INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

PATHOLOGICAL EFFECTS OF *Cryptocotyle lingua* IN DOUBLE-CRESTED CORMORANTS (*Phalacrocorax auritus*) TREATED WITH DEXAMETHASONE

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Master of Science

in the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

María de Jesús Forzán

Charlottetown, P. E. I.

August, 2000

CONDITION OF USE

The author has agreed that the Library, University of Prince Edward Island, may make this thesis freely available for inspection. Moreover, the author has agreed that permission for extensive copying of this thesis for scholarly purposes may be granted by the professor or professors who supervised the thesis work recorded herein or, in their absence, by the Chairman of the Department or the Dean of the Faculty in which the thesis work was done. It is understood that due recognition will be given to the author of this thesis and to the University of Prince Edward Island in any use of the material in this thesis. Copying or publication or any other use of the thesis for financial gain without approval by the University of Prince Edward Island and the author's written permission is prohibited.

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

Chairman of the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Charlottetown, P. E. I.

Canada C1A 4P3

SIGNATURE PAGES

iii-iv

REMOVED

ABSTRACT

An association between large proportions of intestinal parasites, particularly trematodes, found in dead emaciated common loons (*Gavia immer*) and other avian species, suggests that parasites can cause or contribute to a debilitating process that results in the demise of wild, free-ranging seabirds. Since concurrent etiologies can contribute to the mortality of seabirds, determination of the terminal cause of death is a complex process, particularly in chronically debilitated birds. Evaluation of the effects of a single etiology helps assess the extent of its involvement in the death of the birds. One observation reported in dead common loons, the large numbers of *Cryptocotyle lingua* found in weak birds, suggests that an underlying disease problem may cause impairment of the immune system of the birds that leads to an increased degree of parasitism, causing further debilitation. This study intended to replicate the infection caused by *C. lingua*, an intestinal trematode normally present in small numbers in adult seabirds that die in good body condition, with no signs of a debilitating disease. Replication of the infection caused by *C. lingua* was conducted in immunocompetent and immunosuppressed seabirds, specifically double-crested cormorants, *Phalacrocorax auritus*. The birds were collected from a wild colony at 2 weeks of age and raised in captivity until the experimental trial began at 2 months of age. A total of 31 double-crested cormorants (DCC) were divided into 4 different treatment groups: an immunosuppressed/infected group (n=8), an immunosuppressed/non-infected group (n=7), a non-immunosuppressed/infected group (n=8) and a non-immunosuppressed/non-infected group (n=8).

The immunosuppression of the birds involved administration of dexamethasone before and during the experimental infection with *C. lingua* and was confirmed through evaluation of the cellular and humoral responses. A phytohemagglutinin-P skin test showed that DCC treated with dexamethasone had an impaired T cell response ($p<0.01$). A slightly decreased production of antibodies against sheep red blood cells (SRBC) ($p<0.05$), and severe lymphoid depletion of the bursa of Fabricius in treated DCC ($p<0.01$) indicated a detrimental effect on humoral immunity caused by dexamethasone. Heterophilia was also associated with the treatment with dexamethasone.

Infection of the DCC was accomplished by oral administration of large numbers of *C. lingua* metacercariae. A previous pilot study showed that the administration of metacercariae obtained from the fins and tails of fish that inhabit the waters around Prince Edward Island, Canada, successfully produces an infection in DCC. Adult *C. lingua* are present in the DCC 4 days after inoculation of the metacercariae (pre-patent period) and can remain there for at least 6-7 days (patent period).

An increase in the total number of adult *C. lingua* found in the intestine of immunosuppressed DCC suggests that a decreased immune response leads to higher parasite loads in seabirds. However, no pathological or clinical effects were observed in association to the parasite, which seems to indicate the benign nature of infection by *C. lingua*.

ACKNOWLEDGEMENTS

I gratefully acknowledge the financial support from the International Council for Canadian Studies (ICCS).

I also extend my appreciation to the members of my supervisory committee, Dr. Alfonso López, Dr. Caroline Runyon and Dr. Fred Markham. As well as the members of my examination committee, Dr. Shelley Burton, Dr. Richard Cawthron and Dr. Caroline Runyon.

I particularly thank my supervisors, Dr. Pierre-Yves Daoust and Dr. Gary Conboy for their guidance, support and friendship.

I wish to thank Greg Dobbin, Tara Huestis, Robert MacMillan for their technical support and friendship, and Shelley Ebbet for her professionalism and help.

DEDICATION

A Mariuca, Toño, José, Gusa y Tita.

TABLE OF CONTENTS

CONDITION OF USE ii

PERMISSION TO USE POSTGRADUATE THESES iii

CERTIFICATION OF THIS WORK iv

ABSTRACT v

ACKNOWLEDGEMENTS vi

TABLE OF CONTENTS viii

LIST OF TABLES xi

LIST OF FIGURES xii

LIST OF ABBREVIATIONS xiii

1. GENERAL INTRODUCTION 1
   - 1.1 Introduction 1
   - 1.2 Literature Review 2
     - 1.2.1 Parasites and host populations 2
     - 1.2.2 Immune response to intestinal helminths 7
     - 1.2.3 A brief overview of avian immunology 9
       - 1.2.3.1 The avian B complex 12
       - 1.2.3.2 Avian lymphocytes 13
       - 1.2.3.3 Avian phagocytes 15
       - 1.2.3.4 Avian NK cells 16
       - 1.2.3.5 Avian antigen-presenting cells 17
       - 1.2.3.6 Avian complement system 17
     - 1.2.4 Marine birds and their parasites 18
       - 1.2.4.1 Common loons and *Cryptocotyle lingua* 21
       - 1.2.4.2 The double-crested cormorant 22
       - 1.2.4.3 *Cryptocotyle lingua* (Creplin) 1825 28
   - 1.3 Objectives 30

2. MANAGEMENT OF EXPERIMENTAL BIRDS 31
   - 2.1 Introduction 31
   - 2.2 Materials and methods 33
     - 2.2.1 Capture 33

2.2.2 Housing 35
2.2.3 Feeding 38
2.2.4 Health management 39
2.2.4.1 Prophylactic treatment against aspergillosis 39
2.2.4.2 Therapeutic treatment against intestinal parasites, salmonellosis and conjunctivitis 40
2.2.4.3 Bacterial and fungal isolation 40
2.2.4.4 Hematological assessment 41
2.2.5 Statistical analyses 42
2.3 Results 42
2.3.1 Feeding 42
2.3.2 Weight gain 43
2.3.3 Health management 45
2.3.4 Mortality 45
2.3.5 Hematology 52
2.4 Discussion 54

3. IMMUNOSUPPRESSION AND IMMUNOLOGICAL TESTS 61
3.1 Introduction 61
3.2 Materials and methods 63
3.2.1 Control and treatment groups 63
3.2.2 Phytohemagglutinin-P test 64
3.2.3 Sheep Red Blood Cell antibody test 65
3.2.4 Morphometric assessment of bursa of Fabricius atrophy 66
3.2.5 Hematology 67
3.2.6 Statistical analyses 67
3.3 Results 68
3.3.1 PHA-P test 68
3.3.2 SRBC antibody test 71
3.3.3 Morphometric assessment of the Bursa of Fabricius 71
3.3.4 Hematology 78
3.4 Discussion 81

4. *Cryptocotyle lingua*: EXPERIMENTAL INFECTION AND PATHOLOGICAL EFFECTS 85
4.1 Introduction 85
4.2 Materials and methods 88
4.2.1 Origin of metacercariae and administration of infective dose to DCC 89
4.2.2 Viability of the infective metacercariae 92
4.2.3 Egg shedding by, and recovery of, adult *C. lingua* 93
4.2.4 Hematology 94
4.2.5 Pathological effects of *C. lingua* on the intestine of infected DCC 94

4.2.6 Statistical analyses 95
4.3 Results 95
4.3.1 Viability of infective metacercariae 95
4.3.2 Egg shedding by, and recovery of, adult *C. lingua* 96
4.3.3 Hematology 99
4.3.4 Pathological effects of *C. lingua* on the intestine of infected DCC 101
4.4 Discussion 103

5. GENERAL DISCUSSION 109

Appendix 1- Incubation and hatching of double-crested cormorants 116

Appendix 2 - Method of euthanasia 121

LITERATURE CITED 122

LIST OF TABLES

1.1 Parasites of double-crested cormorants, *Phalacrocorax auritus* 26

1.2 Fish hosts for *Cryptocotyle lingua* in Atlantic Canada and the Pacific Ocean 29

2.1 Causes of death in double-crested cormorants (DCC) excluded from the experimental trials (plus 1 bird which died during the main trial) and significant pathological findings in DCC included in both pilot and main experiments 48

2.2 Hematologic values of 29 double-crested cormorants prior to treatment with dexamethasone and infection with *Cryptocotyle lingua* compared to parameters reported in the literature 53

3.1 Treatment and control groups of double-crested cormorants. Dexamethasone was administered to groups A and B. Infective metacercariae of *Cryptocotyle lingua* were administered to groups B and D 64

3.2 Immunosuppressive effect of dexamethasone in double-crested cormorants assessed by the response to subcutaneous injection of phytohemagglutinin-P (PHA-P), the production of antibodies against sheep red blood cells (SRBC), and morphometric evaluation of the bursa of Fabricius (BF). 77

3.3 Hematologic values of double-crested cormorants immunosuppressed (group A) and non-immunosuppressed (group C) compared to baseline values obtained 3 weeks earlier (4 days before the immunosuppressive treatment with dexamethasone started) 80

4.1 Numbers of adult Cryptocotyle lingua recovered from infected double-crested cormorants (DCC) in the pilot experiment (1998) and the main trial (1999) 98

4.2 Hematologic values of double-crested cormorants immunosuppressed and non-infected (group A), immunosuppressed and infected with *Cryptocotyle lingua* (group B), non-immunosuppressed and infected with *C. lingua* (group D) and non-immunosuppressed and non-infected (group C), compared to baseline values obtained 3 weeks earlier 100

LIST OF FIGURES

2.1 Housing of double-crested cormorants showing access to water and perches 37

2.2 Average weights of double-crested cormorants 44

2.3 Multiple granulomas in the spleen of a double-crested cormorant (DCC) from the main trial compared to a healthy DCC spleen 51

3.1 Stimulation index of the response to Phytohemagglutinin-P (PHA-P) skin test in the wing webs of double-crested cormorants 69

3.2 Histological sections of the wing web of double-crested cormorants 24 hrs. following subcutaneous injection of Phytohemagglutinin-P 70

3.3 Antibody response to intraperitoneal injection of Sheep Red Blood Cells in double-crested cormorants 9-10, 14 and 17 days post-inoculation 73

3.4 Histological section of the bursa of Fabricius of a non-immunosuppressed double-crested cormorant showing the normal follicular morphology. . 74

3.5 Histological section of the bursa of Fabricius of a double-crested cormorant 11 days into the treatment with dexamethasone showing moderate lymphoid depletion and formation of cyst-like structures. 75

3.6 Histological section of the bursa of Fabricius of a double-crested cormorant 19 days into the treatment with dexamethasone showing severe lymphoid depletion and collapse of parenchyma. 76

4.1 Life cycle of *Cryptocotyle lingua*, Stunkard (98) 85

4.2 A) Cunners with different degrees of infestation with metacercariae of *Cryptocotyle lingua*. B) Stereoscopic photomicrophotograph of metacercarial cysts of *C. lingua* attached to the tail of a cunner. C) Metacercarial cysts of *C. lingua* in the tail of a cunner, each surrounded by melanin. D) Metacercarial cyst of *C. lingua* in the tail of a cunner 91

4.3 Sections of small intestine of double-crested cormorants infected with *Cryptocotyle lingua*, showing the parasites among intestinal villi. . . . 102

LIST OF ABBREVIATIONS

AVC	Atlantic Veterinary College
BF	Bursa of Fabricius
DCC	Double-crested cormorant
G1	Group 1, collected from a wild colony on June 10, 1999
G2	Group 2, collected from a wild colony on June 22, 1999
H&E	Hematoxylin and eosin
PHA-P	Phytohemagglutinin-P
SRBC	Sheep red blood cells
WBC	White blood cells

1. GENERAL INTRODUCTION

1.1 Introduction

Parasitism in free-ranging wild waterfowl is a common phenomenon. Healthy birds generally carry a parasitic load and keep a low intensity infection through an efficient cellular and humoral immune response (112). However, young naive chicks with an immature immune system and adults with an underlying disease are more severely affected by parasitic infections, especially by tissue-destructive parasites (112). Highly parasitized birds are often in poor body condition and may have other concurrent diseases. Whether the parasites are the cause or the consequence of such a debilitating state is often difficult to determine (112). Examples of poor body condition associated with high intestinal parasite counts are reported in common loons , *Gavia immer*. Forrester *et al.* (34) and Daoust *et al.* (21) described several cases of dead loons found on the coast of Florida, US, and in the Maritime Provinces, Canada, respectively, in which a severe state of emaciation was positively correlated with high intestinal trematode counts. A change in the normal prey consumed by the birds (34) and/or a compromised immune response (21) were speculated to account for the abnormally high intensity of the parasitic infection. To examine the latter possibility, an experimental infection with the most prevalent intestinal trematode found in common loons by Daoust *et al.* (21), *Cryptocotyle lingua*, was conducted on an alternative seabird species, the double-crested cormorant, *Phalacrocorax auritus* . *Cryptocotyle lingua* is a common inhabitant of fish-eating birds and mammals and is acquired through the consumption of fish infested with metacercariae (98). One of the many fish species that acts as an intermediate host for *C. lingua* and is known to carry high loads of metacercariae is the

cunner, *Tautogolabrus adspersus* (94, 98). Thus, double-crested cormorants were chosen as the experimental subject and cunners were chosen as the source of the metacercariae for the experimental infection described in the present study. Before outlining the objectives of the experimental trials that comprised this study, it is important to present a general review of avian immunology, seabird parasites and double-crested cormorants.

1.2 Literature Review

1.2.1 Parasites and host populations

Why do we have parasites?

The theory of natural selection is an important concept in evolution. Natural selection means that the strongest or best adapted forms of life survive while others gradually become extinct. This implies the need for evolutionary modifications due to continuous competition and mutation among the organisms that inhabit any given ecosystem (16).

Evolution leads to differentiation and specialization. Some organisms are seen as prey and others as predators, but most are both. Even the highest ranking predator in the trophic chain eventually dies, becoming prey to scavengers, who are then acting as predators, and later become prey to "more evolved" individuals (16).

Not every living form fits this very organized model, however. Parasitic organisms, whether they are viruses, bacteria, protozoans or metazoans, are neither prey nor predator. A predator feeds on its prey but does not need it for shelter, reproduction, incubation or as a vehicle to reach other organisms (109). Parasites require hosts rather than prey. The symbiosis between two organisms can be beneficial to both parties (mutualism), beneficial

to one party and non-detrimental to the other (commensalism), or beneficial to one part and detrimental to the other (parasitism). The reasons why some interactions have evolved towards mutualism while others involve a competition between two opposite sides are not totally understood. There are two main schools of thought on the subject, consisting of the Gaia hypothesis (87) and the Co-evolutionary theory (16,87).

The Gaia Hypothesis

Proposed by Lynn Margulis in the 1960's (87), the Gaia hypothesis states that planet Earth is a living, self-regulating or "autopoietic" system. This hypothesis derives support from the various examples of organisms that began with the mutually beneficial association of two or more primitive life forms and ended up as a single individual. This is the case of mitochondria in animal cells and chloroplasts in plant cells (87). Genetic evidence has shown that eukaryotic cells were able to acquire the properties of respiration and photosynthesis by incorporating the appropiate bacteria into their cytoplasm by means of a process known as "endosymbiosis" (87). When the concept of endosymbiosis is taken to a larger scale, it is possible to see that pluricellular organisms also benefit from a mutualistic relationship with what could be considered parasitic life forms. For example, 90% of land plants are mycorrhizal, which means that fungi associated with their roots are essential to their ability to draw nutrients from the soil (87). In the animal kingdom, herbivores depend on their gastrointestinal microfauna to digest cellulose (87).

One can think of Earth as one big organism. If a mutual relationship between its various microorganisms could be regarded as optimally beneficial for all, then the concept that as hosts and parasites coevolve and adapt to one another their mutual antagonism should

diminish can be proposed. This should apply to most, if not all, host-parasite associations. A parasite would naturally have a tendency to become less virulent over time. This is well exemplified by a hematogenous parasite, *Trypanosoma sp*, in the red spotted newt. Newts can carry very high numbers of trypanosomes in their blood with no negative effects on their health status or reproductive success (87). The parasite evolved to become a very benign inhabitant of the newt body. That much is obvious, but why? The answer lies in the way the trypanosome is transmitted from one host to the next. Because transmission requires the bite of an amphibious leech, it can only occur in ponds. Newts are born in ponds in the summer but leave at the end of the season to spend approximately six years in the forest before returning to the pond as adults to reproduce (87). If the parasite were a highly virulent one, newts would die in the forest. The trypanosomes would die with them, therefore being unable to infect other individuals (87).

The Coevolutionary Theory

The newt trypanosomiasis is a very good model to support the theory that eventually all parasites and hosts become more tolerant of each other. This is not always the case, however. In some instances, parasitism increases the mortality of a host population. In these cases, the higher mortality index either does not affect the parasite or is beneficial to it. The liver fluke, *Dicrocoelium dendriticum*, which uses sheep and other ruminants as definitive hosts, has a complex life cycle that includes ants as intermediate hosts (87). Cercariae infect ants and at least one of them migrates to the subesophageal ganglion, becoming encysted there. Because this ganglion controls the mouth parts and locomotion of the insect, the

encysted parasite modifies the behavior of the ant. The infected ant crawls to the top of the grass and, if the temperature is low enough, becomes locked onto the plant by its mandibles, being then eaten by the definitive host, thus completing the life cycle of the parasite (1, 75, 87).

The behavior of *D. dendriticum* cercariae is directed toward improving the opportunities of the parasite to complete its life cycle by making the intermediate host more susceptible to predation by the definitive host. There are also other strategies of host exploitation. The parasitic fungus, *Ustilago violacea*, manipulates the reproductive strategy of its host, the catchfly plant, to enhance its own transmission. If the host plant is male, the fungus transforms the pollen-producing stamens in the flowers into organs that shed fungal spores. If the host plant is female, it forces it to grow male-like flowers that will then shed fungal spores. Regardless, the infected plant becomes sterile and the fungus increases its chances of being taken up by insects and infecting other healthy catchfly plants (87).

Dicrocoelium dentriticum and *Ustilago violacea* are two parasites that have not become less virulent with time. Like some other host-parasite relations, they are not at all mutualistic. The very aggressiveness that the parasite expresses towards its host seems to be the key factor in ensuring the completion of its life cycle.

Whether the relationship becomes mutualistic or a continuous conflict between the parasite and the immune system of its host is highly dependent upon the mode of transmission of the parasite (109). A parasite with a complex life cycle, one with a definitive and at least one intermediate host, will tend to become less virulent toward the former, at least until offspring are produced. The parasite will be very virulent toward the intermediate

host since this improves the probability of it being predated upon, thus making it easier for the parasite to reach its definitive host. The coevolutionary theory is based on this continuous adaptation of both parasite and host to survive. The advantage for the parasite is that it can achieve adaptive modifications more rapidly than the host. Parasites are usually a less evolved life form and the interval between generations is, therefore, quite short. The host may not be able to adapt so quickly, but its immune system can. While the parasite is continually trying to avoid being adversely affected by the host's immune response, the immune system is continually trying to improve that response (7, 87).

The ongoing competition between the efforts of the parasite to take more resources from the host and reproduce effectively and the efforts of the immune system of the host to stop it is characterized by a "non-linear model in which coevolution sustains perpetual fluctuations between parasites and hosts" (7). In 1973, ecologist Leigh Van Valen named this model the Red Queen Hypothesis, after the character in Lewis Carroll's book "Through the Looking Glass" (11). In the story, Alice enters a race in the Garden of Live Flowers where everybody is running but nobody is actually moving. When she asks the Red Queen about such odd behavior, the Queen answers: "Now, here, you see, it takes all the running you can do, to keep in the same place". From a coevolutionary point of view, the host is always evolving to become more resistant to a parasite while the parasite evolves to be able to defeat such resistance. Neither the host nor the parasite can afford to relax and, when both sides are evenly matched, neither faces immediate extinction. In this race, they both have to coevolve just to keep in the same place (87).

In 1982, Hamilton and Zuk, (44) took the Red Queen Hypothesis a step further by

suggesting host resistance to parasitism as the main factor of natural selection. They based their hypothesis on a study of American passerine birds that established a relationship between parasite resistance and expression of sexual traits. By expressing sexual traits more fully than a sick or infected one, a resistant male will increase its chances of breeding. This ensures the transmission of resistance-related genes to the offspring (44, 87). Several subsequent studies have supported Hamilton and Zuk's interpretation of the Red Queen Hypothesis (4, 7, 15, 76, 101, 110) Others have concluded that it does not apply to all host species (50, 81). The general consensus is that, if not the most important, resistance to parasitism is definitely one of the factors that greatly influences natural selection and host evolution.

1.2.2 Immune Response to Intestinal Helminths

A host responds to different parasites in different ways. The immune response against an intracellular organism, such as a bacterium, is different from that developed against an extracellular one. The site that the parasite inhabits, and its size, also determine the kind of response it elicits (16). A virus attacking the central nervous system and a trematode lodging in the bile ducts will be dealt with differently.

Intestinal helminths inhabit what is considered one of the major ancestral sites for parasites. The intestinal tract of a host is readily accessible from the outside world and provides a protected and stable habitat, a steady source of nutrition and an exit for reproductive forms (109). A great variety of trematodes, cestodes and nematodes are present in the intestinal tract of most living vertebrates. Intestinal parasites are the most common and varied species found in any host (109). No doubt the adaptation process that intestinal

parasites went through was not an easy one, and protective strategies were evolved to survive in this hostile environment. Some species evolved to enter through an alternative route, such as the skin or mucosae (109).

Because of the large size of helminths, phagocytosis of an entire parasite is impossible (16, 35). Therefore, the main goal of the immune system, when fighting against a helminth, is either to interfere with its metabolic and sensory functions or to cause damage to its external surface so that it can later be phagocytized (9, 16, 109)

When a helminth parasite enters the intestine, the innate immune response (comprised mainly of mucosal enzymes), the presence of secretory immunoglobulin A and the generally inhospitable enteric environment should make adhesion to the epithelium, development and reproduction difficult, if not impossible (102, 109). Usually, however, innate immunity and adverse conditions are not sufficient to prevent helminths from colonizing the intestine of the host (102, 109). When this first line of defense fails, the adaptive immune response responds. The adaptive immune response includes antigen-presenting cells (APC), macrophages and T and B lymphocytes (102). Antigens from the cuticle and those released as part of parasitic metabolism are picked up and processed by APC. Although specialized M cells located above Peyer's patches are the ones normally associated with antigen presentation, cytokine activation during inflammation induces expression of Major Histocompatibility Complex (MHC) Class II in enterocytes, enabling them to act as antigen-presenting cells (109). After the antigens are presented to CD4 T cells, the latter differentiate into helper T cells (Th cells), either as CD4 Th1 or as CD4 Th2 cells. The Th2 cells activate B cells, which then differentiate into plasma cells and produce antibodies, including IgE.

The IgE molecules bind to mast cells and cross-link parasitic surface antigens, thus eliciting mast cell degranulation. This attracts eosinophils to the site and produces inflammation of the intestinal wall. Inflammation allows influx of fluid into the intestinal lumen, along with the passage of higher numbers of antibodies, including IgG. Simultaneously, Th1 cells produce interferon-γ (INF-γ) which activates macrophages. Through antibody linkage, eosinophils and macrophages can bind to the surface of the parasite and release cytotoxic granules, a phenomenon known as antibody-dependent cell cytotoxicity (ADCC). The contents of eosinophilic granules and the nitric oxide released by macrophages during the "respiratory burst" perforate the surface of the parasite, thereby destroying its primary defense against intestinal digestion (16, 102, 109). More importantly, a response directed toward antigens released from the body of the parasite, such as digestive enzymes or anticoagulants, blocks vital metabolic pathways ultimately contributing to its death (109).

The mechanism described above is the basic way in which vertebrate hosts, especially mammals and birds, respond to infection by intestinal helminths. Individual characteristics of the host and the parasite largely influence the outcome of an infection. Processes of "self-cure", reinfection and resistance must be considered in each individual case of parasitism. No rule can be applied to all helminths or all hosts (102, 109).

1.2.3 A brief overview of avian immunology

Although both birds and mammals belong to the Subphylum Vertebrata, each have evolved separately and into different Classes, thereof Avian and Mammalian. Because of this divergence the avian immune response is different from that of mammals (68).

Vertebrates in general have more highly organized and complex defense mechanisms than

invertebrates. Among vertebrates, however, the immune system of less evolved organisms is similar to that of the more evolved ones. Lymphoid tissue in fish is organized differently, but the fundamental cell types of the immune system, such as macrophages, T and B lymphocytes and plasma cells, are similar to those in mammalian species. What varies most between mammals and other vertebrates is the organ distribution of the cellular components of the immune system and the architecture of the lymphoid tissues. In fish and amphibians, in which there are no lymphoid organs *per se*, the lymphoid tissue is located in organs with sinusoidal blood flow such as the kidneys, where the circulation is slow enough for antigens to be detected. In birds, the lymphoid tissue is more organized and some species even have lymph nodes, specialized organs solely dedicated to the immune response (68).

During vertebrate evolution, the cellular immune response may have emerged before the humoral response; this hypothesis is suggested because invertebrates do not have immunoglobulins yet are able to show cellular reactivity to an antigen. The two basic evolutionary changes seen in vertebrates compared to invertebrates are the production of immunoglobulins and the development of the thymus, a primary lymphoid organ (68). All vertebrates have functional T and B cell responses, and, except for the jawless vertebrates (Agnatha), all have a true thymus. In Agnatha, thymus-like functions are performed by the "pharyngeal lymphocytic tissue", a significant concentration of lymphoid cells in an area where pathogens tend to accumulate and can penetrate the epithelium. Even these primitive vertebrates have both T and B cell populations and can produce immunoglobulins, however (68).

Among vertebrates, the avian immune system most closely resembles the mammalian

one. Birds have both primary (central) and secondary (peripheral) lymphoid organs. The primary lymphoid organs are the thymus and the bursa of Fabricius. Secondary lymphoid organs include spleen, bone marrow, nasal-associated lymphoid tissue, bronchial-associated lymphoid tissue, gut- associated lymphoid tissue and head-associated lymphoid tissue (68, 78). The gut- associated lymphoid tissue includes cecal tonsils, Peyer's patches, Meckel's diverticulum, intra-epithelial lymphocytes, and lymphocytes of the lamina propria (68, 78). The head-associated lymphoid tissue includes Harderian or paraocular glands, paranasal glands, lachrymal and lateral nasal ducts and conjunctiva-associated lymphoid tissue (68, 78).

Some avian species, such as ducks and geese, have simple lymph nodes. Others have only lymphoid nodules associated with lymphatic vessels and scattered lymphoid tissue in organs such as pancreas, liver and kidney (68).

The lymphoid nodules of birds are anatomically similar to mammalian lymph nodes: an afferent vessel through which the lymph enters, an efferent vessel through which it exits, germinal centers with B and plasma cells, and aggregates of mature and immature T cells (68). Lymphoid nodules arise from cellular proliferation in the wall of lymphatic vessels, their central sinus representing the main lumen of these vessels (68).

The thymus is responsible for avian T cell production, as in mammals. Production of B cells, however, is different. Instead of originating from the fetal liver and bone marrow, B cells come from the bursa of Fabricius, an organ unique to birds. The bursa is located dorsal to the cloaca. The marked separation of organs for B and T cell maturation is one reason why birds are useful models for immunological experimentation. The opportunity to study

the functions of thymus and bursa separately was a determining factor in the discovery of the two different types of lymphocytes, thymus-dependent (T cells) and bursa-dependent (B cells) (68, 78).

1.2.3.1 The Avian B Complex

In mammals, the group of genes that codifies the expression of glycoproteins holding a key function in the immune response is called the "Major Histocompatibility Complex" (MHC). The MHC was shown to determine compatibility of tissue grafts, hence its name. The gene components of the MHC are divided into Classes I, II and III. The first two classes codify membrane-bound antigens and the third class codifies components of the complement system (49).

In birds, the MHC equivalent is called the B complex since it was first described as a blood group locus that codified erythrocyte antigens (the B locus) (13). The B complex expresses the equivalent of MHC Class I and Class II, plus another class not found in mammals called MHC class IV. As genes that encode the complement components are not linked to the avian MHC, there is no recognized MHC class III in birds (13, 55, 78, 107).

Since the MHC is known as the B complex, the antigens also change names. The MHC class I becomes B-F, MHC class II becomes B-L and MHC class IV is B-G (13). The configurations of B-F and B-L antigens are similar to their mammalian counterparts (13, 78). They also bind T cell receptors (TCR) and the co-stimulatory molecules CD8 and CD4 (13, 78). As with the MHC in mammals, most of their polymorphism is found on the peptide-binding cleft (13,55,78,107).

The B-F antigen is found on the surface of most nucleated cells in birds, including

erythrocytes (13). In mammals, several viral antigens have been shown to decrease MHC Class I expression to avoid recognition by CD8 T cells. A similar effect of viral antigens could be expected toward B-F expression in avian species, but some studies have shown that it is not always so. The B-F expression in cells infected with Marek's disease virus (MDV) is not down-regulated. On the contrary, the virus apparently increases B-F expression during the first stages of infection. The reason for this atypical behavior is unknown. One hypothesis compares MDV with human Cytomegalovirus (CMV), which uses MHC antigens as cellular receptors so that an increase in their expression increases the probability of infection (12).

The B-L antigen is expressed on the surface of B cells, macrophages and activated T cells. It has also been found in MDV-induced T-cell tumors and on endothelial cells in bursal capillary blood vessels (107).

The B-G antigens are expressed mostly on erythrocytes, but also on thrombocytes, leukocytes and intestinal epithelial cells (13). They consist of nonglycosylated transmembrane dimeric or trimeric molecules (13). Although they seem to play no part in the immune response, their function is still not clear (13, 78, 107).

There seems to be another set of histocompatibility genes not included in the B complex, the Rfp-Y (Restriction Fragment Pattern-Y) (73). Rfp-Y has been found in both chickens, *Gallus domesticus*, and ring-neck pheasants, *Phasianus colchicus* (73). Whether or not the Rfp-Y is involved in the immune response has not been determined (73).

1.2.3.2 Avian Lymphocytes

Hematopoiesis begins in the yolk sac and then moves to two intra-embryonic sites.

Hematopoiesis first moves to the intra-aortic cell clusters and later to the para-aortic mesenchyma (68, 78). From the para-aortic cluster, stem cells migrate to lymphoid organs. The B cell precursors pass through the spleen and bone marrow, eventually colonizing the bursa of Fabricius where immunoglobulin (Ig) gene diversification occurs (68, 78). Gene rearrangement happens prior to migration to the bursa (68, 78). The T cell precursors go to the thymus (68, 78).

The B cell precursors give rise to B cells which undergo a selection process and then proliferate. Shortly before and after hatching, B cells migrate or "seed" secondary lymphoid organs, such as spleen and bone marrow (68, 78). Since the bursa undergoes functional involution when the bird reaches sexual maturity, these secondary lymphoid organs will be the main source of B and plasma cells later in life (68,78).

Avian B cells can produce three types of immunoglobulins, consisting of IgM (a pentameric molecule found in serum and on B cell surfaces) , IgA (monomeric in serum, dimeric in bile and mucosal secretions), and IgG (found in serum and egg yolk, also called IgY) (78). Avian IgG strongly resemblances mammalian IgG but it also has similarities to IgE, suggesting that avian IgG predates both mammalian IgG and IgE (78). Antibodies can be passed from mother to offspring via the amniotic fluid in the egg. This amniotic fluid contains IgM and IgA that can potentially be swallowed by the embryo, like colostrum is in domestic mammals (78). However, because amniotic fluid is swallowed in small amounts the antibodies obtained in this manner have no relevance (78). IgG, in contrast, is absorbed from the yolk sac pre- and post-hatching (78).

Avian T cells originate from blood-borne precursors that seed the thymus from the para-

aortic mesenchymal cell cluster (49, 78). Stem cells arrive at the thymus at three different times and each gives rise to a different T cell population. The first wave of stem cells becomes γδT cells (TCR1), the second and third become αβT cells (αVβ1(TCR2) and αVβ2(TCR3), respectively) (49, 78). Cells of the αβT population undergo positive and negative selection for MHC restriction and self-tolerance, but γδT cells seem to leave the thymus before this happens (49, 78). In birds, γδT cells are more abundant than in mammals, 30-50% of avian peripheral blood lymphocytes being γδT cells (24). In the thymus, αβT cells begin as $CD4^{-}CD8^{-}$ (double negative) cells (49, 78). They then become CD4+CD8+ (double positive) cells (49, 78). Later, one of the costimulatory molecules is down-regulated and they become either $CD4^{-}$CD8+(CD8) or CD4+$CD8^{-}$(CD4) cells. Afterward, they migrate to secondary lymphoid tissues and other organs (49, 78). As in mammals, the CD4 cells act as "helper" cells and the CD8 cells as cytotoxic T cells when activated (49, 68, 78).

1.2.3.3 Avian Phagocytes

Phagocytosis in birds is performed mainly by macrophages/monocytes and heterophils (24, 45, 54, 95). Heterophils are the avian equivalent of mammalian neutrophils, although some differences, such as the nature of their granules, exist (45). The microbicidal mechanisms of heterophils are non-oxidative since their granules lack the myeloperoxidase enzyme present in mammalian neutrophils (45). Heterophilic granules contain β-defensins that bind to microbial membranes and form a perforating channel. As part of the innate immune response, heterophils are the first to arrive at a site of inflammation, followed by blood monocytes which, while migrating through tissues, become macrophages (45, 54). Both macrophages and heterophils can phagocytize antigens. Lymphocytes are recruited

mainly by macrophage cytokine (monokine) secretion, approximately 72 hours after antigenic stimulation (54). After phagocytizing the immunogen, heterophils disintegrate and their remnants are taken up by macrophages. Lymphocytes, fibroblasts and macrophages wall off the site of inflammation, producing a granuloma. Granuloma formation is more common than abscess formation in birds due to the rapid walling off of the inflammatory site and to the heterophilic enzymes not being as liquefactive as those from mammalian neutrophils (45,54).

Both the acquired and innate avian immune responses seem to be similar to those of mammals, although avian cytokines, chemoattractants and adhesion molecules have not been described as extensively as in mammals. One difference is found in the production of interferon (IFN), an important Mφ activator. In mammals, IFN-α is produced by monocytes and macrophages, IFN-β by fibroblasts and epithelial cells, and IFN-γ by activated T cells. However, it has been shown that chicken T cells can produce all three kinds of IFN (α, β and γ) (65).

1.2.3.4 Avian NK cells

Avian Natural Killer (NK) cells are located mainly in the spleen (78, 95). Besides the spleen, NK cells are found in peripheral blood, thymus and bursa of Fabricius (78, 95). Natural Killer cells are derived from the para-aortic mesenchymal stem cells that migrate to the bone marrow but are thymus and bursa-independent (78, 95). The CD3 receptors, or TCR3 molecules, of avian NK cells are located in the cytoplasm, as opposed to the mammalian NK cells which have a surface CD3 receptor (78, 95).

Apparently, NK cell activity is similarly regulated in mammals and birds, although

information is limited (95). Among the possible regulators of NK cell activity in birds, IFN and α-fetoprotein are considered relatively important. Some evidence suggests that IFN enhances NK cell activity, as in mammals, but experimental results are inconclusive (95). A molecule naturally present in amniotic fluid, α-fetoprotein, reduces NK cell activity in the spleen of chickens (95).

In humans, high susceptibility to infection by herpes simplex virus is related to low NK cell reactivity (95). In chickens, low NK cell reactivity has been associated with high susceptibility to Marek's disease virus (MDV), although the reason for this is unknown (95). Studies on MDV, a tumor-inducing virus, and NK cells indicate that the latter are important in defense against tumor progression in birds (95).

1.2.3.5 Avian Antigen Presenting Cells

Dendritic cells and macrophages are the main Antigen-Presenting Cells (APC) in mammals, while B cells have a very small contribution (78). In birds, B cells are as active in antigen presentation as avian dendritic cells and macrophages (78).

1.2.3.6 Avian Complement System

As in mammals, the C3 molecule is the key component of the complement system. The C5, C2, C1 (C1q) and C4 components are also found in birds (55). There is no proof of the existence of an avian membrane attack complex (MAC), which, in mammals, is composed of C6, C7, C8 and C9 (49, 55, 78).

Avian complement can be activated through the alternative complement pathway (ACP). Activation of the ACP is triggered by the detection of outer surfaces of foreign organisms (55). Avian ACP requires the presence of factor B, which is also a component of

mammalian ACP (49). However, unlike mammalian ACP, it is calcium independent (49,55).

Classical complement pathway (CCP) activation, which is antigen-antibody and calcium dependent, has not been proven to occur in birds although they are known to have C1q molecules (55). Since the complement components necessary for CCP activation are present in birds, the possibility of its occurrence must be considered (55).

Avian complement can act in three ways. It can act by enhancing phagocytosis (opsonin activity- C3b and C4b), by inducing an immune response (C3a, C5a, C2b and C4b) or by lysing target cells, presumably by using a perforin system similar to mammals (78). Compared to mammals, genes of the complement complex are not linked to the MHC or B complex in birds (49, 55, 78, 107).

1.2.4 Marine Birds and Their Helminth Parasites

Parasitic helminths have been recovered from all major groups of seabirds throughout the world (47). Over the last two centuries, about 50% of seabird species have been examined for parasites, either because of an interest in the effects of parasites on population health status and dynamics or to use parasites as probes in assessments of ecological biodiversity (47).

Most marine birds are secondary or tertiary predators which occupy specific geographic ranges and habitats and, as a consequence, are excellent indicators of the state of marine ecosystems (47). Studies of the biodiversity and ecological history of a given environment can be accomplished by various means. One method is the study of the parasitic helminths of a population to assess contemporary and historical ecology and the long term development of communities. Most studies involving examination of helminth fauna have been conducted

at high latitudes in the South Ocean, Antarctica or the Holartic region (47).

Despite the relatively limited knowledge of seabird parasites (112), trends in diversity and abundance of parasitic fauna occur in certain avian groups. In general, birds inhabiting pelagic (oceanic) environments support faunas of lower diversity than birds found on neritic and littoral waters (47). The diversity in helminth fauna is determined mainly by feeding habits. Piscivorous and planktivorous seabirds inhabiting pelagic ecosystems have a lower diversity of parasites, especially trematodes, due to limitations imposed by life cycles of digeneans. In contrast, birds living in benthic habitats, whether feeding on invertebrates or fishes, have greater numbers of trematode and cestode species since such prey are highly infected with larval (intermediate) parasitic forms (38, 47). An additional factor that affects the diversity of helminth fauna is the nature of the life cycle of the parasite. Parasites whose cycle does not include free-living stages are less affected by detrimental environmental conditions (38). However, helminths with direct life cycles are less competitive than those parasites capable of developing free-ranging life forms (38).

The pathogenic effects of helminth parasites on an individual host and the consequences of infection on an entire seabird population are difficult to assess. Several species of trematodes, cestodes, nematodes and acanthocephalids can cause significant pathological effects in marine birds. Mass mortalities associated with *Eustrongylides* sp., a nematode of the proventricular wall, occur in merganser, ardeids and egrets, resulting from emaciation caused by the parasitic infection (30). Death by starvation of young little penguins, *Eudyptula minor*, affected by heavy loads of hepatic and enteric helminths, occurs regularly in Australia (77). One lesser black-backed gull, *Larus fuscus*, apparently died of

hemorrhagic enteritis caused by a severe *Cryptocotyle lingua* infection (62). In all cases, the helminths involved are normal components of seabird parasitic fauna and cause death, usually of young birds, only when heavy infections occur (30, 37).

An increase in the intensity of helminth infection decreases the resistance of the birds to other stresors, reduces weight gain and results in increased predation of weakened chicks. Occasionally, heavy helminth infection can contribute directly to the death of affected individuals (37).

The reasons behind an unusual rise in the intensity of helminth infections are unclear. However, several contributing factors have been recognized. These factors include an increase in parasitic infection of prey items at certain times of year. For example, the release of cercariae of *Paramonostomum alveatum* from its first molluscan intermediate host and subsequent encystment in the second intermediate host coincides with the time when common eider nestlings, *Somateria mollissima*, begin foraging, thus increasing the number of infective forms being eaten by the young birds (37). A second factor is that parasitized prey may become more abundant and/or available to marine birds. For example, microphallid metacercariae cause behavioral alterations in periwinkles, *Littorina* sp, inducing the latter to creep to open places and thus become easier targets for young eiders (37). A third factor is the modification in the diet of the birds towards an unusual and highly parasitized prey species. An example of this is a switch to high energy prey, such as the shore crab, *Carcinus maenas*, which carries high loads of the highly pathogenic acanthocephalan *Profilicollis botulus*.(8, 34). Finally, a weakened immune system may be unable to fight off invading parasites (21, 37).

Demonstration of a positive correlation between high levels of infection and an impaired immune system could suggest that helminths damage the immune response, resulting in an individual that is more susceptible to other disease processes. However, could a decrease in immune function due to a pre-existing condition produce an increase in parasitism? Would the pathological effects caused by the parasite be enhanced in cases of decreased immune function? Observations supporting the idea of a decreased immunity resulting in higher degree of parasitism are reviewed in the following section.

1.2.4.1 Common Loons and *Cryptocotyle lingua*

Daoust *et al.* (21) correlated the body condition of common loons, *Gavia immer*, from the Maritime provinces of Canada, with the intestinal load of parasitic helminths carried by the birds. The number of intestinal trematodes present in birds in good body condition, with conspicuous fat reserves and well developed pectoral muscles, was significantly lower than that of birds in poor body condition (21). Similar findings were reported by Forrester *et al.* (34) in common loons from Florida, USA.

While Forrester *et al.* (34) suggested that the higher numbers of intestinal trematodes, mainly microphallids, found in dead common loons in Florida were due to an altered diet that made the intermediate form of the parasite more available for consumption, Daoust *et al.* (21) attributed the higher level of infection to impairment of the immune system of the birds secondary to emaciation. These latter investigators proposed that any factor causing debilitation of the loons, such as lead poisoning from ingestion of fishing lures, could cause depression of the immune system which would allow a higher proportion of ingested metacercariae to mature.

Cryptocotyle lingua was the most abundant intestinal trematode found by Daoust *et al.* (21) in common loons. An experimental model using this parasite in a piscivorous bird species was developed to test the hypothesis that a compromised immune system can result in a higher intensity of infection by intestinal trematodes. Experimental infection could also determine what effects *C. lingua* has on infected hosts, to the exclusion of effects from other disease processes. The common loon is not a viable experimental subject due to relative scarcity in the wild and the difficulty of maintaining it in captivity. Therefore, the double-crested cormorant, *Phalacrocorax auritus*, also a piscivorous species, was considered an appropriate alternative species.

1.2.4.2 The Double-crested Cormorant, *Phalacrocorax auritus* (Lesson) 1831

The view held about cormorants seems drastically altered by the subjective perceptions of people. John Milton, in his poem *Paradise Lost*, book IV (74), when referring to Satan says: "*and on the Tree of Life, ...sat like a Cormorant*". The Chinese seemed to agree with the devilish appearance of the cormorant and named it "wu kwei", black devil (63). But it is perhaps catfish farmers and sport fishermen who have a more practical reason to dislike the black birds. Cormorants are opportunistic feeders, highly attracted to concentrated food sources (25, 79). Wintering double-crested cormorants (DCC) forage in commercial channel catfish (*Ictalurus punctatus*) ponds in Mississippi (53). In the summer, DCC prey upon valuable fish species, such as smallmouth bass (*Micropterus dolomieui*), in Lake Ontario (3). A study of farms on the Mississippi Delta estimated that 4% of catfish fingerlings are consumed by DCC each year, despite "fright" strategies used to reduce bird foraging (39). The DCC breeding around Lake Ontario consumed an estimated 87.5 million fish in 1998,

including 1.3 million smallmouth bass (3).

The conflict between people and cormorants around Lake Ontario has intensified since the 1970's, when a very decimated avian population began an extremely successful recovery. Specifically, the use of DDT was banned, and the species became federally protected under a migratory bird treaty signed with Mexico (29). The number of nests went from 10 in 1972, to 18,831 in 1998 (3). Because cormorants are now so numerous, some people, especially those in the sport fishing industry, regard these birds as pests. In July 1998, more than 800 cormorants were illegally shot on Little Galloo Island, Lake Ontario (29, 88).

A suspected shooting incident in the spring of 1996 on Prince Edward Island, Canada, caused the displacement of an entire colony of cormorants from the breeding site that the birds had used for at least 16 seasons. The tree-nesting colony on Ram Island in Malpeque Bay, the largest colony in northeastern North America (10), was found empty in May 1996, a time when cormorants are normally in the incubation stage. The birds had moved to Little Courtin Island, 3 km to the south of Ram Island, a phenomenon that usually results from human harassment (10).

Not all fishermen think badly of the cormorant, however. Eleven hundred years ago, a Chinese fisherman decided to take advantage of the fishing abilities of the great cormorant (*Phalacrocorax carbo)* and taught it to follow two simple commands. These commands consisted of directing birds to dive and catch fish, and to give up its prey upon returning to the boat. The training of cormorants continues today. These birds can be trained to pursue certain species of fish, according to market demands (48, 63). Cormorant keepers fish all year round, except when rain and high winds make the water muddy or when the rivers are

covered with ice (48). Although cormorant fishing is also practiced in Japan, it is the Chinese who "...have brought the cormorant into a complete and perfect state of domestication, breeding and raising it in captivity" (63).

Japanese "Master Cormorant Fishermen", *U-Sho*, fish mainly for "sweetfish" or ayu, *Plecoglossus altivelis*. They obtain their birds from wild populations of the sea cormorant, *Phalacrocorax capillatus*, and submit them to basic training, but this process is never as thorough as that of the Chinese (63). Cormorant fishing in Japan is now merely a tourist attraction performed at night, with the aid of fire lanterns, from May to October (52, 113). Although cormorants are widely distributed throughout the world, nowhere but in China and, to a lesser degree, Japan, have they been domesticated (63).

In North America, four cormorant species occur (40). The great cormorant, *P. carbo*, is found along the southern Atlantic Coast of Canada. Brandt's cormorant, *P. penicillatus*, inhabits the Pacific Coast, from southern British Columbia to Baja California. The pelagic cormorant, *P. pelagicus*, lives on the northern Pacific Coast, from Alaska to Baja California. The species with the widest range, the double-crested cormorant, *P. auritus*, breeds from southwestern Alaska, central Alberta, James Bay, and Newfoundland south to Mexico and the Bahamas (40).

There are five subspecies of double-crested cormorants and they constitute six allopatric breeding populations: *P. auritus floridanus* (Florida population), *P. auritus heuretus* (San Salvador population), *P. auritus cincinatus* (Alaska population), *P. auritus albociliatus*, formerly known as the Farallon Cormorant (West Coast population) and *P. a. auritus*, the double-crested cormorant (Atlantic (Northeast coast) and Interior populations) (46).

Population size varies considerably. In 1995, a total of 220,000 pairs were reported for *P. a. auritus* from the Interior population and 96,000 from the Atlantic population (46). The status of the other populations is not completely known. Partial counts were reported in the same study as 31,000 pairs for the West Coast population, 14,000 pairs for the combined Florida and San Salvador populations and 3,000 pairs for the Alaska population (46). The Interior, Atlantic and West Coast populations are considered to be increasing (46).

Phalacrocorax auritus auritus is, therefore, the most numerous of all five subspecies. It can be found from Alberta to Labrador, from Newfoundland to Massachusetts and the southcentral US during the breeding season, and in southern and northern México and coast of the Gulf of Mexico in winter (51). Several colonies of double-crested cormorants are present in Canada's Maritime Provinces. In Prince Edward Island, most of them (68% in 1995) are found on the north shore. In 1997, a total of 4,714 nests were counted in Malpeque Bay, Prince Edward Island (10). Of these, 1,738 nests were on Ram Island and 2,976 nests were on Little Courtin Island (10).

High population numbers and easy access to rookeries made the DCC a suitable species for an experimental model to study infection with the intestinal trematode *Cryptocotyle lingua*. A list of parasites that infect *P. auritus* is presented in Table 1.1.

Table 1.1 Parasites of the Double-crested Cormorant, *Phalacrocorax auritus*

	Scientific Name	Location
Trematodes	*Amphimerus* sp.[1]	Intestine
	Amphimerus elongatus[13]	Bile ducts
	Ascocotyle sp. *2*[1]	Intestine
	Austrobilharzia variglandis[3]	Blood
	Austrodiplostomum mordax[1]	Intestine
	Clinostomum attenuatum[19]	Trachea
	Clinostomum marginatum[16]	Trachea/lungs
	Cryptocotyle lingua[20]	Intestine
	Drepanocephalus spathans[16]	Intestine
	Galactosomum humbargari[10]	Intestine
	Galactosomum phalacrocoracis[10]	not available
	Hysteromorpha triloba[15,16]	Intestine
	Maritrema sp.[1]	Intestine
	Mesoophorodiplostomum pricei[1]	Small intestine
	Mesostephanus appendiculatoides[16]	Duodenum
	Neogogatea kentuckiensis[14]	Intestine
	Opisthorchis vitellatus [17]	Bile ducts
	Ornithobilharzia sp.[15,16]	Blood
	Parascocotyle diminuta[19]	Intestine
	Parorchis acanthus[15,16]	Cloaca
	Phagicola longus[16]	Intestine
	Phocitremoides butionis[1]	Intestine
	Pseudopsilostoma varium[1]	Esophagus/gizzard
	Renicola sp (*thapari*?)[16]	Kidney

Cestodes	*Paradilepis caballeroi* [12,16]	Intestine
	Paravitaenia eudocimi [12,16]	Intestine
Nematodes	*Capillaria contorta*[1]	Esophagus
	Capillaria spiculata[1]	Large intestine
	Contracaecum microcephalum[9]	Gizzard/pro-ventriculus
	Contracaecum multipapillatum syn. *robustum*[9]	Proventriculus
	Contracaecum rudolphii syn. *Contracaecum spiculigerum*[1,2,9,18]	Gizzard
	Desmidocerca incognita syn. *Desmidocercella incognita*[18]	Air sacs
	Desmidocercella skrjabini[1]	Lung/stomach
	Eustrongylides tubifex[5]	Proventriculus
	Syncuaria squamata[1,6,7]	Under gizzard lining
	Syngamus hexadontus [17]	Trachea
	Synhimanthus sp.[1]	Esophagus
	Tetrameres microspinosa[1]	Gizzard
Acanthocephalids	*Andracantha gravida*[1]	Intestine
Arachnids	*Scutomegninia microfalcifera* [4]	Feather mite
	Ceratophyllus niger[8]	Found in nest

[1] Fedynich, Pence & Bergan 1997, HSOCW 64:176-182; [2]Bartlett 1996, P 3:4,367-376; [3]Barber & Caira 1995, JP 81:4,584592; [4]Mironov 1990, Parazitologiya 24:1,43-55; [5]Measures 1988, CJZ 66:10,2223-2232; [6]Wong & Anderson 1987, CJZ 65:10,2524-2531; [7]Wong, Anderson & Bartlett 1986, CJZ 64:5,1186-1196; [8]Easton 1982, Entomological News 93:5,155-158; [9]Deardorff & Overstreet 1980, JP 66:5,853-856; [10]Pearson 1973, Phil Trans Roy Soc Lond Ser B 266:341-447; [12]Rysavy & Macko 1971, Anal. Inst. Bio. UNAM-Zoologia, 42:1,1-28; [13]Pense & Childs 1972, JWD 8:3,221-224; [14]Stunkard & Olson 1972, JP 58:634-635; [15]Huggins 1956, Proc. South Dakota Acad. Sci. 35:204-206; [16]Threlfall 1982, HSOCW 49:103-108; [17]Chin 1950, JP 36:20-24; [18]Mawson 1957, CJZ 35:213-219; [19]Hutton & Sogandares-Bernal 1960, Bull. Mar. Sci. Gulf Carib. 10:40-54; [20]Conboy, unpubl. data.

1.2.4.3 *Cryptocotyle lingua* (Creplin) 1825

Cryptocotyle lingua has an indirect life cycle, typical of trematodes of the order Digenea. Fish-eating birds and mammals act as definitive hosts, while a gastropod mollusc and various species of marine fish act as the intermediate hosts (98). The adult parasite, found in the intestinal lumen of the definitive host, produces eggs that reach the outside environment in the feces (98). The miracidia, free-living larval stages originating from the eggs, either penetrate the first intermediate host, a marine snail, *Littorina littorea*, or are ingested by it (98). In the snails, the miracidia become lodged in the interhepatic lymph spaces (98). There, they become sporocysts which produce the first generation of rediae. These rediae can produce a second generation of daughter rediae or the next larval stage, the cercaria (98). Cercariae leave the body of the snail and, with their mobile tail, swim until they find the second intermediate host, a fish (98). Once they find it, they lose their tails and encyst in the mouth, gills or skin of the host, becoming metacercariae (98). The definitive host eventually eats the infected fish, allowing the metacercariae to mature into adults (98).

All fish living close to the marine snail, *Littorina littorea*, could potentially become infected by *C. lingua* cercariae. There is an extensive list of fish species that can act as second intermediate hosts for the parasite (Table 1.2). One of these species, *Tautogolabrus adspersus*, or "cunner", is found in waters surrounding Prince Edward Island and seems to have a particularly high intensity of infection resulting from a sluggish nature which facilitates adhesion and encystment of cercariae (94). The availability and high infection levels of cunners make them a good source of infective material.

Table 1.2 Fish hosts for *Cryptocotyle lingua* in Atlantic Canada and the Pacific Ocean (71)

Scientific Name	Common Name
Clupea harengus	Atlantic Herring
Gadus morhua	Atlantic Cod
Glyptocephalus cynoglossus	Witch Flounder
Hemitripterus americana	Sea Raven
Hippoglossoides platessoides	American Plaice
Hippoglossus hippoglossus	Halibut*
Hypomesus pretiosus	Surf Smelt*
Leptocottus armatus	Pacific Staghorn Sculpin*
Limand ferruginea	Sole*
Liopsetta putnami	Smooth Flounder
Melanogrammus aeglefinus	Haddock
Oligocottus maculosus	Sculpin*
Osmerus mordax	Rainbow Smelt
Platichthys stellatus	Starry Flounder
Pleuronectes americanus	Winter Flounder
Pleuronectes ferrugineus	Yellowtail Flounder
Pollachius virens	Pollock
Salvelinus fontinalis	Brook Trout
Scomber scombrus	Atlantic Mackerel
Scopthalmus aquosus	Windowpane
Tautogolabrus adspersus	Cunner

* Hosts found in the Pacific Ocean

1.3 Objectives

This study had four objectives. The objectives were to establish an experimental model infection of a marine bird by *Cryptocotyle lingua*, to describe the prepatent and patent periods as well as the macroscopic and microscopic lesions caused by the parasite, to describe the immunosuppressive effect of dexamethasone in double-crested cormorants and to determine the effects of immunosuppression on an infection with *C. lingua*.

2. MANAGEMENT OF THE EXPERIMENTAL BIRDS

2.1 Introduction

Cormorants are altricial birds, i.e., they are hatched without any feathers, with their eyelids shut, and cannot thermoregulate or move on their own (51). Therefore, raising young cormorants in captivity is extremely difficult. Reports on housing and feeding captive cormorants are generally based on birds obtained as young chicks from wild colonies (22, 56), the exception being those bred by Chinese fishermen. Chinese breeders remove the fertile eggs from the parents and let a hen do the incubation (48). Hatchlings are hand-fed small fish and even blood tofu until they can fish on their own (48, 63). Other than brief descriptions in articles on Chinese cormorant fishing, no published information on breeding cormorants in captivity is available. There is, however, at least one report on artificial incubation of DCC eggs obtained from wild rookeries (82).

The feeding of piscivorous birds is complex. Different species have different nutritional requirements, most of which have not been determined. One of the recommendations when dealing with aquatic birds in general is not to limit the source of fish to a single species. When different species of fish are used, the probability of nutritional deficiencies is reduced (97). Because free-ranging DCC are opportunistic feeders (25, 79), their diet depends on prey abundance and includes several different species of fish (84). Thus, food for captive DCC can consist of any given fish species available. One important consideration is fish body size. If the fish are excessively big or heavy, the birds cannot pick them up and swallow them. Since feeding fresh fish is usually impractical, frozen fish are the usual alternative. Frozen fish are a poor source of vitamins, especially those of the B complex.

Various references on feeding captive marine birds, including San Diego's Sea World Penguin Formula (S. Costelow, pers. comm.), emphasize the importance of vitamin supplementation (18, 97). There are commercially available multivitamin tablets especially formulated for marine birds. Still, considering that interspecific differences exist, there is no specific "DCC Vitamin Formulation".

The amount of food DCC consume varies greatly with age. Adult cormorants consume 10 % of their body weight/day (51) while hatchlings can eat up to 75% of their body weight/day (L. Henry, Sea World, San Diego, CA, USA, pers. comm.).

Another difficulty encountered when keeping captive DCC, or any other marine bird species, is the potential occurrence of infectious diseases. Respiratory aspergillosis has long been recognized as a cause of death in immunosuppressed birds from both wild and captive populations (6, 31). Most affected by the disease are hatchlings and birds suffering from stress due to capture or transport (31, 32, 103). Although cormorants in general are not as susceptible to respiratory aspergillosis as captive penguins seem to be (31), the disease is considered endemic in some wild DCC populations (6). Cormorants in general are considered a "high stress species" and outbreaks in recently captured birds from rehabilitation centers and other holding facilities are frequent (2, 22).

Since stress cannot be completely eliminated, antifungal drugs are commonly used strategically to protect susceptible individuals from aspergillosis (2, 31, 33). A situation where birds are highly susceptible to infection is, undoubtedly, in the early stages of captivity.

The present study was conducted as two separate experiments composed of one pilot

study and one main trial. The pilot study involved four-week-old DCC collected from a wild colony. Because some of the DCC used in the main trial were to be subjected to an immunosuppressive treatment, outbreaks of infectious diseases, such as aspergillosis and bacterial septicemias, were a significant concern. To decrease the probability of occurrence of infectious diseases frequently brought by wild birds into captivity, and to obtain DCC naive to *Cryptocotyle lingua*, the main experimental trial was originally designed to be conducted on DCC artificially incubated and hatched in captivity from eggs collected in the wild. Failure to raise DCC from the egg (see Appendix 1) made it necessary to use two- to three-week-old birds collected from the colony instead.

The present chapter describes the collection and general management, including housing, feeding and basic handling, of the DDC used in both the pilot and main experiments and provides a brief account of the health problems encountered and the corrective measures taken.

2.2 Materials and Methods

The pilot study included the collection of 29 DCC in the summer of 1998. The main experiment, conducted in the summer of 1999, included 47 captured DCC. Experimental protocols in both years were approved by the Animal Care Committee of the University of Prince Edward Island. In the capture, housing and feeding sections, the pilot study is briefly described, followed by a more detailed account of the main experiment.

2.2.1 Capture

The DCC involved in the pilot experiment were collected from their nests on a ground-

nesting colony on the south shore of Little Courtin Island (46°32'N, 63°45'W), Malpeque Bay, Prince Edward Island. A total of 29 DCC were collected, 14 birds on June 15 and the remaining 15 birds on June 22. At the time of collection, the DCC were considered to be approximately 4 weeks old. This was based on the average hatching date of the eggs at the colony and on the size of the birds at the time of capture. Immediately after removing them from their nest, the birds were placed in transport carriers and taken to an isolation unit at the Atlantic Veterinary College (AVC). Upon arrival at the AVC, the cormorants were dusted with a pyrethrin insecticide powder (Diryl® Pitman-Moore, MTC Pharmaceuticals, Ontario, Canada) to eliminate possible arthropod infestations, although no lice were observed. The DCC were tagged with color plastic bands made to fit around their legs.

The DCC involved in the main experiment were collected in two separate groups from their nests on a ground-nesting colony on the south shore of Little Courtin Island (46°32'N, 63°45'W), Malpeque Bay, Prince Edward Island. The first group, collected on June 10, consisted of 20 birds. The second group, collected on June 22, consisted of 27 birds. Whenever possible, only one bird per nest was collected. Cormorants in the first group (G1) were assumed to be no more than 23 days old, based on a previous visit to the colony on May 18, when only eggs were present. For the second collection (G2), birds just starting to show contour feather formation were chosen, since such feathers appear only after the first 2 weeks of age (51). Cormorants obtained on both collection dates were transported and deloused in the same manner as DCC collected for the pilot experiment. All birds were tagged with color plastic spiral poultry leg bands (Agro Co-op, Prince Edward Island, Canada) using a color/number code. The plastic bands were later replaced by sealed adjustable aluminum leg

bands (Agro Co-op, Prince Edward Island, Canada) at the beginning of the experimental trial. The weight of the birds, recorded upon arrival at the AVC and compared to previous data reported by Dunn (28), corresponded to that of 2-3-week-old DCC.

2.2.2 Housing

The DCC in the pilot experiment were kept as a group in a single room in the isolation unit at the AVC. The room was split in two by a partition made of chain-link fencing and a fine vinyl fencing with a 3.8 mm mesh (Vexar™, Dupont Canada Inc. Mississauga, Ontario, Canada) 3 weeks before the experimental infection started. After the division of the room, the DCC were split into a control group (6 birds) and a treatment group (13 birds). Each partition contained a tank with running water and perches made of PVC pipes.

Because deaths from salmonellosis had occurred during the pilot experiment (see 2.3.4), standard quarantine precautions were taken when handling the DCC in the main trial, their enclosure or their food from the time of their initial arrival at the AVC. This included daily disinfection of the epoxy sealed concrete floor with a microbicidal solution (Quatricide®PV15, MTC Pharmaceuticals, Mississauga, Ontario, Canada) which was also placed in a foot bath by the door, and the use of specifically assigned boots and coveralls when working in the enclosure.

Groups G1 and G2 were kept in separate, adjoining rooms in the isolation unit at the AVC. Both rooms had a similar design. Each room had a middle partition made of chain-link fencing and a fine plastic vinyl fencing with a 3.8 mm mesh (Vexar™, Dupont Canada Inc. Mississauga, Ontario, Canada). These midline partitions were later used to divide the

birds into 4 different subgroups: A and B subgroups in one room, C and D in the other. G1 birds were initially housed in room A/B, while G2 birds were in room C/D. Round plastic nests, 10 cm in height, lined with cloth, were set in each room since the cormorants had not yet left their nests at the time of capture. Large plastic mats were placed under the nests so that the birds would not come in contact with the cold floor.

Each of the four subdivisions contained a water tank. The tanks were not filled until the nests had been removed and the cormorants had acquired most of their juvenile plumage. Water in tanks A and B was constantly running while tanks C and D were drained and refilled once a day. In addition, PVC pipes ran along the walls and across the middle of the rooms and had multiple small holes, keeping a constant flow of water on the floor in order to keep it clean of excrement.

In addition to a PVC pipe that ran along the middle portion of the largest length of the tank, a perching "tree" was placed in each subdivision. The tree was made of PVC pipes, one broad pipe supported on a Christmas tree base, and 4 narrow pipes going through it at different levels and directions. The cormorants quickly learned to perch and spent most of their time either on the tree or the rim of the tank (Fig. 2.1).

The temperature in the rooms was 25 °C during the first 2 weeks of captivity. Afterward, due to the water running across the floor and in the tanks, the temperature decreased to around 18 °C.

The DCC had 12-13 hours of light, from 6 am to 6-7 pm.

A

B

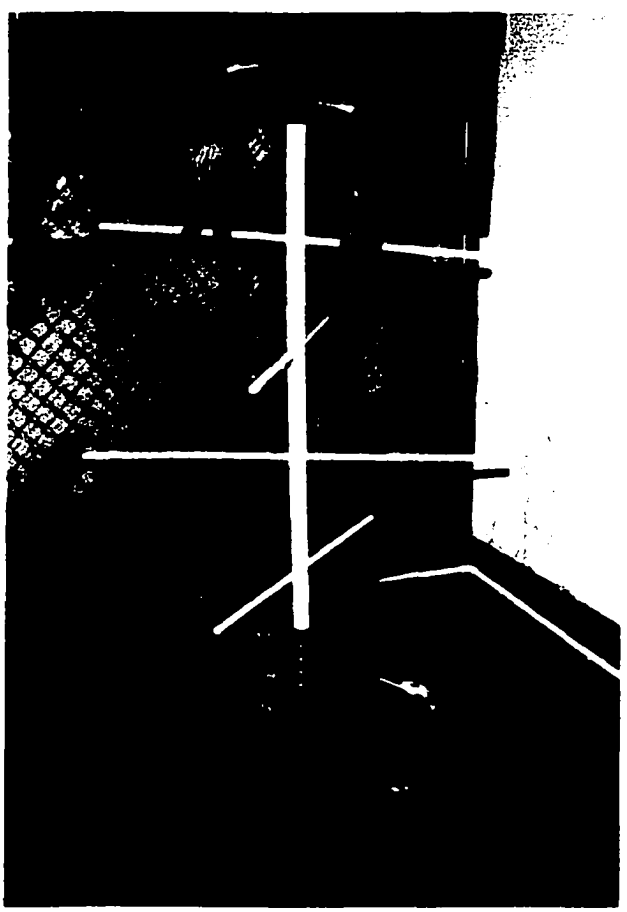

Fig. 2.1 Housing of double-crested cormorants showing access to water (A) and perches (B).

2.2.3 Feeding

The feeding of birds in the pilot study and the main experiment were similar. The diet was based on recommendations reported for either DCC or other piscivorous birds (22, 48, 56, 97). Four species of fish were available as food from commercial sources: Atlantic Mackerel (*Scomber scombrus*), Atlantic Tomcod (*Microgadus tomcod*), Smelt (*Osmerus mordax*) and Atlantic Herring (*Clupea harengus*). The fish were frozen soon after they were caught and stored at -20°C. They were always thawed before feeding, either in the refrigerator or using warm running water. The diets of younger birds consisted almost exclusively of smelts, these being the smallest fish. As the cormorants grew, mackerel, tomcod and, occasionally, herring, were fed. Even when offered to large birds, the fish had to be no more than approximately 20 cm long, otherwise the cormorants would not eat it unless they were force-fed or hand-fed.

At the beginning, all birds were force-fed 3 times a day (at 8 am, 1 pm and 6 pm) by opening their beaks and pushing a fish, head first, down their esophagus. Once they were strong enough, the DCC started picking up fish from trays left on the floor twice a day. In the pilot experiment, the fish were thrown in the water tanks in an attempt to replicate normal feeding behavior, as cormorants chase their prey underwater. However, this accelerated decomposition and complicated the cleaning process and the method was therefore discontinued. In both trials, the fish were always weighed before each feeding and leftovers were handfed before new fish was offered.

Juvenile cormorants were offered an average of 350-400 g of fish per bird per day, starting at over 50% of their body weight (from 2 to 4 weeks of age) and slowly decreasing

to 15-20% (at 4 months of age).

Vitamin supplementation comprised 1/5 tablet of Mazuri® Sea Bird Multi-Vitamin (Richmond, Indiana, USA, formulated for birds eating 2.5 lbs of a marine diet per day), 160 IU of vitamin E (Stanley Pharmaceuticals Ltd., North Vancouver, British Columbia, Canada), 0.6 ml of Vitamin B Complex® (Rhône Mérieux, Québec, Canada), equivalent to 60 mg of thiamine, and 0.25 ml of corn oil (as a source of linoleic acid (18)) per bird. During the pilot experiment, the vitamin mixture was injected into the fish and not given directly to the birds. This proved to be inefficient, as the vitamins oozed out of the fish before the latter could be eaten, and it was impossible to confirm that all cormorants had received an adequate dose. During the main trial, vitamins were mixed together and given orally as a liquid mixture to each bird every morning, before the first feeding, to ensure that all birds received an equivalent dose. It was also hoped that the daily vitamin administration would keep the cormorants used to being handled on a regular basis.

To assess the development of the DCC in the main trial, weekly weighing was performed. Each cormorant was picked up, identified by its leg band, wrapped in a towel (head facing backwards and tucked in between the wings) to restrain its movements, and placed on a Sartorius scale (Sartorius Canada, Mississauga, Ontario, Canada).

2.2.4 Health Management

2.2.4.1 Prophylactic treatment against aspergillosis

The DCC from the pilot experiment did not receive any prophylactic or therapeutic treatment against aspergillosis. Birds from the main trial, however, received prophylactic treatment against aspergillosis consisting of ketoconazole (Nizoral®, Jannsen Pharmaceutica

Inc., Mississauga, Ontario, Canada) at an oral dose of 10 mg/kg, once a day for 8 days, starting on the day of capture. Ketoconazole was suspended in a 1% aqueous solution of methyl cellulose and administered orally to the DCC by means of a plastic syringe just prior to the daily dose of vitamins.

2.2.4.2 Treatment against intestinal parasites, salmonellosis and conjunctivitis

Cormorants from the pilot experiment received a single, oral dose of 50 mg/kg of fenbendazole, at days 28 and 43 of captivity. Fecal samples were collected 13 days after the second dose.

During the main trial, G1, but not G2, birds received a single dose of oral ivermectin at 0.2 mg/bird, 5 days after arriving at the AVC. Both G1 and G2 birds received a single dose of oral fenbendazole at 50 mg/kg, on days 14 and 2 post arrival, respectively. Fecal samples were collected 4 days following each treatment.

Only one cormorant from the pilot experiment was treated for arthritis, thought to have been caused by *Salmonella* sp. This bird received a daily intramuscular injection of enrofloxacin (Baytril®) at 5 mg/kg for 8 days.

Treatment for conjunctivitis consisted of topical applications of 1% Fusidic Acid (Fucithalmic Vet®Leo Pharma Inc., Ajax, Ontario, Canada), once a day for 5 days.

2.2.4.3 Bacterial and Fungal Isolation

All DCC that died before the experimental trials were conducted were submitted to a post-mortem examination. Tissues from DCC that were suspected of having died of a bacterial infection were collected and examined bacteriologically. These tissues were cultured at 35°C in 5% CO_2 on 5% sheep blood and MacConkey agars (Oxoid Inc., Nepean, Ontario,

Canada), and bacterial isolates were identified to genus or species (83). Tissues with gross lesions suggestive of fungal infection were embedded in Sabouraud agar, fungal colonies were wet mounted on lactophenol-cotton blue and identified to genus based on microscopic and culture morphology (61).

Cloacal swabs were collected from all DCC in the main trial on June 28, which was 18 and 6 days after the arrival of G1 and G2, respectively.

2.2.4.4 Hematological assessment

To establish baseline hematological parameters for the experimental DCC in the main trial, whole blood samples were taken before the birds were subjected to the immunosuppressive treatment (3-4 days later) and the infection with *C. lingua* (7 days later). A 3-cc syringe attached to a 23-gauge 25-mm long needle was used to draw blood from the brachial vein, at the level where it crosses the medial proximal surface of the ulna. Prior to insertion of the needle, the area was disinfected with 70% ethanol. No feathers were plucked because this prompts rapid clotting (C. Wheler, Western College of Veterinary Medicine, pers. com.). After the blood was collected, the needle was removed and the sample was placed in a tube containing 0.2 ml of a 15% solution of ethylene diamine tetraacetic acid (EDTA) (Microtainer, Becton Dickinson, Franklin Lakes, New Jersey, USA). After centrifugation, the supernatant plasma was obtained. Total protein was measured in the plasma using a Reichert Refractometer (Cambridge Intruments, Buffalo, New York, USA). Complete blood cell counts (CBC) were obtained using an automatic counter (Cell-Dyn 3500, Abbott Laboratories, Abbott Park, Illinois, USA, 60064). Differential leukocyte counts were performed manually from slides stained with Wright-Giemsa stain (Hema-Tek

1000, Ames Division, Miles Laboratories, Elkhart, Indiana, USA, 46515).

2.2.5 Statistical analyses

Group means (±s.d.), calculated from the food consumption and weight loss of DCC during the main experiment, and the prevalence of aspergillosis in treated and untreated DCC were compared by use of a one-way analysis of variance (ANOVA) test (106). The correlations between body condition and cause of death and between the treatment with dexamethasone and the presence of splenic granulomas at necropsy were evaluated by calculating the 95% confidence interval of the difference in proportions (106). Significance was determined at $p \leq 0.05$.

2.3 Results

2.3.1 Feeding

Based on the amount of food consumed by G1 and G2 DCC during their first 44 days in captivity (prior to initiation of experimental treatment), the mean (±s.d.) food consumption of birds from 2 to 8 weeks of age was calculated at 418±103 g /bird/day, with a range of 147-587 g/bird/day. The DCC consumed a mean (±s.d.) of 27±8.6 % of body weight/day, with a range of 11-42% of body weight/day.

After the experimental trial had begun, the difference in food consumed by dexamethasone-treated and saline-treated DCC (see Chapter 3) and that between infected and non-infected DCC (see Chapter 4) was not statistically significant ($p=0.21$ and $p=0.76$, respectively).

2.3.2 Weight gain

Figure 2.2 shows the average weights of the DCC from G1 and G2 prior to their inclusion in the experimental trial. The weights of birds that died before the experiment was initiated (15 birds) or while it was in progress (1 bird) are not included. There was continuous weight gain observed in all DCC from the time the birds arrived at the AVC up to the beginning of the experimental trial . When the experiment started after week 6, the weights of both G1 and G2 had leveled off. A marked mean weight loss was observed in G1 but not in G2 at week 3, which corresponds to the time when most mortalities were observed in both groups.

After the experimental trial began and the immunosuppressive treatment and infective doses of *C. lingua* were administered (see Chapters 3 and 4), a general weight loss of the DCC was observed. Dexamethasone-treated and saline-treated DCC (see Chapter 3) had mean weight losses of 150 g and 100 g, respectively, over a 2 week-long period. However, there was no significant difference between groups ($p=0.24$). Similarly, mean weight losses of infected and non-infected DCC (see Chapter 4), 102 g and 148 g respectively, were not significantly different ($p=0.29$) over the same 2-week period.

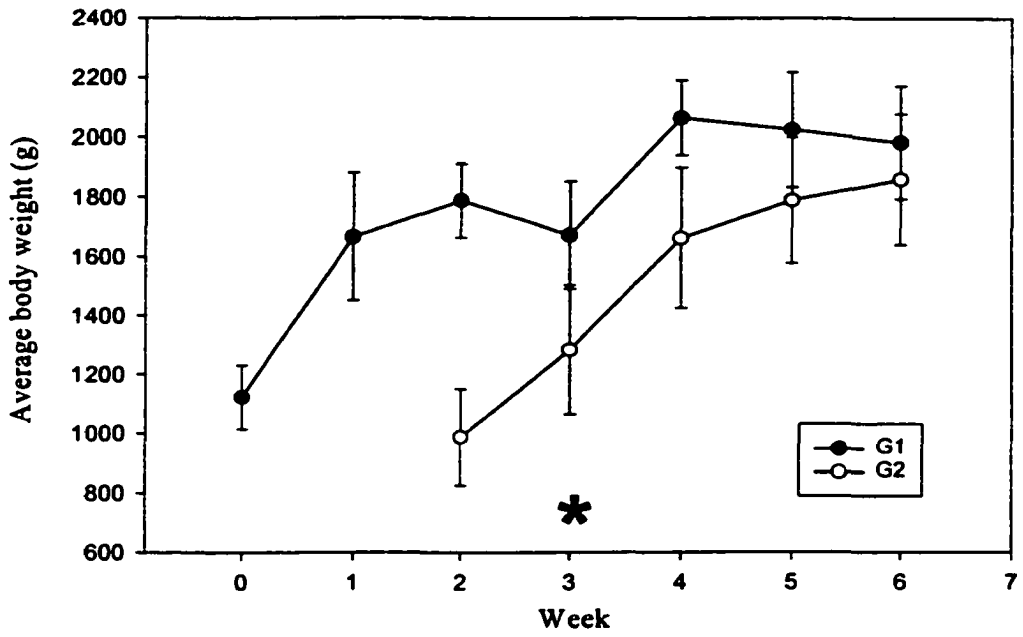

Fig. 2.2 Average weights (mean±s.d.) of double-crested cormorants (DCC). Week 0 and 2 correspond to the weights recorded upon arrival to AVC of Group 1 (G1) and Group 2 (G2), respectively. Week 6 corresponds to the last weight recorded before the birds were submitted to the experimental treatment (immunosuppression/infection) which began 2 days later. The star indicates the week when most mortalities were observed in DCC from both G1 and G2.

2.3.3 Health Management

During both the pilot and main experiments, fecal samples from the DCC were obtained at different times to test for eggs or larvae of parasites.

Flotation in zinc sulfate ($ZnSO_4$) and sedimentation analyses of feces from recently captured birds in the pilot experiment were positive for eggs of *Contracaecum* sp. (5/14) and of spirurid nematodes (1/14). Fecal examination of samples from these DCC, 13 days after fenbendazole administration, revealed no eggs.

Flotation and sedimentation analyses of feces from G1 birds 8 days after ivermectin administration were positive for Echinostome eggs(10/10), eggs of *Cryptocotyle* sp.(2/10) and ascarid eggs (3/10). Samples from G1 and G2 birds, taken 4 days post treatment with fenbendazole, were negative (0/9 and 0/12, respectively) for all these parasite eggs.

2.3.4 Mortality

A total of 29 DCC were collected for the pilot experiment in Summer 1998. Only 19 (66%) survived to be included in the experiment. The remaining 10 (34%) died from emaciation (3 birds, 10%), acute septic shock (1 case caused by *Moraxella* sp., 4%, and 5 by *Salmonella typhimurium*, 17%), and respiratory aspergillosis (1 weak bird that suffocated during handling, 3%). Among the 19 surviving birds, 2 cases of subclinical, focal air sacculitis and 1 case of arthritis due to *Salmonella* sp. were diagnosed at necropsy following euthanasia (Table 2.1). All 3 surviving cormorants with focal lesions caused by *Salmonella* sp. had been observed limping. In two of these birds, the limp resolved on its own, but the third bird developed severe arthritis of the right tibiotarsal-tarsometatarsal joint. Because of the history of salmonellosis in the group, and previous reports of arthritis due to this

pathogen in wild birds (19), *Salmonella* sp. was suspected to be the etiologic agent. This was subsequently confirmed by isolation of *Salmonella* sp. from the affected joint at necropsy, although a sample of synovial fluid taken before antibiotic treatment had revealed no evidence of the bacterium. After treatment with enrofloxacin (see 2.2.4.2), the limp slowly lessened until it almost disappeared one month later. Of the other two birds that had shown signs of limping, one was observed to make coughing noises which resolved a week later. Two DCC showed evidence of non-fatal respiratory aspergillosis at necropsy. One of these died of septic shock, and 1 survived throughout the trial.

For the main trial, 47 DCC were collected. However, only 31 survivors (66%) were submitted to the experimental treatments (immunosuppression/infection). A total of 16 birds (34%) died before (15 birds) or during the experiment (1 bird). The causes of death included acute septic shock (7 cases caused by *Erysipelothryx rhusiopathiae*, 15%; 3 cases caused by *Aeromonas hydrophila*, 6%; and 1 case caused by *Streptococcus* sp., 2%), vegetative valvular endocarditis (1 case due to *Lactobacillus* sp., 2%) and respiratory aspergillosis (4 birds, 9%). Five DCC showed evidence of non-fatal, focal, respiratory aspergillosis at necropsy. Three of these died from septic shock, and 2 survived throughout the trial (Table 2.1). The DCC that received no treatment to prevent aspergillosis (pilot experiment) showed a prevalence of 10.3% (3/29), while those treated prophylactically with ketoconazole (main trial) had a prevalence of 19.1% (9/47). No significant difference was observed in the prevalence of aspergillosis between treated and non-treated DCC.

Several DCC from the main trial exhibited mild to moderate conjunctivitis, with serous to thick, white exudate covering the cornea. A total of 12 birds were affected. However,

only 4 received local treatment with fuscidic acid (see 2.2.4.3); the other 8 DCC recovered without treatment. Cultures of conjunctival swabs from 3 birds yielded inconclusive results, due to secondary bacterial contamination. One conjunctival swab from a fourth bird was positive for *Mycoplasma* sp.

Table 2.1 Causes of death in double-crested cormorants (DCC) excluded from the experimental trials (plus 1 bird which died during the main trial) and significant pathological findings in DCC included in both pilot and main experiments.

DCC not included in trials			DCC included in trials		
Cause of Death	**Pilot Study (n=29)[a]**	**Main Trial (n=47)[a]**	**Significant findings post-euthanasia**	**Pilot (n=19)[b]**	**Main (n=31)[b]**
Emaciation	3 (10%)	0	Granulomas [h] in: liver	3 (16%)	12 (39%)
Respiratory Aspergillosis	1 (3%)	4 (9%)	spleen	0	18 (74%)
Salmonellosis	5 (17%)	0	kidney [i]	0	3 (10%)
Erysipelas	0	7 (15%)	Respiratory Aspergillosis	1 (5%)	2 (6%)
Sepsis, various bacteria	1 (4%) [e]	4 (8%) [f]	Bacterial air sacculitis [j]	2 (10%)	0
Valvular endocarditis	0	1 (2%) [g]	Bacterial arthritis [j]	1 (5%)	0
Total	**10 (34%)[c]**	**16 (34%)[c]**		**7 (37%)[d]**	**23 (74%)[d]**

[a] Total number of DCC collected
[b] Total number of DCC included in trial
[c] DCC that died/ total DCC collected
[d] DCC affected/DCC included in the trials
[e] Bacterium isolated: *Moraxella* sp.
[f] Bacteria isolated: *Aeromonas hydrophila* (3 cases), *Streptococcus* sp. (1 case)
[g] Bacterium isolated: *Lactobacillus* sp.
[h] Bacteria isolated from splenic, hepatic and/or renal granulomas: *Salmonella* sp. (6 cases), *E coli* (6 cases), *Staphylococcus* coagulase neg. (5 cases), *Streptococcus* alpha hemolytic (1 case), *Moraxella* sp. (1 case)
[i] All 3 DCC with renal granulomas also had splenic and hepatic granulomas
[j] Bacterium isolated: *Salmonella* sp.

In addition to determining the terminal cause of death for DCC that were not included in either the pilot or main experiments, the body condition at the time of death of these birds was also evaluated. This body condition was assessed as good (well developed pectoral muscles, conspicuous adipose tissue in subcutis, mesentery and coronary groove), moderate (mild atrophy of the pectoral muscles and reduced adipose tissue) or poor (emaciated birds with atrophic pectoral muscles and total absence of adipose tissue).

In the pilot experiment, the body condition was recorded for only 8 of the 10 DCC that died before the trial. Of these, 5 (62%) were in poor body condition (3 of which actually died from emaciation); 2 (25%) were in moderate body condition and 1 (12%) was in good body condition. Except for the 3 birds dead of emaciation, all other DCC examined died of bacterial sepsis. The body condition of the bird that died of aspergillosis was not assessed.

In the main experiment, the body condition of all 15 DCC that died before the main trial and 1 DCC that died during the experiment was also assessed. Of these, 7 (44%) were in poor body condition at the time of death, 4 (25%) were in moderate body condition and 5 (31%) were in good body condition. The proportion of DCC that were emaciated and died from bacterial sepsis (6/12, 50 %), normally an acute process, was apparently, but not significantly, higher than the proportion of DCC that were emaciated and died from respiratory aspergillosis (1/4, 25 %).

The presence of granulomas in the spleen (Figure 2.3), liver and, less frequently, kidneys, was detected either grossly or through histological examination in 21 (69%) of the 31 DCC that comprised the four experimental groups in the main trial (Table 2.1). Among the affected birds, 11 (55%) were in the dexamethasone-treated group and 10 (45%) were in the

saline-treated group (see chapter 3). No significant difference in the presence of granulomas resulted from the treatment. Bacteria isolated from these granulomas included *Salmonella typhimurium* phage type 195 (6 cases, 43%), *Escherichia coli* (6 cases, 43%), coagulase-negative *Staphylococcus* (5 cases, 36%), alpha hemolytic *Streptococcus* (1 case, 7%) and *Moraxella* sp. (1 case, 7%). The cloacal swabs collected from all DCC in the main trial at approximately 2 weeks of captivity were all negative for *Salmonella* sp. All granulomas positive for *Salmonella typhimurium* belonged to the group of dexamethasone-treated DCC. Apart from the DCC in the dexamethasone-treated group in the main experiment, only one DCC from the pilot experiment had splenic, hepatic and adrenal granulomas, which were caused by *Salmonella* sp.

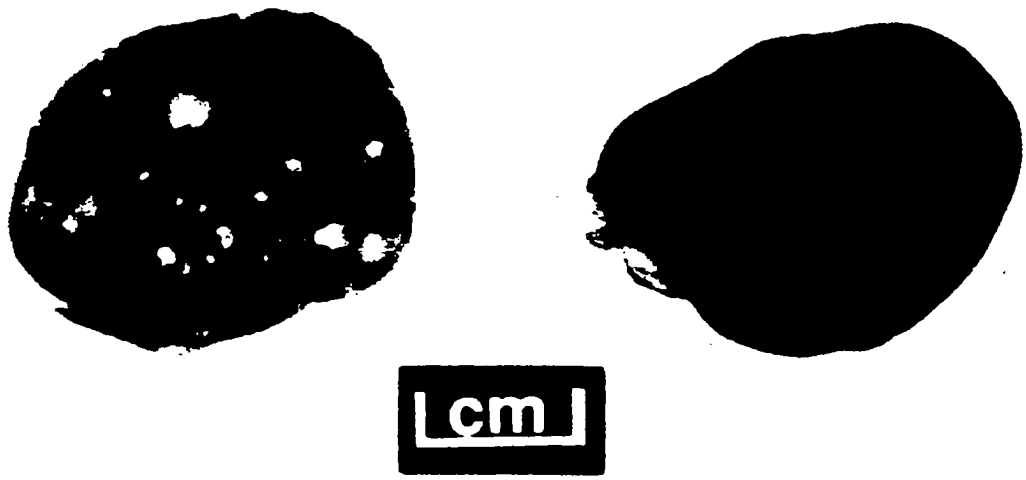

Fig. 2.3 Multiple granulomas in the spleen of a double-crested cormorant (DCC) from the main trial (left) compared to a healthy DCC spleen (right)

2.3.5 Hematology

Hematological data from 29 DCC, obtained 4 days prior to their inclusion in the main trial, are in Table 2.2, with reference values reported by Kuiken *et al.* (57). Although all 32 DCC originally included in the main trial were sampled, three birds were eliminated from the baseline value group, reducing the sample size to 29. The DCC thus excluded were 1 bird that died of vegetative endocarditis 3 days post-sampling and 2 birds whose total white blood cell counts (WBC) were outliers according to the definition by Minitab 10.1® (Minitab Inc., 1994, Pennsylvania, USA). This defines an outlier as any value lying between 1.5-3 times away from the middle 50% of the data, i.e. observations that fall outside the lower and upper limits of the normal distribution. The lower and upper limits are defined as Q1-[1.5(Q3-Q1)] and Q3+[1.5(Q3-Q1)], respectively. In this formula, Q1 is the first quartile and Q3 is the third quartile, Q3-Q1 being the interquartile range which contains 50% of the values obtained.

Table 2.2 Hematologic values of 29 juvenile double-crested cormorants prior to treatment with dexamethasone and infection with *Cryptocotyle lingua* compared to parameters reported in the literature

Parameter	Mean ([a])	s.d. ([a])	Range ([a])
Hematocrit (%)	35 (31)	4 (3)	28-44 (26-36)
Total protein (g/L)	43 (42)	5 (5)	32-53 (34-51)
Absolute leukocyte count ($x10^9$/L)			
Heterophils	17.4 (14.5)	9.7 (5.9)	3.4-44.1 (4.1-24.8)
Lymphocytes	8.5 (9.6)	5.0 (3.4)	2.5-23.7 (3.2-16.8)
Monocytes	0.04 (0.7)	0.1 (1)	0.0-0.7 (0.0-3.6)
Basophils	0.5 (0.3)	1.0 (0.4)	0.0-4.5 (0.0-1.4)
Eosinophils	0 (3.2)	0 (2.9)	0 (0.6-12.1)
Total	26.6 (28.3)	11.7 (9.4)	8.2-55.8 (12.0-45.0)
Distribution of leukocytes (%)			
Heterophils	64 (50)	16 (10)	31-89 (34-77)
Lymphocytes	35 (36)	15 (13)	11-67 (17-59)
Monocytes	0.2 (2)	0.5 (3)	0-2 (0-8)
Basophils	2 (1)	3 (1)	0-9 (0-5)
Eosinophils	0 (11)	0 (7)	0 (3-27)

[a]Previously reported by Kuiken *et al.* (57)

2.4 Discussion

The experience of feeding and handling DCC in the pilot study proved a valuable lesson when dealing with birds in the main trial. The vitamin supplementation and feeding method became more efficient by then and the daily handling of the DCC in the main trial allowed for a closer monitoring of their development and health status. One example of this closer assessment is the data obtained on weight gain.

The weight gain curves for both G1 and G2 DCC (Fig. 2.2) show a steady increase until week 4 in G1 (approximately 7 weeks old) and week 5 in G2 (approximately 5 weeks old) when the DCC have nearly reached adult weights (51). The decline in body weight gain observed in G1 birds on week 3, which corresponded to the 3rd and 1st week post-capture of G1 and G2, respectively, coincided with the time when most mortalities were observed. Interestingly, mean weight gain in G2 was not affected by the disease outbreak, although some birds from both groups died. A possible explanation is that birds in G2 were still being force-fed, which would have eliminated weight loss due to anorexia.

Neither the immunosuppressive drug, dexamethasone, nor the infection with *Cryptocotyle lingua* were responsible for the weight loss observed in DCC during the trial, since both treated and non-treated groups were similarly affected. Weight loss was more likely due to the stress of extra handling to which all birds were submitted (22). If handling during the experiment stressed the DCC enough to cause weight loss, this could indicate that the birds had not become accustomed to the daily routine in the pre-experimental period, as was intended. However, DCC from the main trial were appreciably easier to manage and seemed to be less affected by human interaction than DCC from the pilot experiment, which were

not handled on a daily basis but were also older at the time of capture. Evidence of this difference was that food consumption in DCC in the main trial did not decrease on the days that the birds were weighed, while DCC in the pilot experiment stopped eating after similar handling. Other reports on captive DCC indicate that the birds get used to a routine and can be trained to enter and exit an enclosure when herded by a caretaker (22).

Ivermectin had no effect on the intestinal trematodes or nematodes when administered orally at a dose of 0.2 mg/bird. Ivermectin is not considered effective against trematodes (5). However, its lack of activity against nematodes, specifically ascarids, was unexpected (5, 100). In both experiments, oral administration of fenbendazole at 50 mg/kg completely eliminated gastrointestinal infection by both trematodes and nematodes.

The isolation of *Mycoplasma* sp from a conjunctival swab is consistent with a previous report associating isolation of this bacterium with clinical conjunctivitis in free-ranging DCC (20), suggesting that *Mycoplasma* sp can cause a mild conjunctivitis that can be further exacerbated by other opportunistic agents. There is, however, no conclusive evidence designating *Mycoplasma* sp as the primary etiologic agent. The treatment against conjunctivitis with fuscidic acid was unnecessary; untreated DCC were able to clear the infection on their own.

As indicated in Table 2.1, the mortality rate observed in DCC from both the pilot and main experiments was 34%. Although an 87% survival rate was reported for DCC taken from a wild colony at 2 weeks of age and raised in captivity (22), losing a third of the total birds collected is not an unusual occurrence when bringing wild seabirds into captivity. An approximate 30% mortality rate was reported for horned puffin chicks, *Fraternicula*

corniculata (103) and gentoo penguins, *Pygoscelis papua* (31), when first brought into captivity. The causes of mortality were hypovitaminosis E (103) and aspergillosis (31), respectively. Kuiken *et al.* (58), who raised 20 DCC taken from a wild colony at the age of 0 to 11 days, reported bill malformations in 8 of these birds that were so severe that the birds had to be euthanized. The authors attributed this bill malformation to vitamin D_3 deficiency resulting from the DCC being kept indoors without exposure to ultraviolet light and being fed frozen fish that may have been deficient in this vitamin (58). The fact that DCC with normal bills had been housed and fed similarly to the birds with bill deformities in the report by Kuiken *et al.* (58) could be explained by the age at the time of capture. Affected DCC were younger at the time of capture (only 1 to 3 days old) than DCC with normally developed bills which may have made them more susceptible to hypovitaminosis D. The DCC involved in the pilot and main experiments described in the present study, despite being housed indoors and fed frozen fish without vitamin D_3 supplementation, never showed signs of bill malformation, perhaps because they were at least 14 days old when captured.

Nutritional deficiencies were not a problem for the DCC described in this study; infectious agents were the most common terminal cause of mortality in these birds. Bacterial septic shock caused the majority of mortalities in the pilot experiment (60%) and in the main trial (69%). Respiratory aspergillosis was responsible for 10% of the mortalities in the pilot experiment and 25% in the main trial. Most microorganisms involved are either present in wild populations and brought into captivity by the DCC themselves, such as *Salmonella* sp (112), or are normally found in the environment, such as *Aspergillus* sp (6, 59, 112), *Lactobacillus* sp. Therefore, a depressed immune response seems to be required for these

etiologic agents to cause clinical disease or death of the affected birds (112). Stress due to captivity and handling could contribute to immunosuppression in DCC (2). Alternatively or concurrently, an immature immune system in young birds (all birds that died from sepsis were less than 7 weeks old) could have contributed to the emergence of infectious diseases (14, 112). It is perhaps significant in this respect that mortality in all groups, both in the pilot and main experiments, nearly ceased once the birds had reached 7 weeks of age, with only one death from vegetative endocarditis recorded after that.

Emaciation was the cause of death in 3 DCC from the pilot experiment. The birds died 5, 7 and 13 days after capture. Irreversible emaciation that leads to death regardless of the feeding efforts of animal caretakers has been described in some mammalian species, such as lambs suffering from cobalt deficiency (92). If the DCC were suffering from a similar state of irreversible emaciation at the time of capture, their death was inevitable, force-feeding and supplementation with vitamins notwithstanding. Emaciation can not only be the terminal cause of death in itself, but also contribute to rendering the bird more susceptible to opportunistic diseases, such as bacterial sepsis. Although the number of affected birds was too small for statistical analysis, DCC that died from sepsis were usually in poorer body condition than those that died from respiratory aspergillosis. Since death by septic shock is normally acute, it seems unlikely that the emaciated state of the DCC was due to the bacteremic process. A more probable conclusion would be that the birds became septic due to a pre-existing state of emaciation. In support of this conclusion, DCC dead from respiratory aspergillosis, a chronic disease that would be expected to have a debilitating effect on the body condition of the birds, were mostly in moderate and, in one case, good

body condition. Alternatively, the bacterial septicemia could have resulted in a phenomenon termed septic autocannibalism, the metabolic response that follows severe sepsis in humans (72). Septic autocannibalism entails rapid breakdown of reserves of protein, carbohydrates and fat mediated by inflammatory cytokines such as tumor necrosis factor-α , interleukin 1-β and interleukin 6. If the septic process stimulated the release of such mediators, emaciation could have resulted from the bacterial sepsis, transforming emaciation from underlying to terminal cause of death. Additionally, considering that sepsis does not necessarily mean bacteremia (85) and can be missed if the diagnosis is based only on bacterial isolation from organs such as liver or lung, the 3 DCC which died of emaciation before the pilot study began could have also died from septicemia.

Based on the prevalence of respiratory aspergillosis in treated and non-treated DCC, ketoconazole treatment was ineffective in preventing fungal colonization of the airways. This is consistent with recent literature reports which favors itraconazole and fluconazole over ketoconazole in the prevention and treatment of aspergillosis in marine bird collections and rehabilitation centers (2, 31, 33).

Most of the bacteria isolated from DCC that had granulomas in one or more internal organs are commonly found in the environment. This, however, is not the case for *Salmonella* sp. The birds were housed in isolation rooms, under quarantine conditions which would suggest that the bacterium was brought in by the DCC at the time of capture and was latent in at least one individual. The fact that the cloacal swabs taken 2 weeks into captivity were negative for *Salmonella* sp does not necessarily mean that the birds were not infected since isolation of the bacteria by culture of fecal material is often unsuccessful (80). The

birds could have brought in the pathogen, which may have remained latent through the high susceptibility stage (first 7 weeks of age), and later developed into a subclinical infection partly due to the immunosuppressive treatment.

Hematological parameters followed, in general, those reported for DCC (57), with the exception of the concentration and proportion of heterophils, and the total absence of eosinophils. Elevated numbers of heterophils were due primarily to three individual DCC with very high heterophil counts that necessarily increased the mean and standard deviation. These three DCC could have been undergoing an active, subclinical inflammatory process. A dramatic rise in the white blood cell count, predominantly heterophils, has been reported in cases of aspergillosis in raptors (32). In the present study, however, necropsy of these three DCC at the end of the experiment showed no evidence of aspergillosis, indicating that some other inflammatory etiology may have caused the increased number of heterophils. The fact that eosinophils seemed to be conspicuous in DCC reported by Kuiken *et al.* (57) and were not found in birds from the present study, could be because most of the DCC in the former study were infected with hepatic trematodes. In the present study, the DCC were free from helminths at the time the blood samples were taken (see 2.3.3), therefore, their eosinophil counts were not affected by an ongoing parasitism. Alternatively, the difference in eosinophil counts between DCC in the Kuiken *et al.* (57) study and those in the present report could be due to subjective differences in the manual differential counts, specifically, in the criteria for cellular identification.

In summary, the diet and vitamin supplementation used in this study provided the necessary requirements for captive DCC. Fenbendazole administration was effective against

intestinal trematodes and nematodes in DCC. Captive DCC less than 7 weeks old, especially when suffering from emaciation, were highly susceptible to bacterial septicemia, which caused peracute death or the development of sequellae, such as chronic arthritis. Young DCC were prone to respiratory aspergillosis and prophylactic treatment with ketoconazole was ineffective. Infectious disease and stress decreased the appetite of, and caused delayed growth or weight loss in, juvenile DCC.

3. IMMUNOSUPPRESSION AND IMMUNOLOGICAL TESTS

3.1 Introduction

Evaluation of the immune status of wild animals, particularly in free-ranging populations, is generally aimed at determining possible immunosuppressive effects from environmental pollutants, such as heavy metals (42, 105) and polychlorinated-biphenyls (26). It can also be useful to reproduce immunosuppressive effects under laboratory conditions with the use of immunosuppressive agents to use as a reference when assessing the effect that common environmental pollutant or a given disease process may have on an immunosuppressed individual (93). There are several techniques available to assess the immune response in laboratory animals (49). Applying these techniques to other species raises some difficulties, such as lack of species specific populations of immune cell lines and proper standardization of techniques, in addition to the increased difficulty that working with an alternative species entails. Hence, the *in vivo* assessment of immune function in wild animals is usually restricted to those methods that do not require elaborate reagents. Common techniques include stimulation of T lymphocytes via administration of mitogens and production of antibodies against a known antigen, such as heterologous erythrocytes. These methods have been used in several avian species, such as mallard ducks (*Anas platyrhynchos*) and japanese quail (*Coturnix coturnix*) (42, 92, 105). Phytohemagglutinin-P (PHA-P) is a mitogen which induces T cell proliferation and is commonly used to estimate T cell activity following subcutaneous inoculation. The administration of PHA-P in chickens produces a localized delayed hypersensitivity reaction (49). This reaction is characterized histologically by perivascular accumulation of lymphocytes and migration of macrophages to the inoculation

site; some heterophilic infiltration is also observed, mostly in the early stages post-inoculation (41). The heterologous erythrocytes most commonly used to elicit a humoral response for evaluation of B cell function are washed sheep red blood cells (SRBC).

Immunosuppression of birds can be achieved by various methods including thymectomy, bursectomy, irradiation and drug administration. Neonatal thymectomy (surgical removal of the thymus) causes a decrease in the number and function of circulating T lymphocytes and impairs the production of antibodies against T cell-dependent antigens (41, 49, 102). Surgical removal (bursectomy) or viral destruction of the bursa of Fabricius in chicks significantly decreases the production of antibodies and causes plasma cells to disappear (102). Ionizing radiation from x-ray or γ-ray sources kills lymphoid cells, thus eliminating immune function and enabling researchers to restore and study a particular branch of the immune system by adoptive transfer of specific cells (49). Immunosuppressive drugs have various mechanisms of action. Cytotoxic drugs such cyclophosphamide interfere with DNA synthesis of rapidly dividing cells, including lymphocytes (49). Fungal and bacterial derivatives such as cyclosporin A interfere with signalling pathways by binding to immunophilins, thus downregulating genes that are normally expressed upon T cell activation (49). Corticosteroids such as dexamethasone bind to the target cellular DNA and activate the synthesis of anexins, proteins that block phospholipases from releasing arachidonic acid, thus depriving the cell of the substrate required to produce prostaglandins and leukotrienes (49, 102). Corticosteroids also reduce the production of inflammatory mediators (cytokines, prostaglandins and nitric oxide), inhibit the migration of inflammatory cells to sites of inflammation through downregulation of expression of adhesion molecules,

and induce apoptosis of leukocytes and lymphocytes by activating endonucleases (49, 102).

When working with non-domestic avian species, use of immunosuppressive drugs is the most practical method of causing immunosuppression. A study by Schrank *et al.* (93) on mallard ducks compared three immunosuppressive agents with different mechanisms of action. Dexamethasone, cyclosporine and cyclophosphamide were administered to the experimental ducks and PHA-P skin tests and antibody response to SRBC were measured. The study found that only dexamethasone consistently produced a significant effect on both T and B cell responses and that parameters routinely used to assess the immune response in chickens, such as PHA-P and SRBC antibody tests, may be used to monitor the immunocompetence of mallard ducks.

The present study involved the evaluation of the role of immunosuppression in the colonization of the intestinal tract of DCC by the trematode *Cryptocotyle lingua*. The effect of the immunosuppressive treatment administered to 16 captive DCC, following the general guidelines established by the study of Schrank *et al.* (93), will be described.

3.2 Materials and methods

3.2.1 Control and treatment groups

Thirty-one DCC captured from a wild colony at 2 weeks of age were raised in captivity (see Chapter 2) to be used in an experiment assessing the pathological effects of the intestinal trematode, *Cryptocotyle lingua*, in immunocompetent and immunosuppressed piscivorous birds. Once the DCC had reached an average age of 2 months, 4 different treatment groups, each comprising 8 birds, were established (Table 3.1).

Group	Immunosuppression	Infection
A	yes	no
B	yes	yes
C	no	no
D	no	yes

Table 3.1 Treatment and control groups of double-crested cormorants. Dexamethasone was administered to groups A and B. Infective metacercariae of *Cryptocotyle lingua* were administered to groups B and D.

The immunosuppressive treatment consisted of the administration of 3.5 mg/kg of dexamethasone (Dexamethasone 5®, Vétoquinol, Joliette, Québec, Canada) intramuscularly once daily for 10 treatments in alternating sides of the pectoral muscles, using a 25-gauge needle. Since there is no established dose of dexamethasone for DCC that would ensure a measurable degree of immunosuppression without endangering the bird's life, the dosage was estimated based on the mean dose used by Schrank *et al.* (93) on mallard ducks. Groups A and B were injected with dexamethasone, while control groups C and D were injected with the same volume of 0.85% sterile saline, on days -4, -3, -2, -1, 1, 3, 5, 7, 9 and 11, day 0 corresponding to the first day of the infection with metacercariae of *C. lingua* (Chapter 4).

To assess whether dexamethasone was indeed immunosuppressing the treated DCC, the immune status of all 31 birds was evaluated with a phytohemagglutinin-P test, an antibody production test using SRBC and morphometric assessment of the bursa of Fabricius.

3.2.2 Phytohemagglutinin-P (PHA-P) Test

On day -2, an area in the dorsal and ventral surfaces of the wing webs, measuring

approximately 1.5 cm in diameter, was plucked and marked with a blue ink permanent marker (Lumocolor® Staedtler, Germany). The marked area was disinfected with 70% alcohol. Using a 25-gauge needle, 0.2 ml of PHA-P (ICN Biomedicals, Aurora, Ohio, USA) and 0.2 ml of 0.85% sterile saline solution (SS) were injected subcutaneously in the right and left wing webs, respectively. The thickness of each wing web at the injection site was measured with a micrometer caliper (Fisher Scientific, Nepean, Ontario, Canada) just prior to PHA-P and SS injections (0 hour) and at 24 and 48 hours post-injection. The PHA-P stimulation index was calculated as the difference between the increase in skin thickness from 0 hour due to PHA-P and the increase from 0 hour due to SS.

In addition to assessing the skin response via the stimulation index, 4 DCC from non-infected groups (2 from group A, and 2 from group C) were re-inoculated with PHA-P 24 hours before euthanasia. The stimulation index was obtained just prior to euthanasia and tissue from the wing webs as fixed in 10% buffered formalin and processed routinely for light microscopic examination and stained with heematoxylin and eosin (H&E) (66).

Because the PHA-P test was conducted prior to the experimental infection with *Cryptocotyle lingua* (see Chapter 4), the results were grouped as dexamethasone-treated or saline-treated only, regardless of whether the DCC were later included in an infected (C and D)or non-infected (A and B) group.

3.2.3 Antibody test using Sheep Red Blood Cells (SRBC)

On day -2, all DCC received an intraperitoneal injection of 1.5 ml of a sterile 20% suspension (vol/vol) of packed SRBC in phosphate buffered saline (PBS) using a 22-gauge needle. Blood was obtained from the brachial vein at days 9-10 (n=31), 14 (n=11) and 17

(n=8) post-SRBC inoculation and mixed with 0.2 ml of ethylene diamine tetra-acetic acid (EDTA). The blood was collected and handled as described in 2.2.4.4. The plasma thus obtained was frozen at -80°C until standard hemagglutination tests for determination of antibody titers were performed (102). The antibody titers against SRBC were expressed as the $\log_2$ of the highest dilution of serum that agglutinated an equal volume of 0.5% red blood cells (93).

Although the SRBC antibody test ran concurrently with the infection with C. *lingua* (see Chapter 4), no effects on the antibody titers were expected to come from the presence of the parasite. However, to justify grouping the DCC as dexamethasone-treated and saline-treated, regardless of whether or not the birds were infected, the antibody titers in the 2 main groups, (A and B) and (C and D), were compared to each other and only pooled if no significance was found.

3.2.4 Morphometric assessment of the Bursa of Fabricius

The bursae of Fabricius (BF) from 16 saline-treated DCC (8 from group C and 8 from group D) and from 8 dexamethasone-treated DCC (3 from group A and 5 from group B) were collected at necropsy, between 11 and 19 days after the dexamethasone or saline treatments had begun. The BF were fixed in 10% buffered formalin, processed routinely for light microscopic examination, and stained with H&E, Masson's trichome stain and a modified acid-fast stain for lipofuscin (66). A total of 96 fields from 48 histological sections (16 from dexamethasone-treated and 32 from saline-treated DCC) were examined according to standard stereological procedures (106) to determine length of follicle/area of follicle (Lf/Af), average area per follicle (Af) and follicles/area (f/A). The Lf/Af represents the ratio

between the length of the follicular border and its area, thus assessing the average follicular shape. The higher the ratio, the more irregular the follicles are. The Af represents the average follicular size in mm^2. The f/A is the average number of follicles in a 1 mm^2 area.

No effects on the stereological parameters used to assess bursal architecture were expected to come from the infection with C. *lingua* (see Chapter 4). However, because the morphometric assessment of the BF was performed at the end stages of infection, the Lf/Af, Af and f/A in the 2 main groups, (A and B) and (C and D), were compared to each other and only pooled if no significance was found..

3.2.5 Hematology

Blood samples were obtained immediately before necropsy, 11 (n=12), 16 (n=11) and 19 (n=8) days after commencement of the treatment with dexamethasone or saline. The sampling method and the processing of samples were described in section 2.2.4.4.

To assess whether treatment with dexamethasone affected hematological parameters, total and differential white blood cell counts, hematocrit and total protein values from dexamethasone-treated and saline-treated DCC were compared to baseline values established 4 days prior to treatment (see 2.3.5), and to each other. A maximum of 3 weeks had elapsed from when the baseline values were obtained to when the last blood sample was collected. Because infection with *C. lingua* could potentially produce alterations in hematological parameters (see 4.3.3), only non-infected DCC were included in the analysis.

3.2.6 Statistical analyses

Group means (±s.d.), calculated from values determined during the various tests, were compared with a one-way ANOVA test, except for the ranked SRBC antibody titers where

the non-parametric Mann-Whitney test was used (106). Significance was determined at $p \leq 0.05$.

3.3 Results

3.3.1 PHA-P test

Results of the skin test used in the assessment of T cell function are given in Table 3.2. Stimulation indices from dexamethasone-treated DCC were significantly lower at 24 hr ($p<0.001$) and 48 hr ($p<0.05$) post-inoculation than those from saline-treated DCC (Fig. 3.1).

Skin biopsies from the area of PHA-P inoculation also revealed a difference between dexamethasone-treated and saline-treated DCC. Saline-treated birds had a severe inflammatory response in the dermis and subcutaneous tissue, characterized mainly by multifocal to confluent lymphocytic infiltrates, with numerous heterophils and a few macrophages and plasma cells (Fig. 3.2-B). Dexamethasone-treated DCC had moderate subcutaneous edema and only a mild to moderate lymphocytic response with very few heterophils and fewer macrophages (Fig. 3.2-A).

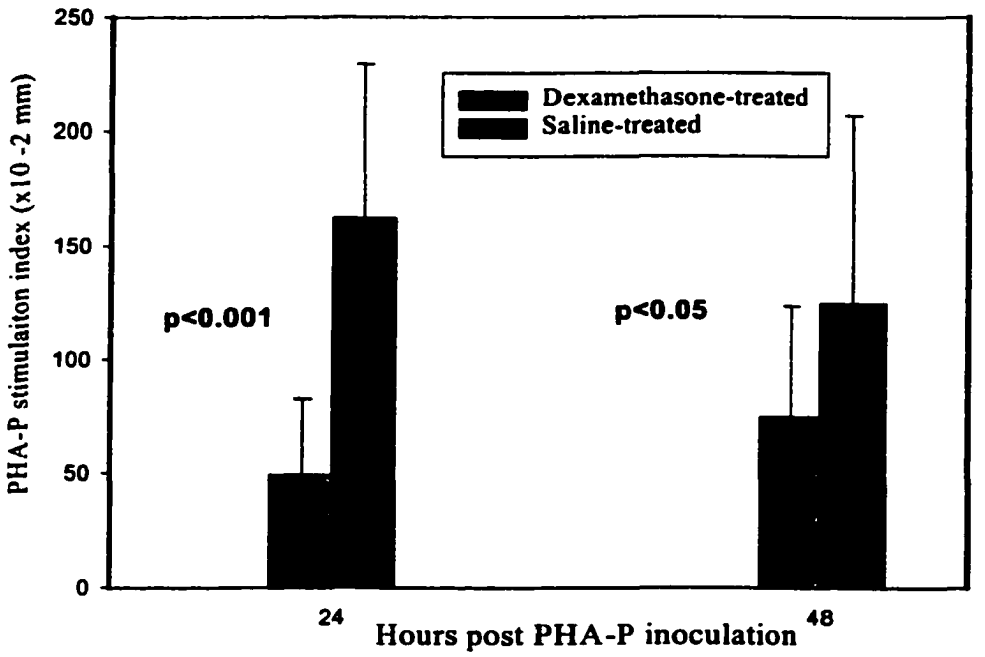

Fig. 3.1 Stimulation index of the response to Phytohemagglutinin-P (PHA-P) skin test in the wing webs of double-crested cormorants (DCC). The index represents the difference between the increase in thickness from 0 hour due to subcutaneous injection of PHA-P and the increase from 0 hour due to subcutaneous injection of saline solution. The response to the mitogen is lower in DCC treated with dexamethasone (grey bars) than in DCC treated with saline (black bars) 24 ($p<0.001$) and 48 hours ($p<0.05$) post-inoculation.

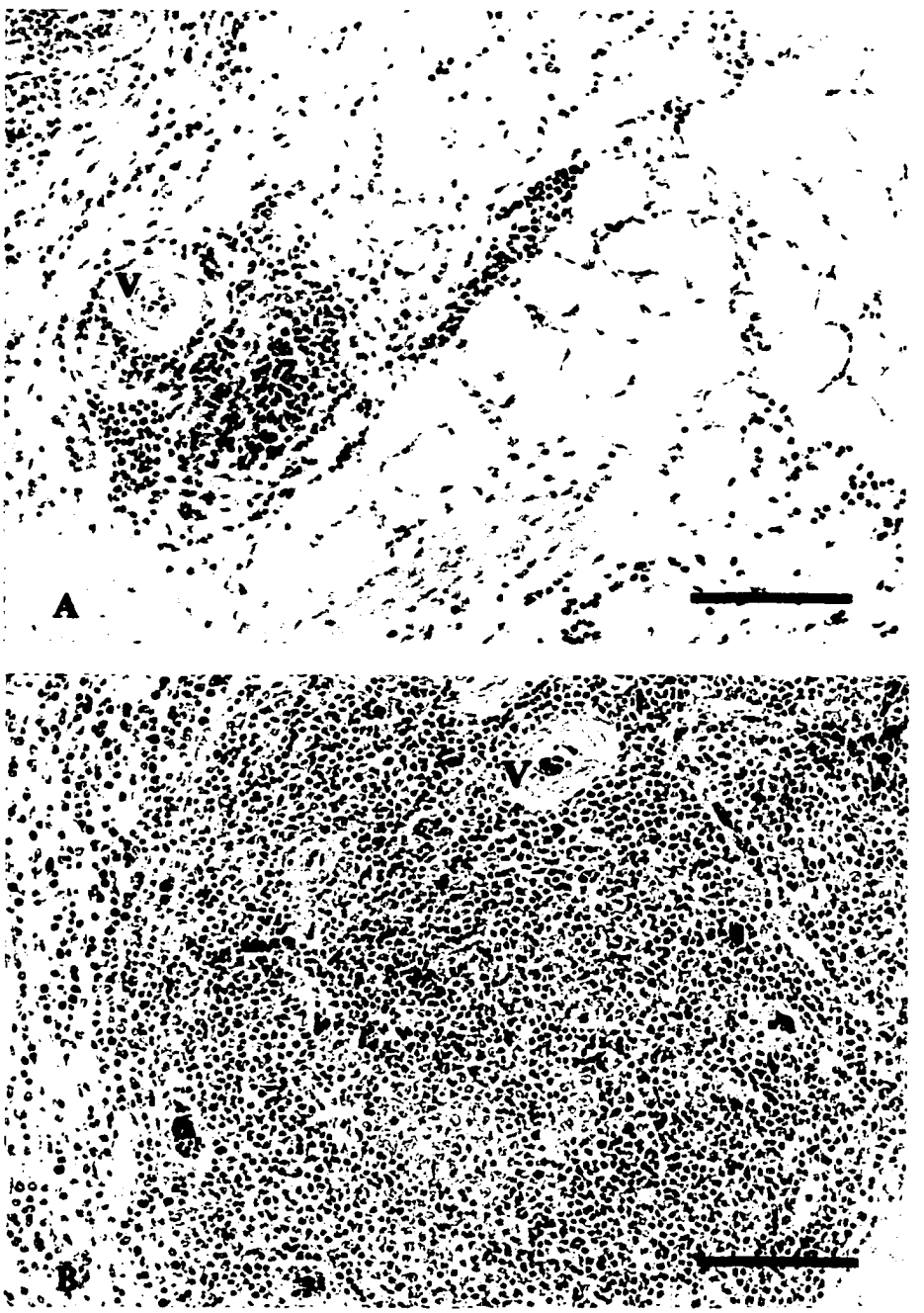

Fig. 3.2 Histological sections of the wing web of double-crested cormorants (DCC) 24 hr. following subcutaneous injection of Phytohemagglutinin-P. A) Dexamethasone-treated DCC with a mild perivascular lymphocytic infiltrate. B) Saline-treated DCC with severe perivascular lymphocytic and heterophilic infiltrates. Hematoxylin & eosin stain. Bar=100μ. V- blood vessel identified as an arteriole.

3.3.2 SRBC antibody test

Confirming that the antibody titers against SRBC were not affected by the infection, no significant difference was observed between groups A and B (dexamethasone-treated/non-infected and dexamethasone-treated/infected, respectively) or between groups C and D (saline-treated/non-infected and saline-treated/infected, respectively). Therefore, the dexamethasone-treated groups (A and B) and the saline-treated groups (C and D) were combined and the results reported in Table 3.2. Because the data was not normally distributed, a non-parametric test was indicated for the statistical analysis. The data were ranked and a Mann-Whitney test performed (Fig. 3.3). There was a statistically significant difference in the antibody titers against SRBC between dexamethasone-treated (groups A and B) and saline-treated DCC (groups C and D) on day 9-10 post-SRBC inoculation ($p=0.029$). Antibody titers in dexamethasone-treated DCC were significantly lower than those in saline-treated DCC. Titers on days 14 and 17 post SRBC inoculation were not significantly different ($p>0.05$).

3.3.3 Morphometric assessment of the Bursa of Fabricius

Histological examination of the BF revealed marked differences in the morphology of the follicles from saline-treated and dexamethasone-treated DCC. Follicles from saline-treated DCC had well demarcated cortical, paracortical, and medullar or germinal regions (Fig.3.4), and high cellularity with some cellular debris consistent with normal apoptotic changes of the lymphoid tissue. The BF from dexamethasone-treated DCC showed moderate to severe atrophy. Four BF contained cyst-like structures in their germinal areas, some of which seemed to correspond to the lumens of degenerated bursal ducts and contained cellular debris

and/or an eosinophilic amorphous material (Fig. 3.5). Three BF contained numerous large cells filled with intracytoplasmic brown material consistent with lipofuscin. Finally, two BF had severe lymphoid depletion and collapse of the parenchyma (Fig. 3.6).

When the results from the morphometric evaluation of the 4 groups were tested individually, no significant difference was observed in the follicular shape among dexamethasone-treated groups (between A and B) and saline-treated groups (between C and D). However, a significant difference in the number of follicles per unit area ($p<0.01$) was found between dexamethasone-treated groups A (non-infected) and B (infected). Bursae from group B DCC had more numerous follicles than those from group A DCC. The follicular size was significantly different among saline-treated groups ($p<0.01$). Group C (saline-treated/non-infected) had significantly smaller follicles than group D (saline-treated/infected). Therefore, only the results from the non-infected groups A and C were used to compare the difference in the architecture of the BF between dexamethasone-treated and saline-treated DCC .

All three morphometric parameters used to evaluate the BF follicles showed significant differences between dexamethasone-treated (group A) and saline-treated DCC (group C), as indicated in Table 3.2. Follicles from dexamethasone-treated DCC were more irregularly shaped, smaller and more densely concentrated ($p<0.001$) compared to those from saline-treated DCC.

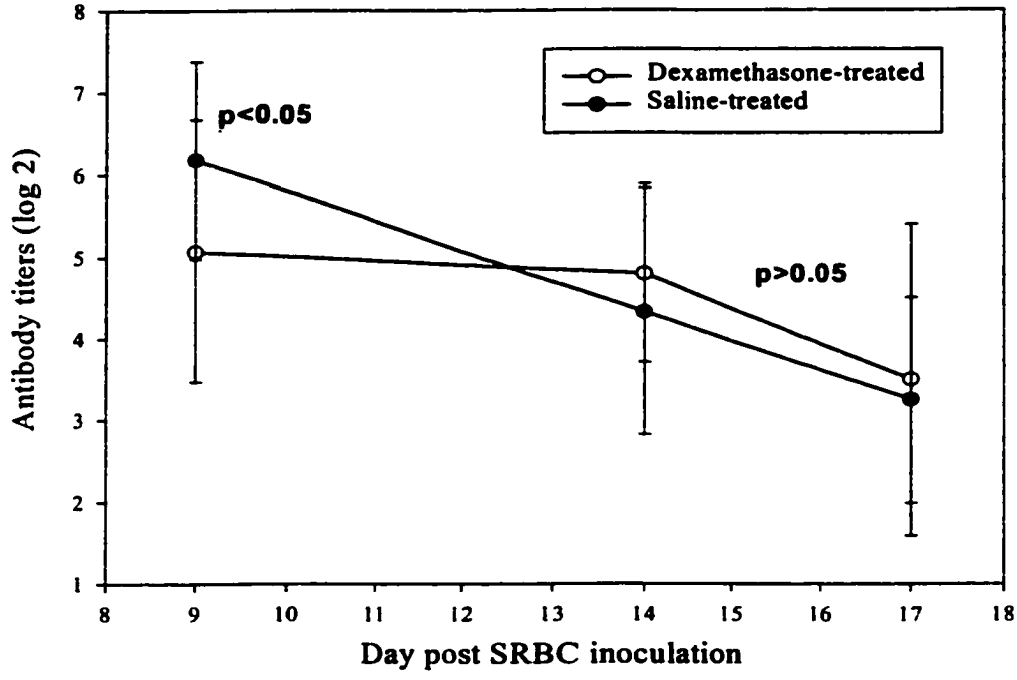

Fig. 3.3Antibody response to intraperitoneal injection of Sheep Red Blood Cells in double-crested cormorants (DCC) 9-10, 14 and 17 days post-inoculation. The response in DCC treated with dexamethasone (white circle) is significantly lower than the response in DCC treated with saline (black circle) on day 9-10 ($p<0.05$).

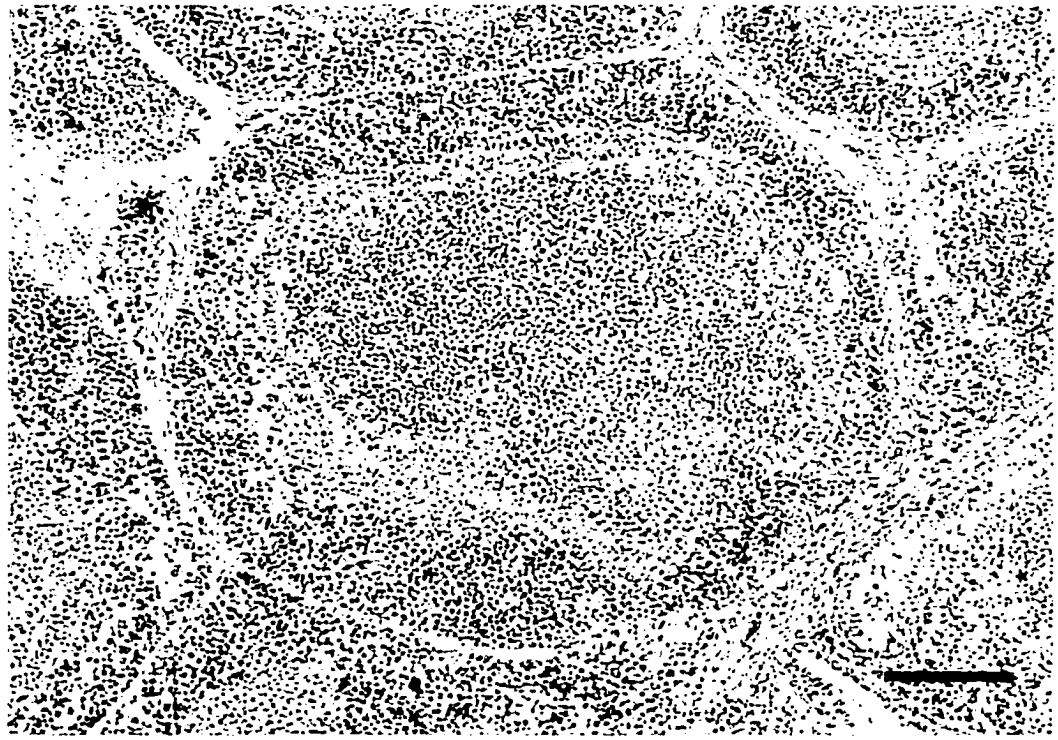

Fig. 3.4 Histological section of the bursa of Fabricius of a saline-treated double-crested cormorant showing the normal morphology of a single lymphoid follicle, hematoxylin & eosin stain. Bar=100μ

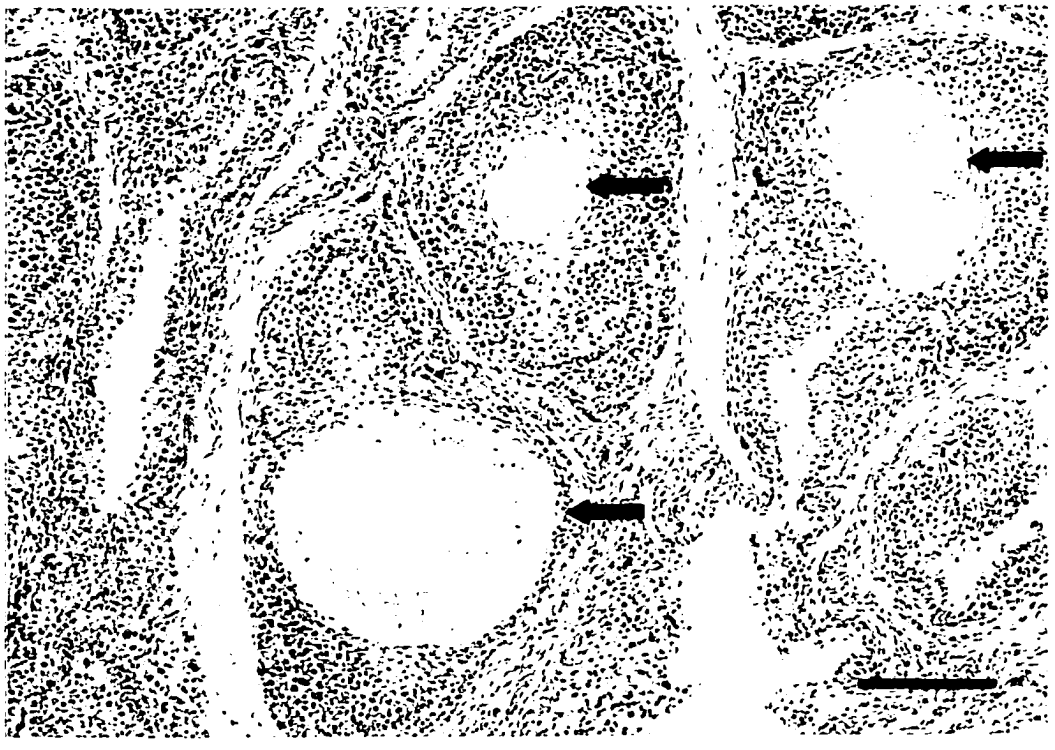

Fig. 3.5 Histological section of the bursa of Fabricius of a double-crested cormorant 11 days into the treatment with dexamethasone showing moderate lymphoid depletion and formation of cyst-like structures (arrows). The size of individual lymphoid follicles in this Figure is markedly smaller than that of the follicle shown in Figure 3.4 at the same magnification, hematoxylin & eosin stain. Bar=100μ

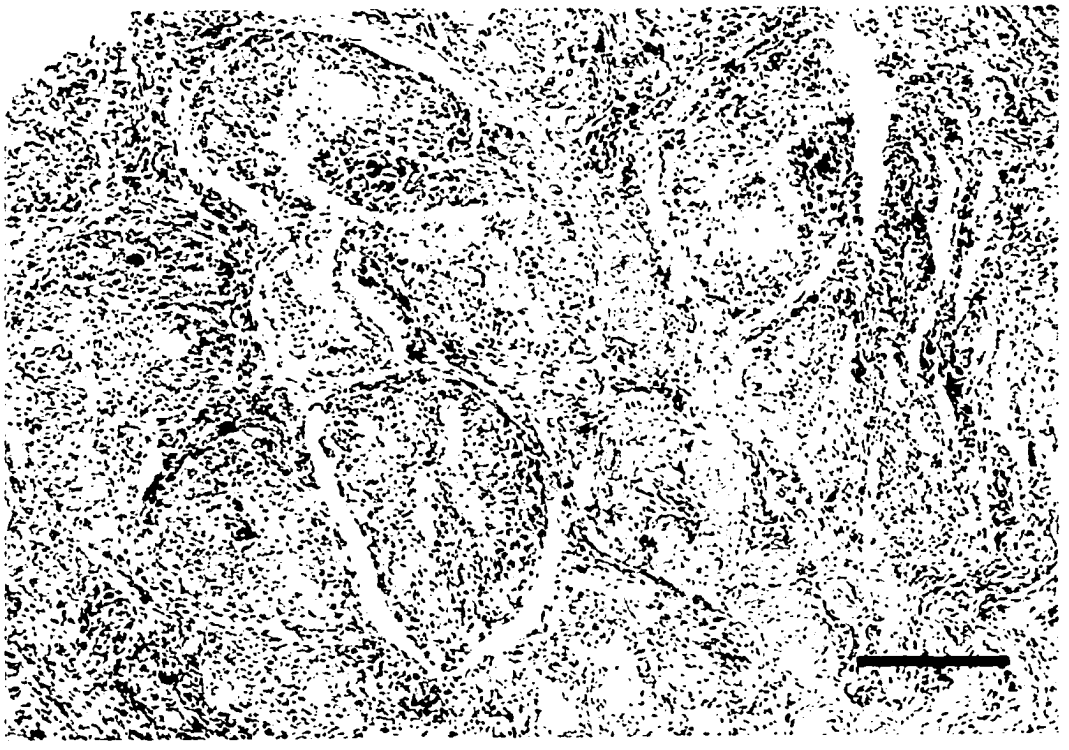

Fig. 3.6 Histological section of the bursa of Fabricius of a double-crested cormorant 19 days into the treatment with dexamethasone showing severe lymphoid depletion and collapse of parenchyma, hematoxylin & eosin stain. Bar=100μ

Table 3.2 Immunosuppressive effect of dexamethasone in double-crested cormorants assessed by the response to subcutaneous injection of phytohemagglutinin-P (PHA-P), the production of antibodies against sheep red blood cells (SRBC), and morphometric evaluation of the Bursa of Fabricius (BF).

	PHA-P stimulation index [a] ($x10^{-2}$ mm) (mean±s.d.)		Antibody against SRBC titers (Log_2) (mean±s.d.)			BF parameters [b f] (mean±s.d.)		
	24 hr [c]	48 hr [d]	day 9-10 [d]	day 14 [e]	day 17 [e]	Lf/Af [c]	Af [c]	f/A [c]
Dexamethasone-treated	48.7±33.9	74.1±49.1	5.07±1.6	4.80±1.5	3.50±1.9	42.1±7.8	0.06±0.02	11.1±3.7
(groups A and B)	(n=16)	(n=16)	(n=15)	(n=5)	(n=4)	(n=3)	(n=3)	(n=3)
Saline-treated	161.7±67.4	124.0±82.4	6.19±1.2	4.33±1.5	3.25±1.3	19.2±4.0	0.16±0.06	5.5±1.8
(groups C and D)	(n=16)	(n=16)	(n=16)	(n=6)	(n=4)	(n=8)	(n=8)	(n=8)

[a] The response to PHA-P is indicated by the stimulation index which is calculated as the difference between the increase in thickness from 0 hour due to subcutaneous injection of PHA-P and the increase from 0 hour due to subcutaneous injection of saline solution

[b] Length of follicle per area of follicle (Lf/Af) represents the ratio between the length of the follicular border and its area, thus assessing average follicular shape. Average area per follicle (Af) indicates follicular size in mm^2. Number of follicles per area (f/A) indicates the number of follicles per mm^2

[c] Significant difference between dexamethasone-treated and saline-treated DCC; $p<0.001$

[d] Significant difference between dexamethasone-treated and saline-treated DCC; $p<0.05$

[e] No significant difference between dexamethasone-treated and saline-treated DCC; $p>0.05$

[f] Only groups A and C in this comparison, see section 3.3.3

3.3.4 Hematology

Table 3.3 shows the hematological baseline parameters for the DCC in this study (see section 2.3.5 and Table 2.2) and mean values for the dexamethasone-treated (n=7) and saline-treated (n=8) groups. Although blood samples were collected on 3 separate dates, corresponding to the time of euthanasia of individual birds from all 4 groups, the results are reported together due to the small sample size.

The mean hematocrit of saline-treated DCC (48±4%) was significantly higher ($p<0.01$) than the mean hematocrit of dexamethasone-treated DCC (43±2%). The mean hematocrit of dexamethasone-treated DCC was significantly higher ($p<0.01$) than the mean hematocrit of baseline values (35±4%). Mean total protein levels in both dexamethasone-treated (56±4 g/L) and saline-treated (53±5 g/L) DCC were significantly higher ($p<0.01$) than mean total protein in the baseline values (43±5 g/L), but not different from each other.

The mean absolute count of heterophils in dexamethasone-treated DCC (20.3±5.3 x 10^9/L) was not significantly different from the mean absolute count of heterophils from the baseline values (17.4±9.7 x 10^9/L). The mean absolute count of heterophils in saline-treated DCC (9.1±2.3 x 10^9/L) was significantly lower than that of dexamethasone-treated DCC ($p<0.01$) and baseline values ($p<0.05$). The mean total leukocyte count in saline-treated DCC (14.5±3.2 x 10^9/L) was significantly lower than that of dexamethasone-treated DCC (27.2±6.2 x 10^9/L), mainly due to the higher heterophil counts in the latter group. The mean absolute count of lymphocytes inf saline-treated DCC was significantly lower ($p<0.5$) than that from the baseline values. The mean heterophil/lymphocyte ratio observed in saline-treated birds (63:36), dexamethasone-treated birds (75:24) and baseline values (64:34)

indicates that heterophils were the predominant leukocyte type found in DCC.

Table 3.3 Hematologic values of dexamethasone-treated (group A) and saline-treated (group C) double-crested cormorants compared to baseline values obtained 3 weeks earlier (4 days before the immunosuppressive treatment with dexamethasone started).

Parameter	Baseline values (n=29)	Saline-treated DCC (group C, n=8)	Dexamethasone-treated (group A, n=7)
Hematocrit (%)	35±4	48±4 [a• b•]	43±2 [a• b•]
Total protein (g/L)	43±5	53±5 [a•]	56±4 [a•]
Absolute counts of leukocytes (x10^9/L)			
Heterophils	17.4±9.7	9.1±2.3 [a# b•]	20.3±5.3 [b•]
Lymphocytes	8.5±5.0	5.3±1.9 [a#]	6.4±2.8
Monocytes	0.04±0.1	0.02±0.05	0.04±0.1
Basophils	0.5±1.0	0.1±0.1	0.4±0.5
Total	26.6±11.7	14.5±3.2 [a• b•]	27.2±6.2 [b•]
Distribution of leukocytes (%)			
Heterophils	64±16	63±10 [b#]	75±11 [b#]
Lymphocytes	35±15	36±10 [b#]	24±9 [b#]
Monocytes	0.2±0.5	0	1±0
Basophils	2±3	2±1	3±1

Eosinophils were not detected. Data on the proportions of monocytes and basophils was too few for statistical analysis.

[a] Significantly different from baseline parameter

[b] Significant difference between treatment and control groups

[#] $p \leq 0.05$

[•] $p \leq 0.01$

3.4 Discussion

The treatment with dexamethasone caused a decreased reaction to PHA-P, dramatic morphological changes in the bursa of Fabricius, a mild reduction in the production of antibodies against SRBC and heterophilia.

The decreased reaction to PHA-P in DCC treated with dexamethasone is consistent with the findings of Schrank *et al.* (93) in mallard ducks in a similar experiment. This decreased skin reaction to a T cell mitogen (PHA-P) in dexamethasone-treated DCC, depicted by a lower stimulation index (93) and lack of lymphocytic proliferation (102) *in situ*, denotes an impaired cellular immune response (41, 49, 93). Determination of the exact mechanisms involved in decreasing cellular immunity in treated DCC is beyond the scope of the present study. However, effects of corticosteroids well recognized in other species (49), such as downregulation of neutrophilic migration to inflammatory sites and reduction of the expression and function of IL-2 which causes inhibition of the proliferation of T cells (69, 102), could cause the impairment of the cellular immune response in dexamethasone-treated DCC.

The antibody response to SRBC in DCC from the present study, where the mean antibody titers for saline-treated birds was only 6.19±1.2 on day 9-10 post-inoculation, appeared to be less affected by the treatment with dexamethasone than that of mallard ducks in the Schrank *et al.* (93) study, where the mean antibody titer for control birds was 9.1±0.5 on day 6 post-inoculation. The only marginally significant difference in antibody titers against SRBC in DCC on day 9-10 post-inoculation and the absence of significant difference between groups on days 14 and 17 suggests that dexamethasone had little to no effect on the

humoral immune response. However, there is no information available on the normal response of DCC to SRBC. The dose of antigen could have been lower than what was required to stimulate a strong response, especially since the DCC were naive to SRBC. Also, like some other avian species (42), DCC may respond poorly to sheep erythrocytes, irrespective of the dose. If the response elicited by SRBC in DCC in general were weak to begin with, as suggested by the low antibody titers in saline-treated birds, it would be difficult to demonstrate an effect of humoral immunosuppression by dexamethasone (42). Furthermore, the evident atrophy of the bursa of Fabricius seen in dexamethasone-treated DCC, also supports a negative effect on the humoral immune response. This organ is intrinsically associated with B lymphocytes and its regression can result from apoptosis of lymphocytes induced by dexamethasone (17). The significant differences in the numbers of follicles per unit area among dexamethasone-treated DCC (A and B) and in the follicular size among saline-treated DCC (C and D) suggested an effect of the infection with *C. lingua* on bursal morphology. However, the results ascribe contradictory effects to the infection. Among the dexamethasone-treated DCC, the number of follicles per square mm increased in infected birds (group B), indicating that the follicular size had decreased. Among the saline-treated groups, however, the follicular size was increased in the infected DCC (group D). Because these effects of the infection in bursal morphology were in contradiction to each other, they were suspected to be the result of a spurious correlation. Therefore, the infection with *C. lingua* was not considered to affect bursal morphology.

The increased hematocrit and hyperproteinemia present in both dexamethasone-treated and saline-treated DCC as compared to baseline values could be explained by simple

dehydration that would result in hemoconcentration (36) unrelated to the treatment. Alternatively, and considering that no clinical evidence of dehydration was observed in any of the birds, the increased hematocrit may have resulted from an age-related increase in erythropoiesis. In some avian species, erythropoiesis increases as individuals mature (89). No information on alterations in the hematocrit of DCC associated with normal aging is available.

Heterophilia and lymphopenia are expected results of corticosteroid treatment (36, 102), and have been reported in other seabird species associated with high cortisone levels during fasting (104). When mean heterophil counts in the experimental DCC were compared to those of baseline values, dexamethasone-treated DCC appeared to have heterophilia while saline-treated DCC had heteropenia. However, heteropenia in birds is normally associated with bacterial sepsis or severe viral disease (36), conditions that would result in clinical signs and/or death of the affected individuals. None of the apparently heteropenic DCC showed signs of disease or died. Furthermore, mean baseline heterophil counts were higher than those previously reported for DCC and other species of cormorants as reviewed by Kuiken *et al.* (57), suggesting that some of the birds that were sampled to obtain these baseline values may have been suffering from a subclinical inflammatory process or stress. Consequently, both baseline heterophil counts and those of dexamethasone-treated DCC may indicate heterophilia due to an inflammatory process and/or stress and dexamethasone treatment, respectively, while there may be no true heteropenia in saline-treated DCC. Therefore, the probable absolute heterophilia observed in dexamethasone-treated DCC was attributed to the treatment with dexamethasone (102, 104). Lymphopenia, also normally

associated with treatment with dexamethasone, however, was not recorded; there was no significant difference in lymphocyte counts between dexamethasone-treated and saline-treated DCC. The reason for this lack of effect of dexamethasone on lymphocyte counts is unknown.

Despite a few contradictory results, particularly with regards to the leukogram, the effects of dexamethasone on the cell-mediated immune response, the morphology of the bursa of Fabricius, and the antibody response to SRBC indicated at least some degree of down-regulation of the immune system of DCC treated with dexamethasone. Dexamethasone-treated DCC will now be referred to as immunosuppressed while saline-treated DCC will be referred to as non-immunosuppressed.

4. *Cryptocotyle lingua*: EXPERIMENTAL INFECTION AND PATHOLOGICAL EFFECTS

4.1 Introduction

Cryptocotyle lingua was first described from the intestine of a great black-backed gull, *Larus marinus*, by Creplin in 1885 (98). Most studies regarding the biology of *Cryptocotyle lingua*, however, date back to the first half of the 20th century. This parasite is widely distributed in the marine environment, both on the American and European continents. Its complete life cycle was described in an extensive paper by H.W. Stunkard in 1930 (98). Like other trematodes of the order Digenea, *C. lingua* has an indirect life cycle in which the successive life stages are harbored by two intermediate and one definitive hosts (Fig. 4.1).

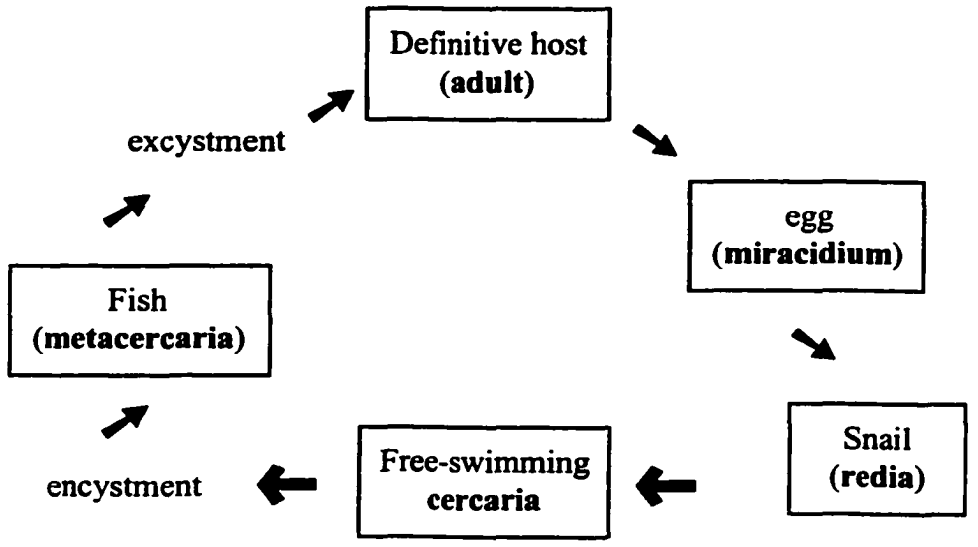

Fig. 4.1 Life cycle of *Cryptocotyle lingua*, Stunkard (98).

Adult *C. lingua* naturally occur in the intestine of fish-eating birds and mammals, where they produce miracidium-containing eggs that are released to the environment with the feces. In water, the miracidium emerges from the egg, penetrates the tissues of the marine snail

Littorina littorea and metamorphoses into a sporocyst (98). Within the sporocyst, numerous rediae are produced. The rediae produce the last larval stage, the cercariae. Cercariae have movable keeled tails which enable them to swim until a fish is detected through a pair of eyespots. The cercariae encyst in the skin, mouth and gills of the fish, thus becoming metacercariae and remaining there until the fish is consumed by the definitive host. The maximum life span of the metacercariae is unknown, but life spans of over 18 months have been documented in codfish (67). Digestive enzymes from the definitive avian or mammalian host stimulate the excystment of the metacercariae, which lodge among the mucosal villi of the small intestine and mature into adults.

Although the second intermediate and definitive hosts can be various fishes (see Table 1.2) and fish-eating birds or mammals, respectively, the role of the first intermediate host is restricted to *Littorina littorea*. This marine snail can carry extremely high infective loads, some laboratory specimens being recorded to emit cercariae for over three years at a rate of approximately 300 per day (90).

Early attempts to describe the complete life cycle of the parasite involved the experimental infection of a definitive host by feeding fish with "black spots" (metacercarial cysts believed to be *C. lingua*) (98). Most attempts were successful, and cat, rats and guinea pigs became infected with adult forms after being fed infested fish (98). Later, less conventional experimental species, such as black-headed gulls, *Larus ridibundus*, were successfully infected and egg shedding was recorded 6 days post-infection, indicating a 6-day pre-patent period (91).

Several avian and mammalian species can serve as definitive hosts of *C. lingua*. The

parasite is often documented as part of the inventory of helminths found in a given species or as an incidental finding at necropsy. Some reports, however, attribute a severe emaciation and death to heavy *C. lingua* infections. Smith (96) and Lauckner (62) reported that the deaths of a bald eagle (*Haliaeetus leucocephalus*) and of a lesser black-backed gull (*Larus fuscus*) were due to a severe *C. lingua* infection. The birds were in poor body condition and their deaths were attributed to *C. lingua*, mainly due to the high numbers found in the small intestine and the lack of other lesions suggesting an alternative etiology. In neither case was emaciation *per se* considered as a possible cause of death. Earlier results of laboratory infections of young and naive terns and dogs resulted in a localized catarrhal enteritis (111). This enteritis was characterized by copious mucus production, hyperemia and cellular infiltrates composed of eosinophils and plasma cells, and attributed to mechanical damage to tissues adjacent to *C. lingua* (111). More recent reports, however, are cautious about implicating *C. lingua* or other small trematodes as terminal, or even underlying, causes of death. Forrester *et al.* (34) and Daoust *et al.* (21) found heavy trematode infections associated with marked emaciation in dead common loons (*Gavia immer*) from Florida, US, and the Maritime Provinces, Canada, respectively. Neither report, however, found conclusive evidence that the parasites were directly responsible for the death of the birds and speculated that the severe infection was due to either an alteration in the diet (Forrester *et al.*, (34)) or an impaired immune response (Daoust *et al.*, (21)). To test the latter hypothesis, the present study aimed at reproducing a *C. lingua* infection in immunosuppressed and immunocompetent DCC, describing the pre-patent and patent periods and the possible pathological effects of the parasite on its host. The collection and processing of infective

metacercariae, development of the infection and hematological and pathological effects of *C. lingua* are described.

4.2 Materials and Methods

Two separate experimental infections of DCC with *C. lingua* were conducted at the AVC. The first, or pilot experiment in 1998, involved 19 DCC (13 infected and 6 controls) captured as nestlings from a wild colony on Little Courtin Island (46°32'N, 63°45'W), Malpeque Bay, PEI (see 2.2), and involved 2 sequential infections. The first infection involved the oral administration of a total of 1,300 metacercariae/DCC divided into 3 doses over a 4-day period (days 0, 1 and 3). The second infection, conducted 2 weeks after the last dose of the first infection was administered and 1 week after the latter had been eliminated (based on the absence of eggs in the feces), consisted of 1,800 metacercariae/DCC administered in 4 doses over a 6-day period (days 0, 2, 3, 5).

The main trial was conducted in 1999 as a single infection of 31 DCC (16 infected, 15 controls) captured from the same colony and divided into 4 experimental groups: A) Immunosuppressed and non-infected; B) Immunosuppressed and infected; C) Non-immunosuppressed and non-infected; and D) Non-immunosuppressed and infected (see 3.2.1). Infected groups (B and D) received a total of 17,560 metacercariae/DCC administered orally over a 4-day period (days 0, 1, 2 and 3). Consecutive dosing was intended to reproduce conditions of natural infection in the wild and was dependent on availability of infested fish. The collection, processing and administration of infective metacercariae were similar in both trials, as is described below.

4.2.1 Origin of metacercariae and administration of infective dose to DCC

The cunner, *Tautogolabrus adspersus*, is known to naturally carry high loads of *C. lingua* metacercariae (94) and was thus chosen as the source of infective material in the main trial. However, the pilot experiment took place in the fall, a time when cunners are not easily caught, and had to rely on rainbow smelts, *Osmerus mordax*, a species also susceptible to *C. lingua* infestation. The smelts were caught by gill nets in the Hillsborough river and Bedeque Bay, Prince Edward Island, during October and November 1998. Cunners came from the north shore of New Brunswick and Prince Edward Island, and were caught in late July and early August 1999 using traps and nets baited with fish. In both the pilot and main trials, the fish were caught and kept at 4 °C until processing, less than 24 hours later. The metacercariae were administered over a period of 4 to 6 days, the parasites having been harvested from the fish a maximum of 24 hours earlier.

For practical reasons, not every single metacercaria was taken from every fish. The smelts used for the pilot experiment had very small numbers of metacercariae (less than 25 per fish), with very few on the body surface; therefore, only the tail and dorsal, pectoral, ventral and caudal fins, being the most accessible infested areas, were removed for processing. The cunners used in the main trial, however, were heavily infested (Fig. 4.2) and, in addition to removing tail and fins, the body was scraped with a necropsy knife to collect metacercariae covering the scales. Due to the marked difference in levels of infestation, over 200 kg of smelts were processed in the pilot experiment, in contrast to 90 kg of cunners required in the main trial. The infested tails, fins and, in the case of cunners, body skin and scales were placed in salt water (Instant Ocean®Aquarium Systems, Mentor,

OH, 440060, USA) and refrigerated at 4°C for up to 12 hours until the infective doses were prepared.

The infective dose consisted of a known concentration of metacercariae suspended in Instant Ocean and placed in 3-cc syringes, one for each DCC. To prepare these infective doses, the infested fish tissues kept in salt water were blended at high speed for 3-5 minutes to detach the metaceracarial cysts from the tissue. After blending, the suspension was filtered through two consecutive sieves. The first sieve (500 μ pore diameter) captured large pieces of tissue to be submitted to a second blending. The second sieve (150 μ pore diameter) retained the metacercarial cysts (200 μ diameter) freed from the tissue. Free metacercariae were re-suspended in Instant Ocean and spun in a TJ-6 Centrifuge (Beckman, Palo Alto, CA, US) at 500 G for 7 minutes. The supernatant was removed, the pellets broken up, pooled together and reconstituted before a second spin (500 G for 7 min.) in order to concentrate the metacercarial suspension and reduce the volume that was to be administered to the DCC. After the second centrifugation, the approximate concentration of metacercariae was calculated based on the manual count obtained from an aliquot placed under a stereoscopic microscope. The infective suspension was then divided by the number of DCC that were to be infected and placed in 3-cc syringes. The infective doses were administered orally to the DCC by emptying the syringe in the birds' esophagus, caudally to the easily visualized larynx.

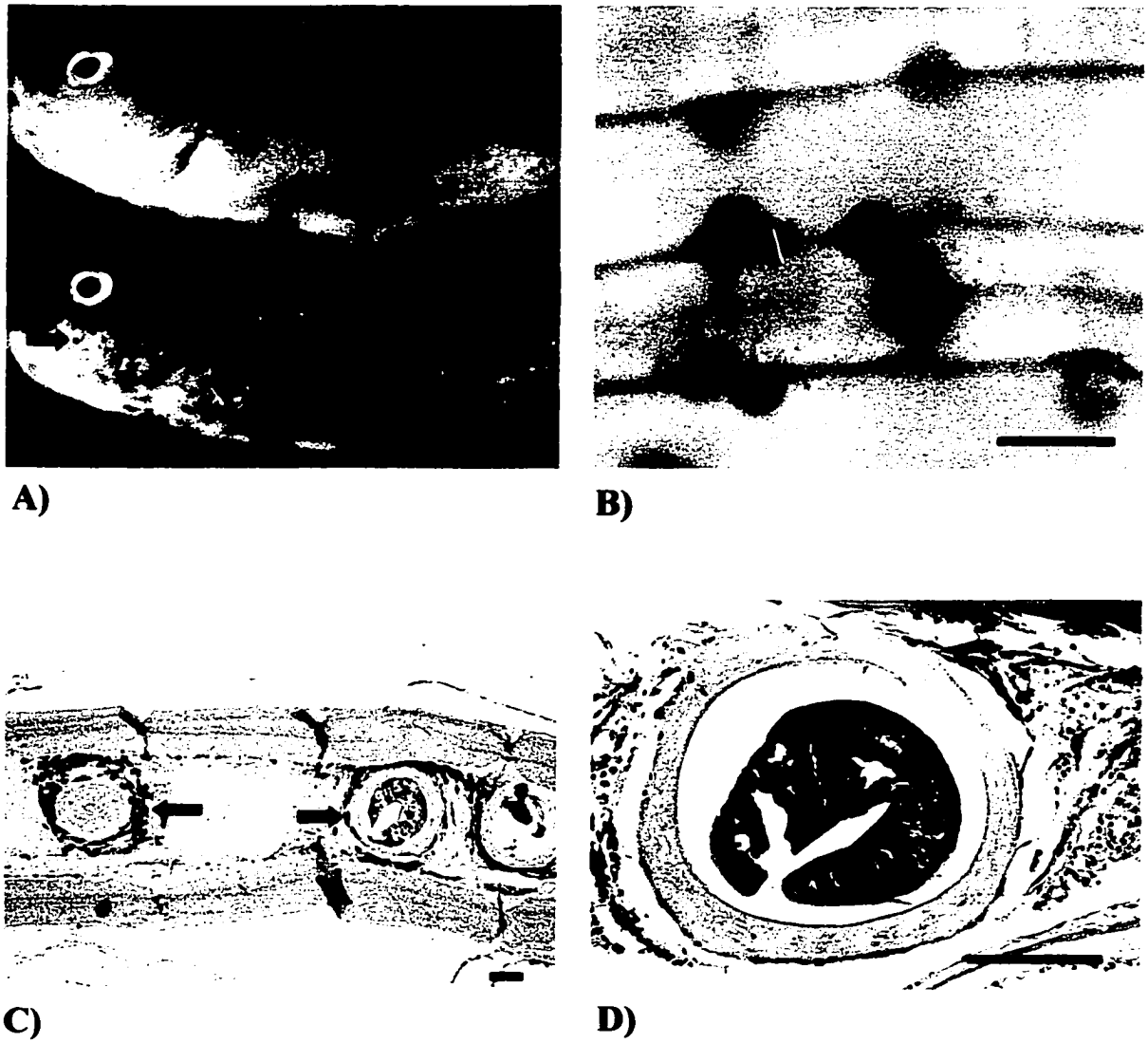

Fig. 4.2 A) Cunners with different degrees of infestation with metacercariae of *Cryptocotyle lingua* (arrows); the lower cunner is more heavily infested than the upper cunner, bar=1 cm. B) Stereoscopic photomicrophotograph of metacercarial cysts of *C. lingua* attached to the tail of a cunner, bar=1 mm. C) Metacercarial cysts of *C. lingua* in the tail of a cunner (arrows) each surrounded by melanin, hematoxylin & eosin stain, bar=100 μ. D) Metacercarial cyst of *C. lingua* in the tail of a cunner, hematoxylin & eosin stain, bar=100 μ.

4.2.2 Viability of the infective metacercariae

The metacercariae contained in the aliquot that was used to estimate the concentration of the infective doses were also submitted to an adaptation of the excystment method developed by McDaniel (70) to calculate what proportion of the total metacercariae administered to the DCC were viable.

Pepsin (1% w/v, at pH 2, adjusted with 1N HCl) was added to a crystal petri dish containing the metacercariae/Instant Ocean suspension and incubated for 30 minutes at 37°C. The suspension was subsequently placed in a test tube for 5 to 10 minutes at room temperature to allow the metacercariae to precipitate so that the supernatant containing pepsin could be decanted and the sediment returned to the crystal plate. In the main experiment the supernatant was not removed from the metacercarial suspension during the evaluation of metacercarial viability. After removing the supernatant, a 1% w/v trypsin (1:250) + 0.1% w/v Sodium Taurochlorate solution at pH 7 (adjusted with 1N NaOH) was added. After a second incubation for 45 minutes at 37°C, the crystal plate was placed under a stereoscopic microscope and live metacercariae were counted. For the pilot experiment, and because of the possibility of having access to only a limited supply of freshly caught fish, assessment of the viability of metacercariae was conducted not only on the day that the corresponding infective dose was processed and/or administered to the DCC, but also 24 hrs, 4- and 14 days later, on samples stored at 4°C. Because of the controversy over whether excystment is an active process only obtained from live parasites or an inactive consequence of the enzymatic effect on the cyst walls (70, 94), metacercariae were not considered alive by merely being freed from the cysts; only metacercariae that were motile were interpreted

as alive and potentially infective.

4.2.3 Egg shedding by, and recovery of, adult *C. lingua*

The pre-patent and patent periods of *C. lingua* were estimated during the pilot experiment. The pre-patent period was defined as the time between infection and the beginning of egg production. The patent period was defined as the period of egg production. This was estimated by either the presence of eggs in feces (first and second infection in the pilot study) or by the presence of adult parasites in the intestine of DCC at the time of euthanasia (second infection in pilot experiment and infection in main trial). During both pilot infections, fecal samples (8 from the infected group and 4 from the controls) were collected daily from the floor of the DCC enclosures and submitted to standard coproparasitological examination (flotation and sedimentation) to look for *C. lingua* eggs. Egg counts were not performed.

Parasite recovery techniques were performed at the time of euthanasia of DCC in both the pilot (6 DCC on day 6, 7 DCC on day 8, and 6 DCC on day 12 post-inoculation) and main experiments (12 DCC on day 7, 11 DCC on day 12, and 8 DCC on day 15 post-inoculation). Two different methods of euthanasia were used in the pilot and main experiments. The DCC in the pilot experiment were killed by an overdose of halothane while the DCC in the main trial were decapitated. The latter method yielded intestinal sections of better quality for histological examination (see Appendix 2). Immediately after euthanasia, a ventral midline incision from the caudal end of the sternum to the caudoventral ischium was performed, thus exposing the gastrointestinal tract and other abdominal and pelvic organs. The duodenum and rectum were clamped prior to the extraction of the intestinal tract as a whole. After tissue samples for histopathological examination were obtained (see section 4.2.5), the

intestinal lumen was exposed and washed by submersion in a water-filled container, with manual scraping of the enteric mucosa. Scraping of the enteric mucosa was aided in the main experiment by the use of a glass slide pressed and brushed against the mucosa to enhance detachment of adult parasites lodged deeply among intestinal villi. The intestinal contents collected in the water-filled container were sedimented for 20 minutes, decanted and the sediment examined in a petri dish using a stereoscopic microscope to count the total number of adult (eggs in uterus) and immature (uterus empty of eggs and smaller size) *C. lingua* present.

4.2.4 Hematology

Blood samples were collected immediately prior to euthanasia. The sampling method and the processing of samples were described in section 2.2.4.4. To assess whether *C. lingua* infection, either *per se* or combined with immunosuppression, affected hematological parameters, total and differential leukocyte counts, total protein values and hematocrit from immunosuppressed/non-infected (group A), immunosuppressed/infected DCC (group B), non-immunosuppressed/infected DCC (group D) and non-immunosuppressed/non-infected DCC (group C) were compared to baseline values established 4 days prior to dexamethasone treatment and parasitic infection (see section 2.3.5), and to each other. A maximum of 3 weeks had elapsed between the time that baseline values were obtained and when the last blood sample was collected.

4.2.5 Pathological effects of *C. lingua* on the intestine of infected DCC

Following extraction, the intestinal tract was placed on a cutting board, extended and arranged in a zigzag pattern. Sections of small intestine were harvested by cutting 1-2 mm

segments (5 in the pilot and 6 in the main experiment) at pre-determined sites with a clean scalpel blade. These sites included 1 (pilot experiment) or 2 (main trial) sections of the middle portion of the duodenum, 3 sections of the jejunum (1 from each third of its length), and 1 just anterior to the ileocecal junction that included the ileum and both ceca. The lumen was exposed and the sections were immediately fixed in 10% buffered formalin for a minimum of 24 hours before routine processing for light microscopic examination and staining with H&E (66). A maximum of 5-7 minutes elapsed from the time of euthanasia until the intestinal sections were immersed in formalin.

4.2.6 Statistical analyses

Group means (±s.d.) were compared by use of a one-way ANOVA test, except for the number of adult *C. lingua* recovered from immunosuppressed and immunocompetent DCC at days 12 and 15 post-infection, where a non-parametric Mann-Whitney test was used (106). Significance was determined at $p \leq 0.05$.

4.3 Results

4.3.1 Viability of the infective metacercariae

Metacercariae obtained from rainbow smelts used in the pilot experiment showed a viability of 80% when harvested and 70% 24 hrs later. The assessment at 4 and 14 days post-harvest involved metacercariae that were processed (detached from the fish and suspended in Instant Ocean Solution) on the day of capture and also metacercariae that remained attached to the fish tissues until the excystment procedure was performed. At 4 days post-harvest, metacercariae processed on the same day of capture had an 80% viability,

compared to 71% for metacercariae that had remained attached to the fish tissues. However, metacercariae that had remained attached to fish tissues subjectively appeared to have a higher degree of motility. At 14 days post-harvest, an average of 62% excystment was observed but viability was considered to be zero since no metacercarial movement was detected. Only 45-50% of metacercariae obtained from Cunners were viable at 24 hrs post-harvest, which corresponded to the time when they were administered to the DCC.

The numbers of metacercariae that were administered to DCC as infective doses were adjusted to the proportion of parasites considered viable at the time of administration and, therefore, potentially infective. Thus, the infective doses in the pilot experiment totaled 975 and 1,350 viable metacercariae/DCC for the first and second infections, respectively. The infective doses in the main trial totaled 8,700 viable metacercariae/DCC. However, one important step was mistakenly omitted in the method for assessment of viability performed in the main trial. The supernatant containing pepsin was not removed before trypsin was added to the metacercarial suspension. Therefore, the proportion of infective metacercariae contained in the dose administered to DCC in the main trial could have been substantially higher than is reported here.

4.3.2 Egg shedding by, and recovery of, adult *C. lingua*

In both infections in the pilot experiment, egg shedding began at day 4 post-infection. Feces stopped being positive for eggs 6 and 7 days after the administration of the last infective dose in the first and second infections, respectively. Therefore, the pre-patent and patent periods were determined to be 4 and 6-7 days long, respectively.

The number of adult *C. lingua* in the intestinal tract of infected DCC from the main trial

was correlated with the immune status of the birds. Immunosuppressed DCC had a significantly higher intestinal population of adult *C. lingua* than non-immunosuppressed DCC on days 7 and 12 post-infection ($p<0.05$) (Table 4.1). On day 15 post-infection, however, there was no statistically significant difference in the number of parasites between immunosuppressed and non-immunosuppressed DCC. All control DCC were negative. Egg shedding was not monitored in the main trial, therefore, the 4-day pre-patent period found in the pilot experiment could not be confirmed or dismissed. However, the fact that adult *C. lingua* were found in the intestine of infected DCC up to day 15 post-infection (corresponding to day 12 after the last infective dose had been administered) suggests a longer patent period than was found in the pilot infections.

Table 4.1 Numbers of adult *Cryptocotyle lingua* recovered from infected double-crested cormorants (DCC) in the pilot experiment (1998) and the main trial (1999).

	Pilot experiment [a]			Main Trial [b]					
				Immunosuppressed DCC			Non-immunosuppressed DCC		
Infection day [c]	6	8	12	7 [e]	12 [e]	15 [f]	7 [e]	12 [e]	15 [f]
Mean±s.d.	80±103	22±32	17±31	3,547±903	640±525	253±332	684±722	4±5	4±6
Range	0-231 [d]	0-78 [d]	0-64 [d]	2,830-4,561	205-1,223	18-488	15-1,450	0-9 [d]	0-8 [d]
n	4	5	4	3	3	2	3	3	2

[a] Average of 1,350 viable metacercariae administered to each DCC over a period of 6 days
[b] Average of 8,700 viable metacercariae administered to each DCC over a period of 4 days
[c] Day 0 corresponds to the administration of the first infective dose
[d] Only one DCC negative for *Cryptocotyle lingua*
[e] Parasite counts in immunosuppressed DCC > parasite counts in non-immunosuppressed DCC ($p<0.05$)
[f] Parasite counts in immunosuppressed DCC ≯ parasite counts in non-immunosuppressed DCC ($p>0.05$)

4.3.3 Hematology

Table 4.2 summarizes the hematological parameters of immunosuppressed/non-infected DCC (group A), immunosuppressed/infected DCC (group B), non-immunosuppressed/infected DCC (group D) and non-immunosuppressed/non-infected DCC (group C), and reference values based on parameters obtained from DCC prior to the experiment (see 2.3.5).

Mean hematocrit and protein values were elevated in all 4 groups of DCC when compared to baseline values ($p<0.01$). Among the 4 groups, non-immunosuppressed DCC (groups C and D) had the highest hematocrit, which was significantly higher than that of immunosuppressed/non-infected DCC (group A) and immunosuppressed-infected DCC (group B), suggesting an negative effect of dexamethasone on red blood cell concentration. The increases in hematocrit and protein levels were not associated with the infection with *C. lingua* since no difference was observed between infected and non-infected saline-treated groups (C and D).

The absolute heterophil counts were significantly higher ($p<0.01$) in immunosuppressed groups (A and B) as compared to non-immunosuppressed groups (C and D).

There was no significant effect on the blood cell counts associated with the infection with *C. lingua* (Table 4.2).

Table 4.2 Hematologic values of double-crested cormorants immunosuppressed and non-infected (group A), immunosuppressed and infected with *Cryptocotyle lingua* (group B), non-immunosuppressed and infected with *C. lingua* (group D) and non-immunosuppressed and non-infected (group C), compared to baseline values obtained 3 weeks earlier.

Parameter	Baseline values(n=29)	A (n=7)	B (n=8)	D (n=8)	C (n=8)
Hematocrit (%)	35±4	43±2 [b* c* d* e*]	38±2 [a* c* d* e*]	48±3 [a* b* e*]	48±4 [a* b* e*]
Total protein (g/L)	43±5	56±4 [e*]	54±6 [e*]	52±4 [e*]	53±5 [e*]
Absolute counts of leukocytes (10^9/L)					
Heterophils	17.4±9.7	20.3±5.3 [c* d*]	36.1±30.4 [c* d* e*]	6.5±2.0 [a* b* e*]	9.1±2.3 [a* b* e*]
Lymphocytes	8.5±5.0	6.4±2.8	10.6±6.5 [c# d#]	4.0±3.3 [b# e#]	5.3±1.9 [b# e#]
Monocytes	0.04±0.1	0.04±0.1	0.2±0.4 [e#]	0.05±0.07	0.02±0.05
Basophils	0.5±1.0	0.4±0.5	0.3±0.3	0.2±0.3	0.1±0.1
Total	26.6±11.7	27.2±6.2 [c* d*]	47.3±34.5 [c* d* e#]	10.8±2.7 [a* b* e*]	14.5±3.2 [a* b* e*]
Distribution of leukocytes (%)					
Heterophils	64±16	75±11	73±18	63±21	63±10
Lymphocytes	35±15	24±9	25±16	34±21	36±10
Monocytes	0.2±0.5	1±0	2±1	1±0	0
Basophils	2±3	3±1	1±0.4	4±2	2±1

[a] Significantly different from group A
[b] Significantly different from group B
[c] Significantly different from group C
[d] Significantly different from group D
[e] Significantly different from baseline parameter

[#] $p \leq 0.05$
[*] $p \leq 0.01$
Eosinophils were not detected. Data on the proportions of monocytes and basophils were too few to be subjected to statistical analysis

4.3.4 Pathological effects of *C. lingua* on the intestine of infected DCC

Among a total of 65 sections (13 birds; 5 sections/bird) of intestine in the pilot experiment and 96 sections (8 immunosuppressed, 8 immunocompetent birds; 6 sections/bird) in the main experiment, parasites were seen in 11 sections, with 1-5 parasites per section and a maximum of 2 parasites per 10x field. *Cryptocotyle lingua* was found in close proximity to the mid region of the mucosal villi (Fig. 4.3). No pathological effects associated with the presence of the parasites were observed in any of the sections examined.

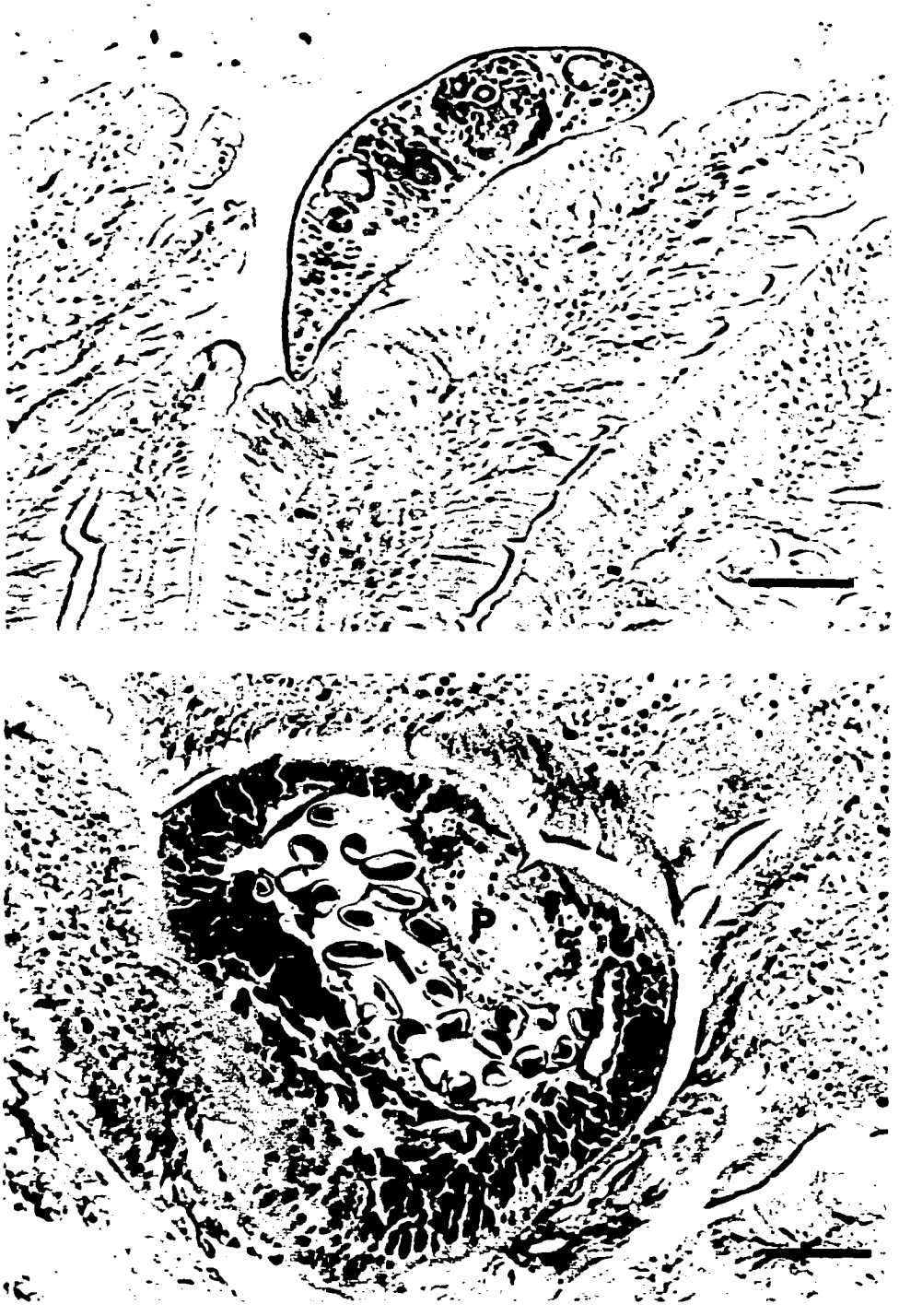

Fig. 4.3 Sections of small intestine of double-crested cormorants infected with *Cryptocotyle lingua*, showing the parasites (P) among intestinal villi. The arrow in B shows one of many eggs within the trematode's uterus. Hematoxylin & eosin stain, bar=100 μ.

4.4 Discussion

There was an unexpected difference in the viability recorded for metacercariae obtained from smelts (over 70%) and cunners (less than 50%). Both groups of metacercariae were processed in the same manner, with only one difference. The excystment procedure indicates that metacercariae are to be incubated in a pepsin solution which is then decanted before trypsin is added prior to the second incubation. In the case of metacercariae from cunners, however, pepsin was mistakenly not decanted before adding trypsin. Therefore, the latter was added to an acidic medium (pH 2) that must have lowered the desired pH at which trypsin requires to function properly, which is approximately 7. The activity of trypsin is appreciably decreased, although not totally absent, at low pH (43, 64, 108). A reduced trypsin activity would explain the reduced excystment of metacercariae from cunners, thus affecting the assessment of their viability. Consequently, the viability of metacercariae from cunners was likely underestimated and the total infective dose administered to the DCC in the main trial was higher than the estimated 8,700 metacercariae/bird.

Alternatively, metacercariae with lower levels of viability may reflect an inconspicuous poor physiological condition in their hosts. This effect of host condition upon the general vitality of its parasites has been observed in infected fish that are kept under laboratory conditions for long periods of time before the metacercariae are collected and used in experimental infections (91). However, this seems unlikely because both smelts and cunners were free-ranging until capture and the time lapse between capture and processing of the metacercariae was the same for both fish species. The exact age of smelts or cunners at the time of capture is unknown. Therefore, the cysts found in the latter could have been older

ones, containing dead metacercariae, which would never excyst, regardless of the procedure. The possibility of many cysts in the cunners being old and, therefore, containing dead metacercariae is unlikely since, although the life span of metacercariae is unknown, long survival rates (over 18 months) do occur (67).

Previous *C. lingua* infections in captive birds and mammals have shown that eggs appear in feces 5-6 days after feeding of infective metacercariae (91, 111) and that adult flukes are not found in the intestine before the fourth post-inoculation day (99). Therefore, the 4-day pre-patent period observed in both pilot infections is consistent with previous reports.

The patent period, however, is difficult to define. Previous experimental infections were generally conducted in a continuous manner, with *C. lingua* metacercariae being administered to the experimental subjects daily, until euthanasia. In a study of young terns fed a diet of infested cunners, the number of eggs detected in feces peaked at day 14 and then, probably due to the development of resistance by the host, they disappeared on day 20, although infested fish were still being consumed by the birds (111). No information on how long adult *C. lingua* can survive in a host is available. Based on what was found in the experimental infections of DCC, patent periods seemed to be either 6-7 days long (pilot experiment) or over 12 days long (main trial). These periods, however, were defined according to different criteria, egg shedding in the pilot experiment, and presence of adults in the intestine of DCC in the main trial. This means that a comparison between the patent periods in pilot and main experiments should consider that the presence of adults in the intestine does not mean they are producing eggs and that eggs are not always detected by fecal examination. Besides the species of fish that served as the source of metacercariae,

which likely does not influence the life span of adult *C. lingua* in the definitive host, the only difference between DCC from the pilot and main experiments was the age of the birds at the time of infection. The DCC from the pilot experiment were 14-15 weeks old at the time of the first infection, while DCC from the main trial were only 9-10 weeks old. Younger terns are more susceptible to heavy *C. lingua* infections presumably due to a less efficient immune response that allows more parasites to mature into adults (111). Older birds maintain a strong immune response and can inhibit reinfection beyond a slight degree (111). Protection against parasites is based on continuous stimulation of the immune system by the maintenance of a low but constant parasitic population, not on immunological memory (102). The DCC in both pilot and main experiments had come into captivity infected with *C. lingua*, but were treated for the infection soon after arrival and had not been in contact with the parasite for at least 4 weeks before the experimental infection. Therefore, the shorter patent period found for *C. lingua* in older DCC cannot be satisfactorily explained by acquired immunity to the parasite. A more probable explanation would be that the immune system of older DCC was more mature, thus being more efficient at controlling the infection than that of younger DCC. This hypothesis is supported by the fact that, in the main trial, immunosuppressed DCC allowed a larger population of adult *C. lingua* to develop in comparison to control DCC. Additionally, fecal samples from 3 and a half-week-old DCC who had been in captivity for13 days with no access to infective food, were positive for *C. lingua* eggs (see 2.3.3), supporting the hypothesis that the parasite has a longer patent period in young birds.

Interpretation of hematological parameters was complicated by the broad range of

baseline values obtained from the small number of normal individuals sampled and large standard deviations. Also, age-related changes in hematological parameters, such as increasing hematocrit values possibly due to enhanced erythropoiesis in growing birds (89), complicates comparison of baseline values to those of the experimental groups evaluated several weeks later. Therefore, although hematological values from experimental groups are compared to baseline values, more credence is given to statistically significant differences among groups (see Table 4.2). Among the non-immunosuppressed groups (C and D) and immunosuppressed groups (A and B), no statistically significant differences were observed in the hematological values between infected and non-infected DCC. Apart from the elevated hematocrit in all 4 groups (A, B, D and C) ascribed to either a state of dehydration (36) or an increased erythropoiesis (89) and the enhanced protein production due to maturation of the birds, effects on the hematological parameters were observed only in dexamethasone-treated DCC (groups A and B). Among the 4 groups, non-immunosuppressed DCC (groups C and D) had the highest hematocrit. It was significantly higher than that of immunosuppressed/non-infected DCC (group A) and immunosuppressed-infected DCC (group B), suggesting that this group experienced subclinical dehydratation or that dexamethasone could have down-regulated the increase in erythropoiesis. However, corticosteroids usually increase red cell production (5, 102), so that this hypothesis would disagree with the literature. Increases in hematocrit and protein levels were not associated with the infection with *C. lingua* since no difference was observed between infected and non-infected saline-treated groups (C and D).

The immunosuppressed DCC had elevated absolute heterophil counts, which is consistent

with the results described in Chapter 3 and ascribed to an effect of dexamethasone (49, 102). The elevated lymphocyte count in DCC in group B, however, is inconsistent with the lymphopenia normally associated with corticosteroids (36, 49, 102), and was only present in DCC treated with dexamethasone and infected with *C. lingua*. The possibility of this lymphocytosis being due to an effect of the parasite is unlikely since DCC in group D, which were also infected, showed no evidence of elevated lymphocyte counts. Therefore, no effects on the hematological profile associated with *C. lingua* infection *per se* were found. Other causes of lymphocytosis, such as lymphocytic leukemia, viral and chlamydial infections, were not detected in any DCC. The cause of lymphocytosis in DCC in group B is unknown.

The pathological effects ascribed to *C. lingua* in the literature range from practically non-existent (111) to massive enteritis (62, 96). Most of the former relate to experimental infections of mammals and birds. The latter are field observations of dead individuals from free-ranging populations that either name *C. lingua* as the cause of death but do not support this with appropriate pathological examination of the affected birds (62, 96) or report its presence as a puzzling incidental finding (21, 34). The experimental infections conducted on both immunosuppressed and non-immunosuppressed DCC showed no evidence of detrimental effects caused by the parasite on the intestinal mucosa or the enteric wall, although a significantly higher number of adult parasites matured in the intestines of immunosuppressed DCC. Thus, an impaired immune response significantly enhanced the level of *C. lingua* infection in DCC but did not increase the pathological effect that the parasite may have on its host.

In summary, the pre-patent period of infection by *C. lingua* in DCC was established at 4

days post-exposure, while the patent period seemed to be influenced by the age of the host at the time of infection. The number of adult *C. lingua* in the intestinal tract of infected birds was assessed and the level of infection correlated with immune status, hematological values and pathological changes. Immunosuppressed DCC fostered higher numbers of adult parasites and had a mild heterophilia, probably due to the dexamethasone treatment. No pathological or hematological effects associated with *C. lingua* infection were found in infected DCC, regardless of the immune status of the host.

5. GENERAL DISCUSSION

When death of free-ranging wild animals is caused by several factors, such as emaciation in combination with various infectious diseases, the distinction between terminal and underlying causes of death is rendered almost impossible. At times, it is tempting to ascribe the cause of death to an abnormality, such as the presence of large numbers of intestinal trematodes in emaciated seabirds, when other disease processes are not found. However, being the most striking finding in a dead bird does not make a heavy trematode infection directly responsible for its death. Therefore, isolating a single etiology and estimating its potential contribution to morbidity and mortality under controlled conditions helps to interpret its significance with respect to other disease entities under more natural conditions. The hypothesis that this study aimed to investigate was whether an impaired immune status would render seabirds, such as *Phalacrocorax auritus* (DCC), more susceptible to infection by an intestinal trematode, *Cryptocotyle lingua*, and to describe the effects that the trematodes would have on the health status of the birds.

The raising of juvenile DCC in captivity and their use as experimental birds proved to be a time-consuming but reasonably simple task; the birds adapted to captivity with relative ease as reported in the literature (22, 58). The first few weeks were the most difficult period, when most of the mortalities were recorded. The proportion of birds that died shortly after being collected from a wild colony was similar to that observed both in DCC (58) and other seabird species, such as penguins (31) and puffins (103) when first brought into captivity. In the present study, however, the main cause of death was not a nutritional imbalance, as has been reported for DCC chicks due to vitamin D_3 deficiency (58) or puffin chicks that

died from hypovitaminosis E shortly after capture (103). The main cause of mortality in the DCC in the present study was bacterial sepsis. The etiologic agents responsible for most cases of septicemia were *Salmonella typhimurium*, which is frequently present in the intestines of some avian species, such as waterfowl (112), and *Erysipelothryx rhusiopathiae*, frequently isolated from fish (23). These bacteria often require an impaired immune response to cause disease in birds (112). The stress caused by the capture and handling of the DCC chicks (2) and their immature, hence inefficient, immune system (14, 112) are suggested as the predisposing factors that resulted in the development of septicemia and death in susceptible birds. Respiratory aspergillosis, another infectious agent responsible for the death of seabirds in rehabilitation centers (2) and a common cause of mortality in penguin collections (31, 86), was also found to cause the death of some DCC. An attempt at preventing this opportunistic fungus from causing disease in the DCC in the main trial involved the administration of ketoconazole to all birds upon arrival to the enclosure. However, there seemed to be no prophylactic value in the ketoconazole treatment since the proportion of treated DCC that developed the disease was similar to that of untreated DCC. These results agree with other reports where ketoconazole is considered ineffective in the prevention or treatment of aspergillosis (31, 86). One report (86) suggests that birds receiving an oral treatment with ketoconazole may have experienced inhibition of pulmonary defense mechanisms; the present study found no evidence to support or refute this hypothesis.

The first step in this study, replicating the infection in captive DCC, was achieved in 1998 in the pilot trial. The DCC were collected from a wild colony, raised in captivity and

infected with *C. lingua* metacercariae obtained from rainbow smelts, *Osmerus mordax*, in two sequential series of inoculations. Eggs were detected in the feces on day 4 post-infection, indicating that *C. lingua* metacercariae matured into adults 96 hours after entering the host (pre-patent period), which corresponded with results of experimental infections in other species, such as gulls (91) and terns (111). The patent period, defined as the life span of adult parasites in the host, was determined to be 6-7 days long in the pilot infections, based on egg shedding in the feces. This patent period was substantially longer in the main trial conducted in 1999 where adults containing eggs in their uterus were found in the intestine of DCC 12 days post-infection. The DCC infected during this second experiment were 4 weeks younger than birds from the pilot trial and the prolonged patent period may have resulted from a less well developed immune system. This is, to some extent, comparable to observations in terns where younger birds allow a larger proportion of *C. lingua* to mature into adults than older birds do (111).

After the model of infection had been established, a second infection was carried out on a different group of DCC, this time including immunosuppression as a variable. Dexamethasone was selected as the immunosuppressive agent based on a previous report of its efficiency in causing immunosuppression in mallard ducks (93). Because there is no information on the effects of dexamethasone in DCC, the experimental dose used in the present study was based on the mean dose that effectively caused immunosuppressive effects in mallard ducks (93). To confirm an immunosuppressive effect from the treatment, lymphocyte stimulation tests (Phytohemmaglutinin-P skin test and antibody production against Sheep Red Blood Cells) were performed along with evaluation of the morphology

of the bursa of Fabricius. The PHA-P stimulation index from DCC treated with dexamethasone was significantly lower than that of saline-treated birds, indicating a depressed T cell response (41, 49) similar to that of mallard ducks treated with dexamethasone (93). Antibody titers against SRBC were only marginally suggestive of an impaired B cell response (49, 93). There was a significant difference between antibody levels of dexamethasone and saline-treated DCC on day 9-10 post-inoculation ($p<0.05$) while no difference was observed on days 14 and 17 post-inoculation. The lack of a significant difference in antibody titers against SRBC between DCC treated with dexamethasone and saline-treated DCC was not consistent with the response found in mallard ducks where production of antibodies against SRBC in treated birds was significantly reduced by the administration of dexamethasone (93). The possibility of the dose of dexamethasone being too low to affect B cells in DCC cannot be discarded. However, a more likely explanation for the lack of difference in the response of dexamethasone and saline-treated DCC is that SRBC may not be sufficiently antigenic in this particular species, as has been observed in Japanese quail (42). The bursae of Fabricius from dexamethasone-treated DCC showed marked lymphoid depletion, with smaller, more irregular and less cellular follicles, further supporting an effect of the treatment on the humoral immune response. Thus, the immunosuppressive effect of dexamethasone was confirmed. Increased heterophil counts were also recorded in dexamethasone-treated DCC ($p<0.01$), both infected and non-infected, which is an expected effect of treatment with corticosteroids (49, 69, 102). Dexamethasone reduces the expression of adhesion molecules on the surface of granulocytes, slowing their migration from blood vessels to tissues, thus elevating the concentration of heterophils

present in the blood (36, 69). Lymphocyte counts, which usually decline mainly due to the decreased production and function of IL-2 caused by corticosteroids (69), were not affected by the treatment with dexamethasone in DCC. Alternatively, a situation causing lymphocytosis, such as epinephrine release, may have acted oppositely on lymphocyte numbers, resulting in no lymphopenia observed in the dexamethasone-treated DCC. The lack of information on the response of DCC to corticosteroids make it possible to speculate that dexamethasone may affect this particular species differently.

Immunosuppression influenced the number of adult *C. lingua* allowed to colonize the small intestine of DCC as indicated by the difference in intensity of infection between dexamethasone-treated and saline-treated birds. The numbers of adult *C. lingua* recovered from the intestine of immunosuppressed DCC on days 7 and 12 post-infection were significantly higher than those recovered from non-immunosuppressed DCC ($p<0.05$). However, no difference in the number of parasites recovered from the intestine of immunosuppressed and non-immunosuppressed DCC was observed on day 15 post-infection. This suggests that immunosuppression allows a larger proportion of *C. lingua* to reach adulthood (111), but has no effect over the life span of the parasite since both immunosuppressed and non-immunosuppressed DCC eliminated the infection at similar times. However, this contradicts the hypothesis that the longer patent period observed in younger DCC resulted from an immature immune system that possibly lengthens the life span of mature *C. lingua*. Further studies to determine the exact life span of *C. lingua* in its final host are needed to clarify this point. An alternative species, easier to raise in captivity, probably from eggs collected in the wild to reduce mortalities from infectious organisms like

those observed in the DCC, with a higher susceptibility to *C. lingua,* would perhaps be more practical as an experimental subject. Herring gulls, *Larus argentatus*, are precocious seabirds, very abundant in the Maritime Provinces, with easily accessible ground-nesting colonies and should be considered for a further study. As well, herring gulls may have a higher susceptibility to *C. lingua* infection (based on the report on enteritis in a different species of gull (62)).

Neither in the pilot infection nor in the main trial were pathological effects found in the intestinal tract of infected DCC, whether or not the birds were immunosuppressed. These negative results raise some doubts about reports of massive enteritis and death in a gull (62) and a bald eagle (96) infected with *C. lingua*. However, the parasites recovered from the experimental DCC were not as numerous as those found in the birds whose death was attributed to *C. lingua*. Although the intensity of *C. lingua* infection was enhanced in DCC by an immunosuppressive dexamethasone treatment, the numbers of adult parasites did not reach intensity levels observed in emaciated loons (21). Furthermore, there was no effect on the body condition of DCC, either due to the immunosuppression or the infection itself. However, the experimental immunosuppression lasted only 19 days, during which time the DCC had easy access to food and shelter, hardly replicating natural conditions for a sick seabird in the wild. In these wild birds, a combination of disease and availability of highly infested prey (cunners are known as sluggish fish and have been reported to carry up to 14,000 metacercariae) (94) would increase their susceptibility and exposure to infective parasitic forms. A prolonged experimental immunosuppression would be required to more accurately replicate what happens to diseased seabirds with access to highly infested fish.

The life span of the parasite is unknown but probably quite short, based on what was described in young terns which cleared the infection only 20 days after receiving the first infective dose (111) and on the results of the present study. Therefore, this would decrease the efficacy of a continuous administration of large quantities of *C. lingua* to immunosuppressed birds over a long period of time. The additive effect would be minimal due to rapid turnover of adult parasites. An alternative explanation for how piscivorous birds reach high levels of infection such as those observed in common loons (21, 34) would be that weak birds, possibly suffering from an underlying disease that impairs their ability to catch fish, turn their attention to marine snails living in the littoral zone, easily accessible even to debilitated individuals, and loaded with thousands of cercariae (90). This hypothesis would require not only the survival of the cercariae during their gastric transit, but also the infection of a definitive host with a larval form designed to infect an intermediate host, which would contradict accepted trematode dogma (35). However, the infection of seabirds with snails infected with *C. lingua* cercariae has not been attempted and, therefore, this remains a possibility. After all, a metacercaria is a cercaria that has lost its tail and is surrounded by a protective cyst (35). Hence, the possibility of a heavy *C. lingua* infection being the terminal cause of death in a bird cannot be disregarded. However, based on the lack of pathological findings in the enteric mucosa of the experimental DCC, even in places of direct contact with adult parasites, a direct or significant involvement in a bird's death seems unlikely.

Appendix 1

Incubation and hatching of double-crested cormorants

On May 6, 1999, DCC from the ground-nesting colony on the south end of Little Courtin Island (46°32'N, 63°45'W), Malpeque Bay, Prince Edward Island, were already in the incubation stage. Nests contained an average of 4 eggs. Approximately 100 eggs were marked with the number "6" using a black wax crayon. On May 18, a total of 77 eggs were collected from 20 different nests. Because hatching success was previously reported to be greater if the eggs are incubated for about a week by the parents (82), marked eggs were preferably chosen, thus ensuring that most of them had undergone at least 12 days of incubation. In some cases, however, one or two unmarked eggs in a nest were taken along with the marked ones in order to promote re-nesting following complete loss of the clutch. Because eggs are generally laid on a daily basis (51), the unmarked eggs were considered to have undergone at least 9 days of incubation. The eggs were placed in egg cartons, surrounded by bubble wrap, put in a styrofoam box, and transported to an isolation unit at the Atlantic Veterinary College. Upon arrival, all eggs were washed and gently scrubbed in warm water to remove the surface feces and dirt and were subsequently dipped in an antibiotic solution (Baytril Turkey Egg Dip Concentrate) containing 500 ppm of enrofloxacin.

After the eggs had been washed and disinfected, they were candled to check for viability. Despite the difficulty associated with evaluating thick-shelled eggs, seven eggs were determined to contained a dead embryo and thus discarded. The remaining 70 eggs were placed in an incubator, on their horizontal axis. Once the eggs were placed inside, the

incubator vents were shut and the fumes from a mixture of formaldehyde and potassium permanganate (1.2 ml of 37.5% formaldehyde + 0.6 g of potassium permanganate/ cubic foot) were used to fumigate both eggs and incubator. Approximately twelve hours later, the vents were opened and the fumes dissipated.

Throughout the incubation process, the eggs were automatically rotated 90° every hour, and manually rotated 180° about their long axis every 24 hours (82). The temperature was maintained close to recommended levels (37.2°C), at 36.7°C dry bulb and 27.8°C wet bulb. However, the humidity was only 51%, not the recommended 60-64% (82).

After only 2 days of being in the incubator, one of the birds started pipping and was thus moved to the hatcher. Subsequently, the incubator was opened twice daily to check for pipping birds which were then removed and placed in the hatcher. The average temperature in the hatcher was 35.8°C dry bulb and 32.6°C wet bulb, with a 81 % humidity.

Out of 70 eggs, 62 birds hatched within a 14 day period. Since the incubation period of DCC eggs is 28 days, it is assumed that all eggs were between 12 and 26 days old when collected. Of the remaining 8 eggs, 5 were removed during incubation (2 because they were determined to be infertile and 3 due to broken shells) and 3 never hatched. The hatching rate was 88%, as compared to the 70% level reported by Powell *et al.* (82).

The mean weight of the birds at hatching was 34.1 g ± 3.3 g, with a range of 23.8 to 40.3 g, consistent with weights of chicks in the wild (28). After being weighed, the cormorant hatchlings were placed in one of four nests, consisting of a plastic bowl lined with cloth towels. The temperature in the nests was 27.8°C (± 2.8°C). The humidity levels were not recorded, but two separate humidifiers were constantly providing water vapor above the

nests.

Body weight was recorded each morning. The birds were fed 10 to 15% of their body weight 5 times a day, at 7 am, 11 am, 2 pm, 5 pm and 8 pm. The food consisted of a semi-liquid fish mash, each batch comprising four herrings, minus heads and tails, weighing approximately 200 g each, 800 ml of tap water, 10 ml of Vitamin B complex® (Rhône Mérieux, Québec, Canada), and 1 g of Electrolytes Plus® (Co-op, Saskatoon, Canada/ Rhône Mérieux, Québec, Canada). All ingredients were blended, strained and refrigerated. Plastic syringes were used to feed the hatchlings with this formula, which was heated to 35°C just before feeding.

Unfortunately, all this work was fruitless. All birds seemed active and healthy at the moment of hatching, but quickly lost their energy. Even with the constant feeding, cleaning and warm temperature, no hatchling lived beyond 9 days of age.

The mortality of the hatchlings was attributed to an insufficiently humid environment, and a second attempt to raise cormorants from eggs was made. Twenty six eggs, presumed to belong to second clutches, were collected from the same colony on Little Courtin Island, on June 18,. These eggs were handled, incubated and hatched similarly to the first group. The incubator was kept at 36.7°C dry bulb and 29.5°C wet bulb; and the hatcher at 37.5°C dry bulb and 33.4°C wet bulb. The housing was slightly different. The nests were placed inside a plastic "tent" that concentrated both temperature and humidity in an attempt to stabilize both parameters. The temperature range in the tent was 26-37°C, with an average of 31-33°C. The humidity range recorded was 67- 93%, with an average of 85%. Out of the 26 eggs collected, 18 hatched within an 11-day period. The eggs had been candled on arrival

at the AVC, but the shells appeared to be too thick to allow an evaluation of their viability. Thus, the resulting hatching rate of 69 % is probably an underestimate. This second group of hatchlings had an average weight of 34.3 g (±2.4 g).

The hatchlings were fed 5 times a day, initially with small trout. When the birds seemed to regurgitate most of the fish they were fed, the diet was changed to the formula previously described for the first group of hatchlings. Even though they had almost the same average weight, this second group of hatchlings seemed less active than the first. None of the birds survived for more than 5 days after hatching.

Post mortem examinations were performed on all cormorant hatchlings. Nothing conclusive was found other than that the cause of death did not appear to be infectious. Several factors could have accounted for the mortality of the hatchlings, including inadequate microenvironment (temperature and/or humidity levels), malnutrition and absence of normal bacterial flora. Because DCC are altricial, and thus incapable of thermoregulation until after 2 weeks of age (27), the microenvironment plays a crucial role in their survival and development. In the wild, parents are never far from the nest and protect the chicks against extreme weather conditions, such as rain, cold or excessive heat. DCC chicks are kept warm by conductive heat coming from the bodies of their parents. The hatchlings in this study were kept warm by radiation heat generated by infrared lamps. Whether or not this was detrimental to the chicks is unknown. The food administered to the hatchlings was thought to be of good nutritional value. However, it may have lacked essential components provided naturally by the parents' saliva and digestive secretions. There are reports on passerine birds, also altricial, indicating that some chicks require that

their diet be supplemented with bacteria-rich saliva from their parents (60).

The cause of death of the hatchlings remains undetermined, but because both attempts at raising DCC from eggs failed, juveniles had to be used in the experiment.

Appendix 2

Method of euthanasia

Proper histopathological evaluation of the intestinal sections from DCC in the pilot experiment was impaired by the large proportion of epithelial cells that had sloughed off the mucosal villi. The need for good quality tissue samples was evident and modifications to the procedure followed in the euthanasia and sample collection in the pilot experiment had to be made . Since the removal of the intestinal tract and fixation of the tissue sections in formalin had immediately followed death, the time which the birds had taken to died was thought to be responsible for the autolytic changes. This hypothesis was tested with chickens. The chickens were euthanized by either halothane administration (3 chickens), cervical dislocation (2 chickens) or exsanguination (1 chicken). Prior to euthanasia with halothane, intestinal sections were surgically obtained from one chicken. Intestinal sections were fixed and processed for histological evaluation in the same way that the DCC samples had been handled, and compared to tissues obtained surgically from live birds by terminal enteric resection. The quality of the samples was directly related to the method of euthanasia that had been used and the length of time that the procedure involved. Tissues from chickens euthanized by halothane overdose and exsanguination showed marked mucosal epithelial sloughing while tissues from a chicken euthanized by quick cervical dislocation were as well preserved as those surgically obtained. For the main trial, therefore, halothane overdose was discarded and decapitation was chosen instead. Decapitation was quick, hence ensured samples of good quality, and made blood collection possible by simply placing a tube under the bird's exposed neck.

LITERATURE CITED

1. ANONYMOUS. Charlie's ant. Veterinary Record 1990; 127: 508.

2. ANONYMOUS. Clinic uses drugs to eliminate aspergillosis. International Bird Rescue Research Center 1994; 3: 3-7.

3. ANONYMOUS. The trouble with cormorants. Field and Stream 1999; March: 16.

4. BAGLA P. Longer tusks are healthy signs. Science 1997; 276: 1972.

5. BARRAGRY TB. Veterinary drug therapy. Philadelphia: Lea & Febiger, 1994.

6. BICKNELL EJ, GREICHUS A, GREICHUS YA, BURY RJ, KNUDTSON WU. Diagnosis and treatment of aspergillosis in captive cormorants. Sabouraudia 1971; 9: 119-122.

7. BOYCE MS. The Red Queen visits the Sage Grouse. American Zoologist 1990; 30: 263-270.

8. BUSTNES JO. Factors influencing the prey choice of marine birds in multiple prey situations. Bulletin of the Scandinavian Society for Parasitology 1996; 6: 1-15.

9. BUTTERWORTH AE. The immunobiology of parasite infection. Royal Society of Medicine Services Limited, 1986.

10. CAIRNS DK, DIBBLEE RL, DAOUST P-Y. Displacement of a large Double-crested Cormorant, *Phalacrocorax auritus*, colony following human disturbance. Canadian Field-Naturalist 1998; 112: 520-522.

11. CARROLL L. Through the looking glass, and what Alice found there. London: J. M. Dent & Sons Ltd., 1871.

12. CHAUSSE AM, BERNARDET N, MUSSET E, THORAVAL P, VAINO O, DAMBRINE G, LOUDERT F. Expression of MHC Class 1 antigens following Marek's disease infection. In: Davison, Bumstead, Kaiser, eds. Advances in avian immunology research. UK: 1995: 135-139.

13. CHEN CHH, PICKEL JM, LAHTI JM, COOPER MD. Surface markers on avian immune cells. Avian cellular immunology. CRC Press, 1991: 1-22.

14. CHUTE HL, RICHARD JL. Fungal infections. In: Calnek B W, Barnes HJ, Beard CW, Reid WM, Yoder HW, eds. Diseases of poultry. Ames, USA: Iowa State University Press, 1991: 326-334.

15. CLAYTON DH. Mate choice in experimentally parasitized Rock Doves: lousy males lose. American Zoologist 1990; 30: 251-265.

16. CLAYTON DH, MOORE J. Host-parasite evolution, general principles and avian models. Oxford University Press, 1997.

17. COMPTON MM, GIBBS PS, JOHNSON LR. Glucocorticoid activation of deoxyribonucleic acid degradation in bursal lymphocytes. Poultry Science 1990; 69: 1292-1298.

18. DANESIK KL. Maternal antibodies to Newcastle Disease Virus, immune response to vaccination with killed Newcastle vaccine and growth parameters of captive Double-crested Cormorant (*Phalacrocorax auritus*) chicks, Western College of Veterinary Medicine Report 1995.

19. DAOUST P-Y. Osteomyelitis and arthritis caused by *Salmonella typhimurium* in a crow. Journal of Wildlife Diseases 1978; 14: 483-485.

20. DAOUST P-Y. Conjunctivitis in cormorants-Prince Edward Island. Canadian Cooperative Wildlife Health Centre Newsletter 1996; 4: 4-5.

21. DAOUST P-Y, CONBOY G, MCBURNEY S, BURGESS N. Interactive mortality factors in Common Loons from Maritime Canada. Journal of Wildlife Diseases 1998; 34: 524-531.

22. DELARONDE GG, GREICHUS YA. Care and behavior of penned Double-crested Cormorants. The Auk 1992; 89: 644-650.

23. DIERAUF LA. Handbook of marine mammal medicine. New York: CRC Press, 1990.

24. DIETERT RR, GOLEMBOSKI KA, BLOOM SE, QURESHI MA. The avian macrophage. Avian cellular immunology. CRC Press, 1997: 71-96.

25. DUFFY DC. Why is the Double-crested Cormorant a problem? Insights from cormorant ecology and human sociology. Colonial Waterbirds 1995; 18: 25-32.

26. DUNIER M, SIWICKI AK. Effects of pesticides and other organic pollutants in the aquatic environment on immunity of fish: a review. Fish and Shellfish Immunology 1993; 3: 423-438.

27. DUNN EH. Growth, body components and energy content of nertling Double-crested Cormorants. The Condor 1975; 77: 431-438.

28. DUNN EH. Development of endothermy and existence energy expenditure of

nestling Double-crested cormorants. The Condor 1976; 78: 350-356.

29. ELIOT JL. Cormorant slaughter in Lake Ontario provokes outrage - and a reward. National Geographic 1999; 195: xii

30. FAGERHOLM H-P. Nematode parasites of marine- and shore birds, and their role as pathogens. Bulletin of the Scandinavian Society for Parasitology 1996; 6: 17-30.

31. FLACH EJ, STEVENSON MF, HENDERSON GM. Aspergillosis in Gentoo Penguins (*Pygoscelis papua*) at Edinburgh Zoo. Veterinary Record 1990; 126: 81-85.

32. FORBES NA. Aspergillosis in raptors. Veterinary Record 1991; 130: 263-263.

33. FORBES NA. Diagnosis of avian aspergillosis and treatment with itraconazole. Veterinary Record 1992; 130: 519-520.

34. FORRESTER DJ, DAVIDSON WR, LANGE RE, STROUD RK, ALEXANDER LL, FRANSON JC, HASELTINE SD, LITTELL RC, NESBITT SA. Winter mortality of Common Loons in Florida coastal waters. Journal of Wildlife Diseases 1997; 33: 833-847.

35. FRIED B, GRACYK TK. Advances in trematode biology. CRC Press, 1997.

36. FUDGE A. Avian clinical pathology, hematology and chemistry. In: Altman R B, Clubb SL, Dorrestein GM, Quesenberry K, eds. Avian medicine and surgery. Philadelphia: WB Saunders, 1997: 142-157.

37. GALAKTIONOV KV. Impact of seabird helminths on host populations and coastal ecosystems. Bulletin of the Scandinavian Society for Parasitology 1996b; 6: 50-64.

38. GALAKTIONOV KV. Life cycles and distribution of seabirds helminths in artic and sub-artic regions. Bulletin of the Scandinavian Society for Parasitology 1996a; 6: 31-49.

39. GLAHN JF, STICKLEY AR. Wintering double-crested cormorants in the Delta region of the Mississippi: population levels and their impact on the catfish industry. Colonial Waterbirds 1995; 18: 137-142.

40. GODFREY WE. The birds of Canada. National Museums of Canada, 1966.

41. GOTO N, KODAMA H, OKADA K, FUKIMOTO Y. Suppression of phytohemagglutinin skin response in thymectomized chickens. Poultry Science 1978; 57: 246-250.

42. GRASMAN KA, SCANLON PF. Effects of acute lead ingestion and diet on antibody and T-cell-mediated immunity in Japanese quail. Archives of Environmental Contamination and Toxicology 1995; 28: 161-167.

43. GUYTON AC. Medical physiology. USA: WB Saunders Company, 1986.

44. HAMILTON WD, ZUK M. Heritable true fitness and bright birds: a role for parasites. Science 1982; 218: 384-386.

45. HARMAN BG. Avian heterophils in inflammation and disease resistance. Poultry Science 1998; 77: 972-977.

46. HATCH JJ. Changing populations of double-crested cormorants. Colonial Waterbirds 1995; 18: 8-24.

47. HOBERG EP. Faunal diversity among avian parasite assemblages: the interaction of history, ecology and biogeography in marine systems. Bulletin of the Scandinavian Society for Parasitology 1996; 6: 65-89.

48. HOH E. Flying fisher. Natural History 1998; 107: 66-71.

49. JANEWAY CHA, TRAVERS P. Immunobiology. Garland Publishing, 1997.

50. JOHNSON SG. Effects of predation, parasites, and phylogeny on the evolution of bright coloration in North American male passerines. Evolutionary Ecology 1991; 5: 52-62.

51. JOHSGARD PA. Cormorants, darters and pelicans of the world. Washington, D.C.: Smithsonian Institution Press, 1993.

52. JOSEPH LE. Man and cormorant. Audubon 1986; 88: 38-41.

53. KING DT. Movements of double-crested cormorants among winter roosts in the Delta region of Mississippi. Journal of Field Ornithology 1996; 67: 205-211.

54. KLASING KC. Avian inflammatory response: mediation by macrophages. Poultry Science 1991; 70: 1176-1186.

55. KOCH C. Complement system in avian species. In: Toivanen A, Toivanen P, eds. Avian immunology: basis and practice, vol. 2. Boca Raton, USA: CRC Press, 1987: 43-56.

56. KUIKEN T Newcastle Disease and other causes of mortality in Double-crested Cormorants (*Phalacrocorax auritus*) 1998; University of Saskatchewan; Doctoral Thesis.

57. KUIKEN T, DANESIK KL. Hematology and serum chemistry of captive juvenile double-crested cormorants (*Phalacrocorax auritus*). Canadian Veterinary Journal 1999; 40: 493-496.

58. KUIKEN T, FOX.G.A, DANESIK KL. Bill malformation in double-crested cormorants with low exposure to organochlorines. Environmental Toxicology and Chemistry 1999; 18: 2908-2913.

59. KUNKLE RA, RIMLER RB. Pathology of acute aspergillosis in turkeys. Avian Diseases 1996; 40: 875-886.

60. KYLE PD, KYLE GZ. An evaluation of the role of microbial flora in the saliva tranfer technique for hand-rearing chimney swifts (*Chaetura pelagica*). Wildlife Rehabilitation 1993; 8: 65-71.

61. LARONE DH. Medically important fungi: a guide to identification. American Society for Microbiology, 1987.

62. LAUCKNER G. Diseases of marine animals. Hamburg: Biologische Anstalt Helgoland, 1985.

63. LAUFER B. The domestication of the cormorant in China and Japan. Studies of Culture History. Chicago, USA: Field Museum of Natural History, 1931: 205-255.

64. LONGLAND AC. Digestive enzyme activities in pigs and poultry. UK: CAB International, 1991.

65. LOWENTHAL JW, YORK JJ, DIGBY MR. Interferon production by avian T cells. In: Davison, Bumstead, Kaiser, eds. Advances in avian immunology research. UK: Carfax Publishing Company, 1995: 179-185.

66. LUNA LG. Manual of histologic staining methods of the Armed Forces Institute of Pathology. New York: McGraw-Hill, 1968.

67. LYSNE DA, HEMMINGSEN W, SKORPING A. Pepsin digestion reveals both previous and present infections of metacercariae in the skin of fish. Fisheries Research 1995; 24: 173-177.

68. MANNING MJ. Phylogeneric development of the lymphoid system. In: Rose, Payne, Freeman, eds. Avian Immunology. UK: British Poultry Science Ltd., 1981: 3-17.

69. MARINO IR, DOYLE HR. Conventional immunosuppressive drugs. In: Thomson A W, Starzl TE, eds. Immunosuppressive drugs. Pittsburgh: Edward Arnold Publisher, 1994: 1-14.

70. MCDANIEL JS. Excystment of *Cryptocotyle lingua* metacercariae. Biology Bulletin 1966; 130: 369-377.

71. MCDONALD TE, MARGULIS L Department of Fisheries and Oceans Synopsis of parasites of fishes of Canada: supplement (1978-1993). Ottawa: NRC. 1995; 122. Canadian Special Public Fisheries and Aquatic Science.

72. MICHIE HR. Metabolism of sepsis and multiple organ failure. World Journal of Surgery 1996; 20: 460-464.

73. MILLER MM, GOTO RM, WAKENELL PS, JARVI SI, BERNOT A, AUFFRAY C, ZOOROB R, BUMSTEAD N, BRILLES WE. The Rfp-Y System - a second system of histocompatibility genes in chicken. In: Davison, Bumstead, Kaiser, eds. Advances in avian immunology research. UK: Carfax Publishing Company, 1995: 95-104.

74. MILTON J. Paradise Lost, a poem in twelve books (1674). Indianapolis, IN: Odyssey Press Bobbs-Merrill Co., 1976.

75. MOORE J. Parasites that change the behavior of their host. Scientific American 1984; 250: May: 108-115.

76. MØLLER AP. Effects of a haematophagus mite on the Barn Swallow (*Hirundo rustica*): a test of the Hamilton and Zuk hypothesis. Evolution 1990; 44: 771-784.

77. NORMAN FI, DU GUESCLIN PB, DANN P. The 1986 "'wreck" of Little Penguins *Eudyptula minor* in Western Victoria. Emu 1992; 91: 369-376.

78. PASTORET PP, GRIEBEL P, BAZIN H, GOVAERTS A. Handbook of vertebrate immunology. Academic Press, 1998.

79. PILON C, BURTON J, MCNEIL R. Summer food of the Great and Double-crested Cormorants on the Magdalen Islands, Quebec. Candian Journal of Zoology 1983; 61: 2733-2739.

80. POMEROY BS, NAGARAJA KV. Fowl typhoid. In: Calnek B W, Barnes HJ, Beard CW, Reid WM, Yoder HW, eds. Diseases of poultry. Iowa: Iowa State University Press, 1991: 87-99.

81. POULIN R, VICKERY WL. Parasite distribution and virulence: implications for parasite-mediated sexual selection. Behav Ecol Sociobiol 1993; 33: 429-436.

82. POWELL DC, AULERICH RJ, NAPOLITANO AC, STROMBORG KL, BURSIAN SJ. Incubation of double-crested cormorant eggs (*Phalacrocorax auritus*). Colonial Waterbirds 1996; 19: 256-259.

83. QUINN PJ, CARTER ME, MARKEY BK, CARTER GR. Clinical veterinary microbiology. UK: Wolfe Publishing, Mosby-Year Book Europe Ltd., 1994.

84. RAIL J-F, CHAPDELAINE G. Food of double-crested cormorants, *Phalacrocorax auritus*, in the gulf and estuary of the St. Lawrence River, Quebec, Canada. Canadian Journal of Zoology 1998; 76: 635-643.

85. RANGEL-FAUSTO MS, PITTET D, COSTIGAN M, HWANG T, DAVIS CS, WENZEL RP. The natural history of the systemic inflammatory response syndrome (SIRS). Journal of the American Medical Association 1995; 273: 117-123.

86. REDIG P. Aspergillosis. Gobbles 1983; August: 11-12.

87. RENNIE J. Living Together. Scientific American 1992; 266: Jan: 123-133.

88. REVKIN A. Cormorants flutter fishermen's feathers. The Globe and Mail 1998.

89. RICKLEFS RE. Avian postnatal development. In: Farner, King, Parkes, eds. Avian Biology, vol. vii. New York, USA: Academic Press, 1983: 1-82.

90. ROTHSCHILD M. A note on the life cycle of *Cryptocotyle lingua* (Creplin) 1825 (Tematoda.). Novitates Zoologicae 1939; 41: 178-180.

91. ROTHSCHILD M. A further note on life history experiments with *Cryptocotyle lingua* (Creplin, 1825). Journal of Parasitology 1942; 28: 91-92.

92. SAVEY M, ESPINASSE J. Identification of enzootic marasmus (cobalt deficiency) in lambs in France. Recueil de Medecine Veterinaire 1983; 159: 623-627.

93. SCHRANK CS, COOK ME, HANSEN WR. Immune response of Mallard Ducks treated with immunosuppressive agents: antibody response to erythrocytes and in vivo response to Phytohemagglutinin-P. Journal of Wildlife Diseases 1990; 26: 307-315.

94. SEKHAR CH, THRELFALL W. Infection of the cunner, *Tautogolabrus adspersus* (Walbaum), with metacercariae of *Cryptocotyle lingua* (Creplin, 1825). Journal of Helminthology 1970; 44: 189-198.

95. SHARMA JM, SCHAT KA. Natural immune functions. CRC Press, 1991.

96. SMITH HJ. *Cryptocotyle lingua* infection in a bald eagle (*Haliaeetus leucocephalus*). Journal of Wildlife Diseases 1978; 14: 163-164.

97. STOSKOPF MK Feeding piscivorous birds, a review. USA: 1986; 69 p. Proceedings of the Annual Meeting of the American Association of Zoo

Veterinarians.

98. STUNKARD HW. The life history of *Cryptocotyle lingua* (Creplin), with notes on the physiology of the metacercariae. Journal of Morphology and Physiology 1930; 50: 143-191.

99. STUNKARD HW, WILLEY CH. The development of *Cryptocotyle* (Heterophyidae) in its final host. American Journal of Tropical Medicine 1929; 9: 117-128.

100. THEODORIDES VJ. Antihelminthic drugs. In: Georgi J R, Georgi, V.J., eds. Parasitology for Veterinarians. Philadelphia: WB Saunders Co., 1990: 225-266.

101. THOMPSON CW, HILLGARTH N, LEU M, MCCLURE E. High parasite load in house finches (*Carpodacus mexicanus*) is correlated with reduced expression of a sexually selected trait. The American Naturalist 1997; 149: 270-294.

102. TIZARD I. Veterinary immunology. WB Saunders, Co., 1992.

103. TOCIDLOWSKI ME, CORNISH TE, LOOMIS MR, STOSKOPF MK. Mortality in captive wild-caught horned puffin chicks (*Fratercula coniculata*). Journal of Zoo and Wildlife Medicine 1997; 28: 298-306.

104. TOTZKE U, FENSKE M, HÜPPOP O, RAABE H, SCHACH N. The influence of fasting on blood and plasma composition of herring gulls (*Larus argentatus*). Physiological and Biochemical Zoology 1999; 72: 426-437.

105. TRUST KA, MILLER MW, RINGELMAN JK, ORME IM. Effects of ingested lead on antibody production in Mallards (*Anas platyrhynchos*). Journal of Wildlife Diseases 1990; 26: 316-322.

106. UNDERWOOD EE. Quantitative stereology. Adison-Wesley Publishing Company, Inc., 1970.

107. VEROMMA T, TOIVANEN P. Cellular cooperation in immune response and role of the MHC. In: Sharma J Meds. Avian cellular immunology. Boca Raon, USA: CRC Press, 1991: 23-34.

108. VONK HJ, WESTERN JHR. Comparative biochemistry and physiology of enzymatic digestion. UK: Academic Press, 1984.

109. WAKELIN D. Immunity to parasites. Cambridge University Press, 1996.

110. WEATHERHEAD PJ, METZ KJ, BENNETT GF, IRWIN RE. Parasite faunas, testosterone and secondary sexual traits in male Red-Winged Black Birds. Behav

Ecol Sociobiol 1993; 33: 13-23.

111. WILLEY CH, STUNKARD HW. Studies on pathology and resistance in terns and dogs infected with the heterophyid trematode, *Cryptocotyle lingua*. Transactions of the American Microscopical Society 1941; 60: 236-253.

112. WOBESER GA. Diseases of waterfowl. New York: Plenum Publishing Corporation, 1997.

113. YAMASHITA Z. Cormorant fishing on the Nagara River. Oceanus 1987; 30: 83-85.