure to NDV, was found in Manitoba, and a DCC with bilateral wing paralysis, consistent with ND, was found in Quebec. Newcastle disease virus also was isolated from a Caspian tern (Hydroprogne caspia) from Ontario. High mortality associated with the disease was reported only in Saskatchewan, where an estimated 32-64% of juvenile birds in a breeding colony on Doré Lake died of ND. In Alberta, about 30 birds were affected, and in Ontario, the mortality in two colonies was about 5 of 500 (1%) birds (Chapter 3; Leighton, 1995).

In August 1996, two juvenile DCC with ND were found in Ontario, but no large-scale mortality was recorded in any Ontario colony monitored by the Canadian Wildlife Service (Campbell and Key, 1996). In October 1996, a juvenile DCC with ND was found on the north shore of New Brunswick (Daoust and McBurney, 1997).

In 1997, ND was diagnosed in juvenile DCC from breeding colonies on Mullet Island, Salton Sea, California, on East Sand Island, Oregon, and on Rock Island, Bear River Migratory Bird Refuge, Utah. Mortality of juveniles varied from "not abnormal" to over 90%. The NDV isolates from each of the colonies were classified as mesogenic (L.

---

[1]A version of this chapter, combined with a version of chapter 1, was submitted for publication as Kuiken (1998).

Glaser, pers. comm.). These outbreaks are the first records of ND in the Pacific population of DCC, which is separated from the Interior population by the Rocky Mountains (Hatch, 1995). ND was also diagnosed in juvenile DCC in a breeding colony on Doré Lake and one on Lavallée Lake, Saskatchewan. Although the mortality rate was not determined, there were only about one-tenth the number of DCC carcasses in the Doré Lake colony than were present at the end of the ND epidemic in 1995 (Chapter 3; Kuiken et al., 1998a).

The NDV isolates in 1995 from Doré Lake, Saskatchewan, and in 1997 from the Salton Sea, California, had similar nucleotide sequences of the genome coding for the fusion protein cleavage site to NDV isolates from ND epidemics in DCC in 1990 and 1992, indicating that DCC were infected from the same source, probably in the wintering range of DCC, and that the NDV had remained stable during the period from 1990 to 1997 (Chapter 4; L. Glaser, pers. comm.).

## 9. 2 Source of Newcastle disease virus for double-crested cormorants

The similarity in nucleotide sequence of the genome coding for the fusion protein cleavage site between NDV isolates from ND epidemics in DCC in 1990, 1992, 1995, and 1997 from widely separated areas in North America (Heckert et al., 1996; Chapter 4; L. Glaser, pers. comm.) suggests that the DCC were infected in the wintering range, where birds from widely divergent nesting areas intermingle (Dolbeer, 1991). However, the origin of NDV in the wintering range is not known. Possible sources are other bird species, including psittacines and domestic poultry. Alternatively, NDV might be endemic in DCC.

Seal et al. (1995) and Seal (1996) suggested that DCC might have become infected by psittacines on the basis of the close relationship between NDV isolates found in DCC from the USA in 1992 and two so-called psittacine isolates FL80 and Largo (see below). Although the wintering range of DCC overlaps with the range of multiple species of psittacines (Forshaw, 1978), it is unlikely that DCC become infected with NDV from this source for the following reasons.

1. There is little chance of close contact between DCC, which inhabit coastal areas, lakes, rivers, and wetlands (Johnsgard, 1993), and psittacines from Mexico and Central America, which are mainly forest birds (Forshaw, 1978, p. 362).
2. There is no direct evidence that NDV is endemic in free-living psittacines. For example, Goodman and Hanson (1988) did not find NDV in 876 birds of 132 species in Costa Rica, including free-living psittacines. Imported exotic birds, which were considered an important source of NDV in North American ND outbreaks in poultry in the 1970s (Walker et al., 1973), are more likely infected with NDV during their transport through numerous hands, where exposure to poultry and other species not encountered in their natural habitat is probable (Goodman and Hanson, 1988).
3. The isolate Largo was not necessarily from a psittacine. It was isolated in Florida in 1971 "from exotic birds with severe disease" (Brugh and Beard, 1984). The isolate FL80 was either Fla-80-1, from a cockatiel (Nymphicus hollandicus), or Fla-80-2, from a green-cheeked conure (Pyrrhura molinae). They were found in 1980 in a commercial facility in Florida with 8223 exotic birds of 89 species (Brugh and Beard, 1984), and it is likely that these two birds acquired NDV from another species. Even if NDV is endemic in free-living populations of these two psittacine species, DCC could not have contact with them, because their ranges do not overlap: cockatiels are from Australia and green-cheeked conures occur in Brazil, Bolivia, and Argentina (Forshaw, 1978).

Domestic poultry from Mexico are another possible source of NDV infection for DCC. Velogenic NDV was isolated from domestic poultry in Mexico until at least 1996 (Office International des Épizooties, 1997). However, the nucleotide sequence of the genome coding for the fusion protein cleavage site of a velogenic NDV isolate found in domestic poultry from Mexico in 1996 differed from NDV isolates found in DCC in 1990 and 1992. The NDV isolate from Mexican poultry did not have the substitution of arginine for glutamine at position 110 of the fusion gene, which uniquely identifies the isolates from DCC (Seal et al., 1995; Seal et al., 1998), suggesting Mexican poultry are not the source of NDV infection for DCC.

The possibility that DCC are infected by species of birds other than domestic poultry or free-living psittacines cannot be excluded. Two potential candidates are the anhinga (Anhinga anhinga) and the neotropic cormorant (Phalacrocorax brasilianus). Both species often associate with DCC in their wintering range (Johnsgard, 1993, p. 197) and may have close contact conducive to virus transmission. Newcastle disease was diagnosed in a captive anhinga from Florida in 1993. The NDV isolate had a nucleotide sequence of the genome coding for the fusion protein cleavage site nearly identical to NDV isolates found in DCC from the USA in 1992 (Seal et al., 1995), suggesting they were infected from the same source. Newcastle disease has never been reported in neotropic cormorants, which range from Tierra del Fuego in the south to southern USA in the north (Johnsgard, 1993). However, there are descriptions of "peste" in neotropic cormorants along the west coast of South America, associated with starvation caused by El Niño/Southern Oscillation events in 1925 and 1932. During these events, affected neotropic cormorants had clinical signs consistent with ND. They became "loco" and "unbalanced", behaving in an unusual manner, and flew beyond the limits of their normal range. Birds also lost their fear of humans. One immature bird "came aboard my launch not far south of Tumbez, Peru, sat on a coiled hawser while it preened its feathers, and subsequently allowed itself to be played with and passed from hand to hand." Other birds "came ashore in hordes, apparently seeking food among the cattle, or in the troughs of swine" (Murphy, 1936, p. 903).

Another possibility is that NDV was already circulating in the DCC species before 1990. This is supported by the isolation of NDV from DCC from Quebec in 1975. Although this isolate had an identical monoclonal antibody pattern to NDV isolates found in DCC and associated species in 1990 and 1992, the nucleotide sequence of the fusion gene signal region and the genome coding for the fusion protein cleavage site differed. Heckert et al. (1996) suggested these differences may be due to: (1) an accumulation of random point mutations while the virus was maintained in the DCC species, (2) introductions of two separate viruses from an unknown source 15 years apart, (3) the existence of more than one type of NDV circulating in the DCC species, or (4) the viruses coming from two different (Interior and Atlantic) populations of DCC.

## 9.3 Epidemiology of Newcastle disease in double-crested cormorants

The epidemiology of ND in DCC has been studied only during the breeding season (Chapter 3). There is no information about ND in DCC in spring or fall migration, or during winter, but circumstantial evidence suggests that DCC carry NDV from the wintering range to the breeding colonies (Heckert et al., 1996). Thus, DCC populations may be able to maintain NDV year-round. As in NDV in wild ducks (Vickers and Hanson, 1982), infection could be maintained in the population through a cycle of transmission between susceptible individuals or through persistent infection in individual birds. This is supported by experimental evidence from the current study that DCC can excrete pathogenic NDV for up to 1 month after infection without clinical signs of ND (Chapter 5). Newcastle disease virus may not be present in individual breeding colonies of DCC every year, as suggested by the absence of antibody to NDV in juvenile DCC from the Doré Lake breeding colony in two of three years (Chapter 3). Similar to domestic chickens (Alexander, 1997), DCC appear to have an age-related resistance to NDV infection. All reported ND epidemics in breeding colonies caused clinical disease and mortality in juvenile birds only (Chapter 3). A NDV isolate, which caused 32-64% mortality in 6-week-old DCC in the wild, caused no mortality and only transient clinical signs in non-vaccinated 16-week-old DCC infected experimentally (Chapter 5). Transmission is more likely by ingestion of infected excreta than by inhalation of infected aerosol, because ND in DCC does not cause respiratory disease, and NDV is isolated more frequently and at higher concentrations from the kidney than from the lungs (Chapters 3 and 4).

Based on the fragmentary information of ND in DCC and the more extensive knowledge of the epidemiology of paramyxovirus and avian influenza virus infections in free-living waterfowl (Wobeser, 1997), it is possible to propose a simple hypothetical model of the epidemiology of NDV infection in DCC in different seasons. This model may help to explain some of the temporal and geographical patterns of ND epidemics in DCC from 1990 to 1997, and to delineate future areas of research.

1. In winter, the ranges of Atlantic, Interior, Florida, and San Salvador populations

of DCC overlap and intermingling of birds from different nesting areas occurs (Hatch, 1995), allowing exchange of NDV among birds from different breeding colonies and different populations. The transmission rate of NDV in the wintering range probably is associated with the rate of contact among birds, the number of susceptible (mainly juvenile) birds, and, to a certain extent, the total number of birds (Fine et al., 1982). If NDV in DCC is similar to avian influenza viruses and paramyxoviruses in free-living ducks, juvenile DCC have a higher prevalence of infection, and the prevalence of infection declines during the course of the winter (Wobeser, 1997).

2. In spring, breeding adults from Atlantic and Interior populations fly north to their breeding colonies, which are dispersed over a wide geographical area (Hatch, 1995). They probably carry NDV to some breeding colonies and not to others. The chance of NDV being carried to a breeding colony may be associated with the prevalence of NDV infection in the population in the preceding winter, length of time between departure from the wintering range and arrival at the colony site, proportion of susceptible breeding birds in the colony, and colony size.
3. In summer, NDV may spread to young of the year in breeding colonies where the virus is present. If this occurs, a large proportion of the young of the year will become infected because of the high contact rate among birds of this age group (Chapter 3). Some of these birds will die, others will survive and develop antibody to NDV. Young of the year in breeding colonies where the virus is not present will not have antibody to NDV.
4. In fall, adults and juveniles from Atlantic and Interior populations fly south to the communal wintering range (Hatch, 1995). Before migration, birds from several colonies may join at certain gathering areas (Palmer, 1962). At such areas, juveniles from breeding colonies where NDV was not present may be infected by birds from breeding colonies where NDV was present. A high prevalence of infection can be anticipated at this time due to the high proportion of susceptible juveniles in the population (Wobeser, 1997).

The rapid increase in DCC numbers in North America, which started in the early 1970s, led to both a large total number of birds and a high proportion of young birds in the wintering range and in breeding colonies (Hatch, 1995). Based on the above model, these changes would predispose the species to rapid transmission of NDV. Widespread mortality from ND in 1990 and 1992 is consistent with this model. It is not known how frequently ND epidemics occurred in the preceding period, when populations were smaller and had a lower proportion of young birds. Epidemics may have been missed because breeding colonies of DCC are relatively inaccessible and human visitation is discouraged (Wobeser et al., 1993). The ND epidemic in 1975 was noticed only because the reproductive success of the affected colony was being studied in detail and juvenile DCC from that colony had been captured for a local zoo (Chapter 1.4).

This model also may explain why ND epidemics were not recorded in the Pacific population until 1997. The range of this population is largely separate from that of the Interior population, where widespread mortality from ND occurred in 1990 and 1992. Newcastle disease virus eventually may have spread to the Pacific population by a small number of infected DCC from the Interior population flying westward; five of 609 (1%) banded DCC from Alberta were recovered west of the Rocky Mountains, and none of 520 (0%) banded DCC from the Pacific coast were recovered east of the Rocky Mountains (Dolbeer, 1991). Now that NDV appears to be present in DCC of the Pacific population, it may spread to DCC in the adjacent Alaskan population, and to other cormorant species (Brandt's, pelagic, red-faced) which occur there (Hatch, 1995).

The model does not provide an adequate explanation for the lack of reported ND epidemics in the Atlantic and Florida populations in 1990 and 1992, when there was widespread mortality in breeding colonies of the Interior population and NDV presumably was carried from affected colonies to the shared wintering range. Possible explanations are that: (1) epidemics occurred but were not observed, (2) birds from different populations did not mix despite the overlap in their wintering ranges, (3) there were crucial differences in age distribution and colony size between the populations, and (4) the epidemiology of NDV infection in the Florida population was different from that in Atlantic and Interior populations due to drawn-out breeding season and lack of

migration, and did not lead to noticeable peaks of mortality.

The 2 to 3-year interval between reported ND epidemics in the Interior population from 1990 to 1997 may be attributed, at least in part, to decreased recruitment of juveniles in years with epidemics. This is consistent with the epidemiology of avian influenza in free-living ducks, where the average prevalence of infection is higher in years with more juvenile birds in the population (Wobeser, 1997). It also fits with a computer model of ND in village chickens, where discernible waves of infection with high mortality occurred every 1.5 to 2 years due to a fluctuating proportion of young, susceptible birds (Spradbrow, 1993/1994).

The severity of ND epidemics in the Interior population appears to have decreased since 1992, both in number of breeding colonies affected and the level of juvenile mortality (Chapter 9.1). Possible reasons for such a change include decreased pathogenicity of NDV, increased resistance of DCC (Anderson, 1981, p. 353), and changes in the size and age distribution of the DCC population. For example, the colony size on Island A, Doré Lake, decreased from about 7,000 breeding pairs in 1994 to 2,500 in 1996 (Chapter 8), and the number of DCC in some areas in the Great Lakes appeared to stabilize in 1996, after many years of rapid increase (Korfanty et al., 1997).

The above discussion of ND in DCC exposes large gaps in knowledge that warrant further study. Areas of interest include: (1) the prevalence of NDV infection and of antibody to NDV in different age groups of DCC at different times of the year, (2) factors determining whether a ND epidemic occurs in a given breeding colony in a given year, (3) the occurrence of ND epidemics in breeding colonies of DCC of the Atlantic and Florida populations, (4) differences in the epidemiology of NDV infection between migratory and sedentary DCC, (5) prevalence of NDV infection in cormorants and allied species cohabiting with DCC, (6) risk of transmission of NDV from DCC to domestic poultry and, if necessary, measures to mitigate the risk, (7) changes in the pathogenicity of NDV isolates found in DCC over time, and (8) the effect of ND on the size of DCC populations, i.e., whether mortality from ND is additive or compensatory.

## 9.4 Conclusions

This study was started when there was only one scientific article on ND in DCC, documenting the unexpected appearance of the disease in wild waterbirds (Wobeser et al., 1993). Since then, several more have been published, reporting mainly on the pathology of ND in DCC (Banerjee et al., 1994; Meteyer et al., 1997) and on the characterization of NDV isolates from DCC and associated species (Seal et al., 1995; Seal, 1996; Heckert et al., 1996). With so much still to be understood, it is difficult to carry out a study in this area and not find something new. Having this in mind, the main contributions of this study to the knowledge of ND in DCC are as follows.

The course of a ND epidemic in a DCC colony on Island A, Doré Lake, in 1995 was monitored closely for one month after it started, in contrast to previous studies, which were usually based on single visits to affected colonies. By measuring the mortality rate during the course of the epidemic, it was found that the number of DCC carcasses present at the end of the epidemic was about 50% of the cumulative mortality. This indicates that the mortality figures based on single visits to colonies affected by ND underestimate the actual mortality from ND. The epidemic started one week after the nestlings left their nests and mingled with other juvenile DCC in the colony, thereby greatly increasing the contact rate among individuals. This sequence of events suggests that departure of juvenile DCC from their nests may be an important factor for the initiation of a ND epidemic.

Sera of juvenile birds were collected from the DCC colony on Island A throughout the breeding season from 1994 to 1996. During the ND epidemic in 1995, 37 of 63 (59%) sera had hemagglutination inhibiting (HI) antibodies to NDV, indicating that there was wide-spread infection with NDV among juvenile birds. In contrast, HI antibodies to NDV were not detected in 1994 (90 sera) nor in 1996 (25 sera). These results suggest that in years without ND epidemics, NDV was not circulating among juvenile DCC in the colony, and correspond with the findings of Wobeser et al. (1993) and Meteyer et al. (1997). In retrospect, it would have been useful to determine whether NDV infection was present in adult breeding birds in the colony. This could have been

done by testing excreta of breeding adults for the presence of NDV by virus isolation or by reverse transcriptase-polymerase chain reaction (RT-PCR) test. Samples of freshly voided excreta could have been collected from inside the tunnel-and-blind (TAB) system by use of a swab attached to the end of a telescopic fishing rod, with minimal disturbance of surrounding birds.

In previous studies, the mortality of juvenile DCC from ND has been estimated (Banerjee et al., 1994, Meteyer et al., 1995), but its importance relative to mortality from other causes has not been determined. In this study, the mortality of juvenile DCC from ND on Island A in 1995 was compared with other causes of mortality from 1994 to 1996. Newcastle disease was the most important cause of death, not only because it caused from two to ten times more mortality than other causes, but also because it killed juvenile birds that had survived the vulnerable nestling period. The survival of juvenile DCC to the end of the breeding season in 1995, when a ND epidemic occurred, was 20% less than in 1996, when ND was absent. This information should be useful for computer models to manage DCC populations. In retrospect, the study of causes of mortality of DCC would have been less confounded by human-induced mortality if human activities around the TAB system had been kept to a minimum. The main source of human disturbance was the collection of serum samples from juvenile DCC, which could have been done on parts of the colony site away from the TAB system.

Double-crested cormorants were experimentally infected with NDV for the first time. Twelve 14 to 16-week-old DCC, raised in captivity, were infected with a NDV isolate from the above ND epidemic on Island A in 1995. No birds died, four birds developed mild transient clinical signs consistent with ND, and NDV was excreted from the cloaca for up to 1 month after infection. These results suggest that DCC have age-related resistance to ND, because the same isolate caused 32 to 64% mortality of 6-week-old DCC in the wild. It also shows that DCC could be a maintenance reservoir for NDV through serial infection of susceptible birds.

The histological lesions of ND in 25 DCC from the above epidemic on Island A in 1995 were described in detail and their prevalence was compared with that in 18 negative control DCC, supplementing the work of Meteyer et al. (1997). Over 40 tissues of each of

five DCC with ND were examined by immunohistochemistry for the presence of NDV antigen, which was found only in nervous system and kidney. Virus isolation procedures were performed on eight individual tissues of each these five birds, but NDV was isolated only from brain, kidney, and jejunum. Surprisingly, kidney had both highest prevalence of infection (4/5) and highest concentration of virus. These data are important because they indicate that: (1) kidney is an important source of excreted NDV in DCC, and (2) the diagnosis of ND in DCC may be improved by including kidney in the set of tissues collected for virus isolation. In past studies of ND in DCC (Wobeser et al., 1993; Banerjee et al., 1994; Meteyer et al., 1997), this was not done.

This study also helped to improve the diagnosis of ND in DCC by showing that NDV isolates from DCC from the ND epidemic on the Island A colony in 1995 did not consistently agglutinate erythrocytes. This property of NDV is utilized in the hemagglutination (HA) test, which is used routinely as the screening test for the presence of NDV. Isolates of NDV that do not agglutinate erythrocytes may therefore be missed. In this study an immunoperoxidase test, based on the specific binding between NDV and anti-NDV antibody, was developed as an alternative to the HA test.

A RT-PCR test was developed and used successfully to detect NDV directly in six types of tissues and cloacal contents from DCC and chickens with ND. In previous studies (Jestin and Jestin, 1991; Stäuber et al., 1995), RT-PCR had been employed only for detection of NDV in allantoic fluid and NDV vaccines. This test allows rapid and inexpensive screening of a large number of diagnostic samples for the presence of NDV, in comparison with virus isolation, which is slow and labour-intensive. However, the detection limit and the specificity of the RT-PCR test need to be improved.

## LITERATURE CITED

AFSHAR, A., G. C. DULAC, AND A. BOUFFARD. 1989. Application of peroxidase labelled antibody assays for detection of porcine IgG antibodies to hog cholera and bovine viral diarrhea viruses. Journal of Virological Methods 23: 253-262.

AL-SHEIKHLY, F. A., AND H. C. CARLSON. 1975. Pathology of velogenic Newcastle disease virus infection in turkeys. Avian Diseases 19: 397-407.

ALEXANDER, D. J. 1988. Newcastle disease diagnosis. In Newcastle disease, D. J. Alexander (ed.). Kluwer Academic Publishers, Boston, Massachusetts, pp. 147-160.

ALEXANDER, D. J. 1989. Newcastle disease. In A laboratory manual for the isolation and identification of avian pathogens, 3rd ed., H. G. Purchase, L. H. Arp, C. H. Domermuth and J. E. Pearson (eds.). Kendall/Hunt Publishing Company, Dubuque, Iowa, pp. 114-120.

ALEXANDER, D. J. 1997. Newcastle disease and other avian Paramyxoviridae infections. In Diseases of poultry, 10th ed., B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald, and Y. M. Saif (eds.). Iowa State University Press, Ames, Iowa, pp. 541-569.

ALTSCHUL, S. F., T. L. MADDEN, A. A. SCHÄFFER, J. ZHANG, Z. ZHANG, W. MILLER, AND D. J. LIPMAN. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25: 3389-3402.

ANALYTICAL SOFTWARE. 1996. Statistix for Windows: User's manual. Analytical Software, Tallahassee, Florida, 333 pp.

ANDERSON, R. M. 1981. Population ecology of infectious disease agents. In Theoretical ecology: principles and applications, 2nd ed., R. M. May (ed.). Blackwell

Scientific Publications, Oxford, U.K., pp. 318-355.

ANONYMOUS. 1949. Outbreaks of fowl pest in south-east Ireland, 1950. Journal of the Department of Agriculture (Dublin) 46: 142-161.

ASDELL, M. K., AND R. P. HANSON. 1960. Sequential changes in the titer of Newcastle disease virus in tissues: a measure of the defense mechanism of the chicken. American Journal of Veterinary Research 21: 128-132.

BANCROFT, J. 1993. Further observations of albinism in birds. Blue Jay 51: 203-204.

BANERJEE, M., W. M. REED, S. D. FITZGERALD, AND B. PANIGRAHY. 1994. Neurotropic velogenic Newcastle disease in cormorants in Michigan: pathology and virus characterization. Avian Diseases 38: 873-878.

BARTON, J. T., A. A. BICKFORD, G. L. COOPER, B. R. CHARLTON, AND C. J. CARDONA. 1992. Avian paramyxovirus type 1 infections in racing pigeons in California. I. Clinical signs, pathology, and serology. Avian Diseases 36: 463-468.

BEARD, C.W. 1989a. Influenza. In A laboratory manual for the isolation and identification of avian pathogens, 3rd ed., H. G. Purchase, L. H. Arp, C. H. Domermuth and J. E. Pearson (eds.). Kendall/Hunt Publishing Company, Dubuque, Iowa, pp. 110-113.

BEARD, C. W. 1989b. Serologic procedures. In A laboratory manual for the isolation and identification of avian pathogens, 3rd ed., H. G. Purchase, L. H. Arp, C. H. Domermuth and J. E. Pearson (eds.). Kendall/Hunt Publishing Company, Dubuque, Iowa, pp. 192-200.

BELÁK, S., AND A. BALLAGI-PORDÁNY. 1993. Application of the polymerase chain reaction (PCR) in veterinary diagnostic virology. Veterinary Research Communications 17: 55-72.

BLAXLAND, J. D. 1951. Newcastle disease in shags and cormorants and its significance as a factor in the spread of this disease among domestic poultry. Veterinary Record 63: 731-733.

BOEKELHEIDE, R. J. AND D. G. AINLEY. 1989. Age, resource availability, and breeding effort in Brandt's cormorant. Auk 106: 389-401.

BOURNE, W. R. P. 1989. The role of birds in the long-distance dispersal of disease. In

Disease and threatened birds, J. E. Cooper (ed.). International Council for Bird Preservation Technical Publication 10: 121-128.

BRECHTEL, S. H. 1983. The reproductive ecology of double-crested cormorants in southern Alberta. M.Sc. Thesis, University of Alberta, Edmonton, Alberta, 119 pp.

BRIGATI, D. J., L. R. BUDGEON, E. R. UNGER, D. KOEBLER, C. CUOMO, T. KENNEDY, AND J. M. PERDOMO. 1988. Immunocytochemistry is automated: development of a robotic workstation based upon the capillary action principle. Journal of Histotechnology 11: 165-183.

BROWN, J., R. S. RESURRECCION, AND T. G. DICKSON. 1990. The relationship between the hemagglutination-inhibition test and the enzyme-linked immunosorbent assay for the detection of antibody to Newcastle disease. Avian Diseases 34: 585-587.

BRUGH, M., AND C. W. BEARD. 1984. Atypical disease produced in chickens by Newcastle disease virus isolated from exotic birds. Avian Diseases 28: 482-488.

CAIRNS, D. K., K. A. BREDIN, AND V. L. BIRT. 1987. A tunnel for hidden access to blinds at high latitude seabird colonies. Journal of Field Ornithology 58: 69-72.

CAMPBELL, D., AND D. KEY. 1996. Newcastle disease in cormorants. Wildlife Health Centre Newsletter 4 (2): 7.

CARTER, G. R., AND J. R. COLE, JR. (EDITORS). 1990. Diagnostic procedures in veterinary bacteriology and mycology. 5th ed. Academic Press, San Diego, California, 620 pp.

CLEARY, L. 1977. Succès de reproduction du cormoran à aigrettes, Phalacrocorax auritus auritus, sur trois Îles du St-Laurent, en 1975 et 1976. M.Sc. Thesis, Université Laval, Ste-Foy, Quebec, 68 pp.

CLIPPINGER, T. L., R. A. BENNETT, AND S. R. PLATT. 1996. The avian neurologic examination and ancillary neurodiagnostic techniques. Journal of Avian Medicine and Surgery 10: 221-247.

COLLINS, M. S., J. B. BASHIRUDDIN, AND D. J. ALEXANDER. 1993. Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease

viruses showing variation in antigenicity and pathogenicity. Archives of Virology 128: 363-370.

COLLINS, M. S., I. STRONG, AND D. J. ALEXANDER. 1996. Pathogenicity and phylogenetic evaluation of the variant Newcastle disease viruses termed "pigeon PMV-1 viruses" based on the nucleotide sequence of the fusion protein gene. Archives of Virology 141: 635-647.

COMMISSION OF THE EUROPEAN COMMUNITIES. 1993. Commission decision of 12 May 1993 laying down the criteria for classifying third countries with regard to avian influenza and Newcastle disease. Official Journal of the European Communities L 137: 24-30.

COYNE, M. J., G. SMITH, AND F. E. MCALLISTER. 1989. Mathematic model for the population biology of rabies in raccoons in the mid-Atlantic states. American Journal of Veterinary Research 50: 2148-2154.

DAOUST, P.-Y., AND S. MCBURNEY. 1997. Probable Newcastle disease in a cormorant. Wildlife Health Centre Newsletter 4 (3): 4-5.

DAVIES, R. 1991. Annelida: leeches, polychaetes, and acanthobdellids. In Ecology and classification of North American freshwater invertebrates, J. H. Thorp and A. P. Covich (eds.). Academic Press, San Diego, California, pp. 437-479.

DESGRANGES, J.-L. 1982. Weight growth of young double-crested cormorants in the St. Lawrence Estuary, Quebec. Colonial Waterbirds 5: 79-86.

DOLBEER, R. A. 1991. Migration patterns of double-crested cormorants east of the Rocky Mountains. Journal of Field Ornithology 62: 83-93.

DRENT, R., G. F. VAN TETS, F. TOMPA, AND K. VERMEER. 1964. The breeding birds of Mandarte Island, British Columbia. Canadian Field-Naturalist 78: 208-263.

DREW, M. L., AND T. J. KREEGER. 1986. Skeletal abnormalities in wings of free-flying juvenile white pelicans (Pelecanus erythrorhynchus) in Minnesota. Journal of Wildlife Diseases 22: 447-449.

DUNBAR, D. L. 1983. The breeding ecology and management of white pelicans at Stum Lake, British Columbia. Fish and Wildlife Report R-6. Wildlife Branch, Ministry

of Environment, Surrey, British Columbia, 85 pp.

DUNN, E. H. 1976. Development of endothermy and existence energy expenditure of nestling double-crested cormorants. Condor 78: 350-356.

ELLISON, L. N., AND L. CLEARY. 1978. Effects of human disturbance on breeding of double-crested cormorants. Auk 95: 510-517.

EMERSON, K. C. 1972a. Checklist of the Mallophaga of North America (north of Mexico). Part 1. Suborder Ischnocera. Deseret Test Center, Dugway, Utah, 200 pp.

EMERSON, K. C. 1972b. Checklist of the Mallophaga of North America (north of Mexico). Part 2. Suborder Amblycera. Deseret Test Center, Dugway, Utah, 118 pp.

EMERSON, K. C. 1972c. Checklist of the Mallophaga of North America (north of Mexico). Part 4. Bird host list. Deseret Test Center, Dugway, Utah, 216 pp.

ERICKSON, G. A., C. J. MARÉ, G. A. GUSTAFSON, L. D. MILLER, S. J. PROCTOR, AND E. A. CARBREY. 1977. Interactions between viscerotropic velogenic Newcastle disease virus and pet birds of six species. I. Clinical and serologic responses, and viral excretion. Avian Diseases 21: 642-654.

ERWIN, R. M. 1995. The ecology of cormorants: some research needs and recommendations. Colonial Waterbirds 18 (Special Publication 1): 240-246.

FINE, P. E. M., J. L. ARON, J. BERGER, D. J. BRADLEY, H. J. BÜRGER, E. G. KNOX, H. P. R. SEELIGER, C. E. G. SMITH, K. W. ULM, AND P. YEKUTIEL. 1982. The control of infectious disease group report. In Population biology of infectious diseases, R. M. Anderson and R. M. May (eds.). Springer-Verlag, Berlin, Germany, pp. 121-147.

FORSHAW, J. M. 1978. Parrots of the world, 2nd ed. Lansdowne editions, Melbourne, Australia, 616 pp.

FOX, G. A., B. COLLINS, E. HAYAKAWA, D. V. WESELOH, J. P. LUDWIG, T. J. KUBIAK, AND T. C. ERDMAN. 1991. Reproductive outcomes in colonial fish-eating birds: a biomarker for developmental toxicants in Great Lakes food chains. II. Spatial variation in the occurrence and prevalence of bill defects in young

double-crested cormorants in the Great Lakes, 1979-1987. Journal of Great Lakes Research 17: 158-167.

GOODMAN, B. B., AND R. P. HANSON. 1988. Isolation of avian paramyxovirus-2 from domestic and wild birds in Costa Rica. Avian Diseases 32: 713-717.

GRAHAM, D.A., T. J. CONNOR, S. J. MCCULLOUGH, E. R. MCKILLOP, D. J. ALEXANDER, R. J. MANVELL, AND L. H. M. RENSTRÖM. 1996. Isolation and characterisation of an avian paramyxovirus type 1 from turkeys in Northern Ireland. Veterinary Record 138: 416-417.

GULLAND, F. M. D. 1995. The impact of infectious diseases on wild animal populations: a review. In Ecology of infectious diseases in natural populations, B. T. Grenfell and A. P. Dobson (eds.). Cambridge University Press, Cambridge, U.K., pp. 20-51.

GUSTAFSON, D. P., AND H. E. MOSES. 1952. Some effects of oral exposure of English sparrows to Newcastle disease virus. American Journal of Veterinary Research 13: 566-571.

HAINES, D. M., AND B. J. CHELACK. 1991. Technical considerations for developing enzyme immunohistochemical staining procedures on formalin-fixed paraffin-embedded tissues for diagnostic pathology. Journal of Veterinary Diagnostic Investigation 3: 101-112.

HANBIDGE, B. A. 1989. Diet, growth, and reproductive success of double-crested cormorants (Phalacrocorax auritus) at Doré Lake, Saskatchewan. M.Sc. Thesis, University of Saskatchewan, Saskatoon, Saskatchewan, 115 pp.

HANBIDGE, B. A., AND G. A. FOX. 1996. Egg characteristics, growth and developmental landmarks of known-age embryos of double-crested cormorants from Manitoba. Colonial Waterbirds 19: 139-142.

HANSON, R. P. 1980. Newcastle disease. In Isolation and identification of avian pathogens, S. B. Hitchner, C. H. Domermuth, H. G. Purchase and J. E. Williams (eds.). American Association of Avian Pathologists, College Station, Texas, pp. 63-66a.

HARDING, M., C. LUTZE-WALLACE, I. PRUD'HOMME, X. ZHONG, AND J.

ROLA. 1994. Reverse transcriptase-PCR assay for detection of hog cholera virus. Journal of Clinical Microbiology 32: 2600-2602.

HATCH, J. J. 1995. Changing populations of double-crested cormorants. Colonial Waterbirds 18 (Special Publication 1): 8-24.

HECKERT, R. A. 1993. Newcastle disease in cormorants. Canadian Veterinary Journal 34: 184

HECKERT, R. A., M. S. COLLINS, R. J. MANVELL, I. STRONG, J. E. PEARSON, AND D. J. ALEXANDER. 1996. Comparison of Newcastle disease viruses isolated from cormorants in Canada and the USA in 1975, 1990 and 1992. Canadian Journal of Veterinary Research 60: 50-54.

HELLER, E. D., D. B. NATHAN, AND M. PEREK. 1977. The transfer of Newcastle serum antibody from the laying hen to the egg and the chick. Research in Veterinary Science 22: 376-379.

HINES, R. K., AND T. W. CUSTER. 1995. Evaluation of an extendable pole-net to collect heron eggs in the canopy of tall trees. Colonial Waterbirds 18: 120-122.

HOBSON, K. A., R. W. KNAPTON, AND W. LYSACK. 1989. Population, diet and reproductive success of double-crested cormorants breeding on Lake Winnipegosis, Manitoba, in 1987. Colonial Waterbirds 12: 191-197.

HOLLAND, G. P. 1949. The Siphonaptera of Canada. Dominion of Canada, Department of Agriculture Technical Bulletin 70, Ottawa, Ontario, 306 pp.

HOWELL, D. C. 1992. Statistical methods for psychology, 3rd ed. PWS-Kent Publishing Company, Boston, Massachusetts, 693 pp.

HUBBARD, C. A. 1947. Fleas of western North America. Iowa State College Press. Ames, Iowa, 533 pp.

HUIZINGA, H. W. 1971. Contracaeciasis in pelicaniform birds. Journal of Wildlife Diseases 7: 198-204.

HUNT, J. D., AND R. M. EVANS. 1997. Brood reduction and the insurance-egg hypothesis in double-crested cormorants. Colonial Waterbirds 20: 485-491.

JESTIN, V., AND A. JESTIN. 1991. Detection of Newcastle disease virus RNA in infected allantoic fluids by in vitro enzymatic amplification (PCR). Archives of

Virology 118: 151-161.

JOHNSGARD, P. A. 1993. Cormorants, darters, and pelicans of the world. Smithsonian Institution Press, Washington, D.C., 445 pp.

JOHNSON, D. H., AND T. L. SHAFFER. 1990. Estimating nest success: when Mayfield wins. Auk 107: 595-600.

JOHNSON, R. F., JR., AND N. F. SLOAN. 1976. The effects of human disturbance on the white pelican colony at Chase Lake National Wildlife Refuge, North Dakota. Inland Bird Banding News 48: 163-170.

JUNGHERR, E. L., E. E. TYZZER, C. A. BRANDLY, AND H. E. MOSES. 1946. The comparative pathology of fowl plague and Newcastle disease. American Journal of Veterinary Research 7: 250-288.

KALETA, E. F., AND C. BALDAUF. 1988. Newcastle disease in free-living and pet birds. In Newcastle disease, D. J. Alexander (ed.). Kluwer Academic Publishers, Boston, Massachusetts, pp. 197-246.

KARZON, D. T., AND F. B. BANG. 1951. The pathogenesis of infection with a virulent (CG 179) and an avirulent (B) strain of Newcastle disease virus in the chicken. I. Comparative rates of viral multiplication. Journal of Experimental Medicine 93: 267-283.

KEAR, J. 1973. Notes on the nutrition of young waterfowl, with special reference to slipped-wing. International Zoo Yearbook 13: 97-100.

KINGSTON, N. 1984. Trematodes. In Diseases of poultry, 8th ed., M. S. Hofstad, H. J. Barnes, B. W. Calnek, W. M. Reid, and H. W. Yoder, Jr. (eds.). Iowa State University Press, Ames, Iowa, pp. 668-690.

KÖLBL, S. 1978. Untersuchungen über Anwendbarkeit und Aussagekraft der Immunofluoreszenz für die Diagnose der Newcastle-Disease bei Hühnern. Wiener tierärztliche Monatsschrift 65: 84-91.

KORFANTY, C., W. G. MIYASAKI, AND J. L. HARCUS. 1997. Review of the population status and management of double-crested cormorants in Ontario. Ontario Ministry of Natural Resources, Fish and Wildlife Branch, Peterborough, Ontario: 34 pp.

KRAMER, T. T., AND H. C. CHO. 1970. Transfer of immunoglobulins and antibodies in the hen's egg. Immunology 19: 157-167.

KREEGER, T. J., AND M. M. WALSER. 1984. Carpometacarpal deformity in giant Canada geese (Branta canadensis maxima Delacour). Journal of Wildlife Diseases 20: 245-248.

KUIKEN, T. 1998. Review of Newcastle disease in cormorants (Phalacrocoracidae). Auk, submitted.

KUIKEN, T., D. FRANDSEN, AND A. CLAVIJO. 1998a. Newcastle disease in cormorants. Canadian Veterinary Journal 39, 299.

KUIKEN, T., R. A. HECKERT, J. RIVA, F. A. LEIGHTON, AND G. WOBESER. 1998b. Excretion of Newcastle disease virus by double-crested cormorants (Phalacrocorax auritus) in absence of mortality or clinical signs of disease. Avian Pathology 27, in press.

KUIKEN, T., F. A. LEIGHTON, G. WOBESER, K. L. DANESIK, J. RIVA, AND R. A. HECKERT. 1998c. An epidemic of Newcastle disease in double-crested cormorants (Phalacrocorax auritus) from Saskatchewan. Journal of Wildlife Diseases 34, in press.

KUIKEN, T., F. A. LEIGHTON, G. WOBESER, AND B. WAGNER. 1998d. Causes of morbidity and mortality in double-crested cormorants (Phalacrocorax auritus) from Saskatchewan and their effect on reproductive success. Journal of Wildlife Diseases, submitted.

KUIKEN, T., G. WOBESER, F. A. LEIGHTON, D. M. HAINES, B. CHELACK, J. BOGDAN, L. HASSARD, R. A. HECKERT, AND J. RIVA. 1998e. Pathology of Newcastle disease in double-crested cormorants (Phalacrocorax auritus) from Saskatchewan, with comparison of diagnostic methods. Journal of Wildlife Diseases, submitted.

KUIKEN, T., G. WOBESER, F. A. LEIGHTON, I. SHIRLEY, AND L. BROWN. 1997. A modular tunnel-and-blind system to reduce investigator disturbance of breeding colonial waterbirds. Colonial Waterbirds 20: 532-536.

KURY, C. R. AND M. GOCHFELD. 1975. Human interference and gull predation in

cormorant colonies. Biological Conservation 8: 23-34.

KWOK, S., AND R. HIGUCHI. 1989. Avoiding false positives with PCR. Nature 339: 237-238.

LANCASTER, J. E. 1966. Newcastle disease: a review of some of the literature published between 1926 and 1964. Monograph no. 3. Health of Animals Branch, Canada Department of Agriculture, Ottawa, Ontario, 188 pp.

LEIGHTON, F. A. 1995. Newcastle disease in double-crested cormorants - summer 1995. Wildlife Health Centre Newsletter 3 (3): 3-4.

LEWIS, H. F. 1929. The natural history of the double-crested cormorant (Phalocrocorax auritus auritus (Lesson)). Ph.D. Thesis, Cornell University, Ithaca, New York, 94 pp.

LUDWIG, J. P., H. KURITA-MATSUBA, H. J. AUMAN, M. E. LUDWIG, C. L. SUMMER, J. P. GIESY, D. E. TILLITT, AND P. D. JONES. 1996. Deformities, PCBs, and TCDD-equivalents in double-crested cormorants (Phalacrocorax auritus) and Caspian terns (Hydroprogne caspia) of the Upper Great Lakes 1986-1991: testing a cause-effect hypothesis. Journal of Great Lakes Research 22: 172-197.

LUNA, L. G. (EDITOR). 1968. Manual of histologic staining methods of the Armed Forces Institute of Pathology, 3rd ed. McGraw-Hill Book Company, New York, New York, 258 pp.

LVOV, D. K., V. N. SYURIN, L. P. NIKIFOROV, V. P. ANDREEV, N. V. PORTYANKO, R. V. BELOUSOVA, N. V. KLOVACH, AND L. A. GIBET. 1975. Isolation of Newcastle disease virus from nestlings in breeding colonies of cormorants, egrets, and herons in the Volga Delta. Sbor. Tr. Inst. Virus Imeni D. I. Ivanovskogo, "Ekologiia Virusov", no. 3: 51-58 (Translated from Russian).

MACPHERSON, L. W. 1956. Some observation on the epizootiology of Newcastle disease. Canadian Journal of Comparative Medicine 20: 155-168.

MAYFIELD, H. F. 1975. Suggestions for calculating nest success. Wilson Bulletin 87: 456-466.

MCDONALD, M. E. 1981. Key to trematodes reported in waterfowl. Resource

Publication 142. Fish and Wildlife Service, United States Department of the Interior, Washington, D.C., 156 pp.

MCFERRAN, J. B., AND R. M. MCCRACKEN. 1988. Newcastle disease. In Newcastle disease, D. J. Alexander (ed.). Kluwer Academic Publishers, Boston, Massachusetts pp. 161-183.

MENDALL, H. L. 1936. The home-life and economic status of the double-crested cormorant Phalacrocorax auritus auritus (Lesson). Maine Bulletin 39 (3): 1-159.

METEYER, C. U., D. E. DOCHERTY, L. C. GLASER, J. C. FRANSON, D. A. SENNE, AND R. DUNCAN. 1997. Diagnostic findings in the 1992 epornitic of neurotropic velogenic Newcastle disease in double-crested cormorants from the upper midwestern United States. Avian Diseases 41: 171-180.

MEYER, M. E., AND M. MEAGHER. 1995. Brucellosis in free-ranging bison (*Bison bison*) in Yellowstone, Grand Teton, and Wood Buffalo National Parks: a review. Journal of Wildlife Diseases 31: 579-598.

MIXSON, M. A., AND J. E. PEARSON. 1992. Velogenic neurotropic Newcastle disease (VNND) in cormorants and commercial turkeys, FY 1992. In Proceedings of the 96th annual meeting of the United States Animal Health Association, Louisville, Kentucky: 357-360.

MUNRO, J. AND J. BÉDARD. 1977. Gull predation and crèching behaviour in the common eider. Journal of Animal Ecology 46: 799-810.

MURPHY, R. C. 1936. Oceanic birds of South America. Macmillan Company and American Museum of Natural History, New York, New York: 1245 pp.

MUTALIB, A. A., AND J. HANSON. 1989. Pasteurellosis (avian cholera) in cormorants. Canadian Veterinary Journal 30: 350-351.

NICHOLS, D., R. J. MONTALI, C. PICKETT, AND C. BUSH. 1983. Rickets in double-crested cormorants (Phalacrocorax auritus). Journal of Zoo Animal Medicine 14: 115-124.

NIETFELD, M. T., M. W. BARRETT, AND N. SILVY. 1994. Wildlife marking techniques. In Research and management techniques for wildlife and habitats, T. A. Bookhout (ed.). Wildlife Society, Bethesda, Maryland, pp. 140-168.

NISBET, I. C. T. 1995. Biology, conservation and management of the double-crested cormorant: symposium summary and overview. Colonial Waterbirds 18 (Special Publication 1): 247-252.

OBENDORF, D. L., AND K. MCCOLL. 1980. Mortality in little penguins (Eudyptula minor) along the coast of Victoria, Australia. Journal of Wildlife Diseases 16: 251-259.

OFFICE INTERNATIONAL DES ÉPIZOOTIES. 1997. World animal health in 1996, part 1. Office International des Épizooties, Paris, France, 390 pp.

OLESIUK, O. M. 1951. Influence of environmental factors on viability of Newcastle disease virus. American Journal of Veterinary Research 12: 152-155.

PALMER, R. S. (EDITOR). 1962. Handbook of North American birds, vol. 1: loons through flamingos. Yale University Press, New Haven, Connecticut, 567 pp.

PARNELL, J. F., D. G. AINLEY, H. BLOKPOEL, B. CAIN, T. W. CUSTER, J. L. DUSI, S. KRESS, J. A. KUSHLAN, W. E. SOUTHERN, L. E. STENZEL, AND B. C. THOMPSON. 1988. Colonial waterbird management in North America. Colonial Waterbirds 11: 129-345.

PELTON, M. R., AND F. T. VAN MANEN. 1996. Benefits and pitfalls of long-term research: a case study of black bears in Great Smoky Mountains National Park. Wildlife Society Bulletin 24: 443-450.

PENSE, D. B., AND G. E. CHILDS. 1972. Pathology of Amphimerus elongatus (Digenea: Opistorchiidae) in the liver of the double-crested cormorant. Journal of Wildlife Diseases 8: 221-224.

PFEFFER, M. M. WIEDMANN, AND C. A. BATT. 1995. Applications of DNA amplification techniques in veterinary diagnostics. Veterinary Research Communications 19: 375-407.

POTTS, G. R. 1969. The influence of eruptive movements, age, population size and other factors on the survival of the shag (Phalacrocorax aristotelis (L.)). Journal of Animal Ecology 38: 53-102.

POTTS, G. R., AND N. J. AEBISCHER. 1991. Modelling the population dynamics of the grey partridge: conservation and management. In Bird population studies:

relevance to conservation and management, C. M. Perrins, J.-D. Lebreton, and G. J. M. Hirons (eds.). Oxford University Press, Oxford, U.K., pp. 373-390.

POTTS, G. R., J. C. COULSON, AND I. R. DEANS. 1980. Population dynamics and breeding success of the shag, Phalacrocorax aristotelis, on the Farne Islands, Northumberland. Journal of Animal Ecology 49: 465-484.

POTTS, G. R., AND P. A. ROBERTSON. 1994. Gamebird research in North America and Europe: the way forward, a critique and a plea. Transactions of the 59th North American Wildlife and Natural Resources Conference: 415-420.

REEKER, W. H. 1975. Cormorants (Phalacrocoracidae)–isolation of velogenic Newcastle disease virus. Memorandum 75-R-MCD-53. Saskatchewan Regional Office, Agriculture Canada, Regina, Saskatchewan, 1 p.

RIDDELL, C. 1987. Avian histopathology, 1st ed. American Association of Avian Pathologists, Kennett Square, Pennsylvania, 152 pp.

RIDDELL, C. 1997. Developmental, metabolic, and other noninfectious disorders. In Diseases of poultry, 10th ed. B.W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald, and Y. M. Saif (eds.). Iowa State University Press, Ames, Iowa, pp. 913-950.

RITCHIE, B. W., G. J. HARRISON, AND L. R. HARRISON. 1994. Avian medicine: principles and application. Wingers Publishing, Lake Worth, Florida, 1384 pp.

ROBERTSON, I. 1971. The influence of brood-size on reproductive success in two species of cormorant, Phalacrocorax auritus and P. pelagicus, and its relation to the problem of clutch-size. M.Sc. Thesis, University of British Columbia, Vancouver, British Columbia, 47 pp.

ROBINSON, H. W. 1913. Great mortality among shags in Orkney. British Birds 6: 348.

ROFFE, T. J. 1992. Neurotropic, velogenic Newcastle disease in double-crested cormorants. In Proceedings of the 96th annual meeting of the United States Animal Health Association, Louisville, Kentucky: 148-150.

ROMANOFF, A. L. 1960. The avian embryo: structural and functional development. Macmillan Company, New York, New York, 1305 pp.

ROMANOFF, A. L. 1967. Biochemistry of the avian embryo: a quantitative analysis of

prenatal development. Interscience Publishers, New York, New York, 398 pp.

ROMANOFF, A. L. 1972. Pathogenesis of the avian embryo. Wiley-Interscience, New York, New York, 476 pp.

ROSS, C. C. 1963. Albinism among North American birds. Cassinia 47: 2-21.

ROSS, P. S., R. L. DE SWART, P. J. H. REIJNDERS, H. VAN LOVEREN, J. G. VOS, AND A. D. M. E. OSTERHAUS. 1995. Contaminant-related suppression of delayed-type hypersensitivity and antibody responses in harbour seals fed herring from the Baltic Sea. Environmental Health Perspectives 103: 162-167.

SAMSON, A. C. R. 1988. Virus structure. In Newcastle disease, D. J. Alexander (ed.). Kluwer Academic Publishers, Boston, Massachusetts, pp. 23-44.

SANDERSON, J. T., R. J. NORSTROM, J. E. ELLIOTT, L. E. HART, K. M. CHENG, G. D. BELLWARD. 1994. Biological effects of polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls in double-crested cormorant chicks (Phalacrocorax auritus). Journal of Toxicology and Environmental Health 41: 247-265.

SEAL, B. S. 1996. Analysis of matrix protein gene nucleotide sequence diversity among Newcastle disease virus isolates demonstrates that recent disease outbreaks are caused by viruses of psittacine origin. Virus Genes 11: 217-224.

SEAL, B. S., D. J. KING, AND J. D. BENNETT. 1995. Characterization of Newcastle disease virus isolates by reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence database for pathotype prediction and molecular epidemiological analysis. Journal of Clinical Microbiology 33: 2624-2630.

SEAL, B. S., D. J. KING, D. P. LOCKE, D. A. SENNE, AND M. W. JACKWOOD. 1998. Phylogenetic relationships among highly virulent Newcastle disease virus isolates obtained from exotic birds and poultry from 1989 to 1996. Journal of Clinical Microbiology 36: 1141-1145.

SHUGART, G. W., M. A. FITCH, AND V. M. SHUGART. 1981. Minimizing investigator disturbance in observational studies of colonial birds: access to blinds through tunnels. Wilson Bulletin 93: 565-569.

SMYTH, J. R., JR. 1990. Genetics of plumage, skin and eye pigmentation in chickens. In Poultry breeding and genetics, R. D. Crawford (ed.). Elsevier, Amsterdam, The Netherlands, pp. 109-167.

SNOW, B. 1960. The breeding biology of the shag Phalacrocorax aristotelis on the Island of Lundy, Bristol Channel. Ibis 102: 554-575.

SOULSBY, E. J. L. 1982. Helminths, arthropods and protozoa of domesticated animals. 7th ed. Baillière Tindall, London, U.K., 809 pp.

SPALATIN, J., AND R. P. HANSON. 1975. Epizootiology of Newcastle disease in waterfowl. Avian Diseases 19: 573-582.

SPRADBROW, P. B. 1993/1994. Newcastle disease in village chickens. Poultry Science Reviews 5: 57-96.

STALLKNECHT, D. E., D. A. SENNE, P. J. ZWANK, S. M. SHANE, AND M. T. KEARNEY. 1991. Avian paramyxoviruses from migrating and resident ducks in coastal Louisiana. Journal of Wildlife Diseases 27: 123-128.

STÄUBER, N., K. BRECHTBÜHL, L. BRUCKNER, AND M. A. HOFMANN. 1995. Detection of Newcastle disease virus in poultry vaccines using the polymerase chain reaction and direct sequencing of amplified cDNA. Vaccine 13: 360-364.

SWAYNE, D. E. 1996. Nervous system. In Avian histopathology, 2nd ed., C. Riddell (ed.). American Association of Avian Pathologists, Kennett Square, Pennsylvania, pp. 183-201.

TAYLOR, E. J. (EDITOR). 1988. Dorland's illustrated medical dictionary. 27th ed. W. B. Saunders Company, Philadelphia, Pennsylvania, 1888 pp.

THORNE, E. T., AND E. S. WILLIAMS. 1988. Disease and endangered species: the black-footed ferret as a recent example. Conservation Biology 2: 66-74.

THRELFALL, W. 1982. Ectoparasites (Mallophaga, Acarina) from the double-crested cormorant (Phalacrocorax auritus) in Florida. Proceedings of the Entomological Society of Washington 84: 369-375.

UNITED STATES DEPARTMENT OF AGRICULTURE. 1996. Exotic Newcastle disease in birds and poultry; chlamydiosis in poultry. Federal Register 61: 56877-56890.

VAN DE VEEN, H. E. 1973. Some aspectes of the breeding biology and demography of the double-crested cormorants Phalacrocorax auritus of Mandarte Island. Final-year Thesis, State University of Groningen, Groningen, The Netherlands, 25 pp.

VAN SCHEIK, W. J. 1985. Thermal aspects of the reproductive ecology of the double-crested cormorant (Phalacrocorax auritus) in southern Alberta. Ph.D. Thesis, University of Alberta, Edmonton, Alberta, 120 pp.

VERMEER, K. 1967. A study of two species of gulls, Larus californicus and L. delawarensis, breeding in an inland habitat. Ph.D. Thesis, University of Alberta, Edmonton, Alberta, 128 pp.

VERMEER, K. 1969. The present status of double-crested cormorant colonies in Manitoba. Blue Jay 27: 217-220.

VICKERS, M. L., AND R. P. HANSON. 1982. Newcastle disease virus in waterfowl in Wisconsin. Journal of Wildlife Diseases 18: 149-158.

VILLEGAS, P., AND H. G. PURCHASE. 1989. Titration of biological suspensions. In A laboratory manual for the isolation and identification of avian pathogens, 3rd ed., H. G. Purchase, L. H. Arp, C. H. Domermuth and J. E. Pearson (eds.). Kendall/Hunt Publishing Company, Dubuque, Iowa, pp. 186-191.

VITOUSEK, P. M. 1992. Global environmental change: An introduction. Annual Review of Ecology and Systematics 23: 1-14.

WALKER, J. W., B. R. HERON, AND M. A. MIXSON. 1973. Exotic Newcastle disease eradication program in the United States. Avian Diseases 17: 486-503.

WELTE, V. R. (EDITOR). 1997. Animal health yearbook 1995. Food and Agricultural Organization, Rome, Italy, 279 pp.

WESELOH, D. V., P. J. EWINS, J. STRUGER, P. MINEAU, C. A. BISHOP, S. POSTUPALSKY, AND J. P. LUDWIG. 1995. Double-crested cormorants of the Great Lakes: changes in population size, breeding distribution and reproductive output between 1913 and 1991. Colonial Waterbirds 18 (Special Publication 1): 48-59.

WESELOH, D. V. C., AND P. J. EWINS. 1994. Characteristics of a rapidly increasing colony of double-crested cormorants (Phalacrocorax auritus) in Lake Ontario:

population size, reproductive parameters and band recoveries. Journal of Great Lakes Research 20: 443-456.

WILCZYNSKI, S. P., M. L. COOK, AND J. G. STEVENS. 1977. Newcastle disease as a model for paramyxovirus-induced neurologic syndromes. II. Detailed characterization of the encephalitis. American Journal of Pathology 89: 649-662.

WILSON, J. E. 1950. Newcastle disease in a gannet (Sula bassana): a preliminary note. Veterinary Record 62: 33-34.

WITTENBERGER, J. F., AND G. L. HUNT, JR. 1985. The adaptive significance of coloniality in birds. In Avian biology, vol. 8, D. S. Farner, J. R. King, and K. C. Parkes (eds.). Academic Press, Orlando, Florida, pp. 1-78.

WOBESER, G., G. R. JOHNSON, AND G. ACOMPANADO. 1974. Stomatitis in a juvenile white pelican due to Piagetiella peralis (Mallophaga: Menoponidae). Journal of Wildlife Diseases 10: 135-138.

WOBESER, G., F. A. LEIGHTON, R. NORMAN, D. J. MYERS, D. ONDERKA, M. J. PYBUS, J. L. NEUFELD, G. A. FOX, AND D. J. ALEXANDER. 1993. Newcastle disease in wild water birds in western Canada, 1990. Canadian Veterinary Journal 34: 353-359.

WOBESER, G. A. 1994. Investigation and management of disease in wild animals. Plenum Press, New York, New York, 265 pp.

WOBESER, G. A. 1997. Diseases of wild waterfowl, 2nd ed. Plenum Press, New York, New York, 324 pp.

WOIWOD, I. P., AND I. HANSKI. Patterns of density dependence in moths and aphids. Journal of Animal Ecology 61: 619-629.

YAMAGUTI, S. 1961. Systema helminthum, vol. 3: The nematodes of vertebrates. Interscience Publishers, New York, New York, 1261 pp.

ZAR, J. H. 1996. Biostatistical analysis, 3rd ed. Prentice Hall, Upper Saddle River, New Jersey, 662 pp.