RISK OF SPILL-OVER OF DISEASES (IN PARTICULAR AVIAN INFLUENZA) FROM WILD AQUATIC BIRDS IN NORTH QUEENSLAND

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Preface

Chapters 2-7 were prepared with the aim of being published in scientific journals. Consequently, some repetition is unavoidable. However, the reference style of the whole document follows the thesis guidelines of James Cook University. Reduction of text, inclusion of an abstract and change of referencing style will be required for submission of each chapter to a journal for publication.

Lauren Cook and Gareth Smith followed by Ai Lee Cheam contributed to Chapter 4: Processing field samples for molecular analysis. Daniel Grace along with Ai Lee Cheam optimised and developed the semi-nested Polymerase Chain Reaction technique that was applied to field samples for avian influenza viruses. Stephen Garland tested the level of inhibitors in the field samples for real time reverse transcriptase polymerase chain reactions. Graham Burgess and David Blair helped analyse avian influenza viral gene sequence data. Above all, Graham Burgess designed all primers used for molecular analysis for this chapter.

Ai Lee Cheam contributed to Chapter 5 by molecular testing for Newcastle disease viruses in field samples.

Andrew Greenhill, Robert Hedlefs, Orachun Hayakijkosol, Laurie Reilly and Ai Lee Cheam were predominantly involved with Chapter 6. Andrew initially helped process sick and dead bird tissue samples for bacteriological evaluation and also gave comments on this chapter. Robert helped in serosubtyping of *salmonella* positive samples with the collaboration of the Queensland Health Pathology Laboratory in Brisbane and also extended his editorial support on this chapter. Orachun contributed by performing post-mortems of bird carcasses followed by histopathological and bacteriological testing on tissues. Laurie provided valuable technical support for the processing of tissues for histological evaluation. Ai Lee Cheam performed molecular testing on tissue samples for avian influenza and Newcastle disease viruses.

The whole thesis was supervised by Lee Skerratt, Graham Burgess and Stephen Garland and as such had significant input in the design, execution and analysis of this research project, as well as reviewing the individual chapters of this thesis.

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I am very grateful to Ai Lee Cheam, Lauren Cook, Orachun Hayakijkosol, Gareth Smith, Desniwaty Karo-Karo, Daniel Grace and Anthony Baker who provided their support in field or lab work. This research would not have been completed without their sincere and active involvement.

I would like to give special thanks to Bob Flemming (Director, Billabong Sanctuary) along with his property manager, as well as all of the rangers for their cooperation. Bob allowed me to use his property to sample wild birds for this study. The manager and rangers occasionally helped bait our traps and collected sick and dead birds for the passive disease surveillance program.

I am very appreciative of Steve Pilla and his family for their untiring support and allowing me to sample wild birds on their property (Green Acres Lagoon, Cromarty). Steve happily baited and closed my traps when necessary for two years which was extra-ordinary and an incredible

help for me capture and then sample sufficient numbers of wild birds. I will never forget this lovely family!

I would like to give my sincere thanks to David Roshier and his team who supported sampling and providing samples of wild birds from Cape York. Samples from this site were very valuable for which we were able to check the spatial distribution of avian influenza viruses (in particular) in north Queensland. I also extend my thanks to staff members (particularly, Dan Hogarth) of Biosecurity Queensland, a service of the Department of Employment, Economic Development and Innovation who helped sample some wild birds on the Atherton Tableland. These samples were also valuable to determine the spatial distribution of AIVs in north Queensland.

David Blair, my assigned research mentor, always encouraged me through the length of my project. I received some crucial help from David in analysing avian influenza viral sequence. He also commented on my concluding chapter. Bruce Corney facilitated opportunities for me to get training on avian influenza molecular techniques at his lab in Brisbane and contributed his important comments on the sero-epidemiology chapter.

I also received necessary training on AI serology at the Tropical and Aquatic Animal Health Laboratory, Oonoonba and Animal Disease Surveillance laboratory, Toowoomba with the assistance of Elizabeth Houston (Toowoomba), Glenn Edmunds and Robert Hedlefs (Oonoonba). Robert also extended his support in commenting on my dead bird, Newcastle disease and concluding chapters.

I am very thankful to Janice Smith who supplied some of the reagents for AI serological testing, chicken eggs for culturing viruses and much needed suggestions on serological techniques all through. I would like to thank Fazlul Karim (Commonwealth Scientific and Industrial Research Organisation) who helped produce a map of north Queensland for my study. I am very grateful to Rae Wiseman for her language and editing support in the writing of my thesis.

I thank the discipline technicians and office and academic staff at the School of Veterinary and Biomedical Sciences and School of Public Health, Tropical Medicine and Rehabilitation Sciences who helped me in different ways during my study. My special thanks to the late Phil Summers who provided sincere advice for my study. He was the most responsible person I met in Australia.

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It would have been great and exciting for my late parents to see my success. My father was so inspirational and all of my achievements so far have been materialized as a result of his love and support. Unfortunately, he died in April 2010 while I was studying in Australia. Many thanks to my other family members, relatives, friends and well wishers scattered in different parts of the world, who blessed and supported my life choices and managed to be close to me even from far away.

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Abstract

Disease surveillance programs and longitudinal studies are uncommon in wild bird populations across the world. But many wild bird species are important sources of pathogens that are of particular importance to animal and human health, for example, avian influenza viruses.

This project included both active and passive surveillance in order to study the diseases and pathogens (in particular avian influenza) in wild aquatic birds of north Queensland. A three-year longitudinal study was conducted on wild aquatic birds at Billabong Sanctuary from April 2007 to March 2010 while a two-year longitudinal study was performed at Green Acres Lagoon (Cromarty), from December 2007 to 2009. Cross sectional studies were also performed on wild aquatic birds at Cape York and on the Atherton Tableland between 2007 and 2009.

The objective of this project was to determine the level of avian influenza and Newcastle disease viral RNA and avian influenza viral antibody, identify the associated potential risk factors and determine the distribution of avian influenza and Newcastle disease viral subtypes and their phylogenetic relationship with other isolates in Australia and overseas. This study also aimed to identify causes of mortality in wild aquatic birds of north Queensland and explore the connection between mortality in birds and avian influenza.

Birds were sampled quarterly at Billabong Sanctuary and Cromarty and sporadically on Cape York and the Atherton Tableland. Birds were captured mostly using funnel traps. A total of 1,555 live birds were captured and this resulted in the collection of 1,522 serum samples, 1,458 cloacal and 1,368 oropharyngeal swab samples. Tissue samples were obtained from 42 sick and dead birds and 1,157 fresh faecal samples of wild aquatic birds were collected from the environment surrounding water bodies. Samples were evaluated by serological, molecular, bacteriological and histopathological examinations where necessary.

Overall avian influenza viral RNA prevalence was ~1.0% in the samples of wild aquatic birds in north Queensland, whereas the avian influenza viral antibody prevalence was 11 times higher. These findings make biological sense given the fact that avian influenza viral shedding periods are relatively shorter than the presence of avian influenza viral antibodies in the blood.

Multivariate regression analysis was performed to identify potential risk factors for avian influenza antibody levels in wild aquatic birds. The odds ratio of being reactive for avian influenza antibodies was 13.1 (95% Confidence interval 5.9-28.9) for Pacific black ducks (53.7%) compared with plumed whistling ducks (10.1%) (Table 3.12; Chapter 3). This result was also supported by the linear regression analysis (Chapter 3; Table 3.11). An identical species pattern was identified in an unadjusted statistical analysis on the viral RNA data of avian influenza (Chapter 4; Table 4.7).

The odds ratios of being reactive for avian influenza antibodies were 2.9 (95% Confidence interval 1.3-6.6) for adult over ≤ sub-adult ducks (Table 3.10; Chapter 3). A similar age pattern was identified in the linear regression analysis (Table 3.9; Chapter 3). This age pattern might be due to more exposure to infections because of more opportunity in addition to longer lasting avian influenza antibodies in older ducks. A different age pattern was, however, identified in unadjusted analysis using the molecular data (Chapter 3; Table 3.11). This analysis indicated that immature birds were more commonly infected which may be due to the fact that they have more frequent infections because they are immunologically naïve whereas adults are more resistant, particularly to viruses to which they may have previously been exposed.

Avian influenza antibodies were at higher levels during warm wet weather (January-April) compared with warm dry weather (September-December) in linear regression analysis (Coefficient 8.3; 95% Confidence interval 3.0-13.6) (Chapter 3; Table 3.7). The warm wet season might reduce the immune status of birds, thus making them more vulnerable to infection which may in turn increase the levels of avian influenza antibodies.

The surveillance programs demonstrated the presence of low pathogenic avian influenza viral subtypes H6 and H9 in samples collected from wild aquatic birds. One of the H6 viruses was likely to have been newly introduced, probably through migratory species of birds such as the sharptailed sandpiper. This migratory bird regularly travels between Australia and Asia. Hence, there is a possibility of highly pathogenic avian influenza exotic viral subtypes such as H5N1 being introduced into Australia. The second H6 virus had a matrix gene similar to those found associated with Australian H7 subtypes. This would suggest an earlier introduction of a H6 subtype which had an opportunity to reassort with local viruses. The low pathogenic avian influenza viral subtype H9 had a matrix gene similar to that found in Asian H9 viruses. Some H9 viruses have been shown to cause mortality in poultry elsewhere in the world. This subtype has also been isolated from pigs and humans in different countries, which indicates its pandemic potential.

At the time that the H6 and H9 subtypes were detected in samples collected from wild birds in north Queensland the serological study demonstrated periods of infection with H6 and H9 serotypes.

The serological study also demonstrated a constant circulation of H5 and sporadic circulation of H7 subtypes in wild aquatic birds. These viruses are perhaps non-pathogenic as evident in other studies elsewhere in Australia. However, these low pathogenic avian influenza viral subtypes have potential to mutate to virulent types once introduced into commercial poultry.

Overall Newcastle disease viral RNA prevalence was 3.5% at the individual bird level which indicates the presence of Newcastle disease viruses in wild bird populations in north Queensland. The prevalence was significantly higher in plumed whistling ducks. Avirulent Newcastle disease viruses (class-one and class-two Australian type) were identified in samples collected from wild aquatic birds. This indicates that wild birds remain a reservoir of paramyxoviruses that could be transmitted to domestic poultry.

A logistic regression model was performed to identify potential risk factors for the level of Newcastle disease viral RNA prevalence in plumed whistling ducks. The odds of reactor samples were 2.7 (95% Confidence interval 1.5-4.9) times more likely in younger than older ducks (Chapter 5; Table 5.5). A similar age pattern of prevalence was observed in the study of avian influenza. This age susceptibility to infection may be due to the fact that young birds are immunologically naïve.

Only univariate logistic analysis indicated birds caught in the warm wet season (January-April) as being significantly associated with a higher prevalence of Newcastle disease viral RNA. This result virtually correlates with an increase in the numbers of immature birds at that time associated with the breeding season of adult birds.

The above identified risk factors will significantly contribute to the design of a targeted avian influenza and Newcastle disease surveillance program in wild aquatic birds in northern Australia by wildlife authorities.

Morbidity and mortality were sporadic and more commonly observed in chicks and juvenile birds in April than other months of the year. Identified bacterial diseases that could be attributable to causing bird mortality were colibacillosis, pasteurellosis and salmonellosis. The investigation identified *Salmonella enterica serotype virchow* and *Salmonella enterica serotype hvittingfoss* from dead bird samples of an Australian white ibis and two plumed

whistling ducks, respectively. These serotypes have been identified as causing disease in Australians and are therefore relevant to public health. No avian influenza viral RNA was detected from any sick or dead birds by the molecular screening assay. There is an opportunity for establishing a long term passive disease surveillance programme for wild aquatic birds in north Queensland.

The project developed a reliable screening assay “competitive enzyme linked immunosorbent assay” (designated as James Cook University-2) and this assay was used to detect avian influenza viral antibodies from serum samples of wild aquatic birds. A semi-nested PCR approach was designed and applied on direct field reactor samples for amplification and sequencing of different avian influenza viral genes (matrix, haemagglutinin and non-structural protein).

Overall findings therefore suggest that there is an opportunity for establishing a long term active and passive surveillance program for monitoring pathogens and diseases of wild aquatic birds in north Queensland, an important region in Australian biosecurity. This would provide valuable information for risk assessment and mitigation and potentially have a significant benefit for public health and the economy for the region and the nation.

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Journal articles

Hoque, M.A., Skerratt, L.F., Rahman, M.A., Rabiul Alam Beg, A.B.M. and Debnath, N.C. (2010) Factors limiting traditional household duck production in Bangladesh. *Trop Anim Health Prod* **42**: 1579-1587

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Hoque, M.A., Skerratt, L.F. and Burgess, G.W. (2007) Epidemiological investigation of avian influenza in waterfowl in northern Queensland. In: The Proc of the AB-CRC Annual National Workshop, Novotel St Kilda, Melbourne, 14-16 November, 2007

Hoque, M.A., Skerratt, L.F., Smith, G. and Burgess, G.W. (2008) A longitudinal study of avian influenza of aquatic birds in northern Queensland. In: The Proc of the AB-CRC Annual National Workshop, Siam City Hotel, Bangkok, 24-26 June, 2008

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Cheam, A.L., Hoque, M.A., Grace, D., Karo-Karo, D., Skerratt, L.F., Garland, S., Baker, A and Burgess, G.W. (2009) Detection and characterization of influenza and Newcastle disease viral RNAs in samples collected from aquatic birds in north Queensland. In: The Proc of the Australian Association of Veterinary Laboratory Diagnosticians 2009 Annual Meeting, Country Club Tasmania – Launceston, Tasmania, 2009

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Other presentations

Hoque, M.A. (2007) Epidemiological investigation of avian influenza in waterfowl in northern Queensland. Presented within the School of Veterinary and Biomedical Sciences, James Cook University, Townsville, August 2007. PhD Confirmation Seminar

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Abbreviations	Elaboration
°C	Degree celsius
AAHL	Australian Animal Health Laboratory
ABI	Applied biosystems
ABTS	2, 2'-Azino-bis: 3-benzthiazoline-6-sulphonic Acid
AF	Allantoic fluid
AGID	Agar gel immunodiffusion
AI	Avian influenza
AIV	Avian influenza virus
ANOVA	Analysis of variance
ASD	Australian shelduck
AWI	Australian white ibis
BHQ	Black hole quencher
BLAST	Basic local alignment search tool
bp	Base pairs
BS	Black swan
BSC	Bush stone curlew
BT	Back titration
BVD	Bovine viral diarrhoea
CC	Cell control
cDNA	Complementary deoxyribonucleic acid
cELISA	Competitive enzyme-linked immunosorbent assay
CI	Confidence interval
CR	Crow
CRBC	Chicken red blood cell
C_T	Threshold
DEEDI	Department of Employment, Economic Development and Innovation
DG	Domestic goose
DM	Dusky moorhen
DMEM	Dulbecco's modified eagle's transport medium
DNA	Deoxyribonucleic acid

dNTP	Deoxynucleotidetriphosphate
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
F	Fusion
FAM	5 (6)-Carboxyfluorescein
GPG	Green pygmy goose
GT	Grey teal
H	Haemagglutinin
HA	Haemagglutination
HAU	Haemagglutination units
HD	Hardhead
H and E	Haematoxylin and Eosin
HI	Haemagglutination inhibition
HIT	Haemagglutination inhibition titre
HPAI	Highly pathogenic avian influenza
HRP	Horseradish peroxidase
iELISAs	Indirect enzyme-linked immunosorbent assays
IgG	Immunoglobulin
IPC	Internal positive control
g	Gravity
GBR	Great Barrier Reef
JCU	James Cook University
Kg	Kilogram
LP	Low pathogenic
LPAI	Low pathogenic avian influenza
LRT	Likelihood ratio test
M	Matrix
MAb	Monoclonal antibody
Max	Maximum
MB	Mutton bird
MD	Muscovy duck
MDPI	Mean difference percent inhibition
MEGA	Molecular evolutionary genetic analysis
MG	Magpie goose
Min	Minimum
ml	Millilitre

MS	Microsoft
MSF	Multiple sequences file
N	Neuraminidase
ND	Newcastle disease
NDV	Newcastle disease virus
mM	Micro molar
nm	Nanometre
NP	Nucleoprotein
nQLD	North Queensland
NS	Negative serum control
NSP	Non-structural protein
NSW	New South Wales
OD	Optical density
OR	Odds ratio
PBD	Pacific black duck
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PIG	Pigeon
PM	Post-mortem
PNG	Papua New Guinea
PWD	Plumed whistling duck
RNA	Ribonucleic acid
RNS	Red-necked stint
ROC	Receiver operating characteristic
RP	Rutland Plains
RPM	Rotation per minute
rRT-PCR	Real time reverse transcriptase-polymerase chain reaction
RSD	Radjah shelduck
RT	Reverse transcription
QLD	Queensland
S	Serum
SD	Serial dilution
Se	Standard error
STS	Sharp-tailed sandpiper

TAE	Tris base, acetic acid and ethylene diamine tetra-acetate
TAS	Tasmania
TMB	3, 3’ 5, 5’-Tetramethylbenzidine
TS	Test serum
UK	United Kingdom
USA	United States of America
VIC	Victoria
VIF	Variance inflation factors
WA	Western Australia
WAB	Wild aquatic bird
WFH	White faced heron
WHO	World Health Organization
WWD	Wandering whistling duck
XLD	Xylose lysine deoxycholate
µl	Micro litre
µM	Micro molar

Chapter 1: General introduction

1.1. Introduction

Many wild bird species are potential reservoir sources of pathogens that are of particular importance to human or animal health such as avian influenza viruses (AIVs), Newcastle disease viruses (NDVs), West Nile Virus, Kunjin virus (and other flaviviruses) and *Salmonella* spp. Each of these pathogens with the exception of NDVs has significant zoonotic potential and has caused illness and deaths in humans (Stallknecht and Shane, 1988; Mackenzie *et al.*, 1995; Alexander, 2000a; Craven *et al.*, 2000; Delogu *et al.*, 2003; Abulreesh *et al.*, 2007).

1.1.1. Avian influenza

Avian influenza (AI) is a disease caused by influenza A type viruses belonging to the family *Orthomyxoviridae*. Wild birds belonging to Anseriformes (such as ducks, geese and swans) and Charadriiformes (such as gulls, terns and shorebirds) are thought to constitute the major natural reservoir for AIVs (Webster *et al.*, 1992; Olsen *et al.*, 2006; Brown *et al.*, 2007; Munster *et al.*, 2007). These wild aquatic birds (WABs) harbour all known subtypes (haemagglutinin: H 1-16 and neuraminidase: N 1-9) of the AIVs (Krauss *et al.*, 2004; Fouchier *et al.*, 2005; Munster *et al.*, 2007).

Some subtypes of H5 and H7 cause highly pathogenic avian influenza (HPAI), with mortality rates as high as 100%, and a drastic decline in poultry production (Alexander, 2000b).

All other viruses (14 H subtypes) cause low pathogenic avian influenza (LPAI) which is milder and is predominantly a respiratory disease (Alexander, 2000b). Some of the LPAI subtypes have jumped permanently into other species and caused mortality. They include H1N1 in humans (Vaillant *et al.*, 2009), H1N1 in swine (Forgie *et al.*, 2011) and H3N8 in horses (Cowled *et al.*, 2009; Virmani *et al.*, 2010; Wei *et al.*, 2010).

1.1.1.1. Avian influenza viral subtype: Haemagglutinin-5

Recently HPAI H5N1 has caused disease outbreaks in domestic poultry across the world (in particular, Asian countries). By September 2010 HPAI H5N1 had spread to 63 countries in Asia, Europe and Africa. Between 2003 and 2010 the human death toll due to the H5N1 reached 299 (N=505 infected cases) (Anon, 2010b). Of the confirmed human cases and deaths,

Indonesia, a close neighbour of Australia, had the highest number of 168 and 139, respectively (Figure 1.1) (Anon, 2010b).

One of the most important points in terms of immediate zoonotic risk from AIVs is that many of those people who died of HPAI H5N1 had substantial direct contact with infected birds and also indirect contact with the H5N1 contaminated water sources (such cases were observed in Indonesia) (Jonathan Bell, personal communication). In addition there is no evidence that this strain of influenza can spread directly from human to human (Martin *et al.*, 2006). There is worldwide concern that the H5N1 virus may evolve the capacity for human-to-human transmission and may be responsible for a pandemic (Alexander, 2007) which highlights the need to understand the epidemiology of AIVs in WABs.

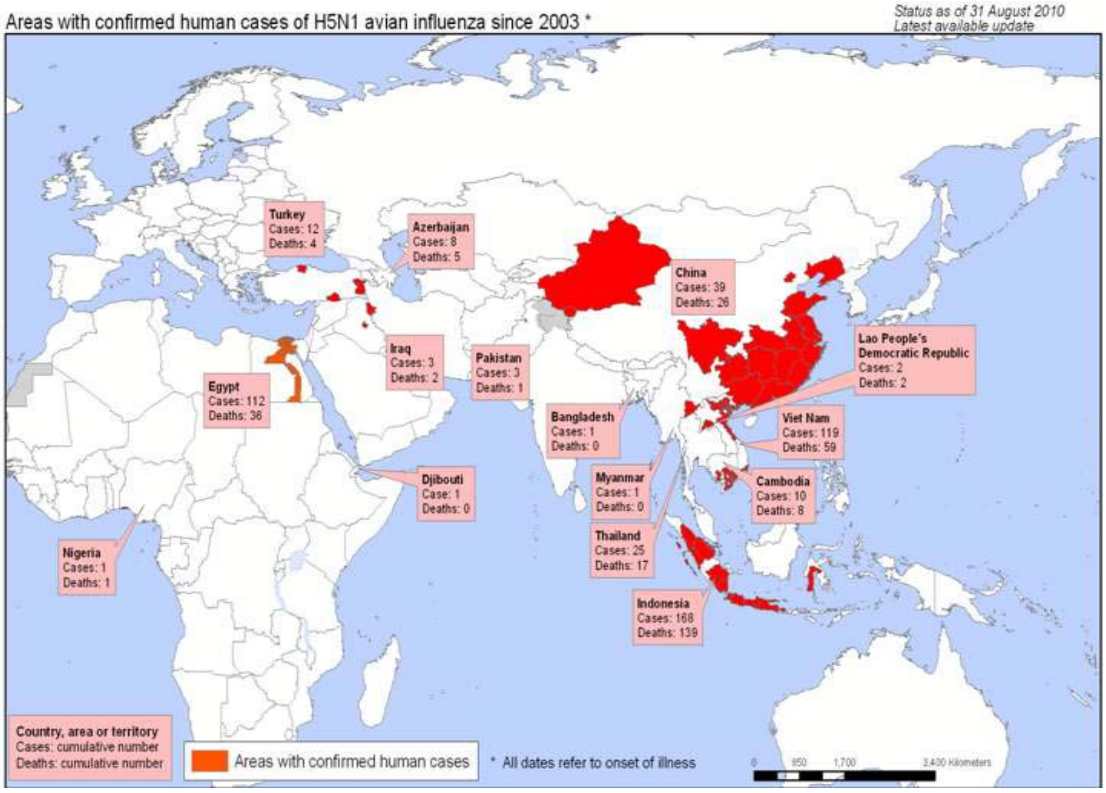

Figure 1.1 Countries reporting confirmed H5N1 human cases and fatalities from 2003 to 2010 (Anon, 2010b)

The first outbreak of the HPAI H5 subtype (H5N3) was recorded in wild birds in South Africa in 1961 when approximately 1,600 common terns (*Sterna hirundu*) died (Alexander, 2000b). After 40 years, the HPAI H5N1subtype was recovered from wild birds in Hong Kong in 2002 (Liu *et al.*, 2005). The H5N1outbreak in wild migratory birds on Qinghai Lake in western China in May 2005 posed serious concerns worldwide because Qinghai Lake is a major breeding site for migratory birds whose flyways extend to south-east Asia, India, Siberia, Australia and

New Zealand (Chen *et al.*, 2005; Lei *et al.*, 2007; Wang *et al.*, 2008). Since 2005, more HPAI H5N1 outbreaks have occurred in wild birds of many different countries (Figure 1.2). In Germany, an HPAI H5N1 outbreak occurred in wild birds in 2006 and again in 2007 (Globig *et al.*, 2009) and these outbreaks caused the deaths of mute (*Cygnus olor*) and whooper swans (*Cygnus cygnus*). The H5N1 subtype has also been isolated from wild birds on Uvs-Nuur Lake in western Siberia and Russia (June 2006) (Sharshov *et al.*, 2010); in wild migratory birds in Korea (2006) (Lee *et al.*, 2008) and in Mongolia (June 2009) (Sharshov *et al.*, 2010); wild birds in Indonesia (Capua and Alexander, 2004; Stoops *et al.*, 2009) and west Papua on the island of New Guinea (part of Indonesia), which is close to Australia (McCallum *et al.*, 2008). Although Australia has not had any HPAI H5N1 outbreaks recorded in wild or domestic birds, there is the potential for the introduction and transmission to humans or birds through wild migratory birds using established migratory routes such as those that transit north Queensland (nQLD) on their way south from New Guinea and south-east Asia. There is on-going debate whether severely affected birds with HPAI H5N1 are capable of continuing migration and spreading viruses.

However, there is evidence that wild migratory birds infected with HPAI H5N1 are capable of disseminating the virus over long distances (Chen *et al.*, 2005) (please see below for more details under Spread and Transmission). This finding is supported by experimental evidence that some wild bird species infected with the H5N1 survived and shed the virus without showing clinical signs (Sturm-Ramirez *et al.*, 2004). It is therefore important to conduct surveillance of migratory and resident aquatic birds in nQLD in order to determine whether nQLD is free from H5N1. It should assess the risk of introduction and transmission of H5N1 and for early detection of any introduction and transmission in order to provide early warning of the increased risk to commercial poultry owners and public health officials.

Both active and passive surveillance programs for AI can play an important role in the development of programs to detect and respond to the introduction of H5N1. One of the aims of this study is to assess the feasibility of conducting the required field surveillance. Highly pathogenic avian influenza H5N1, in particular, has caused mortality events in wild birds and has been detected by passive surveillance in other countries (Komar and Olsen, 2008; Globig *et al.*, 2009; Hesterberg *et al.*, 2009; Willeberg *et al.*, 2010).

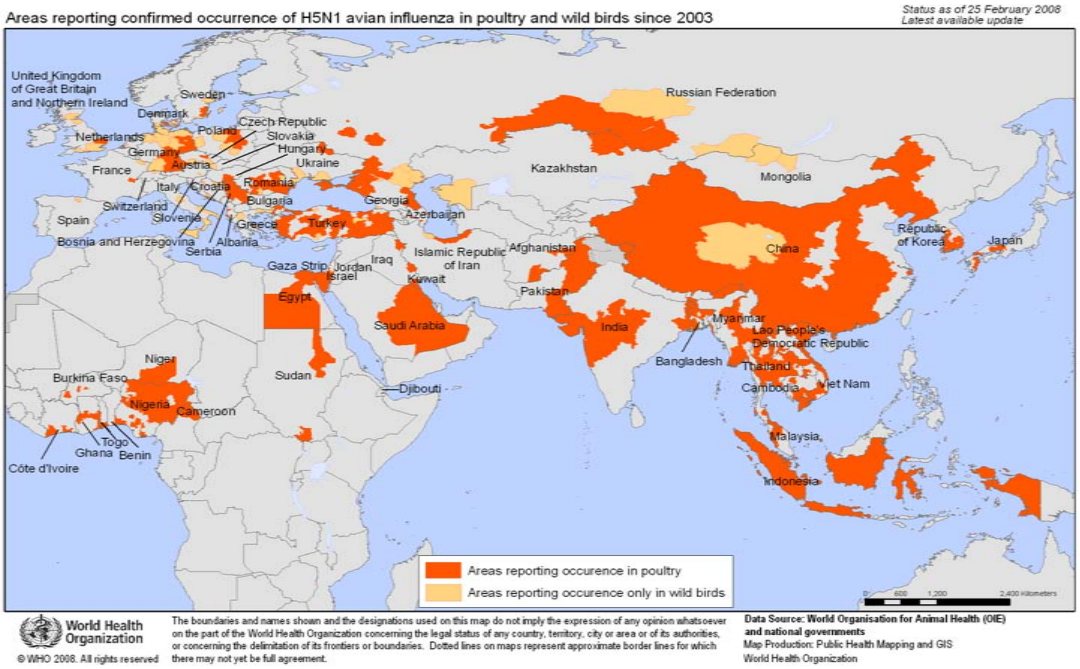

Figure 1.2 Countries reporting confirmed H5N1 in wild and domestic birds from 2003 to 2008

Low pathogenic H5 subtypes have been confirmed in Australian wild birds such as H5N2 in the wedge-tailed shearwater (Downie *et al.*, 1977), H5N2 in the Pacific black duck (PBD), H5N7 in the red-necked stint (RNS) and H5N3 in the Australian shoveler (Haynes *et al.*, 2009) (Table 1.1). These LPAI H5 subtypes could pose a serious threat if introduced to Australian commercial poultry and then allowed to transform into HPAI H5. In one instance, LPAI H5 was identified in the turkey population in Italy during the spring of 1999 and only nine months later it became HPAI H5 (Anon, 2006a). Another aim of this study is to assess the feasibility of conducting surveillance for endemic viruses such as H5 and H7 (please see below) in WABs in nQLD that pose a risk to poultry.

1.1.1.2. Avian influenza viral subtype: Haemagglutinin-7

Australia has had five outbreaks of AI in commercial chickens (Table 1.2). All those outbreaks have been caused by HPAI H7 subtypes, three of which occurred in Victoria (VIC) (1976, 1985 and 1992), one in Queensland (QLD) (1994) and one in New South Wales (NSW) (1997) (Selleck *et al.*, 2003; Westbury, 2003). These H7 subtypes are phylogenetically and antigenically similar and have developed a distinct Australian lineage as compared with overseas H7 isolates (Figure 1.3). Importantly, Australian H7 viruses not only retained the H gene but they also retained all the other seven genes (Bulach *et al.*, 2010) which is also supportive of a much conserved Australian lineage. The fact that there has been no evidence for introduction of H7 into Australia is rather interesting. It is therefore indicating that this virus is circulating within Australian resident wild birds.

Economically, the largest outbreak occurred in Tamworth, NSW in 1997, and led to the death or destruction of over 310,000 birds and 1.2 million fertile eggs on six farms (Selleck *et al.*, 2003). Speculation about the source of the virus in each of the outbreaks has centred on wild birds. The reason for this suspicion is that almost all LPAI subtypes in Australia have been found in Australian wild birds (Table 1.1) (Downie and Laver, 1973; Downie *et al.*, 1977; Mackenzie *et al.*, 1984; Mackenzie *et al.*, 1985; Nestorowicz *et al.*, 1987; Peroulis and O'Riley, 2004; Hurt *et al.*, 2006; Haynes *et al.*, 2009; Hansbro *et al.*, 2010). However, it may raise a question of why only the H7 would transmit to Australian chickens. It possibly means that these viruses have a cleavage site of amino acid sequence that only needs minimal change to become virulent after introduction to chickens and hence readily detectable. The New Zealand experience also suggests that these viruses are constantly being transmitted to domestic birds (Rawdon *et al.*, 2010). Viruses of low virulence could be readily transmitted and then eliminated in an all in, all out management system and therefore not easily detected.

In addition, a precursor virus (IAU20463; Figure 1.3) of Australian HPAI H7 outbreaks was identified in a domestic duck in 1975-76. This evidence suggested that the LPAI H7 subtype might have been circulating in wild birds before introduction to domestic ducks and then into commercial chickens. Moreover, the recently identified LPAI H7 isolates in wild ducks were found to be antigenically similar to the previous HPAI H7 subtype (Figure 1.3) which caused outbreaks in Australian poultry (Bulach *et al.*, 2010). Therefore, the current H7 subtype in wild birds is considered a potential source for the next HPAI H7 outbreak if introduced to resident commercial poultry. Avian influenza surveillance of wild birds is therefore of the utmost importance in determining the avian influenza viral (AIV) subtypes, with their genetic characteristics, which are circulating in WABs in Australia.

Table 1.1 Low pathogenic avian influenza virus isolates in Australian wild birds (1971-2007)

Isolate details	Scientific name of birds	Study site	References
Wedge-tailed shearwater/71/H6N5	*Mutton bird (MB), Puffinus pacificus chlororhynchus*	Tyron Island, Great Barrier Reef (GBR), QLD	(Downie and Laver, 1973)
Wedge-tailed shearwater/71/H6N2 and H6N1	*MB, Puffinus pacificus chlororhynchus*	Tyron Island, GBR, QLD	(Downie *et al.*, 1973)
Wedge-tailed shearwater/72/H6N5	*MD, Puffinus pacificus chlororhynchus*	Tyron Island, GBR, QLD	(Downie *et al.*, 1977)
Wedge-tailed shearwater/75/H5N2 and H3N6	*MD, Puffinus pacificus chlororhynchus*	Tyron Island, GBR, QLD	(Downie *et al.*, 1977)
Wedge-tailed shearwater/77-79/H3N8	*MD, Puffinus pacificus chlororhynchus*	Pelsart Island, WA	(Mackenzie *et al.*, 1984; Mackenzie *et al.*, 1985)
Wedge-tailed shearwater/77-79/H7N6	*MD, Puffinus pacificus chlororhynchus*	Pelsart, WA	(Mackenzie *et al.*, 1985)
Pacific black duck/77-79/H3N8 and H6N4	*Anas superciliosa*	Perth, WA	(Mackenzie *et al.*, 1984; Mackenzie *et al.*, 1985)
Pacific black duck/77-79/H6N5	*Anas superciliosa*	Perth, WA	(Mackenzie *et al.*, 1985)
Pacific black duck/01-02/H3N2	*Anas superciliosa*	Northern irrigation, VIC	(Peroulis and O'Riley, 2004)
Pacific black duck/06/H1, H1N9, H4, H4N6, H5N2 and H11	*Anas superciliosa*	Orange, NSW	(Haynes *et al.*, 2009)
Pacific black duck/06/H3	*Anas superciliosa*	Caroona, NSW	(Haynes *et al.*, 2009)
Pacific black duck/06/H4N6	*Anas superciliosa*	Barilla Bay, TAS	(Haynes *et al.*, 2009)
Pacific black duck/06/H5	*Anas superciliosa*	Herdsman Lake, WA	(Haynes *et al.*, 2009)
Pacific black duck/07/H5, H8 and H9	*Anas superciliosa*	Orange, NSW	(Haynes *et al.*, 2009)
Pacific black duck/06/H6N8, H8 and H12	*Anas superciliosa*	Jerilderie, NSW	(Haynes *et al.*, 2009)
Pacific black duck/07/H7N2	*Anas superciliosa*	Tea Tree, TAS	(Haynes *et al.*, 2009)
Mallard hybrid/06/H3N8	*A. superciliosa x platyrhynchos*	Glenorchy, TAS	(Haynes *et al.*, 2009)
Australian shelduck/77-79/H1N9	*Tadorna tadornoides*	Rottnest Island, WA	(Mackenzie *et al.*, 1984; Mackenzie *et al.*, 1985)
Australian shelduck/77-79/H7N2	*Tadorna tadornoides*	Rottnest Island, WA	(Mackenzie *et al.*, 1985)
Australian shelduck/77-79/H7N9	*Tadorna tadornoides*	Rottnest Island, WA	(Mackenzie *et al.*, 1985)
Eurasian coot/77-79/H6N2	*Fulica atra*	Perth, WA	(Mackenzie *et al.*, 1984; Mackenzie *et al.*, 1985)
Grey teal/77-79/H4N4	*Anas gibberifrons*	Moora, WA	(Mackenzie *et al.*, 1984; Mackenzie *et al.*, 1985)
Grey teal/77-79/H4N6	*Anas gibberifrons*	Moora, WA	(Mackenzie *et al.*, 1985)
Grey teal/77-79/H6N4	*Anas gibberifrons*	Moora, WA	(Mackenzie *et al.*, 1985)
Teal/01-02/H3N2	*Anas gibberifrons*	Northern irrigation, VIC	(Peroulis and O'Riley, 2004)
Grey teal/06/H7N6	*Anas gibberifrons*	Werribee Estuary, VIC	(Haynes *et al.*, 2009)
Grey Teal/06/H3	*Anas gracilis*	Morundah, NSW	(Haynes *et al.*, 2009)
Grey Teal/06/H3	*Anas gracilis*	Tocumwal, NSW	(Haynes *et al.*, 2009)
Grey Teal/05/H3	*Anas gracilis*	Tocumwal, NSW	(Haynes *et al.*, 2009)
Grey Teal/06/H7 and H8	*Anas gracilis*	Jerilderie, NSW	(Haynes *et al.*, 2009)
Grey Teal/06/H7N6	*Anas gracilis*	Werribee Estuary, VIC	(Haynes *et al.*, 2009)
Grey Teal/07/H8	*Anas gracilis*	Orange, NSW	(Haynes *et al.*, 2009)
Grey Teal/06/H8	*Anas gracilis*	Inverell, NSW	(Haynes *et al.*, 2009)
Grey Teal/06/H11N9	*Anas gracilis*	Waterhouse Lake, TAS	(Haynes *et al.*, 2009)
Chestnut Teal/06/H3	*Anas castanea*	Gippsland region: McCloud's Morass, VIC	(Haynes *et al.*, 2009)
Chestnut Teal/06/H3 and H12	*Anas castanea*	Gippsland region: Lake Watt, Victoria	(Haynes *et al.*, 2009)
Pink-eared duck/teal/07/H4N6	*M.membranaceus/Anas spp.*	Werribee Sanctuary, VIC	(Haynes *et al.*, 2009)
Red-necked stint/77-79/H3N8	*Calidris ruficollis*	Perth, Bunbury, WA	(Mackenzie *et al.*, 1985)
Red-necked stint/77-79/H4N8	*Calidris ruficollis*	Perth, WA	(Mackenzie *et al.*, 1985)
Red-necked stint/77-79/H12N9	*Calidris ruficollis*	Broome, WA	(Mackenzie *et al.*, 1985)
Red-necked stint/04/H4N8	*Calidris ruficollis*	Newcastle, VIC	(Hurt *et al.*, 2006)
Red-necked stint/04/H4N8	*Calidris ruficollis*	Fullerton Cove, VIC	(Hurt *et al.*, 2006)
Red-necked stint/04/H5N7	*Calidris ruficollis*	Box Beach, VIC	(Haynes *et al.*, 2009)
Red-necked stint/05/H6	*Calidris ruficollis*	Port Phillip region, VIC	(Haynes *et al.*, 2009)
Sharp-tailed sandpiper/04/H11N9	*Calidris acuminata*	Fullerton Cove, VIC	(Hurt *et al.*, 2006)
Lesser noddy/77-79/H7N2	*Anous tennirostris*	Pelsart, WA	(Mackenzie *et al.*, 1985)
Sooty tern/77-79/H7N6	*Sterna fuscata*	Pelsart, WA	(Mackenzie *et al.*, 1985)
Starling/85/H7N7 (**HPAI**)	*Sturnus vulgaris*	Bendigo, VIC	(Nestorowicz *et al.*, 1987; Westbury, 2003)
Wood duck/01-02/H3N2	*Chenonetta jubata*	Northern irrigation, VIC	(Peroulis and O'Riley, 2004)
Wood duck/06/H1	*Chenonetta jubata*	Orange, NSW	(Haynes *et al.*, 2009)
Australian shoveler/06/H5N3	*Anas rhynchotis*	Gippsland region: McCloud's Morass, VIC	(Haynes *et al.*, 2009)
Silver gull (chick)/06/H13N6	*Larus novaehollandiae*	Hobart, TAS	(Haynes *et al.*, 2009)

QLD: Queensland, *TAS*: Tasmania; *VIC*: Victoria; *WA*: Western Australia; *NSW*: New South Wales; *HPAI*: Highly Pathogenic Avian Influenza

Table 1.2 Avian influenza (subtype haemagglutinin-7) in domestic and wild birds in Australia and New Zealand (1976-2007)

Isolate details	GenBank Entries	Pathogenicity	Study site	References
Domestic chicken/75/H7N7	Z47199	Pathogenic	VIC	(Perdue *et al.*, 1995)
Domestic duck/76/H7	IAU20463	Pathogenic	VIC	(Rohm *et al.*, 1995)
Domestic chicken/76/H7N7	CY024786-CY024793 (eight genes)	Pathogenic	Keysborough, VIC	(Turner, 1976; Alexander *et al.*, 1978; Westbury *et al.*, 1979; Westbury, 1989; Bashiruddin *et al.*, 1992; Bulach *et al.*, 2010)
Domestic duck/76/H7N7	CY061602-CY061609 (eight genes)	Non-pathogenic	Keysborough, VIC	(Turner, 1976; Alexander *et al.*, 1978; Westbury *et al.*, 1979; Westbury, 1989; Bashiruddin *et al.*, 1992; Bulach *et al.*, 2010)
Starling/85/H7N7	CY024778-CY024785 (eight genes)	Pathogenic	Bendigo, VIC	(Barr *et al.*, 1986; Forman *et al.*, 1986; Nestorowicz *et al.*, 1987; Westbury, 1989; Morgan and Kelly, 1990; Bulach *et al.*, 2010)
Starling/85/H7N7	FLAHAASV	Pathogenic	VIC	(Nestorowicz *et al.*, 1987)
Domestic chicken/85/H7N7	CY025069-CY025076 (eight genes)	Pathogenic	Bendigo, VIC	(Barr *et al.*, 1986; Forman *et al.*, 1986; Nestorowicz *et al.*, 1987; Westbury, 1989; Morgan and Kelly, 1990; Bulach *et al.*, 2010)
Domestic chicken/85/H7N7	FLAHAACV	Pathogenic	VIC	(Nestorowicz *et al.*, 1987)
Domestic chicken/92/H7N3	CY025077-CY025084 (eight genes)	Pathogenic	Bendigo, VIC	(Selleck *et al.*, 2003; Heine *et al.*, 2007; Bulach *et al.*, 2010)
Domestic chicken/92/H7N3	GU053079	Pathogenic	VIC	Only published in GenBank
Domestic chicken/92/H7N3	AF20227	Pathogenic	VIC	(Banks *et al.*, 2000)
Domestic chicken/92/H7N3	CY025077	Pathogenic	VIC	Only published in GenBank
Domestic chicken/94/H7N3	CY022685-CY022692 (eight genes)	Pathogenic	Brisbane, QLD	(Westbury, 2003)
Domestic chicken/95/H7N3	AF202231	Pathogenic	QLD	(Banks *et al.*, 2000)
Domestic chicken/95/H7N3	GU053072	Pathogenic	QLD	Only published in GenBank
Domestic chicken/97/H7N4	CY022693-CY022700 (eight genes)	Pathogenic	Tamworth, NSW	(Selleck *et al.*, 2003; Bulach *et al.*, 2010)
Domestic chicken/97/H7N4	CY022701-CY022708 (eight genes)	Pathogenic	Tamworth, NSW	(Selleck *et al.*, 2003)
Domestic emu/97/H7N4	CY022709-CY022716 (eight genes)	Pathogenic	Tamworth, NSW	(Selleck *et al.*, 2003)
Domestic emu/ /97/H7N4	GU053102	Pathogenic	NSW	Only published in GenBank
Domestic chicken/97/H7N4	AY943924	Pathogenic	NSW	Only published in GenBank
Domestic chicken/97/H7N4	GU053094	Pathogenic	NSW	Only published in GenBank
Mallard/05/H7N7	CY061618-CY061625 (eight genes)	Non-pathogenic	Piako River, NZ	(Bulach *et al.*, 2010)
Grey teal/07/H7N6	CY061610-CY061617 (eight genes)	Non-pathogenic	VIC	(Haynes *et al.*, 2009; Bulach *et al.*, 2010)
Wild duck/07/H7N2	CY033161-CY033168 (eight genes)	Non-pathogenic	TAS	(Haynes *et al.*, 2009; Bulach *et al.*, 2010)

VIC: Victoria; *QLD*: Queensland; *NSW*: New South Wales; *TAS*: Tasmania; *NZ*: New Zealand

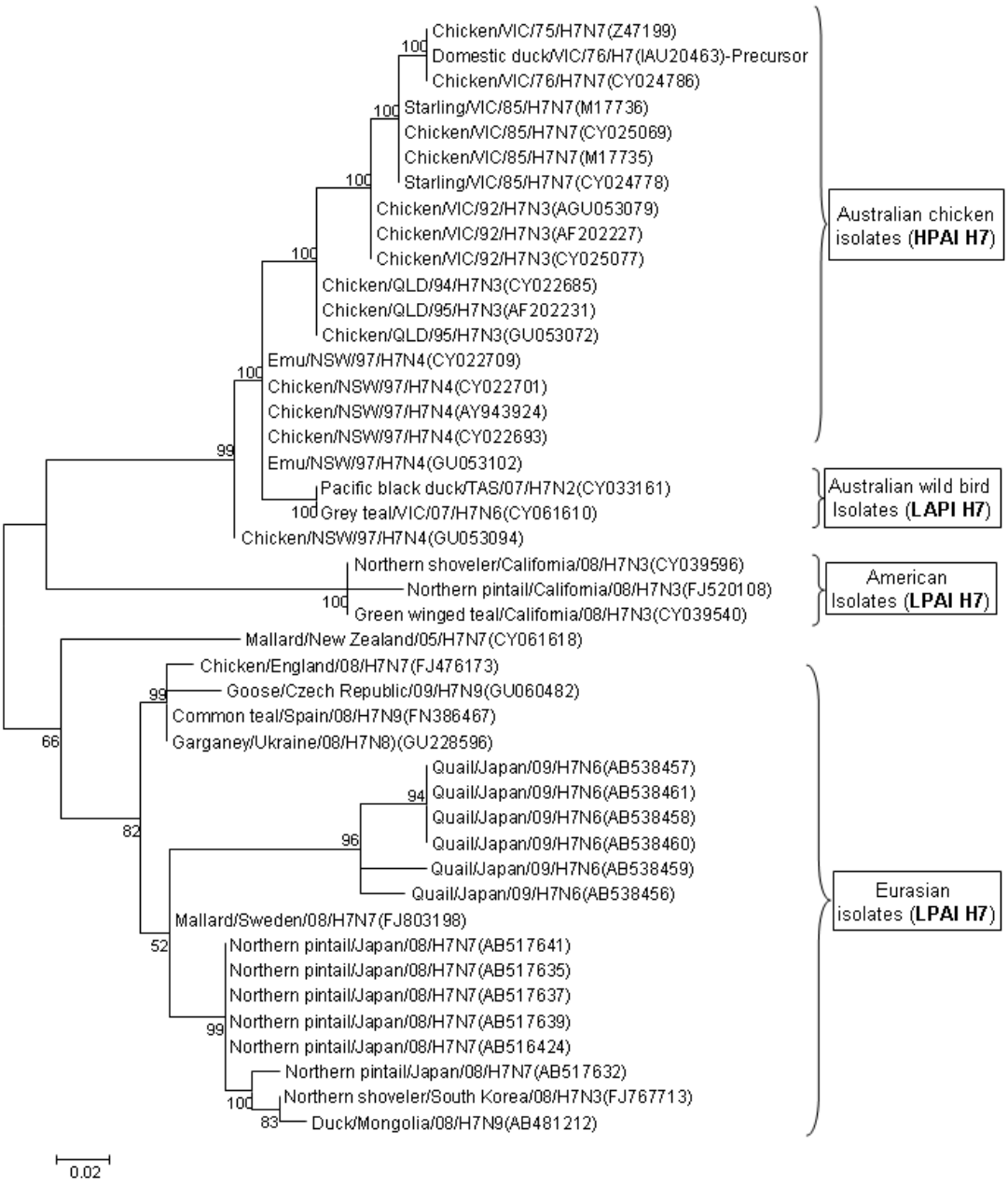

Figure 1.3 Bootstrap consensus trees with 5,000 replications using the maximum likelihood evolution method for avian influenza viral haemagglutinin gene-7 subtypes (nucleotide position between 26 and 1,500 base pairs) (VIC-Victoria, QLD-Queensland, NSW-New South Wales and TAS-Tasmania). This phylogenetic analysis has been performed, based on available GenBank sequences of haemagglutinin-7 isolates in different avian species.

1.1.1.3. Avian influenza viral subtype: Haemagglutinin-9

The H9N2 is another subtype of real concern because it can adapt quickly to the domestic avian species and acquire moderate pathogenic potential (Forrest and Webster, 2010). This subtype caused disease outbreaks in chickens in a number of countries (in particular, Asian countries) (Alexander, 2000b; Naeem *et al.*, 2007; Iqbal *et al.*, 2009; Nagarajan *et al.*, 2009) and poses a threat to poultry industries. This subtype has recently been identified in Australian resident and migratory birds during 2007-2008 (Haynes *et al.*, 2009; Hansbro *et al.*, 2010) which indicates migratory birds are potentially capable of introducing exotic AIVs to Australia (Table 1.1). Moreover, the H9 subtype has been recovered from pigs (Hong Kong, China and Indonesia) (Ninomiya *et al.*, 2002) and humans (Hong Kong) (Peiris *et al.*, 1999; Riedel, 2006). Hence, its ability to cross the taxonomic barrier into mammals highlights its potential threat. The threat of spill-over of H9 from wild birds into domestic animals and humans is another reason to conduct surveillance of AIVs in WABs in nQLD.

Currently, very little is published on the endemic LPAI viruses circulating in WABs in nQLD. There are two main routes for migratory birds into Australia, via Timor to the Northern Territory/Western Australia region and via Papua New Guinea (PNG) to Cape York. Whilst the Timor axis is being monitored, the PNG-Cape York axis is poorly monitored. The Australian Quarantine Inspection Service has been responsible for some sporadic sampling in the region but there has been no systematic study (Jonathan Lee, personal communication). Therefore, this epidemiological study, including active and passive surveillance will provide essential information about the state of AI in the northern part of Australia. This study will also create longitudinal comparisons and identify trends of AI in aquatic birds of this region.

1.1.1.4. Introduction of avian influenza viruses to Australia

There appears to be evidence of the introduction of viruses to Australia from migratory birds. For instance, the H4N8 and H11N9 subtypes were isolated in RNS and sharp-tailed sandpiper (STS), respectively and the origin of these viruses was Asia (Hurt *et al.*, 2006). However, the probability of transferring these viruses, carried through migratory birds to Australian resident wild birds has not yet been examined. Therefore, there is an opportunity to determine the possibility of introduced AIVs transferring to the local wild bird population in the current surveillance study.

1.1.1.5. Evolutionary changes of avian influenza viruses

Emerging novel AIVs through reassortment of genes can easily occur in wild birds. This is because individual birds within the AIV reservoir community are frequently infected with multiple subtypes, generating numerous gene reassortment events and a continuous generation of a nearly endless array of new viruses that could then potentially cross taxonomic barriers (Morens *et al.*, 2009). Previously, a hybrid Australian tern isolate of H2N5 evolved through gene reassortment between viruses of Eurasian and American lineages in free flying birds in nature (Kishida *et al.*, 2008).

This hybrid virus obtained the matrix (M) gene from the American isolates, and the other seven genes of the AIV from Eurasian isolates. Likewise, intercontinental reassortment of viruses was observed in wild ducks, shorebirds and gulls in Canada (Widjaja *et al.*, 2004) and in northern pintails in Alaska (Dugan *et al.*, 2008; Koehler *et al.*, 2008; Ramey *et al.*, 2010a; Ramey *et al.*, 2010b). Some earlier experimental studies reported that infections with two HPAI strains can reassort to a virus of low pathogenicity in chickens (Rott *et al.*, 1979), and infections with two LPAI strains can yield a highly virulent virus in mice (Scholtissek *et al.*, 1979; Vallbracht *et al.*, 1980).

Evolutionary transformation of AIVs in wild birds can also occur due to accumulated mutations of different genes of AIVs over time, resulting in a substantial degree of antigenic drift away from the original AIVs. This mechanism can lead to a distinct subtype of AIVs which have the potential to cause additional mortality. For instance, the 1957 and 1968 human pandemic strains of influenza A both contained drifted H genes originally derived from the 1918 pandemic virus (Taubenberger and Morens, 2006). Study of these two pandemic strains demonstrated that the gradual accumulation of mutation can be antigenically significant. In some cases, it may eventually give rise to an antigenically distinct H subtype as seen with the new, 16^{th} H subtype (Fouchier *et al.*, 2005).

Therefore, it is important to study the evolutionary changes of AIVs as they represent the potential for increased virulence in domestic animals and humans. The first step in this process is to characterise current viruses and their evolutionary history. Therefore, this study aimed to conduct an initial investigation into the diversity and origins of AIVs in nQLD. The study focussed on nQLD because of the region's potential to be a source of novel viruses and because little work had been conducted in this region compared with the rest of Australia and overseas.

1.1.1.6. Spread and transmission

Migration, ranging from short local to intercontinental movement is a common strategy for birds occupying seasonal habitats. Many Anseriformes and Charadriiformes travel a long distance (Hoyo *et al.*, 1996, cited in Olsen *et al.* (2006)). Therefore, they can distribute AIVs between countries or even continents. Birds in one specific breeding area usually follow the same migratory flyways (the east Asian-Australia flyway from eastern Siberia-south to eastern Asia and Australia).

However, there are exceptions. Some birds differ from these common practices (Hoyo *et al.*, 1996, Kam, *et al.*, 2004, cited in Olsen *et al.* (2006)). During migration, birds stop over at different non-specific areas to recharge themselves before reaching the main destination. Consequently, virus-infected birds can transmit their pathogens to resident or other migratory populations which in turn spread the viruses to new areas. However, the transmission of viruses and their geographical spread depends on the ecology of the migrating hosts (Alerstam and Lindstrom, 1990, cited in Olsen *et al.* (2006)). Many species stopover at favourable feeding or resting sites where consequently there is a high density of resident and migratory birds. Such places may be important for transmission of AIVs between wild and captive birds and between species (Olsen *et al.*, 2006).

Waders in the *Charadriidae* and *Scolopacidae* families are well suited to either marine or freshwater wetland areas and often live side-by-side with ducks (Piersma and Oikos, 1997, cited in Olsen *et al.* (2006)), which can create a favourable environment for transmission of AIVs.

Avian influenza viruses remain infectious in lake water at 22°C for four days and at 0°C for more than 30 days. The high AIV prevalence in birds living in aquatic environments may be due in part to efficient transmission through the faecal-oral route via surface water as LPAI viruses preferentially infect cells lining the intestinal tract and are excreted in high concentrations in their faeces (Webster *et al.*, 1978; Webster *et al.*, 1992).

The transmission of the viruses between avian hosts is not well understood but there are various potential routes. A faecal-oral route is one of the most generally assumed routes, but infection via aerosols and perhaps even cloacal drinking may occur (Fouchier and Munster, 2009). Large bodies of water such as lakes that serve as resting places for WABs may also play a role in transmission (Horimoto and Kawaoka, 2001) because all birds shed viruses in faeces (De Jong *et al.*, 2000; Webster, 2002; Sturm-Ramirez *et al.*, 2005).

Low pathogenic AIVs have previously been isolated from different species of Charadriiformes which regularly travel between Asia and Australia. These bird species were ruddy turnstone (*Arenaria interpres*), red knot (*Calidris canutus*), RNS (*Calidris ruficollis*), common tern (*Sterna hirundo*), sooty tern (*Sterna fuscata*), bar tailed godwit (*Limosa lapponica*) and STS (*Calidris acuminate*) (Tracey *et al.*, 2004). Importantly, Charadriiformes can congregate in extremely large concentrations on coastal floodplains and wetlands (Morton *et al.*, 1993), where they regularly interact with Australian resident Anseriformes (Morton, 1990). In contrast, Australian Anseriformes are mostly predominantly resident in Australia and only a few species migrate a short distance to neighbouring countries such as PNG. Therefore, the risk to Australia appears to be in the association between Charadriiformes which potentially harbour overseas AIVs, and resident Anseriformes. These Anseriformes such as wild ducks could potentially spread viruses to domestic free range poultry or commercial poultry as they disperse from coastal areas to inland sites within Australia. Therefore, this study aimed to target surveillance towards Australian Anseriformes as they pose the greatest risk in terms of subsequent spread and spill-over of both exotic and endemic AIVs in Australia.

1.1.1.7. Prevalence

Avian influenza viral and antibody prevalence have previously been estimated in wild birds in many countries.

In Australia various studies have demonstrated a wide variation in AIV and antibody prevalence. A low AIV prevalence of 0.5-1.0% was reported by some studies (Downie and Laver, 1973; Mackenzie *et al.*, 1984; Peroulis and O'Riley, 2004; Haynes *et al.*, 2009). There is one report of AIV prevalence of 5.8% in migratory shorebirds in Australia (Hurt *et al.*, 2006). However, AIV antibody prevalence of 11-19% was estimated in Australian wild birds, which was relatively higher than those of AIV prevalence (Haynes *et al.*, 2009; Curran, 2010; Tracey, 2010).

Countries where studies report low AIV prevalence in wild birds include Argentina (0.4%) (Pereda *et al.*, 2008), Ireland (1.2%) (Raleigh *et al.*, 2009), Europe (~2.1-3.8%) (Munster *et al.*, 2006; Munster *et al.*, 2009) and Germany (4.2%) (Suss *et al.*, 1994).

Countries reporting relatively higher AIV antibody prevalence in wild birds include Pakistan (10%) (Khawaja *et al.*, 2005), Alaska (28%) (Heard *et al.*, 2008), New Zealand (33%) (Stanislawek *et al.*, 2002) and Italy (11-45%) (De Marco *et al.*, 2003a; De Marco *et al.*, 2003b; De Marco *et al.*, 2005).

Overall results, therefore suggest that AIVs commonly occur in global wild bird populations which indicates a likely hazard to animal and human health. Although some Australian studies have documented AIV and AIV antibody prevalence in wild bird population, very little attempt has been made to estimate the burden of AI in endemic resident WABs of nQLD. Hence, it was important to conduct a systematic longitudinal study to measure the level of AI status in WABs of nQLD which may help policy makers assess the need for future surveillance programs in WABs of northern Australia.

1.1.1.8. Potential risk factors

An appropriate risk factor analysis for AIV and antibody prevalence in wild birds has rarely been performed. A number of studies, however, have attempted to explore potential factors associated with AIV or antibody prevalence using crude univariate or meta data analysis or purely theoretical mathematical modelling. Results of some of those studies are presented below.

Mallard ducks (*Anas platyrhynchos*) and or pintail (*Anas acuta*) were the more commonly affected species with AIVs than other Anseriformes in Alberta (29-34% versus 8.0-15%) (Hinshaw *et al.*, 1980b) and southeast Sweden (12% versus 0.5%) (Wallensten *et al.*, 2007). Mallards along with other feral ducks (*A. platyrhynchos, A. Penelope, A. crecca, A. acuta, Clangula hymalis* and *Melanitta fusca*) had higher AIV prevalence than other Anseriformes and non-Anserifomes in Germany (8.7% versus 3.8% versus 0.3%) (Suss *et al.*, 1994). These results were supported by many AI serological studies in different countries (Wood *et al.*, 1985; Stallknecht and Shane, 1988; Ito and Kawaoka, 2000; Stanislawek *et al.*, 2002; De Marco *et al.*, 2003b; Hua *et al.*, 2005; Neumann and Kawaoka, 2006; Heard *et al.*, 2008; Nishiura *et al.*, 2009; Obon *et al.*, 2009). Dabbling ducks such as Pacific black ducks (PBDs) (*Anas superciliosa*) were more commonly affected with AIVs (3.1%) than other Anseriformes such as plumed whistling ducks (PWDs), wandering whistling ducks (WWDs), Australian shelducks (ASDs) and black swan (BS) (0.7-0.9%) in Australia (Tracey, 2010). These results were further supported by other Australian wild bird studies (Mackenzie *et al.*, 1984; Peroulis and O'Riley, 2004). These studies also found that PBDs were a frequently affected species. Pacific black ducks and mallard ducks are likely to differ little in their susceptibility and transmission of AIVs (Tracey, 2010).

Avian influenza viral prevalence was observed to be higher in juvenile (18-60%) than mature WABs (4-27%) in Alberta (Hinshaw *et al.*, 1980b). A similar age pattern of AIV prevalence was estimated in WABs in Siberia (Okazaki *et al.*, 2000), in North America (Munster *et al.*,

2007), Alaska (Ip *et al.*, 2008), northern Europe (Wallensten *et al.*, 2007) and New Zealand (Stanislawek *et al.*, 2002). The higher AIV prevalence in young birds may be due to the fact that they are immunologically naïve whereas adults are more resistant, particularly to viruses to which they may have previously been exposed (Webster *et al.*, 1992). By contrast, AIV antibody prevalence was reported to be higher in adult coats (Italy) and chickens (Bangladesh) than juveniles (De Marco *et al.*, 2003b; Nooruddin *et al.*, 2006), which may be due to the fact that adult birds might have more exposure time to infection and had a longer lasting AIV antibody.

Gender differences in AIV prevalence were observed earlier, where the prevalence was higher in females than male WABs (Runstadler *et al.*, 2007; Ip *et al.*, 2008). It is therefore, speculated that female birds could be immunologically suppressed during the breeding period due to the obvious stress of laying eggs and post-laying stressors and these conditions might have caused birds to be infected more frequently.

An Australian study recorded a higher AIV ribonucleic acid (RNA) prevalence in autumn (March-May) (3.2%) than any other season (1.8-2.7%) which may correspond to an increased number of young birds at that time of year (Hansbro *et al.*, 2010). However, studies in Eurasia and North America estimated AIV prevalence at less than 10% (spring and summer) to between 10-60% just before and during the autumn migration (Krauss *et al.*, 2004; Olsen *et al.*, 2006; Munster *et al.*, 2007; Wallensten *et al.*, 2007). Possible explanations for seasonal variation in prevalence is thought to be driven by the influx and aggregation of naïve juvenile birds following breeding and prior to (and during) migration (Webster *et al.*, 1992).

To identify a robust set of potential factors associated with AIV or antibody prevalence a long term systematic study is required to have sufficient sample size along with accurate measurement of different factors such as bird demographics, spatial, temporal and environmental variables. A higher antibody prevalence compared to AIV prevalence, as observed in the literature, will ensure increased ability to detect risk factors in serological data compared to AIV prevalence data. Therefore, an AI sero-prevalence study is also deemed necessary for an effective risk factor analysis to develop AI surveillance programs in WABs of northern Australia and poultry and public health management plans. For example, a targeted cost-effective surveillance program can be set up if such a risk factor analysis has identified particular bird species, suitable season and appropriate locations for increased risk of AIV infections. The influence of the identified risk factors on increasing the potential interaction between wild bird species and domestic or commercial poultry or humans can be used to develop effective risk minimisation strategies and bio-security measures.

1.1.1.9. Avian influenza sero-dynamism

Understanding the epidemiology of AIVs among wild birds is still in the early stage (Nishiura *et al.*, 2009) and relies on many assumptions regarding the immune response to AIV infection in birds. It is poorly understood how long previously infected birds remain immune in field conditions and remain immune from re-infection by homologous and heterologous AIV subtypes (Fereidouni *et al.*, 2010a). Few studies, to best knowledge, have assessed the immunostatus or sero-conversion of AIV antibodies in the wild under typical field conditions (De Marco *et al.*, 2003b; De Marco *et al.*, 2005; Fereidouni *et al.*, 2009; Fereidouni *et al.*, 2010a). Hence, it is important as one of the aims of this study to investigate AI serology on capture-mark-recapture WABs of nQLD to answer some of the questions about AI sero-dynamism.

Although some studies have previously determined a range of H serotypes in Australian wild birds through AI serological studies (Downie and Laver, 1973; Haynes *et al.*, 2009; Curran, 2010), there is no such investigation in WABs of nQLD. Therefore, the determination of the distribution of H serotypes in WABs of nQLD is one the aims of this study.

The following literature review about the Newcastle disease (ND) and causes of wild bird mortality is briefly presented as these two studies are a spinoff the main AI study.

1.1.2. Newcastle disease

Newcastle disease is a disease of domestic poultry and wild birds, caused by NDVs belonging to the family of *Paramyxoviridae* (Mayo, 2002). Newcastle disease is one of the most devastating diseases of poultry due to enormous economic losses from high mortality; either directly due to the disease or slaughter for disease control (Alexander, 2001a). Wild aquatic birds are recognized as important reservoirs of NDVs and may act as vectors for the transfer of wild viruses to domestic poultry, eventually causing outbreak of disease and economic loss (Alexander, 1995; Stanislawek *et al.*, 2002; Kim *et al.*, 2007a; Jindal *et al.*, 2009a). Virulent NDVs are known to cause high mortality in wild birds including high mortality in juvenile double crested cormorants (*Phalacrocorax auritus*) in North America (Glaser *et al.*, 1999), cormorants, pelicans and ring billed gulls (*Larus delawarensis*) in western Canada (Wobeser *et al.*, 1993) and in teal (*Anas crecca*) in Iran (Bozorgmehri-Fard and Keyvanfar, 1979).

Two classes of NDVs are recognised worldwide. The class-one type viruses are predominantly isolated from wildlife and nine genotypes have been described (Wu *et al.*, 2011). The class-two type viruses have been responsible for most of the outbreaks of ND in domestic poultry and the genetic lineages have been referred to as 1 to 5e (Aldous *et al.*, 2003; Aldous *et al.*, 2010) or I to VII (Lomniczi *et al.*, 1998). The class-two genotype-one viruses represented by V4-QLD, XZ-32-07 China and Ulster 67 are associated with aquatic birds worldwide (Czegledi *et al.*, 2006; Kim *et al.*, 2007a; Mia Kim *et al.*, 2008; Aldous *et al.*, 2010; Wu *et al.*, 2011). With the exception of NDVs isolated from an outbreak in the 1930s all Australian class-two NDVs detected in wild birds and poultry have been classified as genotype-one viruses (Figure 5.3; Chapter 5). A brief history of Australian ND outbreaks with wild bird connections is given below.

Australia has previously experienced virulent ND outbreaks in poultry between 1930 and 2002 (Westbury, 2001; Kattenbelt *et al.*, 2006b). The first appearance of a ND outbreak due to virulent ND virus was in Inverloch (a small seaside village) followed by various suburbs of Melbourne, VIC in 1930. Similar outbreaks reappeared in VIC in 1932 (Johnstone, 1933; Albiston and Gorrie, 1942). The class-two and genotype-three viruses may have been responsible for the 1930s outbreak. The source of the virus for these outbreaks was not determined but it is presumed to have been an imported virus. However, wild bird sources cannot be ignored.

Although the next 35 years (since 1933) were free of virulent ND outbreaks (Geering, 1985), avirulent ND virus (designated as strain V4) was confirmed in chickens near Brisbane, QLD in 1966 (Simmons, 1967) and soon after, evidence of infection was reported in most states (Anon, 1966). A large number of additional isolates of NDVs have been made from Australian poultry or wild birds since 1966, and evidence has been presented that many of these were avirulent (Westbury, 1979), possibly resembling the QLD V4 strain isolated from chickens in 1966 such as I-2 virus or a group of virus isolates related to the European duck Newcastle disease viral (NDV) isolate MC110 taken from wild birds (Spalatin *et al.*, 1976; Kim *et al.*, 1978; Alexander *et al.*, 1986; Hodder *et al.*, 1994; Spradbrow *et al.*, 1995). These viruses were the class-two and genotype-one (Figure 5.3; Chapter 5). This is consistent with these viruses having originated in wild birds.

After an absence of virulent ND outbreak for 66 years, the second Australian virulent ND outbreak in chickens occurred at Dean Park in NSW in 1998 (Gould *et al.*, 2001; Westbury, 2001). It also appeared that the molecular characteristics of the virulent neurotropic Dean Park isolate were almost identical to a low-pathogenicity virus first isolated from birds at Peat's Ridge on the central coast in 1988 (Kirkland, 2000; Westbury, 2001). The third outbreak

occurred at Mangrove Mountain in NSW in 1999 (Kirkland, 2000) followed by the fourth at Tamworth in NSW in 2001 (Westbury, 2001) and the fifth at Meredith in VIC in 2002 (Kattenbelt *et al.*, 2006b). These viruses were all class-two and genotype-one and wild birds were a possible source.

Molecular analysis of the NDV strains from the recent outbreaks suggest that an avirulent precursor of the virulent NDVs identified at each respective outbreak may have mutated to a virulent form to cause the ND outbreaks from 1998 to 2002 in Australia (Kirkland, 2000; Gould *et al.*, 2001; Westbury, 2001). This conclusion has been supported by evidence from sporadic screening of wild birds during or after the ND outbreaks which failed to identify virulent strains in the wild birds. However, avirulent NDV isolates such as strain V4 and AVRL32 were obtained from teals (N=322 wild birds) in VIC, Australia, at this time (Peroulis and O'Riley, 2004). These results were supported by other studies on Australian wild birds (Alexander, 2001a). Therefore, it has been suggested that wild birds may act as a reservoir of avirulent NDVs and transmit these viruses to domestic species (Hinshaw *et al.*, 1980b). Previous evidence for this mutation to virulence has been reported from Ireland where avirulent NDVs (class one-type) in WABs were demonstrated to have mutated to virulence in chickens (Alexander, 1995).

In 2006, the testing of an ibis in Australia that died of unknown causes resulted in the isolation and identification of an avirulent NDV isolate (Ibrahim Diallo, Department of Employment, Economic Development and Innovation, QLD). This NDV isolate belonging to a class-one virus is different to the more widespread and well-described QLD V4-isolate (class-two) (Simmons, 1967). This finding suggests that there is circulation of class-one and class-two NDVs in Australian wild bird populations which is supported by Gould *et al*. (2001), Kattenbelt *et al*. (2006a) and Kattenbelt *et al*. (2006b).

Some sporadic cross sectional studies have documented the serological and virological frequency of various strains of NDVs in Australian wild birds (Mackenzie *et al.*, 1985; Garnett and Flanagan, 1989). However, comprehensive studies on estimating prevalence of NDVs in wild birds and identification of risk factors associated with ND prevalence in Australian wild birds have not been attempted. Therefore, it is important to conduct a systematic longitudinal study on WABs in nQLD to determine the level of NDV RNA prevalence, the associated risk factors and to understand the molecular epidemiology of ND and identify potential threats to domestic poultry in Australia.

1.1.3. Bacterial diseases

Wild avian species are also reservoirs of bacterial pathogens that are of importance to public or animal health, for instance species of *Campylobacter* and *Salmonella*, and toxin-producing strains of *Escherichia coli* (Abulreesh *et al.*, 2007).

Salmonellosis (*Salmonella typhimurium* DT40) and colibacillosis (*Escherichia coli* 086) caused the mortality of wild birds (family-*Fringillidae*) in the United Kingdom (UK) (Pennycott *et al.*, 1998). Similarly, salmonellosis (*Salmonella* spp.) caused wild bird mortality in the United States of America (USA) (Brand *et al.*, 1988; Newman *et al.*, 2007) and Norway (Refsum *et al.*, 2002). In New Zealand, a major outbreak of salmonellosis (*Typhimurium* DT160) occurred during winter and the spring months of 2000 which caused extensive mortality in passerines (*Notimystis cincta*) (Alley *et al.*, 2002). This serotype was also confirmed in humans. Zoonotic and livestock significant pathogens such as *Salmonella* spp., NDVs, AIVs and flaviviruses have been reported in Australian ibis (*Threskiornis molucca*) (Epstein *et al.*, 2006). No attempt has been made to investigate zoonotic potential pathogens of WABs in nQLD. Hence, there is a real opportunity to investigate the zoonotic potential pathogens of WABs in nQLD.

Avian cholera (*Pasteurella multocida*) and or avian botulism (*Clostridium botulinum* Type E) were identified as major causes of sea duck (Mergini) mortality in North America (Skerratt *et al.*, 2005), WABs in the USA (Newman *et al.*, 2007), common eider ducks in the Eastern USA (avian cholera) (Gershman *et al.*, 1964) and wild birds in the USA (avian botulism *C. botulinum* Type C) (Brand *et al.*, 1988). *Pasteurella multocida* was also isolated from the liver of a pelican found dead during a ND outbreak in western Canada in 1990 (Wobeser *et al.*, 1993).

1.1.4. Trauma

Trauma due to collisions has also been responsible for sea duck mortality in North America (Skerratt *et al.*, 2005), wild birds (*Fringillidae*) in UK (Pennycott *et al.*, 1998) and wild birds (such as Passeriformes and Cuculiformes) in Ecuador (Gottdenker *et al.*, 2008). Similarly, Savidge *et al.* (1992) describe trauma (vehicular and predation) as the most common causes of death of avifauna from Guam.

There is no comprehensive report on common causes of mortality in WABs in nQLD, Australia. Identifying significant causes of morbidity and mortality in WABs through passive surveillance is therefore a real opportunity to collect useful information within this study.

Passive surveillance is also useful and cost effective to investigate exotic or emerging or zoonotically important diseases such as AI in dead wild birds, and is a genuine back-up of an active HPAI H5N1 surveillance program. The HPAI H5N1 has caused mortality events in wild birds and has been detected by passive surveillance in other countries (Komar and Olsen, 2008; Globig *et al.*, 2009; Hesterberg *et al.*, 2009; Willeberg *et al.*, 2010).

1.1.5. Conclusion

The above discussion has identified the gap in knowledge regarding biosecurity risks posed by WABs in nQLD. It is unknown whether exotic HPAI or LPAI viruses and virulent or avirulent NDVs are being introduced to northern Australian resident wild birds through migratory birds using nQLD migratory routes. It has also been undetermined whether endemic LPAI viruses or avirulent NDVs have transformed into pathogenic viruses through an evolutionary process in resident wild bird populations in nQLD. Whether novel AIVs or NDVs occur in nQLD resident wild birds through intercontinental reassortment has also not been established. There is a shortage of scientific scrutiny whether HPAI H7 or virulent NDVs, which caused previous outbreaks in Australian commercial poultry, are maintained in wild bird populations in northern Australia. It was therefore, considered opportune to conduct a systematic molecular epidemiological study of AI and ND in WABs in northern Australia.

The AI and ND burden with their sub-type specific distribution as well as their associated risk factors has not been determined in WABs of nQLD through a systematic longitudinal study. Hence, a study was essential to assess those diseases along with their associated risk factors.

No attempt has been made to investigate causes of mortality of WABs in nQLD and explore the connection between the mortality of birds and HPAI viruses (such as H5, H7). No investigation has been performed to investigate potentially zoonotic bacterial pathogens such as *Salmonella* spp. in WABs of this region. The above scenario therefore led to a passive surveillance program to identify common causes of mortality in WABs of nQLD as another aspect of this study.

Moreover, the suitability of surveillance programs (both active and passive) for wild bird pathogens has rarely been tested in nQLD although this area is replete with wetlands, considered as a natural habitat for WABs and also an important entry point for migratory birds.

The above gaps in scientific knowledge of important wild bird pathogens, including those with zoonotic potential, highlight the need for conducting comprehensive epidemiological studies incorporating active and passive surveillance programs on these important pathogens in WABs

of nQLD. In view of these needs a three-year systematic longitudinal study was therefore conducted in WABs of nQLD with the following specific aims.

1.2. Aims of the research project

1.1.6. Specific aims of this project

1. To measure the sero-prevalence of AI in WABs in nQLD.
2. To identify the risk factors associated with the level of AIV antibodies in WABs in nQLD and determine the distribution of AIV serotypes in WABs in nQLD.
3. To measure the AIV RNA prevalence and determine the distribution of AIV subtypes in WABs in nQLD.
4. To relate the viruses in WABs in nQLD to other viruses in Australia and overseas.
5. To estimate the prevalence of NDV RNA and the associated risk factors.
6. To determine the classes of NDVs in WABs in nQLD and relate them to other NDVs in Australia and overseas.
7. To identify common causes of mortality in WABs in nQLD and explore the connection between mortality in birds and AIVs in nQLD.

1.1.7. Anticipated outcomes

1. Identify the importance of a surveillance program for wild bird pathogens (in particular AIVs) in WABs in nQLD and accordingly recommend a surveillance system.
2. Determine the causes of mortality of WABs and identify pathogens that may be a risk to communities and/or biodiversity.
3. Identify risk factors associated with causes of mortality of WABs and important pathogens (in particular AIVs).
4. Make recommendations for a surveillance system and a risk management plan for wild bird diseases that may spill-over to domestic animals and people and/or affect biodiversity.

1.3. Structure of thesis

The thesis includes seven chapters along with appendices. Chapters 2-7 were prepared with the aim of being published in scientific journals. Consequently, some minor repetition is unavoidable. Chapter 1 presented the general introduction focusing briefly on the important features of wild aquatic bird diseases to justify the main aims of this study. More background information was included in each respective chapter where required. The reference style of each chapter follows the current James Cook University (JCU) thesis guidelines. The topics covered in this study and the associated chapters and appendices are arranged as follows.

1.3.1. Evaluation and development of a serological assay for avian influenza (Chapter 2)

The Australian Animal Health Laboratory (AAHL) developed a competitive enzyme-linked immunosorbent assay (cELISA) to investigate equine influenza viral antibodies from horse sera in Australia (Selleck, 2007b). This assay was then adopted for detecting AIV antibodies in chickens followed by wild bird sera in Australia without rigorous testing of its efficacy to detect AIV antibodies in wild bird sera. Therefore, it was necessary to validate this assay with wild bird sera before applying it to the present sero-surveillance program in WABs in nQLD. This chapter includes an equivalence study between the AAHL original cELISA (now designated as AAHL-1) and the modified version of this assay (AAHL-2, JCU-1 and JCU-2), an analytical sensitivity study for these cELISAs and a longitudinal study of the effect of a post-coating on AIV antigen stability in cELISA plates.

1.3.2. Sero-epidemiology of avian influenza (Chapter 3)

Limited sero-epidemiological studies have been conducted in WABs mainly due to the difficulty in collecting serum samples, the lack of reliable serological assays and some reservation about undertaking investigations due to the belief that production of specific antibodies is weak or delayed, compared to chickens (Kida *et al.*, 1980; Suarez and Schultz-Cherry, 2000; De Marco *et al.*, 2003b; Stallknecht and Brown, 2008). Only sporadic cross sectional studies have been conducted on the serology of AI in Australia (Downie *et al.*, 1977; Senne, 2003; Haynes *et al.*, 2009). Therefore, a systematic longitudinal sero-epidemiological study of AI in WABs in nQLD was conducted. This chapter presents the results of seroprevalence of AI, the risk factor analysis for the level of AIV antibodies from sera of WABs and sero-dynamism in sera of capture-mark-recapture WABs. The patterns of distribution of H serotypes according to species and time were presented in this chapter.

1.3.3. Molecular epidemiology of avian influenza (Chapter 4)

This chapter delivers information about the prevalence of AIV RNA and the distribution of AIV subtypes with their genetic analysis. The phylogenetic analysis on different genes of AIV subtypes was performed to identify endemic and exotic AIV subtypes and any evolutionary changes in those subtypes in Australian wild birds. This analysis indicated the recent introduction of exotic AIVs to Australian resident birds through migratory birds. This study also identified suitable samples for a future AI surveillance program in WABs in nQLD.

1.3.4. Monitoring of wild aquatic birds for Newcastle disease (Chapter 5)

This chapter displays the results of the NDV investigation in WABs in nQLD which was a spinoff study from the main AI study. This study explored the prevalence of NDV RNA and the associated risk factors and identified the phylogenetic relationship between the NDVs determined in this study and other viruses in Australia and overseas. It also indicates the possible threat of wild NDVs to Australian domestic or commercial poultry.

1.3.5. Causes of mortality of wild aquatic birds (Chapter 6)

This chapter provides information about common causes (infectious and non-infectious) of wild bird mortality in nQLD through a passive surveillance program. Importantly, bird mortality was not found to be associated with HPAI H5N1. The public health importance of *Salmonella* spp was also reported in this chapter.

1.3.6. Importance of surveillance programs for wild bird diseases (Chapter 7)

This chapter presents discussion on the implications of the integrated results from the whole body of work (Chapter 2-6). The implications for domestic animal and public health are discussed including risk management. This chapter also highlights the importance of future surveillance programs along with recommendations concerning wild bird diseases in regards to animal and public health benefits.

1.3.7. Appendices in brief

Appendices include supplementary tables (Chapter 6) and published and accepted articles and abstracts. A list of poster and oral presentations are also given as appendices.

Four epidemiological articles on household ducks in Bangladesh were published during the PhD, which were included as appendices. These published works were, however, not directly linked to my PhD on wild bird diseases in WABs in Australia. However, similar methodologies (active and passive surveillance programs) were employed to generate data for both cases. Moreover, similar epidemiological data analysis techniques were applied in both instances. Therefore, these publications were indirectly relevant to my PhD on WABs in Australia.

Chapter 2: Development and evaluation of a competitive enzyme-linked immunosorbent assay for immune responses to avian influenza

2.1. Introduction

Enzyme-linked immunosorbent assay (ELISA) is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. Enzyme-linked immunosorbent assay is a simple and highly sensitive method of analysis that allows for simultaneous and rapid quantification of a large number of samples. The assay is based on the specific recognition of the target compound (analyte/antigen) by antibodies which bind to the compound. The antigen-antibody complex is detected and measured with the aid of an enzyme-labelled antibody or antigen. Upon addition of a non-coloured chromogen the enzyme produces a coloured reaction where the colour intensity is directly or inversely proportional to the concentration of the analyte in the sample.

Many different configurations of enzyme-linked immunosorbent assays (ELISAs) are used in serology but all follow the same basic principles. Antigen-antibody complexes are formed, and an enzyme-labelled secondary antibody is added. This conjugate-secondary antibody is specific for the antigen or antibody, which depends on the ELISA platform used.

Indirect ELISAs (iELISAs) were the first technique to detect AIV antibodies in poultry flocks (Snyder *et al.*, 1985; Abraham *et al.*, 1988; Adair *et al.*, 1989). The iELISA requires species-specific conjugated antibodies; therefore a different conjugated antibody is needed for each host species tested. The intensity of colour increases with the titre of antibody.

Competitive ELISAs (cELISAs) are not species-specific like iELISAs. The test antibody competes with an indicator antibody. Frequently, this is a monoclonal antibody (MAb) which is used, regardless of the species being tested (Katz *et al.*, 1995; Shafer *et al.*, 1998; Starick *et al.*, 2006). The intensity of the colour decreases with the increasing titre of test antibody and the result is expressed as percentage inhibition.

In regards to other tests used to detect AIV antibodies, a cELISA was more sensitive and specific than the agar gel immunodiffusion (AGID) test (Katz *et al.*, 1995; Shafer *et al.*, 1998), and as sensitive and specific as the haemagglutination inhibition (HI) test (Katz *et al.*, 1995; Shafer *et al.*, 1998; Jeong *et al.*, 2010). A cELISA was more sensitive and specific than HI

(Starick *et al.*, 2006; Song *et al.*, 2009; Marche and van den Berg, 2010; Perez-Ramirez *et al.*, 2010). A cELISA was also able to detect antibodies at an earlier stage of infection compared with the AGID and HI tests (Zhou *et al.*, 1998). When a MAb is used as the indicator antibody the assay can be epitope specific. The choice of a MAb that will react with an epitope common to all isolates of influenza A virus allows this assay to detect all immune responses to influenza A in a wide variety of species.

In general, cELISA used in this study relies on competition between test serum antibodies and a MAb for binding to an epitope on the influenza A virus nucleoprotein that is conserved in all influenza A viruses. Bound MAb is detected by an anti-mouse immunoglobulin antibody conjugated to horseradish peroxidase and quantified by the addition of the chromogen such as 3, 3' 5, 5'-Tetramethylbenzidine (TMB) and substrate (H_20_2). The action of the peroxidase on the H_20_2 generates oxygen ions that oxidize the chromogen to produce a blue colour (TMB) or a green colour if 2, 2'-Azino-bis: 3-Benzthiazoline-6-Sulphonic Acid (ABTS) is used. Addition of stop solution to TMB (sulphuric acid: H_2S0_4 or phosphoric acid: H_3P0_4) then halts the colour development and converts the blue colour into yellow. The absorbance is read on a plate reader and the percentage inhibition calculated in comparison to the absorbance obtained for a MAb control. 2, 2'-Azino-bis: 3-Benzthiazoline-6-Sulphonic Acid can be allowed to reach a plateau and a stop solution therefore is not required. The percentage inhibition of the binding of the MAb is proportional to the antibody concentration in the test serum.

The AAHL is a central AI referral laboratory. This lab developed a cELISA (now designated as AAHL-1) in order to detect equine influenza viral antibodies from horse sera in Australia (Selleck, 2007b). This assay was then adopted for the detection of AIV antibodies in chickens, followed by wild bird sera, in Australia without a rigorous testing for its ability to detect AIV antibodies using bird sera.

As part of a common agreement, the AAHL provided its protocol (AAHL-1) and necessary reagents to screen AIV antibodies in serum samples from WABs in the present AI surveillance program in northern Australia. As a starting point the AAHL-1 configuration was used without any modification to screen AIV antibodies from 411 field serum samples from WABs and this assay showed low and variable optical density (OD) values for the MAb control (no pre-exposure to polyclonal antibodies, 0% inhibition). In some instances, the OD values for the MAb did not meet the minimum quality control OD value of 0.8 (Selleck, 2007b). It was demonstrated that the concentrations of the AIV antigen, MAb and conjugate as described by Selleck (2007b) were suboptimal and responsible for the low OD of the MAb.

In addition, the mean percentage inhibition between duplicate wells exceeded ±10% which also did not meet quality guidelines (Selleck, 2007b). The use of TMB as a chromogen with a short incubation period (e.g. five minutes according to AAHL-1) can increase background noise, variability between duplicate analyses and possibly generate false positive or negative results.

It was therefore necessary to modify the assay to ensure adequate sensitivity, repeatability and reliability for the analysis of all samples collected in the present AI surveillance program. Reagent concentrations were optimised to obtain OD values (close to 2) for the MAb control (double the recommended reagent concentrations) and ABTS was used as an alternative chromogen to potentially increase reliability. The chromogen ABTS resulted in slower colour development and less background staining potentially reducing variability. An additional step of using post-coating was included to have more stable AIV antigen on plates with the aim of avoiding non specific results and facilitating long term storage and reduced between-run variability.

In order to assess the effects of modifications on sensitivity and reliability of AIV antibody detection, to ensure the modified assays were suitable for use in the surveillance study, the AAHL-1(Selleck, 2007b) technique was compared with the modified techniques, with and without post coating (referred to as JCU-1 and JCU-2, respectively).

A total of 240 sera were obtained from wild PWDs (*Dendrocygna eytoni*) and analysed using the three different versions of the assay. Concurrently, a subset of 160 sera was analysed using the AAHL-1 technique with double the recommended reagent dilutions (now indicated as AAHL-2) and the other three assays.

A separate sensitivity study for four assays (AAHL-1 and 2 and JCU-1 and 2) was performed on the diluted positive field PWD sera to compare analytical sensitivity of these assays. These field sera were initially screened by the JCU-2 assay.

The longitudinal stability of the AIV antigen following post-coating was assessed to determine if storage of large batches of plates was feasible in order to obtain more consistent results between sample batches. No serum sample was used for this experiment as this study wanted to determine only AIV antigen stability on the plates.

2.2. Materials and methods

2.2.1. Sera

Plumed whistling ducks were caught by the JCU avian influenza team led by Md. Ahasanul Hoque (a PhD student) from December 2007 to December 2009 at Billabong Sanctuary (19°22′ S and 146°54′ E) and Green Acres Lagoon (19°34′ S and 147°90′ E), located south of Townsville, Australia. Whole blood samples (0.5-3 ml, less than one percent of body weight) were drawn aseptically from wing veins. The samples were transferred immediately to sterile 15 ml plastic tubes. Blood samples were refrigerated within five hours of sampling. After over-night storage the samples were returned to room temperature (22°C) before centrifugation at 317 *g* for 10 minutes. Supernatant was decanted, transferred to a 1.5 ml microcentrifuge tube and centrifuged at 2,348 *g* for two minutes and stored at -20°C until analysed.

2.2.2. Competitive enzyme-linked immunosorbent assay

Four cELISAs were assessed under an equivalence study that included AAHL-1 (Selleck, 2007b) and three modified versions designated as AAHL-2, JCU-1 and JCU-2. Details of reagents and consumables used are provided in Table 2.1. U-bottom microtitre plates and H_3P0_4 (stop solution) were used instead of the AAHL suggested Nunc Maxisorp plates and H_2S0_4 for this study. Modifications are described in section of 2.2.2.1.

Table 2.1 Reagents, plates and controls with their sources for the competitive enzyme-linked immunosorbent assay

Items	**Compositions**	**Sources**
Antigen	Recombinant AIV antigen is prepared from yeast expressing a recombinant long form of the influenza A nucleoprotein molecule. Yeast cells are transfected with the plasmid and the antigen prepared by sonication of the yeast cells. The cell debris is removed by centrifugation and the supernatant stored at -20°C in aliquots of appropriate volume.	Australian Laboratory Services, AAHL, Geelong, Victoria 3320, Australia
Coating buffer	Sodium bi-carbonate (3.1 ml), sodium carbonate (1.4 ml) and distilled water (1,000 ml), pH-8.4	TropBio Pty Ltd, JCU
Post-coating buffer	Proprietary	TropBio, Cat. No. 05-004-05
Wash buffer	Tris-technical grade-MP Biomedicals, sodium chloride-AR grade (Crown scientific), di-sodium salt-ethylene diamine tetra acetate (EDTA)-AR grade, Pronelis, Tween 20 (Sigma)	TropBio
Serum diluents	Tris-MP Biomedicals, sodium chloride, di-sodium salt-EDTA, casein, Tween 20, bromophenol blue-BioRad in ten buffer, distilled water	TropBio
Antibody	AIV nucleoprotein mouse monoclonal antibody	AAHL
Conjugate	Goat anti-mouse IgG (H+L) (Horseradish peroxidase) HRP conjugate	Blotting grade, Cat. No. 170-6516, Bio-Red Laboratories Pty Ltd, Regents Park, NSW
	Jackson goat anti-mouse immunoglobulin HRPO conjugate	Code #115-035-146, AAHL
Chromogen and substrate solution	2, 2'-Azino-bis: 3-Benzthiazoline-6-Sulphonic Acid (ABTS) peroxidase substrate	KPL, Gaithersburg, MD 20878, USA, 301.948.7755, product code: 50-66-06
	3, 3' 5, 5'-Tetramethylbenzidine (TMB)	TMB Microwell peroxidase substrate system, KPL, Cat. No. 50-76-00
Stop solution	1 M H_3PO_4 Add 16.9 ml of concentrated H_3PO_4 to 250 ml of distilled water	James Cook University
Positive and negative control sera	Chicken sera	AAHL
Negative control sera	Hen and rooster sera	James Cook University
cELISA plate	U-bottom microtitre plate	Cooke Microtitre System

2.2.2.1. Procedures of competitive enzyme-linked immunosorbent assay

1. The recombinant AIV antigen was diluted at a rate of 1 in 800 (for AAHL-1) or 1 in 400 (for the AHHL-2 and JCU assays) in coating buffer and 50 µl was loaded into each well of a round (U) bottom 96-well-microtitre plate. The plate was covered and incubated overnight at 4°C for AAHL-1 and 2 and JCU-1 and at air conditioned room temperature (22°C) for JCU-2 in a sealed humidified box.

2. One of the following steps was then followed: for the AAHL-1 and 2 and JCU-1 assays, the diluted coating buffer was removed and the wells were washed using wash buffer with the aid of a squeeze bottle. Washing consisted of four, five-second rinses, with complete emptying of wells between rinses. After washing, the plate was inverted and tapped on a paper towel to remove any residual wash buffer. The plates were covered with lids immediately after washing to prevent drying; for the JCU-2, the diluted coating buffer was removed and 100 µl of post-coating buffer was dispensed into each well and kept in a humid box for two hours. After incubation, the residual coating buffer was removed and the plate was trap dried with a paper towel and incubated at 37°C for two hours.

3. Each test serum was diluted at a rate of 1 in 10 with serum diluent. Dilutions were made in a 96-wells transfer plate to facilitate mixing.

4. Positive controls (AAHL) were diluted in serum diluents at a rate of 1 in 50 and 1 in 500, whereas the negative control (AAHL and JCU) was diluted at 1 in 10.

5. For the equivalence study, test sera and controls (positive, negative and conjugate) were tested in duplicate, whereas four wells were used for the MAb control. A volume of 50 µl of diluted test serum and all controls were transferred from the dilution plate to the antigen coated plate as described below (Table 2.2). The plate was covered immediately with a lid and incubated at room temperature (22°C) for an hour.

6. Nucleoprotein mouse MAb was diluted at 1 in 800 (for AAHL-1) or 1 in 400 (for the AAHL-2 and JCU assays) in serum diluent and 50 µl added to all wells immediately after the serum incubation step except for the wells specified for the conjugate control wells. The plate was covered and incubated for an hour at room temperature (22°C) and the plate was then washed as described in step 2.

7. Jackson goat anti-mouse immunoglobulin HRPO conjugate for AAHL-1 and 2 and goat anti-mouse IgG (H+L) HRP conjugate for the JCU assays were diluted at a rate of 1 in 2,000 (AAHL-1) and 1 in 1,000 (AAHL-2 and JCU assays) in serum diluent just prior to addition to the plate and 50 µl of diluted conjugate was dispensed to each well. A lid was put on the plate and incubated at room temperature (22°C) for an hour.

8. During the conjugate incubation, the substrate buffer TMB for the AAHL assays and ABTS for the JCU assays were removed from the fridge and left on the bench for 45-60 minutes before use. At the end of the conjugate incubation, the plate was washed as previously described at the end of conjugate incubation. A volume of 100 µl of substrate buffer was dispensed to each well of the plate. After the addition of TMB, the plate was incubated for 10 minutes at room temperature (22°C) followed by the addition of 100 µl of stop solution (1 M H_3PO_4) to each well. The OD of the reactions was read, within five minutes of adding the stopping reagent using an ELISA reader (Multiskan Ex, Labsystems Pathtech) at 450 and 650 nm. At the end of one hour incubation with the ABTS substrate solution, the OD of the reactions was read at 414 and 492 nm using an ELISA reader (Multiskan Ascent Pathtech, Thermo Electron Corporation).

9. The results were expressed as a percentage inhibition relative to the OD of the MAb control using the following formula: 100-(100*(OD test serum mean÷OD MAb control mean)). The calculation was performed using the Microsoft (MS) excel 2003 program. The plate designed for the assay has been presented in Table 2.2.

Table 2.2 The plate designed for the competitive enzyme-linked immunosorbent assay

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Conjugate control		S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	MAb control		S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	MAb control		S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	AAHL Positive control (1 in 50)		S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	AAHL Positive control (1 in 500)		S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	AAHL negative control (1 in 10)		S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	JCU neg control (hen) (1 in 10)		S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	JCU neg control (rooster) (1 in 10)		S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

S: Serum; *neg*: Negative

2.2.3. Equivalence study

The study required 230 samples based on the formula below. It incorporated permitted differences between equivalent tests, their variance and levels of acceptable type 1 and 2 error: $[2*(Z_\alpha + Z_\beta)^2 * SD^2] \div \delta^2$ (Noordhuizen *et al.*, 1997) where, α=1.96 (2-tailed), β=1.28 (2-tailed), SD^2=the variance (.02), δ (permitted difference) =0.04. Variance estimates were obtained from previous serology results. The permitted level of difference was ±10% between wells within a plate (Selleck, 2007b). Therefore, it was conservatively assumed ± 4% permitted difference between assays for sample size calculation.

Two hundred and forty serum samples were randomly selected from 500 samples that were initially screened by JCU-2 for the equivalence study comparing AAHL-1, JCU-1 and JCU-2. A random number table was used to choose the samples. One hundred and sixty of the analysed serum samples were also concurrently tested using AAHL-2.

2.2.4. Analytical sensitivity study

Two pooled plumed whistling duck (PWD) sera samples were made to test the analytical sensitivity of assays AAHL-1 and 2 and JCU-1 and 2: one from 10 individual positive sera samples, each with 80-96% inhibition and one from 10 individual negative sera samples, each with 10% inhibition. The positive and negative sera were randomly selected from 15 positive and 120 negative sera as previously assessed by JCU-2.

A pooled serum sample was then diluted at 1 in 10 with serum diluent before making a dilution series of each. The following eight dilutions, based on the results of two small prior experiments were tested: 1 is to 0 (positive: negative) (0% dilution, undiluted positive pooled sera), 1 is to 0.5 (33%, mixed positive-negative pool), 1 is to 1 (50%), 1 is to 2 (67%), 1 is to 3 (75%), 1 is to 4 (80%), 1 is to 8 (89%) and 0 is to 1 (100%, undiluted negative pooled sera).

Ten replications per dilution using the four assays were used. For quality assurance, the AAHL positive chicken serum was used at the dilutions of 1 in 50, 1 in 500 and 1 in 5,000, each consisting of two replications, and negative serum was used at the dilution of 1 in 10 and 1 in 100, each consisting of one replication. In addition, four conjugate and four MAb controls were included. The first two columns (16 wells) of U-bottom ELISA micro-titre plates were used for all the controls and the remaining 10 columns (80 wells) were used for the test samples. The sensitivity testing was performed concurrently for each assay.

2.2.5. The longitudinal effect of a post-coating buffer on avian influenza viral antigen stability in plates

Eight wells of 10 U-bottom microtitre plates were prepared, as previously described, and the plates were stored at 4°C until testing. The plate coating was as described for the JCU-2 assay (step 1-2). The test procedure was as described for the JCU-2 assay (step 6-8). The only exception was that serum diluents were dispensed at 50 µl per well instead of using test or control sera. The first two wells of a column of a plate were allocated for the conjugate controls and the remaining six wells for the MAb controls. Individual plates were tested at 0, 1, 2, 4, 7, 14, 28, 60, 79 and 214 days after preparation. The OD values for each plate were read at 414 and 492 nm (Multiskan Ascent Pathtech, Thermo Electron Corporation).

2.2.6. Statistical analysis

2.2.6.1. Equivalence study

Data obtained were entered in the MS Excel-2003 program, coded and sorted and then exported into STATA ™ 11.0 (Stata Corporation, College Station, TX) for analysis. Data were cross-checked for validity and consistency.

An acceptable difference in percentage inhibition between wells within a plate was set 10%, as indicated by Selleck (2007b). The average percentage inhibition of an individual sample was calculated. For equivalence testing the mean difference in percentage inhibitions (MDPI) with their corresponding 95% confidence intervals (CIs) calculated using normal approximation method between the assays was calculated. For the equivalence experiment (N=240), the results were compared between the following paired assays: AAHL-1 versus JCU-1; AAHL-1 versus JCU-2; JCU-1 versus JCU-2. For a subset of the results (N=160), all four tests including AAHL-2 were compared. The assays were considered equivalent when the 95% CIs of the difference in average inhibition was within ± 5%.

Categories of negative and positive for AIV antibodies were used as a binary response variable. Sera that produced < 40% inhibition were negative and sera that produced ≥ 40% inhibition were positive (Selleck, 2007b). In addition, a study has found that the percent inhibition values for AIV antibody negative control sera ranged from 0-27% using cELISA for a range of bird species, further supporting the need for a threshold of ≥ 40% inhibition (Starick *et al.*, 2006). Two by two tables were constructed to compare the number of positive and negative results obtained for each assay, in pair-wise comparisons. McNemar tests were performed to compare the proportions of two discordant pairs in each table as a qualitative measure of equivalence.

The results were expressed and interpreted as a difference between the discordant paired proportions with the p value and 95% CIs.

The MAb control results between the assays were also compared using the Wilcoxon rank sum test (unpaired test) followed by a median test (unpaired test). The results were expressed as mean and median OD values with the p values.

2.2.6.2. Analytical sensitivity study

For the sensitivity study a dilution series of pooled PWD sera were tested. A descriptive statistic was carried out on the mean percentage inhibition in relation to different dilutions of serum samples and the results were presented in a line graph.

In the dilution series there were categories: 0% dilution (undiluted positive pooled sera), 33-89% (mixed positive-negative sera) and 100% (undiluted negative pooled sera). Therefore, three linear models were fitted. For the 0% dilution, the one-way analysis of variance (ANOVA) (Model-A) was performed on the mean percentage inhibition between assays. For the 33-89% dilutions, two-way ANOVA (Model-B) was performed on the mean percentage inhibition for the variables of assay and dilution factors. For the 100% dilution, once again the one-way ANOVA (Model-C) was used on mean percentage inhibition between assays.

Contrasts were carried out to determine the differences between cELISA types within each dilution of 0 and 100% and to explain the interaction between dilutions (33-89%) and cELISA types on mean percentage inhibition.

2.2.6.3. The longitudinal effect of a post-coating buffer on avian influenza viral antigen stability in plates

In the post-coating buffer experiment, the OD values of paired wells were used to calculate mean OD values. To test for differences in mean OD of the MAb, a one-way ANOVA was used. The results were expressed as a mean, 95% confidence interval (CI) (normal approximation method) and p value.

2.3. Results

2.3.1. Equivalence study

2.3.1.1. Mean difference in percentage inhibition between the assays

The 95% CIs of the difference in percentage inhibition between the modified versions of cELISAs JCU 1 and 2 (N=240) were 4.5-7.2% (Table 2.3) and therefore it is ambiguous whether the assays are equivalent given that a difference ± 5% was regarded as acceptable. The results for AAHL-1 and JCU-1 were similar having a 95% CI of 4.8-6.8, therefore, also ambiguous with regard to equivalence. The CI for differences between the AAHL-1 assay and JCU-2 assay were greater than the allowable range of ± 5% (9.8-13.3%) and the assays were therefore not quantitatively equivalent.

Table 2.3 Differences in mean percentage inhibition for pair-wise comparisons between the Australian Animal Health Laboratory-1, James Cook University-1 and James Cook University-2 assays in the analysis of plumed whistling duck sera (N=240)

Assay	Mean	Min-Max	Comparative assays	Mean difference (MD)	95% CI of MD
AAHL-1	27.9	-10-99	AAHL-1 versus JCU-1	5.8	4.8-6.8
JCU-1	22.1	-7-98	AAHL-1 versus JCU-2	11.6	9.8-13.3
JCU-2	16.3	-19-98	JCU-1 versus JCU-2	5.8	4.5-7.2

Min: Minimum; *Max*: Maximum; *CI*: Confidence Interval

When only 160 samples were tested, incorporating the testing of AAHL-2, the data showed no quantitative difference between the AAHL-2 and JCU assays. The 95% CIs were 1.2-3.8 for the AAHL-2 versus JCU-1; -0.02-0.5 for AAHL-2 versus JCU-2; 1.4-4.1 for JCU-1 versus JCU-2.

However, the CIs were higher than the acceptable limit ± 5% when the results were compared between the AAHL-1 and the other assays (5.6-8.9 for AAHL-1 versus AAHL-2; 3.6-5.9 for AAHL-1 versus JCU-1 and 5.8-9.2 for AAHL-1 versus JCU-2).

2.3.1.2. Qualitative analysis of percentage inhibition between the assays

Qualitative analysis of the results showed significant differences between AAHL-1 and JCU-1 and between AAHL-1 and JCU-2 assays, but no difference between the JCU assays based on the McNemar test (Table 2.4-2.6) (N=240). However, the criterion for equivalence was again assumed to be within ± 5%, in this case the proportion of discordant pairs. Therefore, it was

ambiguous whether AAHL-1 and JCU-1 and AAHL-1 and JCU-2 assays were equivalent as the 95% CI of the differences overlapped with 5%.

When a subset of the samples was analysed, incorporating the testing of AAHL-2, the results showed no difference in the number of positive sera determined between all pair-wise comparisons except between AAHL-1 and JCU-1 (-0.06-0.01) which was again ambiguously equivalent at a 5% level.

Table 2.4 The assessment of agreement between Australian Animal Health Laboratory-1 and James Cook University-1 assays in the proportions of samples in the categories of≥ 40% inhibition (positive) and < 40% inhibition (negative)

Assay	**AAHL-1**			**Difference between the discordant paired proportions**	**95% CI**	***p* (Exact) (McNemar test)**
JCU-1	≥ 40%	< 40%	Total			
≥ 40%	30	**2**	32	-0.05	-0.08 - -0.01	0.007
<40%	**13**	195	208			
Total	**43**	**197**	**240**			

AAHL: Australian Animal Health Laboratory; *JCU*: James Cook University; *CI*: Confidence Interval

Table 2.5 The assessment of agreement between Australian Animal Health Laboratory-1 and James Cook University-2 assays in the proportions of samples in the categories of≥ 40% inhibition (positive) and < 40% inhibition (negative)

Assay	**AAHL-1**			**Difference between the discordant paired proportions**	**95% CI**	***p* (Exact) (McNemar test)**
JCU-2	≥ 40%	< 40%	Total			
≥ 40%	28	**1**	29	-0.06	-0.09 - -0.02	<0.001
<40%	**15**	196	211			
Total	**43**	**197**	**240**			

AAHL: Australian Animal Health Laboratory; *JCU*: James Cook University; *CI*: Confidence Interval

Table 2.6 The assessment of agreement between James Cook University-1 and James Cook University-2 assays in the proportions of samples in the categories of≥ 40% inhibition (positive) and < 40% inhibition (negative)

Assay	**JCU-1**			**Difference between the discordant paired proportions**	**95% CI**	***p* (Exact) (McNemar test)**
JCU-2	≥ 40%	< 40%	Total			
≥ 40%	27	**2**	29	-0.01	-0.04 - 0.01	0.453
<40%	**5**	206	211			
Total	**32**	**208**	**240**			

CI: Confidence Interval; *JCU*: James Cook University

2.3.1.3. Comparison of monoclonal antibody controls

The OD values obtained for the MAb controls differed significantly between the AAHL-1 and JCU-1 and the AAHL-1 and JCU-2, but there were no significant differences between the JCU assays (Table 2.7) (N=240). When a subset of the samples were analysed (N=160), incorporating the testing of AAHL-2, the OD values obtained for the MAb controls also differed significantly between the AAHL-1 and other assays, but were statistically equal between the AAHL-2 and JCU assays (Table 2.8).

Table 2.7 Comparative optical density values for the monoclonal antibody control (n=12)

Assay	**Mean**	**Median**	**Range**	**Comparative assays**	***p*** **(Wilcoxon rank sum test)**	***p*** **(Continuity corrected) (Median test)**
AAHL-1	0.9	0.8	0.6-1.4	AAHL-1 versus JCU-1	<0.001	<0.001
JCU-1	1.9	2.0	1.2-2.3	AAHL-1 versus JCU-2	<0.001	<0.001
JCU-2	1.8	1.8	1.2-2.1	JCU-1 versus JCU-2	0.133	0.414

Table 2.8 Comparative optical density values for the monoclonal antibody in the assays (n=8)

Assay	**Mean**	**Median**	**Range**	**Comparative assays**	***p*** **(Wilcoxon rank sum test)**	***p*** **(Continuity corrected) (Median test)**
AAHL-1	0.7	0.7	0.6-0.9	AAHL-1 versus AAHL-2	<0.001	<0.001
				AAHL-1 versus JCU-1	<0.001	<0.001
AAHL-2	2.1	2.0	1.9-2.3	AAHL-1 versus JCU-2	<0.001	<0.001
JCU-1	1.8	1.9	1.2-2.3	AAHL-2 versus JCU-1	0.528	0.617
				AAHL-2 versus JCU-2	0.015	0.134
JCU-2	1.9	1.9	1.7-2.1	JCU-1 versus JCU-2	0.793	0.617

2.3.2. Analytical sensitivity study

2.3.2.1. Descriptive results of sensitivity testing

The results of the mean percentage inhibition in relation to different dilution of serum samples are presented in Figure 2.1.

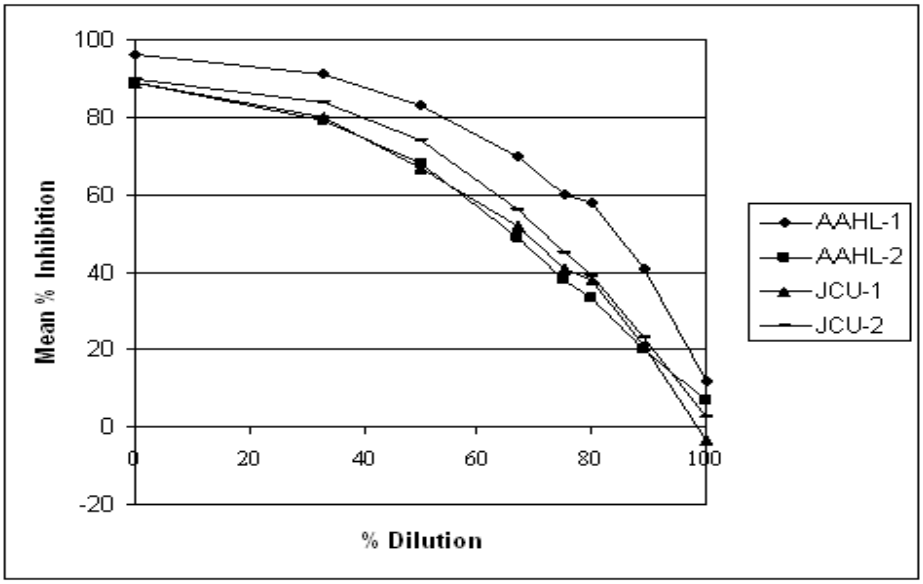

Figure 2.1 The results of the sensitivity testing for the four competitive enzyme-linked immunosorbent assays, Australian Animal Health Laboratory-1 and 2 and James Cook University-1 and 2 showing the mean percentage inhibition versus percentage dilution

2.3.2.2. Model-A one-way analysis of variance testing on mean percentage inhibition with 0% dilution (undiluted positive pooled sera)

The one-way ANOVA testing between cELISA types for mean percentage inhibition values of the undiluted positive PWD sera showed significant differences among the assays ($p<0.001$). The predicted means (95% CIs) for the assays were as follows: AAHL-1 95.6% (94.1-97.2), AAHL-2 89.1% (87.6-90.6), JCU-1 89.5% (88-91.1) and JCU-2 90.2% (88.7-91.7).

Post hoc analysis showed that there was no significant difference between the AAHL-2, JCU-1 and the JCU-2 assays ($p\leq0.420$). The AAHL-1 assay, however, produced a higher mean percentage inhibition for the pooled positive sera compared to the other assays ($p<0.001$).

2.3.2.3. Model-B two-way analysis of variance testing on mean percentage inhibition with 33-89% dilutions

The two-way ANOVA testing on mean percentage inhibition for the variables of assay and dilution factor (33-89%, mixed positive-negative pool) found a significant interaction effect between the cELISA types and the dilution factor (p=0.018). The AAHL-2, JCU-1 and JCU-2 assays were not significantly different at 33%, 80% and 89% (p>0.05) dilutions, however, the AAHL-1 assay gave significantly higher percentage inhibitions than the other assays (p<0.001) at all dilutions (Table 2.9).

The AAHL-2 and JCU-1 assays were not significantly different at 50%, 67% and 75% dilutions (p≥0.21), but the JCU-2 assay differed significantly from them (p≤0.04) at these dilutions.

Table 2.9 Predicted means (95% Confidence intervals using normal approximation method) of the interaction between assays and dilution factors

Dilutions	**AAHL-1**	**AAHL-2**	**JCU-1**	**JCU-2**
33%	90.9 (87.5-94.4)	79.1 (75.7-82.6)	80.4 (76.9-83.8)	83.9 (80.5-87.4)
50%	83.0 (79.6-86.4)	67.4 (64.0-70.9)	67.3 (63.9-70.8)	74.2 (70.8-77.7)
67%	70.4 (66.9-73.8)	49.5 (46.0-52.9)	52.6 (49.1-56.0)	55.6 (52.1-59.0)
75%	60.2 (56.8-63.7)	38.2 (34.7-41.6)	40.4 (37.0-43.9)	44.6 (41.1-48.0)
80%	58.2 (54.7-61.6)	33.1 (29.7-36.5)	37.5 (34.0-40.9)	38.9 (35.5-42.4)
89%	41.3 (37.8-44.7)	20.2 (16.8-23.6)	21.0 (17.5-24.4)	23.0 (19.6-26.4)

AAHL: Australian Animal Health Laboratory; *JCU*: James Cook University

2.3.2.4. Model-C one-way analysis of variance testing on mean percentage inhibition with 100% dilution (undiluted negative pooled sera)

The one-way ANOVA testing on the mean percentage inhibition values of the undiluted negative PWD sera in different cELISA types showed significant differences between all assays (p<0.001). The predicted means (95% CIs) were as follows: AAHL-1 11.5% (9.2-13.8), AAHL-2 7.4% (5.1-9.7), JCU-1 -3.4% (-5.7- -1.1) and JCU-2 2.5% (0.2-4.9). Post hoc analysis also showed significant differences between the assays (p≤0.015).

2.3.3. The longitudinal effect of a post-coating buffer on the stability of avian influenza viral antigen in plates

There was no difference in the mean OD values for the MAb over eight time points and a 60 day period ($p>0.05$), however there was a difference in OD values over 10 time points and a 214 day period ($p<0.001$) (Table 2.10).

Table 2.10 Mean optical density values for the monoclonal antibodies during a 214 day period in the post-coating buffer experiment

Day	Mean OD	95% CI
0	1.8	1.6-1.9
1	1.9	1.8-2.0
2	1.9	1.9-2.0
4	1.9	1.8-2.0
7	1.9	1.7-2.0
14	2.0	1.9-2.2
28	1.9	1.9-2.0
60	1.8	1.6-2.0
79	1.8	1.7-1.9
214	1.5	1.4-1.6

CI: Confidence Interval; *OD*: Optical density

2.4. Discussion

The AIV cELISAs used in this study did not perform equally in the quantitative detection of AIV antibodies in the sera of PWDs. Unequivocal differences were measured between AAHL-1 and JCU-2 where the AAHL-1 assay produced a higher mean % inhibition value in the analysis of 240 serum samples. Higher percentage inhibition values for AAHL-1 over the other assays were also evident in the sensitivity testing.

The discrepancy of the results between the AAHL-1 and either of the JCU techniques corresponds to the use of different reagent dilutions (antigen, antibody and conjugate) along with the types of chromogen (TMB or ABTS) used in the assays. The JCU assays used reagent dilutions optimised for OD (close to 2) for the MAb control, having twice the concentration of antigen and MAb as the AAHL-1 assay. Reduced levels of antigen and MAb in the AAHL-1 procedure would increase the percentage inhibition values for a given serum sample in comparison to other assays by reducing the amount of MAb available to compete with antibodies in the test sera, thus increasing the difference in OD between the MAb control and

that obtained for the test sera. The higher percentage inhibition values observed for AAHL-1 are probably indicative of increased sensitivity although the overall quality of the AAHL-1 cELISA can be considered in doubt due to unacceptable OD values (< 0.8) for the MAb control (Selleck, 2007b).

The OD values for the MAb controls in the JCU assays were satisfactory (median OD close to 2). However, it appears that when the same reagent dilutions are used (AAHL-2) all three assays have equal performances for practical purposes. In regards to quantitative differences between the assays the analytical sensitivity testing also showed a higher sensitivity for AAHL-1 assay over others but this assay once again failed to meet the test quality guideline of 0.8 OD value for MAb control (data are not presented). Analytical sensitivity of the other assays (AAHL-2, JCU-1 and JCU-2) appeared to be identical with satisfactory OD values for the MAb controls (Figure 2.1 and Table 2.9). Moreover, these modified assays showed an adequate relative sensitivity compared to AAHL-1 to detect AIV antibodies field sera for practical purposes as differences were not unambiguously more than 5% (Table 2.4-2.6).

The study to investigate the longitudinal effect of a post-coating buffer treatment on AIV antigens in cELISA plates demonstrated antigen stability in plates at satisfactory level over a 214 day period. This result clearly indicated the benefit of using the post-coating buffer where a large number of plates can be prepared and stored, minimising variation between batches of plates. Therefore, the addition of the post-coating step to cELISAs appears to be beneficial.

Overall results in this study indicated the modified assays (in particular JCU-2) were potentially more reliable, and of adequate relative sensitivity compared to AAHL-1, in the detection of AIV antibodies in wild bird sera. Storage of post-coated plates, to be used between sample batches, would be beneficial in obtaining more consistent results within future longitudinal studies. The JCU assay was therefore applied to the analysis of wild aquatic bird sera in the AI surveillance program.

Moreover, it was the first comprehensive attempt (to author's best knowledge) in improving the AAHL cELISA using wild bird sera in Australia. Adequate time and resources were not available to test various combinations of reagent concentrations and modified versions and therefore a single combination of reagent concentrations optimised for an OD of 2 for the MAb control, was chosen for testing. Further optimization of antigen and MAb concentrations should also be considered to increase the sensitivity of a modified assay, compared to JCU-2, while maintaining acceptable OD values for the MAb control. In addition, the performance of these

versions of cELISAs needs to be compared with commercially available cELISAs to determine their absolute and relative sensitivity and specificity.

Chapter 3: Sero-epidemiology of avian influenza in wild aquatic birds in north Queensland

3.1. Introduction

Avian influenza is a disease caused by influenza A type viruses belonging to the family *Orthomyxoviridae*. This disease was first identified in Italy over 100 years ago (Alexander, 2003). Influenza viruses have been reported in more than 90 species of birds (Alexander, 2000b).

Ducks and other aquatic birds are the recognized reservoir of AIVs and harbour all known subtypes of these viruses (16 H and 9 N types in different combinations). Wild aquatic birds such as shorebirds, carry the LPAI viruses, but at a much lower frequency than other WABs (Alexander, 2000b). The widely distributed influenza virus pool observed in migratory aquatic birds is suggested to be the host reservoir for influenza viruses that cause outbreaks in domestic poultry (Hinshaw *et al.*, 1980a; Hinshaw *et al.*, 1980b).

Avian influenza viruses can be divided into two distinct classes. The very virulent viruses cause HPAI, in which mortality may be as high as 100% and cause a drastic decline in poultry production. This group of viruses is comprised mainly of subtypes H5 and H7, although not all viruses of these subtypes cause HPAI associated diseases. All other viruses (14 H subtypes) cause LPAI which is a milder, primarily respiratory disease. However, LPAI may be exacerbated by other infections or environmental conditions (Alexander, 2000b).

Transmission of AI to humans through close contact with poultry or other birds occurs rarely and only with some subtypes of AIVs. However, some reports have shown evidence of the direct transmission of influenza viruses (for instance H5N1 and H9N2) from birds to humans since 1996 (Kurtz *et al.*, 1996; Peiris *et al.*, 1999). By March 2010 HPAI H5N1 had spread to 63 countries in Asia, Europe and Africa and the human death toll due to the H5N1 was 286 (N=476) between 2003 and 2010 (Anon, 2010a). Of the confirmed human cases and deaths, Indonesia, a close neighbour of Australia, has the highest number of cases (163) and deaths (135) (Anon, 2010a). There is worldwide concern that the HPAI H5N1 may evolve the capacity for human-to-human transmission and the potential emergence of a pandemic (Alexander, 2007).

When epidemics of AI of both HPAI and LPAI viruses occur in domestic species, it is frequently hypothesized that the initial introduction was a virus of low pathogenicity originating from free-living birds. Then, in some instances, a period of circulation among reared birds may have allowed the change of the H5 and H7 subtype strains to HPAI viruses (Alexander and Brown, 2000).

Australia has had five outbreaks of AI in commercial chickens. All those outbreaks have been caused by the HPAI H7 subtype of which three occurred in VIC (1976, 1985 and 1992), one in QLD (1994) and one in NSW (1997) (Selleck *et al.*, 2003; Westbury, 2003). In four of the five AI outbreaks, there was a presumptive association with WABs (Westbury, 2003). In at least two of the five Australian outbreaks of HPAI, surface drinking water contaminated with aquatic bird faeces was suspected to be the source of infection (Selleck *et al.*, 2003; Westbury, 2003). Economically, the largest outbreak occurred in Tamworth, NSW in 1997, and led to the death or destruction of over 310,000 birds and 1.2 million fertile eggs on six farms (Selleck *et al.*, 2003). In many outbreaks of AI in Australia and overseas, the mechanism of introduction of infection into poultry flocks has not been determined; however, direct and indirect contact between WABs (e.g. ducks and geese) and poultry is regarded as an important entry pathway for AI (Anon, 2008b). In addition HPAI viruses are currently circulating in poultry and wild birds with intermittent infection of humans in countries neighbouring Australia like Indonesia and Vietnam.

Although extensive surveillance of AIVs in ducks and shorebirds in North America has been undertaken (Hanson *et al.*, 2003; Krauss *et al.*, 2004), there is limited up-to-date information available for Eurasia, Africa, South America and Oceania, and only for a restricted number of bird species (Suss *et al.*, 1994; Okazaki *et al.*, 2000; De Marco *et al.*, 2003a; De Marco *et al.*, 2003b; Fouchier *et al.*, 2003).

Limited AI serological studies have been conducted in WABs, mainly due to the difficulty in collecting serum samples, lack of reliable serological assays, and some reluctance to undertake investigations due to the belief that production of specific antibodies is weak or delayed in WABs as compared to chickens (Kida *et al.*, 1980; Suarez and Schultz-Cherry, 2000; De Marco *et al.*, 2003b; Stallknecht and Brown, 2008). Studies to determine risk factors for the AIV antibodies in WABs are not very common; however several cross sectional studies have determined species specific prevalence in Alaska, USA (Heard *et al.*, 2008), New Zealand (Stanislawek *et al.*, 2002), China (Hua *et al.*, 2005), Italy (De Marco *et al.*, 2003b), the United Arab Emirates (Obon *et al.*, 2009) and California (Charlton, 2000).

Sporadic cross sectional studies have been conducted on the serology of AI in Australia (Downie *et al.*, 1977; Senne, 2003; Haynes *et al.*, 2009), but longitudinal studies with the aim of estimating seroprevalence and exploring risk factors for increased AIV antibodies and new infection (as indicated by seroconversions in capture-mark-recapture studies) have not been done. The determination of the full set of H serotypes and patterns of occurrence over time have also rarely been investigated in WABs in Australia. Therefore, a three-year systematic longitudinal study was conducted in nQLD from April 2007 on WABs to understand the epidemiology of AI and identify potential factors associated with the presence of AIV antibodies.

3.2. Materials and methods

3.2.1. Study sites and sampling

Epidemiological studies were conducted on WABs from the wetlands of four different study sites of nQLD. Sites were chosen based on their proximity to migratory routes, ease of access, the presence of resident WABs and generally a large bird population. A three-year longitudinal study was performed on WABs at Billabong Sanctuary (19°22′ S and 146°54′ E), 20 km south of Townsville, between April 2007 and March 2010 and a two-year study was performed at Green Acres Lagoon (Cromarty) (19°34′ S and 147°90′ E), 80 km south of Townsville, between December 2007 and 2009. Sporadic cross sectional studies were also carried out on Cape York and the Atherton Tableland between 2007 and 2009. Cape York had eight subsites: Big Red Lily Lagoon, Rutland Plains (RP) (15°59′ S and 141°65′ E); Bullock Lagoon, RP (15°69′ S and 141°65′ E); Cabbage Tree Creek, RP (15°59′ S and 141°69′ E); Unnamed Lagoon, Kenchering outstation, south of Aurukun (13°87′ S and 141°67′ E); Home Lagoon, RP (15°64′ S and 141° 67′ E); RP homestead (15°64′ S and 141°84′ E); Stirling station, Kurumba (17°18′ S and 141° 65′ E) and Ti Tree outstation, south of Aurukun (13°98′ S and 141°67′ E). Atherton Tableland had two sites: a poultry abattoir dam at Mareeba (16°58' S and 145°24' E) and a duck farm at Mount Molloy (16°40' S and 145°19' E).

Longitudinal studies required 138 birds to be sampled per quarter of each year. Accordingly, 1,656 samples were needed for a three-year study at Billabong Sanctuary and 1,104 samples for a two-year study at Green Acres Lagoon. The following formula was applied to calculate sample size assuming a 100% sensitivity and specificity: $N= (Z_{\alpha}^2 * SD^2)/L^2$ (Noordhuizen *et al.*, 1997). Ten percent expected point prevalence, based on the preliminary data analysis and literature (Haynes *et al.*, 2009) and an infinite bird population were assumed. N is the sample size; Z equals 1.96 at 95% confidence level; SD^2 (variance) equals 0.09; L (absolute precision)

equals 0.05. The variance was calculated as follows: P* (1-P), where P is the expected point prevalence (0.10).

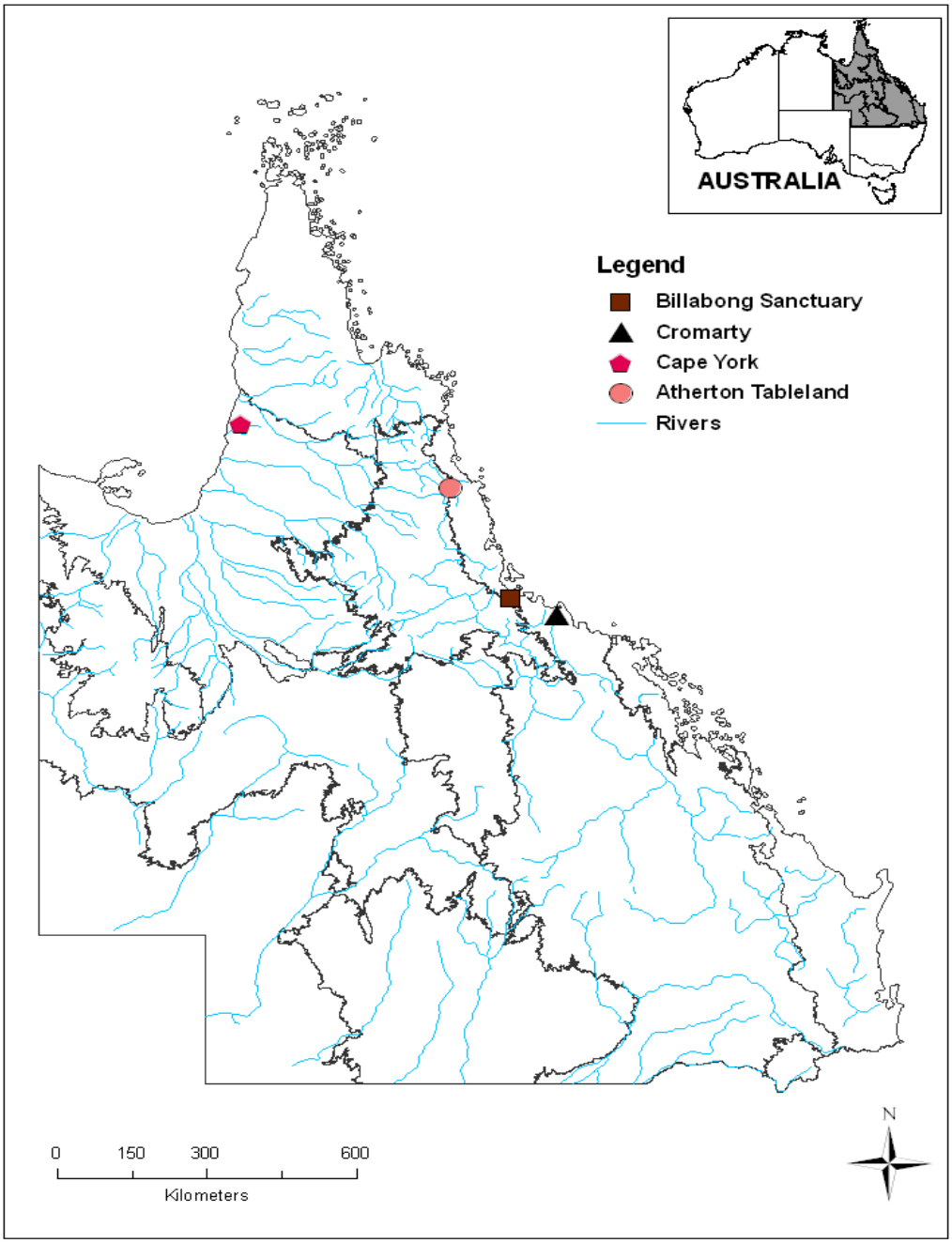

Figure 3.1 Map showing different sampling sites in north Queensland

Birds were sampled quarterly (September-December: Warm dry, January-April: Warm wet and May-August: Cool dry) at Billabong Sanctuary and Green Acres Lagoon in order to study temporal pattern of avian influenza, and sporadically on Cape York and the Atherton Tableland. However, sampling birds, in some instances, were not strictly followed as per time frame due to unavailability of bird population for the longitudinal study. Funnel trapping was mostly used to catch birds at all study sites except Cape York under ethics approval no A 1,175 (Animal Welfare Ethics Committee, JCU). Birds on Cape York were trapped and sampled by mist nets and a net launcher and sampled with the collaboration of Dr David Roshier (Ecologist) of Charles Sturt University, NSW (Currently, School of Life and Environmental Sciences, Deakin University, Waurn Ponds Campus, VIC 3217) under ethics approval number (WISP04524607). Birds were sampled on the Atherton Tableland with the assistance of the staff of Biosecurity Queensland, a service of the Department of Employment, Economic Development and Innovation (DEEDI).

Captured birds were transferred to soft cloth holding bags (pillow cases) for processing. Whole blood samples (0.5-3 ml, less than one percent body weight) were drawn aseptically from wing veins with sterile nontoxic nonpyrogenic syringes (3-5 ml, Terumo) and needle (25 $^{G*5/8''}$or 22 $^{G*3/4''}$, Terumo). A new needle and syringe was used for each bird. Once blood was collected, pressure was applied to the wound until bleeding stopped, usually around 30 seconds. The samples were transferred immediately to the sterile 15 ml plastic tubes with lids (Sarstedt). Each tube was labelled with a unique identity number. Blood samples were transferred to the Virology laboratory at the School of Veterinary and Biomedical Sciences, JCU, within five hours of sampling. Cloacal and oropharyngeal swabs of each bird were taken for the study of AI and NDVs (Chapter 4 and 5). Prior to release, birds were banded using appropriately sized identification bands. Handling and banding methodology were applied as specified by the Australian Bird and Bat Banding scheme (*http://www.deh.gov.au/bidiversity/science/abbbs*). Birds were only recaptured at two sites (Billabong Sanctuary and Green Acres Lagoon).

Demographic data (species, age and gender), body weight, selected body measurements (wing, tarsus, toe, bill, head, knob and body length) and body characteristics were also taken. The ages of sampled birds were determined based on the selected criteria described by Marchant and Higgins (1998). Sex was identified by cloacal plier examination. Body weight was taken with a portable balance (OCS-5A, Hayes; maximum weighing: 10 Kg and minimum weighing 100 g).

3.2.2. Sample processing and analysis

Blood samples were placed at 4ºC within four hours for overnight storage. The next morning the samples were returned to room temperature at 22ºC before spinning down at 317 *g* for 10 minutes. Supernatant was transferred to a 1.5 ml microcentrifuge tube and spun at 2,348 *g* for two minutes and stored at -20°C until analysis. Samples were analysed within six months of freezing.

This study used the cELISA described by Selleck (2007b) and modified by Hoque *et al* (now designated as JCU-2 cELISA) to detect AIV antibodies (details in Chapter 2). The results analyses using JCU-2 were expressed as a percentage inhibition relative to the OD of the MAb control using the following formula: 100-(100*(OD test serum mean÷OD MAb control mean)). The calculation was performed using MS Excel 2003.

3.2.3. Haemagglutination inhibition assay

Serum samples that reacted in the cELISA (≥ mean 40% inhibition) were further assessed by the HI test using a panel of AIV antigens to determine the distribution of AIV serotypes infecting birds. This assay was carried out as per the protocol described by Selleck (2007a), with minor adjustments to the plate incubation period and the amount of AIV antigen used for back titration. Before the HI tests, cELISA reactor and control serum samples were treated with the 10% washed chicken red blood cells (CRBC) to remove non specific haemagglutination (HA), according to the protocols described by Selleck (2007a). Eighteen sera (16 test and two negative chicken sera) were treated per batch. A volume of 25 µl of serum was added to 175 µl CRBC for each sample in a 1.5 ml microcentrifuge tube, then mixed well and incubated for 45 minutes at air-conditioned room temperature (22°C).

All treated samples in the tubes were then intermittently shaken to resuspend cells during incubation. After incubation, tubes were spun at 2,000 *g* for five minutes and supernatant was transferred to the dilution plate, and temporarily stored at 4ºC before testing. The HI antibody titres were evaluated using the 11 reference inactivated AIV antigens provided by AAHL (Table 3.1). The HI titre was expressed as $\log_2$ of the reciprocal of the highest serum dilution.

Table 3.1 Avian influenza viral antigens used for haemagglutination inhibition testing

H type	AIV antigen	Source details
H1	H1N9	Shelduck/Western Australia/1757/1978
H3	H3N8	Black duck/Western Australia/699/1978
H4	H4N4	Grey teal/Western Australia/1840/1979
H5	H5N1	Chicken/Vietnam/8/2004
H6	H6N5	Shearwater/Australia/1975
H7	H7N6	Grey teal/Victoria/512/2007
H9	H9N2	Turkey/Wisconsin/1966
H11	H11N9	Tern/Australia/1975
H12	H12N9	Red-necked stint/Western Australia/5745/1984
H13	H13N6	Gull/Maryland/704/1977
H15	H15N9	Shelduck/Western Australia/1762/1979

The HI assay was performed as follows:

1. Eighteen serum samples (16 test and two negative control chicken serum samples) were run per round bottom microtitre plate, nine sera for each half. The last three columns of each plate were allocated for cell controls (six wells) and back titration (21 wells) (see the layout template Table 3.2).

2. A volume of 25 µl of 1× Phosphate Buffer Saline (1×PBS) was added to all wells of a round bottom microtitre plate except the wells for cell controls and back titration. A volume of 50 µl of 1× PBS was dispensed for cell controls and back titration.

3. A volume of 25 µl of serum was dispensed to the well designated for each test serum followed by mixing and removal of 25 µl for addition to the next well in the dilution series (four dilutions). After completion of the serial dilution 25 µl of solution was discarded from the last well in the series.

4. A volume of 25 µl of the known AIV antigen preparation (diluted in 1× PBS to contain four haemagglutination units (HAU) as determined by the antigen titration) was added to each test serum well. A total of 50 µl of antigen preparation was added to the first three selected wells for back titration, mixed, and serially diluted through the addition of 50 µl to the subsequent well in the series (total of three wells) with 50 µl of diluted product discarded from the last wells.

5. The plate was then incubated for 45 minutes in an air conditioned room (22°C). After incubation, 50 µl of 0.5% CRBC was added to each well and the plate was again incubated for an hour at 4ºC to allow CRBC to settle.

6. The plate was read by tilting to 70º so that non-agglutinated cells stream to the bottom of the well. No agglutination was recorded as a positive result since the antibodies in the sera inactivated the antigen thus inhibiting agglutination.

7. The HI assay was acceptable when the negative control sera in the plate showed a complete HA, no HA in cell control wells where the cells ran when the plate was tilted and the HA titre was four HAU in back titration.

8. Sera that were inhibited at dilutions of 1:16 or greater against four HAU of antigen were considered positive for the antibody.

Table 3.2 The plate layout designed for the haemagglutination inhibition assay

	1	2	3	4	5	6	7	8	9	10	11	12
A (First half)	TS1	TS2	TS3	TS4	TS5	TS6	TS7	TS8	NS1	CC	CC	CC
B	SD	SD	SD	SD	SD	SD	SD	SD	SD	CC	CC	CC
C	SD	SD	SD	SD	SD	SD	SD	SD	SD	CC	CC	CC
D	SD	SD	SD	SD	SD	SD	SD	SD	SD	BT	BT	BT
E (Second half)	TS9	TS10	TS11	TS12	TS13	TS14	TS15	TS16	NS2	SD	SD	SD
F	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD
G	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD
H	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD

BT: Back titration; *CC:* Cell control; *NS:* Negative control serum; *SD:* Serial dilution; *TS:* Test serum

3.2.4. Statistical analysis

Field and laboratory data were stored in the MS-2003 spread sheet. Data were exported into STATA/SE ™ 11.0 (StataCorp, 4905, Lakeway Drive, College station, Texas 77845, USA) for checking data integrity and performing an epidemiological analysis. Descriptive statistics were performed to express individual results of each category as a frequency percentage and 95% CIs where applicable.

3.2.4.1. Risk factor analysis

Both linear and logistic models were applied to the percentage inhibition data to detect any association between AIV antibody levels and year of sampling, season, location, species, age, weight and sex.

3.2.4.2. Linear model

Mean percentage inhibition data were analysed using one-way ANOVA for categorical factors and Spearman's rank tests for continuous factors to identify factors for inclusion into linear models. Factors were selected for inclusion in the model according to the identification of significant univariate analyses ($p \leq 0.20$) or according to a subjective decision to include biologically interesting factors. The model was manually constructed by forward-selection applying the maximum likelihood estimation procedure (Dohoo *et al.*, 2003) and the statistical significance of the contribution of individual predictors (or group of predictors) was determined. Wald's test and the likelihood ratio test (LRT) were used to accomplish this, as described by (Dohoo *et al.*, 2003). Interaction was assessed between factors by constructing two-interaction product terms for the significant main effect factors in the model, forcing them into the model and examining changes in the coefficients and *p* values of the main effects. The presence of confounding factors was investigated by removing one of the variables and assessing changes in the coefficient. A coefficient change of more than 10% was considered to indicate the presence of confounding variables. Variance inflation factors (VIF) for the factors were examined to diagnose collinearity and identify highly correlated factors for avoiding a duplication of effects. The VIF value of more than 10 indicates serious collinearity (Dohoo *et al.*, 2003). The Cook-Weisberg test was used to examine the homogeneity of variance and whether the overall data fitted the model. The results were presented for each adjusted factor as a coefficient, *p* value and 95% CI assuming normal approximation.

3.2.4.3. Logistic model

Categories of negative and positive for AIV antibody were used as a binary response variable. Sera that produced < 40% inhibition were negative and sera that produced ≥ 40% inhibition were positive (Selleck, 2007b). In addition, a study has found that the percentage inhibition values for AIV antibody negative control sera ranged from 0-27% using cELISA for a range of bird species, further supporting the need for a threshold of ≥ 40% inhibition (Starick *et al.*, 2006).

Chi-square tests were performed to assess unconditional relationships between the same set of factors that were used for the one-way ANOVA testing and between the binary categories of mean percentage inhibition. Factors with significant $\chi 2$ results ($p \leq 0.20$) and those used for the linear models were selected for the logistic analysis.

Selection of the most parsimonious model was performed by the same procedure as described for the linear model. Interaction and confounding were assessed between factors using the LRT in a similar procedure as discussed for the linear model analysis. Collinearity was tested between categorical factors using the 2-tailed p value by the $\chi 2$ test. Two factors were considered to be collinear if the *p* value was ≤ 0.05. The model was then assessed for goodness-of-fit using the Hosmer-Lemeshow test, while predictive ability was determined using the receiver operating characteristic (ROC) curve (Dohoo *et al*., 2003). The results were presented for each adjusted predictor variable as an odds ratio (OR), *p*-value and 95% CI.

3.3. Results

3.3.1. Samples collected

From April 2007 to March 2010, 1,522 serum samples from 1,555 live birds captured at four study sites were collected (Table 3.3). Anseriformes were more frequently captured (1,526) than non-anserifomes (29). The samples were collected at the following sites: 741 serum samples at Billabong Sanctuary from April 2007 to March 2010, 604 serum samples at Green Acres Lagoon from December 2007 to December 2009, 143 serum samples at Cape York from July to September 2008 and from June to September 2009 and 34 serum samples on the Atherton Tableland in July 2008 and November 2009 (Table 3.3 and 3.4).

Two hundred and four birds were recaptured (one to five recaptures per individual, a mean recapture rate of two) over the study period, with 114 (15.3%) from Billabong Sanctuary and 90 (14.8%) from Green Acres Lagoon consisting of 176 PWDs, 15 PBDs, nine magpie geese (MGs), two Australian shelduck (ASD), one BS and one dusky moorhen (DM). An additional 295 serum samples were collected from those recaptured birds.

Distribution of serum samples and cELISA reactors, by species and site and time, are also presented in Table 3.3 and 3.4.

Table 3.3 Captured wild aquatic birds with their corresponding number of serum samples and competitive enzyme-linked immunosorbent assay reactors (+), north Queensland (from April 2007 to March 2010)

Common name	**Scientific name (Order)**	**No of birds**	**No of serum samples (+)**	**% Reactor (95% CI)**
Plumed whistling duck	*Dendrocygna eytoni* (Anseriforme)	1,233	1,209 (90)	7.4% (5.9-8.9)
Magpie goose	*Anseranas semipalmata* (Anseriforme)	119	119 (7)	5.9% (2.3-11.7)
Pacific black duck	*Anas superciliosa* (Anseriforme)	114	114 (53)	46.5% (37.2-55.8)
Burdekin duck	*Tadora radjah* (Anseriforme)	15	13 (3)	23.1% (5.0-53.8)
Wandering whistling duck	*Dendrocygna arcuata* (Anseriforme)	10	8 (1)	12.5% (0.3-52.6)
Muscovy duck	*Cairina moschata* (Anseriforme)	10	10 (0)	0% (0-30.8)
Green pygmy goose	*Nettapus pulchellus* (Anseriforme)	7	4 (0)	0% (0-60.2)
Australian white ibis	*Threskiornis molucca* (Anseriforme)	7	7 (1)	14.3% (0.3-57.8)
Grey teal	*Anas gracilis* (Anseriforme)	4	4 (0)	0% (0-60.2)
Hardhead	*Aytha australis* (Anseriforme)	3	2 (0)	0% (0-84.1)
Australian shelduck	*Tadorna tadornoides* (Anseriforme)	2	2 (2)	100% (15.8-100)
Black swan	*Cygnus atratus* (Anseriforme)	1	1 (1)	100% (2.5-100)
Domestic goose	*Anser anser* subsp. *domesticus* (Anseriforme)	1	1 (0)	0% (0-97.5)
Dusky moorhen	*Gallinula tenebrosa* (Gruiforme)	20	20 (0)	0% (0-16.8)
Torresian crow	*Corvus orru* (Passeriforme)	3	3 (0)	0% (0-70.7)
Bush stone curlew	*Burhinus grallarius* (Charadriiforme)	3	3 (1)	33.3% (0.8-90.5)
Nankeen night heron	*Nycticorax caledonicus* (Ciconiiforme)	1	1(0)	0% (0-97.5)
White faced heron	*Egretta novaehollandiae* (Ciconiiforme)	1	-	
Pied imperial pigeon	*Ducula bicolour* (Galliciforme)	1	1(0)	0% (0-97.5)
Total		**1,555**	**1522 (159)**	**10.5% (8.9-12.0)**

Table 3.4 Numbers of sampled wild aquatic birds and their corresponding number of serum samples and competitive enzyme-linked immunosorbent assay reactors (+) by site and month, north Queensland (from April 2007 to March 2010) (- No trapping done)

Time	**No of serum sample tested (+)**					
	Billabong Sanctuary	**Cromarty**	**Cape York**	**Atherton Tableland**	**Total**	**% Total reactor (95% CI)**
Apr 2007	6 (2)	-	-	-	6 (2)	33.3% (4.3-77.7)
May 2007	4 (1)	-	-	-	4 (1)	25% (0.6-80.5)
Jun 2007	19 (1)	-	29 (1)	-	48 (2)	4.2% (0.5-14.2)
Jul 2007	27 (5)	-	-	-	27 (5)	18.5% (6.3-38%)
Aug 2007	11 (1)	-	-	-	11 (1)	9.1% (0.22-41.2)
Sep 2007	19 (2)	-	-	-	19 (2)	10.5% (1.3-33.1)
Oct 2007	73 (3)	-	-	-	73 (3)	4.1% (0.8-11.5)
Nov 2007	8 (1)	-	-	-	8 (1)	12.5% (0.3-52.6)
Dec 2007	24 (3)	7 (0)	-	-	31 (3)	9.7% (2.0-25.7)
Jan 2008	0	11 (2)	-	-	11 (2)	18.2% (2.2-51.7)
Feb 2008	29 (4)	38 (1)	-	-	67 (5)	7.5% (2.4-16.5)
Mar 2008	15 (1)	64 (8)	-	-	79 (9)	11.4% (5.3-20.5)
Apr 2008	5 (0)	42 (2)	-	-	47 (2)	4.3% (0.5-14.5)
May 2008	11 (1)	0	-	-	11 (1)	9.1% (0.2-41.2)
Jun 2008	38 (1)	0	-	-	38 (1)	2.6% (0.06-13.8)
Jul 2008	24 (3)	0	35 (5)	20 (1)	79 (9)	11.4% (5.3-20.5)
Aug 2008	42 (12)	0	-	-	42 (12)	28.6% (15.7-44.5)
Sep 2008	0	26 (4)	36 (6)	-	62 (10)	16.1% (8.0-27.6)
Oct 2008	35 (5)	12 (4)	-	-	47 (9)	19.2% (9.1-32)
Nov 2008	11 (0)	7 (0)	-	14 (1)	32 (1)	3.1% (0.8-90.5)
Dec 2008	0	0	-	-	0	
Jan 2009	5 (1)	0	-	-	5 (1)	20% (0.5-71.6)
Feb 2009	1 (0)	0	-	-	1(0)	0% (0-97.5)
Mar 2009	46 (6)	63 (2)	-	-	109 (8)	7.3% (3.2-13.9)
Apr 2009	65 (3)	53 (2)	-	-	118 (5)	4.2% (1.3-9.6)
May 2009	21 (3)	15 (1)	-	-	36 (4)	11.1% (3.1-26)
Jun 2009	44 (2)	51 (1)	43 (3)	-	138 (6)	4.4% (1.6-9.2)
Jul 2009	16 (0)	0	-	-	16 (0)	0% (0-20.5)
Aug 2009	12 (2)	0	-	-	12 (2)	16.7% (2-48.4)
Sep 2009	30 (5)	11 (4)	-	-	41 (9)	22% (10.5-37.6)
Oct 2009	57 (7)	69 (16)	-	-	126 (23)	18.3% (19-26.1)
Nov 2009	22 (1)	60 (7)	-	-	82 (8)	9.8% (4.3-18.3)
Dec 2009	17 (3)	75 (9)	-	-	92 (12)	13% (6.9-21.6)
Jan 2010	2 (0)	-	-	-	2 (0)	0% (0-84.1)
Feb 2010	0	-	-	-	0	
Mar 2010	2 (0)	-	-	-	2 (0)	0% (0-84.1)
Total	**741 (79)**	**604 (63)**	**143 (15)**	**34 (2)**	**1,522 (159)**	**10.5% (8.9-12)**

3.3.2. Avian influenza viral antibody prevalence

The overall AIV antibody prevalence as determined by cELISA was 10.5% (CI 8.9-12.0) in the wild aquatic bird sera from nQLD. The prevalence was 10.7% (8.4-12.9) at Billabong Sanctuary; 10.4% (8.0-12.9) at Green Acres Lagoon; 10.5% (5.4-15.6) on Cape York and 5.9% (0.1-28.6) on the Atherton Tableland. The prevalence was 46.1% (36.8-55.3) in PBDs; 7.5% (6.0-8.9) in PWDs and 5.9% (1.6-10.2) in MGs. The prevalence in common species at different sites are presented in Table 3.5.

Table 3.5 Avian influenza viral antibody prevalence in the samples of predominantly sampled wild aquatic bird species, north Queensland (from April 2007 to March 2010)

Species	**Billabong Sanctuary**		**Green Acres Lagoon**		**Cape York**		**Atherton Tableland**	
	N	**% (95% CI)**	**N**	**% (95% CI)**	**N**	**% (95% CI)**	**N**	**% (95% CI)**
PWD	567	7.4 (5.3-9.6)	556	7.2 (5.2-9.6)	68	8.8 (2.0-15.7)	17	5.9 (0.1-28.6)
PBD	47	53.2 (38.8-67.6)	45	48.9 (34.9-63.7)	22	27.3 (8.1-46.5)	1	0 (0-97.5)
MG	93	7.5 (2.1-13.0)			26	0 (0-13.2)		

PWD: Plumed whistling duck; *PBD:* Pacific black duck; *MG:* Magpie goose; *CI*: Confidence Interval

3.3.3. Results of competitive enzyme-linked immunosorbent assay for the recaptured wild aquatic birds

A total of 295 serum samples were evaluated. These samples were obtained from 204 recaptured birds (one to five recaptures, mean of two). Of 204 birds, 13 previously non-reactor (negative) birds were newly diagnosed as reactors (positive) (10 at first, two at first and second and one at third recapture); 22 birds were reactors at their first capture and 169 birds were consistently non-reactors. Of 22 birds which were reactive at first capture, 12 birds continued to react at subsequent recaptures (seven at first; four at first and second; and one at first to fourth recaptures) and ten birds were failed to react at recapture.

Samples from 35 recaptured birds (N=204) demonstrated definite changes in AIV antibody titres at different recaptures. An increasing trend, on average, from 27-70% inhibition in cELISA results was noted in 16 birds (11 PWDs and five PBDs) over a mean of 40 days (standard error-se 12 days); a decreasing trend (av. 60-42% inhibition) was recorded in 13 birds (eight PWDs, three PBDs, one BS and one ASD) over a mean of 93 days (se 34 days) and a fluctuating results was also observed in six birds (one PWD, three PBDs, one ASD and one magpie goose: MG) over a mean of 98 days (se 26 days), suggestive of at least two infections at different times. In addition an appreciable level of AIV antibody titre was maintained in one ASD over a period of 399 days. Importantly, the results also indicated that AIV antibody lasted

≥ two months maintaining a high titre of 66-85% mean inhibition in samples from three recaptured PBDs. Samples from the remaining recaptured birds (169) were always non-reactive at subsequent recapture (av. 10-13% inhibition) (Table 3.6).

Table 3.6 Pattern of the potential increasing, decreasing and fluctuating trend of avian influenza viral antibody titre in the samples of recaptured wild aquatic birds

		% Inhibition (Day of capture)					
Bird Number	**Species**	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Pattern of % Inhibition**
1	PWD	3% (0)	50% (70)	NS	NS	NS	Increasing
2	PWD	10% (0)	50% (41)	NS	NS	NS	
3	PWD	1% (0)	50% (104)	NS	NS	NS	
4	PWD	20% (0)	71% (122)	NS	NS	NS	
5	PWD	9% (0)	72% (24)	NS	NS	NS	
6	PWD	27% (0)	72% (7)	NS	NS	NS	
7	PWD	68% (0)	75% (6)	NS	NS	NS	
8	PWD	34% (0)	47% (39)	NS	NS	NS	
9	PWD	13% (0)	89% (171)	NS	NS	NS	
10	PWD	48% (0)	58% (3)	NS	NS	NS	
11	PWD	5% (0)	98% (14)	NS	NS	NS	
12	PBD	30% (0)	63% (26)	NS	NS	NS	
13	PBD	57% (0)	70% (4)	NS	NS	NS	
14	PBD	10% (0)	89% (3)	NS	NS	NS	
15	PBD	30% (0)	88% (13)	93% (20)	NS	NS	
16	PBD	67% (0)	74% (11)	NS	NS	NS	
17	PWD	45% (0)	15% (48)	NS	NS	NS	Decreasing
18	PWD	59% (0)	35% (189)	NS	NS	NS	
19	PWD	42% (0)	21% (54)	NS	NS	NS	
20	PWD	48% (0)	33% (9)	NS	NS	NS	
21	PWD	41% (0)	20% (7)	NS	NS	NS	
22	PWD	59% (0)	23% (431)	NS	NS	NS	
23	PWD	44% (0)	25% (246)	NS	NS	NS	
24	PWD	96% (0)	65% (7)	NS	NS	NS	
25	PBD	96% (0)	85% (56)	NS	NS	NS	
26	PBD	46% (0)	44% (40)	NS	NS	NS	
27	PBD	79% (0)	46% (35)	NS	NS	NS	
28	BS	62% (0)	60% (63)	NS	NS	NS	
29	ASD	65% (0)	68% (28)	NS	NS	NS	
30	PWD	29% (0)	-9% (38)	79% (234)	63% (239)	15% (260)	Fluctuating
31	PBD	76% (0)	77% (24)	80% (28)	66% (31)	80% (45)	
32	PBD	86% (0)	87% (61)	78% (70)	NS	NS	
33	PBD	65% (0)	82% (3)	26% (59)	7% (64)	NS	
34	ASD	85% (0)	45% (101)	94% (399)	NS	NS	
35	MG	2% (0)	-5% (7)	5% (22)	46% (71)	NS	

PWD: Plumed whistling duck; *PBD*: Pacific black duck; *BS*: Black swan; *ASD*: Australian shelduck; *MG*: Magpie goose

3.3.4. Results of risk factor analysis

Due to variability in the numbers of birds captured over time it was necessary to test three subsets of data in order to ensure adequate sample numbers in the classes of the risk factor categories under investigation (species, age, sex, weight, age, site, season and year).

3.3.4.1. Data subset A (N=394)

For subset A this study tested data from the predominantly caught species, PWD at Billabong Sanctuary from June 2007 to May 2009. This study started in April 2007, but few birds were caught from April to May 2007 and therefore those data were excluded from the analysis. This data set was suitable to examine the effect of season on AIV antibody levels. Seasons were defined as warm dry (September-December), warm wet (January-April) and cool dry (May-August) in order to examine seasonal effects. Years were classed as June 2007-May 2008 and June 2008-May 2009. Body weight was not normally distributed even after natural log transformation (skew to the right) and therefore, organized into two categories separated by the 50th percentile (0.20-0.66 and 0.66-1.16 Kg). Age and sex were excluded from the analysis due to missing a significant number of birds in this data that were not assessed for age and sex.

3.3.4.2. Linear model (A)

One-way ANOVA showed a significant effect of year, season and weight on mean percentage inhibition ($p \leq 0.20$). These factors were therefore forwarded to build the linear model. A VIF of < 10.0 suggested collinearity between factors was not a serious problem (Dohoo *et al*., 2003). No interaction was detected in the final model, but all three factors were identified as potential confounders; these were then adjusted in the model. The Cook-Weisberg test indicated that the model fitted the data reasonably well (χ^2=2.14, p=0.144). Year-2, warm wet weather (January-April) and heavier ducks were significantly associated with the rate of increasing mean percentage inhibition values as compared with their respective referent groups (p <0.05) (Table 3.7).

3.3.4.3. Logistic model (A)

Chi-square analysis of univariate data categorised by year, season and body weight are presented in Table 3.10. The proportion of mean percentage inhibition in two categories (< 40 % inhibition versus ≥ 40 % inhibition) varied only for weight ($p \leq 0.20$). However, year and season were also included to build the logistic model. Collinearity between the factors was

significant (χ^2 test, $p \leq 0.05$), but does not affect the model's predictive ability to identify risk factors as supported by Dohoo *et al*. (2003). No interaction was identified in the model. Weight was diagnosed as a potential confounder which was adjusted in the model. The Hosmer-Lemeshow goodness-of-fit test suggested that the model fitted the data (χ^2 test, $p \leq 0.542$). The ROC curve extended reasonably well into the upper left-hand corner of the curve (figure is not presented) and the area under the curve was 0.64. Both of these features indicated the logistic model had moderate predictive ability. The OR of mean percentage inhibition in heavier ducks was 3.2 compared with lighter ducks (p=0.01) (Table 3.8). Results were non-significant for year and season.

Table 3.7 Results of one-way analysis of variance and linear model analysis on mean percentage inhibition values obtained by competitive enzyme-linked immunosorbent assay analysis of wild plumed whistling duck sera for avian influenza viral antibodies (N=394) (from June 2007 to May 2009)

			One-way ANOVA		**Linear Model**	
Factors	**Categories**	**N**	**Mean (normal scale)**	***p***	**β (95% CI)**	***p***
Year	Jun 2007-May 2008	174	8.4	0.002	Referent	
	Jun 2008-May 2009	220	14.0		4.6 (0.5-8.7)	0.029
Season	Sep-Dec (Warm dry)	142	8.2	0.026	Referent	
	Jan-April (Warm wet)	108	16.0		8.3 (3.0-13.6)	0.002
	May-Aug (Cool dry)	144	11.4		3.5 (-0.8-7.9)	0.113
Body weight	0.20-0.659 Kg	196	10.2	0.154	Referent	
	0.66-1.16 Kg	198	12.8		6.8 (3.0-10.6)	0.001
Constant					2.0 (-2.3-6.3)	

Table 3.8 Results of chi-square and logistic model analysis of the proportion of samples in the categories of positive and negative for avian influenza viral antibodies obtained by competitive enzyme-linked immunosorbent assay analysis of wild plumed whistling duck sera (N=394) (from June 2007 to May 2009)

		X^2 test			**Logistic Model**	
Factors	**Categories**	**< Mean 40% inhibition**	**≥ Mean 40% inhibition**	***p***	**OR (95% CI)**	***p***
Year	Jun 2007-May 2008	162	12 (6.9%)	0.633	1.0	
	Jun 2008-May 2009	202	18 (8.2%)		1.5 (0.6-3.7)	0.358
Season	Sep-Dec (Warm dry)	132	10 (7.0%)	0.915	1.0	
	Jan-April (Warm wet)	100	8 (7.4%)		1.4 (0.5-4.5)	0.541
	May-Aug (Cool dry)	132	12 (8.3%)		1.4 (0.5-3.6)	0.499
Body weight	0.20-0.659 Kg	187	9 (4.6%)	0.024	1.0	
	0.66-1.16 Kg	177	21 (10.6%)		3.2 (1.3-7.8)	0.01

3.3.4.4. Data subset B (N=958)

For subset B data from PWDs at Billabong Sanctuary and Green Acres Lagoon from January 2008 to December 2009 were tested. This was the only data set to assess the site effect on AIV antibody levels. A complete two-year dataset was available; therefore, year classes were 2008 and 2009. Age was grouped into two classes (≤ sub-adult and adult) in order to have sufficient samples in each class. Weight was used as either a continuous variable (linear model) or in two classes (0.20-0.64 and 0.65-0.91 Kg) (logistic model). Sex was also included as a category in the analysis.

3.3.4.5. Linear model (B)

Mean percentage inhibition values were not normally distributed. Negative 24% inhibition was the lowest negative value in the data set; therefore 25 was added to each individual percentage inhibition value to make all values positive. The data was normally distributed after log transformation.

One-way ANOVA evidenced significant effects of site, year, age and sex on mean percentage inhibition ($p \leq 0.20$). Scatter plot and Spearman's rank correlation analysis demonstrated an influence of weight on mean percentage inhibition ($p \leq 0.20$). All five factors were, therefore, used to develop the linear model. Site, year, sex and weight were independently associated with mean percentage inhibition values (not seriously collinear) (VIF < 10.0; (Dohoo *et al*., 2003)). A significant interaction was detected between year and sex (p=0.03). Weight was identified as a potential confounder. Interaction and confounding factors were then adjusted in the model. The Cook-Weisberg test showed that the model fitted the data (χ^2=0.91, p=0.341). Year 2009, adult ducks, female and heavier ducks were significantly associated with higher percentage inhibition values (Table 3.9).

3.3.4.6. Logistic model (B)

The chi-square results for the individual categories of site, year, age, sex and weight are presented in Table 3.12. The proportion of results in positive and negative categories varied for age, sex and weight ($p \leq 0.20$). However, all five factors were used to construct the logistic model and examine their combined adjusted effects. Collinearity was detected between site and age, and between site and sex (χ^2 test, $p \leq 0.05$). However, the collinearity problem was ignored as previously discussed. No interaction was detected in the final model. Age was diagnosed as a potential confounder and its effect was adjusted in the model. The Hosmer-Lemeshow

goodness-of-fit test showed that the model fitted the data (χ^2 test, $p \leq 0.903$). The ROC curve extended reasonably well into the upper left-hand corner of the curve (figure is not presented) and the area under the curve was 0.68. Both of these features demonstrated that the logistic model had good predictive ability. The OR of being positive for AIV antibody was 2.9 for adult over ≤ sub-adult (p=0.01); 1.6 for female over male (p=0.187) and 2.3 for heavier ducks over lighter ducks (p=0.005) (Table 3.10).

Table 3.9 Results of one-way analysis of variance and linear model analysis of mean percentage inhibition values obtained by competitive enzyme-linked immunosorbent assay analysis of wild plumed whistling duck sera for avian influenza viral antibodies (N=959) (from January 2008 to December 2009)

		One-way ANOVA			**Linear Model**	
Factors	**Categories**	**N**	**Mean (log scale)**	***p***	**β (95% CI)**	***p***
Study site	Billabong Sanctuary	409	3.6	0.097	Referent	
	Green Acres Lagoon	550	3.7		0.02 (-0.03-0.1)	0.429
Year	2008	312	3.6	0.003	Referent	
	2009	647	3.7		0.3 (0.1-0.4)	<0.001
Age of ducks	≤ Sub-adult	276	3.5	<0.001	Referent	
	Adult	683	3.8		0.1 (0.01-0.1)	0.018
Sex	Male	227	3.5	<0.001	Referent	
	Female	732	3.7		0.2 (0.1-0.3)	<0.001
Weight	Body weight (Kg)	959	0.2* (rho)	0.001	0.6 (0.3-0.9)	<0.001
Year*sex	Interaction between year and sex				-0.2 (-0.3-0.02)	0.028
Constant					2.9 (2.7-3.1)	<0.001

* Spearman's rank correlation test; *rho*: Spearman's rank correlation coefficient

Table 3.10 Results of chi-square and logistic model analysis of the proportion of samples in the categories of positive and negative for avian influenza viral antibodies obtained by competitive enzyme-linked immunosorbent assay analysis of wild plumed whistling duck sera (N=959) (from January 2008 to December 2009)

		X^2 test			Logistic Model	
Factors	**Categories**	**< Mean 40% inhibition**	**≥ Mean 40% inhibition**	***p***	**OR (95% CI)**	***p***
Study site	Billabong Sanctuary	379	30 (7.3%)	0.944	1.0	
	Green Acres Lagoon	509	41 (7.5%)		0.9 (0.6-1.5)	0.716
Year	2008	285	27 (8.7%)	0.304	1.0	
	2009	603	44 (6.8%)		0.9 (0.6-1.5)	0.764
Age of ducks	≤ Sub-adult	269	7 (2.5%)	<0.001	1.0	
	Adult	619	64 (9.4%)		2.9 (1.3-6.6)	0.01
Sex	Male	217	10 (4.4%)	0.048	1.0	
	Female	671	61 (8.3%)		1.6 (0.8-3.0)	0.187
Weight	0.20-0.649 Kg	449	19 (4.1%)	<0.001	1.0	
	0.65-0.91 Kg	439	52(10.6%)		2.3 (1.3-3.9)	0.005

3.3.4.7. Data subset C (N=329)

For subset C, data from PWDs and PBDs at Billabong Sanctuary and Green Acres Lagoon from September to December 2009 were analysed. The two common species were sampled frequently at both sites during this period. This data set allowed us to test the effect of species on AIV antibody levels along with site and age. Age was condensed into two categories (≤ sub-adult and adult) to ensure sufficient sample size. Pacific black ducks are slightly bigger birds than PWDs; therefore, body weight was excluded from this analysis. Sex was not fully determined for sampled PBDs, therefore this factor was excluded from the analysis.

3.3.4.8. Linear model (C)

Mean percentage inhibition values for each sample were not normally distributed. Log transformation was therefore performed after three was added to each individual mean percentage inhibition value (the highest negative value was -2 in this data set). Species had a significant effect on mean percentage inhibition ($p \leq 0.20$), but site and age had no significant effect on mean percentage inhibition. However, considering possible interaction effects between site and age they were included along with species to develop the model. Site, species and age were independently associated with mean percentage inhibition values (VIF <10.0; (Dohoo *et al*., 2003)). Neither interaction nor confounding was detected in the model. The Cook-Weisberg test indicated that the model fitted the data (χ^2=0.11, p=0.741).

According to the final model, PBDs had a strong association with higher percentage inhibition as compared to PWDs (p <0.001) (Table 3.11).

3.3.4.9. Logistic model (C)

The chi-square results are described in Table 3.14. The proportion of mean percentage inhibition results in the positive and negative categories varied for species only ($p \leq 0.20$). However, all three factors were used to develop the logistic model. Collinearity was significant between age and species ($\chi 2$ test, p=0.01) which was not of concern as previously discussed. No interaction was recognized in the model. Species and age were identified as potential confounders; these factors were adjusted in the model. The Hosmer-Lemeshow goodness-of-fit test showed that the model fitted the data (χ^2 test, $p \leq 0.878$). The ROC curve extended reasonably well into the upper left-hand corner of the curve (figure is not presented) and the area under the curve was 0.72. Both of these results demonstrated the logistic model had good predictive ability. According to the model output, the OR of being positive for AIV antibody was 13.1 for PBDs over PWDs (p<0.001) (Table 3.12).

Table 3.11 Results of one way-analysis of variance and linear model analysis on mean percentage inhibition values obtained by competitive enzyme-linked immunosorbent assay analysis of wild plumed whistling duck and Pacific black duck sera for avian influenza viral antibodies (N=329) (from September to December 2009)

			One-way ANOVA		**Linear Model**	
Factors	**Categories**	**N**	**Mean (log scale)**	***p***	**β (95% CI)**	***p***
Study site	Billabong Sanctuary	114	3.2	0.464	Referent	
	Green Acres Lagoon	215	3.3		0.03 (-0.1-0.2)	0.637
Species	Plumed whistling duck	288	3.2	<0.001	Referent	
	Pacific black duck	41	3.9		0.7 (0.5-0.9)	<0.001
Age	≤ Sub-adult	62	3.2	0.552	Referent	
	Adult	267	3.3		0.1 (-0.03-0.3)	0.105
Constant					3.0 (2.9-3.2)	

Table 3.12 Results of chi-square and logistic model analysis of the proportions of samples in the categories of positive and negative for avian influenza viral antibodies obtained by competitive enzyme-linked immunosorbent assay analysis of wild plumed whistling duck and Pacific black duck sera for avian influenza viral antibodies (N=329) (from September to December 2009)

		X^2 test			Logistic Model	
Factors	**Categories**	**< Mean 40% inhibition**	**≥ Mean 40% inhibition**	***p***	**OR (95% CI)**	***p***
Study site	Billabong Sanctuary	99	15 (13.2%)	0.392	1.0	
	Green Acres Lagoon	179	36 (16.7%)		1.2 (0.6-2.5)	0.601
Species	Plumed whistling duck	259	29 (10.1%)	<0.001	1.0	
	Pacific black duck	19	22 (53.7%)		13.1(5.9-28.9)	<0.001
Age	Duckling to sub-adult	55	7 (11.3%)	0.309	1.0	
	Adult	223	44 (16.5%)		3.0 (1.0-8.0)	0.03

3.3.5. Haemagglutination inhibition testing of the competitive enzyme-linked immunosorbent assay reactor samples (≥ 40 % inhibition) from wild aquatic birds

A total of 147 cELISA reactor samples were further evaluated for H serotyping using the HI test. Thirty-five samples reacted with only one serotype and 95 samples reacted with multiple serotypes. The mean and median numbers of types per sample were 1.5 and 1.2 respectively. Seventeen cELISA positive samples were non reactive in HI testing which is probably due to the higher sensitivity of the former test enabling it to detect early stages of serum antibody responses or lower levels of AIV antibody.

Further classification of samples returning multiple H types (95) was performed by identifying the highest HI titre (HIT) produced. This classification produced 54 samples with one dominant serotype and 26 samples with two serotypes, nine samples with three serotypes, four samples with four serotypes and two samples with more than four serotypes (Table 3.13 and Figure 3.2). The subtype H9 was the most frequent type for single reactors (15/35) and H5 and H9 were the most frequently occurring types for the combined data for single reactors and for multiple reactors with a single dominant type (42/89) (Table 3.13). Overall results suggested that birds which were commonly exposed to or infected with H9, H5, H12, H3, H11 and H6, and HI titres, in general, were low (16-128) (Table 3.13).

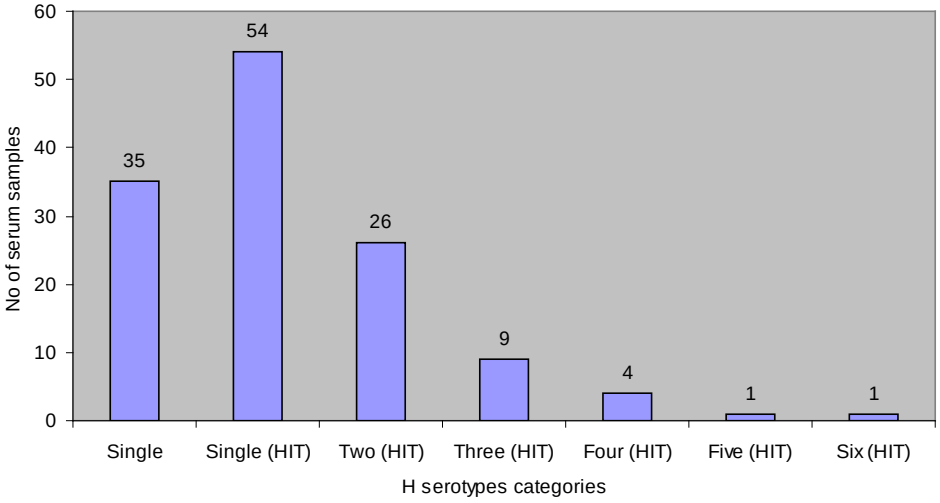

Figure 3.2 The number of serum samples with one to six serotypes. Haemagglutination inhibition titre (HIT)-the number of serotypes as determined by the highest titres observed using haemagglutination inhibition test for haemagglutinin types when multiple types were detected.

Table 3.13 Haemagglutinin serotypes for samples producing a single serotype and the haemagglutinin types with the highest titre for samples producing multiple types (from April 2007 to March 2010)

H serotype	**Number of single serotypes (Titre) (n=35)**	**Number of serotype based on the highest titre (n=95)**	**Species-frequency number**	**Total Number**
H1	3 (16-64)	1 (32)	Pacific black duck (PBD)-3 Magpie goose (MG)-1	4
H3	4 (16)	5 (32-128)	Plumed whistling duck (PWD)-3 PBD-6	9
H4	1 (32)	4 (32-128)	PWD-5	5
H5	3 (32-64)	14 (32-64)	PWD-9 Wandering whistling duck-1 PBD-5 MG-1 Australian white ibis-1	17
H6	2 (32-64)	5 (32-128)	PWD-6 BS-1	7
H9	15 (16-128)	10 (32-128)	PWD-11 PBD-11 Australian shelduck-1 Burdekin duck-2	25
H11	3 (16)	5 (32-128)	PWD-5 PBD-1 MG-2	8
H12	2 (16-64)	8 (32-128)	PWD-9 MG-1	10
H13	1 (16)	1 (16)	PWD-1 PBD-1	2
H15	1 (16)	1 (64)	PWD-2	2
H1-H3		1 (128)	PBD-1	1
H1-H9		1 (16)	PBD-1	1
H1-H12		1 (16)	PBD-1	1
H3-H4		1 (32)	PBD-1	1
H3-H12		3 (16-64)	PWD-3	3
H4-H11		1 (64)	PWD-1	1
H4-H12		1 (16)	PWD-1	1
H5-H9		2 (16-32)	PWD-2	2
H5-H11		1 (128)	Bush stone curlew-1	1
H5-H12		1 (16)	PWD-1	1
H5-H13		1 (32)	PWD-1	1
H5-H15		1 (32)	PBD-1	1
H6-H9		2 (16)	PWD-2	2
H6-H12		1 (128)	PWD-1	1
H7-H11		1 (32)	PWD-1	1
H7-H13		1 (64)	PWD-1	1
H9-H11		2 (16-32)	PBD-2	2
H9-H12		1 (16)	PWD-1	1
H9-H15		1 (32)	PBD-1	1
H12-H13		1 (16)	PBD-1	1
H12-H15		1 (32)	PBD-1	1
H1-H9-H15		1 (32)	PBD-1	1
H3-H12-H15		2 (32-64)	PWD-2	2
H4-H5-H12		1 (64)	PWD-1	1
H4-H9-H11		2 (16-32)	PBD-2	2
H5-H9-H13		1 (16)	PBD-1	1
H6-H11-H12		1 (16)	PWD-1	1
H9-H11-H12		1 (64)	PBD-1	1
H1-H4-H12-H15		1 (16)	PBD-1	1
H5-H9-H11-H15		1 (32)	PBD-1	1
H5-H9-H12-H15		1 (16)	PWD-1	1
H9-H11-H12-H15		1 (16)	PWD-1	1
H3-H6-H12-H13-H15		1 (32)	PWD-1	1
H5-H9-H11-H12-H13-H15		1 (16)	PBD-1	1

3.3.5.1. Longitudinal pattern of haemagglutinin serotypes

Haemagglutination inhibition results suggested that different H types were either detected across the study period, were sporadically, or present for a defined period (Tables 3.14-3.16). Plumed whistling ducks appeared to be exposed throughout the study period to H5, H6, H9, H11 and H12 types. In contrast PBDs were possibly exposed to H1 over the time period but low sample numbers confounded any sound assessment for the other serotypes. The subtype H1 was also detected in MGs but not in PWDs. There was possibly a defined period of detectable levels of antibody to H4 in PWDs from December 2007 to April 2008. This may suggest a wave of infection that was subsequently lost from the population.

Table 3.14 Matrix of time versus haemagglutinin serotype for plumed whistling ducks at Billabong Sanctuary and Green Acres Lagoon (Cromarty) (from April 2007 to March 2010)

	2007									2008												2009												2010		
H	**Ap**	**M**	**J**	**Ju**	**A**	**S**	**O**	**N**	**D**	**Ja**	**F**	**Ma**	**Ap**	**M**	**J**	**Ju**	**A**	**S**	**O**	**N**	**D**	**Ja**	**F**	**Ma**	**Ap**	**M**	**J**	**Ju**	**A**	**S**	**O**	**N**	**D**	**Ja**	**F**	**Ma**
1																																				
3																	•	•						•••••••									•			
4									•••		•	••	•																							
5						•	•	•				•					•								•	•				••	•••		••••			
6							•					•						•												••	•••••	•				
7																			••																	
9				•													•							•			•		•	•	•••••	••• •••	•			
11				•					•										•••			•			•				•		•					
12										••	•	•••• ••••					•	•						••••					•	•	••		•			
13													•				•	•	•																	
15																	•	•						•	•				•	•						
n	**0**		**0**	**2**	**0**	**1**	**2**	**1**	**3**	**2**	**1**	**9**	**2**	**0**	**0**	**0**	**8**	**1**	**4**	**0**		**1**		**7**	**3**	**1**	**1**	**0**	**1**	**4**	**11**	**7**	**6**			**0**
N	**1**	**0**	**17**	**22**	**5**	**17**	**64**	**7**	**26**	**11**	**38**	**71**	**45**	**9**	**25**	**17**	**31**	**17**	**32**	**10**	**0**	**4**	**0**	**101**	**108**	**28**	**74**	**32**	**11**	**31**	**99**	**77**	**82**	**0**	**0**	**3**

n: Number of cELISA reactors tested for H serotype (78); *N*=Number of serum samples tested using cELISA (115)
• Frequency number of serotypes (Multiple serotypes in individual samples were counted individually)

Table 3.15 Matrix of time versus haemagglutinin serotype for Pacific black ducks at Billabong Sanctuary and Green Acres Lagoon (Cromarty) (from April 2007 to March 2010)

	2007									2008											2009											2010		
H	**Ap**	**M**	**J**	**Ju**	**A**	**S**	**O**	**N**	**D**	**Ja**	**F**	**Ma**	**Ap**	**M**	**J**	**Ju**	**A**	**S**	**O**	**N**	**D**	**Ja**	**F**	**Ma**	**Ap**	**M**	**J**	**Ju**	**A**	**S**	**O**	**N**	**D**	**Ja**	**F**	**Ma**
1						•	•									•		•						•					•	•	•					
3					•		•									••	••		••																	
4																		•	•																
5																		•	•					•						•	••		•••			
6																																				
7																																				
9																	•								•	••				•••••	••••••••		••			
11																	•							•						••	••					
12																	•	•										•	•	•	•					
13															•															•	••					
15																		•	•						•	•		•		•	•					
n	**0**				**1**	**1**	**1**		**0**					**0**	**1**	**3**	**4**	**3**	**4**	**0**						**1**	**3**	**0**	**1**	**1**	**5**	**12**	**0**	**5**			
N	**1**	**0**	**0**	**0**	**2**	**2**	**3**	**0**	**1**	**0**	**0**	**0**	**0**	**1**	**1**	**3**	**6**	**9**	**9**	**1**	**0**	**0**	**0**	**0**	**4**	**3**	**1**	**2**	**1**	**10**	**20**	**1**	**10**	**0**	**0**	**0**

n: Number of cELISA reactors tested for HA serotype (46); *N*=Number of serum samples tested using cELISA (91)
• Frequency number of serotypes (Multiple serotypes in individual samples were counted individually)

Table 3.16 Matrix of time versus haemagglutinin serotype for magpie geese at Billabong Sanctuary (from April 2007 to March 2010)

	2007									2008												2009												2010		
H	**Ap**	**M**	**J**	**Ju**	**A**	**S**	**O**	**N**	**D**	**Ja**	**F**	**Ma**	**Ap**	**M**	**J**	**Ju**	**A**	**S**	**O**	**N**	**D**	**Ja**	**F**	**Ma**	**Ap**	**M**	**J**	**Ju**	**A**	**S**	**O**	**N**	**D**	**Ja**	**F**	**Ma**
1																								•												
3																																				
4																																				
5																																				
6																																				
7																																				
9																																				
11				•							•																										
12											•																										
13																																				
15																																				
n	**0**	**0**		**1**	**0**		**0**		**0**		**4**	**0**	**0**		**0**	**0**			**0**			**0**	**0**	**1**	**0**	**0**	**0**				**0**	**0**				**0**
N	**3**	**3**	**0**	**3**	**4**	**0**	**2**	**0**	**3**	**0**	**25**	**8**	**1**	**0**	**9**	**2**	**0**	**0**	**3**	**0**	**0**	**1**	**1**	**7**	**4**	**1**	**1**	**0**	**0**	**0**	**7**	**3**	**0**	**0**	**0**	**2**

n: Number of cELISA reactors tested for HA serotype (6); *N*=Number of serum samples tested using cELISA (93)

• Frequency number of serotypes (Multiple serotypes in individual samples were counted individually)

3.3.6. Haemagglutination inhibition testing of the competitive enzyme-linked immunosorbent assay reactor samples (≥ 40 % inhibition) from recaptured wild aquatic birds

Haemagglutination inhibition results from the recaptured birds commonly identified H9, H6 and H12 serotypes which were also commonly identified in the total bird sample. The pattern also suggests that birds had either multiple concurrent infections or infections with different serotypes at different times (Table 3.17-3.18).

Table 3.17 Results of the haemagglutination inhibition testing of the samples reacting competitive enzyme-linked immunosorbent assay from recaptured wild aquatic birds (from April 2007 to March 2010)

Species	**n**	**Capture history**		
		1st (Day 0) **H type (by the highest titre)**	**2nd** **H type (by the highest titre)**	**3rd** **H type (by the highest titre)**
Plumed whistling ducks	1	cELISA (-ve)	Day 8; cELISA (+ve); but HI (-ve)	
	2	cELISA (-ve)	Day 41; cELISA and HI (+ve): H12 (64)	
	3	cELISA (-ve)	Day 104; cELISA (+ve); but HI (-ve)	
	4	cELISA (-ve)	Day 39; cELISA and HI (+ve): H12 and H15 (32)	
	5	cELISA (-ve)	Day 234; cELISA and HI (+ve): H6 (64)	Day 239; cELISA and HI (+ve): H4 and H6 (32)
	6	cELISA (-ve)	Day 122; cELISA and HI (+ve): H6 and H12 (16)	
	7	cELISA (-ve)	Day 171; cELISA and HI (+ve): H6 (16)	
	8	cELISA and HI (+ve): H9 (16)	Day 3; cELISA and HI (+ve): H9, H12 and H15 (16)	
	9	cELISA (-ve)	Day 24; cELISA (+ve); but HI (-ve)	
	10	cELISA (-ve)	Day 7; cELISA and HI (+ve): H5 and H6 (16)	
	11	cELISA and HI (+ve): H9 (16)	Day 6; cELISA (+ve); but HI (-ve)	
	12	cELISA (-ve)	Day 14; cELISA and HI (+ve): H9 (128)	
	13	cELISA and HI (+ve): H6, H11 and H12 (16)	Day 38; cELISA and HI (+ve): H11 and H12 (32)	
Black swan	1	cELISA and HI (+ve): H6 (64)	Day 63; cELISA and HI (+ve): H1 and H6 (32)	
Australian shelduck	1	cELISA (+ve); but HI (-ve)	Day 101; cELISA and HI (+ve): H9 (16)	c-ELISA and HI (+ve): H9 and H11 (16): Day 399
	2	cELISA and HI (+ve): H9 (64)	Day 28; cELISA and HI (+ve): H5 (64)	
Magpie goose	1	cELISA (-ve)	Day 71; cELISA (+ve); but HI (-ve)	

Table 3.18 Results of the haemagglutination inhibition testing of the samples reacting in competitive enzyme-linked immunosorbent assay from recaptured Pacific black ducks (from April 2007 to March 2010)

n	Capture history				
	1st (Day 0) H type (by the highest titre)	**2nd H type (by the highest titre)**	**3rd H type (by the highest titre)**	**4th H type (by the highest titre)**	**5th H type (by the highest titre)**
1	cELISA (-ve)	Day 13; cELISA and HI (+ve): H1, H3, H4 and H5 (16)	Day 20; cELISA and HI (+ve): H3 (64)	Day 26: cELISA and HI (+ve): H9 (32)	
2	cELISA and HI (+ve): H9 and H15 (32)	Day 4; cELISA and HI (+ve): H5, H9 and H11 (16)			
3	cELISA (-ve)	Day 3; cELISA and HI (+ve): H9 and H13 (64)			
4	cELISA and HI (+ve): H12 and H15 (32)	Day 24; cELISA (+ve); but HI (-ve)	Day 28; cELISA (+ve); but HI (-ve)	Day 31; cELISA and HI (+ve): H9 (64)	Day 45; cELISA and HI (+ve): H9 (16) and H11 (16)
5	cELISA and HI (+ve): H1 and H9 (16)	Day 61; cELISA and HI (+ve): H9 (32)	Day 70; cELISA and HI (+ve): H1 (32)		
6	cELISA and HI (+ve): H5, H9 and H11 (16)	Day 3; cELISA and HI (+ve): H5 (16) and H9 (16)			
7	cELISA and HI (+ve): H1, H9 and H15 (32)	Day 11; cELISA and HI (+ve): H9 (32)			
8	cELISA and HI (+ve): H12 and H13 (16)	Day 56; cELISA and HI (+ve): H3 (128)			
9	cELISA and HI (+ve): H9 (16)	Day 40; cELISA and HI (+ve): H9 (16)			
10	cELISA (+ve); but HI (-ve)	Day 35; cELISA and HI (+ve): H3 and H9 (16)			

3.4. Discussion

3.4.1. Species

This is the most comprehensive study on AI serology of WABs in nQLD, Australia. An average AIV antibody prevalence of 11% is not surprising as wild ducks are known to be a primary reservoir for AIVs (Olsen *et al.*, 2006). Pacific black ducks had an antibody prevalence of 45% and other common species had 6 to 8%. Pacific black ducks also had an increased level of AIV antibody as detected by linear model-C and higher odds of being seropositive in logistic Model-C. The higher antibody levels could be related to a higher level of susceptibility to AIVs or to behavioural differences which affect exposure and infection rates. Tracey (2010) found that AIV prevalence was significantly greater in dabbling ducks such as PBDs (*Anas superciliosa*) and mallards (*Anas platyrhynchos*) (3.1%), compared to other ducks such as PWDs (0.7%) and WWDs (1.0%). Avian influenza viral prevalence data from various studies have also supported mallards as having higher prevalence compared to other diving ducks (e.g., pochards and tufted ducks), for example, 10.1% in mallards and 1.6% in diving ducks (Olsen *et al.*, 2006; Fouchier *et al.*, 2007). Pacific black ducks and mallard ducks are likely to differ little in their susceptibility and transmission of AIVs (Tracey, 2010) and mallard ducks were identified as a species susceptible to AIVs as compared to other dabbling ducks such as, wigeon, teals and gadwall (examination of the susceptibility and or disease severity in inoculation experiments) (Wood *et al.*, 1985; Stallknecht and Shane, 1988; Ito and Kawaoka, 2000; Neumann and Kawaoka, 2006). Epidemiological modelling of field data also supported that mallards were most likely the reservoir community for influenza rather than other dabbling ducks such as wigeons, teals and gadwalls and diving ducks such as pochards and tufted ducks (Nishiura *et al.*, 2009).

Differences in viral prevalence between ecological guilds of ducks are probably partially related to behaviour. Dabbling ducks feed primarily on food in surface waters while diving ducks forage at deeper depths and more often in marine habitats (Del Hoyo *et al.*, 1996). Avian influenza viral prevalence is particularly higher in dabbling ducks, probably because their feeding behaviour favours ingestion of viral particles (Jourdain *et al.*, 2010). Dabbling ducks display a propensity for abmigration, the switching of breeding grounds between years, which is in part due to mate choice (Del Hoyo *et al.*, 1996). This behaviour could provide an opportunity for AIVs to be transmitted between different host sub-populations.

3.4.2. Age

Models (linear model-B and logistic model-B and C) identified an increased level of AIV antibody or higher odds of being seropositive in older ducks which might be due to more exposure to infections because of more opportunity. This result is consistent with the previous finding in coots (*Fulica atra*) in Italy and native chickens in Bangladesh where the antibody prevalence was higher in adults than in juveniles (De Marco *et al.*, 2003b; Nooruddin *et al.*, 2006). However, no reason was suggested for this difference in prevalence. In contrast, no significant age-related difference in antibody prevalence was observed between duck species (*Anas querquedula*) (De Marco *et al.*, 2003b; De Marco *et al.*, 2005).

3.4.3. Body weight

Avian influenza viral antibody level was significantly higher as body weight increased (linear model-A and B) or heavier birds had significantly higher odds of sero-positivity (logistic model-B). This may also be due to more exposure to infections because of more opportunity as weight can be a surrogate for age.

3.4.4. Year

No other comprehensive studies have investigated temporal influences on AIV antibodies in Australian wild birds. However, a previous cross sectional study documented that the AIV antibody prevalence was 17% during 2005-06, and 25% during 2006-07 in NSW, Australia (Haynes *et al.*, 2009). A similar trend of AIV antibody prevalence was observed in TAS: 21% during 2005-06 and 28% during 2006-07 (Haynes *et al.*, 2009). Marked differences in viral prevalence were also found between years in North American aquatic birds (Krauss *et al.*, 2004). These patterns of viral and AIV antibody prevalence are little understood, but an epidemiologic periodicity in immunology has been implicated (Hinshaw *et al.*, 1985). Certain events within the annual cycle of birds may also alter their susceptibility to disease. Energetic trade-offs occur between immune defences and moulting and breeding which could make birds more susceptible to diseases (Hasselquist, 2007; Buehler *et al.*, 2008; Martin *et al.*, 2008). Cyclic patterns have been described for measles and whooping cough in humans, and are determined in part by spatial factors, herd immunity, and population age-structure (Rohani *et al.*, 1999). Variable serology between years for PWDs (linear models-A and B) are therefore supported by these earlier findings. Year was not identified as a significant factor in logistic models-A and B. This may be due to reduced sensitivity of the logistic analysis due to the potential creation of false negatives for samples with inhibition values below 40%. Differences in rainfall between years may explain a possible association of increased antibody levels in

2009 (average rainfall: 131 mm in 2008 and 161 mm in 2009; average temperature: 23°C in 2008 and 24°C in 2009; SILO, Department of Environment and Resource Management, Queensland). These adverse environmental factors (such as warm wet) could suppress the immune system of birds thereby rendering them more susceptible to an infectious disease like AI. An increased bird population density was also observed in the second year as compared to the first year (for example, in dataset C, this study sampled 312 PWDs in 2008 and 647 PWDs in 2009). Therefore, there is more opportunity to transmit infections between individuals thereby increasing the number of AIV antibody positive birds in 2009. Increased local densities of breeding birds may also increase transmission of parasites and other diseases between birds (Poiani, 2006).

3.4.5. Season

Ducks sampled during warm wet weather (January–April) also had higher AIV antibody compared to warm dry weather (September-December) (linear model-A). In logistic model-A, the odds of being seropositive were higher in ducks sampled during the warm wet period, but the difference was not statistically significant which could be due to the use of a binary response variable as discussed above. A similar seasonal effect on AIV antibodies was observed in a study of native chickens in Bangladesh where the highest seroprevalence (15%) was detected in the monsoon season and the lowest (4%) in winter (Nooruddin *et al.*, 2006). They explained that the influence of hot weather and rain during the monsoon season might reduce the immune status of the birds, thus making them more vulnerable to infection. March-April (partly of wet weather) is a breeding period for PWDs as observed in this study. Birds might also have been immunologically suppressed at breeding time which could also encourage exposure to AIV infections.

Australian waterfowl tend to be nomadic with their movements dictated by available food supplies and rainfall, rather than truly migratory (Whitworth *et al.*, 2007). Anatid numbers in northern Australia fluctuate seasonally, where large numbers can congregate during the dry (May-October) period and disperse very widely during the wet (November-April) period (Morton, 1990). A similar seasonal fluctuation of bird abundance was also observed in this study and therefore mixing with other birds (such as shore and migratory birds) during the dispersion period may enable interspecies transmission of viral infections. For example, there are about three million shorebirds birds from 35 species belonging to the Charadriiformes, which regularly migrate from Australia each year (Anon, 2002). Most depart from Australia in March for breeding areas (north-eastern Siberia and Alaska) (Lane and Jessop, 1985) and migrants return in September and spend late spring and summer in coastal and inland Australia (Tulp *et al.*, 1994). Of particular relevance is that Charadriiformes can congregate in extremely

large concentrations on coastal floodplains and wetlands (Morton *et al.*, 1993), where they regularly interact with *Anatidae* (Morton, 1990).

Seasonal variation in the prevalence of infection is a common phenomenon among infectious diseases in humans and wildlife (Altizer *et al.*, 2006). A number of studies reported that the AIV prevalence in wild birds may also exhibit seasonal fluctuations, with a yearly peak in late summer and early fall, followed by low prevalence during the winter period (Hinshaw *et al.*, 1980b; Krauss *et al.*, 2004; Munster *et al.*, 2007; Wallensten *et al.*, 2007). Seasonal differences of AIV prevalence were also observed in wild migratory birds in Alaska (Ip *et al.*, 2008). Prevalence rates were higher in the spring (prior to 1 June) (2.5%), declined in summer (1 June-1August) (0.03%) and increased in the autumn (after 1 August) (3.1%).

Separation of seasonal effects on AIV antibody levels might be difficult if antibodies are detectable for long periods, for example, 6-12 months as observed in captive mallard ducks naturally exposed to AIVs (Fereidouni *et al.*, 2010a). In contrast, this study only detected antibodies lasting for up to a maximum of ~two months. Some experimental studies also demonstrated a shorter antibody persistency e.g. mallard ducks (up to 30 days) (Sinnecker *et al.*, 1982), 42 days in Mallard (Costa *et al.*, 2010), ≥ 40 days in Australian WWDs and grey teal (J. Curran, personal communication).

3.4.6. Gender

Females had higher AIV antibody levels (linear model-B) and higher odds of sero-positivity in logistic model-B suggesting that female birds were more susceptible to AIVs than male birds. Similar findings were supported by Nooruddin *et al*. (2006). Avian influenza viral prevalence was also reported to be higher in female than male waterfowl (Runstadler *et al.*, 2007). Female ducks could be weaker during the breeding period due to shortage of necessary nutrients, the obvious stress of laying eggs and post-laying demands increasing their risk of infection. Birds require more nutrients for their breeding period (Poiani, 2006).

In brief, risk factor analysis identified that PBDs, adult and heavier ducks, female ducks, year 2 and possibly warm wet weather had increased AIV antibody levels. These findings will significantly contribute to the design of a serosurveillance program by wildlife authorities.

3.4.7. Distribution of haemagglutinin serotypes of avian influenza

The present study identified a wide range of H subtypes throughout the study period which is suggestive of constant circulation. This study also suggested the possibility of short periods of infection where the subtype was subsequently lost from circulation. This pattern suggests a wave of infection in this wild bird population.

The serosubtypes of H3, H5, H6, H9, H11 and H12 were common in this study and reflect the diversity of serotypes reported from wild birds in several countries: H9 in mallards, geese and Indian fowls in Pakistan (Khawaja *et al.*, 2005); H1-H3, H5-H6 and H9-H10 in wild waterfowl (such as mallards) in China (Hua *et al.*, 2005); H5 in wild and captive birds in the United Arab Emirates (Obon *et al.*, 2009); H1-H4, H6-H7 and H10 in mallards, sentinel Pekin ducks, swans and geese in Germany (Suss *et al.*, 1994); H1-H3, H5-H6, H8-11 and H13 in mallards, dabbling and diving ducks in Italy (De Marco *et al.*, 2005); H8 in wild ducks in USA (Senne, 2003); H6 in WABs in Australia (Curran, 2010) and H13 in silver gulls in TAS, Australia (Haynes *et al.*, 2009) and H6N5 in noddy terns on Tryon Island on the Australian Great Barrier Reef (Downie and Laver, 1973).

Antibodies corresponding to multiple AIV serotypes were frequently observed in individual birds. In other studies, individual ducks have yielded more than one influenza virus from a single swab sample (Halvorson *et al.*, 1983). Nucleic acid sequencing identified mixed infections of H9-and H6-subtype viruses in mallard ducks (Jackwood and Stallknecht, 2007). These results support the detection of multiple serotypes in this study. In addition Fereidouni *et al*. (2010a) found that ducks immunized with either inactivated LPAI H5N2 or H7N1 did not produce cross-reactive antibodies when serum samples were tested using the HI assays. All of these findings indicate that birds can be infected with more than one AIV serotype throughout their lives.

Low HI antibody titres in this study are in line with the previous findings, showing scarcely detectable HI antibody responses or lack of significant HI antibody responses after AIV infections in ducks as compared to chickens (Kida *et al.*, 1980; Suarez and Schultz-Cherry, 2000; Alexander, 2003; De Marco *et al.*, 2003b; Stallknecht and Brown, 2008).

3.4.8. Seroconversion of avian influenza viral antibody

A definite increasing, decreasing and fluctuating change of AIV antibodies, as well as newly diagnosed cELISA reactor serum samples from recaptured birds, with time, indicate a potential AIV sero-dynamism in that bird population, even though most of the recaptured birds were

negative. A preponderance of recaptured birds having an increased antibody level or newly diagnosed sero-positivity for previously non-reactive birds as recorded in this study parallels the results also obtained from recaptured birds in Italy where most recaptured ducks had increased antibody levels that were suggestive of recent infection by AIVs (De Marco *et al.*, 2005). These results suggest AI sero-dynamism in wild bird populations.

Persistence of AIV antibody for over two months as observed in PBDs (66-85% inhibition) is probably the first observation of persistence for this length of time outside of the laboratory in Australia. However, possible reinfection with different AIV subtypes during this period can not be ruled out.

Some earlier experimental studies provide some evidences for a similar level of antibody persistence. Avian influenza viral antibodies were observed for up to 30-42 days in mallard ducks (Sinnecker *et al.*, 1982; Costa *et al.*, 2010) and ≥ 40 days in Australian WWDs and grey teal (J. Curran, personal communication). Additionally when juvenile mallards were naturally exposed to AIVs, the antibodies lasted for six months during captive monitoring (Fereidouni *et al.*, 2010a). This study also showed that seropositive ducks further infected with the LPAIV H5N2 virus increased the antibody detection period up to 12 months. Upon renewed exposure to H5N2 strain, a boost of AIV antibody titres was also observed in naturally infected ducks compared to naïve mallards. This is assumed to be caused by a priming effect of the proposed natural AIV infection of ducks rather than the effect of the highly infectious doses used. However, this study does not represent a field situation as experiments were carried out in captive birds with the potential influence of captivity and other infections on specific antibody response.

3.4.9. Limitations

The main limitation of the study is that only 45-55% of the targeted sample size was analysed which could decrease the sensitivity and power of the study. However, this study estimated a slightly higher prevalence of 11% than the expected prevalence of 10% reducing the necessary sample size. The main reason for reduced sample numbers was low population densities during some periods, especially during periods of high rainfall, and trap shyness.

In addition the results may not represent the population under investigation because the likelihood of trapping individual birds may be variable. Trapped birds may not be a random sample of the population. Species representation of sampled birds seemed to be correct as per total population of individual species of birds visited the study sites. However, whether AI

positive or AI negative birds were more frequently sampled was not sure with certainty. It was also uncertain whether young or adult birds were frequently caught in this study.

3.4.8. Recommendations

To expedite the identification of the full set of risk factors associated with the AIV antibodies (bird demography, bird species and their ecology, spatial and temporal factors) a long term longitudinal study should be carried out on a wide range of birds.

For example, waders are also thought to be the natural reservoir for LPAI (Stallknecht *et al.*, 1990b) and this species belonging to the *Charadriidae* and *Scolopacidae* families often live side-by-side with ducks in marine and wetland areas. Waders also play an important role in perpetuating certain virus subtypes in North America and Canada (Krauss *et al.*, 2004); therefore an emphasis should be given to waders in coastal wetlands of Australia for investigation in future AI investigations. It can also be specifically tested whether influenza viruses can be perpetuated in ducks alone, or the importance of interaction between ducks and shorebirds as suggested in North America (Krauss *et al.*, 2004). The likely interaction between ducks and shorebirds in areas, where migratory birds from the northern and southern latitudes mix, is of particular interest. It is reported that LPAI infects 100 host species in 26 families, including many whose primary habitat is not aquatic (Olsen *et al.*, 2006). Therefore, there is a possibility that the reservoir species for AI in Australia is not a water dependent species.

A study covering a wide range of aquatic and non-aquatic bird species will classify birds as endemic, opportunistic and an indicator species for AI in Australia or capable of answering which species or group of species are potential reservoirs for these viruses in Australia.

This study is biased towards species that were easy to sample, ubiquitously present, and within reach. This study had sufficient samples from PWDs to draw a reasonable conclusion on the temporal and spatial effect on AIV antibody levels in this study. However, sample sizes for other species (e.g. PBDs and MGs) were not sufficient to test spatial and temporal effects on AIV antibody levels even though AIV antibody prevalence is very high in PBDs. Therefore, in order to ensure sufficient sample sizes from those species an understanding of their ecology is needed to select appropriate study sites. For instance, geese are mainly herbivorous and often congregate in large flocks for grazing in pastures and agricultural fields, especially during the non-breeding season and large flocks of AWI are always seen around garbage areas.

This study did not have the full potential to estimate the true AIV sero-prevalence and the associated risk factors on WABs of the Atherton Tableland WABs even though the Mareeba

area has been identified as a risk area for AI emergence in poultry (Tracey, 2010). Therefore, a further AI study should focus on this particular area.

The sero subtypes of H5 and H7 have been circulating in the bird population of nQLD as indicated by HI testing. These results indicate that LPAI H5 and H7 viral subtypes are active in that population. Therefore, there is a risk that LP H5 or H7 viruses, which can subsequently mutate from LPAI to HPAI subtypes (Kawaoka *et al.*, 1987), may be introduced into poultry flocks through contact with waterfowl (Campitelli *et al.*, 2004).

Chapter 4: Active surveillance and molecular epidemiology of avian influenza in wild aquatic birds

4.1. Introduction

Wild aquatic birds are considered a natural reservoir of AIVs and harbour all known subtypes of the influenza A virus (Brown *et al.*, 2007; Munster *et al.*, 2007). Wild species may play an important role in the epidemiology of AI but little is known about the distribution and dynamics of this pathogen in wild birds throughout the world.

Australia has had five outbreaks of HPAI in commercial chickens (Selleck *et al.*, 2003; Westbury, 2003). All those outbreaks have been caused by HPAI H7 subtypes. Drinking of water contaminated with aquatic bird faeces was suspected to be the source of infection in at least two of the five Australian HPAI outbreaks. Moreover, the HPAI H5N1 strain has been confirmed in one of Australia's closest neighbours Indonesia (Capua and Alexander, 2004) and importantly, due to its proximity to nQLD, its province of west Papua on the island of New Guinea (McCallum *et al.*, 2008).

Transmission of AI to humans, in close contact with poultry or other birds, occurs only with some strains of AIVs. The first occurrence of bird-to-human transmission was reported in Hong Kong in 1997 (Subbarao *et al.*, 1998). The virus re-emerged in poultry in 2003 across Asia and was ultimately transmitted to humans in Thailand and Vietnam (Peiris *et al.*, 2004). By 31st August 2010 HPAI H5N1 had spread to 63 countries in Asia, Europe and Africa and the human death toll due to the H5N1 was 300 (N=505 infected cases) between 2003 and 2010 (Anon, 2010b). Indonesia had the highest number of infected cases (168) and deaths (139) (Anon, 2010b). There is worldwide concern that HPAI H5N1 may evolve the capacity for human-to-human transmission resulting in a pandemic (Alexander, 2007).

Migratory birds are assumed to represent a risk for the spread of HPAI H5N1 worldwide (Fergus *et al.*, 2006). A H5N1 outbreak in wild migratory birds on Lake Qinghai, western China in May 2005 (Lei *et al.*, 2007; Wang *et al.*, 2008) poses serious concerns because this lake is a major breeding site for migratory birds whose flyways extend to south-east Asia, India, Siberia, Australia and New Zealand. Moreover, isolation of the H5N1 virus has been reported in over 60 species of wild birds (Ellis *et al.*, 2004; Chen *et al.*, 2005; Kwon *et al.*, 2005; Liu *et al.*, 2005). Although Australia has been free of HPAI for a number of years, there is an increased concern that the H5N1 virus could be introduced via migratory birds which travel between Australia and

south-east Asia where HPAI is endemic (East *et al.*, 2008b). Specifically, migratory birds could introduce HPAI into nQLD, Australia, as it is on an important flight path. Therefore, the genetic characterization and surveillance of AIV subtypes in WABs in nQLD is important.

Opportunistic cross sectional studies have been conducted on the virology of AI in Australian wild birds (Downie *et al.*, 1977; Senne, 2003; Haynes *et al.*, 2009), but longitudinal studies with the aim of estimating AIV RNA prevalence, determining most suitable samples for future AI surveillance and determining the molecular epidemiology of AIV subtypes have rarely been investigated. Therefore, this study undertook the above during a three-year longitudinal study in nQLD from April 2007 to March 2010.

4.2. Materials and methods

4.2.1. Study sites and sampling

Study sites and sampling birds were described as outlined previously (section 3.2.1; Chapter 3).

4.2.2. Swab sample collection and recording of epidemiological data

Swabs were taken from captured birds by inserting a swab (rayon cotton swab stick, Sarstedt, Copan Italia S.p.A. Italy) deeply into the vent (cloacal) or oropharyngeal airway and swabbing the mucosal wall. The tip of the plastic-shafted swab was immediately placed into a vial containing 1 ml sterile, chilled Dulbecco's modified eagle's transport medium (DMEM) (TropBio. Cat. No 50-020-PB). The viral transport medium was stored in an insulated container with ice packs before use. Samples were transported to the JCU Virology Lab within five to six hours of collection in an insulated container with ice bricks and stored at -80°C. Prior to release, the captured birds were marked by using appropriately sized identification leg bands.

Epidemiological data were recorded and measured as previously described (Section 3.2.1; Chapter 3).

4.2.3. Environmental faecal sample collection and recording of epidemiological data

Faecal samples were collected from WABs on the ground at Billabong Sanctuary and Cromarty (Green Acres Lagoon and McLain Rd Nature Reserve Lagoon) using sterile cotton swabs from October 2008 to January 2010. Only freshly deposited moist samples were collected. The faecal samples were identified to species by observing defecation or observation of birds in an area

immediately prior to collecting samples and using size and shape of the faeces which is distinguishable between different groups of species (duck, goose, ibis, swan etc). Individual samples were given unique identity numbers. Date of collection, site and species were recorded for each sample using structured record keeping sheets. Sample transportation and storage details were identical to methods used for swabs of cloaca or oropharynx.

4.2.4. Sample processing

Frozen field samples were defrosted on the bench in an air conditioned room (22°C) for an hour. Sample tubes were then gently vortexed for 10 seconds and mixed before transfer to a-96 well lysis block. A total of 200 µl of sample (test and controls) was added to each allocated well before being placed into a Corbett Robotics X-tractor Gene™ automated RNA/DNA extraction system (Corbett Robotic, Brisbane, Australia). Sample loading into the lysis block was performed in a class I cabinet. The Universal Liquid Sample Protocol (Viral RNA/DNA Purification Protocol, CorProtocolTM No. 25101 version 03) and Corbett reagent kits were used for processing samples.

In brief, 100 µl of viral digest buffer (VXL) (with 10% digestion buffer additive vol/vol and 1% RNA carrier vol/vol) was added to each well of the lysis plate and the sample was then mixed four times followed by 10 minutes incubation at room temperature. A volume of 400 µl of VX binding buffer (VXB) was added to the lysis plate followed by an addition of 600 µl of the lysate on to the capture plate (pre-mixed five times). A vacuum of 25 kPa was applied for one minute. A total of 200 µl of VXB was again loaded on to the capture plate and then a vacuum of 25 kPa was applied for one minute. After one minute incubation, 600 µl of VX wash buffer (VXW) was loaded on to the capture plate and a vacuum of 25 kPa was used for one minute repeated for two iterations. A total of 600 µl of VX final wash (VXF) was loaded on to the capture plate and a vacuum of 25 kPa was applied for one minute. A vacuum of 25 kPa was applied for five minutes to dry the plate. When the carriage was moved to the elution chamber, 80 µl of elution buffer (E) was loaded on to the capture plate. The sample was then incubated for five minutes and a vacuum of 25 kPa was applied for one minute.

An extraction run contained up to 60 test samples, two AIV negative controls (normal allantoic fluid) and one positive control (either H4N4 positive allantoic fluid (A/chestnut teal/VIC/2004) or Bovine Viral Diarrhoea (BVD) virus positive allantoic fluid (BVD/C5.101/non-cytopathic/nQLD/1987). After extraction the lysis plate was immediately transferred to a -20ºC freezer for storage before further testing. Extraction of RNA followed by real time reverse

transcriptase polymerase chain reaction (rRT-PCR) analysis was performed within six months from the time that the samples were obtained from the field.

4.2.5. Avian Influenza viral ribonucleic acid screening from field samples

Molecular detection of AIV RNA was performed using one step rRT-PCR directed at the M gene, which detected all influenza A subtype viruses. Two parallel assays were used as described by Heine *et al*. (2007) (now designated as AAHL assay) and Ward *et al*. (2004) (designated as Ward assay). The AAHL assay was performed using a TaqMan Dual Labelled Probe FAM/BHQ-1(Bio-search Technologies, USA) while the Ward assay was done using DNA binding fluorescent dye SYTO®9 (Invitrogen, USA).

Invitrogen Superscript™ III Platinum® One step rRT-PCR kits were used. The 20 µl reaction-mix volume for the AAHL assay contained 2 units (U) of Superscript™ III Platinum® Taq polymerase, 0.4 µM forward and 0.8 µM reverse primers and 0.1 µM probe and 5 µl of RNA template extract.

The 20 µl reaction-mix volume for the Ward assay constituted 2-U Superscript™ III Platinum® Taq polymerase, 0.4 µM each Ward forward and reverse primers and 2 µM SYTO9 dye and 5 µl of RNA template extract. The primers and probe details are presented in Table 4.1.

Seventy two-well gene discs (Corbett) were used for polymerase chain reaction (PCR) amplification which contained 60 test RNA samples and 12 control RNA samples per run. Reactions were performed in a RotorGene 3000 or 6000 machine (Corbett Robotic, Brisbane, Australia).

Temperature cycling for the AAHL assay included 30 minutes at 48°C for reverse transcription (RT) and complementary DNA (cDNA) synthesis followed by two minutes at 95°C to activate the hot-start Taq DNA polymerase and 40 cycles of 15 seconds at 95°C for denaturation and 30 seconds at 60°C for primer annealing and extension.

For the Ward assay, the RT step was 30 minutes at 48°C, followed by 10 minutes at 95°C to activate the hot-start Taq DNA polymerase. The cycling conditions consisted of 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C and 20 seconds at 72°C. After the amplification, the melting temperature of the PCR product was determined by progressively increasing the temperature by 0.2°C from 75 to 95°C, with fluorescence acquisition on the FAM channel (excitation at 470 nm, detection at 510 nm) and plots of the rate of change in fluorescence

versus temperature were produced by the RotorGene 3000 or 6000 software. The melting temperature of the amplified product was determined and represents the point of maximum rate of change in fluorescence and the temperature where 50% of the product had denatured. Melting temperature values between 80ºC and 88ºC (Ward assay) were considered indicative of a positive. Melting temperature values below these temperatures were considered to be the result of non-specific amplification or primer interaction.

Real time RT-PCR runs were acceptable when the negative and non-template controls did not fluoresce and the positive controls had fluorescent signals which crossed the auto-set threshold level within 40 cycles.

Any test sample that was reactive to either of the assays with the threshold (C_T) value of ≤ 40 was considered as positive for AIV RNA in this study. A cut-off C_T value of 40 was used for negative samples of wild birds based on the findings that samples that had C_T values ≤ 40 had grown viruses successfully in embryonated chicken embryos (Munster *et al.*, 2009).

Table 4.1 Avian influenza viral real time reverse transcriptase–polymerase chain reaction primers and probe used for avian influenza viral ribonucleic acid screening of field samples

Gene	**Primer sets/Probe**	**Sequence (5'-3')**	**Position**	**Expected product length (bp)**	**Reference**
Matrix	AAHL/F	AGATGAGYCTTCTAACCGAGGTCG	38-61	101	(Heine *et al.*, 2007)
	AAHL/F	TGCAAANACATCYTCAAGTCTCTG	115-138		
	Probe	FAM-TCAGGCCCCCTCAAAGCCGA-BHQ1			
	Ward/F	AAGACCAATCCTGTCACCTCTGA	183-205	95	(Ward *et al.*, 2004; Munster *et al.*, 2005)
	Ward/R	CAAAGCGTCTACGCTGCAGTCC	256-277		

F-Forward, *R*-Reverse; *FAM*-5 (6)-Carboxyfluorescein; *BHQ*-Black Hole Quencher; *bp*: Base pairs

4.2.6. Examination of real time reverse transcriptase-polymerase chain reaction inhibitors

Initially five cloacal and five oropharyngeal swab extracts were assessed for their potential to inhibit PCR using the TaqMan® Exogenous internal positive control (IPC) from Applied Biosystems. A total of 20 µl reactions included 5 µl of extract (single analysis) or water (triplicate), 1x TaqMan® Universal master mix and 1x Exo IPC mix and Exo IPC DNA. The analysis was performed on the RotorGene™ 6000 (Corbett Research) using Gene-Disc 100 tubes.

Temperature cycling involved two minutes at 50°C (carry over prevention using uracil DNA glycosylase), 10 minutes at 95°C (polymerase activation), followed by 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Threshold values (PCR cycle at point of detection) were calculated by the RotorGene™ software 1.7 using a threshold of 0.01. Inhibition was indicated by complete failure of the IPC to amplify or a C_T value significantly higher than that produced by water as the test solution.

A further 42 extracts representing, 21 cloacal and 21 oropharyngeal swab extracts, were tested for their inhibitory effect on PCR using the IPC. In this case, the same polymerase and master mix were used, as used in the rRT-PCR assays (Invitrogen Superscript™ III Platinum® One step rRT-PCR kits) to ensure representative results. A 15 µl reaction-mix contained 3 µl of Superscript™ III RT/Platinum® Taq polymerase mix, 1x Superscript master mix, 1x Exo IPC mix and Exo IPC DNA and 3.75 µl of RNA extract or water. The analysis was performed on the RotorGene™ 6000 (Corbett Research) using Gene-Disc 100 tubes. Duplicate analyses were performed for each sample and non template control (water). Three no amplification control reactions (containing the PCR inhibitor supplied with the IPC kit at the recommended concentration) were also included to ensure that increased fluorescence was due to amplification and not due to probe degradation by some other mechanism. Threshold values (PCR cycle at point of detection) were calculated by the RotorGene™ software 1.7 using a threshold of 0.01. Temperature cycling involved two minutes at 95°C, followed by 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

4.2.7. Viral isolation in chicken embryos

Culturing of AIVs in 9-11 day-old specific pathogen free embryonated chicken eggs was attempted for the reactor samples as determined by the M rRT-PCR assay. The AAHL standard protocols were used for culturing viruses in embryonated chicken eggs (Anon, 2006b). Bacterial contamination was checked by streaking blood agar plates, followed by incubation for 48 hours at 37°C. To remove bacterial contamination, the allantoic fluid (AF) was passed through a 200 nm pore-size syringe filter.

Briefly, 100 µl of DMEM suspensions were injected into the allantoic cavity of 9-11 day-old embryonated chicken eggs, which were then incubated for three days at 37°C. Embryonic death within the first 24 hours of incubation was regarded as non-specific and these eggs were discarded. Embryonated eggs that died after 24 hours of inoculation were refrigerated. Thereafter, the AF was collected and harvested and tested for influenza A virus by using HA assays with 0.5% washed chicken erythrocytes. When the HA titre was negative, the AF was

passaged once again up to three times in embryonated chicken eggs. If HA activity was not detected in any of the passages, the sample was considered negative.

4.2.8. Analysis of avian influenza viral genes

Field samples that potentially reacted in AIV RNA screening tests were further analysed to obtain sequences for the M, H, non structural protein (NSP) and nucleoprotein (NP) genes. Two-step RT-PCR was performed to amplify large fragments of M, H, NSP and NP genes.

4.2.8.1. Complementary deoxyribonucleic acid amplification

Initially, two different kits were tested for cDNA synthesis: Thermoscript RT-PCR system (Invitrogen) and ImProm-II™ Reverse Transcription System (Promega). This comparative trial showed that Promega kits were slightly better than Invitrogen kits in cDNA synthesis followed by PCR (data are not shown). Moreover, Promega kits were economically cheaper than Invitrogen kits. Therefore, Promega kits were used for evaluation of samples in this study.

A template mix of 5 µl was prepared in a 0.2 ml PCR tube for a reaction, which contained 10 mM of a universal 12 bp primer (Table 4.2) and 4 µl of extracted RNA template. The template mixes were then incubated in an Eppendorf Mastercycler at 70°C for five minutes to denature RNA secondary structures. The samples were then rapidly cooled to 4°C for five minutes to prevent renaturation of the RNA template. A universal 12 bp primer targets a conserved sequence located at the 5′ end of each influenza A gene segment, allowing cDNA synthesis of the entire viral genome.

For ImProm-II™, a total volume was 20 µl per reaction that contained 1× ImProm-II reaction buffer, 3 mM $MgCl_2$, 0.5 mM of RNase inhibitor, 1 U/µl of ImProm-II Reverse transcriptase enzyme and 5 µl of chilled template mix.

For GoScript, each reaction volume of 20 µl constituted 1× GoScript reaction buffer, 3 mM $MgCl_2$, 0.5 mM of deoxynucleotidetriphosphate (dNTP) mix, and 1 U/µl of GoScript M-MLV Reverse transcriptase enzyme and 5 µl of chilled template mix.

Reverse transcription reaction conditions were 30 minutes at 45ºC (primer annealing) followed by 60 minutes at 50°C (extension). All RT reactions were carried out using an Eppendorf Mastercycler Gradient thermocycler. Complementary DNA products were stored either in 4ºC or -20ºC.

4.2.8.2. Polymerase chain reaction

Polymerase chain reaction products representing overlapping sequences within the M, H, NSP and NP genes were amplified using the primers supplied in Table 4.2.

Table 4.2 Primer sets used for sequencing different avian influenza viral genes

AI genes	Primer sets	Sequence (5'-3')	Position	Expected product length (bp)	Reference
All genes	Universal primer/F	AGCAAAAGCAGG	1-12	Not applicable	(Hoffmann *et al.*, 2001)
M	JCU-1/F (1st set)	AAGACAAGACCAATCCTG	164-181	767	(Burgess 2009; unpublished)
	JCU-1/R (1st set)	TCATAGACTCAGGCACTC	913-930		
	JCU-2/F (2nd set)	AAGATGAGYCTTCTAACC	23-40	769	
	JCU-2/R (2nd set)	RAGAGGATCACTTGAATC	774-791		
	JCU-3/F (3rd set)	GGAGAYCCAAACAACATG	287-304	504	
	JCU-3/R (3rd set)	RAGAGGATCACTTGAATCG	773-790		
	JCU-4/F (4th set)	GGAGACCCAAACAACATG	287-304	564	
	JCU-4/R (4th set)	AGACGATCAAGAATCCACAATATC	827-850		
Four genes (M, H, N & NSP)	Jindal/F	TATTCGTCTCAGGGAGCAAAAGCAGGDKB	1-14	1,027 (M), 1,770 (H), 1,413 (N) & 890 (NSP)	(Jindal *et al.*, 2009b)
	Jindal/R	ATATCGTCTCGTATTAGTAGAAACAAGGKKSTT	1,758-1,777		
H	Phipps/F	GGAATGATHGAYGGNTGGTATGG	1,110-1,132	668	(Hoffmann *et al.*, 2001; Phipps *et al.*, 2004)
	Hoffman/R	AGTAGAAACAAGGGTGTTTT	1,758-1,777		
H	Gall/F-1	GGRGAATGCCCCAAATAYGT	967-986	164-176	(Gall *et al.*, 2008)
	Gall/F-2	GGRARATGCCCCAGRTATGT	967-986		
	Gall/F-3	GGRGAATGCCCCAARTAYAT	967-986		
	Gall/R-1	CTGAGTCCGAACATTGAGTTGCTATGVTGRTAWCCATACCA	1,142-1,120		
	Gall/R-2	CTGAGTCCGAACATTGAGTTYTGATGYCTGAADCCRTACCA	1,142-1,120		
H6	JCU-1/F	AACAATTCAACAACACAAGT	93-114	1,002	(Burgess, 2009; unpublished)
	JCU-1/R	AGTCCATCCTCCTTCAATA	1,078-1,096		
	JCU-2/F	AAGAACTGAAGGCACTTATTG	363-383	1,291	
	JCU-2/R	CCTACCAAGACCAGRCTG	1,636-1,653		
H9	JCU-1/F	ACACAGAGCACAATGGAA	174-191	1,459	
	JCU-1/R	GCAAGCACAAGAGATGAG	1,615-1,632		
NSP	JCU-1/F	TTCCAACACTGTGTCAAG	32-49	619	
	JCU-1/R	CTCATTACTGCTTCTCCAA	634-652		
NP	JCU-1/F	AATTGGAAGATTCTACATAC	150-169	1,391	
	JCU-1/R	AATTGTCATACTCCTCTG	1,523-1,540		
	JCU-2/F	CYCAAGGCACCAAACGAT	53-70	1,433	
	JCU-2/R	GTCRAAGGAAGGCACGAT	1,468-1,485		

H-Haemagglutinin; *N*-Neuraminidase; *NSP*-Non structural protein; *NP*-Nucleoprotein; *JCU*-James Cook University; *F*-Forward; *R*-Reverse

4.2.8.2.1. Polymerase chain reaction amplification of the matrix gene

Following cDNA synthesis PCR was performed using four sets of nested primers to amplify overlapping sections of the M gene. The first round of PCR was generated either with the first or second sets of M primers followed by a second round of PCR using the first PCR reaction as a template and using the third or fourth sets of M primers. Both the first and second round reactions were then analysed by agarose gel electrophoresis (see details in section 4.2.8.3).

A second strategy was also used where the initial round of PCR amplified the M, H, N and NSP genes using the primers designed by Jindal *et al*. (2009b). A second round of PCR was performed using either the first or second set of M primers followed by a third round of PCR using the third or fourth sets of M primers. The final reactions were then analysed by agarose gel electrophoresis (details in section 4.2.8.3).

Both positive and negative controls were used to check the validity of each PCR run. The primer details are given in Table 4.2. One time GoTaq hot-start PCR mix (Promega) was used in each reaction mix preparation of all PCRs. Other reaction components are given in Table 4.3.

Table 4.3 Components of master mix preparation per reaction of polymerase chain reaction

	Template used		
PCR reaction mix components	cDNA	cDNA	PCR Products
Forward primer	0.4 μM for first or second set of M primers	0.4 μM for Jindal primer	0.4 μM for third or fourth set of M primers
Reverse primer	0.4 μM for first or second set of M primers	0.4 μM for Jindal primer	0.4 μM for third or fourth set of M primers
Template	2.0 μl	2.0 μl	0.5 μl (1in 100 dilution)
Total volume	**30.0 μl**	**15 μl**	**30 μl**

Polymerase chain reaction cycling conditions for the M primers included 95°C for two minutes to activate the enzyme, followed by 40 cycles of 95ºC for 15 seconds, annealing at 50°C (for first or second sets of primers), 57ºC (for third or fourth sets of primers) for 15 seconds and extension at 72°C for 20 seconds, followed by a final extension step of 72°C for 10 minutes.

Cycling parameters for PCR using the primers of Jindal *et al.* (2009b) were 95ºC for two minutes, followed by 40 cycles of 95ºC for one minute, and then 50ºC for 1.5 minutes and extension at 72ºC for five minutes, followed by a final extension step of 72°C for 10 minutes.

4.2.8.2.2. Polymerase chain reaction amplification of the haemagglutinin gene

For the H gene amplification, PCR products were used as a template (either 1 in 50 or 1 in 100 dilution) or cDNA. The PCR products were generated using the Jindal primers (Jindal *et al.*, 2009b) and cDNA was generated using the universal primer (Hoffmann *et al.*, 2001).

Phipps (H1-15) (Phipps *et al.*, 2004), H6 and H9 primers were used in the second round of PCR to amplify the H gene (Table 4.2). In addition, a combination of Gall forward (Gall *et al.*, 2008) and Jindal reverse (Jindal *et al.*, 2009b) primers were also used in a second round of PCR for the H gene amplification.

For the H gene amplification, each reaction volume mix was 30 µl which contained 1× Go Taq mix, 0.4 µM each forward and reverse primer and 0.5 µl template. The PCR cycling parameters were 95ºC for two minutes followed by 35 rounds of 95ºC for one minute, 50°C for 1.5 minutes and 72°C for five minutes, followed by a final extension step of 72°C for 10 minutes.

4.2.8.2.3. Polymerase chain reaction amplification of the non-structural protein gene

Polymerase chain reaction products amplified with the Jindal primers (Jindal *et al.*, 2009b) (1 in 100 dilutions) were used as templates for the NSP gene amplification. A total volume of 30 µl reaction mix was used which included the addition of 2 µl template. The master mix included 1×GoTaq Hot Start PCR mix, 0.4 of each µM NSP forward and reverse primers (Table 4.2), 3 µM $MgCl_2$ and 7 µl molecular grade water.

The PCR cycling parameters were 95ºC for two minutes followed by five cycles at 95°C for 15 seconds, 58°C for 15 seconds and 72°C for 20 seconds and then 35 cycles at 95°C for 15 seconds, 53°C for 15 seconds and 72°C for 20 seconds. The final extension step was at 72°C for 10 minutes.

4.2.8.2.4. Polymerase chain reaction amplification of the nucleoprotein gene

Polymerase chain reaction products generated by the Jindal primers (Jindal *et al.*, 2009b) (1 in 50 dilution) were used as templates for the NP gene amplification. A total volume of 30 µl reaction mix included 1× GoTaq hot-start PCR mix, 0.4 µM each NP-forward and-reverse primers (Table 4.2) and 1 µl of template.

The cycling parameters were 95ºC for two minutes followed by five cycles at 95°C for 15 seconds, 55°C for 15 seconds and 74°C for 105 seconds and then 35 cycles at 95°C for 15 seconds, 50°C for 20 seconds and 74°C for 105 seconds. The final extension step was set at 74°C for 10 minutes.

4.2.8.3. Agarose gel electrophoresis and sequencing

Polymerase chain reactions were visualised in agarose gel electrophoresis to ascertain if the correct fragment size had been amplified and that the product was of sufficient concentration and purity for sequencing. A range of 1-1.5% agarose gels were prepared by adding 1-1.5 g of agarose (Fisher Biotech Australia) to 100 ml of TAE buffer (a mixture of Tris base, acetic acid and ethylene diamine tetra-acetate) and heating in a microwave until the agarose was completely dissolved (approximately two to three minutes). Ethidium bromide was then added to the solution and the gel cast was allowed at least 30 minutes to set. Polymerase chain reaction products were mixed 1:1 with loading dye (comprised of orange G dye in water/glycerol mix) and loaded into wells of the water submerged gel. All gels were cast and run in a high-speed gel system (Biokeystone Company) and run at 200 V for 10-15 minutes. A Bio-Rad 200/2.0 power pack was used to drive electrophoresis. The Syngene InGenius gel documentation system and GeneSnap software were used to capture and record gel images.

Sequencing was performed by Macrogen, Seoul, South Korea. Reactions were purified and sequenced by Macrogen; using ABI 3700 (Applied Biosystems) automated sequencing machines. Sequencing was performed on both strands of DNA. Sequences were analysed using ContiqExpress Project, Vector NTI Advanced 10 (Invitrogen). Chromatograms were loaded, their quality evaluated by visual inspection and the ends trimmed to the reference gene obtained from GenBank. Multiple sequences from each gene, for each sample, were aligned into a single contig and individual chromatograms analysed and edited if required.

Sequences were compared to the available GenBank sequences using a Basic Local Alignment Search Tool (BLAST). Sequences were imported into a multiple sequences file (MSF) in GenDoc and compared with other similar sequences. Where necessary the sequences were trimmed and the protein coding open reading frames were predicted.

Phylogenetic analysis was performed using features of the MEGA4 suite of programs (Tamura *et al.*, 2007). Trees were inferred using the maximum likelihood evolution method (Yang, 2007) and the bootstrap method was used to evaluate statistically the robustness of the trees presented

with bootstrap values above 70%, considered to show a statistically supported node (Felsenstein, 1985).

4.2.9. Statistical analysis

Field and laboratory data were stored in the MS Excel-2003 spread sheet. Data was exported into STATA/SE ™ 11.0 (data analysis and statistical software; StataCorp, 4905, Lakeway Drive, College station, Texas 77845, USA) for checking integrity and performing epidemiological analysis. Descriptive statistics were calculated (frequency number and percentages). The chi-square test was used to compare AIV RNA screening results between or among the selected factors. Both chi-square and McNemar tests were used to compare the difference in the AIV RNA detection rate in the pair-wise comparison of sample categories (cloacal versus oropharyngeal swabs (paired swabs; McNemar test); cloacal swabs versus faecal samples and oropharyngeal swabs versus faecal samples (independent; chi-square test)). The results were expressed as a frequency percentage, discordant proportion, *p* value, and 95% CIs and OR where applicable.

4.3. Results

4.3.1. Samples collected

A total of 1,555 live healthy WABs were captured from four study sites from April 2007 to March 2010. Swab samples were obtained from 1,461 birds. These birds generated 1,458 cloacal and 1,368 oropharyngeal swab samples. The temporal, spatial and species distribution for the live bird samples are presented in Table 4.4 and 4.5. A total of 297 cloacal and 298 oropharyngeal swab samples were also obtained from 204 recaptured birds (one to five recaptures, mean of two) at Billabong Sanctuary (n=114) and Green Acres Lagoon (n=90).

Additionally, 1,157 individual fresh and moist environmental faecal samples of WABs were collected from the ground at Billabong Sanctuary (n=694) and Cromarty (Green Acres Lagoon; n=230 and McLain Rd Nature Reserve Lagoon; n=233) from October 2008 to January 2010. The temporal and species distribution of faecal samples obtained is also shown in Table 4.5.

Table 4.4 Numbers of sampled wild aquatic birds from four north Queensland sites for each month (from April 2007 to March 2010)

Time	Billabong Sanctuary	Cromarty	Cape York	Atherton Tableland	Total
Apr 2007	6	-	-	-	6
May 2007	4	-	-	-	4
Jun 2007	19	-	-	-	19
Jul 2007	27	-	-	-	27
Aug 2007	11	-	-	-	11
Sep 2007	19	-	-	-	19
Oct 2007	68	-	-	-	68
Nov 2007	8	-	-	-	8
Dec 2007	24	7	-	-	31
Jan 2008	0	13	-	-	13
Feb 2008	29	38	-	-	67
Mar 2008	15	64	-	-	79
Apr 2008	5	42	-	-	47
May 2008	11	0	-	-	11
Jun 2008	38	0	-	-	38
Jul 2008	26	0	21	20	67
Aug 2008	42	0	-	-	42
Sep 2008	0	26	22	-	48
Oct 2008	35	12	-	-	47
Nov 2008	11	7	-	-	18
Dec 2008	0	0	-	-	0
Jan 2009	5	0	-	-	5
Feb 2009	1	0	-	-	1
Mar 2009	45	61	-	-	106
Apr 2009	61	53	-	-	114
May 2009	21	15	-	-	36
Jun 2009	26	51	64	-	141
Jul 2009	34	0	-	-	34
Aug 2009	12	0	-	-	12
Sep 2009	30	11	-	-	41
Oct 2009	57	69	-	-	126
Nov 2009	22	61	-	-	83
Dec 2009	17	73	-	-	90
Jan 2010	0	-	-	-	0
Feb 2010	0	-	-	-	0
Mar 2010	2	-	-	-	2
Total	**731**	**603**	**107**	**20**	**1,461**

- No trapping done

Table 4.5 Number of sampled birds and faecal samples for different wild aquatic species at four north Queensland sites (from April 2007 to March 2010)

Species	Billabong Sanctuary	Cromarty	Cape York	Atherton Tableland	Total birds	Live bird samples		Faecal samples
						Cloacal	Oropharyngeal	
PWD	562	556	56	6	1,180	1,178	1,136	712
PBD	47	44	21	0	112	111	92	66
MG	91	0	3	0	94	94	94	128
DM	20	0	0	0	20	20	20	3
WWD	0	0	10	0	10	10	0	0
MD	0	0	0	10	10	10	10	0
RSD	0	0	5	3	8	8	2	0
GPG	0	0	7	0	7	7	0	0
AWI	4	0	0	0	4	4	4	246
BSC	3	0	0	0	3	3	3	0
CR	0	3	0	0	3	3	3	0
HD	0	0	3	0	3	3	0	0
ASD	2	0	0	0	2	2	2	1
BS	1	0	0	0	1	1	1	1
DG	0	0	0	1	1	1	0	0
GT	0	0	1	0	1	1	0	0
WFH	0	0	1	0	1	1	0	0
PIG	1	0	0	0	1	1	1	0
Total	731	603	107	20	1,461	1,458	1,368	1,157

PWD-Plumed whistling duck (*Dendrocygna eytoni*); *PBD*-Pacific black duck (*Anas superciliosa*); *MG*-Magpie goose (*Anseranas semipalmata*); *DM*-Dusky moorhen (*Gallinula tenebrosa*); *WWD*- Wandering whistling duck (*Dendrocygna arcuata*); *MD*-Muscovy duck (*Cairina moschate*); *RSD*-Radjah shelduck (*Tadora radjah*); *GPG*-Green pygmy goose (*Nettapus pulchellus*); *AWI*- Australian white ibis (*Threskiornis molucca*); *BSC*-Bush stone curlew *(Burhinus grallarius)*; *CR*-Crow (*Corvus orru*); *HD*-Hardhead (*Aytha australis*); *ASD*-Australian shelduck (*Tadorna tadornoides*); *BS*-Black swan (*Cygnus atratus*); *DG*-Domestic goose (*Anser anser* subsp. *Domesticus*); *GT*-Grey Teal (*Anas gracilis*); *WFH*-White faced heron (*Egretta novaehollandiae*); *PIG*-Pigeon (*Ducula bicolour*)

4.3.2. Avian influenza viral ribonucleic acid prevalence in wild aquatic birds

The overall apparent prevalence of AIV RNA was 1.4% (95% CI: 0.8-2.1) (21 reactors) in the sampled WABs birds (N=1,461) of nQLD, of which cloacal swabs accounted for 0.3% (0.1-0.7) and oropharyngeal swabs 1.1% (0.6-1.8). In contrast, the overall apparent prevalence was 1% (0.4-1.6) (11 reactors) in faecal samples (N=1,157), which was not significantly different from the prevalence results obtained when cloacal or oropharyngeal swab results were combined (now referred to as the combined swab results) (χ^2=0.12, p=0.731) (Table 4.6). In this case a positive from either of the swab types was registered as a positive result. Of 596 swabs (both

cloacal and oropharyngeal swabs) obtained from 204 recaptured birds, AIV RNA was detected in one cloacal swab of an adult PWD at Cromarty.

The prevalence using the combined swab results from live birds was 1.6% (0.8-2.8) for Billabong Sanctuary, 1.2% (0.4-2.3) for Cromarty, 1.9% (0.2-6.5) for Cape York and 0% (0-16.8) for Atherton Tableland. The results were statistically equivalent when the results were compared between the sites in the longitudinal study (Billabong Sanctuary versus Cromarty) (χ^2=0.26, p=0.613). For faecal samples, the prevalence was, in contrast, higher in Cromarty (1.7%; 0.7-3.3) than Billabong Sanctuary (0.4%; 0.08-1.3) (χ^2=3.70, p=0.05) (Table 4.6).

Table 4.6 Avian influenza viral ribonucleic acid apparent prevalence in the samples from wild aquatic birds for four north Queensland study sites (from April 2007 to March 2010)

Site	**Cloacal swab**		**Oropharyngeal swab**		**Faecal sample**		**Combined: Cloacal and oropharyngeal swab***	
	N	**% (95% CI)**	**N**	**% (95% CI)**	**N**	**% (95% CI)**	**N**	**% (95% CI)**
Billabong Sanctuary	728	0.4% (0.08-1.1)	728	1.2% (0.5-2.3)	694	0.4% (0.08-1.3)	731	1.6% (0.8- 2.8)
Cromarty	603	0% (0-0.6)	599	1.1% (0.4-2.3)	463	1.7% (0.7-3.3)	603	1.2% (0.4-2.3)
Cape York	107	1.9% (0.2-6.5)	21	0% (0-16.1)		-	107	1.9% (0.2-6.5)
Atherton Tableland	20	0% (0-16.8)	20	0% (0-16.8)		-	20	0% (0-16.8)
Overall	**1,458**	**0.3% (0.1-0.7)**	**1,368**	**1.1% (0.6-1.8)**	**1,157**	**1.0% (0.4-1.6)**	**1,461**	**1.4% (0.8-2.1)**

* The positive detection of AIV RNA was determined as a positive result for either the cloacal or the oropharyngeal swab extract for each bird. *CI*: Confidence interval

The prevalence ranged from 0-1.8% for combined swab results and 0-1.2% for faecal samples across the different years. The results were statistically equivalent when results were compared between 2008 and 2009 for the combined swab results (χ^2=1.15, p=0.284) (Table 4.7).

The prevalence varied from 0-2.7% for the combined swab results taken from different species of live birds and 0.4-4.5% for faecal samples (Table 4.7). In the comparison between PWDs and PBDs (common species), the results differed significantly for faecal samples (PWD-0.4% and PBD-4.5%; χ^2=8.14, p=0.004), but were equivalent for the samples taken from live birds (χ^2=1.72, p=0.189) (Table 4.7).

There was no statistically significant difference in prevalence between the age classes (χ^2≥0.01, p≥0.60) or between the sexes (χ^2=1.51, p≥0.219) (Table 4.7).

Table 4.7 Avian influenza viral ribonucleic acid apparent prevalence in the samples taken from wild aquatic birds in north Queensland presented for year, species and age (from April 2007 to March 2010)

Factors	Categories	Live bird samples*		Faecal samples	
		N	% (95% CI)	N	% (95% CI)
Year	2007	193	0% (0-1.8)	-	-
	2008	477	1.0% (0.3-2.4)	265	0% (0-1.3)
	2009	789	1.8% (0.9-2.9)	883	1.2% (0.6-2.2)
	2010	2	0% (0-84.1)	9	0% (0-33.6)
Species	Plumed whistling duck	1,180	1.4% (0.8-2.2)	712	0.4% (0.08-1.2)
	Pacific black duck	112	3.5% (0.9-8.8)	66	4.5% (0.9-12.7)
	Australian white ibis	4	0% (0-60.2)	246	1.6% (0.04-4.1)
	Other species	165	0.6% (0.01-3.3)	133	0.8% (0.01-4.1)
Age	Up to juvenile	271	1.8% (0.6-4.2)		
	Sub-adult	114	2.6% (0.5-7.4)		
	Adult	906	1.4% (0.7-2.4)		
	Unknown	1,170	0% (0-0.3)		
Sex	Male	286	2.4% (0.9-4.9)		
	Female	984	1.2% (0.6-2.1)		
	Unknown	191	0.5% (0.01-2.8)		

Other species for live bird samples: magpie goose, dusky moorhen, wandering whistling duck, muscovy duck, radjah shelduck, green pygmy goose, bush stone curlew, crow, hardhead, Australian shelduck, black swan, domestic goose, grey teal, white faced heron and pigeon. *Other species for faecal samples:* magpie goose, dusky moorhen and Australian shelduck. * The positive detection of AIV RNA was determined as a positive result for either the cloacal or the oropharyngeal swab extract for each bird. *CI*: Confidence interval

4.3.3. Suitable samples for avian influenza viral ribonucleic acid detection

The AIV RNA detection rates were compared among types of samples with the aim of identifying suitable samples for AI surveillance programs in WABs of nQLD. For these comparisons, the study was restricted to the results of 1,148 faecal samples and 705 live bird paired samples (cloacal and oropharyngeal swabs) which were obtained between October 2008 and December 2009 at Billabong and Cromarty (Table 4.8). The species sampled included PWD, PBD, ASD, MG, DM, AWI, BS, CR and BSC. Samples from 629 live birds were excluded due to collection of a single swab only, or if no faecal samples were collected at that time.

Comparisons were made between cloacal and oropharyngeal swabs, cloacal swabs and faecal samples and oropharyngeal swabs and faecal samples. Results obtained from the samples at Billabong Sanctuary and Cromarty were pooled to increase the overall sample size and the same sampling period was used (calendar months, n=12) (Table 4.8) to avoid possible time effects in

this analysis. Transport media, storage, transport operations, testing preparation and testing procedures were identical for all samples.

Table 4.8 Numbers of cloacal swabs, oropharyngeal swabs and faecal samples from wild aquatic birds for each month between October 2008 and December 2009 and the number of samples positive for avian influenza viral ribonucleic acid

Month/Year				**Environmental samples**	
	No. of paired swabs	**Cloacal swabs (+)**	**Oropharyngeal swabs (+)**	**No. of faecal samples**	**+**
Oct 2008	47	0	0	107	0
Nov 2008	18	0	0	158	0
Feb 2009	1	0	0	35	0
Mar 2009	105	0	0	109	0
Apr 2009	114	0	0	14	0
May 2009	36	0	2	51	0
Jul 2009	34	2	0	124	0
Aug 2009	12	0	1	110	2
Sep 2009	41	0	4	92	0
Oct 2009	125	0	0	141	3
Nov 2009	83	0	1	123	3
Dec 2009	89	0	2	84	2
Total	**705**	**2**	**10**	**1,148**	**10**

The McNemar test suggested the AIV RNA detection rate was significantly better in oropharyngeal than cloacal swabs (p=0.038) and the detection rate was five times more likely in oropharyngeal swabs (Table 4.9). The chi-square tests showed that detection rates were similar in comparison between cloacal swabs and faecal samples (p=0.217) or oropharyngeal swabs and faecal samples (p=0.381) (Table 4.10).

Table 4.9 McNemar test for comparing the difference between the discordant paired proportions for the categories of swab type (cloacal or oropharyngeal) and the presence of avian influenza viral ribonucleic acid (yes= -, no= +) for swabs collected from Billabong and Cromarty

Swabs	**Cloacal swab**			**Difference between the discordant paired proportions (95% CI)**	**OR (95% CI)**
Oropharyngeal swab	-	+	Total		
-	693	2	695	0.01* (0.0003-0.02)	5.0 (1.1-46.9)
+	10	0	10		
Total	**703**	**2**	**705**		

**P value*: 0.038; *OR*: Odds ratio; *CI*: Confidence interval

Table 4.10 Chi-square test for comparing the difference between the proportions positive (+) or negative (-) for avian influenza viral ribonucleic acid and different types of samples

Comparisons	Samples	+	-	Total	*p*	OR (95% CI)
Faecal sample (F) versus cloacal swab (C)	F	10 (0.9%)	1,138	1,148	0.217	3.1 (0.6/20.5)
	C	2 (0.3%)	703	705		
Faecal sample (F) versus oropharyngeal swab (O/P)	F	10 (0.9%)	1,138	1,148	0.381	0.6 (0.2/1.6)
	O/P	10 (1.4%)	695	705		

4.3.4. Results of real time reverse transcriptase-polymerase chain reaction inhibitors

In the first run of 10 RNA extracts (five each of cloacal and oropharyngeal swabs), the entire cloacal swab extracts produced slight inhibition with all C_T values being less than two cycles above the result for the triplicate analysis of water. The oropharyngeal extracts produced more inhibition with two complete PCR failures, one producing an increased C_T value of approximately 15 compared to the analysis of water and two extracts elevating the C_T value by between two and five cycles.

But in the second run of 42 RNA extracts (21 of each type of swab) two RNA extracts each from cloacal and oropharyngeal swabs produced complete failure of the PCR, one oropharyngeal extract produced slight inhibition elevating the C_T value by approximately five cycles and one oropharyngeal extract producing an indeterminate result indicating inhibition may be a problem for approximately 14% of extracts from both swab types. If the initial analysis of 10 extracts was considered, problematic inhibition may be higher than 14% and RNA extracts from oropharyngeal swabs may produce more inhibition than that of cloacal swab extracts.

4.3.5. Viral isolation in chicken embryos

The culturing of AIVs in 9-11 day-old chicken embryos was only attempted for six of the 32 PCR reactor samples (PWD: 1-4, PBD: 1-2 in Table 4.11) and no AIVs was cultured after three serial passages in embryonated eggs.

4.3.6. Avian influenza viral sub-typing and sequencing results

Of 33 reactor samples (including one from a recaptured bird) (N=1,461 live birds and 1,157 faecal samples) from the AIV RNA screening assays, sequencing results were obtained (at least for one gene) for nine samples. This study has been unsuccessful in obtaining sequences from 15 reactor samples and sequencing was not attempted for nine samples. Both M

and H gene sequenced in this study were used for the phylogenetic analysis. The NSP gene sequence in this study was of poor quality and therefore was not used for phylogenetic analysis. The details are presented in Table 4.11.

Table 4.11 Details of analysis to obtain gene sequences from avian influenza viral ribonucleic acid reactor samples (+) of wild aquatic birds in north Queensland (from April 2007 to March 2010)

Species	**rRT-PCR (Screening assays)**		**PCR/Sequencing (Attempted/Not)**	**Sequencing results**	**Sample type/Site/Bird ID/Month/Year**
	AAHL (C_T value)	Ward (C_T value)			
PWD-1	-	+ (25.3)	M	Unsuccessful	Op/Cromarty/265/Feb/2008
PWD-2	+ (31.5)	+ (27.4)	M & H	M & H6	Op/Billabong/446/May/2008
PWD-3	-	+ (28.0)	M	Unsuccessful	Op/Billabong/465/Jun/2008
PWD-4	-	+ (12.0)	M & H	H6	Cl/Billabong/480/Jun/2008
PWD-5	-	+ (33.0)	M	Unsuccessful	Op/Billabong/1012/May/2009
PWD-6	-	+ (31.8)	M	Unsuccessful	Op/Cromarty/1022/May/2009
PWD-7	-	+ (33.0)	Not attempted	-	Op/Billabong/1058/Jun/2009
PWD-8	+ (30.4)	+ (30.9)	M, H & NP	Unsuccessful	Op/Cromarty/1068/Jun/2009
PWD-9	+ (27.8)	+ (28.9)	M, H & NSP	M	Op/Billabong/1110/Jun/2009
PWD-10	-	+ (32.6)	M	Unsuccessful	Cl/Billabong/1160/Jul/2009
PWD-11		+ (32.3)	M	Unsuccessful	Cl/Billabong/1178/Jul/2009
PWD-12	-	+ (32.2)	M & H	Unsuccessful	Op/Billabong/1183/Aug/2009
PWD-13	-	+ (31.5)	M	Unsuccessful	Op/Billabong/1211/Sep/2009
PWD-14	-	+ (31.2)	M, H, NSP & NP	M, H6 & NSP	Op/Billabong/1212/Sep/2009
PWD-15	-	+ (31.4)	M	Unsuccessful	Op/Cromarty/1553/Nov/2009
PWD-16	-	+ (32.7)	M	Unsuccessful	Op/Cromarty/1648/Dec/2009
PWD-17	-	+ (32.7)	M	Unsuccessful	Op/Cromarty/1651/Dec/2009
PWD-18	-	+ (30.6)	Not attempted	-	En/Cromarty/999/Nov/2009
PWD-19	-	+ (30.3)	Not attempted	-	En/Cromarty/1012/Nov/2009
PWD-20	-	+ (31.6)	Not attempted	-	En/Cromarty/1055/Nov/2009
PWD-21	-	+ (31.8)	M & H	M	Cl/Cromarty /1716/**Recaptured**/Dec/2009
PBD-1	+ (34.1)	+ (31.8)	M & H	M	Cl/Cape York/11129670/Sep/2008
PBD-2	+ (21.9)	+ (23.5)	M, H, NSP & NP	M & H9	Cl/Cape York/94(11129682)/Jun/2009
PBD-3	-	+ (32.4)	M, H, NSP & NP	Unsuccessful	Op/Cromarty/1213/Sep/2009
PBD-4	-	+ (33.7)	Not attempted	-	Op/Billabong/1214/Sep/2009
PBD-5	-	+ (34.0)	M	M	En/Billabong/610/Aug/2009
PBD-6	-	+ (32.0)	Not attempted	-	En/Cromarty/877/Oct/2009
PBD-7	-	+ (32.2)	Not attempted	-	En/Cromarty/879/Oct/2009
AWI-1	+ (27.9)	+ (28.6)	M, H, NSP & NP	M, H6 & NSP	En/Billabong/686/Aug/2009
AWI-2	-	+ (32.7)	Not attempted	-	En/Cromarty/818/Oct/2009
AWI-3	-	+ (32.1)	Not attempted	-	En/Cromarty/1056/Dec/2009
AWI-4	-	+ (31.6)	Not attempted	-	En/Cromarty/1132/Dec/2009
BS	-	+ (32.5)	M	Unsuccessful	En/Billabong/722/Aug/2009

PWD: Plumed whistling duck; *PBD*: Pacific black duck; *AWI*: Australian white ibis; *BS*: Black swan; *Op*: Oropharyngeal swab; *Cl*: Cloacal swab; *En*: Environmental faecal sample; *M*: Matrix gene; *H*: Haemagglutinin gene; *NSP*: Non structural protein; *ID*: Identity number

4.3.7. Phylogenetic analysis of the matrix gene

The M gene sequenced from eight reactor samples (Table 4.11) was obtained between nucleotide positions 1 and 913 in this study. For phylogenetic analysis, however, sequences of six reactor samples of 479 bp fragments were used, which covered the position between 299 and 777 bp that was obtained for all these sequenced reactor samples. An almost similar length of 414 bp (positions 223-636) of AIV M gene was used by Fusaro *et al.* (2010) for phylogenetic analysis. In addition, this gene region was more polymorphic compared to the first 300 bp of the M gene as observed when aligning these sequences with sequences from GenBank (data are not shown). Therefore, the use of the fragment from position 299 to 777 was suitable for phylogenetic analysis.

Six sequences obtained in this study along with 58 GenBank sequences (23 Australian and 35 overseas isolates) were used for a phylogenetic analysis. These reference sequences were selected as they had the highest similarity to sequences in this study using the BLAST search. Figure 4.1 provides the results of the phylogenetic analysis and presents a subset of the isolates having greatest similarity to the sequences of reactor samples obtained in this study.

The trees had a predominance of Australian and Eurasian isolates (Figure 4.1). This tree was composed of strongly supported groups of similar isolates (81-90 bootstrap values) and many poorly to moderately supported groups (14-61 bootstrap values). The sequences also included North American and African isolates. Sequences of reactor samples obtained in this study were most closely related to Australian isolates (for PBD-1 and PWD-2) or Australian or Eurasian isolates (for PWD-14, AWI-1, PBD-5 and PBD-2) with PWD-14 and AWI-1 being most closely related to each other (as also found for H sequence of H6 subtype). The sequence of PBD-2 was also more closely related to North American isolates than many other Australian and Eurasian isolates.

Australian tern and RNS isolates (AB275864, CY028260 and CY033154) were identified as out groups (Figure 4.1).

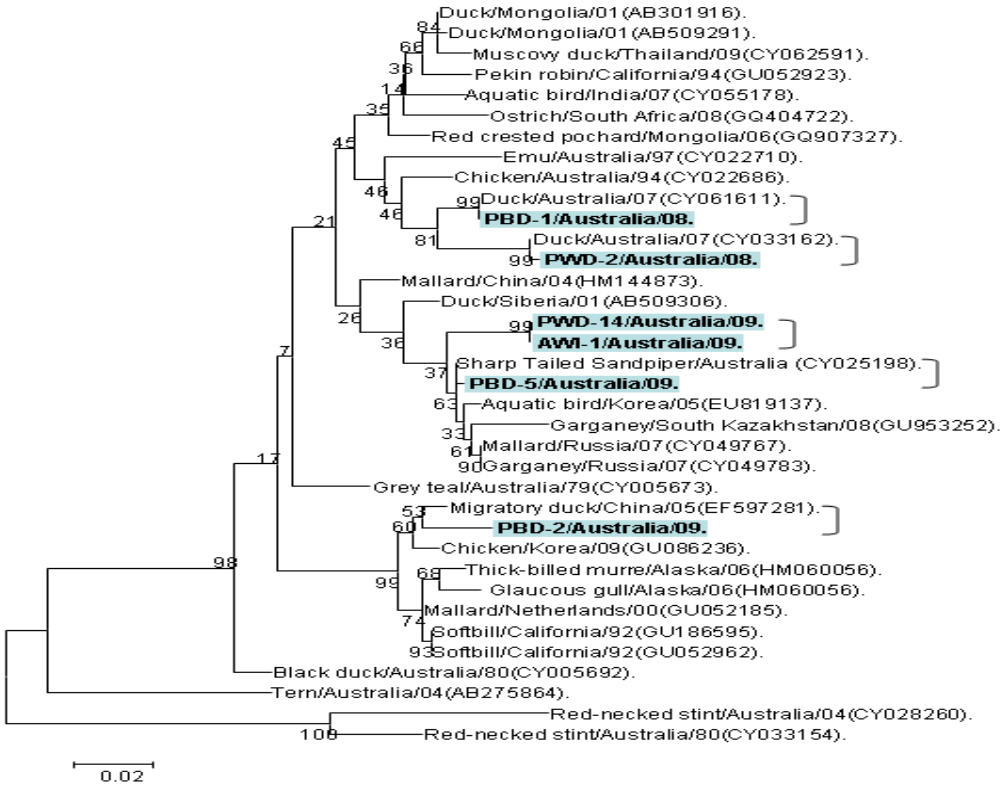

Figure 4.1 Bootstrap consensus trees with 5,000 replications for avian influenza matrix gene (nucleotide positions between 299 and 777 base pairs)

4.3.8. Phylogenetic analysis of the haemagglutinin gene

4.3.8.1. Phylogenetic analysis of the haemagglutinin-6 subtype

The H gene sequence obtained from four H6 reactor samples in this study was obtained between positions 124 and 1,285. For phylogenetic analysis a 563 bp fragment (positions 124-686) was used. This length of fragment was obtained for all sequenced reactor samples. Both Australian and overseas H6 reference sequences were used for phylogenetic comparisons. These reference sequences showed the greatest similarity to the H6 reactor sequences obtained in this study and results are presented in Figure 4.2. The cleavage site of reactor sequences in this study indicated low pathogenic (LP) H6 subtypes (sequences are not shown). The phylogram shows a North American clade (CY033404-CY033356) that is loosely connected with a mixture of North American, Eurasian, Australian (including reactor sample sequences studied), New Zealand and African isolates. Reactor sequences of PWD-14 and AWI-1 were most similar to each other but also a close congener with Eurasian isolates and this relationship is also consistent with the results from the analysis of the M gene sequences in this study. Reactor sequences of PWD-2 and PWD-4 were very similar and moderately related to a New Zealand isolate.

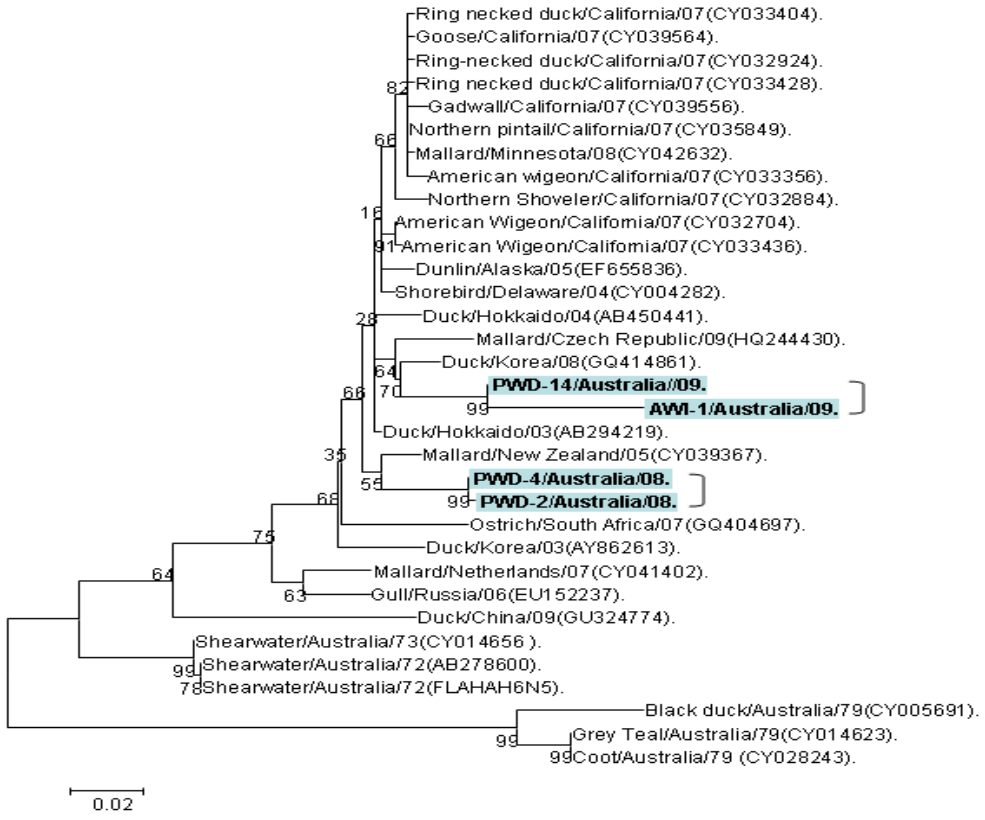

Figure 4.2 Bootstrap consensus trees with 5,000 replications for avian influenza haemagglutinin-6 gene (nucleotide positions between 124 and 686 base pairs)

4.3.8.2. Phylogenetic analysis of the haemagglutinin-9 subtype

This study sequenced 1,350 bases (243-1,592 bp) of the H gene for a single reactor sequence. This sequence had the greatest similarity to the H9 subtype. This sequence along with similar GenBank H9 sequences were therefore used for phylogenetic analysis and the results are presented in Figure 4.3.

Reconstruction of the H9 gene phylogeny showed that the sequence in this study was most similar to isolates from Asia and to a lesser extent European isolates (Figure 4.3). The cleavage site of this isolate indicated a LP H9 subtype (sequences are not shown).

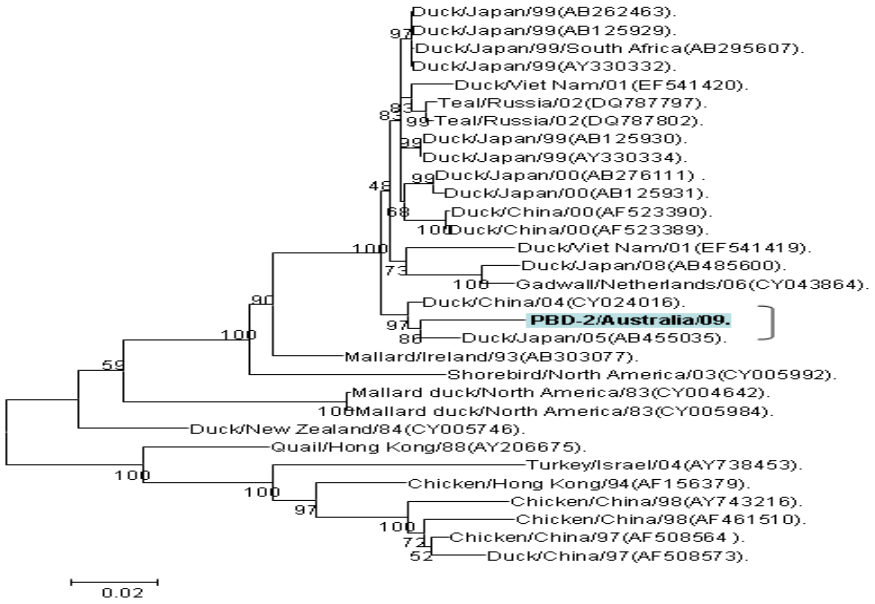

Figure 4.3 Bootstrap consensus trees with 5,000 replications for avian influenza haemagglutinin-9 gene (nucleotide positions between 243 and 1,592 base pairs)

4.4. Discussion

4.4.1. Avian influenza viral subtypes

This study identified the H6 subtype in PWDs and an AWI and the H9 subtype in a PBD, in the years 2008-09 for the first time in nQLD. These subtypes were also commonly found in the AIV serology screening (Chapter 3). The H9 subtype appears to be a recent discovery in Australia which is supported by Haynes *et al*. (2009) and Hansbro *et al*. (2010). They identified the H9 subtype in both Australian resident birds (PBD, GT, chestnut teal and BS) and migratory wild birds (eastern curlew, far eastern curlew and bar-tailed godwit) during 2007-2008.

To put these findings in a wider perspective, all other H subtypes have been identified from Australian wild birds except for H14, H15 and H16 (Mackenzie *et al.*, 1985; Peroulis and O'Riley, 2004; Hurt *et al.*, 2006; Haynes *et al.*, 2009; Hansbro *et al.*, 2010). The AIV serology results also demonstrated a wide range of AIV subtypes in WABs of nQLD (Chapter 3).

4.4.2. Phylogenetic analysis

4.4.2.1. New introductions of avian influenza viral subtypes

The sequences of the M gene of AIV subtype H6 identified in birds PWD-14 and AWI-1 in 2009 had a close similarity to the M gene sequences of a H11 subtype isolated from a STS (Australian migratory bird) (CY025198) (Figure 4.1) whereas the H gene from these samples was a close congener of an AIV subtype H6 isolated from Eurasian ducks (GQ414861 and HQ244430) (Figure 4.2). This suggests that the AIV subtype H6 (2009) in this study may have been introduced recently to Australian resident birds by migratory birds using the nQLD migratory route. The phylogram also indicated a new lineage of the H6 subtypes in Australian wild birds (Figure 4.2). These suggestions of a recent introduction of a H6 subtype (2009) creating a new lineage are supported by other recent studies in Australian wild birds (Aeron Hurt, WHO Collaborating Centre for Influenza, personal communication).

The phylogenetic analysis on the M gene sequence of the H9 subtype (2009) identified in this study had the greatest similarity to that of a H9 subtype isolated from a chicken from Korea (GU086236) and LPAI H5 subtype obtained in a migratory duck from China (EF597281) (Figure 4.2). The H gene sequence of H9 subtype in this study is similar to the sequence of a H9 subtype obtained from ducks in China (CY024016) and Japan (AB455035) (Figure 4.4). Hansbro *et al* (2010) also obtained a similar finding. They identified several H9 isolates in Australian wild birds that formed a clade that grouped more closely to the Asian aquatic bird isolates than to terrestrial or North American bird isolates. They also found the H9 subtype to be most common in migratory shorebirds. Overall results, therefore, suggested the H9 subtype in this study may similarly be recently introduced from Asian regions through migratory birds.

An earlier review identified AIVs from species of Charadriiformes which regularly travel between Asia and Australia (Tracey *et al.*, 2004). The species include ruddy turnstone (*Arenaria interpres*), red knot (*Calidris canutus*), RNS (*Calidris ruficollis*), common tern (*Sterna hirundo*), sooty tern (*Sterna fuscata*), bar tailed godwit (*Limosa lapponica*) and STS (*Calidris acuminate*). Moreover, H4N8 and H11N9 subtypes which were closely related to Asian isolates were isolated in RNS and STS, respectively (Hurt *et al.*, 2006). All these earlier findings support the capability of migratory birds to bring LPAI viruses from Asian regions to Australia. The potential role of spreading exotic HPAI viruses (in particular H5N1) through migratory birds in Australia has, however, been ignored until recently (East *et al.*, 2008a). This study indicated that there is a chance of introducing exotic HPAI viruses to Australia by migratory birds as indicated by the similarity of AIVs identified in this study to a LPAI virus

detected in a STS, a migratory species and viruses detected overseas such as in Eurasia. This likely threat is further strengthened by evidence that wild migratory birds infected with HPAI H5N1 are capable of disseminating the virus over long distances (Chen *et al.*, 2005).

4.4.2.2. Evolution of the highly pathogenic avian influenza viral subtype within Australia

This study identified putative reassortment events between H6 subtypes and M genes previously identified with a H7 subtype virus (CY033162) (Figure 4.1). The phylogenetic analysis therefore indicated a reassortment process could have occurred in Australian resident birds which were concurrently infected with the Australian H7 wild bird and overseas derived H6 subtypes. The overseas H6 subtype was potentially introduced through migratory birds (Figure 4.1 and 4.2).

As mentioned earlier Charadriiformes, which travel regularly between Australia and Asia and can congregate in extremely large concentrations on coastal floodplains and mainland wetlands (Morton *et al.*, 1993), regularly interact with Australian resident Anseriformes (Morton, 1990). An environment is created that is conducive for birds being infected concurrently with two different AIV subtypes with the potential for producing a recombinant subtype through a reassortment process of whole gene fragments.

There is evidence of reassortment events in wild birds. For example, the analysis of the Australian tern H2N5 isolates suggested the occurrence of intercontinental reassortment between viruses of Eurasian and American lineages in free flying birds in nature (Kishida *et al.*, 2008). They identified that the M gene of a tern isolate belonged to the American lineage and the other seven genes to the Eurasian lineage AIVs. Likewise, intercontinental reassortment was observed in wild ducks, shorebirds and gulls in Canada (Widjaja *et al.*, 2004) and in northern pintails in Alaska (Dugan *et al.*, 2008; Koehler *et al.*, 2008; Ramey *et al.*, 2010a; Ramey *et al.*, 2010b).

There is also a possibility of multiple reassortment events. Hatchette *et al*.(2004) identified multiple genotypes of H6 subtypes in feral Canadian ducks in different years which were indicative of multiple reassortment events. The formation of novel gene combinations through introduction of AIVs from Asia, followed by reassortment, may increase the chance of the development of unique gene combinations which produce HPAI viruses in Australia.

A second potential evolutionary mechanism involves the accumulation of mutations resulting in a substantial degree of antigenic drift away from the prevalent subtypes in Australian wild birds.

Although AIV gene mutation is relatively slow in its natural hosts (Olsen *et al.*, 2006), this process can occur rapidly in humans. For instance, the 1957 and 1968 human pandemic strains of influenza A both contained drifted H genes originally derived from the 1918 pandemic virus (Taubenberger and Morens, 2006). These two pandemic strains demonstrate that gradual accumulation of mutation can be antigenically significant. In some cases, it may eventually give rise to an antigenically distinct H subtype. The discovery of a new, 16th H subtype illustrates this process (Fouchier *et al.*, 2005).

In addition, highly pathogenic AIVs could evolve from Australian subtypes through intermolecular recombination. Non-homologous recombination occurs between two heterologous genes or RNA fragments and homologous recombination occurs between segments with similar sequences from related or unrelated genes or RNA fragments (Chetverin *et al.*, 1997). Although recombination in AIVs occurs infrequently, this phenomenon has been identified elsewhere. For example, a non-homologous recombination between H and NP genes of a LPAI virus is thought to be responsible for an outbreak of HPAI H7N3 in chickens that occurred in Chile in 2002 (Suarez *et al.*, 2004). Experimental studies using chicken embryo cells also provide evidence for various forms of non-homologous recombination in influenza viruses and subsequently increased virulence (Khatchikian *et al.*, 1989; Orlich *et al.*, 1994). Evidence from the field and the laboratory therefore suggests that, although rare, non-homologous recombination does take place in influenza viruses, and may play an important role in altering the pathogenicity of circulating strains.

Homologous recombination, although controversial, is thought to involve template switching while the polymerase is copying the RNA (Boni *et al.*, 2008). In 2004, an HPAI H7N3 virus arose after an outbreak of LPAI on an index farm in British Columbia; the HPAI virus had acquired a 7-amino acid M gene insertion at the cleavage site in the H through homologous recombination (Pasick *et al.*, 2005).

However, the exploration of the novel H6 subtypes through AIV gene reassortment should be considered cautiously as this assessment was based on only two short genes (N=8) (M gene: 479 bp and H gene: 563 bp). A more comprehensive and complete genotypic analysis of these subtypes is therefore needed to examine some of the concepts arising from the phylogenetic analysis and to determine how AIVs reassort or mutate in WABs.

4.4.2.3. Implications of the adaptation of the recently introduced avian influenza viral subtypes and hybrid subtypes to Australian poultry

The H6 and H9 subtypes identified in this study may be of concern if they were introduced to Australian domestic and commercial poultry. This is because these subtypes have previously been shown to adapt quickly to domestic avian species and acquire moderate pathogenicity (Forrest and Webster, 2010).

In particular the H9N2 subtype can cause serious disease outbreaks in domestic chickens when certain enteric bacteria are present (Kishida *et al.*, 2004). This subtype has caused worldwide disease outbreaks in chickens (Alexander, 2000b; Iqbal *et al.*, 2009; Nagarajan *et al.*, 2009) and was also recovered from pigs in Hong Kong, China and Indonesia (Ninomiya *et al.*, 2002) and humans in Hong Kong (Peiris *et al.*, 1999; Riedel, 2006). The crossing of the taxonomic barrier from birds to mammals highlights the pandemic potential of the H9N2 subtype.

4.4.3. Avian influenza viral ribonucleic acid prevalence

Overall AIV RNA prevalence as determined by the M rRT-PCR was 1.0-1.4% in the sampled WABs of nQLD. This prevalence is consistent with other studies in Australian WABs that found 1.0-2.4% prevalence (Haynes *et al.*, 2009; Hansbro *et al.*, 2010; Tracey, 2010). Similar results as assessed by the M rRT-PCR were observed in WABs of Argentina (0.4%) (Pereda *et al.*, 2008) and Ireland (1.2%) (Raleigh *et al.*, 2009).

Pacific black ducks had an AIV RNA prevalence of 3.5-4.5% and other species had 0-1.6% in this study. The results differed significantly for faecal samples when comparing PBDs (4.5%) with PWDs (0.4%). This variability in prevalence between different species is well supported by Tracey (2010) who reported 3.1% prevalence for PBDs, 0.9% for BS and 0.7% for PWDs in Australia. An analogous species pattern was also observed in the AIV antibody prevalence (Chapter 3).

Pacific black ducks and mallard ducks are likely to differ little in their susceptibility and transmission of AIVs (Tracey, 2010) and mallard ducks were identified as a species more susceptible to AIVs compared to other dabbling ducks such as wigeon, teals and gadwall (determined through the examination of the susceptibility and or disease severity in inoculation experiments) (Wood *et al.*, 1985; Stallknecht and Shane, 1988; Ito and Kawaoka, 2000; Neumann and Kawaoka, 2006). These findings further support PBDs to be highly susceptible to AIVs. These findings provide a possible cause for the current study observation of higher

prevalence in PBDs. This study also estimated the prevalence of 1.6% in AWI which is, to the author's knowledge, the first quantification of AIV RNA prevalence in nQLD.

This study was not capable of testing the seasonal effect on prevalence of AIV RNA as reactor samples were either absent or in very low numbers in each season. An Australian study, however, recorded a higher AIV RNA prevalence in autumn (March-May) (3.2%) than other seasons (1.8-2.7%) (Hansbro *et al.*, 2010), which may correspond to an increased number of young birds at that time. Increased viral prevalence following breeding is often observed or assumed for animal pathogens including AIVs (Hinshaw *et al.*, 1985; Alfonso *et al.*, 1995). However, Australia is dry with irregular rainfall and as a consequence, breeding and movements of WABs are irregular (Tracey *et al.*, 2004; McCallum *et al.*, 2008; Tracey, 2010). In addition, most Australian anatids do not breed during severe drought (Frith, 1982), which is likely to limit the AIV prevalence. A similar lower AIV antibody prevalence was also observed in the warm dry season (September-December) in the AI serology study (Chapter 3).

A number of overseas studies assessed the seasonal effects on LPAI prevalence in WABs demonstrating higher levels of prevalence compared to that of Australia. For example, the prevalence among mallards varied seasonally from less than 10% (spring and summer) to between 10% and 60% just before and during the autumn migration. This pattern was seen in both Eurasia and North America (Krauss *et al.*, 2004; Olsen *et al.*, 2006; Munster *et al.*, 2007; Wallensten *et al.*, 2007). Possible explanations provided for seasonal variation in prevalence in addition to the influx and aggregation of naïve juvenile birds following breeding and prior to (and during) migration (Webster *et al.*, 1992) are the environmental conditions that determine viral survival outside the host (Stallknecht *et al.*, 1990a).

No significant age difference in AIV RNA prevalence was observed in the present study which may be due to a low AIV RNA detection rate. This finding however, remains consistent with an earlier study in WABs in the USA (Deibel *et al.*, 1985), but differs with studies in Canada (Hinshaw *et al.*, 1980b), Siberia (Okazaki *et al.*, 2000), North America (Munster *et al.*, 2007), northern Europe (Wallensten *et al.*, 2007), Alaska (Ip *et al.*, 2008) and New Zealand (Stanislawek *et al.*, 2002). These latter studies observed a significantly higher prevalence in juveniles than adult WABs, but these results were determined by unadjusted univariate statistical analysis without accounting for confounding and other potential factors using multivariable analysis. The higher prevalence in young birds, however may be due to the fact that they are immunologically naïve whereas adults are more resistant, particularly to viruses to which they may have previously been exposed (Webster *et al.*, 1992).

No difference in AIV RNA prevalence between the sexes was observed in this study. Similar findings of no difference in prevalence between the sexes were reported by Deibel *et al*. (1985), Munster *et al*. (2007) and Wallensten *et al*. (2007). However, some studies observed a significantly higher prevalence in females than that of male WABs (Runstadler *et al.*, 2007; Ip *et al.*, 2008). An identical gender pattern was observed in the study of AIV antibody prevalence (Chapter 3).

Some complex biological mechanisms might have contributed to the identification of a low AIV RNA prevalence in free living birds. For example, WABs are frequently exposed to multiple AIV subtypes concurrently or at different times which significantly decrease the viral shedding period (Fereidouni *et al.*, 2009; Costa *et al.*, 2010; Fereidouni *et al.*, 2010b) and therefore, the opportunity to detect AIVs from field samples. The AI serological result suggested that this population was exposed to multiple AIV subtypes (Chapter 3). In addition, samples containing inhibitors might reduce the success of the AIV RNA detection. This study found 14% or more of sample extracts produced significant inhibition of PCR.

When a bird is infected with influenza, viruses will be excreted in faeces and it can be found in oropharyngeal samples. The viral genome can be detected using RT-PCR. Experimental evidence suggests that the maximum time that this will be detected will probably not exceed seven to ten days (Heckert *et al.*, 1999; VanDalen *et al.*, 2010). Subsequent infections with homologous or heterologous strains of virus are likely to also result in a viral genome being detected. However, the duration of detection is likely to be shorter than that seen with the primary infection. If samples collected from birds and the viral genome can be detected for seven days a prevalence of 1% would indicate that the birds had been infected at least once every two years. If a period of detection is less than this, it would suggest a more frequent infection rate. It is reasonable to suggest that the observed prevalence of between 1% and 2% should equate to an annual incidence of somewhere between one and two infections with influenza virus. The point prevalence appears to be quite low. However, when this is translated to an annual incidence it becomes apparent that influenza infections are frequent events for this population.

4.4.4. Suitable samples for avian influenza viral ribonucleic acid detection

This study observed better AIV RNA detection rates from oropharyngeal than cloacal swabs, but similar levels for faecal samples and cloacal swabs, indicating that the LPAI viruses may have a higher replication in the respiratory tracts compared to the intestinal tracts of wild birds. However, these findings are different to that of others. Sturm-Ramirez *et al*. (2005) found significantly higher recovery of HPAI H5N1 viruses from oropharyngeal as compared with

cloacal swabs. Tracey (2010) observed the same AIV detection rate between cloacal and oropharyngeal samples (2.3% cloacal and 2.2% oropharyngeal) and a lower detection rate from faecal samples (0.3%). In contrast, a European study observed a significantly higher detection rate from cloacal (8.0%) than oropharyngeal swabs (3.7%) (Munster *et al.*, 2009). No difference in AIV RNA detection rates was observed between cloacal and faecal samples in the present study, which corresponds to a previous study in northeast Germany (Pannwitz *et al.*, 2009).

It is not unexpected that results vary between different studies. The site of infection may differ between species, for example, there was no difference in detection probability between cloacal and oropharyngeal swabs observed in free-living Eurasian wigeons (*Anas penelope*) (Munster *et al.*, 2007). In contrast, white-fronted geese (*Anser albifrons*) were two times more likely to have infection detected in the oropharyngeal than cloacal swabs (Munster *et al.*, 2009). In addition, the exact contribution of the LPAI viral replication in the respiratory tracts of wild birds compared to that in the intestine may differ between viral subtypes and needs to be elucidated.

Other factors may have also contributed to the findings in this study, and the interpretation of the findings of others, such as increased PCR inhibition in the extracts from the cloacal swabs or increased RNA extraction efficiency or viral-particle sampling efficiency for the oropharyngeal swabs. However, the analysis of rRT-PCR inhibition on 52 random RNA extracts (26 for both oropharyngeal and cloacal swabs) in this study found no evidence of increased inhibition from cloacal swab extracts over oropharyngeal swab extracts.

Overall results suggest that both live bird swabs and faecal sampling are useful for future AI surveillance programs. Live bird sampling reduces the chance of incorrect identification of the bird species and the sampling of faeces from more than one bird. In addition, there is a simultaneous opportunity to record bird demographic information along with the collection of serum samples. Contrarily, faecal sampling is easier and a cheaper option (Tracey, 2010) increasing the number of samples that can be collected when live bird trapping is difficult, labour intensive and costly.

4.4.5. Viral isolation in chicken embryos

This study was not successful in growing and isolating AIVs using the AIV RNA reactor samples of WABs in embryonated chicken eggs. However, the present study only attempted viral isolation for 19% of AIV RNA reactor samples (N=33). Many earlier studies also observed poor performance of this conventional technique in isolating AIVs using AIV RNA reactor field samples of wild birds such as 0% in Ireland (Raleigh *et al.*, 2009), 3% in Australia (Haynes

et al., 2009), 8% in Argentina (Pereda *et al.*, 2008) and 25% in the USA (Deliberto *et al.*, 2009). Considerable difficulty has also been experienced in obtaining high titres for 28 of 44 haemagglutinating agents (such as AIVs, NDVs) for identification, even after 20 passages in the allantoic and/or amniotic cavity of embryonated chicken eggs (Mackenzie *et al.*, 1985).

Low viral loads in field samples were previously identified as a cause of poor performance of this conventional technique (Munster *et al.*, 2007; Munster *et al.*, 2009). In addition, the failure of viral isolation in this study may be due to failure in maintaining samples at a sufficiently cool temperature in the field and multiple freeze-thaws which might have degraded or killed viruses. In addition, it was not possible to inoculate samples into chicken embryos directly from fresh field samples due to the unavailability of chicken eggs due to the expense and the intensive labour needed. Therefore, field samples were screened first by rRT-PCR and reactor samples (six) were then inoculated into chicken eggs. However, for future studies, samples could be inoculated directly into embryonated chicken eggs without screening. The pooled samples (four to five swabs per pool) could be used instead of using individual samples which would reduce costs and labour.

4.4.6. Limitations

The H sub-typing has not been possible for 79% of the reactor samples which may be due to lower sensitivity of the sequencing PCR as compared to the rRT-PCR screening assay. An earlier study experienced the same difficulties in AIV sub-typing (Hansbro *et al.*, 2010). They were unsuccessful in 64% reactor samples for sub-typing. The present study, however, used semi-nested PCR to try to improve the sensitivity of PCR sequencing, but a low concentration of RNA in reactor samples or deterioration of the RNA after repeated freezing and thawing may have limited the success of this semi-nested PCR approach.

4.4.7. Conclusions

This study determined a 1-1.4% apparent AIV RNA prevalence in WABs of nQLD which appears to be quite low. However, when this is translated to an annual incidence it becomes apparent that influenza infections are frequent events for this population.

Importantly, the prevalence was higher in PBDs (3.5-4.5%) which indicates higher susceptibility of this species to AIVs. Both live bird and faecal samples appeared to be equally important for future AI surveillance programs. This study identified the recent introduction of H6 and H9 subtypes to nQLD wild bird populations through migratory birds as well as H6 that probably had reassorted. There is also the chance of introducing exotic HPAI viruses to

Australia by migratory birds as indicated by the similarity of AIVs identified in this study to a LPAI virus in a migratory STS and viruses found in Eurasian WABs. Adaptability of the newly established H6 and H9 subtypes to domestic avian species is unknown. However, these subtypes can cause mortality after quickly adapting to domestic poultry (Forrest and Webster, 2010). Additionally this study developed a reasonable semi-nested PCR method for sequencing different AIV genes (M, H and NSP) from field samples.

A successful network was established between the property owners and the JCU AI research team through which live bird samples, with accompanying information, were readily obtained for analysis. Therefore, there is an opportunity for establishing a long term AI surveillance program for WABs in nQLD of Australia. The experience gained in this study may inform Government policy and implementation of a high-quality overall health surveillance system for WABs in northern Australia.

Chapter 5: Monitoring of wild birds for Newcastle disease virus in north Queensland

5.1. Introduction

Wild aquatic birds are recognized reservoirs of NDVs belonging to the family of *Paramyxoviridae* and may act as a vehicle for transference to poultry, eventually causing outbreaks of disease (Alexander, 1995; Stanislawek *et al.*, 2002). Virulent NDVs can cause high mortality in wild birds such as juvenile double crested cormorants (*Phalacrocorax auritus*) in North America (Glaser *et al.*, 1999), western Canada (Wobeser *et al.*, 1993) and teals (*Anas crecca*) in Iran (Bozorgmehri-Fard and Keyvanfar, 1979).

Two classes of NDVs are recognised worldwide. The class-one type viruses are predominantly isolated from wildlife and nine genotypes have been described (Wu *et al.*, 2011). The class-two type viruses have been responsible for most of the outbreaks of ND in domestic poultry world-wide and the genetic lineages have been referred to as 1 to 5e (Aldous *et al.*, 2003; Aldous *et al.*, 2010) or I to VII (Lomniczi *et al.*, 1998).

Australia has previously experienced ND outbreaks due to virulent NDVs in poultry between 1930 and 2002 (Johnstone, 1933; Albiston and Gorrie, 1942; Westbury, 2001). With the exception of the NDVs isolated from an outbreak in the 1930s all Australian NDVs isolated from chickens were defined as class-two genotype-one viruses (Figure 5.3). The source of viruses was presumed to be originated from wild birds because avirulent class-two NDVs were commonly found in wild bird populations. The class-two genotype-one viruses include V4 like viruses (Australia) and XZ-32-07 (China) and Ulster 67 (Simmons, 1967; Westbury, 1979; Spradbrow *et al.*, 1995; Alexander, 2001a; Peroulis and O'Riley, 2004; Czegledi *et al.*, 2006; Kattenbelt *et al.*, 2006a; Kattenbelt *et al.*, 2006b; Kim *et al.*, 2007a; Mia Kim *et al.*, 2008; Aldous *et al.*, 2010; Wu *et al.*, 2010; Wu *et al.*, 2011).

Therefore, it has been suggested that wild birds may act as a reservoir of avirulent NDVs and transmit these viruses to domestic species (Hinshaw *et al.*, 1980b). These viruses continue to evolve as they are rapidly passed in chickens. They acquire a furin cleavage site in the fusion (F) gene resulting in an increase in virulence (Gould *et al.*, 2001). In contrast, only one published example of class one-type NDVs from WABs mutating to a virulent form in chickens in Northern Ireland has been documented (Alexander, 1995).

Comprehensive studies on estimating the prevalence of NDV RNA and determination of risk factors associated with the prevalence in Australian wild birds have not been attempted. Some opportunistic studies, however, documented the serological and virological prevalence of NDVs in Australia such as 13% sero-prevalence in grey teal (Garnett and Flanagan, 1989) and 0.4% NDV prevalence in mixed wild birds (Mackenzie *et al.*, 1985).

Therefore, the monitoring of NDVs was included in the three-year longitudinal study in nQLD beginning April 2007 on WABs to understand the molecular epidemiology of NDVs and identify potential threats to domestic poultry. The specific aims were to estimate NDV RNA prevalence and the associated risk factors and determine the distribution of NDV subtypes by genetic analysis.

5.2. Materials and methods

5.2.1. Study sites and sampling

Study sites and sampling details were described as previously outlined (section 3.2.1; Chapter 3).

5.2.2. Swab and environmental faecal sample collection and recording of epidemiological data

Collection, transportation and storage of samples obtained from WABs and recording of epidemiological data were explained as previously outlined (section 4.2.2 and 4.2.3; Chapter 4).

5.2.3. Sample processing

Sample processing and RNA extraction procedures were followed as described earlier (section 4.2.4; Chapter 4).

5.2.4. Molecular detection of Newcastle disease viral ribonucleic acid

A multiplex one-step rRT-PCR was performed using the DNA saturated binding dye SYTO9® (Invitrogen, USA). This assay is based on the M gene of NDVs to detect NDV RNA from both class-one and class-two type NDVs. Two sets of primers were used for this assay: one set targeted the M gene of class-one type (Forward-GGAACCGCATGTTATCCGATTG and Reverse-GTGTGCCAGCTTGAATGATCAC) (Burgess, 2006; unpublished) and the other set

targeted the M gene of class-two type (Forward-AGTGATGTGCTCGGACCTTC and Reverse-CCTGAGGAGAGGCATTTGCTA) (Wise *et al.*, 2004).

Invitrogen Superscript™ III Platinum® One-step rRT-PCR kits were used for this assay. Reaction mixture, thermal cycling conditions and melt curve analysis for this assay were followed as described previously for the Ward assay (Section 4.2.5; Chapter 4).

Real time RT-PCR runs were acceptable when the negative and non-template controls did not fluoresce and the positive controls had fluorescent signals which crossed the auto-set threshold level within 40 cycles. Any test sample that was reactive in the rRT-PCR assay with the C_T values of ≤ 40 was considered to be reactive. A cut-off C_T value of 40 was set, based on a prior experiment with known control samples at different dilution concentrations (data are not shown). Classification of NDV RNA class types (one and two) for field samples were determined, based on the melt curves produced by the respective positive controls in the assay. The Australian class-one and class-two type NDVs produced clearly different peaks in the assay.

5.2.5. Analysis of Newcastle disease viral genes

Field samples that reacted in the screening test were further analysed to obtain sequences for M and F genes. A two-step nested RT-PCR was performed to amplify these genes. Primers are outlined in Table 5.1.

ImProm-II™ Reverse Transcription System kits (Promega) were used for the first-strand cDNA synthesis. A template mixture of 5 µl containing 10 mM of a forward primer for each gene (Table 5.1) and 4 µl of extracted RNA template was prepared in a 0.2 ml PCR tube. The template mix was then incubated in an Eppendorf Mastercycler at 70°C for five minutes to denature RNA secondary structures. The sample was then rapidly cooled to 4°C for five minutes.

A total volume of 20 µl per reaction contained 1× ImProm-II reaction buffer, 3 mM $MgCl_2$, 0.5 mM of RNase inhibitor, 1 U/µl of ImProm-II Reverse transcriptase enzyme and 5 µl of chilled template mix.

Reverse transcription reaction conditions included 30 minutes at 45ºC for primer annealing followed by 60 minutes at 50°C for extension. All RT reactions were performed using an

Eppendorf Mastercycler Gradient thermocycler. Complementary DNA products were stored either in 4ºC or -20ºC.

5.2.6. Polymerase chain reaction

Polymerase chain reaction products showing overlapping sequences within the M and F genes were amplified using the primers provided in Table 5.1.

5.2.6.1. Polymerase chain reaction amplification and sequencing of the matrix and fusion genes

Following cDNA synthesis, PCR was performed using the primers presented in Table 5.1 to amplify overlapping sections of the M and F genes. A volume of 30 µl per reaction was composed of 1×GoTaq hot-start PCR mix (Promega), 0.4 mM each forward and reverse primer and 2 µl template (M gene) or 5 µl template (F gene). The PCR cycling parameters were 95ºC for two minutes followed by 40 cycles of 95ºC for 15 seconds, 60°C (M-1) or 55°C (M-2 and M-3) or 50°C (F-1 and F-2) for 15 seconds and 72°C for 15 seconds (M-1) or 72°C for 45 seconds (M-2 and M-3) or 72°C for 30 seconds (F-1 and F-2), followed by a final extension step of 72°C for 10 minutes.

All samples were kept at 4°C until the PCR product was analysed through electrophoresis on 1% agarose gel. Polymerase chain reaction products on the gel were visualized through ethidium bromide staining. The apparent size of each PCR product was compared with DNA markers of known size. Both positive and negative controls were used to check the validity of each PCR run.

Table 5.1 Primer sets used for sequencing different Newcastle disease viral genes (Burgess, 2009; unpublished)

NDV genes	Primer	Sequence (5'-3')	Position	Product length (bp)
Matrix (M)	M-1 (NDV RNA class-one type)-one of the screening primer sets	GGAACCGCATGTTATCCGATTG-F	891-915	109
		GTGTGCCAGCTTGAATGATCAC-R	979-1,000	
	M-2 (NDV RNA class-one type)	CGTTTACAAGATTCCAACCGCAGC-F	555-578	549
		GCGAGTGCTTACTTCTTGAACGGG-R	1,080-1,103	
	M-3 (NDV RNA class-two type)	CTGTCGGCATGATCAATGATAATCC-F	222-248	706
		GGAGAGGCATTTGCTATGGGATAGC-R	909-934	
Fusion (F)	F-1 (NDV RNA class-one type)	CCCTCTTGGTGATTCTATC-F	279-297	648
		CTTACAGACAAGGTTTCC-R	909-926	
	F-2 (NDV RNA class-two type)	AATATGCCCAAGGATAAAG-F	202-220	876
		AATACCAGGAGACATAGG-R	1,060-1,077	

F: Forward; *R*: Reverse; *bp*: Base pairs

Reactions were purified and sequenced by Macrogen (Seoul, South Korea) using ABI 3700 (Applied Biosystems) automated sequencing machines. Sequencing was performed on both DNA strands. Sequences were analysed using the ContiqExpress Project, Vector NTI Advanced 10 (Invitrogen). Chromatograms were loaded, their quality evaluated by visual inspection and the ends trimmed to the reference gene obtained from the GenBank. Multiple sequences from each gene, for each sample, were aligned into a single contig and individual chromatograms analysed and edited if required.

Sequences were compared to the available GenBank sequences using a BLAST. Sequences were imported into an MSF file in GenDoc and compared with other similar and dissimilar sequences. Where necessary the sequences were trimmed and the protein coding open reading frames were predicted.

Phylogenetic analysis was performed using features of the MEGA4 suite of programs (Tamura *et al.*, 2007). Trees were inferred using the maximum likelihood evolution method (Yang, 2007) and the bootstrap method with 5,000 iterations was used to evaluate statistically the robustness of the trees presented with bootstrap values above 70% considered to show a statistically supported node (Felsenstein, 1985).

5.2.7. Statistical analysis

Field and laboratory data were managed in the MS Excel-2003 spread sheet. Data was exported into STATA/SE ™ 11.0 (StataCorp, USA) for checking integrity and performing an epidemiological analysis. Descriptive statistics were used to express individual results of each category as a frequency percentage and with 95% CIs.

5.2.7.1. Risk factor analysis

Univariate and multivariate logistic regression analysis were performed to detect any association between the binary response variable (reactor or non-reactor NDV RNA samples obtained in screening rRT-PCR) and site, year of sampling, season, age, weight and sex.

5.2.7.1.1. Univariate logistic regression

Chi-square tests followed by univariate logistic regression were performed to identify potential risk factors for a binary response variable using the data set-A (data descriptions are given in the result section).

5.2.7.1.2. Multivariate logistic regression

Chi-square tests were performed to assess unconditional relationships between the selected factors and the binary response variable using the data set-B (data descriptions are given in the result section). Factors with significant $\chi 2$ results ($p \leq 0.1$) were selected for the multivariate logistic regression.

The model was manually constructed by forward-selection applying the maximum likelihood estimation procedure by Dohoo *et al*. (2003) and the statistical significance of the contribution of individual predictors was determined. Wald's test and the LRT were used to complete this, as described by Dohoo *et al*. (2003). Interaction and confounding between factors were assessed by constructing two-interaction product terms for the significant main effect factors in the model, forcing them into the model and examining changes in the coefficients and p values of the main effects. The presence of confounding factors was investigated by removing one of the variables and assessing changes in the coefficient. A coefficient change of > 10% was considered to indicate the presence of confounding variables. Collinearity between categorical factors was tested using the 2-tailed *p*-value by the $\chi 2$ test. Two factors were considered to be collinear if the *p* value was ≤ 0.05. The model was then assessed for goodness-of-fit using the Hosmer-Lemeshow test, while predictive ability was determined using the ROC curve (Dohoo *et al*., 2003). The results were presented for each adjusted predictor variable as an OR, *p* value and with a 95% CI.

5.3. Results

5.3.1. Samples collected

Number of sampled birds (by time and site), sample distribution (by species and site) and samples obtained from the recaptured birds were presented earlier (section 4.3.1 and Table 4.4 and 4.5; Chapter 4).

5.3.2. Newcastle disease viral ribonucleic acid prevalence in wild aquatic birds

The overall apparent prevalence of NDV RNA was 3.4% (0.4% NDV RNA class-one and 3.0% class-two) in the sampled WABs (N=1,461) of nQLD. The prevalence of RNA from swabs was 3.0% for cloacal (n=1,458) and 0.4% for oropharyngeal (n=1,368). The prevalence by site of collection was 4.6% for Cromarty, 3.0% for Billabong Sanctuary and 0% for Cape York and Atherton Tableland. Prevalence in PWDs was higher (4.2%) compared with PBDs (0.9%) (Table 5.2).

By contrast, the overall prevalence was 0.4% of the faecal samples of WABs (N=1,157) of which Cromarty accounted for 0.6% (0.2% NDV RNA class-one and 0.4% class-two type) (n=695) and Billabong Sanctuary accounted for 0.2% NDV RNA class-two type (n=462).

The prevalence was 1.6% (n=246) for AWIs, 0.8% for MGs and 0% for other species.

Table 5.2 Newcastle disease viral ribonucleic acid prevalence in the samples obtained from wild aquatic birds in north Queensland according to different factors (N=1,461) (from April 2007 to March 2010). The positive detection of Newcastle disease viral ribonucleic acid was determined as a positive for either the cloacal or the oropharyngeal swab extract for each bird.

Factors	Categories	Number of samples tested	% (Number of reactors)	95% CI
Site	Billabong Sanctuary	731	3.0% (22)	1.8-4.5
	Cromarty	603	4.6% (28)	3.1-6.6
	Cape York	107	0% (0)	0-3.3
	Atherton Tableland	20	0% (0)	0-16.8
Year	2007	193	0.5% (1)	0.01-2.8
	2008	477	4.6% (22)	2.9-6.8
	2009	789	3.3% (26)	2.1-4.7
	2010	2	50% (1)	1.2-98.7
Species	Plumed whistling duck	1,180	4.2% (49)	3.0-5.4
	Pacific black duck	112	0.9% (1)	0.02-4.8
	Others	169	0% (0)	0-2.1
Age	Up to Juvenile	271	7.0% (19)	4.2-10.7
	Sub-adult	114	2.6% (3)	0.05-7.4
	Adult	906	3.0% (27)	1.9-4.3
	Unknown	170	0.6% (1)	0.01-3.2
Sex	Male	286	4.9% (14)	2.7-8.1
	Female	984	3.7% (36)	2.5-5.0
	Unknown	191	0% (0)	0-1.9

Other species: Magpie goose (*Anseranas semipalmata*); Dusky moorhen (*Gallinula tenebrosa*); Wandering whistling duck (*Dendrocygna arcuata*); Muscovy duck (*Cairina moschate*); Radjah shelduck (*Tadora radjah*); Green pygmy goose (*Nettapus pulchellus*); Australian white ibis (*Threskiornis molucca*); Bush stone curlew *(Burhinus grallarius)*; Crow (*Corvus orru*); Hardhead (*Aytha australis*); Australian shelduck (*Tadorna tadornoides*); Black swan (*Cygnus atratus*); Domestic goose (*Anser anser* subsp. *Domesticus*); Grey teal (*Anas gracilis*); White faced heron (*Egretta novaehollandiae*) and Pigeon (*Ducula bicolour*)

5.3.3. Real time reverse transcriptase-polymerase chain reaction results for the recaptured birds

This study evaluated 297 cloacal and 298 oropharyngeal swab samples that were obtained from 204 recaptured birds (one to five recaptures, mean of two). Of 204 birds, 16 previously non-reactor birds (15 cloacal and one oropharyngeal swabs) were newly detected as reactors (10 at first, four at second, one at third and one at fourth recapture). Of the 16 reactors, two were NDV RNA class-one and 14 were class two-type. The rest of the birds (188) remained negative to the testing at each recapture.

5.3.4. Results of risk factor analysis

Due to variability in the numbers of birds captured over time two subsets of data were analysed in order to ensure sufficient sample numbers in each category of risk factor under investigation (site, year, season, age, sex and weight).

5.3.4.1. Data subset A (N=394)

This study generated data from the predominantly caught species, PWDs at Billabong Sanctuary from June 2007 to May 2009. Although the present study commenced in April 2007, few birds were caught during April to May 2007 and therefore that data was excluded from the analysis. This data set was suitable for examining the effect of season on the dichotomous results of rRT-PCR on samples evaluated for NDV RNA. Seasons were classed as warm dry (September-December), warm wet (January-April) and cool dry (May-August). Years were categorized as June 2007-May 2008 and June 2008-May 2009. Age was grouped into three categories: 1. chicks and juveniles, 2. sub-adults and adults and 3. unknown age class. Body weight was not normally distributed even after natural log transformation (it remained skewed to the right) and therefore, it was organized into two categories separated by the 50^{th} percentile (0.20-0.65; 0.66-1.16 Kg). Gender was excluded from the analysis due to a significant number of birds in this data which were not assessed for sex. The analysis for this data set was restricted to univariate logistic regression due to an insufficient number of reactor samples in each factor category.

5.3.4.2. Univariate logistic regression (A)

The results of rRT-PCR on samples evaluated for NDV RNA varied significantly between years, weight categories and among seasons and age classes (χ^2, $p<0.05$) (Table 5.3). The odds ratios of being reactor samples for NDV RNA were 10.9 for the second year over the first year of the study ($p=0.022$), 9.8 for warm wet over warm dry seasons ($p=0.034$), 9.4 for younger over older ducks ($p=0.004$) and 6.4 for lighter over heavier ducks ($p=0.016$) (Table 5.3).

Table 5.3 Univariate chi-square and univariate logistic regression analysis of the proportion of samples in the categories of reactor and non-reactor for Newcastle disease viral ribonucleic acid obtained by real time reverse transcriptase-polymerase chain reaction analysis of swabs of plumed whistling ducks (N=394) (from June 2007 to May 2009)

		NDV RNA		χ^2	Univariate logistic regression	
Factors	**Categories**	**-**	**+**	***p***	**OR (95% CI)**	***p***
Year	Jun 2007-May 2008	173	1 (0.6%)	0.005	Referent	
	Jun 2008-May 2009	207	13 (5.9%)		10.9 (1.4-83.9)	0.022
Season	Sep-Dec (Warm dry)	141	1 (0.7%)	0.045	Referent	
	Jan-Apr (Warm wet)	101	7 (6.5%)		9.8 (1.2-80.7)	0.034
	May-Aug (Cool dry)	138	6 (4.2%)		6.1 (0.7-51.6)	0.095
Age	Chick and juvenile	94	11 (10.5%)	<0.001	9.4 (2.0-43.4)	0.004
	Sub-adult and adult	161	2 (1.2%)		Referent	
	Unknown	125	1 (0.8%)		N/A	
Weight	0.20-0.659 Kg	184	12 (6.1%)	0.006	6.4 (1.4-28.9)	0.016
	0.66-1.16 Kg	196	2 (1.0%)		Referent	

OR: Odds ratio; *CI*: Confidence interval; - Non-reactor; + Reactor

5.3.4.3. Data subset B (N=958)

This study also produced a data set from PWDs at Billabong Sanctuary and Cromarty (Green Acres Lagoon) from January 2008 to December 2009. This was the only data set to examine the site effect on the results of rRT-PCR on samples evaluated for NDV RNA. This study had a complete two-year dataset; therefore year classes were 2008 and 2009. Age was regrouped into two classes, 1. chicks and sub-adults and 2. adults in order to have sufficient samples in each class. Weight was not normally distributed even after natural log transformation (it remained skewed to the right) and therefore, was categorized into two groups, based on the 50^{th} percentile (0.20-0.65; 0.66-0.91 Kg). Gender was also included as a category in the analysis. The analysis for this data set was extended to multivariate logistic regression due to a sufficient number of reactor samples of each category of factors being examined. This was because the effects of season were not examined.

5.3.4.4. Logistic model (B)

The chi-square test results for the individual categories of site, year, age, sex and weight are shown in Table 5.4. The proportion of results in NDV RNA reactor and non-reactor categories of rRT-PCR results varied for year and age ($p \leq 0.10$). Therefore, these two factors were put forward to construct the logistic model and their combined adjusted effects were examined.

Collinearity was not detected between year and age (χ^2 test, p>0.05). No interaction or confounding was detected in the final model. The Hosmer-Lemeshow goodness-of-fit test demonstrated that the model fitted the data (χ^2 test, $p \leq 0.865$). The ROC curve extended reasonably well into the upper left-hand corner of the curve and the area under the curve was 0.64 (curve is not shown). Both of these model characteristics indicated that the model had good predictive ability. The odds of reactor samples of WABs was 2.7 (95% CI 1.5-4.9) more likely in younger than older ducks (p=0.001) (Table 5.5).

Table 5.4 Univariate chi-square analysis of the proportion of samples in the categories of reactor and non-reactor for Newcastle disease viral ribonucleic acid obtained by real time reverse transcriptase-polymerase chain reaction analysis of swabs of plumed whistling ducks (N=959) (from January 2008 to December 2009)

Factors	**Categories**	**NDV RNA (-)**	**NDV RNA (+)**	***p*** **($\chi^{2)}$**
Site	Billabong Sanctuary	390	19 (4.7%)	0.752
	Cromarty	522	28 (5.1%)	
Year	2008	291	21 (6.7%)	
	2009	621	26 (4.0%)	0.068
Age	Chick and juvenile	191	20 (9.5%)	<0.001
	Sub-adult and adult	721	27 (3.6%)	
Weight	0.20-0.65 Kg	470	21 (4.3%)	0.359
	0.66-0.91 Kg	442	26 (5.6%)	
Sex	Male	214	13 (5.7%)	0.509
	Female	698	34 (4.6%)	

- NDV RNA non-reactor samples; + NDV RNA reactor samples

Table 5.5 Results of a logistic model analysis of the proportion of samples in the categories of reactor and non-reactor for Newcastle disease viral ribonucleic acid obtained by real time reverse transcriptase-polymerase chain reaction analysis of swabs of plumed whistling ducks (N=959) (from January 2008 to December 2009)

Factors	**Categories**	**OR**	**95% CI**	***p***
Year	2008	1.6	0.9-3.0	0.111
	2009	1.0	Referent	
Age	Chick and juvenile	2.7	1.5-4.9	0.001
	Sub-adult and adult	1.0	Referent	

OR: Odds ratio; *CI*: Confidence interval

5.3.5. Newcastle disease viral ribonucleic acid sequencing results

Of 71 reactor samples (including 16 from recaptured birds) (N=1,641 live birds and 1,157 faecal samples) from the NDV RNA screening assay, sequencing results were obtained for only three samples (at least the M gene). Sequences from 17 reactor samples were unsuccessful. Sequencing was not attempted for 51 reactor samples. The details are shown in Table 5.6.

Table 5.6 Details of analysis to obtain gene sequences from Newcastle disease viral ribonucleic acid reactor samples of wild aquatic birds in north Queensland (from April 2007 to March 2010)

Species	rRT PCR (Screening assay)		PCR/Sequencing (Attempted/Not)	Sequencing results	Sample type/Site/Bird ID/Month/Year
	NDV RNA Class one type (C_T value)	NDV RNA Class two type (C_T value)			
PWD-1		+ (21.4)	M-3	Unsuccessful	Cl/Billabong/92/Sep/2007
PWD-2		+ (22.6)	M-3	Unsuccessful	Cl/Billabong/195/**Recaptured**/Nov/2007
PWD-3		+ (26.9)	Not attempted	-	Cl/Cromarty/237/Jan/2008
PWD-4		+ (27.4)	Not attempted	-	Cl/Cromarty/405/Apr/2008
PWD-5		+ (24.8)	M-3	Unsuccessful	Cl/Cromarty/418/Apr/2008
PWD-6		+ (26.0)	Not attempted	-	Cl/Cromarty/421/Apr/2008
PWD-7		+ (29.9)	Not attempted	-	Cl/Cromarty/422/Apr/2008
PWD-8	+ (31.1)		Not attempted	-	Op/Cromarty/423/Apr/2008
PWD-9	+ (27.3)		Not attempted	-	Op/Cromarty/429/Apr/2008
PWD-10	+ (26.6)		M-2	Unsuccessful	Op/Cromarty/436/Apr/2008
PWD-11	+ (26.6)		Not attempted	-	Cl/Cromarty/438/Apr/2008
PWD-12		+ (22.3)	M-3	Unsuccessful	Cl/Billabong/468/Jun/2008
PWD-13		+ (23.5)	Not attempted	-	Cl/Billabong/480/Jun/2008
PWD-14		+ (22.8)	Not attempted	-	Cl/Billabong/610/Aug/2008
PWD-15		+ (24.3)	Not attempted	-	Cl/Billabong/612/Aug/2008
PWD-16		+ (23.8)	Not attempted	-	Cl/Billabong/614/Aug/2008
PWD-17		+ (23.2)	Not attempted	-	Cl/Cromarty/620/Sep/2008
PWD-18		+ (22.4)	Not attempted	-	Cl/Cromarty/625/Sep/2008
PWD-19		+ (22.6)	Not attempted	-	Cl/Cromarty/626/Sep/2008
PWD-20		+ (22.7)	Not attempted	-	Cl/Cromarty/632/Sep/2008
PWD-21		+ (21.4)	M-3	Unsuccessful	Cl/Cromarty/640/Sep/2008
PWD-22		+ (24.0)	Not attempted	-	Cl/Cromarty/641/Sep/2008
PWD-23		+ (26.8)	M-3	Unsuccessful	Cl/Billabong/668/**Recaptured**/Oct/2008
PWD-24		+ (23.1)	M-3	Unsuccessful	Cl/Cromarty/726/Nov/2008
PWD-25		+ (27.7)	Not attempted	-	Cl/Billabong/763/Mar/2009
PWD-26		+ (29.8)	Not attempted	-	Cl/Billabong/796/Mar/2009
PWD-27		+ (27.6)	Not attempted	-	Cl/Cromarty/802/Mar/2009
PWD-28		+ (27.5)	Not attempted	-	Cl/Billabong/806/Mar/2009
PWD-29		+ (23.8)	Not attempted	-	Cl/Billabong/882/Apr/2009
PWD-30		+ (28.2)	Not attempted	-	Cl/Billabong/884/Apr/2009
PWD-31		+ (25.5)	M-3	Unsuccessful	Cl/Billabong/890/Apr/2009
PWD-32		+ (24.0)	Not attempted	-	Cl/Billabong/961/Apr/2009
PWD-33		+ (28.8)	M-3	Unsuccessful	Cl/Cromarty/1000/**Recaptured**/Apr/2008
PWD-34		+ (28.8)	Not attempted	-	Cl/Cromarty/1005/Apr/2009
PWD-35		+ (27.5)	Not attempted	-	Cl/Billabong/1008/May/2009
PWD-36		+ (29.1)	Not attempted	-	Cl/Billabong/1061/**Recaptured**/Jun/2009
PWD-37		+ (27.4)	Not attempted	-	Cl/Cromarty/1073/**Recaptured**/Jun/2009
PWD-38		+ (26.5)	Not attempted	-	Cl/Billabong/1117/**Recaptured**/Jun/2009
PWD-39		+ (29.2-29.3)	Not attempted	-	**Cl-Op**/Cromarty/1137/Jun/2009
PWD-40		+ (28.7)	Not attempted	-	Cl/Billabong/1145/Jul/2009
PWD-41		+ (25.5)	Not attempted	-	Cl/Billabong/1205/**Recaptured**/Sep/2009
PWD-42	+ (24.1)		Not attempted	-	Cl/Billabong/1221/Sep/2009
PWD-43		+ (26.6)	Not attempted	-	Cl/Cromarty/1222/Sep/2009
PWD-44		+ (28.7)	Not attempted	-	Cl/Billabong/1626/**Recaptured**/Dec/2009
PWD-45		+ (26.4)	M-3	Unsuccessful	Cl/Cromarty/1639/**Recaptured**/Dec/2009
PWD-46		+ (23.8)	M-3 and F-2	M-3 and F-2	Cl/Cromarty/1643/Dec/2009
PWD-47		+ (29.2)	Not attempted	-	Cl/Cromarty/1651/Dec/2009
PWD-48	+ (30.8)		M-1	M-1	Op/Cromarty/1657/**Recaptured**/Dec/2009
PWD-49		+ (27.6)	Not attempted	-	Cl/Cromarty/1662/Dec/2009
PWD-50		+ (26.9)	Not attempted	-	Cl/Cromarty/1666/Dec/2009
PWD-51		+ (29.4)	Not attempted	-	Cl/Billabong/1670/Dec/2009
PWD-52		+ (29.7)	Not attempted	-	Cl/Billabong/1675/Dec/2009
PWD-53		+ (30.2)	Not attempted	-	Cl/Billabong/1676/Dec/2009
PWD-54		+ (28.9-31.3)	M-3 and F-2	Unsuccessful	**Cl-Op**/Billabong/1677/Dec/2009
PWD-55	+ (28.7)		M-1	M-1	Cl/Cromarty/1694/**Recaptured**/Dec/2009
PWD-56		+ (29.7)	Not attempted	-	Cl/Cromarty/1696/**Recaptured**/Dec/2009
PWD-57		+ (30.0)	Not attempted	-	Cl/Cromarty/1698/**Recaptured**/Dec/2009
PWD-58		+ (27.0)	Not attempted	-	Cl/Cromarty/1705/Dec/2009
PWD-59		+ (30.3)	Not attempted	-	Cl/Cromarty/1707/Dec/2009
PWD-60		+ (30.0)	Not attempted	-	Cl/Cromarty/1709/**Recaptured**/Dec/2009
PWD-61		+ (29.2)	Not attempted	-	Cl/Cromarty/1712/**Recaptured**/ Dec/2009
PWD-62		+ (29.8)	Not attempted	-	Cl/Cromarty/1713/Dec/2009
PWD-63		+ (30.4)	Not attempted	-	Cl/Billabong/1715/Dec/2009
PWD-64		+ (26.4)	M-3	Unsuccessful	Cl/Cromarty/1716/**Recaptured**//Dec/2009
PWD-65		+ (27.1)	M-3 and F-2	Unsuccessful	Cl/Billabong/1720/Mar/2010
PBD-1	+ (29.7)		M-2	Unsuccessful	Op/Cromarty/651/Oct/2008
MG-1	+ (29.1)		M-2	Unsuccessful	En/Billabong/865/Oct/2009
AWI-1	+ (30.6)		Not attempted	-	En/Cromarty/866/Oct/2009
AWI-2		+ (30.6)	M-3	Unsuccessful	En/Cromarty/867/Oct/2009
AWI-3	+ (26.4)		Not attempted	-	En/Cromarty/1130/Dec/2009
AWI-4	+ (26.7)		Not attempted	-	En/Cromarty/1131/Dec/2009

PWD: Plumed whistling duck; *PBD*: Pacific black duck; *MG*: Magpie goose; *AWI*: Australian white ibis; C_T: Threshold value*; M-1*: Matrix gene (NDV RNA class-one type, short sequence); *M-2*: Matrix gene (NDV RNA class-one type); *M-3*: Matrix gene (NDV RNA class-two type); *F-1*: Fusion gene (NDV RNA class-one type); *F-2*: Fusion gene (NDV RNA class-two type); *Op*: Oropharyngeal swab; *Cl*: Cloacal swab; *En*: Environmental swab; +: Reactor.

5.3.5.1. Phylogenetic analysis of the matrix gene sequences

The short fragment of M gene sequenced from two reactor samples (PWD-48 and 55) (Figure 5.1 and Table 5.6) was obtained between nucleotide positions 900 and 1,008 in this study. For phylogenetic analysis, however, 106 bp fragments covering the positions 901 and 1006 were used and this length was available for both reactor sample sequences. For comparative phylogenetic analysis, the reference sequences for class-one and class-two type NDVs along with two reactor sample sequences obtained in this study were included (Figure 5.1). The phylogram shows the reactor sample sequences (PWD-48 and 55) were closely related to each other (69 bootstrap value) and more similar to the Australian RNA NDV class-one type of ibis isolate (Queensland/2006) and duck-3245 isolate (Western Australia/1977) than it was for any other sequence (Figure 5.1.).

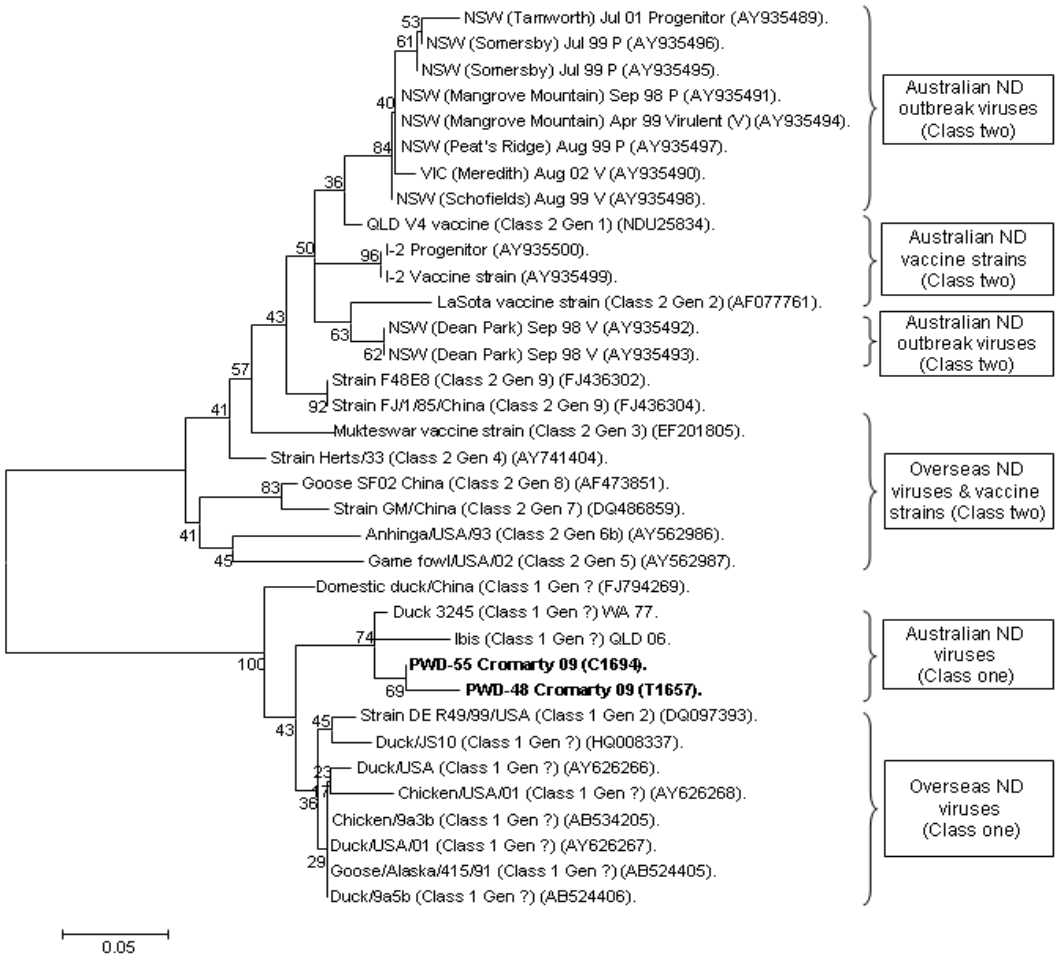

Figure 5.1 Bootstrap consensus trees with 5,000 replications for the Newcastle disease viral matrix gene (nucleotide positions between 901 and 1,006 base pairs)

A long fragment of M gene sequence from one reactor sample (PWD-46) (Figure 5.2 and Table 5.6) was obtained between nucleotide positions 237 and 908 in this study. Therefore, a

672 bp fragment sequence of this reactor sample, along with reference sequences was used for constructing phylogenetic trees. The phylogram shows the reactor sequence (PWD-46) clustered with the sequences of Australian I-2 progenitor virus and I-2 vaccine strain (Figure 5.2).

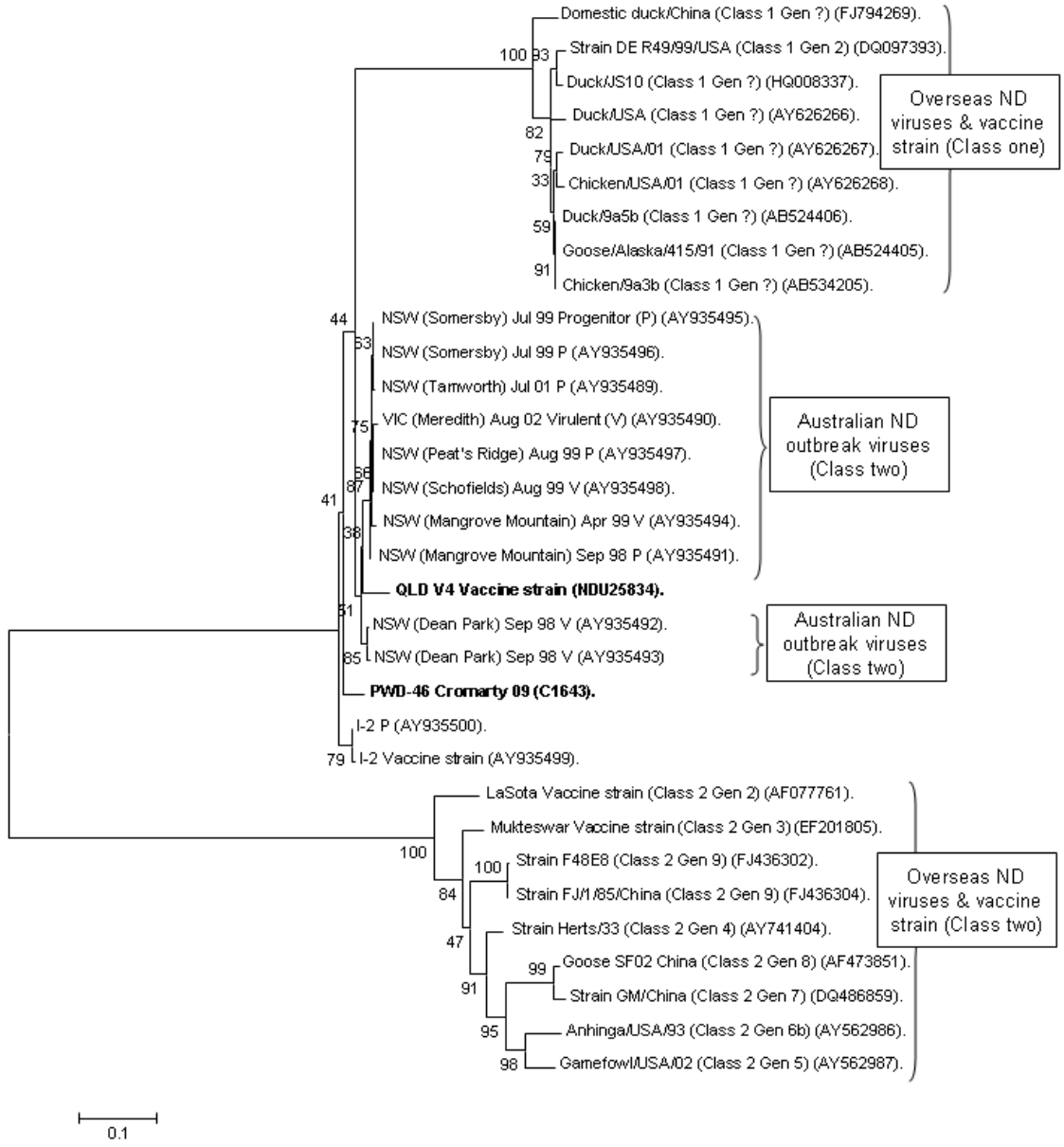

Figure 5.2 Bootstrap consensus trees with 5,000 replications for the Newcastle disease viral matrix gene (nucleotide positions between 237 and 908 base pairs)

5.3.5.2. Phylogenetic analysis of the fusion gene (class-two type) sequences

This study also obtained a long fragment of fusion gene sequence (843 bp, nucleotide positions: 220 and 1,062) for the PWD-46 reactor sample. This sequence with reference sequences was combined. The results are presented in Figure 5.3.

The F gene phylogeny demonstrated that the reactor sequence (PWD-46) was similar to sequences from Australian I-2 progenitor and I-2 vaccine strains as well as the chicken isolates AP1 and SP3. It is distinct from the V4 cluster and the outbreak viruses. All of these viruses are NDV class-two genotype-one viruses (Figure 5.3). The F gene and M gene sequences both indicate that this reactor sequence clustered with the I-2 progenitor and I-2 vaccine viruses. The I-2 progenitor, AP1 and FP3 were previously isolated from chickens and have been classified as lentogenic or apathogenic viruses (Kim *et al.*, 1978; Spradbrow *et al.*, 1995). The I-2 progenitor was then developed as a vaccine (Bensink and Spradbrow, 1999) and extensively used in developing countries (Tu *et al.*, 1998; Bensink and Spradbrow, 1999; Nasser *et al.*, 2000; Illango *et al.*, 2005; Henning *et al.*, 2009).

The cleavage site of the reactor sample sequence indicated an avirulent ND class-two type virus (EGQGRL) (Table 5.7).

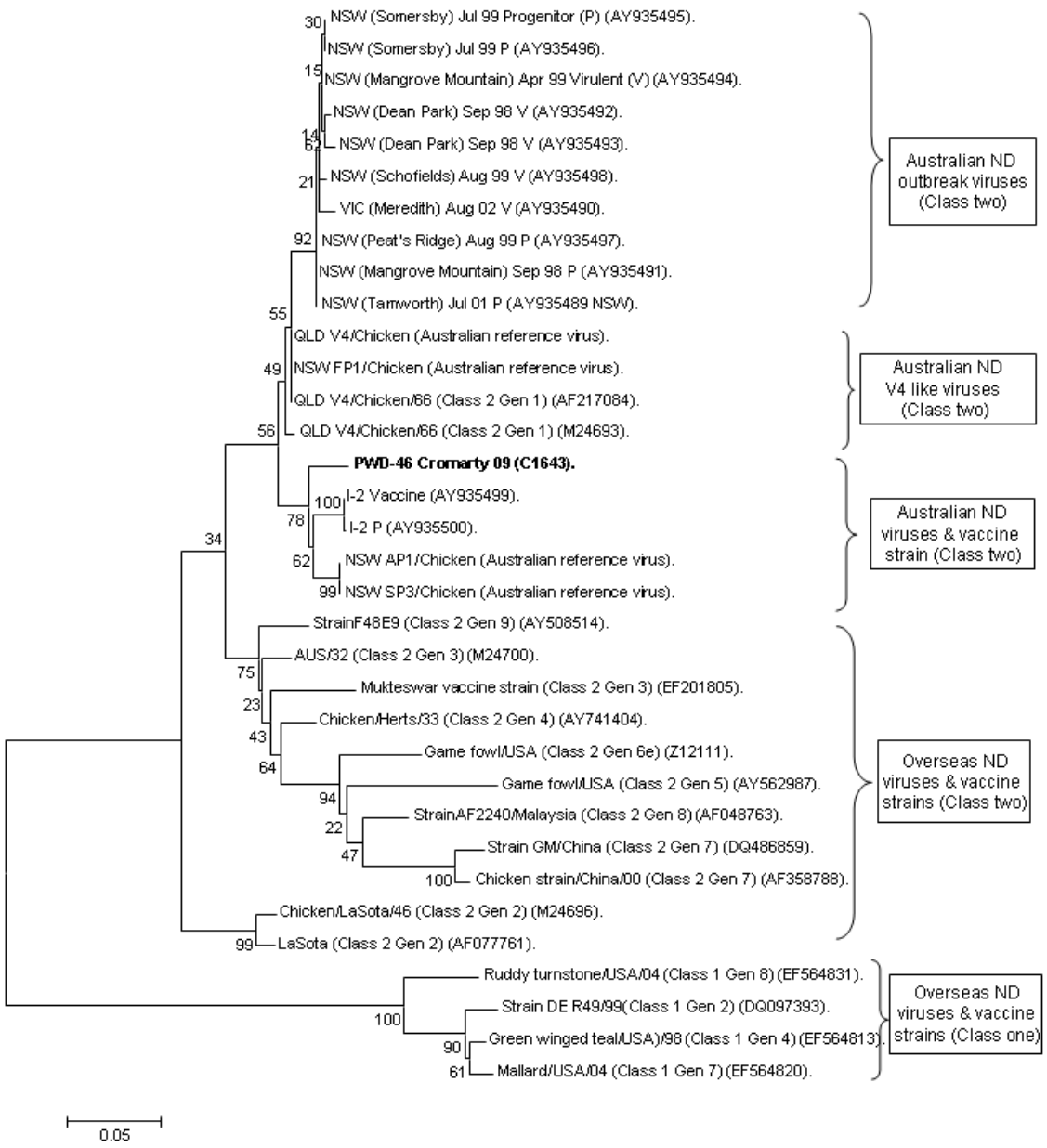

Figure 5.3 Bootstrap consensus trees with 5,000 replications for the Newcastle disease viral fusion gene (nucleotide positions between 220 and 1,062 base pairs)

Table 5.7 The amino acid sequences at the cleavage sites of Newcastle disease viral fusion genes (Australian Newcastle disease viruses with a reactor sample sequence obtained in this study)

GenBank accession or sample number	Date isolated/reactor sample	Region isolated	F cleavage site	Pathogenicity
AY935491	September 1998	Mangrove Mountain, NSW	RRQGRL	Progenitor virus
AY935494	April 1999	Mangrove Mountain, NSW	RRQRRF	Virulent
AY935495	July 1999	Somersby, NSW	RRQGRL	Progenitor like virus
AY935496	July 1999	Somersby, NSW	RRQRRL	Progenitor like virus
AY935497	August 1999	Peats Ridge, NSW	RRQGRF	Progenitor like virus
AY935492	September 1998	Dean Park, NSW	RRQRRF	Virulent
AY935493	September 1998	Dean Park, NSW	RRQRRF	Virulent
AY935498	August 1999	Schofields, NSW	RRQRRF	Virulent
AY935489	July 2001	Tamworth, NSW	RRQGRL	Progenitor like virus
AY935490	August 2002	Meredith, VIC	RRQRRF	Virulent
AF217084 (V4)	1966	Brisbane, QLD	GKQGRL	Avirulent
AY935499 (I-2 progenitor)		Australia	RKQGRL	Avirulent
AY935500 (I-2 vaccine strain)		Australian vaccine strain	RKQGRL	Avirulent
AP1/Chicken		Australian reference virus, NSW	GKQGRL	Avirulent
SP3/Chicken		Australian reference virus, NSW	GKQGRL	Avirulent
FP1/Chicken		Australian reference virus, NSW	GKQGRL	Avirulent
* PWD-46 (C1643)		Cromarty, nQLD	EGQGRL	Avirulent

* Reactor sample; *F*: Fusion gene

5.4. Discussion

5.4.1. Descriptive results

Overall NDV RNA prevalence at the individual bird level was 3.4% in this study, which corresponds to the proportionate prevalence encountered in the dead bird study (4.8%, N=42) (Chapter 6). The prevalence, however, was very low (0.4%) for faecal samples in this study, which conforms to an Australian wild bird study using swab samples (0.4%) (Mackenzie *et al.*, 1985). A similar level of 0.5% prevalence was reported in composite fresh faecal samples of northern pintails (*Anas acta*) in Japan (Jahangir *et al.*, 2009). Overall findings, therefore, suggest NDVs have been circulating at low prevalence in WABs of nQLD. A higher prevalence has been estimated in waterfowl in Finland (5.2%) (Lindh *et al.*, 2008), China (8.2%) (Zeng *et al.*, 2008) and wild birds in central Nigeria (8.0%) (Ibu *et al.*, 2009).

This suggests the presence of NDVs worldwide in wild bird populations at variable levels. This study predominantly detected NDV RNA reactor swab samples from captured and recaptured PWDs which were supported by successfully obtaining NDV sequences from some reactor samples of PWDs. Previously, ND virus has also sporadically been isolated from samples of PWDs in Australia (Mackenzie *et al.*, 1985). Some cross sectional studies in Australia, however, have suggested NDVs are absent in PWDs (Mackenzie *et al.*, 1984) (Ibrahim Diallo 2007, DPIF, Queensland, personal communication). To the author's knowledge this is the first time the level of NDVs has been quantified in PWDs through a systematic longitudinal study in nQLD. Interestingly, PWDs had the highest NDV RNA prevalence where PBDs had the highest prevalence for AIV RNA (Chapter 4).

Sporadic NDV RNA reactor samples were determined in the species other than PWDs such as one reactor swab of PBD (live bird) and five reactor faecal samples of AWI (four) and MG (one) in this study. These findings are in agreement with the previous Australian wild bird studies for NDVs (Mackenzie *et al.*, 1984; Mackenzie *et al.*, 1985) (Ibrahim Diallo 2006, DPIF, Queensland, personal communication).

5.4.2. Risk factors

Both univariate and multivariate logistic regression using the data sets-A and B identified younger PWDs as being significantly associated with a higher prevalence of NDV RNA in swab samples. Only univariate logistic analysis using data set A indicated lighter PWDs and birds caught in the warm wet season (January-April) as being significantly associated with a higher prevalence of NDV RNA. This result virtually correlates with younger birds because lighter birds represent immature birds and these birds were commonly sampled in the warm wet season. A similar age pattern of prevalence was observed in the AI study (Chapter 5). In addition, age specific prevalence was also observed earlier in domestic chickens where clinical disease or mortality due to virulent or less virulent NDVs was higher in younger birds than adults (Ezeokoli *et al.*, 1984; Janviriyasopak *et al.*, 1989; Martin, 1992). The higher prevalence in young birds may be due to the fact that they are immunologically naïve whereas adults are more resistant, particularly to viruses to which they have previously been exposed (Webster *et al.*, 1992). Contrarily, pheasants, a highly susceptible species to NDVs, were affected at all ages (Higgins, 1982; Alexander, 2001b).

Reports of seasonal influence on the occurrence of ND or prevalence of NDVs have not been readily available for wild birds but seasonal effect has varied geographically for domestic chickens. For example, ND incidence was observed to peak at the end of the dry season

(February-April) in Thailand (Ratanasethakul, 1989), winter season (December-March) in Bangladesh (Asadullah, 1992), hot dry season (September-November) and hot humid season (January-March) in Zambia (Sharma *et al.*, 1986) and dry hot season in Kenya (Njagi *et al.*, 2010). Although the ND incidence varied according to seasons in these referral studies, these seasons anonymously provided adverse conditions which might reduce the immune status of the birds, thus making them more vulnerable to infection.

A year effect on the NDV RNA prevalence was only determined in univariate logistic regression for data set-A and not in multivariate analysis for data set-B in this study. Therefore, the year effect may be an artefact of unadjusted analysis. Marked differences in AIV prevalence were, however, found between years in North American aquatic birds (Krauss *et al.*, 2004). These patterns of viral prevalence are little understood, but an epidemiologic periodicity in resistance has been implicated (Hinshaw *et al.*, 1985).

5.4.3. Newcastle disease viral ribonucleic acid classes

Both classes of NDV RNAs were evident in this study according to screening and sequencing results. Overall results suggested the NDV RNA class-two type predominantly circulated in the wild bird population in nQLD (Table 5.6) and this finding is supported by many wild bird studies throughout the world (Alexander, 1995; Aldous *et al.*, 2003; Jindal *et al.*, 2009a). (Aldous *et al.*, 2003; Kim *et al.*, 2007a; Kim *et al.*, 2007b; Lindh *et al.*, 2008; Jindal *et al.*, 2009a; Aldous *et al.*, 2010).

The presence of dual class NDV RNAs in this study was supported by earlier studies in Australia (Russell and Alexander, 1983; Mackenzie *et al.*, 1985; Alexander *et al.*, 1986) (Ibrahim Diallo 2006, DPIF, Queensland, personal communication), the USA (Spalatin *et al.*, 1976) and France and England (Russell and Alexander, 1983) and Finland (Lindh *et al.*, 2008). These studies identified the class-one type NDVs in PBD, AWI, lesser and common noddy, sooty tern and black-fronted plover (Australia), feral shelduck (French), mallard and berk duck (England), common teal and pochard (Finland). These studies also reported the class-two type NDVs in chestnut-breasted manikin, STS, red-kneed dotterel and comb-crested jacana (Australia), migratory WABs (the USA), feral ducks (French) and common teal (Finland).

5.4.4. Sequencing results

Phylogenetic analysis of a short fragment of M gene (106 bp) on two reactor sample sequences (PWD-48 and 55) showed a greater similarity to isolates of duck 3245 and AWI and (class-one) than any other isolates belonging to NDV RNA class-one type sequences (Figure 5.1). The reactors sequences were also distantly placed from the sequences of Australian virulent ND outbreak viruses (class-two); moreover, they were located at a separate node from the sequences of class two-type (Figure 5.1). The overall pattern, therefore, suggests these reactor sample sequences may represent Australian avirulent class-one type like NDVs.

Phylogenetic evaluation of the long fragments of M (672 bp) and F (843 bp) genes on another reactor sample sequence (PWD-46) showed they clustered with the sequences of Australian I-2 progenitor virus and I-2 vaccine strain which are the class-two genotype-one viruses (Figure 5.2 and 5.3). The fusion cleavage site of this reactor sequence was shown to have an avirulent motif in their F_0 cleavage site (amino acid position: 109-119) (EGQGRL) which is little different to the cleavage site sequences of avirulent I-2 progenitor and vaccine strain (RKQGRL). This difference may indicate the reactor sample sequence is even less virulent than that of the I-2 progenitor and vaccine strain (Table 5.7). However, the cleavage site of the reactor sequence is much closer to the V4 sequence (GKQGRL).

Therefore, this avirulent ND virus identified in PWD in this study would be unlikely to pose a direct threat to Australian domestic and commercial poultry. However, there is a theory that if avirulent NDVs from wild birds are introduced to domestic poultry, then after several generational cycles avirulent viruses can be mutated to virulent ones and could be capable of causing considerable mortality. For example, there is evidence that avirulent class-one NDVs in WABs mutated to virulent forms in chickens in Ireland (Alexander, 1995).

5.4.5. Limitations

The sequencing success for NDV RNA reactor samples was very low in this study (15%, N=20) which may be due to a deterioration of RNA after repeated freezing and thawing of those samples and low RNA starting concentrations. In addition, inherent inhibitors in those reactor field samples may restrict the full potential of conventional PCR to amplify PCR products for sequencing (evidenced in the AI study, Chapter 4). The alternative was to grow viruses in embryonated chicken eggs followed by sequencing; however, commitment to the major AI studies hindered us taking that further step. However, the strategy of growing viruses using embryonated chicken eggs did not work in the AI study.

Sequencing was not attempted for 51 reactor samples due to time constraints. Analysing these reactor samples at a later date could give a better picture of the viral genetic distribution details in the wild bird population in nQLD.

The relative sensitivity and specificity for the screening rRT-PCR test were studied with known control samples and found to be acceptable (data are not shown). The absolute sensitivity of this test has yet to be determined. This procedure however, does not necessarily apply to field samples. Therefore, the estimated NDV RNA prevalence might be an over or under estimation.

5.4.6. Conclusions

This study estimated 0.4-3.4% apparent NDV RNA prevalence in WABs of nQLD through a systematic longitudinal study in Australia. The prevalence was significantly higher in PWDs (4.2%). Young birds had a higher prevalence. Newcastle disease viral RNA class-two type was predominant in PWDs. An avirulent NDV RNA class-two genotype-one determined in PWD in this study appears to be of low risk to domestic and commercial poultry. Overall results obtained in this study will assist in developing future surveillance programs for NDVs of WABs in northern Australia. Continued sampling of wild birds will also assist in maintaining the understanding of the type of viruses circulating in wild birds and their potential to threaten the Australian poultry industry.

Chapter 6: Morbidity and mortality of wild aquatic birds in north Queensland

6.1. Introduction

Mortality in wild birds due to infectious and non-infectious diseases has been common across the world (Skerratt *et al.*, 2005; Bunbury *et al.*, 2008; Gottdenker *et al.*, 2008). Disease is receiving increasing recognition as a potential contributory factor to global avian decline and extinction (Weimerskirch, 2004).

Wild birds can share common pathogens with humans, for example a species of *Salmonella*, and toxin-producing strains of *Escherichia coli* (Abulreesh *et al.*, 2007). In New Zealand, a major outbreak of salmonellosis (*Typhimurium* DT160) caused extensive mortality in passerines (Stichbirds) (*Notimystis cincta*) in 2000 (Alley *et al.*, 2002) and the same *Salmonella* serotype was confirmed in humans in the region of the bird mortality. In Australia, zoonotic and livestock significant pathogens such as *Salmonella* spp., ND virus, avian influenza virus and Flaviviruses were reported in healthy AWIs (*Threskiornis molucca*) (Epstein *et al.*, 2006).

The H5N1 outbreak on Qinghai Lake in western China caused the death of wild migratory birds in 2005 (Chen *et al.*, 2005). This outbreak identified a serious risk of this virus spreading world-wide because this lake is a major breeding site for migratory birds whose flyways extend to south-east Asia, India, Siberia, Australia and New Zealand.

The human death toll due to the HPAI H5N1 between 2003 and 2010 (to 31st August 2010) was 300 (N=505 cases) (Anon, 2010b). Of the confirmed human cases and deaths, Indonesia, a close neighbour of Australia, had a high number of deaths at 139 (N=168 cases) (Anon, 2010b). There is worldwide-concern that the HPAI H5N1 may evolve the capacity for human-to-human transmission and the potential emergence of a pandemic (Alexander, 2007).

Although Australia has not recorded any HPAI H5N1 outbreaks in wild or domestic birds, there is the potential for wild migratory birds to introduce this subtype into Australia followed by transmission to local birds or humans. Established migratory routes between nQLD, PNG and south-east Asia potentially provide a direct pathway for introduction.

Therefore, disease surveillance has an important role to play in investigating the HPAI H5N1 in Australian WABs and providing early warning for the emergence of viruses in these populations prior to spill-over events in domestic stock and humans. The HPAI H5N1 has caused mortality events in wild birds and has been detected by passive surveillance in other countries (Komar and Olsen, 2008; Globig *et al.*, 2009; Hesterberg *et al.*, 2009; Willeberg *et al.*, 2010). To date, HPAI H5N1 has not been detected in Australia, but given that it has been detected in migratory birds in countries that share migratory routes with Australia, ongoing surveillance is required. Thus, a three-year passive surveillance program was conducted on sick and dead WABs at Billabong Sanctuary near Townsville, nQLD to investigate the causes of bird mortality with special reference to mortality due to the HPAI H5N1. In conjunction with this study an active surveillance program was conducted for comparison (Chapter 3).

6.2. Materials and methods

6.2.1. Collection of sick and dead birds and epidemiological data

Passive surveillance was undertaken on sick and dead WABs at Billabong Sanctuary only (19°22′ S and 146°54′ E), 20 km south of Townsville, in nQLD from April 2007 to March 2010. Sanctuary rangers actively participated with the reporting and collection of sick birds and carcasses. Dead and sick birds were collected from sanctuary rangers during unscheduled field visits. Sick birds were euthanased using a standard procedure described in the Australian Registry of Wildlife Health field manuals (*http:/www.arwh.org*) before transferring them to JCU. Carcasses were temporarily stored in the field refrigerator (4°C) or freezer (-12°C) in air-tight plastic bags before transportation to the post-mortem (PM) laboratory at JCU. Each carcass was given a unique identity number. Observable clinical signs along with the identity information of the birds' (dates of death and species) were recorded through a ranger interview or by clinical examination if the bird was still alive.

6.2.2. Collection, preservation and storage of tissue samples

A complete necropsy of each carcass was performed. Any pathological changes observed were recorded.

Tissue samples from the oesophagus, trachea, lung, brain, muscle (neck/thigh/subcutaneous), heart, liver, spleen, pancreas, bursa, intestine, proventriculus, gizzard, caecal tonsil, kidney and testes or ovary were collected if possible, regardless of whether visible lesions were observed. Individual tissue samples from each carcass were taken and tested individually for histological,

bacteriological and virological examinations. Samples, approximately ≤ 1.0 cm thick per tissue slice, for histological study were placed in vials containing 10% buffered neutral formalin. Samples for bacteriological and virological study, 0.5-1.0 g per tissue, were separately placed in 2 ml sterile tubes (virology) or sterile plastic bags (bacteriology). Impression smears were immediately made from the liver and heart and the smears were fixed with methanol in order to identify if *Pasteurella multocida* was present. For virological investigation, both cloacal and oropharyngeal swab samples were also collected from all sick and suitable dead birds before necropsy.

Each specimen and slide smear was then given a unique identity number which corresponded to each carcass identification number. Sampling date, sample name, species, age, sex (determined by physical examination during PM) and other morphological features were recorded for each carcass. The age of sampled carcasses was determined based on the criteria described by Marchant and Higgins (1998). Samples for bacteriological analysis were kept temporarily at 4°C (up to four hours) and those for virological examinations at -80°C in the laboratory until processing.

6.2.3. Histopathology

Samples preserved in 10% neutral formalin were left for 24 hours before further processing for histological examination according to the standard procedures described by Bancroft and Gamble (2008). Tissues embedded in paraffin wax were sectioned at 5 μm and stained with Haematoxylin and Eosin (H and E) before examination using 1000× magnification on a light microscope.

6.2.4. Bacteriology

6.2.4.1. Sample processing

A 0.5-1.0 g tissue sample was homogenised with 1 ml of 0.9% sterile saline using a laboratory blender (Seward Stomacher 400, Lab System, John Morris Scientific Pty Ltd.) for two minutes. Suspensions were immediately used to inoculate appropriate selective and/or differential bacteriological media to enable the detection of the aetiological agents of colibacillosis, pasteurellosis and salmonellosis.

6.2.4.2. Isolation of bacterial pathogens

E. coli, P. multocida and *Salmonella* spp., the aetiological agents of colibacillosis, pasteurellosis and salmonellosis respectively, were identified using standard bacteriological techniques (Barrow and Felthan, 2004). Suspensions of tissue samples (as described above) were inoculated onto blood agar, MacConkey agar and xylose lysine deoxycholate (XLD) agar (Oxoid Limited, Australia). Plates were examined after incubation at 37°C for 18-24 hours. Approximately 1 ml of suspension was also used to inoculate mannitol selenite broth (Oxiod Limited, Australia) for enrichment of *Salmonella* spp. Following incubation at 37ºC for 18-24 hours a loopful of broth was streaked onto the XLD medium and incubated as above.

Suspect colonies were sub-cultured onto blood agar for purity and tests conducted to identify the isolates. *E. coli* was identified based on lactose fermentation, Gram stain (- rod), oxidase (-), catalase (+), citrate (-), urease (-) and indole (+). *P. multocida* was identified based on colony morphology on blood agar, Gram stain (- rod), oxidase (+) and catalase (+) tests. *P. multocida* was also diagnosed based on the histopathological results: the observation of characteristic bipolar organisms under microscopic examination of Dip Quick stained liver or heart imprints is suggestive of pasteurellosis (Richard and Glisson, 1997). *Salmonella* spp. was identified based on the typical growth on XLD (red colonies with black centres) and a positive serum agglutination test using poly-O and poly H antisera (Oxoid Limited, Australia). *Salmonella* isolates were typed using the Kauffmann-White method (Popoff *et al.*, 2000) at the Queensland Health Pathology Laboratory in Brisbane, Queensland.

6.2.5. Virology (molecular investigation)

6.2.5.1. Sample processing

The Universal Tissue Sample DNA Extraction CorProtocol^{TM} (14202 Version 3) and Corbett reagent kits were used for sample processing and extraction of RNA using the Corbett Robotic X-tractor Gene^{TM} automated RNA/DNA extraction system (Corbett Robotic, Brisbane, Australia, currently Qiagen). Frozen tissue samples were defrosted in an air-conditioned room (22°C), then 20-30 mg of each tissue was added to 420 µl volume of tissue digest buffer and 4.2 µl digest enzyme in 2 ml screw cap tubes. Sample tissues were processed in batches of 60 test sample tissues and one known negative sample tissue. Sample tubes were placed in a rack and covered with self adhesive PCR plastic sealing film, then incubated in a Vortemp (Hybaid Ltd Integrated sciences) at 55°C and 40 rate per minute (RPM) overnight to complete

digestion. Digested samples were then cooled to room temperature and centrifuged at 2,500 *g* for five minutes.

A total of 200 µl of tissue suspension (test and controls) was added to each allocated well before being placed into a Corbett Robotics X-tractor Gene™ automated RNA/DNA extraction system (Corbett Robotic, Brisbane, Australia). Sample loading into the lysis block was performed in a class I cabinet. An extraction run contained up to 60 test samples, one AIV or NDV known negative tissue suspension and one positive (either AIV positive allantoic fluid-A/chestnut teal/VIC/2004/H4N4 or BVD virus positive allantoic fluid-BVD/C5.101/non-cytopathic/nQLD/1987). After extraction, the lysis plate was immediately transferred to a -20°C freezer for storage, before further testing. Ribonucleic acid extraction followed by the rRT-PCR was performed for screening AIV RNA and NDV RNA within six months from the time that the samples were obtained from the field.

Procedure of RNA extraction from swab samples is given in details in section 4.2.4 (Chapter 4).

6.2.5.2. Avian influenza viral ribonucleic acid screening

Molecular detection of AIV RNA was performed as previously discussed (section 4.2.5; Chapter 4).

6.2.5.3. Newcastle disease viral ribonucleic acid screening

Molecular detection of NDV RNA was carried out as previously referred (section 5.2.4; Chapter 5).

6.2.6. Trauma

Trauma was suspected when haemorrhagic lesions on the skull of the head and/or blood in the nostril and massive haemorrhages in the neck muscle were observed during autopsy.

6.2.7. Statistical analysis

Field and laboratory data were stored in the MS Excel-2003 spread sheet. Data was then exported into STATA/SE ™ 11.0 (StataCorp, 4905, Texas, USA) for checking data integrity and performing epidemiological analyses. A descriptive analysis was performed on the data of morbid and dead birds for year, month, species, age and gender. Descriptive statistics were also generated on clinico-pathological, histological and bird diseases or other data. The results were expressed as frequencies and percentages or frequencies alone for histopathology.

6.3. Results

6.3.1. Bird mortality

Overall bird mortality numbers were low and sporadic. The mortality was higher (22) in the first year than the second and third years of collection (seven and 13, respectively). The mortality numbers were higher in April 2007 and April 2009 than the other months of the years. Plumed whistling ducks had higher mortality numbers (23) than other species (one to five). Bird mortality was higher (26) for chicks and juveniles than for adults (16). By gender, females recorded higher mortality (28) than males (14) (Table 6.2). The term 'mortality' includes both dead birds and sick birds that were euthanased due to terminal illness.

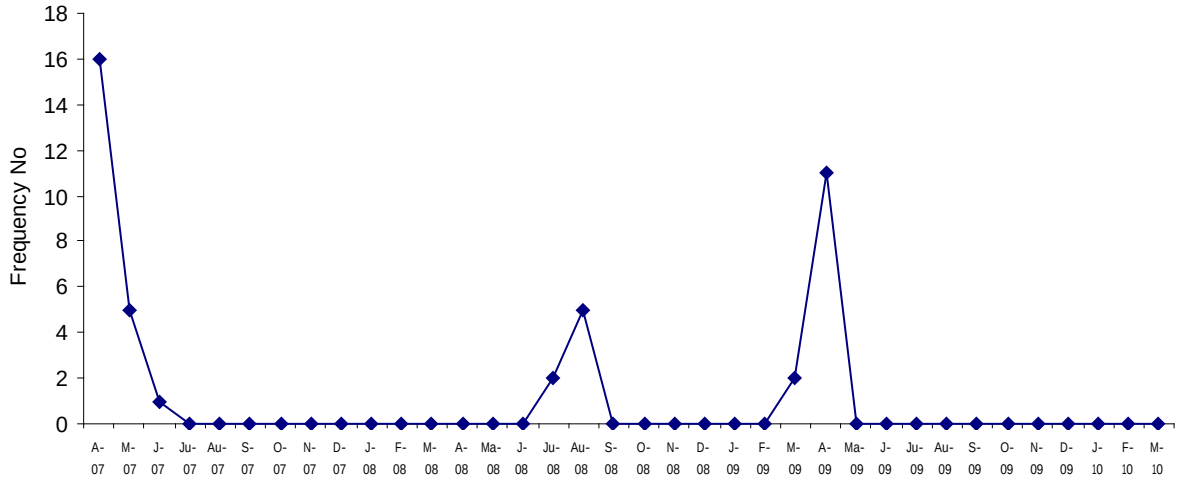

Figure 6.1 Temporal pattern of proportional bird mortality (including sick birds) at Billabong Sanctuary, Townsville, north Queensland (from April 2007 to March 2010)

Table 6.2 Number of sick and dead wild birds obtained from Billabong Sanctuary in Townsville, north Queensland (from April 2007 to March 2010)

Variable	Category	No. of sick birds	No. of dead birds	Total
Species	Plumed whistling duck (*Dendrocygna eytoni*)	4	19	23
	Pacific black duck (*Anas superciliosa*)	0	1	1
	Magpie goose (*Anseranas semipalmata*)	0	3	3
	Australian white ibis (*Threskiornis molucca*)	3	2	5
	Dusky moorhen (*Gallinula tenebrosa*)	0	2	2
	Australian pelican (*Pelecanus conspicillatus*)	0	3	3
	Australian king parrot (*Alisterus scapularis*)	0	2	2
	Pigeon (*Ducula bicolour*)	0	1	1
	Tawny frogmouth (*Podargus strigoides*)	0	1	1
	Sulphur crested cockatoo (*Cacatua galerita*)	0	1	1
Age	Chick	2	7	9
	Juvenile	2	15	17
	Adult	3	12	15
Sex	Male	5	9	14
	Female	2	26	28

6.3.2. Clinico-pathological syndromes and histological changes in tissues of bird carcasses

Clinical signs were observed from seven morbid birds. The remaining birds (35) were found dead, therefore, there was no opportunity to record signs. Inability to walk, paralysis of wings, bent neck, greenish faeces and dyspnoea were common signs for chick and juvenile PWDs. Inability to walk and dyspnoea along with depression were also recorded from adult AWI (Table 6.3).

A wide range of pathological changes were observed in different organs of birds at necropsy. Haemorrhagic lesions in different organs were frequently observed in all species (Frequency of lesions 67). Some examples of these lesions are presented in Figure 6.2. Other changes were a pale colour of the oesophagus (1) and liver (3), friable liver (3) and swollen spleen (1) and kidney (1) in PWDs. A whitish membrane covering the heart was observed in one MG and one Australian pelican. Oedematous abdomen, an asymmetrical liver and necrotic foci on the surface of the spleen were also recorded in an Australian pelican. A pale lung, friable heart and liver were found in a parrot (Table 6.4).

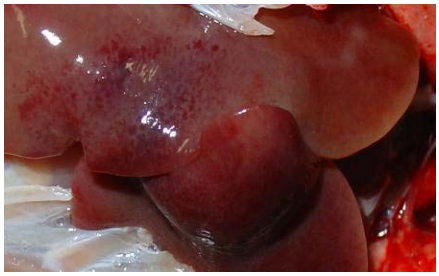
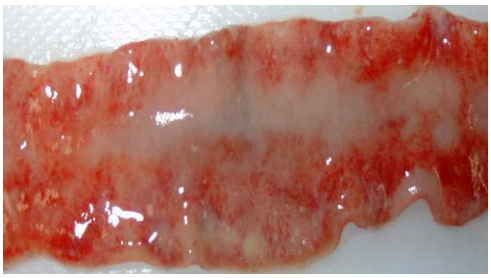

Figure 6.2 Pin point haemorrhages in the liver of a plumed whistling duckling April 2009 (Left) and profuse haemorrhages in the intestine of a sick adult Australian white ibis (Right)

A range of histological changes were observed in different organs of birds with H and E staining. Infiltration of lymphocytes and monocytes or heterophils was commonly recorded in different tissues of PWDs. Fat degeneration, necrosis of cells and muscle and haemorrhages were also generally observed in different tissues of PWDs. Inflammatory conditions of air sacculitis, pericarditis and peritonitis were also recorded. Oedema or exudates were present in the trachea and lungs. Bacterial cells, nematodes, fungus, inclusion bodies and haemosiderin pigment were sporadically observed in organs of PWDs (Appendix A).

Bacterial cells, nematodes and haemosiderin pigment were observed in organs of the AWI as well as infiltration of lymphocytes, cellular degeneration and necrosis and haemorrhages (Appendix A). Histological changes for other species were similar to those of the main species represented and are listed in detail in Appendix A.

Table 6.3 Clinical signs observed from sick wild aquatic birds (7 of 42) at Billabong Sanctuary in Townsville, north Queensland (from April 2007 to March 2010)

Signs	**No. of birds**	**Species**	**Age**
Unable to walk, paralysis of wings and bent neck	2	Plumed whistling duck	Chick
Unable to walk and yellow discoloration observed under wings	1	Plumed whistling duck	Chick
Unable to walk and vent covered with greenish faeces and dyspnoea	1	Plumed whistling duck	Juvenile
Unable to walk and depression	1	Australian white ibis	Adult
Unable to walk and dyspnoea	1	Australian white ibis	Adult
Unable to walk and depression	1	Australian pelican	Adult

Table 6.4 Pathological changes observed in different organs of wild bird carcasses at post-mortem at Billabong Sanctuary near Townsville, north Queensland (from April 2007 to March 2010)

Species (Total numbers)	**Types of lesions (Frequency number of lesions)**
Plumed whistling duck (23)	▪ Haemorrhage in the trachea (1), lung (2), heart (5), liver (9), spleen (6), pancreas (2), kidney (1), intestine (1), caecum (1), caecal tonsil (1), proventriculus (3), cerebrum (3) and neck muscle (1) ▪ Congestion in the lung (1) ▪ Enlarged and pale oesophagus (1) ▪ Fat infiltration in the liver (1) ▪ Pale and fragile liver (3) ▪ Swollen spleen (1) and kidney (1)
Pacific black duck (1)	▪ Haemorrhage in the pancreas (1), testes (1) and brain (1)
Magpie goose (3)	▪ Haemorrhage in the oesophagus (1), trachea (1), lung (2), pancreas (1), proventriculus (2), kidney (2) and neck muscle (1) ▪ Blood clot in the nostril (1) ▪ Heart covered with whitish membrane (1)
Australian white ibis (5)	▪ Haemorrhage in the lung (1), spleen (1), intestine (1), caecum (1), cerebrum and/or skull of the brain (4)
Dusk moorhen (2)	▪ Haemorrhage in the heart (1), liver (1), spleen (1), proventriculus (1) and kidney (1) ▪ Pale coloured lung
Australian pelican (3)	▪ Heart covered with whitish membrane (1) ▪ Asymmetric liver (1) ▪ Necrotic foci on the surface of the spleen (1) ▪ Oedematous abdomen (1)
Australian king parrot (2)	▪ Haemorrhage in the intestine (1) ▪ Pale lung (1) ▪ Friable heart (1) and liver (1)
Pigeon (1)	▪ Haemorrhage in the lung (1), liver (1) and brain (1)
Sulphur crested cockatoo (1)	▪ Haemorrhage in the kidney

6.3.3. Bird diseases and conditions or organisms identified

Colibacillosis accounted for the highest frequency of bird diseases at 19%. Pasteurellosis was 12% and concurrent colibacillosis and salmonellosis was 7.1%. Newcastle disease viral RNA with colibacillosis was 2.4% and NDV RNA alone was 2.4%. No AIV RNA was detected in the sampled birds (Table 6.5).

Salmonella positive isolates were further assessed for serotyping. Of the three infected birds, one AWI chick (from caecal tonsil) had *Salmonella enterica serotype virchow* and two juvenile

PWDs (one from bursa of Fabricius, liver and proventriculus; one from proventriculus) had *Salmonella enterica serotype hvittingfoss*.

Table 6.5 Common diseases and conditions or organisms isolated among 42 morbid and dead wild aquatic birds at Billabong Sanctuary in Townsville, north Queensland (from April 2007 to March 2010)

Name of diseases/conditions/ organisms identified	**Frequency No. (%)**	**Species**	**Age**	**No. (%) with diseases/conditions/ organisms identified**
Colibacillosis (*E. coli*) alone or Colibacillosis with trauma	8 (19%)	Plumed whistling duck	Juvenile	4 of 13 (30.8%)
		Plumed whistling duck	Adult	1 of 2 (50.0%)
		Pacific black duck	Adult	1 of 1 (100.0%)
		Magpie goose	Adult	1 of 2 (50.0%)
		Australian king parrot	Adult	1 of 1 (100%)
Pasteurellosis (*P.multocida*) alone or Pasteurellosis with trauma	5 (11.9%)	Plumed whistling duck	Chick	1 of 8 (12.5%)
		Australian pelican	Juvenile	1 of 1 (100.0%)
		Australian white ibis	Adult	3 of 4 (75.0%)
Both colibacillosis and salmonellosis (*S. enterica serotype virchow or hvittingofoss*)	3 (7.1%)	Australian white ibis	Chick	1 of 1 (100.0%)
		Plumed whistling duck	Juvenile	2 of 13 (15.4%)
Colibacillosis and Newcastle disease	1 (2.4%)	Australian pelican	Adult	1 of 2 (50.0%)
Newcastle disease	1 (2.4%)	Plumed whistling duck	Juvenile	1 of 13 (7.7%)
Avian influenza	0 (0%)	All species listed in Table 6.2	Chick to adult	0% (0)
Negative to above diseases/conditions/ organisms identified	24 (57%)	Plumed whistling duck	Chick	7 of 8 (87.5%)
			Juvenile	7 of 13 (53.8%)
			Adult	1 of 2 (50.0%)
		Magpie goose	Adult	2 of 2 (100.0%)
		Australian white ibis	Adult	1 of 4 (25.0%)
		Pigeon	Adult	1 of 1 (100.0%)
		Australian king parrot	Juvenile	1 of 1 (100.0%)
		Dusky moorhen	Adult	1 of 2 (50.0%)
		Tawny frogmouth	Adult	1 of 1 (100.0%)
		Sulphur crested cockatoo	Adult	1 of 1 (100.0%)
		Australian pelican	Adult	1 of 2 (50.0%)

6.4. Discussion

Although overall bird mortality rate was low and sporadic during the three-year surveillance program, the detection probability was quite good for the Sanctuary because of sufficient number of rangers. The frequency of bird mortality was, however, high in April 2007 and 2009 as compared with other months. This could be due to the increased number of immature birds after the breeding period. Carcasses (mostly PWDs) that were collected in April were immature birds with *E. coli*, *P. multocida* and *Salmonella* spp. detected in tissue samples. There was no clear link between these organisms and some clinical signs and lesions recorded in birds. However, some of the clinical signs and tissue lesions observed in birds such as the inability to walk, dyspnoea, haemorrhage, congestion, necrosis and lymphocytic infiltration in different organs can be seen in pasteurellosis (Leotta *et al.*, 2006; Hoque *et al.*, 2011a; Hoque *et al.*, 2011b) (Appendix B). Greenish faeces, whitish membrane covering the heart and congestion in different organs can be seen in colibacillosis (Hoque *et al.*, 2011a; Hoque *et al.*, 2011b) (Appendix B). Off-feed, whitish faeces, whitish foci on the surface of the liver and haemorrhage and congestion in different organs can be observed in salmonellosis (Hoque *et al.*, 2011a) (Appendix B).

The organisms isolated are likely to have contributed to bird mortality. In China, *E. coli* was identified as the attributable cause of the mortality of young crested ibises (*Nipponia nippon*) (Xi *et al.*, 2007) as for finches (*Fringillidae*) in Britain (Pennycott *et al.*, 1998). In Bangladesh, young waterfowl such as ducks were found to be more susceptible to various infectious diseases including duck viral hepatitis, duck viral enteritis, colibacillosis and salmonellosis (Hoque *et al.*, 2010; Hoque *et al.*, 2011a; Hoque *et al.*, 2011b). *E coli* along with *Candida trapicalis* have been previously seen in a dead adult Australian pelican (Anon, 2007a). The identification of *P. multocida* in the present study corresponds to the finding of the same organism confirmed in tissue samples of dead and sick waterfowl (Anseriformes and Charadriiformes) in China (Wang *et al.*, 2009). These findings may indicate a general low level of circulating infection with occasional deaths in more susceptible groups.

The identification of *S. enterica serotype virchow* in the caecal tonsil of AWI is supported by previous serological evidence of exposure to *Salmonella* spp. in AWI (Epstein *et al.*, 2006). The serotype seen in this study was reported in humans in nQLD between 1978 and 1988 (Ashdown and Ryan, 1990) and south eastern Australia between 1997 and 1998 (Bennett *et al.*, 2003).

Sporadic *S. enterica serotype virchow* infection has also been reported in humans in Britain and Japan and the sources of infection were chickens and a dog respectively (Mani *et al.*, 1974; Sato *et al.*, 2000). *S. enterica serotype hvittingfoss* was isolated from PWDs in this study and this serotype has been previously isolated from humans, animals and environmental sources in nQLD, south QLD, NSW, the Northern Territory, the Australian Capital Territory and western Australia in 2005 (Oxenford *et al.*, 2005).

This current study confirms that these zoonotic *salmonella* serotypes are still active in wild animal reservoirs and remain a significant concern to public and animal health in Australia. The particular concern is that an easy intermingling between WABs (such as PWDs) from Billabong Sanctuary and domestic poultry was frequently observed at common scavenging places. This environment can create an opportunity to transfer the *salmonella* organism from WABs (if infected) to domestic poultry which may then pass the organism to consumers (humans).

The present study did not identify AIV RNA from tissue samples of birds collected. This result remains consistent with the earlier findings of no AIV RNA or HPAI H5N1 detected from samples of dead wild birds in Australia (Haynes *et al.*, 2009; Anon, 2010c; Anon, 2010d). Therefore, these results along with the active surveillance study (Chapter 4) and previous investigations elsewhere in Australia confirm that the H5N1 is likely to be absent from Australian WABs. This negative result for H5N1 is encouraging for commercial animal health and public health in Australia.

Bird species can be classified as carrier and indicator species of H5N1. Mallard can be considered as a carrier species and the H5N1 infection in this species can produce clinical manifestations without causing mortality (Sturm-Ramirez *et al.*, 2004). This bird is genetically similar to the PBD (Tracey, 2010); however no AI specific clinical signs were observed during the three-year surveillance program in this species at Billabong Sanctuary. Goose and swan can be classified as an indicator species where the H5N1 infection causes high mortality (Brown *et al.*, 2008). However, no such high mortality was observed in MG and BS at the study site during the whole study period. This evidence, therefore suggests an unlikely introduction of H5N1 to the nQLD wild bird populations.

Newcastle disease viral RNA class-two type was only detected from the lung of a PWD and liver and pancreas of one Australian pelican. The detail sequence of this reactor sample was, however, not determined. Some earlier reports excluded ND as the cause of mortality of Australian WABs and other wild birds (Anon, 2009b; Anon, 2010c). Different subtypes of NDV have been reported to be circulating in healthy WABs in Australia (Mackenzie *et al.*,

1985; Peroulis and O'Riley, 2004) and overseas (Stanislawek *et al.*, 2001; Stanislawek *et al.*, 2002) for a number of years. Moreover, the active surveillance for ND identified avirulent NDV RNA class-one and class-two type reactor sequences in healthy WABs in nQLD (Chapter 5). There is no compelling evidence that NDV infection contributed to the disease of this bird. However, a study in North America reported that the virulent NDV strain caused mortalities in juvenile double crested cormorants (*Phalacrocorax auritus*) (Glaser *et al.*, 1999).

Trauma likely caused many deaths. This study recorded the following lesions which supported trauma as the cause of death: distinct haemorrhagic lesions observed on the skull, clot in the nostril and severe haemorrhages in the neck muscle of three AWIs and one MG. This damage is possibly due to collision with trees or vehicles during flight or intra-specific fighting. Similar mortality events due to trauma have been reported in a number of overseas countries, for example, in sea ducks in North America (Skerratt *et al.*, 2005), wild birds (*Fringillidae*) in the UK (Pennycott *et al.*, 1998) and wild birds (such as Passeriformes, Cuculiformes) in Ecuador (Gottdenker *et al.*, 2008).

Avian botulism (*Clostridium botulinum*) could not be excluded as a cause of bird mortality in this study. Botulism intoxication has occurred frequently in wild birds in Australia such as wild ducks (unknown species) in NSW (Anon, 2007a; Anon, 2009a), PWDs, WWDs and PBDs in NSW (Anon, 2010c), sick PWD in Northern Territory (Human, 2005) and AWI in Queensland (Anon, 2007b).

Botulism is more likely in April in nQLD as water levels become low and stagnant in the dry season which creates a favourable environment for *C. botulinum* to grow and produce toxins. Aquatic birds scavenge on aquatic insects, molluscs and crustaceans or decaying organic matter contaminated with the bacterial toxin from *C. botulinum* (Milton and Christian, 1999). Botulism intoxication has been reported as an important waterfowl toxin (Degernes, 2008) and the toxin of *C. botulism* is very stable in the environment.

Some of the clinical signs observed in this study such as signs of the inability to walk, paralysis of wings and bent neck can be produced by avian botulism intoxication (Appendix B). However, these signs are non-specific and may be indicative of other diseases such as duck viral hepatitis and duck plague (Hoque *et al.*, 2011a; Hoque *et al.*, 2011b). However, these specific diseases have not been reported in Australia.

The presence of bacterial cells, nematodes, fungus, inclusion bodies and haemosiderin pigment (possibly due to the effect of toxins) in tissues under histological examination indicated that multiple causal factors may be associated with bird mortality in this study (Appendix A).

Previous investigations reported infections with larval nematodes (microfilaria) and adults of *Cotracaecum* spp. and lung lesions consistent with aspergillosis in Australian pelicans (Anon, 2007a; Anon, 2008a). This study observed nematodes in the proventriculus of PWDs and AWI on histological examination. *Acanthocephalus* spp. was also observed while sampling cloacal swabs from healthy PWDs and PBDs for the active surveillance program for AI investigation in nQLD. These kinds of parasites can play a contributory role in bird mortality.

A successful network was established between the property owner and the JCU AI research team through which dead birds, with accompanying information, were readily obtained for analysis. Therefore, there is an opportunity for establishing a long term passive disease surveillance program for WABs in the Townsville region of nQLD.

6.4.1. Limitations

A total of 42 bird carcasses were collected during the three-year study period at Billabong Sanctuary which may raise the question of under-reporting in the passive surveillance program. However, Billabong Sanctuary is a small area and there was a strong collaborative relationship between the sanctuary rangers and JCU AI research team to successfully identified sick and dead birds. In addition, a concurrent active surveillance program for AI was operational at this site. It is therefore unlikely that many dead bird carcasses were missed during the study period.

Billabong Sanctuary is a very suitable site for Australian resident WABs dominated by PWDs. The bird population varied from 100 to 1,500 in a year based on estimation by regular head counting of birds using the naked eye or binoculars. These populations disperse widely during the wet season (November-February) and therefore some bird mortality events and carcasses were likely to have been missed during time outside of Billabong Sanctuary.

This study processed both frozen and fresh samples for bacteriological investigation. Using frozen samples could degrade sample quality and consequently undermine the true estimates of organisms identified from samples of bird carcasses in this study. For instance, this study identified *Salmonella* serotypes from the freshly collected bird carcasses only in April 2007. Therefore, frozen bird carcasses obtained around that time in April 2007 might have prohibited the detection of more *Salmonella* positive birds.

The HPAI H5N1 had not been detected as a possible cause of bird mortality. However, this study mostly analysed frozen samples for AI molecular investigation which could downgrade sample quality. It is therefore, unsure if AIVs would have been detected if fresh samples were processed.

The sporadic and low level of mortality in this study along with the results of previous investigative findings of no HPAI H5N1 in Australian dead wild birds implies that the detection of H5N1 in this passive surveillance project was unlikely. Autolytic changes and damage due to freezing some bird carcasses might have also hampered the histopathological evaluation of different tissues. Other diseases could have been investigated which might have been associated with bird mortality such as avian botulism, aspergillosis, chlamydiosis, duck viral hepatitis and duck plague, but time and economic constraints prohibited the inclusions of these diseases.

6.4.2. Conclusions

This study identified low and sporadic mortality in WABs, but the mortality was higher in immature birds in April. Important discoveries were the isolation of zoonotically important *Salmonella* serotypes and the lack of detection of AIVs (H5N1) in the mortality events of WABs in Townsville of nQLD. These overall findings will significantly contribute to the design of passive surveillance programs on wild dead birds in northern Australia by wildlife authorities.

Chapter 7: Importance of surveillance programs for wild bird diseases and spill-over of these diseases to domesticated birds and humans in north Queensland

7.1. Introduction

Many wild bird species carry or are reservoirs for important pathogens that are of particular importance to human or animal health such as AIVs, NDVs, West Nile Virus and Kunjin virus (other flaviviruses) (Stallknecht and Shane, 1988; Mackenzie *et al.*, 1995; Alexander, 2000a; Delogu *et al.*, 2003; Abulreesh *et al.*, 2007).

Wild birds are considered a natural reservoir of AIVs and harbour all known subtypes of the AIVs (Munster *et al.*, 2007). Wild species may play a crucial role in the epidemiology of AI but the distribution and dynamics of this pathogen in the wild bird population across the world has been little explored. Previous HPAI H7 outbreaks in Australian commercial poultry (Westbury, 2003) and HPAI H5N1 outbreaks in wild birds, poultry and humans in many countries, particularly Indonesia (Subbarao *et al.*, 1998; Capua and Alexander, 2004; Peiris *et al.*, 2004), highlight the importance of a comprehensive epidemiological study on wild bird populations in Australia. Moreover, there is world-wide concern that HPAI H5N1 may evolve the capacity for human-to-human transmission and the potential emergence of a pandemic (Alexander, 2007).

The H5N1 outbreak on Qinghai Lake in western China caused considerable deaths of wild migratory birds in 2005 (Chen *et al.*, 2005). This outbreak identified a serious risk of this virus spreading world-wide because this lake is a major breeding site for migratory birds whose flyways extend to south-east Asia, India, Siberia, Australia and New Zealand.

Wild avian species are also reservoirs of bacterial pathogens that are significant for public or animal health, for instance species of *Campylobacter* and *Salmonella*, and toxin producing strains of *Escherichia coli* (Abulreesh *et al.*, 2007).

Therefore, both active and passive surveillance programs along with a longitudinal study were conducted on WABs from April 2007 to March 2010 in nQLD to understand the overall epidemiology of wild bird diseases (in particular AI and enteric bacteria). This chapter describes the implications of important outcomes obtained from the different studies and emphasizes the

importance of surveillance programs for wild bird pathogens in managing the risk of spill-over of these pathogens to domestic animals and humans in northern Australia.

7.2. Characterisation of viral and bacterial pathogens and implications for human and poultry health

7.2.1. Avian influenza

7.2.1.1. Subtypes haemagglutinin-6 and 9

The AIV subtypes H6 and H9 were confirmed in WABs in nQLD through molecular and serological investigations (Chapter 3 and 4). The HI serology demonstrated both H6 and H9 subtypes constantly circulated in PWDs (2007-2009) whereas the H9 subtype was commonly found in PBDs in 2009. These temporal and species patterns of subtype identification in HI serology also conform to that of the molecular testing. The H6 and H9 subtypes identified in this study may be of concern if introduced to Australian domestic and commercial poultry. This is because these subtypes have been previously shown to adapt quickly to domestic avian species and can acquire moderate pathogenic potential (Forrest and Webster, 2010). The H9N2 subtype, in particular, caused disease outbreaks in chickens in a number of countries (Alexander, 2000b; Naeem *et al.*, 2007; Iqbal *et al.*, 2009; Nagarajan *et al.*, 2009). This subtype was also recovered from pigs (Hong Kong, China and Indonesia) (Ninomiya *et al.*, 2002) and humans (Hong Kong) (Peiris *et al.*, 1999; Riedel, 2006). Hence, crossing the species barrier to mammals highlights the pandemic potential of H9N2 subtype in the future.

The phylogenetic analysis suggested the H6 isolate (2009; non-pathogenic) was similar to the isolates from STS for the M gene and Eurasian ducks for the H gene and the H9 (2009; non pathogenic) was a close relative of Asian duck-like isolates for both genes. These patterns are therefore indicative of the recent introduction of H6 and H9 subtypes to Australian wild birds from overseas (in particular Eurasian regions) through migratory birds such as STS.

These patterns were also supported by some recent Australian studies (Haynes *et al.*, 2009; Hansbro *et al.*, 2010) (Aeron Hurt, personal communication). Furthermore, LPAI viruses have previously been isolated from species of Charadriiformes which regularly travel between Asia and Australia. The species include ruddy turnstone (*Arenaria interpres*), red knot (*Calidris canutus*), RNS (*Calidris ruficollis*), common tern (*Sterna hirundo*), sooty tern (*Sterna fuscata*), bar tailed godwit (*Limosa lapponica*) and the STS (*Calidris acuminate*) (Tracey *et al.*, 2004). In particular, H11N9 identified previously in the STS was closely related to the Asian isolates (Hurt *et al.*, 2006). All these earlier findings and the results of the present

study support the capability of migratory birds to bring LPAI viruses from Asian regions to Australia. Therefore, migratory birds may also have the capacity to introduce exotic HPAI viruses to Australian wild bird populations if they are infected. This possibility becomes strengthened further when there is evidence that wild migratory birds infected with HPAI H5N1 are capable of disseminating the virus over long distances (Chen *et al.*, 2005). This result is supported by experimental evidence that some wild bird species infected with this virus survived and shed the H5N1 virus without showing clinical signs (Sturm-Ramirez *et al.*, 2004).

However, the roles of migratory birds in spreading exotic viruses like H5N1 in Australia have been ignored earlier (East *et al.*, 2008a). The main concern with the Asian lineage HPAI H5N1 is that this virus has the capacity to cross species barriers and has previously caused human mortality across the world (Subbarao *et al.*, 1998; Peiris *et al.*, 2004; Anon, 2010b). As a result, there is worldwide concern that HPAI H5N1 may evolve the capacity for efficient human-to-human transmission and there is the potential for the emergence of a pandemic (Alexander, 2007).

Of particular relevance is that Charadriiformes can congregate in extremely large concentrations on coastal floodplains and mainland wetlands (Morton *et al.*, 1993), where they regularly interact with Australian resident Anseriformes (Morton, 1990). Therefore, the risk to Australia appears to be in the association between Charadriiformes which potentially harbour overseas AIVs, and resident Australian Anseriformes. If affected, Anseriformes such as wild ducks could potentially spread viruses to domestic free-range poultry or commercial poultry as they disperse from coastal areas.

Therefore, the AI surveillance program of Australian wild birds should continue in order to provide “early warning” signals for the introduction of exotic viruses (in particular HPAI H5N1) and provide access to virus strains for characterization, monitoring of viral evolution and detection of emerging HPAI viruses. Moreover, migratory bird sampling during migratory and non-migratory seasons (adjacent to migratory routes and coastal areas where shore and Anseriformes mix) should be targeted to exploit the specific roles of these birds in introducing exotic AIV subtypes to Australia. These strategies could potentially help construct proper risk analyses and the development and location of appropriate biosecurity measures to prevent any HPAI outbreak in Australian commercial poultry in future.

7.2.1.2. Subtypes haemagglutinin-5 and 7

Molecular investigation excluded the presence of the HPAI H5 or H7 subtype in samples obtained from live, sick and dead birds in this study (Chapter 4 and 6). This result remains consistent with the earlier findings of no HPAI H5 or H7 detected from samples of live and dead wild birds in Australia (Haynes *et al.*, 2009; Anon, 2010c; Anon, 2010d). The serological study, however, identified a constant circulation of the H5 subtype in PWDs (2007-09) and PBDs (2008-09) while serological evidence for the transmission of the H7 subtype was identified in PWDs in October, 2008 only. The pathotype of these sero-subtypes was not determined, but the LPAI H5 and H7 subtypes were previously isolated from samples of wild ducks in Australia (Haynes *et al.*, 2009; Bulach *et al.*, 2010). Therefore, it can be assumed the H5 and H7 subtypes confirmed in the serological study may be non pathogenic subtypes.

Current knowledge holds that the AIVs may mutate from LPAI to HPAI viruses after the introduction of the LPAI virus to poultry by wild birds (Kawaoka *et al.*, 1987; Campitelli *et al.*, 2004). Moreover, the recently identified LPAI H7 isolates in wild ducks were found to be antigenically similar to the previous HPAI H7 subtype which caused outbreaks in Australian poultry between 1975 and 1997 (Bulach *et al.*, 2010). Therefore, it appears a real concern that LPAI H5 or H7 subtypes can mutate to be virulent subtypes if introduced to domestic poultry in Australia. Hence, the serological evidence of H5 and H7 subtypes in this study along with their presence elsewhere in Australia justifies continuing wild bird sampling in nQLD under surveillance programs.

7.2.1.3. Evidence of multiple serotypes in haemagglutination inhibition serology and newly evolved avian influenza viral subtypes

Avian influenza viral antibodies corresponding to multiple AIV subtypes were frequently detected in individual birds in this study (Chapter 3). This pattern was not however, observed in the AIV molecular investigation which may be due to limited sub-typing success on the AIV RNA reactor samples or due to a shorter viral shedding period compared with a longer AIV antibody persistence period (Chapter 4). However, some earlier studies confirmed more than one AIV subtype from individual wild ducks (unspecified subtypes) (Halvorson *et al.*, 1983) and mallard ducks (H6 and H9) (Jackwood and Stallknecht, 2007). These previous results along with the sero-typing results in this study indicate that wild birds can be infected with more than one AIV subtype throughout their lives.

This study identified putative reassortment events between H6 subtypes and M genes previously identified with a H7 subtype virus. The phylogenetic analysis therefore indicated a reassortment process could have occurred in Australian resident birds which were concurrently infected with the Australian H7 wild bird and overseas derived H6 subtypes. The overseas H6 subtype was potentially introduced through migratory birds (Chapter 4; Figure 4.1 and 4.2).

As mentioned earlier, migratory Charadriiformes can congregate in extremely large concentrations on coastal floodplains and mainland wetlands (Morton *et al.*, 1993) where they regularly interact with Australian resident Anseriformes (Morton, 1990). These migratory birds travel regularly between Australia and Asia (Morton *et al.*, 1993). An environment is created that is conducive for birds being concurrently infected with two different AIV subtypes with the potential for producing a recombinant subtype through a reassortment process. There is also a high potential for multiple reassortment events as successive reassortment events have been reported previously in wild birds (Hatchette *et al.*, 2004). Hatchette *et al*. (2004) identified multiple genotypes of H6 subtypes in feral Canadian ducks in different years which were suggestive of multiple reassortment events. The formation of novel gene combinations through introduction of AIVs from Asia, followed by reassortment, may increase the chance of the development of HPAI viruses in Australia.

However, the exploration of the novel H6 subtypes through AIV gene reassortment should be considered cautiously as this assessment was based on only two short genes (N=8) (M gene: 479 bp and H gene: 563 bp). A more comprehensive and complete genotypic analysis of these subtypes is therefore needed to examine some of the concepts arising from the phylogenetic analysis and to determine how AIVs reassort in WABs.

Adaptability of the newly established H6 subtype (2008) to domestic avian species is unknown. However this subtype can cause mortality of domestic poultry with quick adaptation as also found for the H9 subtype (Forrest and Webster, 2010).

7.2.2. Newcastle disease

Newcastle disease viral RNA class-one and two-type was identified in WABs in nQLD (Chapter 5). These findings corroborate the results of other studies on Australian wild birds (Russell and Alexander, 1983; Mackenzie *et al.*, 1985; Alexander *et al.*, 1986) (Ibrahim Diallo 2006, DPIF, QLD, personal communication) and overseas studies (Spalatin *et al.*, 1976; Russell and Alexander, 1983; Lindh *et al.*, 2008). The overall picture, however, indicated the NDV RNA class-two type commonly circulated in wild bird populations in nQLD which is supported by many overseas studies (Alexander, 1995; Aldous *et al.*, 2003; Jindal *et al.*, 2009a).

Molecular analysis of a few reactor samples in this study indicated they were avirulent NDV RNA class-one type in PWDs, similar to AWI and duck 3245 isolates, and class-two genotype-one in PWD, close to Australian I-2 progenitor virus and I-2 vaccine strain. These identified avirulent ND viruses would not therefore be a direct threat to Australian domestic and commercial poultry. However, there is a theory that avirulent NDVs from wild birds that are introduced to domestic poultry, after certain generational cycles, can mutate to become virulent and capable of causing heavy mortalities. Previous evidence for this mutation to virulence has been reported from Ireland where avirulent NDVs class-one in WABs were demonstrated to have mutated to virulence in chickens (Alexander, 1995).

7.2.3. Bacterial diseases

The dead bird study identified the public health importance of *Salmonella* spp. in WABs (Chapter 6). *Salmonella enterica serotype virchow* was identified in an AWI chick which is supported by previous serological evidence of exposure to *Salmonella* spp in AWI (Epstein *et al.*, 2006). This sero-subtype was also reported earlier in humans in nQLD (Ashdown and Ryan, 1990) and south eastern Australia (Bennett *et al.*, 2003) and sporadically in Britain (Mani *et al.*, 1974) and Japan (Sato *et al.*, 2000). *S. enterica serotype hvittingfoss* was isolated from PWDs in this study and this serotype has been commonly isolated from humans, animals and environmental sources across Australia in 2005 (Oxenford *et al.*, 2005). This study confirms that these zoonotic *salmonella* serotypes are still active in wild animal reservoirs and remain a significant concern to public and animal health in Australia and overseas.

7.3. Prevalence of detection

7.3.1. Avian influenza

Overall AIV RNA prevalence was low (~1.0%) in the sampled WABs of nQLD (Chapter 3), whereas the AIV antibody prevalence was 11 times higher (Chapter 4). These results remain consistent with similar studies in Australia and overseas (Haynes *et al.*, 2009; AIV RNA and antibody prevalence), (Pereda *et al.*, 2008; Raleigh *et al.*, 2009; Hansbro *et al.*, 2010; Tracey, 2010; AIV RNA prevalence only). The low AIV RNA prevalence in this study may be due to a number of reasons. Wild aquatic birds are frequently exposed to more than one AIV subtype concurrently or at different time, which significantly decreases the viral shedding period (Fereidouni *et al.*, 2009; Costa *et al.*, 2010; Fereidouni *et al.*, 2010b) and therefore, the opportunity to detect AIV RNA from the field samples is reduced. The serological results in this study also suggested the wild bird population in nQLD was exposed to multiple AIV subtypes (Chapter 3). In addition, samples containing possible inhibitors might reduce the success of the

AIV RNA detection rate as this study observed ~14% of the extracts produced a significant inhibition of PCR (Chapter 4). Moreover, field samples were processed after up to six months of storage at -80°C which could degrade sample quality at thawing. Therefore, the true AIV RNA prevalence in WABs in nQLD might be higher than the estimated prevalence.

A higher AIV antibody prevalence, on the other hand, could be due to a longer persistence of antibodies. Analysis of serum samples obtained from captured-marked-recaptured birds in this study indicated long lasting AIV antibodies. For example, persistence of AIV antibodies for over two months as observed in PBDs (66-85%) is probably the first observation of persistence for this length of time outside of the laboratory in Australia. Some earlier experimental studies observed AIV antibodies for up to 32-42 days in mallard ducks (Sinnecker *et al.*, 1982; Costa *et al.*, 2010) and ≥ 40 days in Australian WWDs and grey teal
(J. Curran, personal communication).

When a bird is infected with AIVs, particles will be excreted in faeces and it can be found in oropharyngeal samples. The viral genome can be detected using RT-PCR. Experimental evidence suggests that the maximum time that virus will be detected will probably not exceed seven to ten days (Heckert *et al.*, 1999; VanDalen *et al.*, 2010). Subsequent infections with homologous or heterologous strains of virus are likely to also result in a viral genome being detected. However, the duration of detection is likely to be shorter than that seen with the primary infection. If samples collected from birds, where the viral genome can be detected for seven days, a prevalence of 1% would indicate that the birds had been infected at least once every two years (Chapter 4). If the period of detection is less than this, it would suggest a more frequent infection rate. It is therefore reasonable to suggest that the observed prevalence of between 1% and 2% should equate to an annual incidence of somewhere between one and two infections with influenza virus. The point prevalence appears to be quite low. However, when this is translated to an annual incidence it becomes apparent that influenza infections are frequent events for this population.

7.3.2. Newcastle disease

Overall NDV RNA prevalence was, however, higher (3.5%) (Chapter 5) than the AIV RNA prevalence (1.4%) for live bird samples (Chapter 4). This level of NDV RNA prevalence corresponds to the proportionate prevalence encountered in the dead bird study (4.8%) (Chapter 6). A low level of NDV prevalence (0.4%) was previously estimated in the Australian wild bird populations through a cross sectional study (Mackenzie *et al.*, 1985) and 0.5% in wild birds in Japan (Jahangir *et al.*, 2009). By contrast, a higher prevalence has been estimated in waterfowl in Finland (5.2%) (Lindh *et al.*, 2008), China (8.2%) (Zeng *et al.*, 2008) and wild

birds in Nigeria (8.0%) (Ibu *et al.*, 2009). Moreover, ND is a serious concern for Australian poultry due to previous virulent ND outbreaks (Westbury, 2001; Kattenbelt *et al.*, 2006b). The relatively high prevalence of NDV RNA supports the concern of the Australian poultry industry and supports the implications of appropriate biosecurity precautions to minimise contact with WABs.

7.4. Risk factor analysis: Avian influenza and Newcastle disease

7.4.1. Species

The AIV RNA prevalence was observed to be significantly higher in the samples of PBDs (3.5-4.5%) than PWDs (0.4-1.4%) from the separate analysis of live bird swabs combined and faecal samples of WABs in nQLD (Chapter 4). An identical species pattern was observed in the serology screening for AIV antibodies (Chapter 3). The odds ratio of being positive for AIV antibodies was 13.1 for PBDs (53.7%) over PWDs (10.1%) (Chapter 3). Similarly, Tracey (2010) found that AIV prevalence was significantly greater in dabbling ducks such as PBDs (*Anas superciliosa*) and mallards (*Anas platyrhynchos*) (3.1%), compared to other ducks such as PWDs (0.7%) and WWDs (1.0%). Overall results, therefore suggest that PBDs could be considered to be a highly susceptible species to AIVs. By contrast, the NDV RNA prevalence was estimated to be significantly higher in PWDs (4.2%) than PBDs (0.9%) which was indicative of a higher susceptibility of PWDs to NDVs.

7.4.2. Age

An apparent trend of higher AIV RNA prevalence was observed in younger as compared with older birds (Chapter 4). This finding remains consistent with the studies in Canada (Hinshaw *et al.*, 1980b), Siberia (Okazaki *et al.*, 2000), North America (Munster *et al.*, 2007), northern Europe (Wallensten *et al.*, 2007), Alaska (Ip *et al.*, 2008) and New Zealand (Stanislawek *et al.*, 2002). A similar age pattern of prevalence was observed in the study of NDVs in the samples of WABs and other ND studies in domestic chickens elsewhere in the world (Ezeokoli *et al.*, 1984; Janviriyasopak *et al.*, 1989; Martin, 1992). A higher prevalence in young birds may be due to the fact that they are immunologically naïve whereas adults are more resistant, particularly to viruses to which they may have previously been exposed (Webster *et al.*, 1992).

The AI serological study, however, determined a higher AIV antibody prevalence in older than younger birds which might be due to more exposure to infections because of more opportunity in addition to long lasting AIV antibodies (Chapter 3). This result agreed with the previous

finding in coots (*Fulica atra*) in Italy and native chickens in Bangladesh where the AIV antibody prevalence was higher in adults than in juvenile birds (De Marco *et al.*, 2003b; Nooruddin *et al.*, 2006).

7.4.3. Season

The AIV molecular study was not capable of testing the seasonal effect on the prevalence of AIV RNA as reactor samples were either absent or in very low numbers in each season class (Chapter 4). An Australian study, however, recorded a higher AIV RNA prevalence in autumn (March-May) (3.2%) than other seasons (1.8-2.7%), which may correspond to an increased number of young birds at that time of year (Hansbro *et al.*, 2010). A similar higher prevalence of NDV RNA was determined in the warm wet season (January-April) in the ND investigation, also correlating with the increased numbers of younger birds (March-April) sampled at that time of the year (Chapter 5).

However, a seasonal effect was identified by the linear regression analysis of the AIV antibody levels in PWDs (Linear model-A, Chapter 3). This model identified ducks sampled during warm wet weather (January–April) also had higher AIV antibody compared to warm dry weather (September-December) (Linear model-A, Chapter 3). A similar seasonal effect on AIV antibody was observed in a study of native chickens in Bangladesh where the highest seroprevalence (15%) was detected in the monsoon season and the lowest (4%) in winter (Nooruddin *et al.*, 2006). They explained that the influence of hot weather and rain during the monsoon season might reduce the immune status of the birds, thus making them more vulnerable to infection. March-April (the part of the wet season) is a breeding period for PWDs as observed in the present study. Birds might also have been immunologically suppressed at breeding time which could also encourage exposure to AIV infections.

7.4.4. Location

No site effect (only tested Billabong Sanctuary versus Cromarty) has been evident on the prevalence of AIV RNA (Chapter 4) or NDV RNA (Chapter 5) or AIV antibody (Chapter 3) in the different studies. The Atherton Tableland and Cape York were not considered in assessing the site effect due to insufficient sample sizes for each. The Mareeba area has been identified as a risk area for AI emergence in poultry (East *et al.*, 2008a; East *et al.*, 2008b; Tracey, 2010) and Cape York is a potential entry point for migratory birds into nQLD. Therefore, a further AI study should focus on these two particular areas.

7.4.5. Conclusions and recommendations for future studies and surveillance programs

Overall results indicated that PBDs may be more susceptible to AIVs and PWDs may be more susceptible to NDVs. Therefore, these species should be considered for future surveillance programs which also target the warm wet season (January-April). This will facilitate sampling of more immature birds. Additionally, the sampling period should be extended to the early part of the cool dry season (May-August) which could guarantee more young birds being sampled. Important sites such as the Atherton Tableland and Cape York should be incorporated in future AI epidemiological studies in nQLD.

To expedite the identification of the full set of risk factors associated with the AIV antibody or AIV RNA (bird demography, bird species and their ecology, spatial and temporal factors) a long term systematic longitudinal study should be carried out to ensure sufficient sample sizes on a wide range of birds. For example, waders are also thought to be the natural reservoir for LPAI (Stallknecht *et al.*, 1990b) and these species belonging to the *Charadriidae* and *Scolopacidae* families often live side-by-side with ducks in marine and wetland areas. Waders also play an important role in perpetuating certain virus subtypes in North America and Canada (Krauss *et al.*, 2004); therefore an emphasis should be given to investigate waders in coastal wetlands in future AI investigations. Such a study could also specifically test whether influenza viruses can be perpetuated in ducks alone, or the importance of interaction between ducks and shorebirds as suggested by the study in North America (Krauss *et al.*, 2004). The likely interaction between ducks and shorebirds in areas, where migratory birds from the northern and southern latitudes mix, is of particular interest. The chance of detecting AIVs in shorebirds increases five-times, if WABs are in contact with those shorebirds (Suss *et al.*, 1994). It is reported that LPAI infects 100 host species in 26 families, including many whose primary habitat is not aquatic (Olsen *et al.*, 2006). Therefore, there is a possibility that the reservoir species for AI in Australia are not only water dependent species.

This study is biased towards species that were easy to sample and ubiquitously present. Samples from PWDs were sufficient to draw reasonable conclusions on temporal and spatial factors affecting AIV antibody levels only (Chapter 3). However, sample sizes for other species (e.g. PBDs and MGs) were not sufficient even though AIV antibody prevalence is very high in PBDs. Therefore, in order to ensure sufficient sample sizes from those species, their ecology needs to be considered in order to select appropriate study sites. For instance, geese are mainly herbivorous and often congregate in large flocks grazing in pastures and agricultural fields, especially during the non-breeding season, and large flocks of AWI are always seen around rubbish areas.

A study considering a wide range of aquatic and non-aquatic bird species as hosts for AI in Australia will answer which species or group of species are important reservoirs for these viruses in Australia.

Avian influenza and ND surveillance in wild birds could be more useful if conducted where waterbirds have a greater risk of interacting with poultry, for instance around free-range poultry establishments, 'backyard' operations, or where biosecurity measures are lacking. The interaction between these farms and other commercial operations is also important in understanding the potential emergence and spread of AIVs within poultry.

It is also important to understand the interaction of AIV infection with climate and environment with the prospect of a variable climate in future.

7.5. Risk assessment for the introduction or establishment of highly pathogenic avian influenza in Australia

An apparent risk of the introduction of exotic HPAI viruses to Australian birds was identified in the AI molecular study. This study identified the likely introduction of foreign LPAI viruses (H6 and H9, 2009, Chapter 4) to Australian resident wild birds. Migratory birds which travel regularly between Australia and Asia are likely to be the vectors introducing exotic HPAI viruses to Australia. This study determined a novel AIV H/M gene sequence association (H6, 2008, Chapter 4) which probably evolved through AIV gene reassortment between two different subtypes that co-infected cells of Australian resident birds. Similarly, novel HPAI subtypes may be produced in wild bird populations in the future which would be a significant risk for animal and public health. The serological presence of H5, H7 and H9 subtypes (probably non pathogenic, Chapter 3) in this study indicated their active circulation in the Australian wild bird populations. These subtypes can easily be transformed into HPAI viruses after introduction to poultry as evidenced in earlier studies (Kawaoka *et al.*, 1987; Campitelli *et al.*, 2004).

Based on the above findings two different risk pathways should be considered when conducting systematic risk analyses for the threat of AI to animal and public health in Australia. The first one requires evaluating the possibility of endemic LPAI viruses (e.g. H5, H7 and H9) in resident wild birds (in particular Anseriformes) transferring to free range domestic or commercial poultry. The possibility of LPAI viruses then mutating to HPAI viruses in poultry which could then pose a threat to humans should also be examined.

The second route is the possibility of the introduction of exotic LPAI or HPAI viruses through migratory birds to domestic or commercial poultry via resident Anseriformes which ultimately could be a threat to humans.

For these analyses, the following minimum relevant information should be obtained: the LPAI prevalence and population abundance of resident birds and the factors that affect the poultry density, movement of resident birds (through banding telemetry or based on available literature), the level of interaction between resident birds and domestic or commercial poultry (direct or indirect) and the level of LPAI exposure from WABs to poultry.

For the second study it is necessary to know the following additional information: migratory patterns of shore birds between Australia and overseas, the probability of migratory birds carrying different exotic viral subtypes (based on sampling studies or the literature), the interaction between shore and resident birds and the extent of the exposure of resident aquatic birds to AIVs from migratory shore birds.

Two earlier studies suggested a zero to medium level of risk of introducing exotic HPAI viruses to Australian poultry through nomadic waterfowl and migratory birds (East *et al.*, 2008a; East *et al.*, 2008b). Of those risk areas identified, Mareeba (nQLD) has been identified as being at risk for the emergence of HPAI to poultry. East *et al*. (2008a) and East *et al*. (2008b) proposed the following guidelines to reduce the level of risk: tightening overall biosecurity measures such as ensuring no direct and indirect (water contamination with wild bird faeces) contact of wild birds with free range domestic or commercial poultry. Previous HPAI H7 outbreaks in Australia occurred in farms where unprotected surface water sources were used or where there were significant populations of wild *Anatidae* in the area (Westbury, 2003).

This study provides information on some of the factors that affect the risk of spill-over of AIVs from WABs and can be used to alert poultry owners to times and places of higher risk. This leads to the adaptation of biosecurity measures which better match and mitigate the level of risk. Of course many important risk factors as outlined above remain to be determined.

7.6. Recommendations for diagnostic methods used in future avian influenza surveillance programs

7.6.1. Suitable samples for avian influenza surveillance in wild aquatic birds

Overall results have suggested that both live bird swabs (cloacal and oropharyngeal) and faecal sampling would be useful for future AI surveillance programs (Chapter 4). Live bird sampling reduces the chance of the incorrect identification of the bird species and the sampling of faeces from more than one bird. In addition, there is a simultaneous opportunity to record bird demographic information along with the collection of serum samples. However, faecal sampling is an easier and cheaper option (Tracey, 2010) thereby increasing the number of samples that can be collected when live bird trapping is difficult, labour intensive and costly. This study suggested that faecal sampling may not result in a loss of sensitivity.

7.6.2. Screening test for avian influenza viral antibodies in wild aquatic birds

The AAHL developed a cELISA (now designated as AAHL-1) in order to detect equine influenza viral antibodies from horse sera in Australia (Selleck, 2007b). This assay was then adopted for the detection of AIV antibodies in chickens, followed by wild bird sera, in Australia without a rigorous testing for its ability to detect AIV antibodies using bird sera. As a part of a common agreement, the AAHL provided its protocol (AAHL-1) and necessary reagents to screen AIV antibodies in serum samples from WABs in the large AI surveillance program in northern Australia. As a starting point, the AAHL-1 was used with minimal modification to screen AIV antibodies from 411 field serum samples from WABs and observed this assay did not meet the test quality guidelines set by Selleck (2007b). Therefore, it was necessary to optimise this assay, adjusting reagent concentrations and replacing the chromogen TMB with ABTS. Three modified versions (designated as AAHL-2, JCU-1: without post-coating and JCU-2: with post-coating) were designed and these versions required double the amount of reagent concentrations as used for AAHL-1 to satisfy the AAHL test quality guidelines. These modified versions performed equally (Chapter 2). The analytical sensitivity study also demonstrated reliable performance with an adequate relative sensitivity for the modified assays which also satisfy the AAHL test quality guidelines (Selleck, 2007b) (Chapter 2). The JCU-2 version of assay was therefore used to screen AIV antibodies from the serum samples of WABs obtained under the large scale AI surveillance study in nQLD.

The longitudinal effect of a post-coating buffer was assessed on stability of AIV antigens in the cELISA plates (Chapter 2). This study demonstrated AIV antigen stability at satisfactory level over a 214 day period which indicated the benefit of using the post-coating, allowing a large

number of plates to be prepared and stored, minimising variation between batches of plates. Homogenous results are one of the important prerequisites to estimate unbiased AI sero-prevalence and identify the associated risk factors which will ultimately help develop an effective future AI surveillance program on WABs in this region.

The use of stored post-coated plates for performing cELISA on samples obtained from the field was not possible due to time constraints. However, the JCU-2 (with post-coating) can be reliably used with further optimisation to increase the level of sensitivity for future AI sero-surveillance in wild birds in Australia.

7.6.3. Screening test for avian influenza viral ribonucleic acid in wild aquatic birds

Two parallel assays were used to screen AIV RNA from samples of WABs (Chapter 4). There were concerns that the AAHL assay described by Heine *et al.* (2007) would fail to detect any samples that deviated from the sequence for which they were designed. The Ward assay was therefore used and this assay is more likely to detect samples that are heterologous (Ward *et al.*, 2004). Using assays in parallel was justifiable in this study, for example, some samples reactive in the Ward assay were not reactive in the AAHL assay, but have been successful in sequencing of AIV genes (Table 4.11; Chapter 4). It is therefore important to use both assays for AIV RNA screening under future AI surveillance programs in wild birds in Australia.

7.6.4. Semi-nested polymerase chain reaction for avian influenza viral ribonucleic acid reactor samples of wild aquatic birds

This project developed a reasonable semi-nested PCR approach which was applied on field reactor samples for sequencing different AIV genes (M, H and NSP) (Chapter 4). A universal 12 bp primer, targets a conserved sequence of each influenza A gene segment, allowing cDNA synthesis of the entire viral genome. Complementary DNA was then used as a template for the next round of PCR using a pair of primers developed by Jindal *et al.* (2009b) followed by the gene targeted primers for the third round of PCR. Jindal primers produce PCR products for four AIV genes: M, H, N and NSP.

This semi-nested PCR was very effective for the molecular epidemiological study when AIV RNA concentrations in extracts from reactor field samples were low. Moreover, growing viruses in chicken embryos was not successful using reactor field samples. This PCR assay, however, needs further optimisation to improve its sensitivity for molecular evaluation of field samples and monitoring viral evolution for future AI surveillance programs in this region of northern Australia.

7.6.5. Viral isolation in chicken embryos

As mentioned earlier growing and isolating AIVs was not successful using the AIV RNA reactor samples of WABs in embryonated chicken eggs (Chapter 4). Only 19% of AIV RNA reactor samples were tested for growth in chicken embryos. Some earlier studies observed poor performance of this conventional technique in isolating AIVs using AIV RNA reactor field samples of wild birds such as 0% in Ireland (Raleigh *et al.*, 2009), 3% in Australia (Haynes *et al.*, 2009) and 8% in Argentina (Pereda *et al.*, 2008). Low viral loads in field reactor samples were previously identified as a cause of poor performance of this conventional technique (Munster *et al.*, 2007; Munster *et al.*, 2009). The unsuccessful viral isolation in this study may be due to failure in maintaining samples at an insufficiently cool temperature in the field and multiple freezing-thawing which might have degraded viruses. Therefore, for future studies, an appropriate cool temperature should be maintained for samples in the field and inoculated into embryonated chicken eggs directly without delay. Pooled samples can be used instead of using individual samples which could reduce cost and labour. This strategy might enhance the success of viral isolation. Live virus can be potential candidates for future vaccine production. Isolation of live viruses will also ensure available samples to analyse a full genome analysis of AIVs.

7.7. Overall conclusions

Studies identified the risk of spill-over of several diseases such as AI, ND and *Salmonella* from WABs to domestic animals and people and factors that were associated with the increased risk of spill-over of these diseases such as PBDs and the warm wet season for AIV. The risk of spill-over of HPAI AIV or LPAI capable of becoming HPAI appears greater due to the large diversity of circulating viral subtypes. There was also evidence to suggest the risk of introduction of novel exotic viruses which adds to this capability. This information should enable better risk mitigation of spill-over of diseases from WABs to domestic animals and people. Additional information collected on common causes of bird mortality also provides background information which greatly helps identify causes of future disease outbreaks in WABs. Moreover, aspects of wild bird diseases in northern Australia that are important for further study have been identified.

A successful surveillance network was established between property owners and the JCU AI research team through which live, sick and dead bird samples, with accompanying information, were readily obtained for analysis.

Competent diagnostic assays were developed for evaluation of samples of WABs for different diseases (in particular AI and ND). Therefore, there is an opportunity to establish and maintain a long term active and passive disease surveillance program of wild birds in northern Australia as well as investigating other risk factors for the spill-over of diseases from WABs to domestic animals and people.

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Appendices

Appendix A. Histological changes observed in different organs of wild aquatic bird carcasses at Billabong Sanctuary in Townsville, north Queensland (April 2007-March 2010)

Species (Total No.)	Types of lesions (Frequency number of lesions)
Plumed whistling duck (21)	▪ Infiltration of lymphocytes in the oesophagus (1 of 1), trachea (7 of 11), lung (13 of 20), heart (6 of 19), liver (11 of 21), spleen (6 of 13), pancreas (6 of 20), kidney (5 of 9), proventriculus (7 of 15), gizzard (5 of 8), intestine (5 of 8), caecal tonsil (4 of 10), bursa (4 of 8) and brain (5 of 8) ▪ Infiltration of mono/heterophils in the lung (1 of 20) and liver (3 of 21) and infiltration of fat in the liver (1 of 21) ▪ Degeneration of cells/muscle/blood vessel in the trachea (4 of 11), heart (3 of 19), liver (4 of 21), spleen (2 of 13), pancreas (6 of 20), kidney (6 of 9), proventriculus (1 of 15), gizzard (6 of 8), intestine (5 of 8), caecal tonsil (2 of 10), bursa (2 of 8) and brain (3 of 8) ▪ Necrosis of cells in the lung (2 of 20), heart (4 of 19), liver (9 of 19), pancreas (6 of 20), kidney (6 of 9), proventriculus (4 of 15), gizzard (1 of 8), intestine (2 of 8), caecal tonsil (1 of 10), bursa (2 of 8), brain (6 of 8) and neck muscle (1 of 1) ▪ Haemorrhage in the trachea (2 of 11), lung (2 of 20), heart (4 of 19), liver (6 of 21), spleen (3 of 13), pancreas (3 of 20), kidney (2 of 9), proventriculus (2 of 15), intestine (2 of 8), caecal tonsil (1 of 10), bursa (1 of 8), brain (1 of 8) and neck muscle (1 of 1) ▪ Air saeculitis (2 of 20), pericarditis (2 of 19), peritonitis (spleen, 1 of 13; pancreas, 1 of 20; proventriculus, 4 of 15) and loss of villi of caecal tonsil (1 of 10) ▪ Oedema/exudate in the trachea (1 of 11) and lung (3 of 20) and granulomatous tissues in the lung (1 of 20) and intestine (1 of 8) ▪ Bacterial cells in the lung (3 of 20), pancreas (1 of 20), gizzard (1 of 8), intestine (2 of 8), caecal tonsil (2 of 10) and bursa (3 of 8), fungus in the caecal tonsil (1 of 10), nematodes in the proventriculus (1 of 15) and gizzard (1 of 8), inclusion bodies in the liver and haemosiderin pigment in the liver (2 of 21) and spleen (1 of 13)
Pacific black duck (1)	▪ Infiltration of lymphocytes and heterophils in the trachea, heart, liver, spleen, pancreas, proventriculus and brain
Magpie goose (3)	▪ Infiltration of lymphocytes and or neutrophils in the oesophagus (1 of 1) and kidney (1 of 3), necrosis in the liver (1 of 3) and kidney (1 of 3), bacterial cells in the lung (1 of 3), heart (1 of 3), liver (1 of 3) and kidney (1 of 3), oedema in the lung (2 of 3) and pancreas (1 of 3)
Dusky Moorhen (2)	▪ Bacterial cells in the trachea (1 of 2)
Australian white ibis (5)	▪ Infiltration of lymphocytes in the trachea (3 of 5), lung (3 of 5), heart (3 of 5), liver (3 of 5), spleen (3 of 5), pancreas (3 of 5), kidney (3 of 5), proventriculus (2 of 5), gizzard (4 of 4), intestine (3 of 3), caecal tonsil (3 of 4), bursa (2 of 3) and brain (3 of 4) ▪ Degeneration in the trachea (2 of 5), heart (2 of 5), liver (2 of 5), spleen (2 of 5), pancreas (1 of 5), kidney (3 of 5), proventriculus (1 of 5), gizzard (4 of 4) and bursa (2 of 3) ▪ Necrosis in the trachea (1 of 5), heart (1 of 5), liver (4 of 5), pancreas (2 of 5), proventriculus (1 of 5), intestine (3 of 3), caecal tonsil (2 of 4) and brain (3 of 4) ▪ Haemorrhage in the trachea (1 of 5), lung (2 of 5), heart (1 of 5), liver (1 of 5), spleen (1 of 5), pancreas (1 of 5), kidney (1 of 5), proventriculus (1 of 5), gizzard (1 of 4), intestine (1 of 3), caecal tonsil (1 of 4), bursa (1 of 3) and brain (1 of 4), oedema in the lung (3 of 5), granulomatous tissues in the brain (1 of 4), loss of villi of the intestine (1 of 3) and caecal tonsil (2 of 4) ▪ Bacterial cells in the lung (1 of 5), proventriculus (1 of 5) and nematodes in the proventriculus (1 of 5) and haemosiderin pigment in the spleen (1 of 5)
Australian pelican (3)	▪ Infiltration of lymphocytes in the trachea (1 of 3), heart (1 of 3), liver (1 of 3), kidney (1 of 2) and brain (1 of 2), necrosis in the spleen (2 of 2) and kidney (1 of 2), haemorrhage in the liver (1 of 3) and oedema in the lung (1 of 3)
Australian king parrot (1)	▪ Necrosis in the liver, oedema in the lung and bacterial cells in the lung
Pigeon (1)	▪ Infiltration of lymphocytes in the trachea and liver
Sulphur crested cockatoo (1)	▪ Infiltration of lymphocytes in the heart and brain, necrosis in the heart and oedema in the liver and kidney
Tawny frogmouth (1)	▪ Infiltration of lymphocytes in the trachea, lung, heart, pancreas, kidney, proventriculus, intestine and brain, degeneration of cell/muscle in the liver, spleen, gizzard, caecal tonsil and brain, necrosis in the liver, spleen, kidney and brain, haemorrhage in the liver and bacterial cells in the bursa

Appendix B. Clinico-pathological syndromes and histological changes observed for different diseases of wild aquatic bird carcasses at Billabong Sanctuary in Townsville, north Queensland (April 2007-March 2010)

Diseases (Bacteriological/ molecular investigation)	**Tissues for the identification of organisms**	**Signs**	**Lesions**	**Histological changes**
Colibacillosis (*E. coli*)	Trachea, bursa, lung, liver, heart, spleen, pancreas, caecal tonsil and proventriculus	Non-observable	Whitish membrane covering the heart, nostril filled with blood, pale liver, congestion in the liver, infiltration of fat in the liver and haemorrhage in the oesophagus, trachea, lung, heart, liver, pancreas, proventriculus, cecum, kidney, brain, testes and neck muscle	Infiltration of lymphocytes in the oesophagus, trachea, lung, heart, liver, pancreas, proventriculus, kidney and brain, infiltration of monocytes and or heterophils in the oesophagus, trachea, heart and liver, haemorrhage in the lung and heart, necrosis in the heart, liver, kidney, oedema in the lung, liver and pancreas, bacterial cells in the liver and inclusion bodies in the liver
Pasteurellosis (*P. multocida*)	Trachea, lung, spleen, caecal tonsil, intestine, gizzard and bursa	Depression, dyspnoea, dehydration and unable to walk	Pale and friable liver, necrotic foci on the surface of the liver and haemorrhage in the liver, cerebrum and on the skull	Infiltration of lymphocytes in the trachea, lung, heart, liver, spleen, pancreas, intestine, proventriculus, gizzard, caecal tonsil, bursa, kidney and brain, haemorrhage in the lung and liver, necrosis in the heart, liver, spleen, pancreas, kidney and brain, muscle degeneration in the trachea, proventriculus, gizzard, intestine and bursa, cell degeneration in the liver, spleen and kidney, granulomatous cells in the lung and brain, oedema in the lung, lost of villi in the gizzard and bacterial cell in the lung and proventriculus
Salmonellosis (*S. enterica serotype virchow*)	Caecal tonsil	Non-observable	Soiled vent and haemorrhage in the trachea	Cell cilia lost in the trachea, oedema in the lung, infiltration of macrophage in the spleen, haemosiderin pigment in the spleen and nematodes in the proventriculus
Salmonellosis (*S. enterica serotype hvittingfoss*) and Colibacillosis (*E. coli*)	Liver, proventriculus and bursa	Non-observable	Pale heart and liver, haemorrhage in the trachea, lung, heart, liver, pancreas and neck muscle and congestion in the heart	Exudate in the trachea, infiltration of fat in the liver, infiltration of heterophils, haemorrhage and necrosis in the neck muscle, infiltration of lymphocytes in the trachea, lung, liver and proventriculus and nematodes in the proventriculus
Newcastle Diseases (Newcastle disease virus)	Lung, liver and pancreas	Non-observable	Haemorrhage in the heart and spleen	Infiltration of lymphocytes in the trachea, lung, heart, pancreas, kidney and brain, necrosis in the kidney and oedema in the lung

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ORIGINAL RESEARCH

Factors limiting traditional household duck production in Bangladesh

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Abstract A cross sectional survey of duck production was carried out in 2002 on 771 traditional, semiscavenging household duck farms on the coastal Island of Hatia. We determined the socioeconomic characteristics of duck farmers and their management systems, identified the factors associated with egg production, and measured the level of selected duck diseases and current preventive strategies. Household family size varied from 1 to 14 individuals and women were the main caretakers of ducks. Around 34% of keepers were illiterate. Most duck products (eggs and meat; 85%) were sold at the local market. Duck houses were poorly ventilated and a variety of bedding materials were used. Feed was available in nearby scavenging areas; however, additional feed was frequently supplied by farmers. Almost all farmers (96%) ranked the rainy season as the best time for rearing ducks due to greater feed availability. The annual egg production was 79 eggs per layer with a weight of 48 g and a hatchability rate of 87%. Egg production varied by zone ($p<0.05$). The odds of suboptimal egg production was 0.5 times lower in educated farmers ($p=0.001$). The odds of suboptimal egg production was 2.5 times more likely in ducks that attained sexual maturity at >22 weeks ($p<0.001$). Most farmers ranked duck plague as the most important disease, followed by duck cholera, botulism, and duck viral hepatitis. Preventive vaccination was sporadic and used by few farmers (28%). There are significant opportunities for improved duck production on the Island of Hatia and in Bangladesh generally.

Keywords Indigenous duck (*Anas platyrhynchos*) · Traditional rearing · Risk factors · Egg production · Diseases · Bangladesh

Abbreviations

DANIDA	Danish International Development Agency
DUS	Dip Unnayan Sangstha
NGO	Nongovernment Organization
OR	Odds Ratio
P	Probability
SLDP-2	Smallholder Livestock Development Project-2

Introduction

Bangladesh is comprised of several large rivers and their tributaries which create a river delta covering nearly 90% of the country. The Bay of Bengal forms the southern border of Bangladesh. This coastal belt is particularly well suited for duck rearing, an activity which is an important source of income and animal protein among low income families (Amber and Mia 2002). Bangladesh, with duck stocks of

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38.1 million, has the third largest duck population in the world (Dolberg 2008).

Over the past two decades, the "Bangladesh Poultry Model" has entailed a very successful collaboration between private enterprise, governmental agencies, and nongovernmental organizations (Permin 2003). This project aims to reduce poverty among women who are the predominant gender involved in the poultry industry by improving productivity and is financially supported by the Danish International Development Agency.

The gradual success of this poultry model, targeting chicken production, encouraged the relevant authorities to also focus on small-scale duck rearing. Consequently, in 2002, the government with the support of the Smallholder Livestock Development Project-2 (SLDP-2) developed a duck model for the water belt areas in five southern-central districts of Bangladesh (Amber and Mia 2002). The model included incentives to encourage household duck farming to become profitable and self supporting among small scale enterprises. As an extension of the program, a research project was developed with the goal of assessing the current systems of semiscavenging household indigenous duck (*Anas platyrhynchos*) farming. The broad aims of the study were to describe the socioeconomic status of the farmers, identify key management practices and factors associated with production potential, and assess the importance of common duck diseases and the level of control practices.

Several studies have previously addressed management practices and health in traditional rural chickens and ducks in other parts of this country (Huque et al. 2001; Biswas et al. 2005; Das et al. 2005; Biswas et al. 2006). However, there is only limited information available on duck production in the coastal belt area. The present study allows comparisons with other areas and adds to our understanding of duck management and diseases globally, which is important in managing emerging and re-emerging diseases and biosecurity.

Materials and methods

Study area

Hatia Island is one of the subdistricts known as an "Upazilla" of the Noakhali district in Bangladesh. The duck model implementation organizations, Dip Unnayan Sangstha (DUS), which was a local NGO and SLDP-2 divided the island into four zones: Tumurrudi-1 (1), Tumurrudi-2 (2), Charking (3), and Charishwar (4). Division of the island was based on the following: proximity to large and permanent water bodies (river/sea), nature of farmer's houses, and ease of communication.

Sampling

We used a list of households (≥3 indigenous ducks (*A. platyrhynchos*) per household; N=4,000) developed by DUS and SLDP-2 and then validated by us. We chose 1,000 households in total using 25% proportional probability and random sampling from each of the four zones to ensure the sample was representative of the target population. Zone 1 contributed 380 (n=1,520); zone 2, 128 (n=512); zone 3, 242 (n=968); and zone 4, 250 (n=1,000). The field survey was conducted during September and October 2002.

Questionnaire design and data collection

To train field workers and to validate and improve the questionnaire, a pilot study was conducted on 15 randomly selected households before the main cross sectional survey. The questionnaire included closed, semiclosed, with space available to record alternatives to the options given, and open-ended questions. The questions focused on five areas of interest: socioeconomic conditions of farmers and their families, flock composition and housing, feed, production (average rate of egg production per duck per household farm, egg weight, and hatchability), and diseases. Eggs were weighed if available at interview with a portable balance to check the accuracy of the farmer's response.

Farmers were requested to give a rank of importance against four selected duck diseases (Table 1). Farmers were shown pictures depicting the characteristic clinical signs specific for the selected diseases to help farmers recall and recognize diseases their ducks might have had during the previous year. Most of the diseases encountered in these ducks were expected to be infectious and field diagnosis is commonly based on clinical signs and/or postmortem examination (Aini 1993). Farmers were also told duck viral hepatitis commonly occurs in 1–2-week-old ducklings, duck cholera occurs from 1 month of age and onward and duck plague and avian botulism occurs at any age (Milton and Christian 1999).

Four field interviewers were trained and evaluated for acceptable efficiency using the simulated client's method as described by Radford et al. (2003). They were supervised by the local veterinarian, working for the Development of Livestock Services, and the area manager of SLDP-2.

Statistical methods

Data obtained were entered in MS Excel-2000 and then exported into STATA™ 9.0 (Stata Corporation, College Station, TX, USA) for epidemiological analysis. Descriptive

Table 1 Criteria set for the tentative diagnosis of duck diseases at household farms on Hatia Island

Name of the disease	Clinical signs commonly observed
Duck viral hepatitis (Enterovirus or astrovirus)	Ducklings: ≤1-week-old, stop moving with eyes partially closed, fall on their sides, kick spasmodically with both legs, die ≤4 days with head back
Duck plague or duck virus enteritis (Alphaherpesvirus)	Ducklings: diarrhea, blood stained vent, dehydration, cyanotic bill
	Breeder ducks: sudden, high, persistent flock mortality, prolapsed penis in males
	Laying ducks: marked drop in egg production, photophobia, half closed pasted eyelids, ruffled feathers, nasal discharge, soiled vents, watery diarrhea, inability to stand
Duck Cholera (*Pasteurella multocida*)	Loss of appetite, thirst, frequent green or yellow watery droppings, swollen joints, staggering gait, mucus discharge from the mouth and dyspnea, death common at 3–6 months of age
Botulism (*Clostridium botulinum*)	Progressive flaccid paralysis of the neck, loss of neck feathers, diarrhea with staining of the tail and vent region, die ≤48 h

statistics were performed to express individual results of each coded category as frequency percentage, mean with standard error, median, and range where applicable.

The average egg production rate per duck per household farm in a year was divided into two categories: ≤75 eggs (suboptimal) and >75 eggs (optimal) based on advice from the literature and the fact that egg production was not normally distributed even after various transformations such as log transformation (Huque et al. 1993). Univariate chi-square tests were performed to assess associations between the categorized response variable of egg production and the selected independent variables: zone (1/2/3/4), farmer's education classed in terms of three categories (illiterate/school class level attended: I–IV/school class level attended: V+), presence of a duck house (yes/no), and duck age at sexual maturity as measured in terms of two categories (≤22/>22 weeks).

Predictor variables were selected for inclusion in the multivariable logistic model when univariable associations were significant ($p<0.1$). The variable of farmer's education was further condensed into two categories (education (schooling)/noneducation). All variables produced significant p values and were considered for inclusion in the logistic model. The model was constructed by forward selection applying the maximum likelihood estimation procedure and statistical significance contribution of individual predictors (or group of predictors) to the models tested using the Wald's test and likelihood ratio test as described by Dohoo et al. (2003). We assessed for interaction between variables by constructing two interaction product terms for the significant main effect variables in the model, forcing them into the model and examining changes in the odds ratio (OR) and p values of the main effects. Evidence of confounding was checked by dropping one of the variables and assessing the changes of odds ratio (≤10% change meant no confounding). The model was then assessed for goodness-of-fit using the Hosmer–Lemeshow test while the predictive ability was determined using the receiver operating characteristic (ROC) curve (Dohoo et al. 2003). The results were presented for each adjusted predictor variable as an OR, p value, and 95% confidence interval.

Results

Incomplete data and nonrespondents

The percentage of households providing incomplete questionnaires and nonrespondents were 22% and 1%, respectively. Therefore, 77% ($n=771$) of households were included in the final analysis, which represented 19% of the target population (Table 2).

Table 2 Response of duck farm households on Hatia Island to the questionnaire

Zone	Households in the sampling frame	Sampled households	Households with incomplete data	Nonrespondents	Households analyzed
Tumurrudi-1	1,520	380	45		335
Tumurrudi-2	512	128	7	1	120
Charking	968	242	100		142
Charishwar	1,000	250	69	7	174
Total	4,000	1,000	221	8	771

Socioeconomic characteristics

In the subset of complete questionnaires, duck farmers were predominantly female (92%). Their education status varied from primary (49%), secondary (14%), and higher secondary (2%) to a few college graduates (1%). About 34% were illiterate and were only able to write their name. In the incomplete subset of questionnaires, the education status was more or less similar (53% primary, 9% secondary, 4% higher secondary, 1% graduation level, and around 33% illiterate). For the remainder of the text only data from complete questionnaires are reported (please see tables for incomplete questionnaire data).

The main livelihood appeared to be mixed farming consisting of crop production and livestock "rearing" including ducks, chickens, cattle, and goats. Around 37% of farmers did not have cultivable land other than their homestead. Most of the duck farmers (97%) had common possession of vegetation around their homestead, which consisted mostly of fruit and wood production and medicinal plants.

The median age of the duck farmer was 45 years (Table 3). The experience of households involved in duck farming ranged from 1 to 50 years of traditional duck raising. The median family size on farms was five, consisting of three males and two females. A median of two members per household were receiving some level of education (Table 3).

Size and composition of flocks on duck farms

The median and range of household duck flock size for the complete and incomplete data sets were seven (5–45) and six (3–30), respectively, whereas the median and range of chicken flock size were five (1–20) and five (1–15), respectively (Table 4).

Sources of ducks, rearing purpose, and marketing of the products

The local market (45%) and the farmer's own production (55%) were the two main sources of ducklings and replacement stock. Farmers reared ducks for semicommercial purposes. The local market (85%) was the major venue for selling eggs and live birds, aside from occasional sales to neighbors (15%).

Existing management practices

Duck housing

Out of 771 farmers, 83% kept their ducks in a separate duck house at night while 17% kept their ducks in bamboo made small crates. The ventilation facilities of the traditional duck houses were graded as poor (no windows; 86%), moderate (small windows on two sides; 9%), and good (small windows on three sides; 5%).

Rice husk, paddy straw, sawdust, sand, ash, and chot (made of jute) were the commonly used bedding materials. Most (85%) farmers continued to use the same litter for 1–2 months at a time without changing or sun drying. Most farmers (86%) did not use conventional waste disposal methods, rather they threw old litter and droppings onto the nearby ground and some (14%) emptied manure into a deep pit.

Table 3 Age of duck farmers and family composition on household duck farms on Hatia Island

Category	Complete data set			Incomplete data set		
	Mean±SE	Median	Range	Mean±SE	Median	Range
Age of duck farmers	41.2±0.46	45	15–75	45.9±0.85	50	16–70
Family size	5.6±0.07	5	1–14	5.5±0.14	5	1–17
No. of males	3.0±0.05	3	1–8	2.9±0.09	3	1–8
No. of females	2.6±0.04	2	1–8	2.7±0.1	2	1–9
No. of members with education	2.4±0.05	2	1–7	2.4±0.11	2	1–7

Table 4 Size and composition of flocks on household duck farms on Hatia Island (complete and incomplete questionnaire data sets)

Categories	No. of households	Complete data set				Incomplete data set		
		Mean± SE	Median	Range	No. of households	Mean± SE	Median	Range
Flock size	771	8.1±0.15	7	5–45	221	8±0.30	6	3–30
No. of layers	692	4.2±0.09	4	1–25	162	4.1±0.20	4	1–23
No. of immature developing females	564	3.4±0.09	3	1–17	165	3.7±0.16	3	1–15
No. of drakes	559	1.9±0.05	2	1–9	169	1.9±0.13	1	1–20
No. of ducklings	108	4.7±0.33	3	1–22	33	5.8±0.62	5	1–15
No. of chickens	771	5.5±0.15	5	1–20	221	5.2±0.16	5	1–15

Feeds, feeding, and rearing season

Almost all farmers (98%) provided broken snails and duck weed when available to the ducklings before allowing them to scavenge. Around 86% of the farmers supplied feed enriched with grain (rice and wheat), protein (snail and duck weed), and minerals (snail shells and calcium carbonate) during the laying period. The frequencies of feed offered per day to ducks and proportion of farmers using these regimes were once (1%), twice (60%), and thrice (39%). Feed supplements were from farmers' own sources (38%), bought at the local market (18%), or both (44%). Almost all the duck farmers (96%) reported the rainy season as the best season for duck rearing.

Production potential

Around 74% of the farmers reported that the sexual maturity of the local indigenous ducks was reached at >22 weeks, whereas 26% were reported to mature at ≤22 weeks. The average number of eggs produced per layer per household farm per year was 79 (22–200), egg weight 48 g (40–60), and hatchability 87% (20–100%). Most of the farmers (99%) reported to have set more than ten eggs at a time under one broody hen. More than half (53%) of the farmers surveyed reported that if the broody hen did not complete their brooding, they were replaced by another broody hen of the same flock when available. Otherwise, 44% of the farmers reported they transferred the hatchable eggs to a broody hen from a neighboring house and 3% did not take any steps.

Risk factor analysis

Univariate analysis

The average egg production per duck per household farm at two different levels (suboptimal/optimal) varied significantly among zones, whether the farmer was educated, if there was a duck house and according to the time it took to reach sexual maturity ($p<0.001$). The farms in zone 1 had the highest suboptimal egg production as compared with the other zones. Farmers without education and without duck houses had higher suboptimal egg production. Ducks which took longer to reach sexual maturity were more likely to have suboptimal egg production (Table 5).

Final logistic model

Neither interaction nor confounding was detected in the model. The Hosmer–Lemeshow goodness-of-fit test showed that the model fitted the data ($X^2=1.75$, $p=0.63$). The ROC curve extended reasonably well into the upper left-hand corner of the curve and the area under the curve was 0.77 (Fig. 1). Both of these indicated the logistic model had a good predictive ability.

After adjustment of factors (zone/farmer's education/duck house/sexual maturity), the odds ratio of egg production in the zone categories 2, 3, and 4 were 0.3, 0.1, and 0.6 compared with zone 1, respectively ($p<0.05$). The odds ratio was 0.5 in educated compared with noneducated farmers ($p=0.001$). The odds of suboptimal egg production was 2.5 times more likely in ducks that attained sexual maturity at >22 weeks than in ducks that attained sexual maturity at ≤22 weeks ($p<0.001$; Table 6).

Disease frequency and preventive approach of the household farm

Most of the farmers gave duck plague the highest rank of importance, followed by duck cholera, botulism, and duck viral hepatitis (Table 7). Only 28% of the farmers said they vaccinated their ducks against duck diseases.

Discussion

The overall participation in the questionnaire survey was very good (99%). However, we were unable to fill in the

Table 5 The association of selected variables and rate of egg production in household duck farms on Hatia Island (n=771)

Variables	Categories	Number of farms (%)		p value
		≤75 eggs (Suboptimal)	>75 eggs (Optimal)	
Zone	1	244 (72.9%)	91 (27.5%)	<0.001
	2	68 (56.7%)	52 (43.4%)	
	3	30 (21.2%)	112 (78.9%)	
	4	97 (55.8%)	77 (44.3%)	
Farmer's education	Illiterate to signature capability	204 (76.4%)	63 (23.6%)	<0.001
	Class: I–V	174 (46.6%)	200 (53.5%)	
	Class: +V	61 (47%)	69 (53.1%)	
Duck house	Yes	324 (51.1%)	311 (49%)	<0.001
	No	115 (84.6%)	21 (15.4%)	
Attainment of sexual maturity	≤22 weeks	94 (47%)	106 (53%)	<0.001
	>22 weeks	345 (60.5%)	226 (39.6%)	

The p values are for unconditional significance tests for differences among categories within each variable

gaps of information of 22% households mainly because the male field workers were unable to gain access to conservative Muslim households a second time to interview female duck farmers. However, the data provided in incomplete questionnaires did not differ significantly from completed ones and therefore their exclusion did not bias the final logistic analysis.

Duck rearing on Hatia Island was carried out primarily by female family members, with both younger and older members participating equally. This is because the male household owners (husband/father/elder brother, etc.) were involved with activities outside the home. Women also dominate rural poultry rearing in Western Kenya (Okitoi et al. 2007) and the Garhwal Himalayas of India (Selvam 2004; Singh et al. 2009).

The median family size recorded in the present study mirrors the national figure. Similar and slightly higher family size have been reported elsewhere in the country (Shamsuddoha and Sohel 2005).

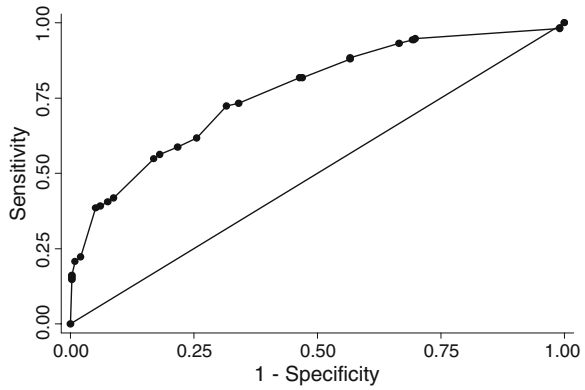

Fig. 1 Plot of sensitivity versus 1-specificity for a receiver operating characteristic curve (ROC) of various parameters of the logistic model of egg production for duck household farms on Hatia Island. The *point* of the curve which is most towards the *upper left* of the figure indicates the maximum accuracy of the model

A slightly lower average age of farmers (36 years) has been recorded in other parts of Bangladesh (Hoque and Itohara 2008). The education status of the farmers in this study corresponds with the previous study on rural poultry farmers in Bangladesh (Biswas et al. 1999). However, a study in India reported 95% were illiterate and 5% had primary level of education which was different from our study (34% of farmers were illiterate farmers; Gajendran and Karthickeyan 2009). Since rural duck farmers along with their family members are usually born into poverty, they are compelled to contribute to family income instead of gaining further education. Typically, households are completely dependent on agricultural enterprises including small-scale poultry rearing.

Farmers typically possessed a small patch of cultivable land next to their homestead in the present study, which is in accordance with the earlier studies (Huque et al. 2001; Hoque and Itohara 2008). The median number of ducks reared per household farm is similar to other duck raising areas of Bangladesh (Das et al. 2008). The average flock size of chicken per rural household in this study agrees with the study carried out in India (Mandal et al. 2010). However, flock size varies according to season, availability of scavenging feed, market price, natural calamities like floods, and infectious disease outbreaks and nondisease causes of mortality like predation.

As there was no organized commercial breeding farm at Hatia, farmers depended on the local market and on their own private sources for increasing or renewing their stocks. Ducks were reared both for income and food for the family, even though eggs are not commonly included in the daily diet for rural families. The local market remained the only choice for selling eggs and live ducks. Farmers commonly had separate duck houses, but their standard and management were very poor in terms of ventilation and bedding materials used. This enables infectious agents to be sustained easily in the production environment.

Table 6 The association of selected variables and rate of egg production in a conditional logistic model for duck household farms on Hatia Island (n=771)

Variables	Categories	OR	p value	95% CI
Zone	1	1.0		
	2	0.33	<0.001	0.20, 0.54
	3	0.10	<0.001	0.06, 0.17
	4	0.60	0.015	0.39, 0.90
Farmer's education	Illiterate to signature capability	1.0		
	Some education	0.53	0.001	0.37, 0.79
Duck house	Yes	1.0		
	No	3.28	<0.001	1.90, 5.67
Attainment of sexual maturity	≤22 weeks	1.0		
	>22 weeks	2.47	<0.001	1.68, 3.63

OR odds ratio, *CI* confidence interval

In scavenging areas, ducks had a variety of protein feeds (small fish and slug during the year in addition to snails, duck weed, earth worms, crabs, and land and water insects). Scavenging feed may supply 50% of the total feed requirement (Amber and Mia 2002). Scavenging feed depends on the season of the year, and almost all farmers stated the rainy season was the best for duck rearing due to access to large amounts of scavenging feed rather than water availability. Kabir et al. (2007) reported similar availability of scavenging feed for ducks during the rainy season and suggested no supplementary feeding was needed during that period.

Farmers in this study gave feed dominant in carbohydrate and poor in protein, even though protein is essential for stimulating production. Kabir et al. (2007) reported that providing improved supplemental food to growing ducklings and laying ducks has been suggested as a means to boost growth and laying performances. Almost every farmer reported to have provided ducklings with seasonal broken snails before letting them scavenge on their own. This kind of feed supplementation might enhance duckling's growth and strength and improve their resistance to infectious diseases. The predominant age of sexual maturity recorded in the present study corresponds with Das et al. (2008; Bangladesh) and Gajendran and Karthickeyan (2009; India).

Production levels achieved by local indigenous ducks, as recorded in this survey, are in accordance with the work done by Das et al. (2008). Khanum et al. (2005) recorded a slightly lower level of egg production in rural ducks in Bangladesh. But Das and Hoq (2000) reported greater production results for exotic and cross bred ducks reared in traditional systems where Khaki Campbell was the best performer laying 221 eggs per year followed by Jinding with 176 eggs and then the indigenous cross Khaki Campbell with 174. In contrast, a previous report stated the white local indigenous duck to be a better egg producer (150 to 200 eggs per layer for a year) than the above-mentioned breeds (Gajendran and Karthickeyan 2009). Variability in egg production was also recorded based on feeding practices where scavenging indigenous ducks and semiscavenging ducks laid 42 and 138 eggs per layer per year, respectively (Huque et al. 1993).

A better egg weight was reported for Khaki Campbell (60 g), Jinding (56 g) (Das and Hoq 2000), indigenous ducks (62 g) in India (Gajendran and Karthickeyan 2009), and local ducks (57–65 g) in Bangladesh (Kabir et al. 2007; Das et al. 2008) compared with the indigenous ducks in the present study (48 g). This difference in egg weight could be attributed to the genetic potential among different breeds, different environmental conditions between geographical regions and years. Alternatively, farmers might have given extra care to exotic breeds than local indigenous breeds.

Farmers surveyed in this study considered ten or more duck eggs as an appropriate number of eggs to set under one broody hen. Even though farmers occasionally needed to transfer an "abandoned" clutch to a neighbor's broody hen, the hatchability of eggs within this traditional system appears to be satisfactory. Hatchability performance corresponds to the earlier work done by Das et al. (2008).

Table 7 Relative ranking of importance of duck diseases for 771 household farms on Hatia Island

Note: As reported by farmers based on their recollection and after viewing pictorial descriptions of typical clinical signs of duck diseases

Diseases	Number of farms			
	Rank-1 (Highest)	Rank-2	Rank-3	Rank-4 (Lowest)
Duck viral hepatitis	22	49	383	317
Duck plague (duck virus enteritis)	463	296	12	0
Duck cholera	198	251	139	183
Botulism	88	40	279	364

Risk factors for egg production

Although the study was confined to Hatia Island, there were substantial differences among zones in terms of egg production with zone 3 better than the other zones. This might have been due to this particular area being closely located to big rivers which could provide better scavenging (in particular protein and trace elements). In spite of the fact that the more educated farmers used traditional duck keeping practices, their ducks produced significantly more eggs than ducks kept by less educated farmers. It may be that educated farmers are comparatively more aware of management practices and supplying necessary supplementary feeds to their ducks due to their reading skills which help them to readily gather useful information. Ducks accommodated in separate duck houses had better average egg production than ducks accommodated in temporary small night shelters such as crates. Farmers generally used creates because they did not have enough land for building duck houses. However, a duck house is a good indicator for better management and greater egg production. Ducks that attained earlier sexual maturity produced comparatively more eggs possibly again indicating better management of these ducks.

Duck diseases

As clinical signs are similar among our selected duck diseases, the differential diagnosis based on only clinical signs might be problematic. However, farmers in our study appeared to be able to confidently differentiate among these diseases. In addition, the local livestock office and other local NGOs involved with these families on Hatia Island reported similar disease prevalence among poultry. Moreover, our prospective studies on Hatia Island and in other coastal areas in Bangladesh agreed with the level of duck diseases reported in this study (Hoque and Rahman 2004; Hoque 2006). Obviously postmortem examinations and laboratory tests could provide more accurate diagnosis but this study provides a baseline to encourage more sophisticated investigations.

Duck plague and duck cholera have been reported to occur commonly in ducks irrespective of breed and management system in Southeast Asia (Hossain et al. 2004; Chellapandian et al. 2005; Das et al. 2005; Prasad and Singh 2008; Gajendran and Karthickeyan 2009) where vaccination is performed at irregular intervals as seen in the present study. Duck cholera caused by *Pasteurella multocida* spp. has also been reported in 7% free ranging ducks in Tanzania (Muhairwa et al. 2001). The occurrence of duck viral hepatitis in our study is in accordance with an earlier report (Das et al. 2005; Gajendran and Karthickeyan 2009). Therefore, preventive husbandry practices and vaccination against prevalent duck diseases should be practiced on a regular basis. Government and nongovernment agencies and industry should endeavor to provide adequate supplies of safe and effective vaccines and technical advice for their use.

Avian botulism was ranked lower than duck plague and duck cholera and similar to duck viral hepatitis in terms of importance but could occur if ducks scavenge aquatic insects, mollusks, and crustacea or decaying organic matter contaminated with the bacterial toxin from *Clostridium botulinum* (Milton and Christian 1999). Botulism has been reported as an important waterfowl toxin (Degernes 2008) and toxin of *C. botulinum* is very stable in the environment. It can be ingested by insect larvae with only two to three maggots capable of causing clinical signs if eaten by a duck (Smith 2008).

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ORIGINAL RESEARCH

Monitoring the health and production of household Jinding ducks on Hatia Island of Bangladesh

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Abstract Duck rearing is an important component of sustainable living in poor rural communities, especially as a source of subsistence. A study was conducted on 118 households (N=1,373 Jinding ducks, *Anas platyrhynchus*) from December 2002 to February 2004 on Hatia Island in Bangladesh with the aim of identifying the factors that limit the health and production of Jinding ducks. Overall duck mortality was 29.3%, with disease (19.7%) being a more significant factor than predation (9.6%; p=0.001). Duck mortality also varied significantly among study zones (p<0.001). Common diseases were duck plague (21.1%) and duck cholera (32.1%). Helminth infection was prevalent, with endemic trematode (*Prosthogonimus* spp., *Trichobilharzia* spp., *Echinostoma* spp.) and nematode (*Cyathostoma bronchialis*, *Amidostomum anseris*, *Heterakis gallinarum*, *Capillaria* spp., and *Echinuria* spp.) infections and epidemic cestode infections due to *Hymenolepsis setigera*. The median egg production rate per duck per household was 93 for a 6-month laying period. The odds of diminished egg production (average≤93 eggs per duck per household for a 6-month laying period) was 25.4 times higher in ducks that were kept in traditional duck houses (p<0.001) and 14.2 times higher in ducks that experienced delays in the onset of sexual maturity (days 191 and 280; p<0.001). Ducks that were provided snails for a shorter period of time over the laying period were 18.2 times more likely to produce fewer eggs than their longer fed peers (p=0.002). In conclusion, duck mortalities due to diseases and predation and parasitic infections appear to be common constraints on household duck production on Hatia Island. Additionally, improving duck housing and providing longer nutritional supplementation with snails increased the production capabilities of household-raised Jinding ducks on Hatia Island.

Keywords Jinding duck · Production · Mortality · Risk factor · Hatia Island of Bangladesh

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Introduction

Poultry rearing is an integral part of rural farming communities in Bangladesh, a country with the third largest population of ducks in the world (38.1 million; Dolberg 2008). Rural duck rearing, owned and managed by women

and children, is an important means of reducing poverty in resource-poor smallholder families with low income and livelihood conditions (Pym et al. 2002). Over the past two decades a production system known as the "Bangladesh Poultry Model" has been running in collaboration with private enterprise, governmental, and non-governmental organizations (NGOs). The success of the poultry model in Bangladesh has encouraged the government and international aid agencies to trial and establishes a similar model for ducks. This model promotes household duck raising as a source of income. The environment and duck raising knowledge of the southern coastal regions of Bangladesh provide natural business advantages, which ensure that a well designed model will be highly beneficial in these areas. The main goal of the model program was to help increase women's social standing by engaging them in profitable duck rearing. Our study aimed to identify factors which limited duck production within this model program. Hatia Island was chosen as the location for our study because the duck model program had been primarily started establishing from this Island in the southern coastal regions. A layer type exotic breed "Jinding," which was thought to be a better production performer than the local indigenous duck, was introduced for household rearing for the first time on Hatia Island under this model program. There were two types of farmers: "duckling-rearers," who reared day old Jinding ducklings up to 8 weeks and "key-rearers," the participants of the present study, who received the 8-week-old ducks. The initial credit support for buying 8-week-old ducks was anticipated to be returned primarily through the sale of eggs. The economic return was therefore dependent on the rate of egg production of ducks per household.

Known limiting factors for rural poultry production are high mortality rate due to infectious diseases and predation and untimely sale and family consumption of stock (Hoque and Rahman 2004; Biswas et al. 2005; Biswas et al. 2006; Hoque 2006; Hoque et al. 2006; Biswas et al. 2008; Hoque et al. 2010b). Besides viral and bacterial duck diseases, helminth infections are thought to be an important factor in limiting the production potential of the duck rearing program in Bangladesh. Some earlier workers have reported the incidence of parasites in household ducks elsewhere in Bangladesh (Ahmed 1969) and Tanzania (Muhairwa et al. 2007), but epidemiological approaches in assessing the temporal and spatial pattern of helminth infections in household ducks are rarely attempted.

Factors limiting production potential were assessed for indigenous ducks on Hatia Island (Hoque et al. 2010a), but were not evaluated for Jinding ducks, which was essential for further development of a production model. The present study describes the level of Jinding duck mortality in households associated with diseases and predators and the pattern of parasitic infections in household ducks. The study also describes production potential of ducks and associated risk factors. This study provides the information necessary for future studies to compare the duck production model in different regions in Bangladesh, as well as contributing to our understanding of duck management practices and limitations to duck production in a developing economy.

Materials and methods

Study area

Hatia is one of several coastal islands located in the Noakhali district of Bangladesh. Duck model implementation bodies included the local NGO Dip Unnayan Sangstha (DUS) and the Smallholder Livestock Development Project-2 (SLDP-2). The study area comprised four zones: Tumurrudi (1), Tumurrudi (2), Charking (3), and Charishwar (4).

Sampling

DUS selected 400 households (designated as key rearers) from four zones, taking 100 households from each zone. Households were selected for inclusion into the program by DUS according to the following criteria: at least 1 year's experience with rearing ducks and successful completion of a training program focused on methods for improving traditional duck rearing. All participants had to abide by the model operating rules and conditions as prescribed by DUS. However, at the starting point of our study, only 118 households had been recruited by DUS and therefore, our study was limited to these households. Of those selected households, Tumurrudi-1, Tumurrudi-2, Charking, and Charishwar represented 34 households (419 ducks), 26 (345), 35 (353), and 23 (256), respectively. As part of the program, DUS provided 5 to 20 8-week-old Jinding ducks to each household from the duckling rearers. Duckling rearers were compensated at a set rate, based on duck body weight.

Ten households from each zone were randomly chosen to participate in a longitudinal study, which was nested within the main study. It aimed to determine the level of parasitic infection in household ducks on Hatia Island. In total, 40 households participated in the longitudinal study from February 2002 to January 2004.

Questionnaire design and data collection

Questionnaires were designed in order to obtain the information listed in Table 1. A mixture of closed, semi-

Table 1 The information collected in the questionnaire and the record keeping sheet (December 2002–February 2004)

Topic	Information collected
Basic identification information	Farmer household identity numbers and zone numbers (given by DUS).
	The date and number of ducks received, and duck age (in days).
Population dynamic information	The number of living and dead ducks.
	The number of weekly duck deaths due to predation.
	The number of weekly duck deaths due to possible disease contributory.
	The number of suitable duck carcasses collected by field investigators weekly.
	Farmers were also requested to note any observable clinical signs they could identify before death of their ducks.
Vaccination	Whether ducks with a booster dose of duck plague vaccine.
Duck housing	Whether farmers had improved duck houses designed by DUS and SLDP-2.
Nutrition	Whether broken snails were provided to ducks at least a week per month during a 6-month laying period.
Sexual maturity	The date of first egg laying.
Duck production	The number of laying and non-laying ducks and total number of eggs laid per month per household.

open, and open questions were included in the questionnaire. A trial run of the questionnaire was conducted with eight households (two per zone) to help refine the questionnaire and train field investigators. The responses collected were not incorporated into the subsequent study results.

During the study period, farmers used a notebook designed by DUS to record relevant information pertaining to the study. Farmers were also trained by DUS on how to recognize clinical signs of disease. One trained field investigator was dedicated to each zone. Investigators obtained information relevant to the study from interviews with farmers and records maintained by farmers. This information was collected on a twice-weekly basis. The farmers cooperated with the investigation as a part of their agreement to the credit supplier and the program developer. The activities of the field workers were supervised by the local veterinarian employed by the Government of Bangladesh and also by the area manager of SLDP-2.

Collection of dead ducks and their post-mortem examination

Due to limitations of resources and post-mortem facilities, only 142 duck carcasses were collected from different studied households were suitable for necropsy. Zone representation of collected dead ducks was 27% in Tumurrudi-1, 26% in Tumurrudi-2, 27% in Charking, and 21% in Charishwar. During the scheduled household visits, field investigators collected duck carcasses and temporarily stored them in an ice-packed cool box. Investigators asked farmers about clinical signs of the dead ducks and for age and booster duck plague vaccine history. Farmers were paid Bangladeshi Taka 20 for each dead duck, a price lower than the market price for healthy ducks. Each duck was necropsied by a trained local veterinarian.

The following sets of clinical signs and post-mortem lesions were considered as indicative of the following diseases:

Duck plague (a disease caused by Alphaherpesvirus) Clinical signs—marked drop in egg production, photophobia, half-closed pasted eyelids, ruffled feathers, nasal discharge, soiled vent, watery or greenish–yellow diarrhea sometimes stained with blood, inability to stand up, and prolapsed penis

Post-mortem lesions—hemorrhagic proventriculus, hemorrhagic bands and sloughing of a thick yellow white membrane on mucosa in intestine, pinpoint hemorrhages in kidney, varying degree of hemorrhage on heart surface, multiple pale foci in liver, necrotic plaques in the cloaca

Duck cholera (a disease caused by Pasteurella multocida) Clinical signs—loss of appetite, thirst, frequent green or yellow watery droppings, swollen joints, staggering gait, mucus discharge from mouth, and dyspnea; death common at 3–6 months of age

Post-mortem lesions—hemorrhagic esophagus, heart, mesentery and abdominal fat, congestion and blackish coloration of lungs, enlarged and asymmetric liver covered with whitish membrane, and pinpoint white foci on the surface, sometimes copper colored and friable

Aspergillosis (caused by Aspergillus fumigatus) Clinical signs—labored breathing, listlessness, dehydration, lack of appetite, occasional diarrhea, and under-weight

Post-mortem lesions—congestion and yellowish color of the lungs, thickened air sacs with the presence of fibrin, and caseous mass present in the trachea

Non-specific—mortality which resulted from causes that could not be identified based on the clinical signs and lesions observed

Time to onset of duck sexual maturity within individual households

The number of days until attainment of duck sexual maturity was calculated by counting the days between the date of hatching and the date of the first egg laid as reported by farmers. The average period of days until attainment of sexual maturity was calculated as the sum of the days of all individual ducks at sexual maturity divided by the number of ducks that attained sexual maturity.

Average rate of egg production per duck within individual households

The average rate of egg production in an individual household for each month was calculated by dividing the total number of eggs laid at month's end by the number of laying ducks present during that month. Each month's average rate of production over the 6-month period was then summed, and the total number of eggs was divided by the total number of ducks during the whole 6 months.

Fecal sample collection and examination for identification of helminths in household ducks

A total of 12 pooled fecal samples were collected monthly from each household over 1 year. Each pool contained two to three fecal samples. Fresh voided feces were collected from ducks early in the morning, before they had begun scavenging. The samples were preserved in 10% formalin before forwarding to the Chittagong Veterinary and Animal Sciences University laboratory. Qualitative assessment was performed to detect the presence of parasitic infection in ducks using direct smear, flotation, and sedimentation techniques. A standard criterion was then followed to identify eggs of different species of parasites microscopically (Theinpont et al. 1986).

Statistical evaluation

Descriptive statistics

Microsoft Excel 2003 was used to manage study data. Data was exported into STATA®, version 11 (data analysis and statistical software; StataCrop, 4905 Lakeway Drive, College Station, Texas 77845, USA) for checking data integrity and performing epidemiological analysis. A descriptive analysis was performed on duck mortality data among study zones. A chi-square test was carried out to compare the proportions of duck mortality among zones and between diseases and predation.

Descriptive statistics were also performed on parasitic infections. A chi-square test was used to assess the proportion of households having ducks that were affected by each parasite class according to month, age group, and zone. Descriptive statistics were also generated for household duck production.

Logistic regression model

Distribution of the average egg production rate per duck per household was not normal, even after various transformations were performed. Therefore, we constructed a logistic model based on the category of average egg production rate per duck per household (≤93 eggs versus >94 eggs) for the selected factors. When attempting to define a binary response variable for the level of egg production from Jinding ducks, we lacked published examples. This is due to the fact that the use of Jinding ducks for household rearing is a relatively recent development in Bangladesh and other countries. Therefore, the classification of the rate of egg production was performed based on the 50th percentile.

The average age of ducks at sexual maturity failed to approximate a normal distribution, even after logarithmic transformation and was instead organized into two categories, separated by the 50th percentile. The duration of nutritional supplementation using snails, during a 6-month laying period was also needed to be categorized. The 50th percentile of the data was again used.

Chi-square tests were performed to assess associations between the response variable of two categories of egg production and the selected factors: zone (1/2/3/4), duck house (whether farmers had improved duck houses or traditional duck houses), average age of ducks per household at sexual maturity in two categories (av. 150–190 days versus 191–280 days), and whether farmers provided broken snails to their ducks at least 1 week in a month during a 6-month laying period, over terms of 3 months, or >3 months.

All variables producing p values of <0.1 were considered for inclusion in the logistic model. The variable of zone was further pooled into two categories (Tumurrudi 1 and 2/Charking and Charishwar) to increase the sample size needed for fitting the model. The model was constructed by applying forward selection to the maximum likelihood estimation procedure and applying the statistical significance contributions of individual predictors (or group of predictors) to the models tested. Wald's test and likelihood ratio test were used to accomplish this, as described by Dohoo et al. (2003). We assessed the interaction between variables by constructing two interaction product terms for the significant main effect variables in the model, forcing them into the model and examining changes in the odds

ratio (OR) and p values of the main effects. The presence of confounding factors was investigated by removing one of the variables and assessing changes in the OR. An OR change greater than 10% was considered to indicate the presence of confounding factors. The model was then assessed for goodness-of-fit using the Hosmer–Lemeshow test, while predictive ability was determined using the receiver operating characteristic curve (ROC; Dohoo et al. 2003).

Results

Duck mortality

The overall duck mortality on Hatia Island was 29.3%, of which disease accounted for 19.7% and predation 9.6%. These results differed significantly from each other ($p<0.001$). The proportion of duck mortality among zones varied significantly ($p=0.001$; Table 2). According to the farmer's responses, the predominant predators were the civet cat *Viverricula indica*, fox *Vulpes bengalensis*, crab-eating mongoose *Herpestes urva*, Bengal lizard *Varanus bengalensis*, and indigenous dogs. Eagles and kites were possible unseen predators.

Frequency of duck diseases among dead ducks collected

A total of 142 fresh duck carcasses were collected over the study period, which represented 53% of the total dead ducks ($N=270$). Of the 142 intact carcasses, 21.1% had duck plague, 32.1% duck cholera, and 2.1% aspergillosis. Post-mortem reports on 44.6% carcasses were inconclusive due to a lack of characteristic lesions specific to the diseases under investigation.

Helminth species recorded in household ducks

A total of nine different species were identified upon examination of feces. Of the species identified, five were nematodes, three trematodes, and one cestode (Table 3). The samples from each household were found to harbor at least one species of helminth. The majority of samples indicated mixed worm burdens.

Temporal and spatial pattern of parasitic infection in household ducks

Parasitic infection of household ducks varied significantly across both the months of the year and hence also age group of ducks ($p<0.05$). Trematodal infection was significantly more frequent during September 2003, when compared with other months ($p<0.001$). During this period, ducks that were 302 days old had a significantly higher incidence of infection than ducks from other age groups ($p<0.001$). Cestodal infection was first detected in April 2003 in 149-day-old ducks, peaked in August (271 days) and then sharply declined in September (302 days). Nematodal infection displayed an endemic pattern, with a peak in infections recorded in November–December 2003 (363–393 days old ducks; Fig. 1). A small amount of variability was noted in the level of parasitic infection between household ducks from different zones, but the variance in trematodal and nematodal infections was not significant. The level of cestodal infection, however, did vary significantly between zones, with household ducks in Charishwar experiencing the highest frequency of cestodal infection ($p=0.002$; Fig. 2).

Attainment of sexual maturity and production of Jinding ducks

Overall, the median number of days required to attain sexual maturity of ducks per household was 190 on Hatia Island. By zone, the median number of days required to attain sexual maturity of ducks per household were 185 days (Tumurrudi-1), 205 days (Tumurrudi-2), 210 days (Charking), and 190 days (Charishwar; Table 4).

The overall median egg production rate per duck per household, for a 6-month laying period, was 93. The median egg production rate per duck per household was 110, 76, 83, and 77 in Tumurrudi-1, Tumurrudi-2, Charking, and Charishwar, respectively (Table 4).

Risk factor analysis

Univariable analysis

The average egg production rate per duck per household at two different levels (average ≤93 eggs versus >94 eggs)

Table 2 Frequency of duck mortality by study zones on Hatia Island (December 2002-February 2004)

Zone	Number of ducks	Cause of death		
		Disease (%)	Predation (%)	Total mortality (%)
Tumurrudi (1)	419	17.2	5.7	22.9
Tumurrudi (2)	345	20.0	10.4	30.4
Charking (3)	353	20.7	9.6	30.3
Charishwar (4)	256	21.9	14.8	36.7
Total	1373	19.7	9.6	29.3

Table 3 Types of worm species recorded on examination of pooled fecal samples of household ducks on Hatia Island (December 2002–February 2004)

Worm class	Genus and species
Trematode	*Prosthogonimus* spp., *Trichobilharzia* spp., *Echinostoma* spp.
Nematode	*Cyathostoma bronchialis*, *Amidostomum anseries*, *Heterakis gallinarum*, *Capillaria* spp. *and Echinuria* spp.
Cestode	*Hymenolepsis setigera*

varied significantly among zones, between types of duck houses, and according to the time it took to reach sexual maturity and the duration of providing snails to ducks during a 6-month laying period ($p \leq 0.001$; Table 5).

Final logistic model

No interaction was detected in the final model. The Hosmer–Lemeshow goodness-of-fit test showed that the model fitted the data ($\chi^2=16.22$, $p=0.133$). The ROC curve extended well into the upper left-hand corner and the area under the curve was 0.9 (Fig. 3). Both of these features indicated the logistic model had strong predictive ability.

The odds ratio for the average egg production of ducks per household (≤93 eggs versus >94 eggs) was 25.4 for ducks that were kept in traditional duck houses when compared to ducks that were kept in improved duck houses ($p<0.001$). The odds of average egg production rate per duck per household was 14.2 times more likely in ducks that attained sexual maturity later than in ducks that attained sexual maturity earlier ($p<0.001$). The odds ratio was 18.2 in ducks provided snails for a longer period of time compared to ducks provided snails for a shorter time, over 6 months of laying period ($p=0.002$; Table 5).

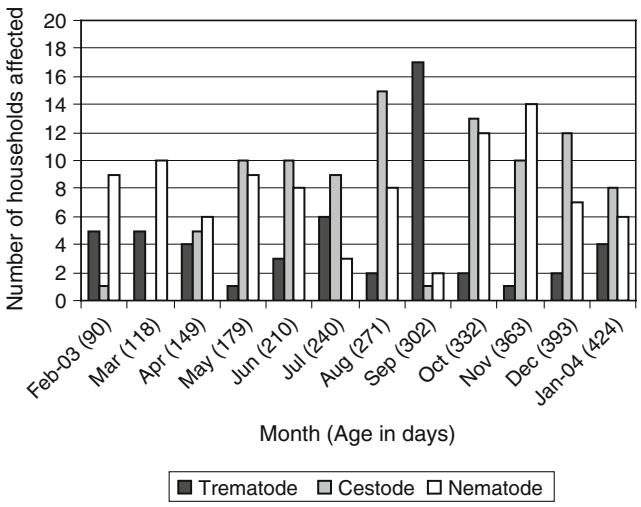

Fig. 1 Temporal pattern of parasitic infection in household ducks on Hatia Island (December 2002–February 2004)

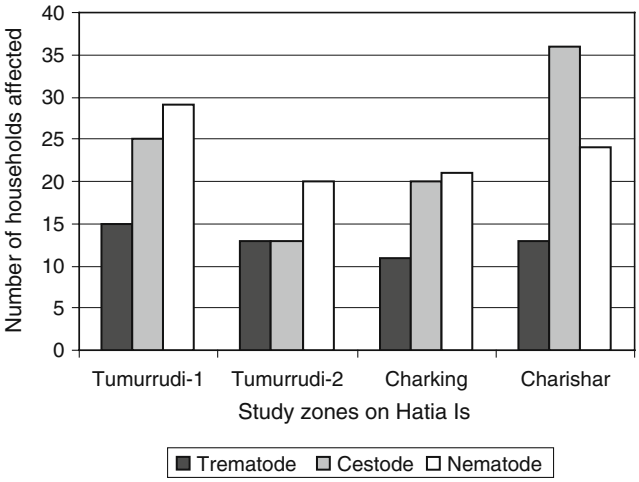

Fig. 2 Spatial pattern of parasitic infection in household ducks on Hatia Island (December 2002–February 2004)

Discussion

Duck mortalities due to diseases and predation

A high duck mortality rate was observed in this study, especially with respect to disease. This suggests that duck health management on Hatia Island is of poor quality. The mortality rate observed was similar to rates encountered in comparable studies in Bangladesh (Biswas et al. 2006; Hoque 2006; Hoque et al. 2006; Biswas et al. 2008; Hoque et al. 2010b) and India (Iqbal and Pampori 2008). In agreement with our study, their investigations found predation and disease to be significant contributors to household poultry mortality, both in Bangladesh (Biswas et al. 2006; Hoque 2006; Biswas et al. 2008; Hoque et al. 2010b) and elsewhere in the world (Iqbal and Pampori 2008; Gajendran and Karthickeyan 2009). Predation by baboons in Zimbabwe (Kusina et al. 2001), mongoose in Bostswana (Mushi et al. 2005), and kites, jackals, and mongooses in India (Iqbal and Pampori 2008) is also of concern for producers in these regions. Unfortunately, mongoose attacks cannot be easily prevented unless the ducks are monitored while scavenging for feed, which is a time-consuming process that carries a relatively high opportunity cost, compared with the apparent benefits. Ducks typically scavenge around the ponds and paddy fields found near many homesteads. Allowing ducks to scavenge presents a number of risks, including predation by the Bengal lizard, eagles, and kites. The latter two predators are capable of killing ducks of any age and size.

Globally, dogs and feral cats are common household poultry killers (Kusina et al. 2001; Stahl et al. 2002;

Table 4 Descriptive statistics for the onset of duck sexual maturity per household (by zone) and the rate of egg production of ducks per household (by zone) over a 6-month laying period (N=118 households; December 2002–February 2004)

Zone	Onset of sexual maturity of ducks per household (days)			Average egg production rate per duck per household over a 6-month laying period		
	Mean	Median	Range	Mean	Median	Range
Tumurrudi (1)	188	185	155–280	106	110	76–131
Tumurrudi (2)	212	205	155–272	80	76	47–131
Charking (3)	206	210	158–271	91	83	56–123
Charishwar (4)	212	210	150–276	77	77	34–123
Overall	203	190	150–280	90	93	34–131

Moberly et al. 2004; Mushi et al. 2005; Iqbal and Pampori 2008). Nocturnal predators such as civet cats and foxes can also gain entry to duck housing by digging under the walls at night. Both the traditional and improved duck houses used in this study were not well protected against this type of attack. Fortifying the housing would be expected to reduce the mortality caused by predation and should be a priority for farmers in the future. On Hatia Island, the types and population sizes of predators, as well as the frequency of attacks, vary according to geographical location and the habitat surrounding the affected households.

Duck diseases

Duck plague and duck cholera appeared to be common diseases and associated with significant mortality on Hatia Island. This corresponds well with previous studies that found those two diseases to be common and widespread across Southeast Asia (Das et al. 2005; Prasad and Singh 2008; Gajendran and Karthickeyan 2009). Duck cholera has also been reported previously in households in other coastal regions in Bangladesh (Hoque et al. 2006; Hoque 2006; Hoque et al. 2010b) and in the free-ranging ducks in Tanzania (Muhairwa et al. 2001). Additionally, the occurrence of aspergillosis in our study is in accordance with an earlier report in Bangladesh (Das et al. 2005).

Interestingly, administration of a booster dose of duck plague vaccine during the study failed to reduce duck mortality significantly. Possible explanations for vaccine failure include vaccination during sub-clinical infection, breaks in cold-chain transport and storage of the vaccine, and delayed booster injection.

Table 5 The association of selected variables and the rate of egg production in the χ^2 test and adjusted logistic model for duck household farms for a 6-month of laying period on Hatia Island (N=118; December 2002–February 2004)

Factors	Categories	Number of households	Univariate analysis (χ^2 test) % of egg production rate		Final logistic model		
			Category-1 (34–93 eggs)	Category-2 (94–131 eggs)	p	OR (95% CI)	p
Zone	Tumurrudi (1)	34	23.5	76.5	<0.001		
	Tumurrudi (2)	26	69.2	30.8			
	Charking (3)	35	62.9	37.1			
	Charishwar (4)	23	73.9	26.1			
Zone (Pooled)	Tumurrudi 1 and 2	60	43.3	56.7	0.009	1.0	
	Charking and Charishwar	58	67.2	32.8		1.9 (0.6, 6.6)	0.293
Duck house	Improved	62	25.8	74.2	<0.001	1.0	
	Traditional	56	87.5	12.5		25.4 (6.7, 96.2)	<0.001
Attainment of sexual maturity	Av. 150–190 days	60	28.3	71.7	<0.001	1.0	
	Av. 191–280 days	58	82.8	17.2		14.2 (4.1, 49.2)	<0.001
Nutrition (broken snail)	4–6 months	23	21.7	78.3	<0.001	1.0	
	0–3 months	95	63.2	36.8		18.2 (2.8, 119.4)	0.003

The p values (χ^2 test) are for unconditional significance tests for differences among categories within each variable

Av. average, *OR* odds ratio, *CI* confidence interval

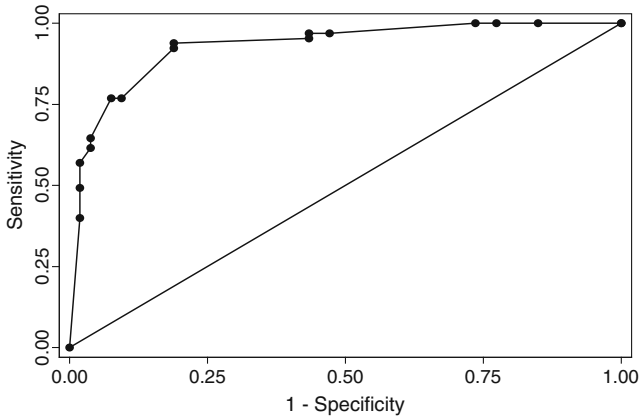

Fig. 3 Plot of sensitivity versus 1-specificity for a receiver operating characteristic curve (ROC) of various parameters of the logistic model of average egg production of ducks per household on Hatia Island. The *point of the curve* which is most towards the *upper left* of the figure indicates the maximum accuracy of the model

In the future, in order to better control diseases, it is important that farmers have access to affordable and robust diagnostic assays. Judicious selection of suitable antimicrobials with timely intervention may help controlling bacterial diseases. In addition, preventive husbandry practices and vaccination against prevalent duck diseases should be practiced regularly. Government and non-government agencies and industry should endeavor to provide adequate supplies of safe and effective vaccines and technical advice for their use.

Parasitic infections

Our longitudinal study on parasitic investigation identified a total of nine species of helminth and is the first record of duck helminths on Hatia Island in Bangladesh. Parasitic infections are a common problem for household ducks and may have indirectly contributed to duck mortality and lost productivity, in combination with duck plague and cholera. The parasite species observed in Jinding ducks in this study (Table 3) are similar to parasites identified in other studies of household ducks in Bangladesh (Baki and Mondal 1994) and Tanzania (Muhairwa et al. 2007), in household chickens in Zambia (Phiri et al. 2007) and India (Puttalakshmamma et al. 2008), and in wild birds in Spain (Cordon et al. 2009). The common endoparasitism observed in this study may be a result of continuous exposure of ducks to parasite vectors in soil such as insects and earth worms. These organisms serve as intermediate/paratenic/transport hosts for helminth parasites that are capable of infecting ducks (Pandey and Jiang 1992).

During our study, peak trematodal infection occurred in ducks during September 2003. It has been postulated that the rainy season is more suitable for snails, which are intermediate hosts for trematodes and a food source for ducks. The differences in the number of trematodal infections among age groups of ducks could be attributed to changes in the feeding patterns as ducks age resulting in a greater exposure to infective stages of the parasite. Age specific variation was also observed for cestodal and nematodal infections, possibly for the same reasons.

For cestodal infections, an epidemic pattern of infection was observed. In contrast, an endemic pattern of infections was observed for nematodal infections. The significantly higher level of cestodal infection observed in Charishwar could be explained by an abundance of intermediate hosts compared with other zones. Variations in the frequency of infection over time for both cestodes and nematodes could be attributed to the seasonal availability of intermediate hosts to ducks. Variation in host susceptibility is another possibility. The seasonal trend of increased cestodal and nematodal infection in household ducks during the rainy season suggests that a season-specific control strategy be implemented and its effects tested in future. Our observations suggest that treatment programs be conducted in the lead up to the rainy season and maintained through autumn for control of nematodes. Given the limitations of the diagnostic method used for detecting worm infections, it is probable that the magnitude of infections is greater than reported here. The use of a more sensitive diagnostic method in future would help clarify this issue.

Production potential of ducks and risk factors

The average number of days required for Jinding ducks to reach sexual maturity varied, possibly as a result of variable feed quality during different stages of development. The zone specific differences in the age of onset of sexual maturity could be due to differences in the types of foods available for ducks to scavenge on. Snails, duck weed, and slugs for example, provide additional protein and aid growth (Amber and Mia 2002). The optimum age of onset of sexual maturity found in this study is consistent with those reported by Das et al. (2008) in Bangladesh and Gajendran and Karthickeyan (2009) in India. Significantly, when compared with indigenous ducks raised at home, the median rate of egg production by household-raised Jinding ducks over a 6-month laying period is greater (Hoque et al. 2010a). This suggests that Jinding ducks have a greater potential to ensure the economic viability of the household-based approach to farming and should be preferred wherever the necessary infrastructure, resources, and training are available.

The risk factor analysis indentified improved nutrition and housing as important factors associated with increased egg production in Jinding ducks. It should be noted that the availability of snails depends on the presence of scavenging sources and the time of year. The risk factor analyses may not be demonstrating causation in this data set; however, a

plausible explanation for the outcomes seen in this study is that improved duck housing and nutrition lead to better health and as a result, an earlier onset of sexual maturity. Earlier sexual maturity was found to be associated with a greater rate of egg production ($p \leq 0.001$), and greater production was most pronounced when all three factors were present (improved housing and nutrition and early sexual maturity). These results are also consistent with findings from an earlier study that was conducted with indigenous ducks on Hatia Island (Hoque et al. 2010a). Given the predictive strength of the logistic model constructed, the age of onset of sexual maturity could be used as a reliable indicator of both the health and productivity of household flocks on Hatia Island in the future.

This study identified that the high rate of duck mortality due to disease and predation, as well as helminth infection, is a major constraint on the productivity of household Jinding ducks on Hatia Island. The study also found that use of improved duck houses and extended feeding of snails as a nutritional supplement during the laying period significantly improves egg production rates. It is recommended that when designing parasite control programs, seasonal trends in infection rates should be taken into account and that greater resources and technical advice be made available to farmers, especially for vaccination and disease-monitoring programs.

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ORIGINAL RESEARCH

Factors limiting the health of semi-scavenging ducks in Bangladesh

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Abstract Duck rearing is well suited to coastal and lowland areas in Bangladesh. It is an important component of sustainable livelihood strategies for poor rural communities as an additional source of household income. An epidemiological study was conducted during January 2005–June 2006 on 379 households in Chatkhil of the Noakhali District, Bangladesh which were using the recently devised "Bangladesh duck model". The overall objective of the study was to identify factors that significantly contributed to mortality and constrained productivity and to generate sufficient knowledge to enable establishment of a disease surveillance system for household ducks. The overall mortality was 15.0% in Chatkhil, with predation causing a significantly higher mortality compared with diseases ($p<0.001$). Common diseases were duck plague and duck cholera. Morbid ducks frequently displayed signs associated with diseases affecting the nervous and digestive systems. Haemorrhagic lesions in various organs and white multiple foci on the liver were frequently observed in dead ducks. Epidemiological analysis with a shared frailty model that accounted for clustering of data by farm was used to estimate the association between survival time and risk factors. The overall mortality rate due to disease was significantly lower in vaccinated than in non-vaccinated ducks in all zones except zone 2 ($p<0.001$). Only vaccinated ducks survived in zone 1. In conclusion, duck mortality and untimely sale of ducks appeared to be important constraints for household duck production in Chatkhil. Vaccination against duck plague appears to be an effective preventive strategy in reducing the level of associated duck mortality. A successful network was established amongst farmers and the surveillance team through which dead ducks, with accompanying information, were readily obtained for analysis. Therefore, there is an opportunity for establishing a long-term disease surveillance programme for rural ducks in Chatkhil of the Noakhali District of Bangladesh.

Keywords Duck (*Anas platyrhynchus*) · Mortality · Survival · Risk factor · Surveillance · Bangladesh

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Introduction

Household poultry production in Bangladesh occurs all over the country. It is a low input–low output profitable system and forms an integral part of poor rural communities in Bangladesh, a country with the third largest world population of ducks (38.1 million; Dolberg 2008). Duck

rearing is well suited to the southern coastal and lowland areas of the country. Rural duck rearing facilities are owned and managed by women and children and are an important means of reducing poverty for this demographic in resource-poor low-income areas (Pym et al. 2002). The predominate limiting factors in poultry production are mortality, untimely sale (such as before laying eggs) and family consumption of ducks (Hoque and Rahman 2004; Biswas et al. 2005, 2006, 2008; Hoque 2006; Hoque et al. 2006, 2010b).

Over the past two decades, a production system known as the "Bangladesh Poultry Model" has led to a very successful collaboration amongst private enterprises, government agencies and non-governmental organizations (Permin 2003). A main goal of the model is to increase women's social standing by engaging them in profitable poultry rearing. This model is financially supported by the Danish International Development Agency. The success of the poultry model has encouraged the government and international aid agencies to trial and establishes a similar model for ducks. Consequently, in 2002, the government of Bangladesh with the support of the Smallholder Livestock Development Project-2 (SLDP-2) developed a duck model for the water belt areas in five southern-central districts of Bangladesh (Amber and Mia 2002). The model was simplified in 2004 for Chatkhil (the study area) and included only two instead of the initial five to six components. Hence, some people in rural communities acted as duck rearers and others as duck vaccinators (Sarkar et al. 2005).

Like many other developing countries, the animal health information system in Bangladesh is poorly developed, especially in relation to duck rearing. The factors associated with loss of potential productivity such as whether economically important diseases occur in ducks remain unidentified. However, likely constraints in optimizing the production system include poor infrastructure and a shortage of skilled workers and resources. The existing data collection systems are poorly organized, making collection, collation, management, analysis and dissemination of information difficult. This severely limits the development of effective disease surveillance and intervention policies. Therefore, an epidemiological project was undertaken in 2005–2006. The overall objectives of the study were to identify mortality problems and other factors that constrain productivity and to generate sufficient knowledge to enable establishment of a disease surveillance system for household ducks. We also hoped to provide recommendations to improve the production and health status of ducks. This study will likely enable future comparative studies to be done in other areas and will add to our understanding of duck health management globally, which is important in managing emerging and re-emerging diseases.

Materials and methods

Study area

Chatkhil is one of the sub-districts known as an "Upazilla" of the Noakhali District in Bangladesh. The duck model implementation organizations were the local NGO Community Development Centre (CODEC) and the SLDP-2. The study area was composed of four zones: (1) Mohammadpur and Pachgoan, (2) Porcoat and Hatpukuria, (3) Badal Coat and (4) Nowakhula. Division of the Upazilla into different zones was necessary for ease of development and operation of the duck model. The distance between zones was around 10–15 km by road.

Sampling and study population

The CODEC primarily selected 400 households (designated as duck rearers) for inclusion into the duck model programme from four zones, 100 households each. Households were chosen based on the following criteria: at least 1 year of experience with duck rearing and successful completion of a training programme focused on improved methods of traditional duck rearing. All participants were required to adhere to the operating rules of the model as prescribed by CODEC. As part of the programme, CODEC provided day-old Jinding ducklings to each household from the Naryanganj government breeding farm, at a fixed price. Each household received 5–20-day-old Jinding ducks. The selected households were entered into the programme on one of nine possible entry dates between November 2004 and December 2005. There were two factors for the variable entry dates: shortage of day-old birds supplied from the duck breeding farms and unavailability of necessary funds for CODEC to include all households at one point in time into the model programme. This study began in January 2005 and set selection criterion of having ≥4 Jinding ducks along with or without indigenous ducks per household. As a result, we had 379 households, some of which already had ducks whilst others received their ducks during the study period. Therefore, data for a household varied from as little as 7 months to as much as 18 months between January 2005 and June 2006. Age of Jinding ducks when recruited into the study ranged from 1 to 187 days and age of indigenous ducks ranged from 1 to 900 days. In zone 1, 98 households participated in the study (1,426 ducks), in zone 2 there were 85 households (1,080 ducks), zone 3 had 96 households (1,421 ducks) and in zone 4 100 households were recruited (1,274 ducks). However, for the purpose of this paper, we restricted the analysis to 238 households that were monitored for a period of 12 months.

Vaccination

A vaccination programme against duck plague was implemented with vaccine supplied by the government at the defined price and vaccine administered by four trained vaccinators. One vaccinator was assigned per zone. An initial dose of duck plague vaccine was to be injected between the 15th and 30th days of life, followed by a second dose within 2 weeks of the first inoculation. However, irregular vaccine supply resulted in deviation from this regime.

Data and dead duck collection

A record-keeping sheet was designed with open and semi-closed questions as per targeted objectives, with space available to record alternatives to the options given. We refined the record-keeping sheet through formal and informal testing. One trained field investigator was dedicated to each zone. Investigators obtained the required information through farmer interviews or review of records that were maintained by the farmers (listed below). This information was collected on a twice-weekly basis. Farmers used a notebook designed by CODEC to record the necessary information pertaining to their farms. Farmers were also trained by CODEC on how to recognize clinical signs and record them in the notebook. The farmers cooperated with the investigation as a part of their agreement to the credit supplier and the programme developer. The activities of the field workers were supervised by the local veterinarian working for the department of livestock services and the area manager of SLDP-2.

Zones, household enrolment date and initial flock size along with breed type and age were registered at the beginning of the study. The number of ducks, their corresponding age, breeds type and vaccination history and the amount of duck loss per household were recorded over the study period. The following reasons were recorded for duck loss: death (possibly due to disease or predation), stolen, malicious or accidental loss, sudden disappearance, sale and family consumption.

During the scheduled visit to the households, the field investigators collected dead ducks and temporarily stored them in an ice-packed cool box. Investigators asked farmers for clinical signs of the dead ducks. Farmers were paid Bangladesh Taka 20 (approximately US $0.40) for each dead duck (set at less than the market price for healthy ducks). Farmers were also provided vitamin supplements and antimicrobial drugs for their remaining ducks to treat possible stress and concurrent bacterial infection if a disease problem appeared. These strategies, to some extent, helped prevent further mortality, and they encouraged farmers to participate more fully in the study.

Postmortem, collection of tissue samples and preparation of slides

Each duck was necropsied by a trained veterinarian at the temporary field postmortem room, whereupon internal examination of the carcasses was carried out. Any characteristic changes that were observed were recorded.

Samples from the liver, heart and kidney were collected, whether visible lesions were detected or not. Further samples were collected from other tissues with gross lesions. One pooled (approximating equal weight of each tissue) and individual tissue samples from each carcass were taken for virological and bacteriological examinations. These samples were then placed in vials containing 1% phosphate-buffered saline. Impression smears were immediately made from the liver and heart during postmortem for microscopic examination in order to diagnose duck cholera. A unique identity number was given for each carcass. We also recorded sampling date, sample name, zone, age and sex for each carcass. Samples obtained were forwarded in a cool box packed with ice to the laboratory at the Chittagong Veterinary and Animal Sciences University within 6–8 h for laboratory testing. Samples for bacteriology were kept temporarily at 4°C and those for virology at 70°C in the laboratory until processing was possible. All data were entered into a computer database.

Diagnosis of diseases

Duck viral enteritis

Pooled tissue samples obtained from an individual carcass were cultured in three embryonated 10-day-old duck eggs (specific pathogen free) by injecting a 20% tissue sample suspension in 0.2 ml amounts for each chorioallantoic membrane (CAM). Eggs were incubated at 37°C and embryo mortalities were monitored for 6 days. Eggs with dead embryos after 24 h were stored at 4°C in a fridge. In the first passage of eggs, if at least two of three eggs died and at least one dead embryo showed typical lesions (thickened CAM, extensive petechial haemorrhages of head, neck, wing or legs; Tu et al. 2003), the virus isolation was considered as positive. The specific antiserum used could differentiate between antigens from duck viral hepatitis and duck viral enteritis. As duck viral hepatitis is primarily a disease of ducks less than 2 weeks old and duck viral enteritis is a disease of both young and adult ducks (Milton and Christian 1999), then ducks which died by 2 weeks of age and produced lesions in duck embryos by inoculation of pooled tissues, these ducks had evidence of viral disease, most likely had duck hepatitis.

Bacterial diseases

Duck cholera (*Pasteurella multocida*) was identified by the presence of characteristic bipolar organisms under microscopic examination of Giemsa stained liver or heart imprints made in the field (Richard and Glisson 1997).

Colibacillosis (*Escherichia coli*) was identified through isolation of the causative organism on MacConkey agar followed by eosin methylene blue agar and typical biochemical characteristics shown in a triple sugar iron slant. Isolates suspected to be *E. coli* were confirmed by the positive ring of the indole test (Wray and Woodward 1994).

Salmonellosis (*Salmonella* spp.) was diagnosed by isolating the organism on Salmonella–Shigella agar and typical biochemical characteristics shown in triple sugar iron agar slants. Test positives were further assessed by the maltose fermentation test. Samples were first cultivated in mannitol selenite cysteine broth before using the selective media.

Censor

Ducks that exited the study were defined as censored for the survival analysis. Reasons for ducks exiting the study included study completion, sale and slaughter, predation, sudden unexplained disappearance, as well as malicious and accidental deaths caused by neighbours.

Statistical evaluation

Data obtained were entered into a spreadsheet created in Microsoft Excel-2003 and then exported into STATA®, version 11 (StataCorp 2010) for cleaning and epidemiological analysis. A descriptive analysis was performed on the data of duck loss, clinicopathological syndromes and duck diseases. The results were expressed as frequencies and percentages.

The incidence rates (rate per 1,000 duck-days) and survival times (days) between the different categories of predictor variables were calculated. Variables were established as zone (1/2/3/4), breed (Jinding/indigenous), entry age class of ducks (1–2/>2 days) and day of vaccination for duck plague (no vaccine/≤20/21–30/≥31).

Univariate and multivariate shared frailty survival analyses were used to assess associations between the selected predictors and the survival time and to construct the final model. Predictor variables were selected for inclusion in the model when univariate associations were significant ($P \geq 0.1$). Selection of the most parsimonious multivariable frailty model was performed by backwards stepwise elimination of non-significant ($P > 0.05$, two-tailed) factors. A forward-selection procedure was also considered and results compared to those of the backwards elimination approach. The confounding and interaction variables were investigated in the model as described previously (Hoque et al. 2010a, b).

The best model to explain the data included the farm identification as the frailty effect and zone, vaccination and the interaction between them as fixed effects. Breed was confounded with the farm identification. The model was checked by Schoenfeld residuals (Grambsch and Therneau 1994) for the proportional hazards assumption. The global test p value of 0.149 indicated a reasonable fit of the data into the model. The adjusted predicted means and contrasts were applied to investigate the interaction between zone and vaccination. The results were presented as the mean Cox regression coefficient and 95% confidence interval.

Results

Descriptive analyses of duck loss (N=3,237)

The overall duck mortality was 15.0% in Chatkhil, of which disease accounted for 5.9%, predation 8.3% and malicious or accidently killed 0.8%. Importantly, predation caused a significantly higher level of duck mortality than diseases ($p<0.001$). Stealing and sudden disappearance accounted for 1.8% and 0.3% of duck loss, respectively. Around 60.0% and 9.0% of ducks were sold out and slaughtered for family consumption, respectively (Table 1).

Clinicopathological syndromes of ducks

According to the farmers' responses, 94 and 98 ducks died without and with clinical signs, respectively. Clinical signs were most frequently associated with diseases affecting the nervous and digestive systems (Table 2). A wide range of pathological changes observed in 119 ducks on autopsies (N=192). However, no pathological changes were observed in various tissues of 73 ducks. Of the 15 categories of lesions, pin point and or haemorrhagic lesions in various organs had the highest frequency (166) followed by white multiple foci on the surface of the liver (50), a flabby heart (23) and a fragile liver and kidney (22) and then other categories (1–11; Table 3).

Duck diseases

Duck plague (*Alphaherpes virus*) and concurrent occurrence of both duck plague and duck cholera (*P. multocida*) accounted for the highest frequency of duck diseases at around 32% for each. Duck cholera only was 21% and duck viral hepatitis 1.6%. Concurrent colibacillosis (*E. coli*) and salmonellosis (*Salmonella* spp.) accounted for 5.2%,

Table 1 Causes of mortality, loss, sale and family consumption of rural ducks in Chatkhil (2005–2006)

Zone	*N*	*n*	Cause of mortality of ducks			Causes of loss of ducks		Sale and family consumption of ducks	
			Disease	Predation	Malicious killed	Stolen	Disappeared (no trace)	Sold	Slaughtered
1	49	713	55	72	0	40	1	336	86
2	62	788	44	96	1	1	7	404	94
3	57	844	45	69	20	9	0	536	94
4	70	892	48	32	4	9	0	680	21
Total	238	3,237	192	269	25	59	8	1,956	295
%			5.9	8.3	0.8	1.8	0.3	60.4	9.1

N number of households, *n* number of ducks

whereas only colibacillosis was 1.6%. Results for around 3.6% ducks were inconclusive (Table 4).

Univariate analyses of mortality and survival time of ducks

Zone 3 had the highest mortality rate, only 25% of ducks lived more than 99 days. Ducks in zone 4 lived longer than in other zones with 50% of ducks living more than 123 days. There was a higher mortality rate in Jinding ducks than in indigenous ducks. Fifty percent of Jinding ducks died before 81 days in contrast to 75% of indigenous ducks that survived more than 80 days. There were some apparent differences in mortality rates and survival times between the two study entry ages of ducks. Ducks at entry age of >2 days had a lower mortality rate, with 75% of ducks living more than 230 days, whereas 75% ducks at entry age of 1 to 2 days did not survive more than 181 days (Table 5).

The mortality for the categories of vaccination against duck plague was similar. However, the survival times were different. Fifty percent of the non-vaccinated ducks died before 20 days. Seventy-five percent of the vaccinated ducks (at 21–30 days) survived more than 86 days compared to 50% of the vaccinated ducks (at ≤20 days) which survived more than 90 days. Seventy-five percent of the vaccinated ducks (at >31 days) survived more than 104 days compared to only 50% of the vaccinated ducks (at 21–30 days) which survived more than 102 days (Table 5).

Frailty model

We found that there were significant differences of mean coefficients between the ducks vaccinated at ≤20 days (−2.3 and −2.9) and non-vaccinated ducks (−0.6 and −0.1) in both zone 3 ($p<0.001$) and zone 4, respectively ($p<0.001$), but there were no significant differences of mean coefficients between the ducks vaccinated at ≤20 days (−2.8) and non-vaccinated ducks (−1.5) in zone 2 ($p=0.062$). Only vaccinated ducks survived in zone 1 (Table 6).

Table 2 Clinical signs observed in rural morbid ducks in Chatkhil (according to the farmers' responses; 2005–2006)

Signs associated with body systems	Types of clinical signs	Frequency number
Nervous	Unable to walk or walk with sternum or lameness	29
	Dull and depressed	24
	Shivering or convulsing or circling around	9
	Unable to hold up the head with beak touching the ground or bend neck	7
Digestive	Watery faeces (green or white or yellow)	43
	Off feed	15
	Sore on the tongue	2
	Thirsty	2
Respiratory	Nasal discharge	5
	Drowsiness and dyspnoea	3
Reproductive	Prolapse of the penis	3
	Drop in egg production	1
Integumentary	Ruffled feathers	2

Table 3 Pathological changes observed in different organs of duck carcasses at postmortem in Chatkhil (2005–2006)

Type of lesions	Name of organs	Frequency number
Haemorrhage	Trachea or vocal cord or lung or heart or atrium or small intestine or proventriculus or kidney	111
Pin point haemorrhage	Heart or liver or kidney	55
White multiple foci	Liver	50
Flabby	Heart	23
Fragile	Liver or kidney	22
Pale	Liver or kidney	11
Black colouration	Lung or heart or liver or kidney	11
Copper colour	Liver	10
Congestion	Trachea or lung or liver or kidney	9
Whitish membrane covered the organ	Heart/liver	9
Dehydration and emaciation of carcass		6
Clotted blood	Atrium or ventricle or heart	4
Enlargement/asymmetric organs	Liver or heart	3
Necrotic foci	Liver	2
Red colouration	Liver	1

We only found differences of mean coefficients in ducks between the ducks vaccinated at ≤20 days (−2.4) and the ducks vaccinated at 21–30 days (−1.2; $p=0.04$) and between the ducks vaccinated at 21–30 days (−1.2) and ducks vaccinated at ≥31 days (−2.5; $p=0.024$) in zone 3. There were no differences of mean coefficients in zone 4 for the same comparisons (Table 6).

Discussion

Discussion on descriptive results

Overall duck mortality rate was high in this study, which indicated that duck health management in Chatkhil is poor. The mortality rate observed was similar to rates encountered in comparable studies in Bangladesh (Biswas et al. 2006, 2008; Hoque 2006; Hoque et al. 2006, 2010b) and India (Iqbal and Pampori 2008). In agreement with our study, their investigations found predation and clinical diseases to be significant contributors to household poultry mortality both in Bangladesh (Biswas et al. 2006, 2008; Hoque 2006) and elsewhere in the world (Iqbal and Pampori 2008; Gajendran and Karthickeyan 2009).

Table 4 Common duck diseases amongst 192 dead ducks in Chatkhil (2005–06)

Name of diseases	Frequency number	Percent
Duck plague	61	31.8
Duck plague and duck cholera	60	31.3
Duck cholera	41	21.4
Colibacillosis and salmonellosis	10	5.2
Colibacillosis	10	5.2
Duck viral hepatitis	3	1.6
Inconclusive	7	3.6

Globally, dogs and feral cats are common household poultry killers. Predation by baboons in Zimbabwe (Kusina et al. 2001), mongooses in Botswana (Mushi et al. 2005) and kites, jackals and mongooses in India (Iqbal and Pampori 2008) is also of concern for rural poultry farmers in these regions. According to the farmers' survey responses, the predominant predators were the civet cat *Viverricula indica*, fox *Vulpes bengalensis*, crab-eating mongoose *Herpestes urva*, Bengal lizard *Varanus bengalensis* and indigenous dog. These findings correspond to our earlier study on Hatia Island (Hoque et al. 2010b). Unfortunately, mongoose attacks cannot be easily prevented unless the ducks are monitored whilst scavenging for feed, which is a time-consuming process that carries a relatively high cost, compared with the immediate benefits.

Nocturnal predators such as civet cats and foxes can also potentially gain entry to duck housing by digging under the walls at night. Both types of duck houses (traditional and improved) used in this study were not well protected against this type of attack. Rectifying the housing would be expected to reduce the mortality caused by predation and should be a priority for farmers in the future. In Chatkhil, the types and population sizes of predators, as well as the frequency of attacks, vary according to the geographical

Table 5 Mortality rates due to disease and survival times of ducks in Chatkhil (N=3,237; 2005–2006)

Predictor	Category	Total ducks	Total duck days at risk	Total recorded death	Mortality rate per 1,000 ducks-days	Survival time (days)		
						25%	50%	75%
Zone	1	713	102,778	55	5.7	51	96	202
	2	788	116,956	44	5.5	53	94	219
	3	844	78,183	45	9.9	21	47	99
	4	892	141,485	48	5.6	39	123	258
Duck breed	Jinding	2,732	351,220	166	6.9	39	81	173
	Indigenous	505	88,182	26	4.5	80	180	271
Entry age (days)	1–2	1,882	236,449	107	7.0	41	82	181
	>2 days	1,355	202,953	85	5.7	64	112	231
Days at duck plague vaccine (days)	No vaccination	796	113,067	37	5.8	6	20	150
	≤20	2,001	272,862	114	6.4	46	90	205
	21–30	280	30,195	18	8.7	286	102	172
	≥31	160	23,278	23	5.7	104	134	290

location and the habitat surrounding the affected households. Given the limitations of witness reports, it is probable that the data collected is not a comprehensive account of all of the possible livestock predators (such as eagles and kites); however, it likely includes some of the most common poultry predators. For this reason, predator-specific mortality rates could not be determined. However, given the monetary constraints placed upon studies in developing countries, this data collection method remains both useful and cost-effective. This is evidenced by similar studies looking at predation of household poultry in the regions (Hoque and Rahman 2004; Hoque et al. 2010b; Biswas et al. 2006, 2008).

Stealing and either maliciously or accidental killing of ducks were not found to be as serious a cause of duck loss as predation and diseases; however, these factors remain a concern. People at the poorest level in rural society might be involved with stealing because economic crises are a constant. Further, if ducks pollute a neighbour's yard by defecating and destroying crop fields, neighbours could kill the ducks accidently or maliciously (by poison). However, poisoning was not considered in this study. Farmers experienced situations in which ducks sometimes did not return home from a distant field after scavenging. Farmers thought this could have happened due to predation, stealing or even sudden death due to disease or snake bite.

The results of premature sale and family consumption of ducks in this study are similar for rural chickens (Biswas et al. 2006, 2008). Under the new duck production model, they had prior training on how to improve traditional duck rearing practices, but were impeded from fully implementing these strategies due to economic constraints. Ducks were sold at inopportune times (just not the time when the maximum financial gain could potentially have been made) in order to raise enough money to pay for urgently needed food, family clothing, medical treatment and medicines and to pay monthly loan instalments to credit suppliers. Jinding ducks had a good market value because of their higher laying capability as compared with indigenous ducks (Hoque et al. 2010a) possibly encouraging farmers to sell them. Disease outbreak could be another reason for the sale of ducks in order to recoup some of their expected economic losses, and rural farmers do not like to allow their sick ducks to die slowly. They would rather sell them at minimum prices to their neighbours or slaughter the

Table 6 The adjusted means of the Cox regression coefficients for ducks in Chatkhil (2005–2006)

Day of vaccination	Mean coefficients (95% confidence interval)			
	Zone 1	Zone 2	Zone 3	Zone 4
No vaccine		−1.48 (−2.8, −0.2)	−0.6 (−1.8, 0.7)	−0.1 (−1.4, 1.3)
≤20	−2.5 (−3.7, −1.4)	−2.3 (−3.4,−1.1)	−2.4 (−3.6, −1.1)	−2.9 (−4.1, −1.7)
21–30			−1.2 (−2.5, 0.1)	−3.5 (−4.9, −2.1)
≥31	−2.2 (−3.5, −0.8)		−2.5 (−4.1, −1.0)	−3.4 (−5.0, −1.8)

ducks for family consumption even though there is a risk of zoonosis from infectious diseases like salmonellosis. Also farmers usually consumed duck meat at different festivals (religious and non-religious) and shared the meat with their relatives. In addition, there is a scarcity of scavenging feed for ducks during the dry season which might have forced farmers to sell their ducks.

In summary, farmers need greater income security and stability in order to enable long-term planning of duck sales and more duck health care knowledge to prevent the untimely selling of ducks due to outbreak of disease. To obtain maximum benefits out of duck rearing, farmers must stop the unplanned sale and slaughter of ducks.

Signs and pathological changes recorded in this study are useful in the presumptive diagnosis of duck diseases where laboratory diagnosis is not readily available (Hoque et al. 2010b). Several studies have reported similar clinical signs and lesions when making diagnoses such as duck plague (Campagnolo et al. 2001; Hien et al. 2004; OIE 2008a), duck viral hepatitis (OIE 2008b), duck cholera (Islam et al. 2003; Rahman et al. 2004; Mbuthia et al. 2008), salmonellosis and colibacillosis (Rahman et al. 2004). More sensitive and specific diagnostic techniques are available; however, they are too expensive for rural farmers in Bangladesh. Hence, we employed pragmatic solutions to enable at least some quantification of losses due to major diseases. We were able to confirm many presumptive diagnoses in the laboratory.

Duck plague and cholera appear to be common diseases associated with mortality in Chatkhil. This corresponds well with previous studies that found those two diseases to be common and widespread across Southeast Asia (Hossain et al. 2004; Chellapandian et al. 2005; Das et al. 2005; Hoque et al. 2006; Prasad and Singh 2008; Gajendran and Karthickeyan 2009; Hoque et al. 2010a, b). Duck viral hepatitis has also been reported in the rural ducks of Bangladesh (Hoque et al. 2006, 2010a). As there is no vaccination practiced against duck cholera and duck viral hepatitis, these diseases are likely to occur. Salmonellosis and colibacillosis have been reported previously in growing ducks in Bangladesh (Hoque et al. 2006). Further, colibacillosis, salmonellosis and fowl cholera are common endemic diseases for both chickens and ducks, and some bacterial diseases may be transmitted between chickens and ducks because farmers prefer mixed rearing where close contact, droppings, contaminated feed and water may be potential ways of exchanging diseases (Biswas et al. 2006).

It is important in the future that cheap and robust diagnostic methods are available for all farmers. Judicious selection of suitable antimicrobials with timely intervention may control bacterial diseases. In addition to preventive husbandry practices, vaccination against prevalent duck diseases should be practiced on a regular basis. Government and non-government agencies and industry should endeavour to provide adequate supplies of safe and effective vaccines and technical advice for their use.

Discussion on the results obtained from the frailty model

The survival of ducks was variable amongst zones. In general, the first dose of vaccine reduced the mortality rate of ducks in all zones except zone 2. This is possibly due to the success of maintaining the vaccine cold chain and vaccinating healthy ducks. Vaccination did not work in zone 2 possibly due to failure of maintaining vaccine cold chain. It also appeared that vaccination of ducks between ≤20 and 30 days reduced the mortality rate in zones 3 and 4, perhaps due to the adherence to the recommended schedule of vaccination and vaccinating healthy ducks. A similar vaccine effect on the mortality of ducks was evident on Hatia Island in Bangladesh (Hoque and Rahman 2004).

Sub-clinical infections already present prior to vaccination may also account for the lower than expected reduction in vaccinated duck mortality. The survival rate of ducks was zero without vaccination at zone 1 and was perhaps due to poor farm management practices in addition to a communication gap between farmers and the village vaccinator in regards to the need for vaccination.

Overall, vaccination against duck plague reduced the level of mortality in this study; however, this could be improved. Therefore, we recommend conducting a field vaccine trial for the predominant duck disease (duck plague) in order to devise an appropriate vaccine schedule.

A new epidemiology unit has recently been established within the Bangladesh Government livestock services, and authority has been granted to begin recruiting relevant personnel, equipment etc. for collecting, collating, analysing and producing reports and finally disseminating results. The experience gained in this study may inform Government policy and implementation of a high-quality health surveillance system for livestock in Bangladesh.

Conclusion

Our study identified that the high rate of duck mortality due to disease and predation is a major constraint for household duck production. The study also demonstrated that duck plague vaccine (given ≤20 days) may be effective in reducing mortality rates of ducks.

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A descriptive study of the health of ducklings on smallholdings, Hatia Island, Bangladesh

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Monitoring the Health of Wild birds in North Queensland of Australia

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Avian Influenza (AI) is a viral disease that primarily affects birds and is caused by influenza A viruses belonging to the family Orthomyxoviridae. AI is endemic in aquatic birds worldwide. Migratory birds could introduce highly pathogenic avian influenza into Australia as north Queensland is on an important flight path. A three-year epidemiological study has been carried out in north Queensland since 2007 to monitor AI in wild aquatic birds. The study was predominantly conducted at two sites close to Townsville and concentrated on plumed whistling ducks, *Dendrocygna eytoni*; Pacific black ducks, *Anas superciliosa;* and magpie geese, *Anseranus semipalmata*. Sporadic cross sectional studies have also been carried out at Cape York and the Atherton Tableland. Tracheal and cloacal swabs as well as serum samples were collected from each bird. Serum samples from recaptured birds were tested in an attempt to detect sero-conversion. Concurrently, general causes of mortality are also being investigated as well as the epidemiology of Newcastle disease (ND) as a beneficial spinoff. Serum samples were screened for AI using a competitive enzyme-linked immunosorbent assay (c-ELISA). Samples that reacted in the c-ELISA were then tested using a panel of haemagglutinins (11) in an attempt to determine the distribution of serotypes infecting birds in North Queensland. Swabs were screened using reverse transcriptase polymerase chain reaction (RT-PCR) and potential reactors were further sequenced for AI and ND typing.

From April 2007 until September 2009, a total of 2,095 samples (1,237 live birds; 35 dead birds; 15 euthanised sick birds; and 808 environmental faeces samples) have been obtained. Of the first 1,506 tested, approximately 1-2% samples reacted in the screening assays for AI and ND. H6 and H9 sequence types were identified from the reactor swabs in plumed whistling and Pacific black ducks, respectively. Around 10% of serum samples reacted in the c-ELISA. Adult birds, Pacific black ducks and warm-wet weather (January-April) were identified as potential risk factors for increased AI sero-prevalence. A minority of samples reacted with only one serotype. Multiple reactions were common with the predominant serotypes being H4, H5, H9 and H11. These results suggest that AI commonly infects aquatic birds in North Queensland and that a variety of serotypes infect these populations. Both increases and decreases in titres were observed in samples from recaptured birds. AI antibody lasted [3] 2 months maintaining a high level of titre of % inhibition (66-85%) observed in samples from three recaptured Pacific black ducks. *Salmonella* sp. was isolated from three dead bird samples (one Australian white ibis, *Tadorna tadornoides;* and two plumed whistling ducks).

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Monitoring of wild birds for Newcastle disease virus in north Queensland, Australia

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Abstract

Wild aquatic birds (WABs) are considered as reservoir hosts for Newcastle disease viruses (NDVs) and may act as vectors for transferring these viruses to poultry, causing outbreaks of disease. A three-year epidemiological study was conducted on WABs of north Queensland from April 2007 to March 2010. Swab and fresh moist faecal samples of WABs were screened to detect Newcastle disease viral (NDV) RNA by one-step real time reverse transcriptase polymerase chain reaction (rRT-PCR) in multiplex primers, targeting the matrix gene. The potential reactor samples in rRT-PCR were processed for sequencing of the different NDV genes using conventional PCR. The overall NDV RNA prevalence was 3.5% for live bird samples (N=1,461) and 0.4% for faecal samples (N=1,157). Plumed whistling ducks (PWDs) had a higher prevalence (4.2%) than Pacific black ducks (PBDs) (0.9%) (χ^2 test, p=0.001). Univariate and multivariate logistic regression analyses were used to estimate the association between the proportion of reactor and non-reactor NDV RNA samples of PWDs and potential risk factors. The odds of reactor samples were 2.7 (95% Confidence interval 1.5-4.9) times more likely in younger than older ducks (p=0.001) (data set B, multivariate analysis). Both NDV RNA class-one and class-two types were identified in samples of WABs (12 and 59, respectively) (Supplementary Table 1). Phylogenetic analysis of the matrix gene identified two reactor sequences of class-one type NDV RNA (PWD-48 and 55) which were closely related to the sequences of Australian Ibis and duck isolates (Figure 2). Another reactor sample sequence was determined as class-two type NDV RNA (PWD-46, avirulent) based on analysis of the matrix and fusion genes which was more similar to the sequences of Australian I-2 progenitor virus and vaccine strain virus (Figure 3 and 4). Our findings of higher prevalence in PWDs along with confirmation of class-one and class-two type NDV RNAs will significantly contribute to the design of surveillance programs for NDVs in northern Australia.

Key words: Wild aquatic birds; Newcastle disease virus; Monitoring; North Queensland of Australia

1. Introduction

Wild aquatic birds are recognized reservoirs of NDVs belonging to the family of *Paramyxoviridae* and may act as a vehicle for transference to poultry, eventually causing outbreaks of disease (Alexander, 1995; Stanislawek et al., 2002). Virulent NDVs can cause high mortality in wild birds such as juvenile double crested cormorants (*Phalacrocorax auritus*) in North America (Glaser et al., 1999), Western Canada (Wobeser et al., 1993) and teal (*Anas crecca*) in Iran (Bozorgmehri-Fard and Keyvanfar, 1979).

Two classes of NDVs are recognised worldwide. The class-one type viruses are predominantly isolated from wildlife and nine genotypes have been described (Wu et al., 2011). The class-two type viruses have been responsible for most of the outbreaks of Newcastle disease (ND) in domestic poultry world-wide and the genetic lineages have been referred to as 1 to 5e (Aldous et al., 2003; Aldous et al., 2010) or I to VII (Lomniczi et al., 1998).

Australia has previously experienced ND outbreaks due to virulent NDVs in poultry between 1930 and 2002 (Johnstone, 1933; Albiston and Gorrie, 1942; Westbury, 2001). With the exception of the NDVs isolated from an outbreak in the 1930s all Australian NDVs isolated from chickens were defined as class-two genotype-one viruses (Figure 4). The source of viruses was presumed to be originated from wild birds because avirulent class-two NDVs have been commonly found in them. The class-two genotype-one viruses include V4 like viruses (Australia) and XZ-32-07 (China) and Ulster 67 (Simmons, 1967; Westbury, 1979; Spradbrow et al., 1995; Alexander, 2001a; Peroulis and O'Riley, 2004; Czegledi et al., 2006; Kattenbelt et al., 2006a; Kattenbelt et al., 2006b; Kim et al., 2007a; Mia Kim et al., 2008; Aldous et al., 2010; Wu et al., 2011).

It has been suggested that avirulent class two-type NDVs that spill-over from wild birds into poultry continue to evolve as they are rapidly passed in chickens (Hinshaw et al., 1980b). They acquire a furin cleavage site in the fusion (F) gene resulting in an increase in virulence (Gould et al., 2001). In contrast, only one published example of class one-type NDVs from WABs mutating to a virulent form in chickens in Northern Ireland has been documented (Alexander, 1995).

Comprehensive studies on the epidemiology of NDVs and determination of risk factors associated with the prevalence in Australian wild birds have not been attempted. Some opportunistic studies have documented the serological and virological prevalence of NDVs in Australia such as 13% sero-prevalence in grey teal (Garnett and Flanagan, 1989) and 0.4% NDV prevalence in mixed species of wild birds (Mackenzie et al., 1985).

Therefore, the monitoring of NDVs was included in a three-year longitudinal study on WABs in nQLD beginning April 2007 in order to assess the burden of disease, identify potential factors associated with NDV RNA prevalence, determine the distribution of NDV subtypes by genetic analysis and then their molecular epidemiology, and identify potential threats from NDVs to domestic poultry in the region.

2. Materials and Methods

2.1. Study sites and sampling

Epidemiological studies were conducted on WABs from the wetlands of four different study sites of nQLD (Figure1). Sites were chosen based on their proximity to migratory routes, ease of access, the presence of resident WABs and generally a large bird population. A three-year longitudinal study was performed on WABs at Billabong Sanctuary, 20 km south of Townsville, between April 2007 and March 2010 and a two-year study was performed at Green Acres Lagoon (Cromarty), 80 km south of Townsville, between December 2007 and 2009. Sporadic cross sectional studies were also carried out on Cape York and the Atherton Tableland between 2007 and 2009.

Birds were sampled quarterly at Billabong Sanctuary and Cromarty led by the first author of the paper. Birds were captured mostly using funnel traps under ethics approval No A 1,175 (Animal Welfare Ethics Committee, James Cook University: JCU). Faecal samples were also collected quarterly at those study sites between 2008 and 2009.

Birds at Cape York were sporadically sampled with the collaboration of Dr. David Roshier of Charles Sturt University, New South Wales (currently School of Life and Environmental Sciences, Deakin University, Waurn Ponds Campus, Geelong, VIC 3217) between 2008 and 2009. These birds

were captured by both mist nets and a net launcher under licence No. WISP04524607. On the Atherton Tableland, birds were opportunistically sampled during 2008 with the assistance of staff from Biosecurity Queensland, a service of the Queensland Department of Employment, Economic Development and Innovation.

2.2. Sample collection and recording of epidemiological data

Swab samples were taken from each bird by inserting a swab (Rayon cotton swab stick, Sarstedt, Capan Italia S.p.A. Italy) deeply into the vent (cloacal) or oropharyngeal airway and swabbing the wall. Faecal samples were also swabbed by sterile cotton sticks. The tip of the plastic-shafted swab was instantly placed into a vial containing 1 mL sterile chilled Dulbecco's modified eagle's transport medium (TropBio. Cat. No. 50-020-PB). Sample tubes were then immediately stored in an insulated container with ice packs until transferring to -80°C at the laboratory. Samples were transported to the JCU Virology laboratory within five to six hours of collection. Birds were marked by using appropriately sized identification leg bands before releasing the captured birds. Handling and banding methodology were followed as described by the Australian Bird and Bat Banding scheme (*http:/www.deh.gov.au/biodiversity/science/abbbs*).

Capture date, site, species, age, gender, weight, sample type and other morphological features were noted for each bird. The ages of sampled birds were assessed based on the features described by Marchant and Higgins (1998). Gender was determined by examination of the cloaca with cloacal pliers. Weight was measured with a portable balance (OCS-5A, Hayes).

The faecal samples were identified as belonging to a species by observing defecation or observation of birds in an area immediately prior to collecting samples and using size and shape of the faeces which is distinguishable among some species. Individual samples were given unique identity numbers. Date of collection, site and species were recorded for each sample.

2.2. Molecular detection of Newcastle disease viral RNA from field samples obtained

The Universal Liquid Sample Protocol (Viral RNA/DNA Purification Protocol, CorProtocol™ No.25101 version 3) and Corbett reagent kits were used for extraction of RNAs using the Corbett

Robotic X-tractor Gene™ automated RNA/DNA extraction system (Corbett Robotic, Brisbane, Australia).

A multiplex one-step rRT-PCR was performed using the DNA saturated binding dye SYTO9® (Invitrogen, USA). This assay is based on the matrix (M) gene of NDVs to detect NDV RNA from both class-one and class-two type NDVs. Two sets of primers were used for this assay: one novel set targeted the M gene of class-one type (Forward-GGAACCGCATGTTATCCGATTG and Reverse-GTGTGCCAGCTTGAATGATCAC) and the other set targeted the M gene of class-two type (Forward-AGTGATGTGCTCGGACCTTC and Reverse- CCTGAGGAGAGGCATTTGCTA) (Wise et al., 2004).

Invitrogen Superscript™ III Platinum® One-step rRT-PCR kits were used for this assay. The 20 μL reaction mixture constituted 2-U of Superscript™ III Platinum® Taq polymerase, 0.4 μM each forward and reverse primer and 0.2 μM SYTO9® dye and 5 μL of RNA template extract. Reactions were done in the RotorGene 3000 or 6000 machine (Corbett Robotic, Brisbane, Australia).

Thermal cycling included 30 minutes at 48°C followed by 10 minutes at 95°C to activate the hot start Taq DNA polymerase. The cycling conditions consisted of 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C and 20 seconds at 72°C. After the amplification, the melting temperature of the PCR product was determined by progressively increasing the temperature at 0.2°C from 75 to 95°C, with fluorescence acquisition on the FAM (5(6)-carboxyfluorescein) channel (excitation at 470 nm, detection at 510 nm) and plots of the rate of change in fluorescence against temperature were produced by the RotorGene 3000 or 6000 software. Melting temperatures between 80°C and 88°C were considered indicative of a reactor. The Australian class-one and class-two reference NDVs produced clearly different peaks in melt curve analysis of this assay. These results are presented in supplementary Figures 1-2.

Real time RT-PCR runs were acceptable when the negative and non-template controls did not fluoresce and the positive controls had fluorescent signals which crossed the auto-set threshold level within 40 cycles. Any test sample that was reactive in the rRT-PCR assay with the threshold (C_T)

values of ≤40 was considered as reactive. A cut-off C_T value of 40 was set, based on a prior experiment with known Australian referral control samples of allantoic fluid (HA titre 2^{12}) at different dilution concentrations (Supplementary Figure 3). Classification of NDV RNA types (one and two) for field samples were determined based on the standard melt curves produced by the respective positive controls in the assay.

2.3. Analysis of Newcastle disease viral genes (matrix and fusion)

Field samples that reacted in the screening test were further analysed to obtain sequences for the M and F genes. A two-step nested RT-PCR was performed to amplify these genes. Primers are outlined in Table 1.

ImProm-II™ Reverse Transcription System kits (Promega) were used for the first-strand cDNA synthesis. A template mixture of 5 μL containing 10 mM of a forward primer for each gene (Table 1) and 4 μL of extracted RNA template was prepared in a 0.2 ml PCR tube. The template mix was then incubated in an Eppendorf Mastercycler at 70°C for five minutes to denature RNA secondary structures. The sample was then rapidly cooled to 4°C for 5 minutes.

A total volume of 20 μL per reaction contained 1× ImProm-II reaction buffer, 3 mM $MgCl_2$, 0.5 mM of RNase inhibitor, 1 U/μL of ImProm-II Reverse transcriptase enzyme and 5 μL of chilled template mix.

Reverse transcription (RT) reaction conditions included 30 minutes at 45ºC for primer annealing followed by 60 minutes at 50°C for extension. All RT reactions were performed using an Eppendorf Mastercycler Gradient thermocycler. Complementary DNA products were stored either at 4º C or -20º C.

Following cDNA synthesis, PCR was performed using the primers presented in Table 1 to amplify overlapping sections of the M and F genes. A volume of 30 μL per reaction composed of 1×GoTaq hot-start PCR mix (Promega), 0.4mM each forward and reverse primer and 2 μL template (M gene) or 5

μL template (F gene). The PCR cycling parameters were 95ºC for 2 minutes followed by 40 cycles of 95ºC for 15 seconds, 60°C (M-1) or 55°C (M-2 and M-3) or 50°C (F-1 and F-2) for 15 seconds and 72°C for 15 seconds (M-1) or 72°C for 45 seconds (M-2 and M-3) or 72°C for 30 seconds (F-1 and F-2), followed by a final extension step of 72°C for 10 minutes.

All samples were kept at 4°C until the PCR product was analysed through electrophoresis on 1% agarose gel. Polymerase chain reaction products on the gel were visualized through ethidium bromide staining. Apparent size of each PCR product was compared with DNA markers of known size. Both positive and negative controls were used to check the validity of each run.

Reactions were purified and sequenced by Macrogen (Seoul, South Korea) using ABI 3700 (Applied Biosystems) automated sequencing machines. Sequencing was performed on both DNA strands. Sequences were analysed using ContiqExpress Project, Vector NTI Advanced 10 (Invitrogen).

Chromatograms were loaded, their quality evaluated by visual inspection and the ends trimmed to the reference gene obtained from GenBank. Multiple sequences from each gene, for each sample, were aligned into a single contig and individual chromatograms analysed and edited if required.

Sequences were compared to the available GenBank sequences using a Basic Local Alignment Search Tool. Sequences were imported into an MSF file in GenDoc and compared with other similar and dissimilar sequences. Where necessary the sequences were trimmed and the protein coding open reading frames were predicted.

Phylogenetic analysis was performed using features of the MEGA4 suite of programs (Tamura et al., 2007). Trees were inferred using the maximum likelihood evolution method (Yang, 2007) and the bootstrap method with 5,000 iterations was used to evaluate statistically the robustness of the trees presented with bootstrap values above 70% considered to show a statistically supported node (Felsenstein, 1985).

2.4. Statistical evaluation

Field and laboratory data were managed in the Microsoft Excel-2003 spread sheet. Data were exported into STATA/SE ™ 11.0 (StataCorp, USA) for checking data integrity and performing an epidemiological analysis. Descriptive statistics were used to express individual results of each category as a frequency percentage and 95% confidence intervals.

2.6. Risk factor analysis

Univariate and multivariate logistic regression analysis were performed to detect any association between the binary response variable (reactor or non-reactor NDV RNA samples obtained in screening rRT-PCR) and site, year of sampling, season, age, weight and sex.

2.6.1. Univariate logistic regression

Chi-square tests followed by univariate logistic regression were performed to identify potential risk factors for the binary response variable using the data set A (data description in result section).

2.6.1. Multivariate logistic regression

Chi square tests were performed to assess unconditional relationships between the selected factors and the binary response variable using the data set B (data description in result section). Factors with significant $\chi 2$ results ($p \leq 0.1$) were selected for the multivariate logistic regression.

The model was manually constructed by forward-selection applying the maximum likelihood estimation procedure (Dohoo et al., 2003) and the statistical significance of the contribution of individual predictors was determined. Wald's test and the likelihood ratio test (LRT) were used to complete this, as described by Dohoo et al. (2003). Interaction and confounding between factors were assessed by constructing two-interaction product terms for the significant main effect factors in the model, forcing them into the model and examining changes in the coefficients and *p* values of the main effects. The presence of confounding factors was investigated by removing one of the variables and assessing changes in the coefficient. A coefficient change of > 10% was considered to indicate the presence of confounding variables (Hoque et al. 2010; Hoque et al. 2011a, b, c).

We tested for collinearity between categorical factors using the 2-tailed *p* value by the $\chi 2$ test. Two factors were considered to be collinear if the *p* value was ≤ 0.05. The model was then assessed for goodness-of-fit using the Hosmer-Lemeshow test, while predictive ability was determined using the receiver operating characteristic (ROC) curve (Dohoo et al., 2003). The results were presented for each adjusted predictor variable as an odds ratio (OR), *p* value and 95% confidence interval.

3. Results

3.1. Samples collected

A total of 1,555 live healthy WABs were captured from four study sites from April 2007 to March 2010. Swab samples were obtained from 1,461 birds (Billabong Sanctuary-731 birds; Cromarty-603, Cape York-107 and Atherton Tableland-20). These birds generated 1,458 cloacal and 1,368 oropharyngeal swab samples. The temporal and species distribution for the live bird samples are presented in Table 2 and 3.

A total of 297 cloacal and 298 oropharyngeal swab samples were also obtained from 204 recaptured birds (1-5 recaptures, mean of 2) at Billabong Sanctuary (n=114) and Green Acres Lagoon (n=90).

Additionally, 1,157 individual fresh and moist environmental faecal samples of WABs were collected from the ground at Billabong Sanctuary (n=694) and Cromarty (Green Acres Lagoon; n=230 and McLain Rd Nature Reserve Lagoon; n=233) from October 2008 to January 2010. The temporal and species distribution of faecal samples obtained is also shown in Table 2 and 3.

3.2. Newcastle disease viral RNA prevalence in wild aquatic birds

The overall apparent prevalence of NDV RNA was 3.4% (0.4% NDV RNA class-one and 3.0% class-two) in the sampled WABs (N=1,461) of nQLD. The prevalence of RNA from swabs was 3.0% for cloacal (n=1,458) and 0.4% for oropharyngeal (n=1,368).

297 The prevalence by site of collection was 4.6% for Cromarty, 3.0% for Billabong Sanctuary and 0%
298 for Cape York and Atherton Tableland. Prevalence in PWDs was higher (4.2%) compared with PBDs
299 (0.9%) (Table4).
300
301 By contrast, the overall prevalence was 0.4% in the faecal samples of WABs (N=1,157) of which
302 Cromarty accounted for 0.6% (0.2% NDV RNA class-one and 0.4% class-two types) (n=695) and
303 Billabong sanctuary accounted for 0.2% NDV RNA class-two type (n=462).
304
305 The prevalence was 1.6% (n=246) for Australian white ibises, 0.8% for magpie geese (MG) and 0%
306 for other species.
307
308 *3.3. Real time RT-PCR results for the recaptured birds*
309 We evaluated 297 cloacal and 298 oropharyngeal swab samples that were obtained from 204
310 recaptured birds (1-5 recaptures, mean of 2). Of 204 birds, 16 previously non-reactor birds (15 cloacal
311 and 1 oropharyngeal swabs) were newly detected as reactors (10 first, 4 second, 1 at third and 1 at
312 fourth recaptures). Of the 16 reactors, 2 were NDV RNA class-one and 14 were class-two types. The
313 rest of the birds (188) remained negative to the testing at each recaptures.
314
315 *3.4. Results of risk factor analysis*
316 Due to variability in the numbers of birds captured over time 2 subsets of data were analysed in
317 order to ensure sufficient sample numbers in each category of risk factor under investigation (site, year,
318 season, age, sex and weight).

319 *3.4.1. Data subset A (N=394)*
320 We generated data from the predominantly caught species, PWDs at Billabong Sanctuary from June
321 2007 to May 2009. Although we started our study in April 2007, few birds were caught during April to
322 May 2007 and therefore that data were excluded from the analysis. This data set was suitable to
323 examine the effect of season on the dichotomous results of rRT-PCR on samples evaluated for NDV
324 RNA. Seasons were classed as warm dry (September-December), warm wet (January-April) and cool
325 dry (May-August). Years were categorized as June 2007-May 2008 and June 2008-May 2009. Age was
326 grouped into three categories: 1. chicks and juveniles, 2. sub-adults and adults and 3. unknown age

class. Body weight was not normally distributed even after natural log transformation (it remained skewed to the right) and therefore, it was organized into two categories separated by the 50^{th} percentile (0.20-0.65; 0.66-1.16kg). Gender was excluded from the analysis due to a significant number of birds in this data which were not assessed for sex. The analysis for this data set was restricted to univariate logistic regression due to an insufficient number of reactor samples in each factor category.

3.4.1.1. Univariate logistic regression (A)

The results of rRT-PCR on samples evaluated for NDV RNA varied significantly between years, weight categories and among seasons and age classes (χ^2, $p<0.05$) (Table 5). The odds ratios of being reactor samples for NDV RNA were 10.9 for the second year over the first year of the study ($p=0.022$), 9.8 for warm wet over warm dry seasons ($p=0.034$), 9.4 for younger over older ducks ($p=0.004$) and 6.4 for lighter over heavier ducks ($p=0.016$) (Table 5).

3.4.2. Data subset B (N=958)

We also produced a data set from PWDs at Billabong Sanctuary and Cromarty (Green Acres Lagoon) from January 2008 to December 2009. This was the only data set to examine the site effect on the results of rRT-PCR on samples evaluated for NDV RNA. We had a complete two-year dataset; therefore year classes were 2008 and 2009. Age was regrouped into two classes 1. chicks and sub-adults and 2. adults in order to have sufficient samples in each class. Weight was not normally distributed even after natural log transformation (it held skewed to the right) and therefore, categorized into two groups, based on the 50^{th} percentile (0.20-0.64; 0.65-0.91kg). Gender was also included as a category in the analysis. The analysis for this data set was extended to multivariate logistic regression due to sufficient number of reactor samples of each category of factors being examined. This was because the effects of season were not examined.

3.4.2.1. Logistic model (B)

The chi-square test results for the individual categories of site, year, age, sex and weight are shown in Table 6. The proportion of results in NDV RNA reactor and non-reactor categories of rRT-PCR results varied for year and age ($p\leq0.10$). Therefore, these two factors were put forward to construct the logistic model and their combined adjusted effects were examined.

Collinearity was not detected between year and age (χ^2 test, $p>0.05$). No interaction or confounding was detected in the final model. The Hosmer-Lemeshow goodness-of-fit test demonstrated that the model fitted the data (χ^2 test, $p \leq 0.865$). The ROC curve extended reasonably well into the upper left-hand corner of the curve and the area under the curve was 0.64 (curve not shown here). Both of these model characteristics indicated that the model had good predictive ability. The odds of reactor samples of WABs was 2.7 (95% CI 1.5-4.9) more likely in younger than older ducks (p=0.001) (Table 7).

3.5. Newcastle disease viral RNA sequencing results

Of 71 reactor samples (including 16 from recaptured birds) (N=1,641 live birds and 1,157 faecal samples) from the NDV RNA screening assay, we only obtained sequencing results (at least the M gene) for three samples. We were unsuccessful in obtaining sequences from 17 reactor samples. Sequencing was not attempted for 51 reactor samples. The details are shown previously in Table 2 and 3 and supplementary Table 1.

3.5.1 Phylogenetic analysis of the matrix gene sequences

The short fragment of M gene sequenced from two reactor samples (PWD-48 and 55) (Figure 2 and supplementary Table 1) was obtained between nucleotide positions 900 and 1,008 in this study. For phylogenetic analysis, however, we used 106 bp fragments covering the positions 901-1006 that were available for both reactor sample sequences. For comparative phylogenetic analysis, the reference sequences for class-one and class-two type NDVs along with two reactor sample sequences obtained in this study were included (Figure 2). The phylogram shows the reactor sample sequences (PWD-48 and 55) were closely related to each other (69 bootstrap value) and more similar to the Australian RNA NDV class-one type of ibis isolate (Queensland/2006) and duck-3245 isolate (Western Australia/1977) than it was for any other sequence (Figure 2).

A long fragment of M gene sequence from one reactor sample (PWD-46) (Figure 3 and supplementary Table 1) was obtained between nucleotide positions 237 and 908 in this study. Therefore, a 672 bp fragment sequence of this reactor sample, along with reference sequences was used for constructing phylogenetic trees. The phylogram shows the reactor sequence (PWD-46) clustered with the sequences of Australian I-2 progenitor virus and I-2 vaccine strain (Figure 3).

3.5.2. Phylogenetic analysis of the fusion gene (F-2, class-two type) sequences

This study also obtained a long fragment of fusion gene sequence (843 bp, nucleotide positions between 220 and 1,062) for the PWD-46 reactor sample. This sequence with reference sequences was combined. The results are presented in Figure 4.

The F gene phylogeny demonstrated that the reactor sequence (PWD-46) was similar to sequences from Australian I-2 progenitor and I-2 vaccine strains as well as the chicken isolates AP1 and SP3. It is distinct from the V4 cluster and the outbreak viruses. All of these viruses are NDV class-two genotype-one viruses (Figure 4). The F gene and M gene sequences both indicate that this reactor sequence clustered with the I-2 progenitor and I-2 vaccine viruses. The I-2 progenitor, AP1 and FP3 were previously isolated from chickens and have been classified as lentogenic or apathogenic viruses (Kim et al., 1978; Spradbrow et al., 1995). The I-2 progenitor was then developed as a vaccine (Bensink and Spradbrow, 1999) and extensively used in developing countries (Tu et al., 1998; Bensink and Spradbrow, 1999; Nasser et al., 2000; Illango et al., 2005; Henning et al., 2009).

The cleavage site of the reactor sample sequence indicated an avirulent ND class-two type virus (EGQGRL).

The sequences in fasta format of three reactor samples in this study have been provided as supplementary information.

4. Discussion

4.1. Descriptive results

Overall NDV RNA prevalence at the individual bird level was 3.4% in this study, which corresponds to the proportionate prevalence encountered in our dead bird study (4.8%, N=42) (Hoque 2011, A PhD Thesis). The prevalence, however, was very low (0.4%) for faecal samples in this study, which conforms to an Australian wild bird study using swab samples (0.4%) (Mackenzie et al., 1985). A similar level of 0.5% prevalence was reported in composite fresh faecal samples of northern pintail

(*Anas acta*) in Japan (Jahangir et al., 2009). Overall findings, therefore, suggest NDVs have been circulating at low prevalence in WABs of nQLD. A higher prevalence has been estimated in waterfowl in Finland (5.2%) (Lindh et al., 2008), China (8.2%) (Zeng et al., 2008) and wild birds in central Nigeria (8.0%) (Ibu et al., 2009). This suggests NDVs occur worldwide in wild bird populations at variables levels.

We predominantly detected NDV RNA reactor swab samples from captured and recaptured PWDs. We successfully obtained NDV sequences from some of these reactor samples. Previously, ND virus has been isolated sporadically from samples of PWDs in Australia (Mackenzie et al., 1985). Some cross-sectional studies in Australia, however, have suggested NDVs are absent in PWDs (Mackenzie et al., 1984) (Ibrahim Diallo 2007, DPIF, Queensland, personal communication). To the authors' knowledge this is the first time the level of NDVs has been quantified in PWDs through a systematic longitudinal study in nQLD.

Sporadic NDV RNA reactor samples were determined in the species other than PWDs in our study such as one reactor swab of PBD (live bird) and five reactor faecal samples of Australian white ibises (four) and MG (one) in this study. These findings are in agreement with previous Australian wild bird studies for NDVs {Mackenzie, 1984 #317} {Mackenzie, 1985 #203} (Ibrahim Diallo 2006, DPIF, Queensland, personal communication).

4.2. Risk factors

Both univariate and multivariate logistic regression using the data sets A and B identified younger PWDs as being significantly associated with higher prevalence of NDV RNA in swab sample. Only univariate logistic analysis using data set A indicated lighter PWDs and birds caught in the warm wet season (January-April) as being significantly associated with a higher prevalence of NDV RNA. This result correlates with sampling younger birds because lighter birds represent immature birds and these birds were commonly sampled in the warm wet season. A similar age pattern of prevalence was observed in our avian influenza study (Hoque 2011, A PhD Thesis). In addition, age specific prevalence was observed earlier in domestic chickens where clinical disease or mortality due to NDVs was higher in younger birds than adults (Ezeokoli et al., 1984; Janviriyasopak et al., 1989; Martin,

1992). The higher prevalence in young birds may be due to the fact that they are immunologically naïve whereas adults are more resistant, particularly to viruses to which they have previously been exposed (Webster et al., 1992). Contrarily, pheasants, a highly susceptible species to NDVs, were affected at all ages (Higgins, 1982; Alexander, 2001b).

Reports of seasonal influence on the occurrence of ND or prevalence of NDVs have not been readily available for wild birds but seasonal effect has varied geographically for domestic chickens. For example, ND incidence was observed to peak at the end of the dry season (February-April) in Thailand (Ratanasethakul, 1989), winter season (December-March) in Bangladesh (Asadullah, 1992), hot dry season (September-November) and hot humid season (January-March) in Zambia (Sharma et al., 1986) and dry hot season in Kenya (Njagi et al., 2010). Although ND incidence varied according to seasons in these referral studies, these seasons provided adverse conditions which might reduce the immune status of the birds, thus making them more vulnerable to infections.

A year effect on the NDV RNA prevalence was only determined in univariate logistic regression for data set A and not in multivariate analysis for data set B in this study. Therefore, the year effect may be an artefact of unadjusted analysis. Marked differences in AIV prevalence were, however, found between years in North American aquatic birds {Krauss, 2004 #311}. These patterns of viral prevalence are little understood, but an epidemiologic periodicity in resistance has been implicated {Hinshaw, 1985 #392}.

4.3. Newcastle disease viral RNA classes

Both classes of NDV RNAs were evident in this study according to screening and sequencing results. Overall results suggested the NDV RNA class-two types predominantly circulated in the wild bird populations in nQLD (Table 2 and supplementary Table 1) and this finding is supported by many wild bird studies throughout the world (Alexander, 1995; Aldous et al., 2003; Jindal et al., 2009)

The presence of dual class NDV RNAs in this study is supported by earlier studies in Australia (Russell and Alexander, 1983; Mackenzie et al., 1985; Alexander et al., 1986; Ibrahim Diallo 2006, DPIF, Queensland, personal communication), the USA (Spalatin et al., 1976) and France and England (Russell and Alexander, 1983) and Finland (Lindh et al., 2008). These studies identified the class-one

type NDVs in PBD, Australian white ibis, lesser and common noddy, sooty tern and black-fronted plover (Australia), feral shelduck (France), mallard and berk duck (England), common teal and pochard (Finland). These studies also reported the class two-type NDVs in chestnut-breasted manikin, sharptailed sandpiper, red-kneed dotterel and comb-crested jacana (Australia), migratory WABs (the USA), feral ducks (French) and common teal (Finland).

4.4. Sequencing results

Phylogenetic analysis of a short fragment of the M gene (106 bp) from 2 reactor sample sequences (PWD-48 and 55) showed a greater similarity to isolates of duck 3245 and Australian white ibis (class-one) than any other isolates belonging to NDV RNA class-one type sequences (Figure 2). Our sequences were also distantly placed from the sequences of Australian virulent ND outbreak viruses (class-two) (Figure 2). The overall pattern, therefore, suggests our reactor sample sequences may represent Australian avirulent class-one type like NDVs.

Phylogenetic evaluation of the long fragments of M (672 bp) and F (843 bp) genes on another reactor sample sequence (PWD-46) showed they were close congeners to the sequences of Australian I-2 progenitor virus and I-2 vaccine strain which are under the class-two type viruses (Figure 3 and 4). The F cleavage site of our reactor sequence was shown to have an avirulent motif in its F_0 cleavage site (amino acid position: 109-119) (EGQGRL) which is little different to the cleavage site sequences of avirulent I-2 progenitor and vaccine strain (RKQGRL). This difference may indicate our reactor sample sequence is even less virulent than that of the I-2 progenitor and vaccine strain. The cleavage site of the reactor sequence is much closer to the V4 sequence (GKQGRL).

Therefore, this avirulent ND virus identified in PWD in this study would be unlikely to pose a direct threat to Australian domestic and commercial poultry unless it were able to evolve rapidly once introduced into poultry.

4.5. Limitations

The sequencing success for NDV RNA reactor samples was low in this study based 15% of reactor samples analysed (N=71) which may be due to a deterioration of RNA after repeated freezing and thawing of those samples and low RNA starting concentrations. In addition, inherent inhibitors in those reactor field samples may restrict the full potential of conventional PCR to amplify PCR products for sequencing (seen in our avian influenza study). The alternative was to grow viruses in embryonated chicken eggs followed by sequencing; however, that strategy did not work in our avian influenza study.

Sequencing was not attempted for 51 reactor samples due to time constraints, lack of resources and low sequencing success rates previously.

The relative sensitivity and specificity for our screening rRT-PCR test were assessed with known control samples of Australian reference NDVs and found to be acceptable (supplementary Figures 1-3). The assay was able to detect 1/1000 dilutions of the original reference viruses. The absolute sensitivity of this assay has yet to be determined. Supplementary figure 1 and 2 show the Australian class-one and class-two reference NDVs produced clearly different peaks in melt curve analysis of this assay which can confidently differentiate the class-one and class-two NDVs. These figures show very good specificity of the test in differentiating classes of the Australian reference viruses.

4.6. Conclusions

This study estimated 0.4-3.4% apparent NDV RNA prevalence in WABs of nQLD through a systematic longitudinal study in Australia. The prevalence was significantly higher in PWDs (4.2%). Young birds had a higher prevalence. Newcastle disease viral RNA class-two type was predominant in PWDs. An avirulent NDV RNA class-two found in PWD in this study appears to be of low risk to domestic and commercial poultry. Overall results obtained in this study will assist in developing future surveillance programs for NDVs of WABs in northern Australia. Continued sampling of wild birds will also assist in maintaining our understanding of the type of viruses circulating in wild birds and their potential to threaten the Australian poultry industry.

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Table 1

Primer sets used for sequencing different NDV genes

NDV genes	Primer	Sequence (5'-3')	Position	Product length (bp)
Matrix (M)	M-1 (NDV RNA class one type)-one of the screening primer sets	GGAACCGCATGTTATCCGATTG-F	891-915	109
		GTGTGCCAGCTTGAATGATCAC-R	979-1,000	
	M-2 (NDV RNA class one type)	CGTTTACAAGATTCCAACCGCAGC-F	555-578	549
		GCGAGTGCTTACTTCTTGAACGGG-R	1,080-1,103	
	M-3 (NDV RNA class two type)	CTGTCGGCATGATCAATGATAATCC-F	222-248	706
		GGAGAGGCATTTGCTATGGGATAGC-R	909-934	
Fusion (F)	F-1 (NDV RNA class one type)	CCCTCTTGGTGATTCTATC-F	279-297	648
		CTTACAGACAAGGTTTCC-R	909-926	
	F-2 (NDV RNA class two type)	AATATGCCCAAGGATAAAG-F	202-220	876
		AATACCAGGAGACATAGG-R	1,060-1,077	

F: Forward; *R*: Reverse

Table 2

Numbers of sampled wild aquatic birds and sample types, cloacal and oropharyngeal swabs and faeces, along with the number of NDV RNA reactors samples (+) in north Queensland (from April 2007 to March 2010) (All samples were positive for class-two viruses unless specified)

Time	**No. birds (+)***	**Cloacal swab (+)**	**Oropharyngeal swab (+)**	**Faecal sample (+)**
Apr 2007	6 (0)	6 (0)	6 (0)	
May 2007	4 (0)	4 (0)	4(0)	
Jun 2007	19 (0)	19 (0)	19 (0)	
Jul 2007	27 (0)	27 (0)	27 (0)	
Aug 2007	11 (0)	11 (0)	11 (0)	
Sep 2007	19 (1)	19 (1)	19 (0)	
Oct 2007	68 (0)	67 (0)	66 (0)	
Nov 2007	8 (0)	8 (0)	8 (0)	
Dec 2007	31 (0)	31 (0)	31 (0)	
Jan 2008	13 (1)	13 (1)	13 (0)	
Feb 2008	67 (0)	66 (0)	66 (0)	
Mar 2008	79	78 (0)	78 (0)	
Apr 2008	47 (8)	47 (5: **1 Class 1**)	47 (3: **Class 1**)	
May 2008	11 (0)	11 (0)	11 (0)	
Jun 2008	38 (2)	38 (2)	38 (0)	
Jul 2008	67 (0)	67 (0)	67 (0)	
Aug 2008	42 (3)	42 (3)	42 (0)	
Sep 2008	48 (6)	48 (6)	26 (0)	
Oct 2008	47 (1)	47 (0)	47 (1: **Class 1**)	107 (0)
Nov 2008	18 (1)	18 (1)	18 (0)	158 (0)
Dec 2008	0	0	0	
Jan 2009	5	5 (0)	5 (0)	
Feb 2009	1	1 (0)	1 (0)	35 (0)
Mar 2009	106 (4)	106 (4)	106 (0)	111 (0)
Apr 2009	114 (5)	114 (5)	114 (0)	14 (0)
May 2009	36 (1)	36 (1)	36 (0)	51 (0)
Jun 2009	141 (1)	141 (1)	77 (1)	
Jul 2009	34 (1)	34 (1)	34 (0)	127 (0)
Aug 2009	12 (0)	12 (0)	12 (0)	114 (0)
Sep 2009	41 (2)	41 (2: **1 Class 1**)	40 (0)	92 (0)
Oct 2009	126 (0)	126 (0)	126 (0)	141 (3: **2 Class 1**)
Nov 2009	83 (0)	83 (0)	83 (0)	123 (0)
Dec 2009	90 (12)	90 (12)	88 (1)	84 (2: **Class 1**)
Jan 2010	0	0	0	
Feb 2010	0	0	0	
Mar 2010	2 (1)	2 (1)	2 (0)	
Total	**1,461 (50)**	**1,458 (45)**	**1,368 (6)**	**5 (1,158)**

* Birds were positive for NDVs if NDV RNA was detected in either cloacal or oropharyngeal swabs. Results of recaptured birds were not included in the above table.

Table 3

Numbers of sampled wild aquatic birds and sample types, cloacal and oropharyngeal swabs and faeces, along with the number of NDV RNA reactors samples (+) in north Queensland (from April 2007 to March 2010) (All samples were positive for class-two viruses unless specified.)

Species	**Scientific name (Order)**	**No. birds (+)***	**Cloacal (+)**	**Oropharyngeal (+)**	**Faecal Samples (+)**
Plumed whistling duck	*Dendrocygna eytoni (Anseriforme-A)*	1,180 (49)	1,178 (45)	1,136 (6)	712
Pacific black duck	*Anas superciliosa (A)*	112 (1)	111 (0)	92 (0)	66 (1)
Magpie goose	*Anseranas semipalmata (A)*	94 (0)	94 (0)	94 (0)	128
Dusky moorhen	*Gallinula tenebrosa (Gruiforme)*	20 (0)	20 (0)	20 (0)	3
Wandering whistling duck	*Dendrocygna arcuata (A)*	10 (0)	10 (0)	0	0
Muscovy duck	*Cairina moschate (A)*	10 (0)	10 (0)	10 (0)	0
Radjah shellduck	*Tadora radjah (A)*	8 (0)	8 (0)	2 (0)	0
Green pygmy goose	*Nettapus pulchellus A)*	7 (0)	7 (0)	0	0
Australian white ibis	*Threskiornis molucca (A)*	4 (0)	4 (0)	4 (0)	246 (4)
Bush stone curlew	*Burhinus grallarius (Charadriiforme)*	3 (0)	3 (0)	3 (0)	0
Crow	*Corvus orru ((Passeriforme)*	3 (0)	3 (0)	3 (0)	0
Hardhead	*Aytha australis (A)*	3 (0)	3 (0)	0	0
Australian shellduck	*Tadorna tadornoides (A)*	2 (0)	2 (0)	2 (0)	1
Black swan	*Cygnus atratus (A)*	1 (0)	1 (0)	1 (0)	1
Domestic goose	*Anser anser* subsp. *Domesticus (A)*	1 (0)	1 (0)	0	0
Grey teal	*Anas gracilis (A)*	1 (0)	1 (0)	0	0
White faced heron	*Egretta novaehollandiae (Ciconiiforme)*	1 (0)	1 (0)	0	0
Pigeon	*Ducula bicolour (Galliciforme)*	1 (0)	1 (0)	1 (0)	0
Total		**1,461 (50)**	**1,458 (45)**	**1,368 (6)**	**1,157 (5)**

* Birds were positive for NDVs if NDV RNA was detected in either cloacal or oropharyngeal swabs. Results of recaptured birds were not included in the above table.

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Table 4

Newcastle disease viral RNA prevalence in the samples obtained from WABs in nQLD according to different factors (N=1,461) (from April 2007 to March 2010). *The positive detection of NDV RNA was determined as a positive for either the cloacal or the oropharyngeal swab extract for each bird.*

Factors	Categories	Number of samples tested	% (Number of reactors)	95% CI (assuming exact binomial distribution)
Site	Billabong sanctuary	731	3.0% (22)	1.8-4.5
	Green Acres lagoon (Cromarty)	603	4.6% (28)	3.1-6.6
	Cape York	107	0% (0)	0-3.3
	Atherton Tableland	20	0% (0)	0-16.8
Year	2007	193	0.5% (1)	0.01-2.8
	2008	477	4.6% (22)	2.9-6.8
	2009	789	3.3% (26)	2.1-4.7
	2010	2	50% (1)	1.2-98.7
Species	Plumed whistling duck (*Dendrocygna eytoni*)	1,180	4.2% (49)	3.0-5.4
	Pacific black duck (*Anas superciliosa*)	112	0.9% (1)	0.02-4.8
	Others	169	0% (0)	0-2.1
Age	Up to Juvenile	271	7.0% (19)	4.2-10.7
	Sub-adult	114	2.6% (3)	0.05-7.4
	Adult	906	3.0% (27)	1.9-4.3
	Unknown	170	0.6% (1)	0.01-3.2
Sex	Male	286	4.9% (14)	2.7-8.1
	Female	984	3.7% (36)	2.5-5.0
	Unknown	191	0% (0)	0-1.9

Other species: Magpie goose (*Anseranas semipalmata*); Dusky moorhen (*Gallinula tenebrosa*); Wandering whistling duck (*Dendrocygna arcuata*); Muscovy duck (*Cairina moschate*); Radjah shellduck (*Tadora radjah*); Green pygmy goose (*Nettapus pulchellus*); Australian white ibis (*Threskiornis molucca*); Bush stone curlew *(Burhinus grallarius)*; Crow (*Corvus orru*); Hardhead (*Aytha australis*); Australian shellduck (*Tadorna tadornoides*); Black swan (*Cygnus atratus*); Domestic goose (*Anser anser* subsp. *Domesticus*); Grey Teal (*Anas gracilis*); White faeced heron (*Egretta novaehollandiae*) and Pigeon (*Ducula bicolour*)

Table 5

Univariate chi square and univariate logistic regression analysis of the proportion of samples in the categories of reactor and non-reactor for NDV RNA obtained by rRT-PCR analysis of swabs of PWDs from Billabong Sanctuary, north Queensland (N=394) (from June 2007 to May 2009, data set A)

		NDV RNA		χ^2	**Univariate logistic regression**	
Factors	**Categories**	**-**	**+**	**p**	**OR (95% CI)**	**p**
Year	Jun 2007-May 2008	173	1 (0.6%)	0.005	Referent	
	Jun 2008-May 2009	207	13 (5.9%)		10.9 (1.4-83.9)	0.022
Season	Sep-Dec (Warm dry)	141	1 (0.7%)	0.045	Referent	
	Jan-Apr (Warm wet)	101	7 (6.5%)		9.8 (1.2-80.7)	0.034
	May-Aug (Cool dry)	138	6 (4.2%)		6.1 (0.7-51.6)	0.095
Age	Chick and Juvenile	94	11 (10.5%)	<0.001	9.4 (2.0-43.4)	0.004
	Sub-adult and adult	161	2 (1.2%)		Referent	
	Unknown age class	125	1 (0.8%)		N/A	
Weight	0.20-0.659kg	184	12 (6.1%)	0.006	6.4 (1.4-28.9)	0.016
	0.66-1.16kg	196	2 (1.0%)		Referent	

OR: Odds ratio; *CI*: Confidence interval; - Non-reactor; + Reactor

Table 6

Univariate chi square analysis of the proportion of samples in the categories of reactor and non-reactor for NDV RNA obtained by rRT-PCR analysis of swabs of PWDs from Billabong Sanctuary and Green Acres Lagoon, north Queensland (N=959) (from January 2008 to December 2009, data set B)

Factors	**Categories**	**NDV RNA (-)**	**NDV RNA (+)**	**P ($\chi^{2)}$**
Site	Billabong Sanctuary	390	19 (4.7%)	0.752
	Green Acres Lagoon (Cromarty)	522	28 (5.1%)	
Year	2008	291	21 (6.7%)	
	2009	621	26 (4.0%)	0.068
Age	Chick and Juvenile	191	20 (9.5%)	<0.001
	Sub-adult and adult	721	27 (3.6%)	
Weight	0.20-0.649kg	470	21 (4.3%)	0.359
	0.65-0.91kg	442	26 (5.6%)	
Sex	Male	214	13 (5.7%)	0.509
	Female	698	34 (4.6%)	

- NDV RNA non-reactor samples; + NDV RNA reactor samples

Table 7

Results of logistic model analysis of the proportion of samples in the categories of reactor and non-reactor for NDV RNA obtained by rRT-PCR analysis of swabs of PWDs from Billabong Sanctuary and Green Acres Lagoon, north Queensland (N=959) (from January 2008 to December 2009, data set B)

Factors	**Categories**	**OR**	**95% CI**	**P**
Year	2008	1.6	0.9-3.0	0.111
	2009	1.0		
Age	Chick and Juvenile	2.7	1.5-4.9	0.001
	Sub-adult and adult			

OR: Odds ratio; *CI*: Confidence interval

Figure 1

Map showing different sampling sites in north Queensland

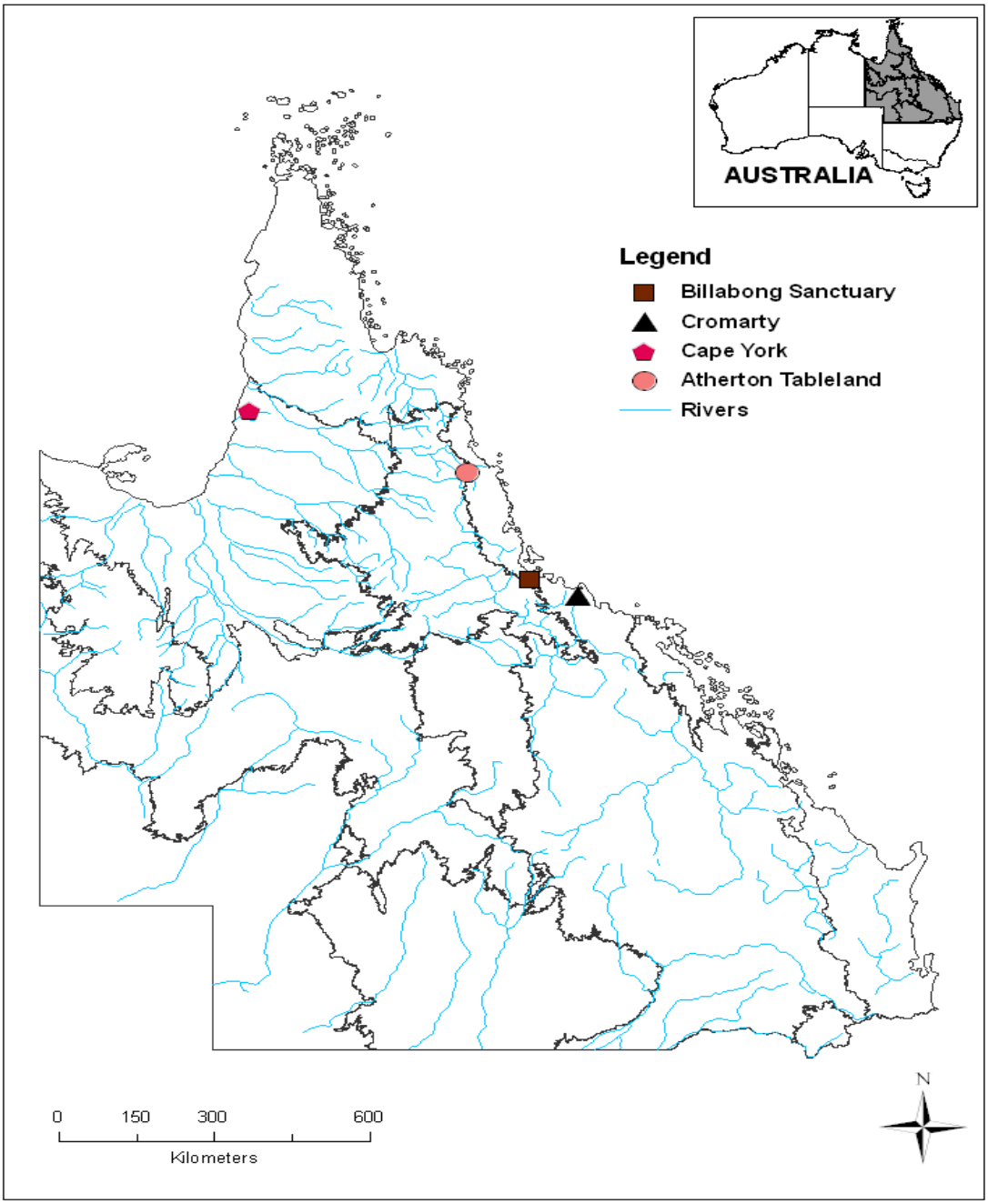


Figure 2

Bootstrap consensus trees with 5,000 replications for the Newcastle disease viral matrix gene (nucleotide positions between 901 and 1,006 base pairs)

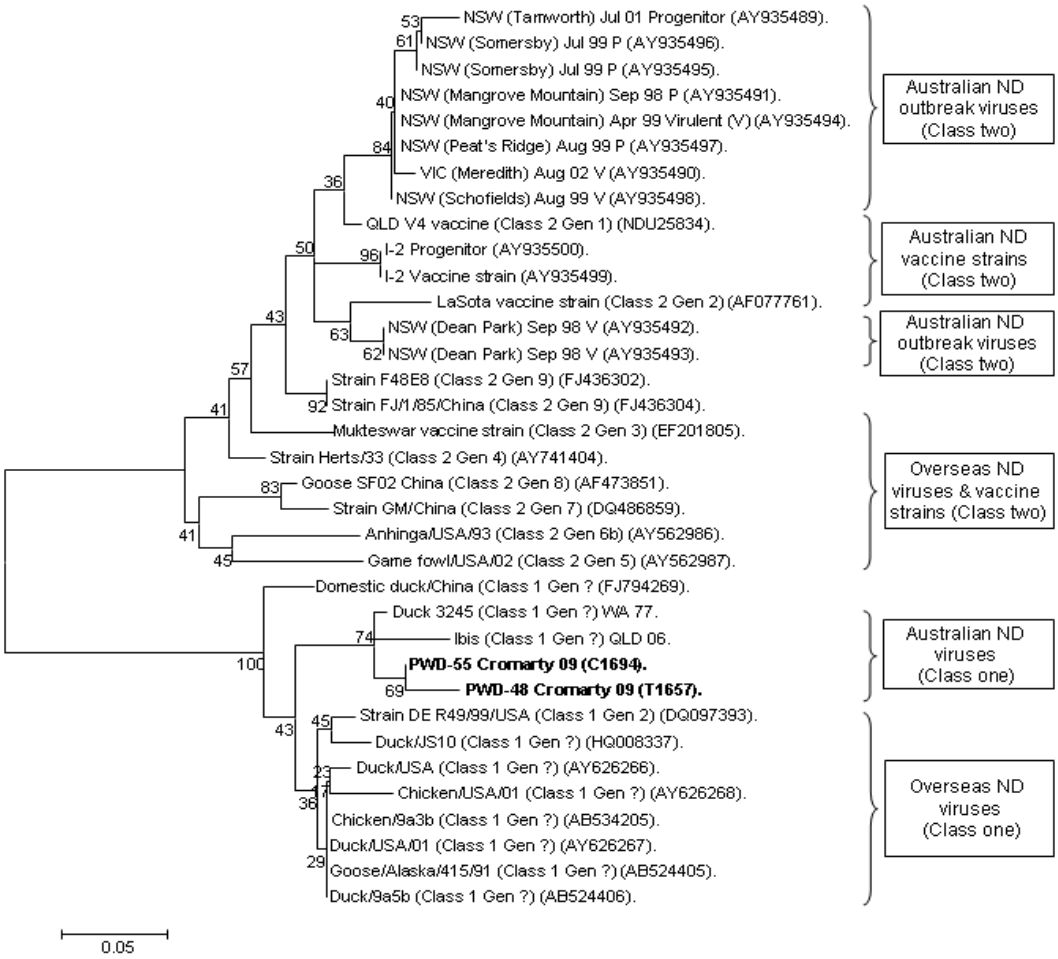


Figure 3

Bootstrap consensus trees with 5,000 replications for the Newcastle disease viral matrix gene (nucleotide positions between 237 and 908 base pairs)

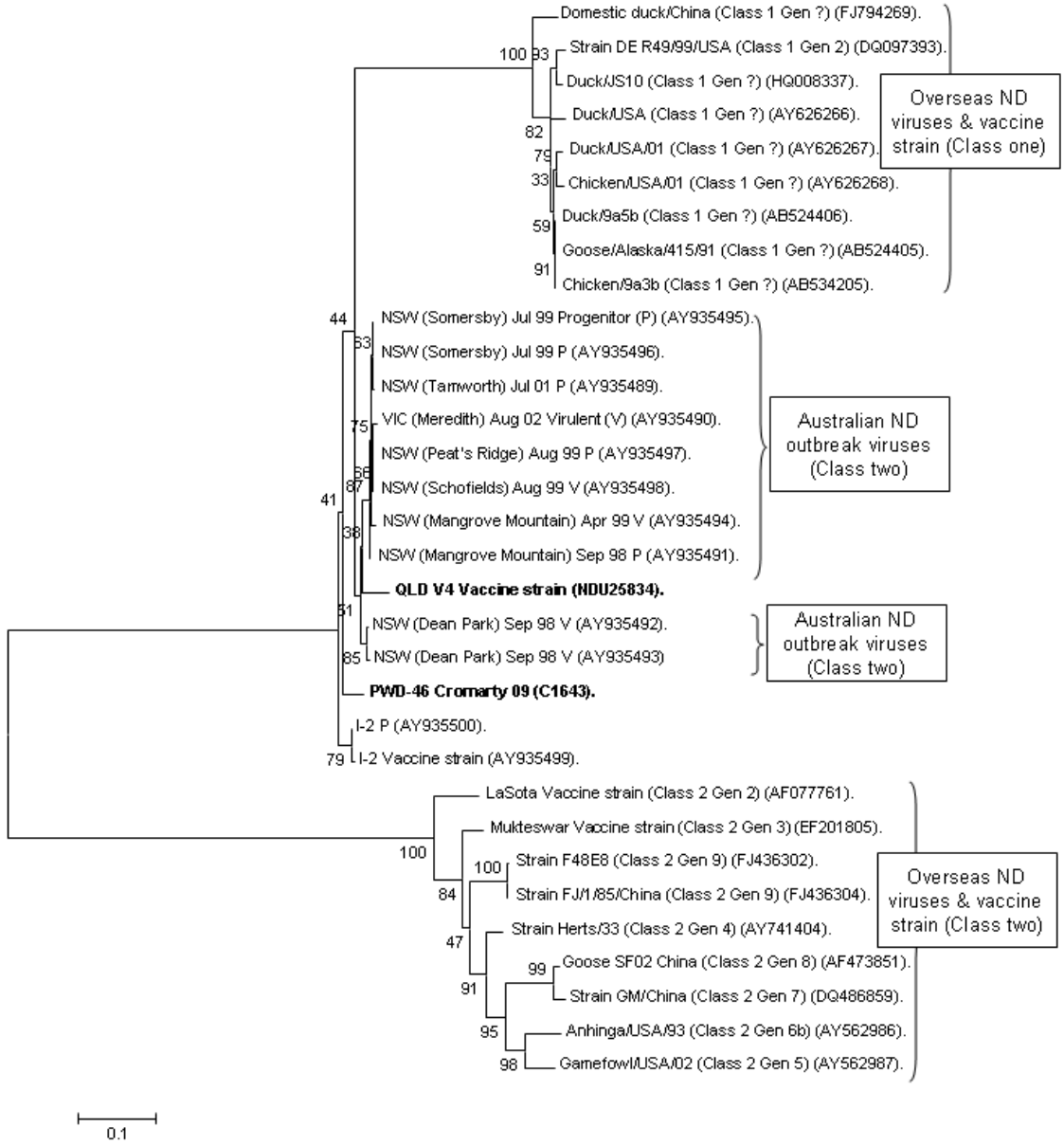
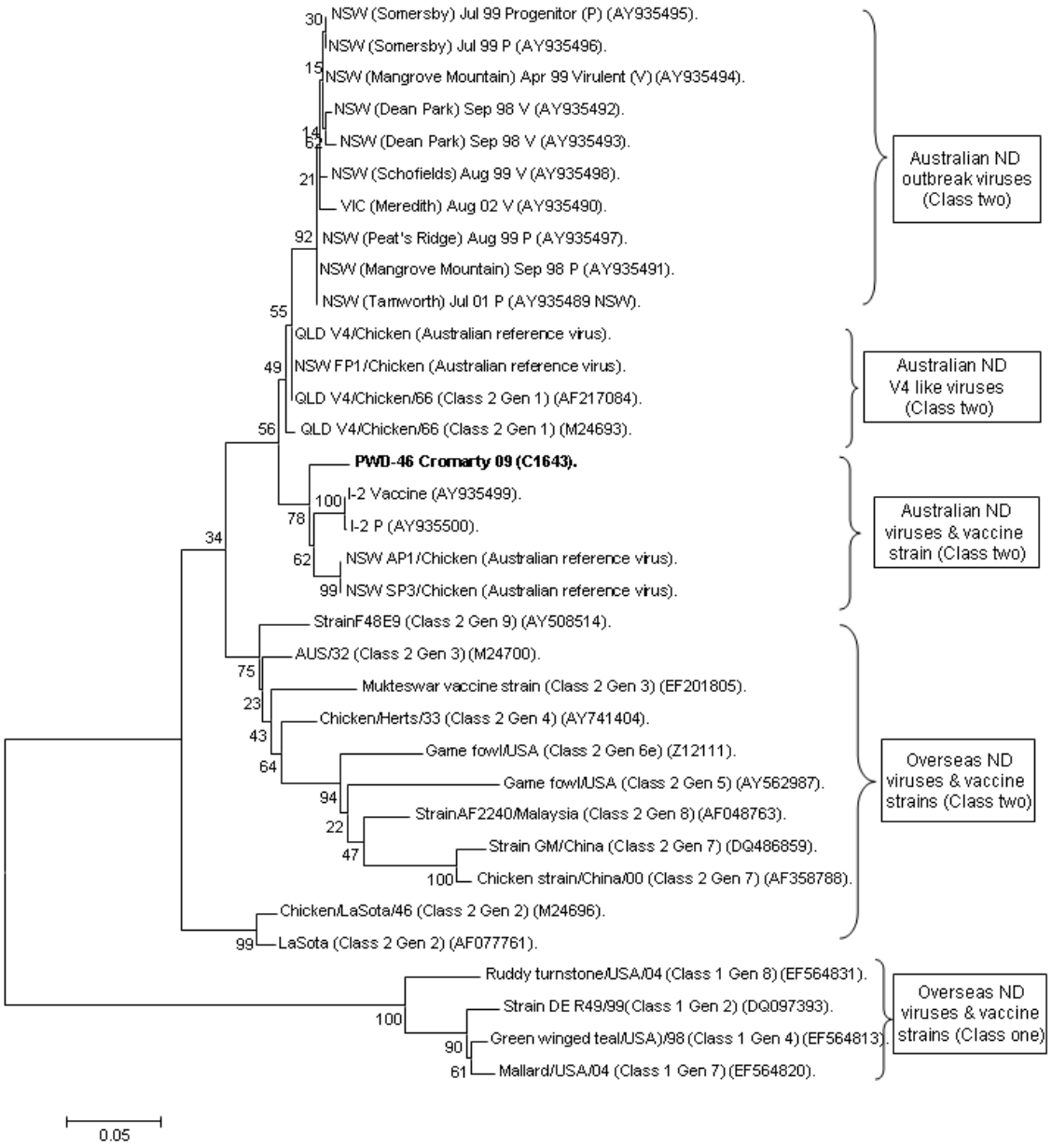


Figure 4

Bootstrap consensus trees with 5,000 replications for the Newcastle disease viral fusion gene (nucleotide positions between 220 and 1,062 base pairs)



Supplementary Table 1

Details of analysis to obtain gene sequence from NDV RNA reactor samples of WABs in nQLD (from April 2007 to March 2010)

Species	rRT PCR (Screening assay)		PCR/Sequencing (Attempted/Not)	Sequencing results	Sample type/Site/Bird ID/Month/Year
	NDV RNA Class one type (C_T value)	NDV RNA Class two type (C_T value)			
PWD-1		+ (21.4)	M-3	Unsuccessful	Cl/Billabong/92/Sep/2007
PWD-2		+ (22.6)	M-3	Unsuccessful	Cl/Billabong/195/**Recaptured**/Nov/2007
PWD-3		+ (26.9)	Not attempted	-	Cl/Cromarty/237/Jan/2008
PWD-4		+ (27.4)	Not attempted	-	Cl/Cromarty/405/Apr/2008
PWD-5		+ (24.8)	M-3	Unsuccessful	Cl/Cromarty/418/Apr/2008
PWD-6		+ (26.0)	Not attempted	-	Cl/Cromarty/421/Apr/2008
PWD-7		+ (29.9)	Not attempted	-	Cl/Cromarty/422/Apr/2008
PWD-8	+ (31.1)		Not attempted	-	Op/Cromarty/423/Apr/2008
PWD-9	+ (27.3)		Not attempted	-	Op/Cromarty/429/Apr/2008
PWD-10	+ (26.6)		M-2	Unsuccessful	Op/Cromarty/436/Apr/2008
PWD-11	+ (26.6)		Not attempted	-	Cl/Cromarty/438/Apr/2008
PWD-12		+ (22.3)	M-3	Unsuccessful	Cl/Billabong/468/Jun/2008
PWD-13		+ (23.5)	Not attempted	-	Cl/Billabong/480/Jun/2008
PWD-14		+ (22.8)	Not attempted	-	Cl/Billabong/610/Aug/2008
PWD-15		+ (24.3)	Not attempted	-	Cl/Billabong/612/Aug/2008
PWD-16		+ (23.8)	Not attempted	-	Cl/Billabong/614/Aug/2008
PWD-17		+ (23.2)	Not attempted	-	Cl/Cromarty/620/Sep/2008
PWD-18		+ (22.4)	Not attempted	-	Cl/Cromarty/625/Sep/2008
PWD-19		+ (22.6)	Not attempted	-	Cl/Cromarty/626/Sep/2008
PWD-20		+ (22.7)	Not attempted	-	Cl/Cromarty/632/Sep/2008
PWD-21		+ (21.4)	M-3	Unsuccessful	Cl/Cromarty/640/Sep/2008
PWD-22		+ (24.0)	Not attempted	-	Cl/Cromarty/641/Sep/2008
PWD-23		+ (26.8)	M-3	Unsuccessful	Cl/Billabong/668/**Recaptured**/Oct/2008
PWD-24		+ (23.1)	M-3	Unsuccessful	Cl/Cromarty/726/Nov/2008
PWD-25		+ (27.7)	Not attempted	-	Cl/Billabong/763/Mar/2009
PWD-26		+ (29.8)	Not attempted	-	Cl/Billabong/796/Mar/2009
PWD-27		+ (27.6)	Not attempted	-	Cl/Cromarty/802/Mar/2009
PWD-28		+ (27.5)	Not attempted	-	Cl/Billabong/806/Mar/2009
PWD-29		+ (23.8)	Not attempted	-	Cl/Billabong/882/Apr/2009
PWD-30		+ (28.2)	Not attempted	-	Cl/Billabong/884/Apr/2009
PWD-31		+ (25.5)	M-3	Unsuccessful	Cl/Billabong/890/Apr/2009
PWD-32		+ (24.0)	Not attempted	-	Cl/Billabong/961/Apr/2009
PWD-33		+ (28.8)	M-3	Unsuccessful	Cl/Cromarty/1000/**Recaptured**/Apr/2008
PWD-34		+ (28.8)	Not attempted	-	Cl/Cromarty/1005/Apr/2009
PWD-35		+ (27.5)	Not attempted	-	Cl/Billabong/1008/May/2009
PWD-36		+ (29.1)	Not attempted	-	Cl/Billabong/1061/**Recaptured**/Jun/2009
PWD-37		+ (27.4)	Not attempted	-	Cl/Cromarty/1073/**Recaptured**/Jun/2009
PWD-38		+ (26.5)	Not attempted	-	Cl/Billabong/1117/**Recaptured**/Jun/2009
PWD-39		+ (29.2-29.3)	Not attempted	-	**Cl-Op**/Cromarty/1137/Jun/2009
PWD-40		+ (28.7)	Not attempted	-	Cl/Billabong/1145/Jul/2009
PWD-41		+ (25.5)	Not attempted	-	Cl/Billabong/1205/**Recaptured**/Sep/2009
PWD-42	+ (24.1)		Not attempted	-	Cl/Billabong/1221/Sep/2009
PWD-43		+ (26.6)	Not attempted	-	Cl/Cromarty/1222/Sep/2009
PWD-44		+ (28.7)	Not attempted	-	Cl/Billabong/1626/**Recaptured**/Dec/2009
PWD-45		+ (26.4)	M-3	Unsuccessful	Cl/Cromarty/1639/**Recaptured**/Dec/2009
PWD-46		+ (23.8)	M-3 and F-2	M-3 and F-2	Cl/Cromarty/1643/Dec/2009
PWD-47		+ (29.2)	Not attempted	-	Cl/Cromarty/1651/Dec/2009
PWD-48	+ (30.8)		M-1	M-1	Op/Cromarty/1657/**Recaptured**/Dec/2009
PWD-49		+ (27.6)	Not attempted	-	Cl/Cromarty/1662/Dec/2009
PWD-50		+ (26.9)	Not attempted	-	Cl/Cromarty/1666/Dec/2009
PWD-51		+ (29.4)	Not attempted	-	Cl/Billabong/1670/Dec/2009
PWD-52		+ (29.7)	Not attempted	-	Cl/Billabong/1675/Dec/2009
PWD-53		+ (30.2)	Not attempted	-	Cl/Billabong/1676/Dec/2009
PWD-54		+ (28.9-31.3)	M-3 and F-2	Unsuccessful	**Cl-Op**/Billabong/1677/Dec/2009
PWD-55	+ (28.7)		M-1	M-1	Cl/Cromarty/1694/**Recaptured**/Dec/2009
PWD-56		+ (29.7)	Not attempted	-	Cl/Cromarty/1696/**Recaptured**/Dec/2009
PWD-57		+ (30.0)	Not attempted	-	Cl/Cromarty/1698/**Recaptured**/Dec/2009
PWD-58		+ (27.0)	Not attempted	-	Cl/Cromarty/1705/Dec/2009
PWD-59		+ (30.3)	Not attempted	-	Cl/Cromarty/1707/Dec/2009
PWD-60		+ (30.0)	Not attempted	-	Cl/Cromarty/1709/**Recaptured**/Dec/2009
PWD-61		+ (29.2)	Not attempted	-	Cl/Cromarty/1712/**Recaptured**/ Dec/2009
PWD-62		+ (29.8)	Not attempted	-	Cl/Cromarty/1713/Dec/2009
PWD-63		+ (30.4)	Not attempted	-	Cl/Billabong/1715/Dec/2009
PWD-64		+ (26.4)	M-3	Unsuccessful	Cl/Cromarty/1716/**Recaptured**//Dec/2009
PWD-65		+ (27.1)	M-3 and F-2	Unsuccessful	Cl/Billabong/1720/Mar/2010
PBD-1	+ (29.7)		M-2	Unsuccessful	Op/Cromarty/651/Oct/2008
MG-1	+ (29.1)		M-2	Unsuccessful	En/Billabong/865/Oct/2009
AWI-1	+ (30.6)		Not attempted	-	En/Cromarty/866/Oct/2009
AWI-2		+ (30.6)	M-3	Unsuccessful	En/Cromarty/867/Oct/2009
AWI-3	+ (26.4)		Not attempted	-	En/Cromarty/1130/Dec/2009
AWI-4	+ (26.7)		Not attempted	-	En/Cromarty/1131/Dec/2009

PWD: Plumed whistling duck; *PBD*: Pacific black duck; *MG*: Magpie goose; *AWI*: Australian white ibis; C_T: Threshold value; *M-1*: Matrix gene (NDV RNA class one type); *M-2*: Matrix gene (NDV RNA class two type); *M-3*: Matrix gene (NDV RNA class one type, short sequence); *F-1*: Fusion gene (NDV RNA class one type); *F-2*: Fusion gene (NDV RNA class two type); *Op*: Oropharyngeal swab; *Cl*: Cloacal swab; *En*: Environmental swab; +: Reactor.

Figure 1.

The Melt curve showing the 100% specificity of multiplex one step real time RT-PCR to detect Newcastle disease viral RNA from the Australian class-one (D 3245; Mackenzie *et al*., 1984; Mackenzie *et al*., 1985) and class-two (V4; Simmons, 1967) reference viruses. Assay of these viruses results in clearly different peaks. The X- and Y-axis of this figure represent melting temperature and the level florescent.

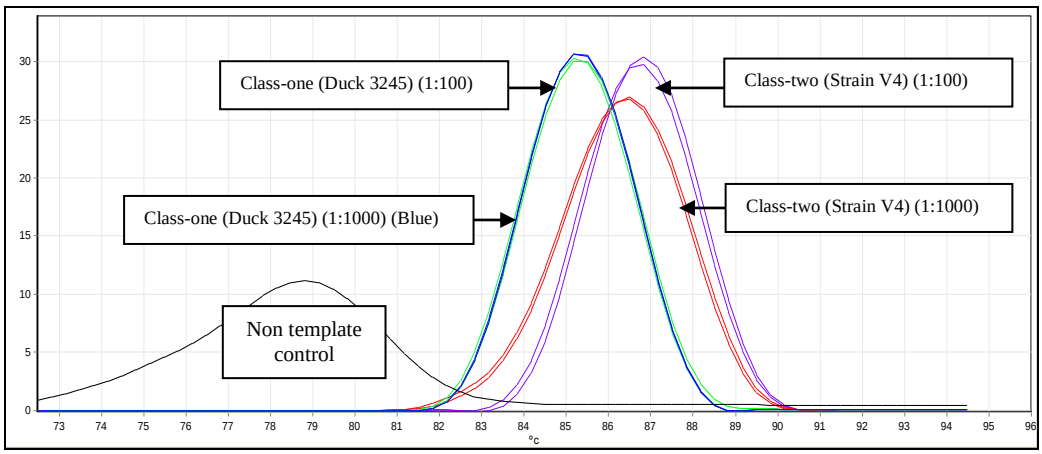


Figure 2.

The High Resolution Melt curve showing the 100% specificity of multiplex one step real time RT-PCR to detect Newcastle disease viral RNA from the Australian class-one and class-two reference viruses (Simmons, 1967; Mackenzie *et al*., 1984; Mackenzie *et al*., 1985; Spradbrow *et al*., 1995; Gould *et al*., 2001; Kattenbelt et al., 2006). These viruses have produced clearly different peaks. The X- and Y-axis of this figure represent melting temperature and the level florescent.

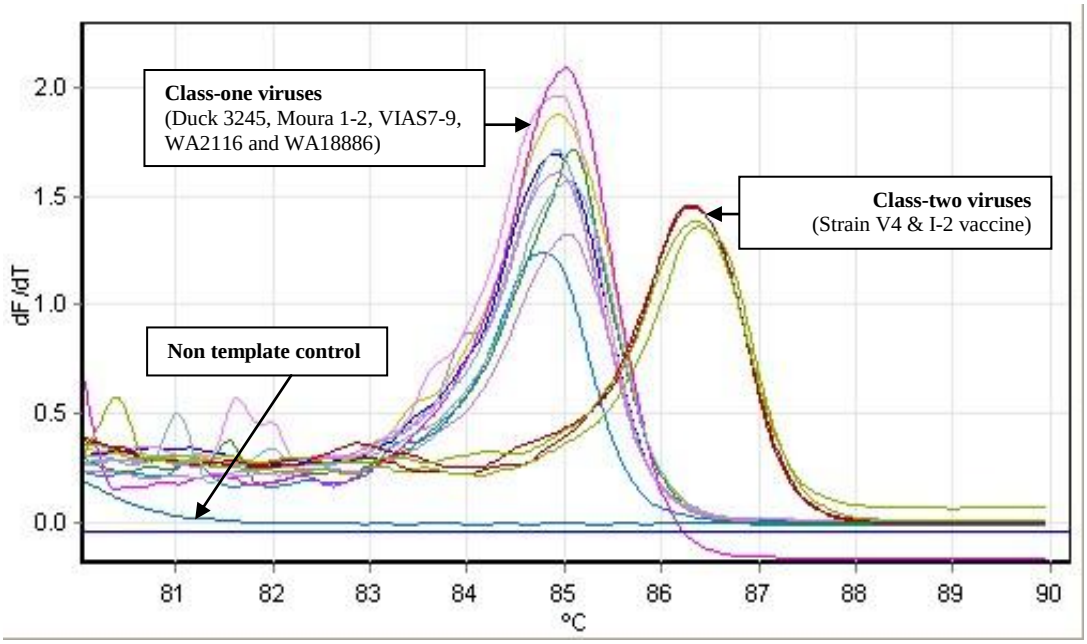
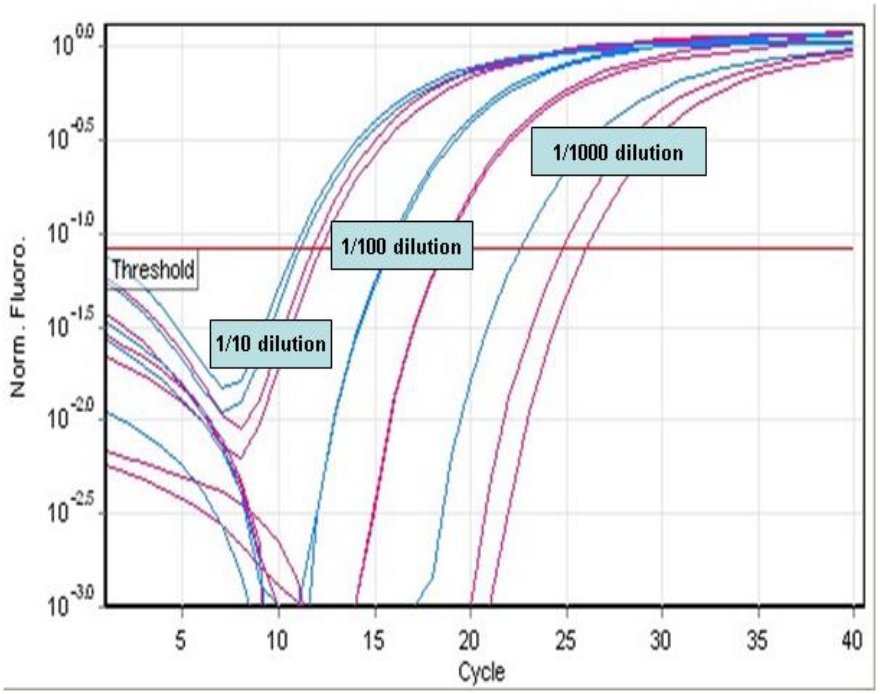


Figure 3.

Performance of the tenfold dilution quantitation of multiplex one step real time RT-PCR for the detection of Newcastle disease viral RNA. Blue and purple lines represent the Australian class-one (Duck 3245; Mackenzie *et al*., 1984; Mackenzie *et al*., 1985) and class-two (V4; Simmons, 1967) reference Newcastle disease viruses, respectively. No attempt was made to determine the number of copies being detected.



Supplementary sequence data

Fasta sequence of matrix gene of class-one Newcastle disease virus:

Plumed whistling duck (PWD-48)/Cromarty/ 09 (T1657)

GAACCGCATGTTATCCGATTGCCAATGCATTCCCTCAGGTGGCGAAGATATTGAGGAGTC
AGACAGCGAGTCTGCGTAGTGTCAAGGTGATCATTCAAGCTGGCACACA

Plumed whistling duck (PWD-55)/Cromarty/ 09 (C1694)

AACCGCATGTTATCCGATTGCCAATGCATCCCCTCAGGTGGCGAAGATATTGTGGAGTCAG
ACAGCGAGTCTGCGTAGTGTCAAGGTGATCATTCAAGCTGGCACA

Fasta sequence of matrix gene of class-two Newcastle disease virus:

Plumed whistling duck (PWD-46)/Cromarty/ 09 (C1643)

TGATAATCCCAAGCGCGAGTTACTTTCCCCTGCCATGCTCTGCCTAGGGAGTGTACCGAAT
GTCGGGGATCTTGTTGAACTGGCAAGAGCCTGCCTCACTATGGCGGTGACATGCAAGAAG
AGTGCAACTAACACTGAGAGAATGGTCTTCTCAGTAGTACAGGCACCCCAGGTGCTGCAA
AGTTGTAGAGTTGTGGCAAACAAATACTCGTCGGTGAATGCAGTCAAACACGTGAAAGCG
CCGGAGAAGATTCCTGGGAGTGGAACTCTAGAGTACAAAATAAACTTTGTCTCTCTGACC
GTGGTGCCGAAAAAGGATGTCTACAAGATACCAACTGCAGCACTTAAGGTTTCTGGCTCA
AGCCTGTACAATCTTGCGCTCAATGTCACTATTGATGTGGAGGTAGACCCGAAGAGCCCGT
TGGTCAAATCCCTTTCCAAGTCAGACAGTGGGTACTATGCTAATCTCTTCTTGCATATTGG
GCTTATGTCCACTATAGATAAGAAGGGGAAGAAAGTAACATTTGACAAGCTGGAAAAGA
AGATAAGGAGACTTGATCTGTCTGTAGGGCTCAGTGACGTGCTCGGACCTTCCGTACTTGT
AAAGGCGAGAGGTGCACGGACTAAGCTGCTGGCACCTTTCTTCTCCAGCAGTGGGACAGC
CTGCTATCC

Fasta sequence of fusion gene of class-two Newcastle disease virus:

Plumed whistling duck (PWD-46)/Cromarty/ 09 (C1643)

AGGCATGTGCAAAAGCCCCGTTGGAGGCATACAACAGGACATTGACTACCTTGCTTACTC
CTCTTGGTGATTCTATCCGTAGGATACAAGAGTCTGTGACCACGTCCGGAGGAGAGGGAC
AGGGACGTCTTATAGGAGCCATCATCGGTGGTGTAGCTCTCGGGGTTGCGACCGCTGCAC
AAATAACAGCAGCCTCGGCTTTGATACAAGCCAATCAAAATGCTGCAAACATCCTCCGGC
TCAAAGAGAGCATTGCTGCAACCAATGAGGCTGTGCACGAGGTCACTGACGGATTATCAC
AACTAGCAGTGGCAGTTGGGAAGATGCAGCAATTTGTTAATGACCAGTTTAATAAGACAG
CCCAGGAATTGGACTGTATAAAAATTACACAGCAGGTTGGTGTAGAACTCAACCTGTACT
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TCGTACTGTATAGAGACCGATTTGGATCTATATTGTACAAGAATAGTGACATTCCCTA