Final Report on

A Cross-Jurisdictional Model for Targeted Surveillance of Wild Bird Species

to the

Wildlife and Exotic Disease Preparedness Program,
The Department of Agriculture, Fisheries and Forestry

Submitted by

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Project Details

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Project Summary

Between 1/11/05 and 30/6/06 the laboratory processed 47 submissions of wild birds, comprising 103 individuals of 20 species, and encompassing 20 mass mortality events, as well as six individual bird mortalities. Diagnoses for the mass mortality events included:

- Organophosphorus compound (fenthion, fenamiphos and diazinon) poisoning
- Botulism
- Misadventure
- Necrotic enteritis

Just under half of the mass mortality events remained undiagnosed. This was attributed to a combination of poor sample quality and incomplete diagnostic workup as a consequence of funding constraints. In particular, the routine use of pesticide screens in these cases requires a formal cost-sharing agreement.

Most birds were tested at Biosecurity Sciences Laboratory (BSL) for the presence of Avian Influenza (AI) viruses using a real time RT-PCR developed at Australian Animal Health Laboratory (AAHL). Samples from a small number of healthy wild birds were also included for AI surveillance during an investigation into one of the larger mass mortality events. Two of 155 samples, derived from76 birds, gave low reactivity by PCR at BSL, but subsequently tested negative at AAHL. No AI viruses were isolated, and AI was excluded as a cause of mortality in all cases.

About half the submissions were screened for Newcastle Disease (ND) viruses, also using a real time RT-PCR developed at AAHL. All samples were negative. However, a ND virus was isolated, as an incidental finding, from cloacal swab samples which had tested consistently negative in the real-time PCR at both BSL and AAHL. The isolated virus was also consistently PCR-negative. This led to the suspension of the PCR for NDV screening of wild birds, pending the development of more appropriate primers.

Selected cases were investigated for the presence of West Nile Virus, with negative results. Material has also been referred to the Australian Registry of Wildlife Health for continuing investigations.

Cross-jurisdictional collaboration improved over the course of this project, with a greater range of participants than originally anticipated. This model was considered effective in gaining surveillance data on AIV in sick and dead wild birds. It was less effective in providing alternate diagnoses, and thereby increasing confidence in negative AIV findings. Some specific recommendations are made to improve the diagnostic rate and enhance disease surveillance; adequate funding is foremost amongst these.

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1. Introduction

1.1 Background

The pattern of avian influenza infection in the 2003-5 Asian epidemic represents a disturbing evolutionary development in the behaviour of the influenza A virus. The H5N1 virus emerged in (or prior to) 1996 when it caused an outbreak of influenza in geese in Guangdong with consequent high mortality. Over the last decade the virus has shown a high rate of evolution, and unusually wide and expanding host range. Its future behaviour, both as an animal pathogen and as a potential source of a human pandemic virus, is of particular concern[8].

Historically, wild bird submissions from Queensland Parks & Wildlife Service (QPWS) to DPI&F Queensland have focused on confirming suspected malicious poisonings[*]. Thus reliance on these submissions to support absence of exotic avian infections of primary industry and/or public health significance to Queensland and Australia is misplaced. QPWS and DPI&F have now developed protocols for the submission of samples positively biased towards identified high-risk AI species and morbidity/mortality events more likely associated with infectious disease[10]. To a large extent, these are the water fowl (Anseriformes) and migratory shore birds (Charadriiformes) previously identified as the most likely carriers of AI viruses[16].

1.2 Objectives

The aim of this project is to trial a new cross-Departmental approach to wild bird disease surveillance. The primary objective is to survey high-risk species and events for virulent AIV and NDV. The primary outcome will be improved confidence in negative AIV and NDV findings in Australian avifauna. A secondary outcome will be the provision of improved 'cause of morbidity/mortality' data to QPWS. The intent of this second outcome is not to necessarily provide a definitive aetiology in each submission, but to better classify the aetiology and thus provide improved identification of wildlife disease trends and a more informed basis for wildlife health management.

1.3 Acknowledgements

Many staff members of BSL, across different sections, contributed to this project. We thank duty pathologists Wafa Shinwari, Greg Storie, Shirley Turner, Selina Ossedryver and Bruce Hill for their handling of wild bird cases. The contribution of Ibrahim Diallo and Bruce Corney in meticulously testing many samples by PCR is gratefully acknowledged. Diagnostic support was provided by Paul Burrell and Paul Duffy (Bacteriology), Patrick Seydel (Chemical Residues), Mo Amigh (Histology), Barry Rodwell (Virology/Serology), Howard Prior (EM), Chris McCarthy and Ralph Stutchbury (Parasitology) and Brian Burren (Biochemistry). Administrative support was provided by Carol Kurylewski, Rosa Farrow, Helen Standfast, John McCarthy and David Waltisbuhl. Thanks also to Jeffrey Sayer and Sally Fox for assistance in the

[*] A finding of the post –WDA meeting *The Australian Wildlife Health Network in Queensland,* DPI&F Animal Research Institute, July14 2005.

necropsy room. Pat Kelly kindly explained the intricacies of courier transport and how to comply with UN3373 for the transport of diagnostic specimens.
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1.4 Abbreviations

AAHL	Australian Animal Health Laboratory, Geelong
AI	Avian Influenza
AWHN	Australian Wildlife Health Network
BSL	Biosecurity Sciences Laboratory
DPI&F	Department of Primary Industries and Fisheries, Queensland
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EPA	Environmental Protection Agency
FAT	Fluorescent antibody test
GC/MS	Gas chromatography/mass spectrometry
GIT	Gastrointestinal tract
HPAI	Highly pathogenic avian influenza
ND	Newcastle Disease
OC	Organochlorine
OP	Organophosphate
QPWS	Queensland Parks and Wildlife Service
RT-PCR	Reverse transcriptase - polymerase chain reaction
SEQ	Southeast Queensland
WNV	West Nile Virus
VTM	Virus Transport Medium

2. Methods

2.1 Necropsy

Birds were necropsied in a Class 2 biohazard hood when a necropsy assistant was available. Species identifications were based on descriptions in standard field guides[9,13], and nomenclature followed that recommended by Birds Australia[3].

A range of samples were collected at necropsy depending on the degree of autolysis, and variation between pathologists. For most submissions, samples were collected to exclude AI, at a minimum.

2.2 Serology/Virology

2.2.1 PCR

Samples of cloacal swabs and tissues (usually trachea, plus some or all of lung, brain, pancreas and intestine) in virus medium (VTM) were tested for the presence of AI and ND viruses using a real-time RT-PCR (Taqman) developed at AAHL[6] (Heine pers. comm.). Any AI reactive samples were also tested for H5 and H7 sub-types by real-time RT-PCR[6] (Heine pers. comm.), and duplicate samples were forwarded to AAHL for investigation.
Screening of wild birds for ND viruses by PCR test ceased midway through the project, when it was demonstrated that some Newcastle Disease viruses of wild birds could not be detected by this method (see Section 3.4.2).

2.2.2 Virus isolation

In a few cases, PCR testing was accompanied by attempts to isolate viruses in chicken eggs, using routine methods. Briefly, tissue homogenates and/or swab media were inoculated into the allantoic sac of 7-11 day old embryonated eggs (2 x 7 day passages). Allantoic fluids from the second passage were tested for haemagglutination activity and examined by electron microscopy.

2.3 Bacteriology

2.3.1 Botulism testing

Samples of whole blood, serum, fresh liver, gastrointestinal tract and environmental samples (water, invertebrate prey) were tested for *Clostridium botulinum* types C and D toxin using an ELISA developed by DPI&F[14]. Selected samples were also tested using the mouse bioassay at DPI&F's Tropical and Aquatic Animal Health Laboratory (TAAHL).

2.3.2 Other bacteriology

Swabs of selected tissues were collected at necropsy using aseptic technique. Culture for aerobes, spore-forming anaerobes and *Salmonella* were undertaken using routine methods.
The Serology laboratory of BSL tested impression smears of liver from two cases for the presence of *Chlamydia psittaci* by direct immunofluorescence, using a commercial test kit (Imagen™ ,Dako Diagnostics Ltd., UK).

2.4 Toxicology testing

2.4.1 Pesticides

Samples of fresh liver and/or gastrointestinal tract from 13 submissions were screened for organophosphorus (OP), organochlorine (OC) and synthetic pyrethroid pesticides by the DPI&F Biosecurity Chemical Residues laboratory, using a solvent extraction method, with Florisil cleanup followed by GC/MS. Samples from a further two submissions were tested by Queensland Health Scientific Services for a range of OP/OC pesticides.

To interpret the significance of any pesticide residues detected, concentrations in liver and/or GIT were firstly converted to an estimate of minimum whole body exposure, using an actual or estimated body weight for the bird. If these exposures were within an approximate order of magnitude of published LD50s for birds[15], they were considered significant (S.Ossedryver, pers.comm).

2.4.2 Other toxins

Stomach contents from three submissions were tested for strychnine by Biochemistry section of BSL.

Heavy metal (arsenic, cadmium, copper, lead, mercury) concentrations were determined for two submissions. Concentrations of arsenic, copper and lead were determined for one case by Biochemistry section of BSL. For the other case, concentrations of arsenic, cadmium, mercury and lead were determined by Queensland Health Scientific Services.

3. Results

3.1 Cross-jurisdictional collaboration

3.1.1. Submission of samples

The majority of submissions were made by officers of the EPA and DPI&F following notification from the public. However, many of the QPWS officers initially identified as being participants did not submit any birds during the course of this project, while officers from other branches of the EPA (eg Environmental Operations) became involved serendipitously. In addition, accessions were received from a wider range of collaborators than listed in the original proposal. These included submissions direct from private veterinary practitioners, and members of the public, as well as the following organisations:

- Queensland Health
- Currumbin Wildlife Sanctuary
- RSPCA
- Brisbane City Council
- Ipswich City Council
- Redlands Shire Council
- Laidley Shire Council

Many parties (including The Australian Wildlife Hospital at Beerwah, and the Gold Coast City Council) expressed interest in participation, but no submissions were made.

3.1.2 Testing of samples

Material from this project was referred to the following organisations for testing:

- AAHL
- Australian Registry of Wildlife Health
- Queensland Health Scientific Services
- Museum Victoria

3.2 Overview of submissions

3.2.1 Bird numbers and species

There were 52 submissions of wild birds between 1/11/2005 and 30/6/2006. There were 47 submissions, comprising 101 individuals of 20 species, from morbidity or mortality events mostly in SEQ (Table 3.1). In addition there were five submissions from 12 healthy wild birds of 4 species, which were sampled during mortality event# 10 (see Section 3.2.2 below) (Table 3.2).

Waterfowl, waders and shorebirds were well-represented amongst submissions. These included free-living (feral) domestic ducks, which were generally not identified to species. Although the initial intent of the project was to prioritise these bird groups for investigation, there were also many submissions of Australian magpies and related passerines. These were investigated in light of the large mortality of magpies and other wild birds in the Sydney basin in early 2006[2].

3.2.2 Mass mortality events

There were 20 mass mortality events, defined as multiple bird deaths within a limited geographic area (< 1 km^2, but generally confined to a single property) over a continuous time frame (from one day to several weeks). Multiple species were affected in ten events, a single species in six events, and insufficient history was available for four events. The number of dead birds ranged from three to approximately 100, but only five mortality events involved 15 or more birds. The laboratory received 1-13 submissions per event. A summary of these events is provided in Appendix 1, and some of the larger mortality events are described in Appendix 2.

3.2.3 Individual mortality events

Birds were sampled from six individual mortality events, summarised in Appendix 3. These cases involved species of wader or waterfowl for which the agreed EPA/DPI&F trigger for investigation is a single dead bird[10].

3.2.4 Quality of submissions

For mass mortality investigations, the majority of birds (62%) received were moderately to severely autolysed, and a couple had been frozen. Only 36% of submissions were fresh enough for worthwhile histopathology and/or microbiology. In contrast, all of the birds received for individual mortality investigations were suitable for a full diagnostic workup; five had been euthanased, and one was found reasonably freshly dead.

Table 3.1 Submissions of wild birds from single and mass mortality events in southeast and central Qld between 1/11/2005 and 30/6/2006

Family	Scientific name	Common name	No. submissions	No. birds received	No. birds sampled
Anatidae	*Anas superciliosa*	Pacific black duck	4	4	4
	Chenonetta jubata	Australian wood duck	2	18	7
	-	Duck (unidentified)	4	6	4
Sulidae	*Morus serrator*	Australasian gannet	1	3	2
Phalacrocoracidae	*Phalacrocorax varius*	Pied cormorant	3	4	4
Ardeidae	*Egretta garzetta*	Little egret	1	1	1
	Egetta novaehollandiae	White-faced heron	1	1	0
	Egretta sp.	Egret (unidentified)	1	2	2
Threskiornithidae	*Threskiornis molucca*	Australian white ibis	6	12	11
	Threskiornis spinicollis	Straw-necked ibis	2	2	1
	Threskiornis sp.	Ibis (unidentified)	1	2	2
Rallidae	*Gallinula tenebrosa*	Dusky moorhen	1	1	1
Laridae	*Sterna bergii*	Crested tern	1	1	1
Cacatuidae	*Eolophus roseicapilla*	Galah	1	1	1
Psittacidae	*Trichoglossus haematodus*	Rainbow lorikeet	2	2	2
Cuculidae	*Eudynamys scolopacea*	Common koel	2	2	2
Meliphagidae	*Manorina melanocephala*	Noisy miner	2	4	4
Dicruridae	*Grallina cyanoleuca*	Magpie-lark	2	2	1
Artamidae	*Cracticus torquatus*	Grey butcherbird	1	1	1
	Gymnorhina tibicen	Australian magpie	5	16	15
	Strepera graculina	Pied currawong	1	9	3
Corvidae	*Corvus orru*	Torresian crow	2	5	2
Sturnidae	*Sturnus vulgaris*	Common starling	1	2	2
TOTAL			47	101	70

Table 3.2. Healthy birds sampled during investigations for mortality event #10

Family	Scientific name	Common name	Submissions	No. of birds sampled
Threskiornithidae	*Threskiornis molucca*	Australian white ibis	1	4
Dicruridae	*Grallina cyanoleuca*	Magpie-lark	1	1
Corvidae	*Corvus orru*	Torresian crow	2	6
Sturnidae	*Acridotheres tristis*	Common myna	1	1
TOTAL			5	12

3.3 Diagnostic Overview

Of the twenty mass mortality events, the causes were considered to be:

- Organophosphorus compound poisoning (5 events; fenthion 3, fenamiphos 1, diazinon 1 presumptive)
- Botulism (presumptive in 4 events; suspected in 2 events)
- Misadventure (1 event)
- Necrotic enteritis (1 event)
- Undiagnosed (9 events)

Of the six individual mortality cases, the causes were determined to be:

- Trauma (3 cases)
- Inanition/parasitism (2 cases)
- Dermatitis (1 case)

3.4 Exotic viral exclusions

3.4.1 Avian Influenza

155 samples from 76 birds of 19 species were tested for Influenza A viruses using the Taqman PCR at BSL (Table 3.3).
153 samples were negative.
Two cloacal swab samples from two ibis which died in mortality event #10 yielded a low reactivity result. The first of these also yielded a low reactivity result in the H5 Taqman PCR assay at BSL. In both cases, further testing at AAHL was undertaken for the same and additional samples. All were confirmed negative by Taqman PCR at AAHL. This discrepancy was attributed to a difference in the sensitivity of the PCR as performed in each of the laboratories. The level of viral RNA detected in the BSL Taqman suggested a low viral load, which is not uncommon in wild birds as they are recognised reservoirs of all types of avian influenza virus[17].
No influenza viruses were isolated either at BSL or AAHL.
No samples for PCR testing or virus isolation were received from the koel mortality in central Queensland (mortality event #8), however a serum sample tested negative at AAHL in the competitive ELISA for antibodies to Influenza A virus.

3.4.2 Newcastle Disease

85 samples from 42 birds of 15 species were tested for Newcastle Disease viruses using the Taqman PCR at BSL (Table 3.3).
All samples were negative.
However, a paramyxovirus was isolated from cloacal swabs from an Australian white ibis from mortality event #10. At both BSL and AAHL a haemagglutinating agent was detected in allantoic fluid from passage 1 and 2, respectively. Electron microscopy at BSL identified a paramyxovirus, which was confirmed as a Newcastle Disease virus by the haemagglutination inhibition test at both BSL and AAHL. The isolated virus gave consistently negative results in the Taqman PCR at both BSL and AAHL. It was believed that the primers used did not sufficiently match the target sequence of the isolate being examined, as wild bird NDV isolates can be genetically different from poultry viruses[1].
As a consequence, screening of wild birds for Newcastle Disease viruses by Taqman PCR ceased for the remainder of the project.

Table 3.3. Screening of wild birds for AI and ND viruses by real-time RT-PCR at BSL between 1/11/05 and 30/6/06.

Species tested	AI PCR			ND PCR		
	Birds	Samples	Positives	Birds	Samples	Positives
Pacific black duck	4	8	0	2	4	0
Australian wood duck	7	16	0	2	6	0
Duck (unidentified)	4	10	0	4	10	0
Australasian gannet	2	4	0	-		
Pied cormorant	4	7	0	3	6	0
Little egret	1	2	0	1	2	0
Egret (unidentified)	2	2	0	-		
Australian white ibis	15	41	*1	8	19	**0
Straw-necked ibis	1	2	0	-		
Ibis (unidentified)	2	2	*1	-		
Dusky moorhen	1	3	0	1	3	0
Crested tern	1	2	0	1	2	0
Galah	1	2	0	-		
Rainbow lorikeet	2	4	0	1	2	0
Noisy miner	4	2	0	3	1	0
Magpie-lark	2	4	0	1	2	0
Grey butcherbird	1	1	0	1	1	0
Australian magpie	8	21	0	4	13	0
Pied currawong	3	6	0	-		
Torresian crow	8	11	0	7	9	0
Common starling	2	4	0	2	4	0
Common myna	1	1	0	1	1	0
TOTAL	76	155	2	42	85	0

* Tested negative at AAHL (see text)
** Newcastle Disease virus isolated at BSL and AAHL (see text).

3.4.3 West Nile virus

Paraffin blocks of tissues from two magpies from mortality event #7 were referred to AAHL for immunohistochemical exclusion of WNV. Sections of a wide range of tissues were stained with a monoclonal antibody, 4G4, against a conserved epitope of the NS1 protein of flaviviruses (including WNV and Kunjin virus), and no antigen was detected in any tissue.

A serum sample from a koel from central Queensland (mortality event #8) tested negative at AAHL in the competitive ELISA for antibodies to flavivirus. Paraffin blocks of tissues from the two submissions received from this mortality event were referred to the Australian Registry of Wildlife Health at Taronga Zoo for further testing, but no results have been received to date.

3.5 Bacteriology results

3.5.1 Botulism testing

The botulism testing undertaken during this project is presented in Table 3.4.

Table 3.4 Results of botulism testing for nine mass and one individual mortality event in wild birds between 1/11/05 and 30/06/06

Mortality event #	Species tested (no. birds)	ELISA			Comment
		No. samples	No. suspect	No. positive	
*0	Pacific black duck (1)	2	0	0	
3	Duck (unidentified 1)	4	0	0	
4	Pacific black duck (1)	2	0	0	
5	Duck (unidentified 1)	2	0	0	Clinical signs consistent with botulism
6	Duck (unidentified 1)	2	0	2	Carcase in advanced autolysis
9	Wood duck (2)	2	0	0	Diazinon poisoning
10	Australian white ibis (4)	7	1	2	Two ELISA +ves from one bird in moderate autolysis (#1). ELISA suspect result from one bird in advanced autolysis (#4). Mouse inoculation test on samples from 3 birds (#1-3): #1 suspect, #2,3 neg.
10	Little egret (1)	1	0	0	Mouse inoculation test: neg
10	Polychaete worms/water	1	0	0	
11	Pied cormorant (1)	2	0	0	
16	Pacific black duck (1)	1	0	0	
18	Torresian crow (1)	2	0	2	Carcase in advanced autolysis

* Represents an individual mortality event

A presumptive diagnosis of botulism was made for mortality event #5, 6, 10 and 18 on the basis of clinical signs or ELISA results. Botulism was strongly suspected as a cause of mortality events #3, 4 on the basis of clinical history, and remains amongst the differential diagnoses for mortality events #16, 20.

3.5.2 Other bacteriology results

Clostridium perfringens (untyped), together with *E. coli* and streptococci, were isolated from the intestine and peritoneum of a rainbow lorikeet with necrotic enteritis (mortality event #14). No significant isolates were obtained from liver swabs of a Pacific black duck (mortality event #16).

Liver impression smears from a rainbow lorikeet and a galah (mortality event #14) stained negatively in a direct immunofluorescence test for *Chlamydia*.

3.6 Toxicology Results

3.6.1 Pesticides

Fifteen submissions, from twelve of the 20 mass mortality events, were screened for pesticides. Five mortality events were attributed to OP compound poisoning. Concentrations recovered from these cases are given in Table 3.5.

Table 3.5. Concentrations of OP compounds recovered from wild bird poisonings between 1/11/05 and 30/06/06. NT, not tested.

Mortality event #	Species	Compound	Concentration (ppm wet weight) Liver	GIT
9	wood duck	Diazinon	0.32	NT
12	magpie-lark	Fenthion	0.26	12.00
12	Australian magpie	Fenthion	0.14	12.50
13	Torresian crow	Fenthion	0.05	150.00
15	pied currawong	Fenthion	NT	60.00
19	duck (unidentified)	Fenamiphos	NT	8.00

Although the concentration of diazinon in the livers of wood ducks from mortality event #9 was relatively low, it was considered likely that GIT concentrations would have been substantially higher; therefore a presumptive diagnosis of diazinon poisoning was made in this case.

Low levels of OC compounds (*pp*DDE, dieldrin, heptachlor epoxide) were demonstrated in liver or GIT from 4 submissions; these were consistent with bioaccumulation, rather than acute toxicity.

3.6.2 Other toxins

Stomach contents from birds in mortality events #7, 12 and 13 were tested for strychnine, and all were negative.

No abnormally high levels of heavy metals (some of arsenic, cadmium, copper, mercury and lead) were detected in tissues from two submissions (kidney, liver and GIT of one dusky moorhen from mortality event #3, and liver of one koel from mortality event #8).

4. Discussion

4.1 Sample submission

4.1.1 Submission kits

It was originally planned to lodge submission kits with several QPWS centres in SEQ. They were to contain suitable packaging, guidelines for sample submission and pre-printed courier consignment notes, and their intent was to encourage submissions by defraying courier costs. However, the logistics proved challenging, and the attempt was abandoned in the face of a steady stream of submissions arriving via DPI&F and QPWS officers.

Bird carcases are classified as “Biological Substance, Category B” and therefore assigned to UN3373 under the current IATA Dangerous Goods Regulations[7]. They require both specialised packaging and training of staff responsible for packing. Suitable packaging is available from the BSL Store, but the provision of a simplified packing instruction to submitters with no training or experience required labour in excess of what was available for the current project. It is not an insurmountable obstacle to future projects.

4.1.2 Sample quality

The majority of submissions from mass mortality events were in moderate to advanced autolysis. The proportion was skewed by mortality event #10, for which 12 of 13 submissions comprised markedly autolytic birds (see Appendix 2). Most submitters were aware of the requirement for freshly dead carcases, and could distinguish these from autolysed ones. Submission of decomposing birds, unsuitable for anything but PCR testing, was driven by Departmental imperatives outside of this project's protocol. It is likely that fresher carcases (of sick or euthanased birds) could be obtained from wildlife hospitals and carers; several such institutions had expressed interest in participating, but few or no submissions were received from them. It is likely that the strategic placement of submission kits with these organisations would have encouraged submission of carcases suitable for diagnostic workup. An alternative option would be to have an intermediary, such as the QPWS, deliver suitable carcases to the laboratory from these institutions. An informal arrangement like this has recently started (D. Stewart, QPWS, pers. comm.).

4.2 Laboratory investigations

4.2.1 Incomplete diagnostic workup

Apart from autolysis, several factors contributed to incomplete diagnostic workups of submitted birds. Foremost amongst these were funding constraints, which dictated that more expensive assays, such as pesticide screens, were not run routinely, even when other findings were negative. A pesticide screen at BSL costs $200 per sample, and ideally >1 sample (liver and GIT contents) is tested per bird. Although QPWS regularly pays for pesticide screens of selected submissions, financial liability became unclear during this project, since QPWS were often acting as an intermediary to deliver birds to the laboratory, but had not initiated the investigation. For future projects, it is important to budget more realistically for the costs of a full diagnostic investigation that covers a range of disciplines, including pesticide screens. A formal cost-sharing arrangement between interested parties at all levels of government (local council, state, and federal) may be required.

In addition, although the standard pesticide screen in use at BSL detects a wide range of OP, OC and synthetic pyrethroid pesticides, it is likely that some intoxications will not be detected. For example, there was a suggestion that mortality event #7 (undiagnosed mortalities of 25 Australian magpies and one magpie-lark) was associated with use of imidacloprid ("Confidor"), a chloronicotinyl insecticide. Approaches to a private pharmaceutical company for help assaying this compound were unproductive, and intoxication by this product remains a diagnostic possibility.

Finally, there was substantial variation in diagnostic approach between the six pathologists involved in processing these submissions, as well as confusion in communications to submitters. Having a single pathologist primarily responsible for these investigations would result in a more consistent approach to the types and extent of testing undertaken. It would also result in more consistent communications with other project participants.

4.2.2 PCR limitations

Two findings from this project have implications for the continued use of the Taqman PCR for screening wild birds for AI and ND viruses. Firstly, differences in sensitivity of the AI PCR between institutions were demonstrated (Section 3.4.1). This matter has been referred to AAHL for resolution. Secondly, the insensitivity of the ND PCR as a

screening test in wild birds has also been demonstrated (Section 3.4.2), and is the subject of proposed further research[4].

4.2.3 Botulism diagnosis

Botulism diagnosis was problematic in this study. Since the ELISA used has low sensitivity[14], negative results do not rule out botulism. A presumptive diagnosis of botulism was made for mortality event #5, despite the negative ELISA, since the submitted bird presented with classical signs of botulism, including generalized flaccid paralysis, "limber-neck", and diarrhoea. Similarly, negative ELISAs were obtained for samples from mortality events #3 and 4, but in these cases there were either anecdotal reports of "limber-neck" amongst affected waterfowl (event #4), or other investigations, including pesticide screens, were negative (event #3). The mouse bioassay has higher sensitivity than the ELISA[14], but ethical constraints have led to a widespread reduction in its use. It was only used in the present study to investigate mortality event #10 (Table 3.4), where it was unhelpful in confirming botulism as the diagnosis.

The significance of the positive ELISA results obtained for mortality events #6, 10 and 18 (Table 3.4) is unclear, since all samples were obtained from carcasses which were moderately to markedly autolytic. Many birds carry Type C botulism spores; these can germinate and produce toxin after the bird's death, regardless of the cause of death[11,12]. Despite this, ELISA false-positives resulting from *post-mortem* production of toxin are rarely encountered at BSL (R. Thomas, pers. comm.). Botulism was diagnosed presumptively for these events, because other findings, including pesticide screens, were negative (#10, 18), or because the history (species affected, time of year) was highly suggestive of botulism (#6).

The reliability of future botulism diagnosis could be improved by limiting ELISA testing to freshly-dead carcases, as well as the use of more sensitive diagnostic tests, such as PCR[5].

4.3 Summary and recommendations

This project was effective in establishing a network of cross-jurisdictional submitters, and in gaining surveillance data on AI viruses in sick and dead wild birds. It was less effective in providing alternate diagnoses, and thereby increasing confidence in negative AI findings. It is important to make a clear distinction between birds sampled for the sole purpose of exotic viral exclusions, and those where a disease investigation is required (i.e. determining cause of mortality). Distinct submission and testing protocols should apply to these two categories. Where disease investigations are required, a higher diagnostic rate would be achieved by adopting the following recommendations, in order of priority:

1. Secure adequate funding to cover expensive diagnostic workups, including screening for pesticides where appropriate. Cost-sharing between governments/organisations may be required.
2. Appoint one pathologist to be primarily responsible for disease investigations, to ensure a consistent approach to the testing undertaken.
3. Increase the proportion of freshly dead carcases through (i) Strategic placement of submission kits with wildlife hospitals, including a simplified packing instruction so that staff without training can package carcases in compliance with UN3373 and/or (ii)

formalising an arrangement between QPWS and wildlife hospitals to deliver suitable carcases to the laboratory.
4. Avoid use of the botulism ELISA on decomposing carcases. Support development of more sensitive diagnostic assays for Type C botulism.
5. Investigate possibilities for assaying chloronicotinyl pesticides, either in-house, or by referral.

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Appendix 1. Summary of mass mortality events in wild birds investigated between 1/11/2005 and 30/06/2006

	Location	Date	Species	Mortality	No. submissions	Diagnosis	Comments
1	Sherwood	15/11/05	Australian magpie (2) pigeon (unidentified) (1)	3	1	No diagnosis Peritonitis in one magpie	No pesticide screen
2	Kilcoy	19/11/05	noisy miner	3	1	No diagnosis	Autolysed; limited workup
3	Hugh Muntz Lake (Gold Coast)	Feb 06	domestic duck (feral) dusky moorhen	6	2	No diagnosis (suspect botulism)	Associated with fish kill Negative pesticides
4	Runaway Bay (Gold Coast)	16/06/06	Pacific black duck swan (unidentified)	8	1	No diagnosis (suspect botulism)	No pesticide screen
5	Morayfield	Jan/Feb 06	wild ducks (unidentified) Muscovy ducks	6 or 7	1	Presumptive botulism	Associated with fish kill
6	Narda Lagoon, Laidley	16/02/06-23/02/06	wild ducks (unidentified)	est 100	1	Presumptive botulism type C/D	Autolysed No pesticide screen
7	Thorneside	17/03/06-23/03/06	Australian magpie (25) magpie-lark (1)	26	3	No diagnosis	Adjacent to wader roosts Negative pesticides Negative WNV IHC See Appendix 2
8	Tannum Sands (central Qld)	Mar 06	common koel (10) channel-billed cuckoo (1)	11	2	Myelomalacia	Aetiology uncertain Referred to ARWH
9	Glenwood	5/04/06	wood duck	10	1	Presumptive diazinon poisoning	Autolysed
10	Wynnum	27/03/06-29/05/06	Australian white ibis (22) egret sp. (5) white-faced heron (1) heron sp. (1) Torresian crow (2) magpie-lark (1) common starling (1) rainbow lorikeet (1)	34	13	Misadventure Presumptive botulism type C/D	Adjacent to wader roosts See Appendix 2

	Location	Date	Species	Mortality	No. submissions	Diagnosis	Comments
11	Moreton Island	13/04/06-19/04/06	pied cormorant	4	3	No diagnosis	Possible parasitic gastritis Negative pesticides Negative botulism ELISA
12	Capalaba	30/04/06-2/05/06	Australian magpie (12) magpie-lark (1) rainbow lorikeet (1) grey butcherbird (1)	15	3	Fenthion poisoning	
13	Aspley	14/04/06-27/04/06	Torresian crow (13) Australian magpie (3) pigeon sp. (5) butcherbird sp. (2) kookaburra (1) lorikeet (1)	25	1	Fenthion poisoning	
14	Regents Park	10/05/06-11/05/06	rainbow lorikeet (6) galah (1)	7	2	Necrotic enteritis	No diagnosis for galah See Appendix 2
15	Aroona (Sunshine Coast)	16/05/06	pied currawong	10	1	Fenthion poisoning	
16	Belmont	Apr/May 06	Pacific black duck	8	1	No diagnosis	Autolysed No pesticide screen Negative botulism ELISA
17	Castaway Bay (Sunshine Coast)	25/05/06	Australasian gannet	3	1	No diagnosis	Autolysed; limited workup
18	Clontarf	30/05/06	Torresian crow	Several (not specified)	1	Presumptive botulism type C/D	Negative pesticides
19	Chambers Flat	1/06/06	ducks (unidentified)	6	1	Fenamiphos poisoning	
20	Carole Park	9/06/06	wood duck	10	1	No diagnosis	Autolysed; limited workup

Appendix 2: Details of Selected Mass Mortality Events

Mortality Event #7

History: Deaths of 25 Australian magpies and 1 magpie-lark were reported on adjacent properties at Thorneside between 17/3/06 and 23/3/06. These properties back onto tidal flats close to Moreton Bay, and a wader roost site was reported to be nearby. Affected magpies were reported to be unable to fly, and then progressively unable to stand, with a tendency to fall forwards. Some were found dead. Several birds were treated or euthanased by a private veterinary practitioner.

Laboratory findings: Three dead magpies were received in separate submissions. The first had been exhumed, and was of limited use.
Gross/histopathology: No significant findings
Viral exclusions: Eight samples from 3 birds tested negative in the AI and ND PCR. Selected tissues from two birds were negative for WNV immunohistochemistry, undertaken at AAHL.
Toxins: Liver and GIT of one magpie contained low levels of *pp*DDE; this was considered an incidental finding, consistent with bioaccumulation. Strychnine was not detected in stomach contents of this same bird.

Diagnosis: Open.

Comment: There was anecdotal evidence of a high level of imidacloprid ("Confidor") use in the area around that time. This group of compounds (chloronicotinyl pesticides) are not detected by the pesticide screens currently in use at BSL, though they may be in the future.

Mortality Event #10

History: A sewerage treatment plant at Wynnum experienced increased mortalities of wading birds (primarily Australian white ibis, and a few herons and egrets) during April and May, 2006. The usual background mortality rate at this site is 1 bird/month, whereas 29 waders were found dead between 27/03/06 and 29/05/06. The plant abuts Moreton Bay, and is in close proximity to a roost site for migratory waders; several bird species are known to interchange between the two sites, sparking concerns about HPAI. At the start of the outbreak, plant workers reported increased numbers of polychaete worms (*Namalycastis abiuma*; Polychaeta, family Nereididae) accumulating in the shallow concrete channels surrounding the six secondary treatment tanks (biological filter tanks). The water in these shallow channels undergoes variable flow rates, and drains via 1m deep sumps and pipes with baffles to the centres of two tertiary tanks. Waders were observed gathering within the channels to feed on the polychaetes, and wader carcases were found in the centres of the two tertiary tanks. The consistently autolysed state of these carcases suggested that they had emanated from the secondary tank channels, and were delayed by the presence of baffles in the pipes. Most carcases were recovered from the tertiary tank draining the channels containing the majority of feeding birds. A few passerine carcases were found on the surrounding grass. No sick birds were observed at any stage of the outbreak.

Mortalities ceased following the installation of wire mesh over the sump leading from the secondary tank channels to the tertiary tanks (Fig. 1).

Fig. 1. Shallow channel surrounding secondary treatment tank at Wynnum waste water treatment plant, showing wire mesh in place over sump draining to tertiary tank.

Laboratory findings:
Twelve submissions of autolysed waders, and a few passerines, were made to the laboratory. In addition, an autolysed rainbow lorikeet was submitted from a nearby property.
Gross/histopathology: Multifocal, granulomatous, parasitic, intestinal serositis was an incidental finding in one Australian white ibis, which was the freshest bird examined. Advanced autolysis in the remaining submissions precluded satisfactory histopathology.
Serology/Virology: AI PCR: 27 samples from 15 birds were tested, with two positive (both from ibis) at BSL, but negative at AAHL. No AI viruses were isolated from these two cases. An additional 18 samples, derived from 12 healthy birds at the site (see Table 3.2) all tested negative for AI viruses.
ND PCR: 15 samples from six birds were tested, with no positives. However a ND virus was isolated from cloacal swabs of one of these birds (an Australian white ibis) at both BSL and AAHL. PCR testing of this isolate was also negative at both BSL and AAHL.
Botulism testing: Nine samples (tissues from five birds, polychaete worms, channel water) were tested by ELISA, and four bird tissue samples were tested by mouse inoculation. Samples of liver and GIT from the first bird (an Australian white ibis) received from the outbreak were strongly positive by ELISA, but only suspect by mouse test. Other mouse tests were negative. A suspect ELISA result was also obtained from GIT of another Australian white ibis.
Pesticides: Low levels of heptachlor epoxide and dieldrin were detected in liver, but not GIT, of one Australian white ibis; this was considered an incidental finding, consistent with bioaccumulation.

Diagnosis: Misadventure (drowning). Possible botulism Type C or D early in the outbreak. HPAI was ruled out as a cause of mortalities.

Comment: It was considered likely that waders feeding on polychaetes in the secondary treatment tank channels fell into the sump, and were trapped in the pipe by the baffles, leading to their delayed appearance in the tertiary tank. Botulism may have played a role in allowing weakened birds to drown more readily, however the absence of clinically affected birds casts doubt on this hypothesis. The cause of death for the few passerines found dead on the grass is unknown.

Mortality Event #14

History: Six rainbow lorikeets and one galah were found dead on two properties in the same street in Regents Park between 10/05/06 and 11/05/06.

Laboratory findings: One rainbow lorikeet and one galah were submitted to the laboratory.
Gross/histopathology: The lorikeet had severe, acute, transmural necrotising enteritis, with gross evidence of peritonitis. There were no significant gross or histological findings in the galah.
Bacteriology: A mixed culture of *E. coli*, streptococci and *Clostridium perfringens* was cultured from both intestine and peritoneum of the lorikeet. Both birds were negative in the FAT for *Chlamydia psittaci.*
Serology: Both birds were negative in the AI PCR.
Pesticides: The GIT contents of the galah were negative for pesticides.

Diagnosis: Rainbow lorikeet: Necrotic enteritis
Galah: Open

Appendix 3. Summary of individual mortality events investigated between 1/11/2005 and 30/06/2006

	Location	Date	Species	Diagnosis	Comments
1	Ipswich	08/02/06	Pacific black duck	Trauma	Tibiotarsal fracture
2	Carina	08/02/06	Australian white ibis	Trauma	Bilaterial tarsometatarsal fractures
3	South Brisbane	14/02/06	Pacific black duck	Dermatitis of undetermined aetiology	Negative botulism ELISA
4	Clear Island Waters (Gold Coast)	28/02/06	Crested tern	Inanition/parasitism	
5	Banyo	16/05/06	Straw-necked ibis	Inanition/lice infestation	Found dead
6	Murphys Creek (Toowoomba)	27/06/06	Straw-necked ibis	Trauma	Femoral fracture