THE CREATION OF AN AVIAN VACCINE FOR WEST NILE VIRUS

by

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Abstract

West Nile Virus (WNV) arrived in North America in 1999 and has since caused significant morbidity and mortality, mainly in birds but also in horses and humans. Many families of birds, especially corvids, are highly susceptible to WNV, with infections often resulting in fatalities. Avian species susceptible to WNV infection also include endangered species, such as the Greater Sage-Grouse (*Centrocercus urophasianus*) and the Eastern Loggerhead Shrike (*Lanius ludovicianus migrans*). Although WNV is now endemic throughout the continent, to date there is no veterinary vaccine available for birds. This thesis focuses on the use of a recombinant adenovirus to construct vaccines against WNV, that would contain either the envelope or the NS3 ‘genes’ from WNV.

To assist in assessing the vaccines, work was undertaken to assess to what extent avian antibody reagents could be used in an avian species for which the antibody was not created. The duck specific CD8 antibody, Du-CD8-1 and the chicken/turkey specific CD4 antibody, CT4, bound to Japanese Quail T cells. The CD4 and CD8 antibody reagents were used to analyse Japanese quail T cell populations, establishing the proportions of CD4+ and CD8+ cells, and discovering a previously unreported population of CD4/CD8 double positive cells. An anti-bird IgG antibody was found to bind to chicken, House Sparrow and Japanese Quail IgY; the anti-bird IgG antibody was able to detect IgY from these three species when used as part of a serum ELISA assay.

Results from initial vaccine testing in Japanese Quail (*Coturnix japonica*), indicated that the vaccines activated more T cells and triggered production of higher levels of antibodies in vaccinated birds compared to unvaccinated controls. This was achieved using an intracellular interferon gamma (IFN-γ) assay to assess T cell activation and a serum ELISA to measure
levels of WNV specific antibodies. During a challenge assay, using a wild population of House sparrows (*Passer domesticus*) following infection with WNV, vaccinated birds showed overall reduced levels of viremia compared to unvaccinated controls.
Preface

All the work on infections of House Sparrows with West Nile virus, done as part of the challenge assay for the vaccines and detailed in chapter 6, was done in collaboration with Dr. Robbin Lindsay, Head of Field Studies in the Zoonotic Diseases and Special Pathogens Section of the National Microbiology Laboratory, Public Health Agency of Canada. The work was undertaken at the biocontainment level 3 facility at Arlington Road, Winnipeg, that falls under the remit of the Public Health Agency of Canada (PHAC). All experimental protocols involving House Sparrows, were conducted within guidelines issued by the PHAC Animal Care Committee. The committee also issued an animal use document, numbered H-11-014, approving all experimental work and housing conditions for the birds.

Dr. Lindsay provided advice on experimental set up, running of assays and troubleshooting problems with the assays, as well as a lot of practical help with injecting the birds and harvesting samples. Whilst I undertook the majority of the day to day care of the birds and their environment, Dr. Lindsay also undertook those tasks for me on a few occasions. Other members of his team, notably Ms. Antonia Dilberardo, also provided guidance and practical assistance with the running of the assays in the lab. However, the final decisions on all assays and protocols, within set guidelines, were mine; all practical set up of assays, running of samples once harvested and analysis of data was also all my own work.

All experimental protocols involving Japanese quail were undertaken at UBC and were approved by the UBC Animal Care Committee. The committee issued certificate number A08-0628 as approval for all experimental work and housing conditions for the birds.
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<tr>
<td>APC</td>
<td>allophycocyanin, a fluorescent marker used during flow cytometry</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate (used in polyacrylamide gels for western blots)</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD4</td>
<td>cell surface marker to identify helper T cells</td>
</tr>
<tr>
<td>CD8</td>
<td>cell surface marker to identify cytotoxic T cells</td>
</tr>
<tr>
<td>CEF</td>
<td>chicken embryonic fibroblasts</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxyribonucleotide triphosphate (equimolar mix of the four bases)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescent-activated cell sorter</td>
</tr>
<tr>
<td>FB</td>
<td>FACS Buffer</td>
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<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate, a fluorescent marker used during flow cytometry</td>
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<td>HOSP</td>
<td>standard four-letter abbreviation for House Sparrows (<em>Passer domesticus</em>) [1]</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
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<tr>
<td>IFU</td>
<td>infection forming unit, a measurement for quantifying viruses (see glossary)</td>
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<tr>
<td>IgG/IgY</td>
<td>immunoglobulin G or Y family, often used synonymously with reference to avian antibodies, although avian IgY is different from mammalian IgG</td>
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<tr>
<td>JAQU</td>
<td>standard four-letter abbreviation for Japanese Quail (<em>Coturnix japonica</em>) [1]</td>
</tr>
<tr>
<td>Kb</td>
<td>kilo base</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MCMV</td>
<td>murine cytomegalovirus</td>
</tr>
<tr>
<td>mfu</td>
<td>mean fluorescent unit</td>
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<td>ml</td>
<td>millilitre</td>
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MOI: multiplicity of infection, the number of viral particles per cell
MSL: Michael Smith Laboratories
NML: National Microbiology Lab
pAb: polyclonal antibody
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PE: phycoerythrin, a fluorescent marker used during flow cytometry
PFU: plaque forming unit, a measurement for quantifying viruses (see glossary)
qRT-PCR: quantification real-time PCR
rpm: revolutions per minute
RPMI 1640 medium: Roswell Park Memorial Institute, tissue culture medium
SDS: sodium dodecyl sulphate
U.S.: United States (of America)
WCSP: standard four-letter abbreviation for White Crowned Sparrow (Zonotrichia leucophrys) [1]
WN(V): West Nile (Virus)
Glossary

All terms are defined in relation to their specific use within this document.

293 Cells: is the name of a cell line stably transfected with adenoviral DNA. These cells are able to support replication of adenovirus particles that are missing certain ‘genes’ necessary for the virus to replicate in normal, untransfected, cells. Such viruses are replication-deficient, see below.

Autofluorescent cells: cells that automatically generate high background levels of fluorescence when analysed by flow cytometry. This means that any signal specifically from fluorescent antibodies on the cell cannot be easily seen; the cell has to be excluded from analysis.

Epitope: a localised region on the surface of an antigen that binds to products of the immune response. It also refers to the T cells or B cells specific to that region of the antigen.

IFU (infection forming unit): a measurement used to quantify viruses, whereby the number of viral particles that can infect cells are quantified. This is a measure of infectious viral particles, not a count of total viral particles present. IFU is related to PFU.

Infect (an animal): introduction of a virus (adenovirus or WNV) into the animal’s body, at levels sufficient to allow expression of viral proteins and, if the virus is pathogenic (WNV), to induce signs of disease. Viral replication will also occur, if the virus is able to do so.

Infect (a cell or cell line): the virus is able to penetrate the cellular membrane and enter the cell.

Infectious: containing levels of virus sufficiently high that transfer of the virus to another individual could occur, for WNV this would be if the infectious animal was bitten by a mosquito.

PFU (plaque forming units): a measurement used to quantify viruses, whereby the number of viral particles that can infect cells and cause visible deleterious effects (plaques) are quantified. This is a measure of fully functional viral particles, not a count of total viral particles present.

Replication-deficient virus: the virus retains the ability to infect cells but is unable to replicate itself once inside normal cells. This is because ‘genes’ essential for either replication or packaging of the viral particles have been removed. The recombinant adenoviruses are of this type and are only able to replicate in cells transfected with the missing viral ‘genes’, such as 293 cells (see above).
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Dedication

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Chapter 1: Introduction

West Nile virus (WNV) is endemic throughout North America and is a cause for concern to both human and avian populations. With birds being an essential host for the virus, they are uniquely targeted for infection, with devastating results for highly susceptible species, particularly if such species are also rare or endangered. An avian vaccine against WNV would be desirable, especially for the latter group of birds to assist in preventing possible extinction. As no such vaccine is currently available specifically for birds, the main aim of this thesis was to develop and test candidate vaccines using avian species as animal models.

This introductory chapter provides background information on WNV and its effects on birds as well as details of the avian immune system and WNV vaccines already tested on birds. The selected vaccine platform (of live recombinant adenoviruses) is discussed, including details of how these viruses have already been shown to act as vaccines. Toward the end of this chapter, details of the precise designs of the candidate WNV vaccines are described and specific objectives and hypotheses for the thesis are stated.

1.1 West Nile Virus

WNV was first isolated in Uganda in 1937, from a woman presenting a mild febrile illness. This, as well as additional human infections demonstrating similar symptoms, served to indicate that infection with WNV was fairly harmless for humans [2]. It was in studies run during the 1950’s, that researchers undertaking serological surveys of the same region, found most WNV human infections to be asymptomatic. It was around the same time that basic details of the transmission cycle were first identified, namely that mosquitoes act as vectors
and birds as the main amplifying hosts for WNV [2]. Over the ensuing decades there have been several sporadic outbreaks of WNV, most causing little illness in humans, until a spate of outbreaks occurred around the Mediterranean Basin during the 1990’s. Starting in Algeria in 1994, other recorded WNV outbreaks followed, in Romania in 1996, Tunisia in 1997, Israel in 1998 and Russia in 1999 [3]. These later outbreaks differed from the earlier reports as the virus now caused more disease signs in humans, including fatal encephalitis cases, mostly in elderly humans, which had previously only rarely been seen. Not only were new virulence patterns being seen in WNV itself, new geographical regions were also being opened up to the virus as well, as in 1999 the first reports of WNV infections in North America were reported [4]. It was apparent that by some means the virus had arrived in New York City, likely via an infected bird or mosquito arriving at the airport or seaport. From the higher levels of disease seen in people in New York infected with WNV, it seemed likely that it was the more virulent form of the virus that had arrived; genetic analysis of the New York strain (NY-Q99) found that its RNA sequence was almost identical to an isolate from the outbreak in Israel in 1998 [2]. The method of its arrival may never be discovered but the effects of its arrival soon became apparent. As early as mid-August 1999 unusual die offs of birds, mostly of crows and related species, were seen in wild bird populations around the New York area. Throughout the late summer local zoos were seeing deaths in a wide range of avian species, including, flamingos, cormorants, Bald Eagles and Snowy Owls [5]. By October 1999, 62 human cases of encephalitis had been reported with 7 confirmed fatalities, all of which were subsequently found to be due to infection with WNV. With this new more virulent form of WNV, human symptoms now demonstrated a wider range, although 80% of human infections still showed no signs or symptoms at all. Approximately 20% of human
cases present the non-neurological form of the disease called West Nile fever and can show symptoms of mild fever, muscle fatigue and anorexia. The acute neurological form of disease caused by WNV can include symptoms such as headaches, acute fever, encephalitis, haemorrhage and flaccid paralysis, but this occurs in less than 1% of human cases [6]. These initial deaths were followed annually by higher levels of human infections and deaths for several years and an on-going spread of WNV, until as early as 2002 it was considered epidemic in nearly all of North America and had reached as far as Mexico and the Caribbean as well as into Canada [3]. So far, annual numbers of human infections and deaths peaked in 2003 for the U.S., with values of 9,862 and 264 respectively [7] and in 2007 in Canada when 2,215 cases were recorded but no deaths [8]. Since these peak years, annual cases have fallen to an average of only 38 in Canada and approximately 2,000 in the U.S., although for the last 3 years (2009-2011) in the U.S. total case numbers have been less than 1,000 [7, 8]. Numbers have likely fallen due to the prior exposure to WNV that most areas, and therefore most residents, of the U.S and Canada have now had, however despite this WNV is still able to cause considerable problems throughout the continent.

The arrival of WNV in North America also initiated increased interest in the virus from a molecular point of view and many studies were undertaken to explore the structure, genetics and mechanisms of infection and replication of WNV. WNV is a Flavivirus, the same family as Dengue Virus, Murray Valley Encephalitis Virus and Yellow Fever Virus, but within the Japanese Encephalitis serocomplex, which also contains Kunjin Virus. WNV consists of encapsulated isosahedral particles of 50 nm in diameter that contain a single positive sense strand of RNA which is approximately 11 kb long [9]. A single polyprotein is produced, which is subsequently processed by cellular and viral proteases to produce ten mature viral
proteins, three structural, capsid (C), membrane (prM/M) and envelope (env or E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [10], see Figure 1.1.

The single strand of viral RNA is contained within the viral capsid which is surrounded by a host-cell derived membrane, with both membrane and envelope viral proteins embedded in the membrane. The structural genes begin from the 5’ end of the genome with the non-structural ones nearer the 3’ end. The ten proteins are initially produced as a single polypeptide which is subsequently processed by cellular and viral proteases to produce the ten mature viral proteins [9]. Figure used with permission, John Wiley and Sons publisher.

Following infection, WNV enters cells through receptor mediated endocytosis, whereby the domain III of the E protein binds to αvβ3 integrin on host cells [11], which triggers internalisation of the viral particle through a clathrin-mediated endocytic pathway [12] and allows subsequent amplification and transcription of the viral RNA genome (Figure 1.2).
Following entry, viral particles have been tracked from early endosomes to lysosomes, where low pH causes a conformational change in the E protein, which in turn causes the viral host-derived membrane to fuse with the lysosomal membrane. The fusion of the two membranes serves to free the viral capsid from the lysosome. The capsid subsequently releases the WNV genomic RNA into the perinuclear region of the host cell’s cytoplasm, close to the endoplasmic reticulum (ER) [10, 12]. As well as being responsible for binding to the cellular receptor, changes in the E protein may also be one cause of the change in virulence seen in WNV from 1998. When sequences of WNV strains from before the virulence change were compared to those from after, such as the NY99 strain, although the amino acid sequence is only 0.6% different, a lot of the differences were centred around the E protein. The more virulent strain was found to have acquired a glycosylation site within the E protein. When the E protein was glycosylated the virion as a whole was more stable at mildly acidic pH; therefore fewer capsids were inactivated during de-capsulation [13].

Following release of the viral RNA into the cytoplasm, a host cell RNA polymerase is then utilised to transcribe a complementary minus strand of RNA from the WNV genomic, positive sense, RNA strand [10]. The viral RNA-dependent RNA polymerase is essentially the NS5 protein, although it likely works in conjunction with other viral non-structural proteins, and is responsible for subsequent production of further genomic RNA from the minus RNA strand. Such replication is ten times more efficient than the creation of the initial minus strand, with studies in the related Kunjin virus indicating that for any infected cell possibly only a single minus strand is present from which all genomic RNA is copied [10]. Once present, the nascent genomic RNA strands can act as templates for translation or transcription. Early in the replication process it is likely that they alternate between these two
functions, as a sufficient pool of structural proteins would need to be accumulated prior to virion assembly [10].

Figure 1.2 WNV Replication Cycle.

The figure depicts the steps from the entry of a viral particle into a cell, to the release of new viral particles from the infected cell. A.) Endocytosis of viral particle and release of viral RNA into cytoplasm. B.) Translation of viral RNA. C.) Proteolysis of the polyprotein by NS3 and cellular proteases. D.) Synthesis of initial minus RNA strand. E.) Synthesis of nascent genomic positive sense RNA strands. F.) Transport of structural proteins to vacuole membranes. G.) Encapsulation of viral genomic RNA in vacuoles. H.) Movement of viral particles to the cell surface. L.) Release of viral particles (containing viral RNA) and virus-like particles (lacking RNA payload). Figure republished from [10], with permission of Annual Reviews; permissions conveyed through Copyright Clearance Center, Inc.

Initial translation of the WNV polyprotein occurs in association with the rough ER with the pre-M, E, NS1 and NS4B proteins being co-translationally inserted into the ER membrane to localise them inside the ER lumen [9, 14]. Proteases from the host cell and the viral serine protease, which consists of the NS2B and NS3 proteins, both act to cleave the polyprotein at
multiple sites to generate the individual mature viral proteins (Figure 1.3 (1.)). More recent studies have shown that the NS3 protein not only possesses the serine protease domain, at its N terminal, but also an RNA triphosphatase, an NTPase domain and an RNA helicase at its C terminal; it is thus involved in viral replication as well as polyprotein processing. The RNA triphosphatase likely contributes to RNA capping and the NTP-helicase activity works to unwind the RNA 3’ region secondary structure, to separate the nascent strand of RNA from the template and also facilitates the initiation of viral replication; the viral replication functions of NS3 are achieved by interactions with the viral NS5 protein [15]. Although NS3 is also the enzymic protein of the protease, without the presence of NS2B, NS3 cannot function properly; when synthesised alone the NS3 protease domain is folded significantly differently and enzyme function is inactive. The NS2B region also plays an important role in directing the specificity of the whole complex, the NS3 helicase domain alone is capable of unwinding both DNA and RNA, whilst the full length NS2B-NS3 complex will only unwind RNA. This may aid the virus by specifically focusing on RNA templates instead of DNA, as 20% of NS5 RNA polymerase activity, and associated NS3 functions, are resident within the nucleus of infected cells, although their exact function there is still not clear [15].

Formation of new virions begins when the pre-M and E proteins dimerise on the ER membrane, their conformation leads to production of the icosahedral scaffold and continued accumulation leads to budding of the structure which includes some host membrane, into the ER lumen. When WNV genomic RNA is present it is packaged by the capsid protein into a core particle, which is then incorporated into the structural scaffold as it buds off, thus forming a virion; however particle budding can still take place efficiently in the absence of available genomic RNA, resulting in virus-like particles being produced containing the
pre-M and E proteins but no RNA containing capsid (Figure 1.3) [14]. All particles are transported to the cell plasma membrane in ER vesicles and are released by exocytosis. During or shortly after release of the particles from the host cell, the pre-M region is cleaved, leaving the M and E proteins as separate molecules, although the E protein does tend to oligomerise into trimers as well as persisting as monomers [16]. Once released the new virions are able to go on to infect new cells and thus perpetuate the amplification cycle.

Figure 1.3 Formation of WNV Virions and Virus-Like Particles.

1. WNV genomic RNA is translated into the polyprotein, which is cleaved by the cellular signal peptidase within the lumen (horizontal arrows) or by viral NS2B-NS3 protease (when present) in the cytoplasm (arrow heads). 2. Virus-like particles will be formed if only pre-M and E proteins are present. 3. and 4. Particles are formed by budding of the ER membrane which is driven by the pre-M and E proteins, independent of the presence of the capsid protein or preformed genomic RNA. Virions are formed when capsid proteins and RNA are available (3.) and virus-like particles when they are not (4.). 5. All particles follow the same exocytic pathway for secretion from the cell. ‘Cy’ indicates the cytoplasmic side of the ER membrane. Reprinted by permission from Macmillian Publishers Ltd: Immunology and Cell Biology [14], copyright 2004.
Compared with the larger DNA viruses, small RNA viruses such as WNV, due to their size restriction tend to evolve multifunctional genes, as demonstrated by the NS3 protein. Another WNV protein that is also highly multifunctional is the NS1 protein. Its main initial role was found to be regulation of the synthesis of the negative sense strand of WNV RNA, and WNV without NS1 was unable to replicate within infected cells [17]. However, unusually for a viral protein, NS1 has been found to be secreted from WNV infected cells and has been isolated from both infected tissue culture supernatant [18], and at high levels (up to 50 µg/ml) from the serum of WNV infected patients [19]. Even if not present inside the infected cell, NS1 was able to facilitate its intracellular RNA replication function by an interaction with the viral NS4B protein [17], however this did not explain why NS1 was secreted. It was suggested that secretion may be a mechanism by which WNV is able to modulate the host immune system to reduce the response to infection. Extracellular NS1 is able to disrupt production of an antiviral state in and around infected cells, via interaction with the Toll-like receptor (TLR) 3 molecule. TLR3 is a cell surface receptor on fibroblast cells and is found within endocytic vesicles in dendritic cells; its activation through ligand binding leads to a signalling cascade finalising with pro-inflammatory events such as production of IL-6, IL-8 and IFN-β; NS1 functionally blocks the ligand binding to TLR3 thus preventing the whole cascade of events [20]. Secreted NS1 has also been found to adversely modulate the complement system, which is a part of the innate immune response. Complement acts to assist in viral clearance by enhancing priming of B and T cells, by forming pore complexes in infected cells which result in cell lysis and by binding the complement component C3b to the surface of viral particles and infected cells, which assists in targeting them for
destruction. Secreted NS1 binds to the cell surface of infected cells and prevents the binding of the complement pore complexes and/or the C3b molecules, thus saving infected cells from destruction. NS1 also acts to activate factor H, which is an inhibitor of the complement system. With factor H activated the overall effects of complement to act against the WNV infection are reduced [19]. In contrast to these negative effects on the immune response NS1 also triggers antibody production. Antibodies to NS1 from other Flaviviruses have been found following infection [18], so it is likely that the secreted NS1 is also prompting an antibody response during WNV infection. In mice, some of the antibodies against NS1 can provide high levels of protection against a lethal WNV infection, when the antibodies are administered as a prophylactic [21]. With regards to the immunological functions of other WNV proteins, the E protein, likely due to its position on the exterior of the encapsulated virion, has been found to be highly antigenic, with the majority of neutralising antibodies mapping to epitopes within its DIII domain [22]. Injection of the E protein itself into the muscle of chickens was found to result in significant levels of antibodies being produced and to significantly lower levels of viremia following infection with WNV [23]. The WNV protein NS3 has also been shown to contribute highly to the creation of the pool of antibodies tested so far, although following an infection some level of antibody can be detected to almost all of the WNV proteins. The NS3 protein contained the dominant epitopes for cytotoxic T cells, although again T cell epitopes were also found in NS1, NS2A, E, NS4A and NS4B [10].

On a more macroscopic level, methods of transmission of WNV to new hosts has also come under scrutiny, with further elucidation on the original transmission cycle as well as novel methods of transmission being discovered (Figure 1.4.). The original transmission cycle,
first discovered in the 1950’s, introduced birds as the main amplifying hosts and mosquitoes as the main amplifying vectors [24]. A cycle begins when a bird becomes infected by the virus, as WNV can easily and effectively replicate in birds, the number of viral particles in the blood eventually becomes high enough that the bird is considered infectious; if the bird is subsequently bitten by a mosquito it is likely the mosquito will take up some virus with its bloodmeal. WNV can also replicate in mosquitoes, in a temperature dependent manner over two to seven days, until sufficient viral particles are present when some migrate to the salivary glands of the mosquito [25]. Virus is transmitted from the infectious mosquito to another bird when the mosquito takes another blood meal, as virus is expectorated as the mosquito feeds.

With viruses that use a vector, such as the mosquito, to spread to new hosts, the type and behaviour of the vector can have a major affect on transmission of the virus and the level of disease caused by it. The *Culex pipiens* complex of mosquitoes are the main vector for WNV both in Europe and North America; the complex is so named because it contains many ‘species’ that show a high variation in physiological and behavioural traits without noticeable morphological differences to clearly distinguish them. Bloodmeal sources for the different groups of *Cx. pipiens* were ascertained: in Europe different groups within the complex feed among species of birds while other groups bite mainly humans or other mammals. However in the U.S., hybrids between these two groups of mosquitoes were present in all areas tested; with such hybrids willing to feed on mammals and on birds, they would be the ideal bridging vector to facilitate the rapid spread of WNV seen across North America [26].
Figure 1.4 Transmission Cycle of West Nile Virus.

The amplification cycle is indicated by the arrowed circle, dotted arrowed circle for possible additional amplification and unidirectional transmission by single arrows. Intermosquito transmission occurs through laying infected eggs, as male mosquitoes were found to be positive for WNV. Mammal to bird and direct bird to bird transmission occurs through birds eating WNV infected prey or carcasses. Interhuman transmission routes are all indicated in the figure. Modified from [24], used with implicit permission from Cambridge University Press.

Behaviour of avian vectors is also important in the spread of the virus and its related disease. In northeast and north central U.S., Cx. pipiens, prefer to feed on American robins (Turdus migratorius) during the summer months, which aids the amplification of the virus. However, when the birds begin to migrate away from their summer breeding grounds in the late summer, the mosquitoes switch their feeding behaviour and become seven fold more likely to
feed on humans than birds. This change in behaviour of the mosquito vector, due to the lack
of birds to feed on, is reflecting in the fact that the highest disease incidence in humans is
evident during the late summer and early autumn [25]. The most well known dead-end hosts
for WNV are humans and horses, which can become infected when bitten by an infectious
mosquito such as the Cx. pipiens hybrids [2]. More recently WNV has also been found to
infect cats, dogs, domestic rabbits, some squirrel species and even bats [27]. Although signs
and symptoms of disease may be present, usually the resulting infections do not produce high
enough levels of viremia for the animal to be considered infectious, this is why they are
considered ‘dead-ends’ for the transmission of the virus. Since the spread of WNV across
North America, other methods of transmission have come to light, such as the consumption
of WNV contaminated food, or particular to humans is transmission as a result of organ
transplant or blood transfusion [28]. That such transmission routes existed was first
established in 2002, and screening of pooled plasma samples was starting from 2003 to
prevent them from continuing; however some positively infected samples were still able to
slip through the net. Because WNV could adhere to red blood cells, the levels of virus in a
few of the plasma samples were below detectable levels [29]. Infection from contaminated
food was found to occur not only in birds that eat either WNV infected mosquitoes or other
birds, but was also implicated in an outbreak of WNV in an alligator farm in 2002 when it
was discovered that meat fed to the alligators had come from WNV infected horses [24].
As well as being key in the amplification of the virus, once infected, birds play a key role in
the spread of the virus, both geographically and over time. Many birds are migratory and
although the adverse effects of a viral infection and the high amount of energy and stamina
required for a long migration do not at first appear compatible, migrating birds can carry
WNV with them. A 2009 study of the Atlantic and Mississippi flyways found not only WNV antibodies in both resident and migratory birds, but also WNV viremia. Although percentages appear small, at 1.9% for presence of antibodies and 0.1% for viremia, the numbers of birds tested out of the entire numbers of birds migrating means that the actual numbers of birds infected is likely to be highly relevant in transmitting the virus [30]. It was also in migrating storks, arriving in Israel, that the first avian cases of the more virulent form of WNV were seen [31]. As the amplification cycle involves mosquitoes, which need heat to be active and multiply, cycles of amplification and infection are highly seasonal for activity in the warmer months; thus the virus has to find some method of surviving each winter when mosquitoes are not active. Birds, or the small mammals that they feed on, may be one such method used by WNV, as a dead hawk was found in New York state in February of 2000 that tested positive for active viremia. The hawk itself, or something it had recently eaten, must have been harbouring the virus since the end of the mosquito season the previous summer, with the levels of virus slowly increasing over the ensuing five months [32].

1.1.1 West Nile Virus and Birds

Although birds are known to be amplifying hosts central to the WNV transmission cycle, for many years even this position did not result in high levels of avian mortality and provided very little signs of disease. However all that changed in 1998 in Southern Israel. In the late summer of 1998 a flock of White Storks landed near the town of Eilat, due to strong winds they had been blown off their usual migration course and were weak and stressed when they landed; when their health status was checked some birds were obviously sick and dead birds were also found. Samples sent for testing showed positive for WNV in both tissue from the dead birds and sera from sick ones. Most of the flock were considered fledglings and as such
were migrating for the first time, so it was likely they picked up the new more virulent strain of WNV on their migration route over Europe [31]. This was the first time high levels of mortality associated with WNV had been seen in birds. The virus also appeared to be more easily transmitted, as it was found in flocks of domestic geese, in the same area in the same year. Although no deaths were reported in the geese that first year, it was found that some of the geese farmers and veterinarians working with the geese were seropositive for WNV antibodies when tested [33]. This more virulent form of WNV arrived in New York in 1999, as was demonstrated by the high mortality shown in birds in the area surrounding New York, where several thousand crow deaths were attributed to WNV [5]. Although corvids, such as crows and their relations, appear to be most susceptible to the effects of WNV, within two zoos in the New York area many other exotic species, such as owls, penguins and ibis were also found to succumb to disease [34]. In many birds, as with humans, infection shows little sign of disease, but when signs are present they can be quite varied. Signs can range from lethargy, ruffled feathers and weight loss, to unusual twisting of the head and/or neck (torticollis), loss of voluntary muscle control (ataxia), recumbency, paralysis and uncoordination when the infection becomes neurological [24]. One study, specifically looking at WNV infections in species of owl, found torticollis, tremors, seizures and uncoordinated flight or inability to fly as well as the more general weakness, depression and recumbency [35].

There are currently approximately 320 species of birds in North American that are susceptible to WNV infection including several rare and endangered species [36]. One such susceptible rare species is the Eastern Loggerhead Shrike (*Lanius ludovicianus migrans*). This species is considered rare throughout North America, and to maintain population
numbers two captive breeding colonies were set up, one of which is at the Toronto zoo. It was there, in 2002, that five birds died shortly after contracting WNV and with none of the remaining 37 birds testing positive for WNV antibodies it suggested a 100% mortality rate for this species [37]. Another endangered species, important in Alberta, is the Greater Sage-Grouse (*Centrocercus urophasianus*), whose habitat has now become severely restricted to several sites across regions of North Eastern U.S. as well as in Alberta. WNV arrived in many Sage-Grouse areas in 2003 and caused 25% decline in survival of females in four WNV infected populations and a comparison of 20% survival at one infected site compared to 76% at two sites without WNV. By 2004, this had changed somewhat, to 86% survival at sites with WNV and 96% at those without; however at the time it was too early to attribute this to either increased WNV immunity due to prior exposure or to it being a bad year for mosquitoes [38]. In a later study, following a natural infection with WNV, some Sage-Grouse were testing positive for antibodies, so not all infected birds were dying; however they also calculated infection rates were only between 4-29%, implying that most sage-grouse had not been exposed to WNV and were still susceptible to its effects [39].

### 1.2 The Avian Immune System

When an animal is threatened with a disease-causing pathogen, such as WNV, the main line of defence is provided by the individual’s own immune system. If that system could be enhanced by exposure to the pathogen, without disease, or by vaccination, the subsequent immune response would be increased. As birds are central to the transmission cycle they form a group highly targeted for infection by WNV, as well as being susceptible to the
disease caused by WNV infections. Thus, their response to the virus and their immune systems in general are also important and will be discussed here.

Due to the demands on the poultry industry, most studies concerning the avian immune systems relates to the chicken (Gallus gallus domesticus) specifically, which is reflected in the information available. Although there are many basic similarities between the mammalian model immune system, namely the mouse, and its avian counterpart, there are also many differences. General similarities include, the presence of an adaptive immune response, the presence of the lymphocyte sub-populations of NK cells, T cells and B cells and the existence of the MHC (major histocompatibility complex) molecule [40]. More specifically, in comparison to mammals, chickens have different repertoires of Toll-like receptors, defensins, cytokines, chemokines and antibodies as well as lacking functional eosinophils (granular phagocytic cells) [41].

However, even some of the similarities are different. Whilst both groups have B cells, unlike mammals, where B cells develop in the bone marrow, in birds the main primary lymphoid organ associated with the B cell is the Bursa of Fabricius. B cells migrate to the bursa quite early in embryo development (8 to 14 days) and the B cell repertoire is generated there during late embryonic development until shortly after hatching. B cell variation in the chicken is achieved through somatic gene conversion, where part of one allele is copied to the other allele. However, in mammals gene rearrangement is involved whereby alleles are extensively rearranged to generate high levels of B cell variation. Also unlike mammals, in birds the final mature B cell repertoire is fixed at around five to seven weeks post-hatch, when the bursa is fully mature, after which time the bursa begins to regress [40].
Birds and mammals both also have MHC class I and II molecules and although the function of peptide presentation to T cells, is the same, many details are different. First is the size of the MHC region in the genome: in humans, the MHC regions consist of about four million base pairs encoding at least 280 gene, in chickens the MHC region, known as the B locus, spans only 92 thousand base pairs and encodes 19 genes. As these numbers suggest, compared to mammals, variation within the MHC of the chicken is severely restricted. Only two copies of both MHC class I and class II genes are present which also restricts the variety of peptides that can be presented. Chicken MHC type is closely related to a survival or death response to infection by a number of important pathogens, thus the presence of a particular MHC allele can be used to predict the outcome of an infection. This differs from humans where the strong associations with the MHC regions are related to autoimmunity or particular biochemical defects rather than resistance to pathogens. It is suggested that the more complex mammalian MHC might provide a lower level of protection from a range of pathogens but at the cost of higher autoimmunity (as some of the MHC variants would likely recognise self-peptides) whereas the single dominant MHC that is expressed in chickens would either confer resistance or susceptibility to any particular pathogen but incidences of autoimmunity would be much reduced [40, 42]. However, whether this is an adopted strategy for avian immunity or should be considered a step in the evolution of a ‘fully developed’ immune system remains to be determined.

One definitive difference between the mammalian and avian immune systems is the lack of encapsulated lymph nodes in birds, which instead, develop diffuse lymphoid tissue in particular areas of the body where antigenic stimulation is likely to occur. Birds possess several regions of ‘associated lymphoid tissue’ (ALT) including several regions in the gut
(GALT) such as Peyer’s patches. Bronchial-associated tissues (BALT) are also present, as are ALTs associated with the conjunctiva (CALT), the latter includes the avian specific Harderian gland in the eye [40]. However, birds do possess rudimentary lymph nodes, called mural lymph nodes (MLN), that associate with the deep lymphatic system near the blood vessels in the legs and emerge approximately six weeks after hatch. They differ from true mammalian lymph nodes in that they are not encapsulated and they do not interrupt the flow of the lymphatic vessel; instead they lie laterally to the vessel, with the larger MLNs containing sinuses in which the flow of lymph may be slowed. Avian MLNs also lack the reticular fibres and macrophages to filter the afferent lymph. With the reduced definition of the organs themselves and a lack of a filtering system, avian MLNs are generally considered an evolutionary stage in development of the lymphoid system. Antigen presentation has been shown to occur in the MLNs with activated cells then entering the circulation, although the proportion of B cells to T cells is higher in MLNs than in the mammalian lymph node [43].

Other immune studies, done specifically in birds, demonstrate that hormones can affect the immune system. This is one area of study where avian species other than chickens are used, namely Japanese quail (Coturnix japonica, JAQU) (hereafter also referred to as ‘quail’). One study looked into the effects of testosterone levels on a generalised T cell immune response, in which they compared the response of castrated birds to those with intact testicles; there was a significantly higher immune response in the castrated birds compared to the intact birds. However the researchers also highlighted the difficulties in attributing decreased immunity directly to higher testosterone levels; resting metabolic rates (RMR) were also higher in the intact birds, implying they were using the additional energy to maintain sexually related tissue, thus reducing the energy available to raise an immune response. This
indirect effect of testosterone may also have been the cause of the decreased immune response in quail with high testosterone levels [44]. Another study used quail to look at the effects of melatonin on the immune system. Melatonin is a hormone related to light/dark cycles, circadian rhythms and mammalian breeding cycles but it is also known to regulate the immune response. In mice the inhibition of melatonin synthesis leads to a decreased response in both cellular and humoral immunity and the same has been shown in quail, with responses being restored by administration of a melatonin replacement. In mammals melatonin acts by binding to receptors on CD4+ lymphocytes causing them to secrete opioid peptides, which further modulate the immune response. In quail, melatonin also functions via opioid peptides but the cells that secrete them were not ascertained [45].

Possibly, again because of the link between avian related research and the poultry industry, avian immunity studies, especially when involving chickens, often tend towards research into disease resistance, including vaccine research. The poultry industry has available to it an extremely large range of vaccination techniques including: intramuscular or subcutaneous injection, application via mucus membranes (ocular, nasal or oral), administration in either drinking water or liquid or gel sprayed directly onto the birds or by introduction into feather follicles [46] or even directly into eggs to vaccinate the developing embryos. The industry also appear to have an equally long list of diseases relating to poultry flocks, which cover the full range of pathogens from viruses, bacteria and parasites to mycoplasmas, fungi and protozoa [47] of which the diseases from some can be prevented by vaccination.
1.3 Assessment of West Nile Virus Vaccines in Birds

With regards to vaccines against WNV, many potential strategies have been investigated and some have now run the gauntlet through to commercialisation, although none are yet available specifically for humans, or for birds [48].

It was not long after the change of virulence in WNV before the first vaccines against it were being tested. By 2001, in Israel, both an inactivated WNV antigen preparation and a vaccine for another Flavivirus (turkey meningoencephalitis virus (TME)) had been tested in a challenge assay in goslings. Results were varied, with protection ranging from 39% - 72% for the TME vaccine and 52% - 80% for the WNV vaccine, although this variation may have been caused by prior exposure to pathogens, as the birds were reared and vaccinated on local farms [49]. The first commercially available vaccine of killed WNV was targeted for use in horses, but when this was tested in birds, results were not spectacular. Of 16 Chilean flamingos (*Phoenicopterus chilensis*) and 10 red-tailed hawks (*Buteo jamacencis*) vaccinated, neutralising antibodies were not found in any of the samples tested, even when a booster vaccination had been administered three weeks after the first [50]. Later, when tested in a wider range of avian species from zoos in the U.S., the same vaccine produced better results, possibly due to an increased dose and one, or even two, booster injections. Out of five exotic avian species vaccinated, including the Chilean flamingo as before, three showed significant increases in WNV antibodies over time, in those birds that seroconverted. However, the fraction of birds that did seroconvert, was also variable: 80% of black-footed penguins (*Spheniscus demersus*), 41% of Chilean flamingos and 30% of American flamingos (*Phoenicopterus ruber*) [51]. However, in neither of these antibody related studies, was the cellular immune response to the vaccine also assessed.
A recent study provided details of a recombinant subunit protein antigen, which consisted of 80% of the WNV E protein, expressed in *Drosophila* cells, combined with an adjuvant, being tested in geese. An initial vaccination with the protein, followed by a similar booster 4 weeks later, gave significantly higher levels of antibodies in groups vaccinated with two different doses of the protein compared with control groups. It also provided protection from viremia in the geese following challenge with WNV [52].

Currently, there are five WNV vaccines commercially available for use in horses in Canada: one is a live chimera of WN and Yellow Fever Virus (a related Flavivirus), one is a killed Flavivirus/WNV chimera, one is a live recombinant canarypox virus and two are killed WNV preparations [48]. However, at present none are licensed for use in birds.

### 1.4 Recombinant Adenoviruses for Protein Expression and as Vaccines

One vaccine platform proven to be successful and safe is the chimera, or recombinant virus vector, whereby two different viruses, or a virus and another pathogen, are combined to form a vaccine. A virus, called the backbone or vector virus, is used to provide the main genetic component for the vaccine; it is rendered non-infectious by removal of a part of the viral genome, which makes the virus unable to replicate. Specific genes from the pathogen that the final vaccine will target, in our case WNV, are then added back into the backbone of the first virus, using molecular techniques to cut and then ligate DNA from the pathogen. The vector virus still retains the ability to infect a cell, that is, to be able to penetrate the cellular membrane, and once inside a cell it allows expression of the proteins from the target pathogen. The target proteins thus expressed would be recognised as foreign by the immune system and would trigger an immune response, without the associated chance of disease.
possible from the native form of the target pathogen. The WNV/Yellow Fever Virus chimera vaccine mentioned above is this kind of virus vector vaccine, and regarding success and safety for such a platform, as of January 2011, it was one of the few candidate WNV vaccines to have completed phase II trials for human use in the U.S. [53]. The same platform, but using an adenovirus as the backbone, has been used successfully in our laboratory to express components of the antigen presentation complex, this enhanced the T cell response and provided protection from viral infection [54]. Adenoviruses have also been tested, as the vector in recombinant virus vaccines, in birds with good success, as described below.

As well as WNV, another virus that may prove problematic for birds and humans is the avian influenza virus (AI), with vaccination of birds being looked into as a possibility to try to stop a pandemic in humans. One study used recombinant adenoviruses as vaccines in chickens, one adenovirus variant expressed a protein from the H7 strain of AI and the other a protein from an H5 strain of AI. Either vaccine alone produced substantial antibody titres against AI, but when the AI H5 vaccine was injected, followed by a booster injection of the AI H7 vaccine 27 days later, birds produced high levels of antibodies to both strains and were protected from the adverse effects of an AI infection [55].

As well as being used to express pathogen target proteins as part of a vaccine, recombinant adenoviruses can also be used to express genes that are missing or defective, as part of a gene therapy program. One such recombinant adenovirus was constructed to express the human acid maltase gene, and was tested in a quail breeding line that had an acid maltase deficiency. When the acid maltase gene is non-functional in humans, which is an autosomal recessive trait, early death ensues due to dysfunction of cardiac and respiratory muscle caused by a build up of excess glycogen. The same thing happens in the quail line used, but when the
adenovirus was injected into one pectoral muscle of each bird and not the other, there was a marked reduction in glycogen accumulation in that muscle compared to the non-vaccinated side [56]. This study is a proof of principle demonstrating that recombinant adenoviruses are able to significantly express proteins, \textit{in vivo} in birds, and specifically in quail.

\textbf{1.5 The Requirement for an Avian West Nile Virus Vaccine}

As birds are one of the group of animals most highly affected by the change of virulence that WNV underwent in the late 1990’s, as well as being key to the amplification and spread of the virus, a way to reduce the deleterious effects on birds would be desirable. Corvids, which includes crows, ravens and jays, are the family of birds that demonstrate the highest mortality levels to WNV. Although there is generally no shortage of crows, the family also includes the Stellar’s Jay (\textit{Cyanocitta stelleri}) and Blue Jay (\textit{Cyanocitta cristata}) which, as representatives of less common species of native North American birds, it would be desirable to save. As well as common species there are also rare and endangered species of birds that are susceptible to WNV, such as the Eastern Loggerhead Shrike and the Greater Sage-Grouse already mentioned. There are usually several different pressures on rare populations of any endangered animal that drives their numbers down, often, as in the case of the Sage-Grouse, the main adversity is caused by habitat destruction [57]. If the additional pressure caused by the deleterious effects of a WNV infection could be removed, as would be possible if the birds were vaccinated against the disease, it would help the species persist. Due to the possible fatal effects of a WNV infection in such endangered species, with bird numbers so low anyway, some groups have not waited to find out if their species of interest is even susceptible to WNV before having deemed it prudent to vaccinate the birds against
the virus. Such was the case for the endangered Californian condor (*Gymnogyps californianus*) when WNV arrived in California in July of 2003; the condors were considered likely targets, partly because other raptors had been shown to be susceptible to WNV and partly because condors are also scavengers, which would expose them to the possibility of ingestion of WNV infected carcasses. Initially, two pilot studies were undertaken. First, birds related to the condor were vaccinated to make sure the vaccine was safe; when it was found to be, a few condors not so essential to the breeding program, were vaccinated, and again it proved safe and effective in producing antibodies in the birds. By the end of 2004, all Californian condors, both in the captive breeding program and the free-living birds, had been vaccinated against the effects of WNV, a very necessary precaution when at the time there were only 273 individuals in existence [58]. Similar results were obtained in Island Scrub-Jays (*Aphelocoma insularis*), a species of Scrub Jay found only on the island of Santa Cruz, off the Californian coast. This species was deemed vulnerable when other Scrub-Jay species were found to be susceptible to WNV, but mostly due to their restricted location. As with the condor, vaccines were first tested in other related species before vaccination of some of the free-living birds was undertaken. Even though vaccinating the birds two or three times was found to produce WNV antibodies in the birds, it was also found to be impracticable to recapture the same individuals for booster vaccination on a regular basis. Instead a one-dose DNA vaccine was tested and although 86 birds were vaccinated, this vaccine did not raise any detectable antibodies in the 10 birds later tested. However, it was still recommended that annual vaccination of birds continue in subsequent years, as the vaccine had been shown to reduce viremia and improve survival in a closely related species [59]. The aim of continuing vaccination in the island population would be to establish a sub-population of vaccinated
birds that would be more likely to survive a WNV epidemic and be able to re-populate the island again afterwards.

Although many birds have been vaccinated against WNV, the vaccines used were either still experimental ‘prototypes’ or a vaccine licensed for horses being used ‘off-label’ in birds. Despite the vaccination of some rare species there are still those for which this precaution has not yet been undertaken, such as the Loggerhead Strike and the Greater Sage-Grouse, two species that would benefit from the protection a vaccination would provide; thus it would be relevant and useful if a vaccine could be specifically developed, and licensed, for use in birds.

A vaccine against WNV for use in birds was considered a worthwhile goal, with the vaccine being primarily aimed for use in rare and endangered species of bird. As such birds are often either in captive breeding facilities, or are frequently caught and/or re-caught for monitoring purposes, this would facilitate administration of an injectable vaccine. The same reasons, that of captivity and ease of administration, would also apply to the second target population, namely susceptible species of birds held in zoos or animal parks or in specialised captive breeding colonies. In such places, an additional reason to provide protection from WNV would be that some species would be exposed to threats outside of their natural environment; other than the Galapagos Islands and parts of South Africa, it is hard to think of a place other than a zoo where penguins are likely to encounter a mosquito.

1.5.1 Identifying a Model for the Avian Immune System

When considering an animal model for use in assessing the efficacy of a vaccine, several points had to be considered, including: choosing a species relevant to the study, ease of
handling and housing the animals, availability of animals and gaining sufficient knowledge to be able to work with and care for the selected animal species.

Although mice are the most studied species, having been used in the laboratory since at least 1664 [60], as the vaccine was aimed for use in birds, it was decided that a suitable avian model should be used for testing. Chickens are arguably the most widely used research bird, however chickens are not susceptible to WNV infections [61] which was considered a potential drawback to using chickens as a model. Next, Japanese Quail were considered as an avian model and although not extensively used for immune systems studies, they are used for laboratory based research purposes. They were finally selected as the species to use, for the following reasons:

- a source of birds from quail breeding lines specifically for scientific research, were available and accessible,
- their smaller size would made them easier to house and handle compared to chickens,
- it was considered that their smaller size would mean less vaccine would be required compared to that required for an equivalent dose in a chicken, and
- a collaborator was found who was prepared to assist with this project, namely Dr. D. Bennet, an Assistant Professor in the Faculty of Land and Food Systems at UBC, who had extensive knowledge and experience of working with quail.

After the initiation of the use of quail within this project, Japanese quail were shown to be susceptible to WNV infection only at a low level. When compared to several other species of birds, quail had lower viremia (3.4 log PFU/ml) over a fairly short period of time (1.3 mean days) [62]. Although results of testing the vaccines in quail would still be valid, it did mean that another species would need to be selected for use in a challenge assay situation, where
the response to an infection would need to be clearly visible. One study looking at WNV infection in House Sparrows (*Passer domesticus*, HOSP) (hereafter referred to as ‘sparrows’ or HOSP) found them much more susceptible to WNV infection than Japanese quail. The sparrows demonstrated higher levels of viremia (10.3 log PFU/ml) over a longer period of time (4.5 mean days) following infection, when compared to quail, as well as showing a 50% mortality rate [62]. Any work, such as a challenge assay, involving working with WNV, would need to be done in a level 3 biocontainment facility. The level 3 facility in the National Microbiology Laboratory in Winnipeg was also able to house birds and had been recruited to collaborate on the project. Sparrows were also readily accessible there, as wild birds caught in the Winnipeg area. For these reasons, it was necessary to switch to HOSP for the challenge assay part of the vaccine testing.

### 1.6 Scientific Rationale for Vaccine Design

A human recombinant adenovirus containing WNV ‘genes’ was decided upon as the platform for the vaccine. This was chosen partly because of prior experience in the lab with recombinant adenoviruses, but also because human adenoviruses had served to express proteins *in vivo* in birds, specifically in quail, the species in which initial vaccine testing was to be undertaken [56]. WNV genes would be inserted into the adenovirus DNA and the recombinant adenovirus would function as a vaccine by entering the cells of vaccinated birds and then expressing the WNV proteins from the inserted region of DNA. These WNV proteins would be recognised by the bird’s immune system as foreign and would trigger an immune response against them. This WNV specific response would prime the immune system for early recognition of any subsequent WNV infection and should provide the bird
with protection from disease. It was also decided a human adenovirus platform might be advantageous for designing a vaccine for use in birds as it would be unlikely that birds had experienced any previous exposure to the human virus. This would avoid any possible complications arising from pre-existing antibodies against the adenovirus itself that may have existed if prior exposure had occurred. If anti-adenovirus antibodies are present they would act to recognise the adenovirus very quickly and trigger an immune response to it; this would impinge upon the adenoviruses’ ability to enter cells and express the recombinant proteins, which is the main function of the vaccine.

When selecting the ‘genes’ of WNV for insertion into the adenovirus, production of proteins that were highly immunogenic was required to enable stimulation of a strong immune response against them. An ideal vaccine would stimulate both the humoral and the cellular immune response, to elicit a effective overall response. While searching academic literature to discern the effects on the immune system of the different WNV proteins, it became apparent, as detailed in the following references, that a protein which triggered one type of response was not necessarily efficient at triggering the other type of response. It seemed that these two distinct parts of the immune system might be best triggered by two different WNV proteins, so it was decided to construct two different variations of the vaccine, one aimed at triggering an antibody response and one at triggering a cytotoxic T cell response. As the antibody response is more often aimed towards extracellular pathogens, for a virus the antibody response would occur when the viral particles were inside the host body but not yet inside the cells. Thus the proteins on the outside of the viral particle would be more highly targeted; for WNV this would be the pre-membrane (pre-M), membrane (M) and envelope (E) proteins. When an antigen was produced using just the E protein, serum samples from
human patients were found to strongly interact with the antigen. In fact, sera from mice
immunised with the same antigen were sufficient to provided other mice with protection
from a WNV infection, following passive administration of the sera [63]. Therefore the E
protein was a likely candidate. However, as the WNV genome is expressed as a single
polypeptide, using E DNA by itself would not be sufficient [64]. For Japanese Encephalitis
Virus, which is closely related to WNV, the envelope protein is not fully functional in the
absence of the pre-membrane protein, due to improper folding of the E protein [64]. To
make use of the WNV E protein as a vaccine the final protein should be folded as it would be
during an infection, to enable the vaccine to induce an absolutely specific immune response.
This was considered especially important for the E protein, because as a structural protein it
should be a target for the antibody response, so the full 3D structure of the final protein
would be critical. To ensure correct folding, and hence proper final structure, of the E
protein, it was decided to include DNA for the full M protein, which included the pre-M
region, with the E DNA for insertion into the recombinant adenovirus. The effectiveness of
this strategy was indicated in two studies using a DNA vaccine containing DNA for pre-M
and E from WNV; the DNA gave protective results when used in mice, horses and even Fish
Crows (Corvus ossifragus) [65][66].

As opposed to the antibody response, viral proteins that might trigger a better T cell response
would likely be internal to the viral particle, as they would only become exposed for immune
surveillance by T cells once processed into peptides by antigen presenting cells. Using WNV
and the closely related Flavivirus Kunjin Virus, Hill et.al. investigated the close
cross-reactivity known to occur between different flaviviruses [67]. They used an assay to
test T cell specific responses in mice using T cells from WNV infected animals, and target
cells that had already been primed with a range of Kunjin virus peptides. When the T cells and the target cells were mixed together the WNV specific T cells would lyse more target cells expressing peptides from a region around the NS3 protein than cells expressing peptides from other regions of the Kunjin virus genome. As Kunjin virus and WNV have an overall amino acid identity of 93%, this implies the same results would apply if the target cells had been primed with WNV peptides. So it seemed appropriate to include the WNV NS3 protein in the adenovirus vaccine, as a potential inducer of a T cell response.

When using an adenovirus as a vector for foreign DNA, it is possible that the adenovirus itself could trigger an immune response. To enable testing of this scenario an empty vector control recombinant adenovirus, containing no additional DNA, was also created.

1.7 Overall Objectives and Main Hypotheses

1.7.1 Objectives

i. To assess which available reagents for monitoring the immune system will work in the model avian organisms selected.

ii. To create two recombinant adenovirus vaccines one containing DNA from WNV M and E ‘genes’ and the other containing WNV NS3 DNA.

iii. To confirm that both recombinant adenovirus vaccines are able to complete protein synthesis from the WNV genes, in both human and avian cells.

iv. To assess immune responses in a Japanese Quail model following vaccination, of different groups of birds, with the two recombinant adenoviruses, by measuring levels of T cell activation and amount of anti-WNV antibodies produced.
v. To assess any level of protection provided by the recombinant adenovirus vaccines, against an induced WNV infection in a House Sparrow model.

vi. To test for any synergistic effect that using both of the recombinant adenoviruses together, as a single vaccine, may have.

1.7.2 Main Hypotheses

For clarity, the recombinant adenoviruses containing either WNV ‘gene’ will be referred to as vaccines.

i. Following infection of both human and avian cell lines with either vaccine, mRNA and protein expression of the relevant WNV ‘gene’ will be at detectable levels.

ii. Following intramuscular injection into birds, the vaccines will trigger a significant WNV specific immune response, as measured by antibody levels and T cell activation.

iii. Following intramuscular injection into birds, the vaccines will provide significant protection from the harmful effects of a WNV infection, as measured by increased survival or lower viremia compared to unvaccinated birds.

1.8 Synopses of Subsequent Chapters

Chapter 2 contains methods for experiments described in all following data chapters.

Chapters 3 to 6 are the main data chapters for this thesis and contain results and discussions relevant to the topics indicated below.

1.8.1 Chapter 2.

Chapter 2 contains full detailed methods for all experimental procedures dealt with in subsequent data chapters within this thesis. It covers animal handling and housing protocols
as well as details of all statistical analyses made. Rationales and objectives for all experiments undertaken are provided in the relevant data chapter, rather than in chapter 2.

1.8.2 Chapter 3.

Chapter 3 covers the screening of avian based reagents to assess their efficacy in Japanese Quail and House Sparrows; the two species used for *in vivo* testing of the vaccines. White Crowned Sparrow (*Zonotrichia leucophrys*, WCSP) (hereafter referred to as WCSP) were also screened for reagent reactivity with available samples, as representatives of a different avian species. Screening was undertaken to establish reagents suitable for assessing immune responses to the vaccines following *in vivo* testing. All screening was conducted using a flow cytometry approach. This chapter also includes a section on using working reagents to assess the normal T cell population levels in naive quail, using flow cytometry.

1.8.3 Chapter 4.

Chapter 4 contains details of the creation of the recombinant virus vaccines, assessments of the newly produced plasmids and initial testing of the subsequent recombinant viruses. Techniques used include cDNA synthesis, PCR, DNA ligation, amplification and purification as well as RNA extraction and western blots. Results include screening of the plasmids for the presence of WNV DNA and confirmation of both mRNA and protein expression of the WNV relevant ‘genes’ from the recombinant viruses.

1.8.4 Chapter 5.

Chapter 5 covers the assessment of the effects of vaccination on quail, by measuring the numbers of activated T cells and the amount of WNV specific antibodies present. T cells were assessed by quantifying cells triggered to produce intracellular interferon-γ (IFN-γ), and antibody levels were quantified by a WNV specific serum ELISA. Results for both assays,
from three different doses of each vaccine are included, as well as from samples taken between day five and day fifteen post vaccination as part of a time course study.

1.8.5 Chapter 6.

Chapter 6 provides details of work completed in House Sparrow, to assess the ability of the recombinant virus vaccines to provide birds with protection from the adverse effects of a WNV infection. This includes an infection response study, to confirm the effects of a WNV infection in sparrows under the protocols used. A challenge assay was also undertaken, where birds were vaccinated and later infected with WNV. Results were measured by survival and level of viremia in both blood and tissue, with viremia being measured using a qRT-PCR technique.

1.8.6 Chapter 7.

Chapter 7 is the concluding chapter of the thesis and contains an overview and final assessment of work completed, as well as details of possible future direction for research stemming from this thesis.
Chapter 2: Experimental Rationales and Methods

2.1 Assessing the Avian Immune System for Vaccine Testing

As the overall aim was to use the vaccines in birds, it was necessary to establish which assays for measuring immune responses would be feasible to use in birds and what reagents would work sufficiently in an avian system.

2.1.1 Antibody Screens for Flow Cytometry Analysis

Table 2.1 lists the antibodies tested for use by flow cytometry experiments, on splenocytes and peripheral blood if available, from three different bird species, Japanese Quail, HOSP and WCSP. See the following sections 2.1.1.1, 2.1.1.2 and 2.1.1.3 for details of how the cells were harvested from the different species. Following collection, cells were counted using a haemocytometer to establish lymphocyte numbers per sample, based on a cell morphology of small, round, bright cells under the light microscope. Cells were transferred to wells of a 96-well round bottomed plate for staining with antibodies, with one well per antibody and $1 \times 10^6$ lymphocytes per well. If sufficient cells were available samples were tested in duplicate. Cells were centrifuged in a bench top centrifuge at 1,500 rpm for 5 minutes and resuspended in 100 ul of antibody (see Table 2.1 for concentrations) diluted in FACS buffer (phosphate buffered saline; PBS) containing 2% FBS (foetal bovine serum). For some antibody/bird species combinations where the initial concentration of antibody appeared positive, assays were re-run, with different samples from the same species, using different concentrations of the antibody, to see if it was possible to determine an optimal concentration for that particular combination. The cells were incubated with the antibody at 4°C for 30 minutes, centrifuged as before and washed twice in 200 ul per well of FACS buffer. Cells were transferred to 1.2 ml cluster tubes and kept on ice until ready to analyse by flow cytometry, cells were assayed on a BD FACS Calibur flowcytometer at the Michael Smith Laboratories.
<table>
<thead>
<tr>
<th>Avian Species</th>
<th>Antibody Specificity</th>
<th>Antibody Clone Name</th>
<th>Species Reactivity</th>
<th>Antibody Amount per 10^6 cells</th>
<th>Fluorescent Conjugate</th>
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<tr>
<td>WCSP</td>
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<td>CT-4</td>
<td>Chk/Tky</td>
<td>0.2 µg</td>
<td>PE</td>
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<tr>
<td></td>
<td>CD4</td>
<td>Du-CD4-1</td>
<td>Duck</td>
<td>0.2 µg</td>
<td>None - secondary Ab used</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>Du-CD8-1</td>
<td>Duck</td>
<td>0.2 µg</td>
<td>APC</td>
</tr>
<tr>
<td></td>
<td>CD8α</td>
<td>3-298</td>
<td>Chk</td>
<td>0.2 µg</td>
<td>PE</td>
</tr>
<tr>
<td>HOSP</td>
<td>CD4</td>
<td>CT-4</td>
<td>Chk/Tky</td>
<td>0.2 µg</td>
<td>APC</td>
</tr>
<tr>
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<td>CD4</td>
<td>EP96</td>
<td>Chk</td>
<td>0.2 µg</td>
<td>APC</td>
</tr>
<tr>
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<td>Du-CD8-1</td>
<td>Duck</td>
<td>0.2 µg</td>
<td>APC</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>3-298</td>
<td>Chk</td>
<td>0.2 µg</td>
<td>PE</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>CT-8</td>
<td>Chk</td>
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<td>Duck</td>
<td>0.1 µg</td>
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<td>Chk</td>
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<td>PE</td>
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<td>APC, PE or FITC</td>
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<tr>
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<td>EP96</td>
<td>Chk</td>
<td>0.2, 0.4, 0.6 µg</td>
<td>APC</td>
</tr>
</tbody>
</table>

Table 2.1 Description of Avian Species/Antibody Combinations for Staining.

Species Reactivity Abbreviations: chk – chicken, tky – turkey. The fluorescent conjugate refers to the fluorescent marker attached to each antibody to identify its presence during analysis in the flow cytometry, each marker relates to a different fluorescent channel: FITC relates to the FL1-H channel, PE relates to the FL2-H channel and APC to the FL4-H channel. (for explanation of fluorescent marker abbreviation see Abbreviation List)

Results were analysed and displayed using Flowjo software (Tree Star Inc.). To establish criteria for criteria for comparison of results and to indicate positive or negative binding results for each antibody antibody tested,
Figure 2.1 was constructed; it represents an ideal fluorescent antibody staining profile for a sample of splenocytes. The blue line represents the negative control sample of cells to which no antibody was added. This is used to establish a baseline level of fluorescence usual for the cell type being assessed. The orange line represents a sample stained with a relevant antibody. The peak in the orange line, on the left of the graph, is the negative population of cells, that do not possess the molecule specific to the antibody. This negative peak may however be shifted to the right somewhat, when compared to a sample where no antibody was added, this is indicative of a slight increase in baseline fluorescence levels for the cells following antibody treatment. Such an increase may be due to how the stained samples were handled during staining or to non-specific interactions between the cells and the antibodies during incubation. The peak on the right, in the stained population, represents the cells bound by the antibodies and therefore considered positive for the marker for which the antibody was specific. The height of this peak is likely to vary considerably depending on the antibody tested. There are a range of values for the positively stained population, in the example from just over $10^2$ to $10^4$, due to the different number of antibody molecules that may have bound to a single cell. This is why the concentration of antibody can also make a difference to fluorescent profiles. In an ideal profile the peak of fluorescence for the negative sample/population would be centred around $10^1$ mfu. The positive population would have higher values, there would be good separation between the positive and negative populations within the same sample and fluorescence should fall to nearly zero at the lowest point between the two populations.
2.1.1.1 **Japanese Quail – Antibody Screen**

Japanese Quail were housed at the UBC South Campus (see section 2.3 for more detail on sourcing and housing of quail) where all manipulation of live animals was also carried out. Prior to collection of samples, naive quail were rendered unconscious by an inhalation overdose of isoflurane, they were then decapitated by use of a guillotine, following guidelines provided by the UBC Animal Care Committee. Trunk blood was collected in 4 ml EDTA Vaccutainer tubes (BD Bioscience) which were inverted 8 times and placed on ice. Spleens were harvested and placed in tubes containing PBS and placed on ice until transported back to the lab at MSL. Blood was diluted with an equal volume of PBS and layered over a volume of Ficoll-Paque Plus (GE HealthCare Life Sciences) equal to that of the diluted blood. Tubes were centrifuged in a bench top centrifuge at 1,300 rpm, at approximately 24°C for 30 minutes and cells were harvested from the ficoll/plasma interface.
Cells were washed once in RPMI 1640 medium and resuspended in 2-3 ml of RPMI 1640 medium ready for counting. Spleens were mashed through a 70 µm mesh cell strainer and resuspended in 3 ml RPMI 1640 medium for counting.

2.1.1.2 House Sparrow – Antibody Screen

Six birds were captured, using mist nets, from a wild population in the suburban area of the North End of Winnipeg. Birds were rendered unconscious by inhalation of isoflurane and were exsanguinated by bleeding from the jugular vein, using a 26g needle and 3 ml syringe. Spleens were harvested from the birds and placed directly into tubes containing PBS on ice. As soon as possible after processing all birds, each spleen was mashed through a 70 µm mesh cell separator and re-suspended in 5 ml of RPMI 1640 medium. Tubes of cells were sealed with parafilm and placed in an insulated box with cold packs for shipment to the MSL in Vancouver. This work was undertaken by Dr. R. Lindsay and Ms. A. Dilbernardo of the NML in Winnipeg. As soon as possible following arrival at MSL, the cells were unpackaged and centrifuged in a bench top centrifuge at 1,200 rpm for 5 minutes. They were resuspended in 3 ml of fresh RPMI 1640 medium ready for counting.

2.1.1.3 White-Crowned Sparrow – Antibody Screen

WCSP were used as representative of another bird species for testing of antibodies, to see how much cross reaction of the antibodies across avian species there might be. They were selected mainly because birds were available as they were being collected and euthanised for a study by Sarah Heimovics, a post doctoral fellow in Dr. Soma’s lab in the Department of Psychology at UBC. Spleens were collected between 5.30 am and 10 am each morning the study was run, place in PBS on ice as soon as harvested and delivered to the lab at the Michael Smith Laboratory for processing as soon as possible after collection. Spleens were
mashed through a 70 µm cell strainer, to dissociate them into a single cell suspension, into 1.5 ml of FACS buffer (2% FBS in PBS) ready for counting.

2.1.2 Antibody Screens and Optimisations for West Nile Virus ELISA

It was planned to use an indirect ELISA, using serum samples from vaccinated birds, to detect any WNV specific antibodies produced by the birds following vaccination. In the final form of the assay, WNV specific antigens were bound to a plate which was then incubated with the test sera. During the incubation any WNV antibodies in the sera should bind to the antigen already on the plate. Any bound antibody is subsequently colourmetrically detected and measured by the amount of light absorbed by each sample. For this assay to work, a detecting antibody is needed that can recognise and bind to the WNV antibodies produced by the vaccinated birds. An anti-bird IgG HRP conjugated antibody (Benthyl Labs, #A140-110P, 1 mg/ml) was tested for use as the final ‘detecting’ antibody in this assay, as it should recognise the Fc region of a range of avian antibodies, and be easily measurable when a suitable substrate is added, due to the action of the HRP enzyme. The anti-bird IgG antibody was produced by vaccination of a goat with a combination of purified IgG from chicken, dove, duck and sparrow. The accompanying paperwork stated that the antibody would also recognize quail IgG. The WN antigen, to be used to bind any WNV specific antibodies to the plate, would also need to be assayed for its suitability for use with samples specifically from vaccinated birds. This is to confirm that any antibodies produced in vaccinated birds are able to bind to the antigen, as the antibodies cannot be measured if they are incompatible with the antigen.

Using the anti-bird IgY ‘detection’ antibody a checkerboard titration was set up in conjunction with normal quail IgY (Gallus Immunotech Inc, #QuIgY-010) as the antigen,
this was to define a likely dilution of the detection antibody to use for set dilutions of serum. The dilution of detecting antibody was then confirmed using quail serum samples from vaccinated quail in conjunction with WNV antigen (NML). To assess whether the anti-bird antibody would recognise HOSP antibodies, the HOSP serum itself was used as the antigen and bound directly to the plate, prior to adding the anti-bird antibody. The suitability of the WN antigen was assessed by comparison to an antigen more specific to the vaccines, to assess which would bind more WN specific antibodies when assayed in conjunction with sera from vaccinated and unvaccinated groups of quail, as well as positive control serum from a WNV infected bird.

2.1.2.1 Japanese Quail - ELISA

The initial screen, to assess the detecting antibody concentration to use, was achieved by binding purified quail IgY directly onto the wells of the ELISA (Immulon 4HBX) plate at different concentrations, by serial dilution across the plate; this was to simulate the different amounts of avian antibodies that may be present in the final samples. To find the optimal concentration of the detecting antibody, different concentrations were added to the plate by serial dilution going down the plate. This combination, of diluting one component across the plate and another component down the plate, constitutes a checkerboard titration.

The quail IgY was diluted in carbonate buffer (0.05 M, pH 9.6) to a concentration of 10 µg/ml. To set up the serial dilutions 50 µl of carbonate buffer was added to each well of the plate and 50 µl of diluted IgY was added to all the wells in column 1 to give 100 µl total volume in those wells. 2-fold dilutions were achieved by using a multi-channel pipette to first mix the contents of the wells in column 1 and then to transfer 50 µl to the wells of column 2 of the plate. The contents of the wells in column 2 were then mixed and a subsequent 50 µl
again transferred to column 3 and so on across all columns of the plate, the final additional
50 µl left from the final column was discarded. The plate was then incubated at 37 °C for
2 hours to allow the IgY to bind to the plate. The plate was washed by adding 200 µl wash
buffer (0.05% tween-20 in PBS) per well, the plate was then flicked to remove the buffer and
blotted on absorbent paper. This washing step was repeated four times. To set up the plate for
the serial dilutions of the detecting antibody, 50 µl blocking buffer (2% BSA in PBS) was
added to all wells of the plate. Detecting antibody was diluted in blocking buffer to a 1:200 dilution and 50 µl of this was added to all the wells in row A of the plate. As before, the
contents of these wells were mixed and then 50 µl transferred to the next row down the plate
and so on down the entire plate, with the final additional 50 µl being discarded. The plate
was incubated at 37 °C for 80 min and then washed four times as before. To measure the
amount of detecting antibody bound to the plate, 50 µl of substrate for the HRP enzyme
(1-Step Turbo TMB Substrate (ThermoScientific #34022)) were added to each well of the
plate and left at room temperature for approximately 2.5 min for sufficient colour
development to occur. The reaction was then stopped by adding 50 µl of 1M H₂SO₄ to each
well, in the same order in which the substrate was added, and the plate was read at 450 nm.
Statistical analyses were conducted using Microsoft Excel.

A small scale pilot study was run to provide serum samples that should be positive for
antibodies to the WNV proteins expressed by the recombinant adenoviruses. This was to
confirm the assay would work in a full scale study. Groups of three quail were vaccinated
with either rAdE or rAdNS3 (1.3×10⁸ IFU/bird in Emulsigen-P adjuvant (MVP
Technologies), or PBS only as a negative control group, a final group received an injection of
the WN antigen (25 µg/bird in Emulsigen-P), which it was hoped would act as a positive
control group. All birds were boosted with half of the dose of the initial injection 14 days later with the negative control group again being injected with PBS only. For an ELISA assay, to confirm that the concentration of detecting antibody established using the IgY as antigen, would work with actual serum samples, blood was collected from the quail at 24 days post boost directly to 4 ml serum vaccutainer tubes (BD Bioscience). Blood was held for approximately 2.5 hours at room temperature and then centrifuged in a bench top centrifuge at 3,870 rpm for 12 min. Sera were aliquoted and stored at -80°C until required for use in the following test ELISA.

WN antigen (NML) was diluted in 0.05M carbonate buffer (ph 9.6) to a final concentration of 60 µg/ ml, 50 µl (3 µg) were added to relevant wells of an ELISA plate and the plate was incubated at 4°C in a humid chamber overnight. The plate was washed by adding 200 µl per well of wash buffer (0.1% tween-20 in PBS) to all wells and the plate was then flicked to remove the buffer and blotted on absorbent paper. This wash step was repeated four more times. To block any non-specific binding sites, 200 µl blocking buffer (5% milk powder in PBS) was added to all wells and the plate incubated in a humid chamber at 37°C (same temperature used for all subsequent incubations) for 40 min, after which the plate was again washed five times. Fifty µl of blocking buffer were added to all relevant wells and 2-fold serial dilutions of all sera were set up across the plate: 50 µl serum were added to the first column of wells giving a 1:2 dilution, these were mixed and 50 µl transferred to the next column of wells for a 1:4 dilution and so on to a 1:256 dilution. The additional 50 µl from the final column of wells was discarded. The plate was incubated for 3 hours and 10 min and then washed five times as before. The anti-bird HRP conjugated antibody, used for detection, was diluted in blocking buffer and dilutions of 1:400, 1:600 and 1:800 were tested for all
dilutions of each sample. One hundred µl of the diluted antibody were added to the relevant wells and the plate was incubated for 1 hour and subsequently washed five times. To measure the bound detecting antibody, 100 µl of 1-Step Turbo TMB substrate for the HRP, were added to each well and the plate left at room temperature for 31 min before 100 µl 1M H₂SO₄ were added well to stop the reaction. The level of colour on the plate was measured by reading at 450 nm on a plate reader. Results were analysed using Microsoft Excel.

The above method was also employed when comparing two different forms of antigen for use to capture the WNV specific antibodies from serum samples. Some samples were run using the ELISA exactly as described above and some were run on a plate set up using a combination of WNV env and NS3 proteins, as antigen. The env protein (Abcam, # ab48960) and the NS3 protein (R&D Systems, cat # 2907-SE) were diluted in carbonate buffer and aliquoted to individual wells of an Immulon plate, such that there was 1.5 µg of each protein in each well, 3 µg total protein per well as for the standard ELISA assay, with an approximate volume of 60 µl per well. The plate was placed at 4 °C to allow the proteins to bind to the plate, 2 days later the plate was removed from 4 °C and washed, blocked and serum and antibodies added and incubated as per the ELISA protocol using the standard antigen. However due to the limited availability of the proteins being used as antigen, only 2 dilutions of serum could be included in this version of the assay, serum samples were directly diluted in blocking buffer to 1:40 and 1:100 dilutions before being added to the plate, so no further serial dilutions were required.

2.1.2.2 House Sparrow - ELISA

HOSP were captured and euthanised as detailed in section 2.1.1.1.2. The blood collected was transferred into 500 µl EDTA microtainer tubes (BD Bioscience), which were left at room
temperature for at least 30 minutes and then centrifuged in a microcentrifuge at 13,000 rpm for 5 min. Sera were removed, placed into fresh 1 ml tubes and held on ice, prior to shipping to the MSL in an insulated container on cold packs. On arrival at the MSL, the sera were placed at -80°C as soon as possible for storage until required. The sera were used to assess whether the anti-bird antibody would recognise HOSP serum when used in an ELISA assay. As the birds were WNV negative, the serum components were bound to the plate to act as the antigen and the detecting antibody was subsequently added to the plate.

To set up the 2-fold serial dilutions of sera, 90 µl of carbonate buffer (0.05 M, pH 9.6) were added to the first column of wells of an ELISA plate and 50 µl to subsequent well across the plate. One sample each of chicken and quail serum where included to act as positive controls for comparison, those and all HOSP sera were pre-diluted in carbonate buffer to a 1:40 dilution. Ten µl of the 1:40 dilutions of sera were added to the first column of wells giving a 1:400 dilution, the contents of the wells were mixed and 50 µl transferred across the plate with the final 50 µl being discarded. 2-fold dilutions in the range 1:400 – 1:102,400 were thus set up, although not all dilutions were developed in the assay. The plate was incubated, in a humid chamber, at 4°C for 2 days. Plates were washed by adding 200 µl wash buffer (0.1% tween-20) per well and then flicking the plate to remove the buffer and blotting on absorbent paper, this wash step was repeated four more times. Anti-bird antibody was diluted to 1:600 in blocking buffer (5% milk powder in PBS) and 100 µl was added to all wells, the plate was incubated in a humid chamber for 1hr at 37°C and then washed five times as before. One hundred µl 1-Step Turbo TMB substrate were added to each well and the reaction was left to develop for approximately 3 min before 100 µl 1M H₂SO₄ was added as a
stop. The amount of colour that had developed on the plate was then read at 450nm on a plate reader, results were analysed using Microsoft Excel.

2.1.3 Assessment of CD4 and CD8 Lymphocytes in Japanese Quail

Because Japanese quail have not been used extensively in detailed immunological studies, there have been no direct studies to establish T cell population analysis with regards to percentage of CD4+ and CD8+ cells. To this end available avian antibodies to the T cell markers CD4 and CD8 were used to identify these relevant sub-populations, from both splenocytes and PBMC samples from naive quail, using flow cytometry analysis to quantify the different sub-populations.

Naive quail were selected at random from the population housed in the UBC South Campus Annex (see section 2.3. for housing details). Birds were euthanised by an isoflurane overdose and trunk blood was collected from the carotid artery, following decapitation, directly into 4 ml EDTA vacutainer tubes (BD Bioscience) which were then placed on ice. Spleens were also harvested from each bird and placed into approximately 5 ml of PBS on ice. Organs and blood were returned to the MSL for processing as soon as possible. Spleens were mashed through a 70 µm nylon cell strainer into 3 ml RPMI 1640 medium ready for counting. Three ml of blood from each bird was layered over 3 ml Ficoll-Paque Plus (GE HealthCare Life Sciences) and tubes were centrifuged in a bench top centrifuge at 1,300 rpm, at approximately 24 °C for 30 minutes. Cells were harvested from the ficoll/plasma interface and placed in fresh RPMI 1640 medium, cells were washed once in RPMI 1640 medium and resuspended in 2 ml of RPMI 1640 medium ready for counting. Cells from spleens and blood were counted using a haemocytometer and leukocytes were identified and counted. Cells, in RPMI 1640 medium, were transferred to wells of a 96-well round bottom plate at a
concentration of $1 \times 10^6$ cells per well and were washed once; this was achieved by adding 200 µl FACS buffer (2% FBS in PBS) per well the plate was then centrifuged (1,500 rpm bench top centrifuge, 5 min, 4°C) and flicked to remove the buffer, cells were then ready for antibody staining. The antibodies used had been previously shown to recognise T cell specific CD markers in quail. They were the duck specific CD8 antibody (Du-CD8-1) with an APC in house conjugate and the chicken, turkey specific CD4 antibody (CT4) with either an APC or a FITC in house conjugate added. Antibodies were diluted in FACS buffer, Du-CD8-1 was used at 0.1 µg per $1 \times 10^6$ cells and CT4 at 0.2 µg per $1 \times 10^6$ cells concentrations, and 100 µl volume of diluted antibody were added to each relevant well. Unstained control wells received FACS buffer only and the plate was left at 4°C for 30 min wrapped in aluminium foil. Cells were then washed as before and 200 µl FACS buffer was added to each well, cells were re-suspended and transferred to 1.2 ml cluster tubes ready for FACS analysis (BD FACS Calibur). Results were analysed and displayed using Flowjo software (Tree Star Inc.). Statistical analyses to calculate standard deviation and coefficient of variation (standard deviation/mean) were calculated using Microsoft Excel. Coefficient of variation is considered a more comparable value for variation than standard deviation for populations where the means are very different.

2.2 Generation and Testing of Recombinant Adenovirus Vaccines

Two different recombinant adenoviruses were created as WNV vaccines. Each adenovirus variant contained different WNV cDNA: one contained cDNA for both pre-M and Env (M-E) and the other cDNA for NS3. The recombinants were named rAdE and rAdNS3 respectively. For previously mentioned control purposes, a recombinant adenovirus was also
created which contained no additional DNA; this empty vector control was named rAdMT. Following recombination between any additional DNA and the adenovirus genomic plasmid, several viral plaques for each construct were selected and screened for inclusion of the correct DNA. These viral colonies were further purified and the stocks expanded to allow initial testing of the recombinant adenoviruses. Following positive assessment of both mRNA and protein expression in both recombinants, amounts of both adenoviruses were further expanded and purified to create working vaccine stocks.

2.2.1 Adenovirus Infections in Avian Cell Lines

To check whether or not avian cells could be infected by human adenoviruses, two different avian cell lines were infected with a recombinant adenovirus that expresses the eGFP protein (rAdeGFP). Cells were infected at different virus concentrations and then assessed by flow cytometry.

The human epithelial cell line A549 was used as a positive control, as these cells are known to be easily infected by adenoviruses and are able to express high levels of the recombinant proteins produced by the infected cells. The avian cells lines used were embryonic fibroblast cells (CEF) from chickens and the QT35 fibrosarcoma cell line from Japanese quail. A549 cells were grown in DMEM (supplemented with 10% FBS, 1× l-glutamine, 1× pen/strep and 1× HEPES (Invitrogen)), CEF cell in DMEM (supplemented with 5% FBS, 5% tryptose phosphate broth, 1× l-glutamine and 1× pen/strep (Invitrogen)) and QT35 cells in Ham’s F12 medium (supplemented with 10% FBS, 5% tryptose phosphate broth, 1× HEPES and 1× pen/strep (Invitrogen)). All cell lines were incubated at 37 °C at 5% CO₂.

Cells were grown in 10 cm diameter tissue culture dishes until approximately 80% confluent, medium was then removed and replaced with 9 ml of the corresponding medium but with the
FBS supplement reduced to 2%. For CEF and A549 cells, rAdeGFP was diluted 10-fold in the respective medium and 1 ml of dilutions $10^{-2}$, $10^{-4}$ and $10^{-6}$ were added to one plate each of the different cell lines. QT35 cells were tested at a later date, again rAdeGFP was diluted in medium and used to infect cells at MOI of 1, 5 and 100. As a control, cells were infected with MOI10 of rAdNS3 (a non-fluorescent adenovirus). For all experiments some cells were left uninfected as controls and cells were incubated for 2 days post-infection before being harvested. As all cell lines were adherent, cells were washed directly in the plates, by adding 5 ml PBS and gently rocking the plates before removing the PBS. One ml of FACS buffer (2% FBS in PBS) was added to each plate, cells were carefully scraped to removed them from the plate and gently resuspended in the FACS buffer by pipetting and were then transferred to tubes ready to be analysed by flow cytometry (BD FACS Calibur). As the fluorescent protein, if present, is made directly by the adenovirus inside of the cells, there is no need for later antibody staining prior to measuring fluorescence levels by flow cytometry. Results were analysed and displayed using Flowjo software (Tree Star Inc.).

### 2.2.2 Generation of Plasmids and Their Recombination

The recombinant adenoviruses to be used as vaccine vectors were created using the Admax™ Adenoviral Vector Creation System from Mircobix Biosystems Inc (Ontario, Canada). The system was supplied as plasmid DNA consisting of an adenovirus genomic plasmid, called pBHGloxΔE1,3Cre (size approx. 35 Kb) and several different shuttle plasmids (see Appendix A for plasmid maps). The genomic plasmid (BHG) is missing both the E1 and E3 adenovirus genes, which renders any viral particles created unable to replicate. It does however contain a Cre gene and a loxP site, both of which are used during recombination. The shuttle plasmids contain a loxP site, a multiple cloning site containing different
restrictions enzyme sites and are with or without a promoter; the shuttle plasmid selected for use was pDC316 (size 3.9 Kb) which included the MCMV promoter. The system is designed to allow the user to insert their DNA of choice into the selected shuttle plasmid, based on ligation of complementary restriction enzymes sites on both the insert DNA and the shuttle plasmid. Re-combination between the shuttle plasmid (pDC316) and adenovirus genomic plasmid (BHG) is then undertaken using a co-transfection technique in ‘293 cells’ (see glossary), see Figure 2.2, for a schematic. The Cre, to stimulate re-combination, is created from BHG itself, and recombination should occur between the loxP sites on both plasmids to incorporate the novel DNA from pDC316 into BHG. The 293 cells used are already stably transfected with the adenovirus E1 gene which provides conditions suitable for replication, thus allowing any recombinant viruses to replicate when they would not normally be able to do so. As well as two WNV expressing recombinant adenoviruses, another recombinant (rAdMT) was also created through recombination of the genomic plasmid and pDC316. However, pDC316 for rAdMT did not contain any WNV DNA, thus the adenovirus created would act exactly as the other recombinants but would only contain adenoviral DNA and could be used as a vector only control.

To enable creation of WNV DNA for insertion into pDC316, inactivated WNV RNA from an infected bird was obtained from Mike Drebot of the Public Health Agency of Canada in Winnipeg. The RNA was in Qiagen RNA lysis buffer and the Qiagen kit, RNeasy Mini Kit, was used to extract the WNV RNA from the buffer. Briefly an equal volume of 70% ethanol was added to precipitate the RNA and the samples were then added to a filter column. The column was centrifuged (15 sec at 8,000 g) and washed twice with specific buffers from the kit, to
remove the lysis buffer and salt components. The RNA was eluted from the filter column by adding 30 µl of RNase free water and spinning (1 min at 8,000 g), the quantity and quality of the RNA was assessed using a spectrophotometer and the vial of RNA was then placed at -80°C.

Figure 2.2 Schematic of Recombination from the Admax™ System.

Foreign DNA is inserted into the shuttle plasmid (on the right) prior to recombination with the genomic plasmid, which occurs at the loxP sites in each plasmid when plasmids are co-transfected into 293 cells. The lower section of the figure shows the linear recombinant adenoviral DNA containing the foreign DNA and the viral packaging region (ψ).

The WNV RNA was converted into cDNA by use of the Invitrogen Superscript II RT kit. 20 ng of RNA, diluted as required in DEPC treated water were added to microtubes containing 1 µl 10 mM dNTP mix and 2 pmole of gene specific primer (3’ end primers only) for each of the ‘genes’ of WNV, namely capsid, membrane, envelope or NS1 through NS5. The tubes were incubated at 65°C for 5 min and chilled briefly on ice. Four µl 5× first strand
buffer, 2 µl 0.1M DTT and 1 µl RNase (40 U/µl) were added to each tube and tubes were incubated at 42°C for 2 min before 200 U (1 µl) of superscript reverse transcriptase was added to each tube. Tubes were further incubated at 42°C for 50 min after which time the reactions were inactivated by heating to 70°C for 15 min. cDNA was stored at -20°C until required. The cDNA created using the membrane and the NS3 primers were used as DNA templates in subsequent PCR reactions to create inserts for the recombinant adenoviruses that would express either the preM-E proteins or the NS3 protein, respectively.

Specific PCR primers were designed for synthesis of the preM-E or NS3 DNA to be inserted into pDC316. The primer sets contained a different restriction enzyme site for the forward (EcoRI) and reverse (BglII) primers, as well as Kozak sequences and a start codon prior to the WNV sequence specific region on the forward primer (preM-E fwd: GCATCTTAAGTACCAATGGGAGAGATT, preM-E rev: GCATTCTAGATTAAGCGTCACGTTCA, NS3 fwd: AGGAACCTTAAGACCCTCCGCACAACAC and NS3 rev: AGAAAGATCTTTAACGTTTTCCCGAGG).

PCR reactions were set up using Invitrogen Platinum Pfx DNA Polymerase. Extensive optimisation resulted in the following volumes of reagents being used in each 0.2 ml PCR reaction tube: 5 µl 10× Pfx buffer, 5 µl 10× PCRx enhancer, 1.5 µl 10 mM dNTP mix, 1 µl 50 mM MgSO₄, 1 µl 0.1 mM primer for both forward and reverse priming, approx 100 ng of the relevant cDNA template and 0.5 µl Pfx polymerase, with the total volume brought to 50 µl with nanopure water. The contents were gently mixed by pipetting up and down. Cycler conditions were used as follows: 1 cycle of 94°C for 5 min, 35 cycles of 94°C for 15 sec and 68°C for 2 min and 15 sec, 1 cycle of 68°C for 10 min. PCR products were run on a 1% agarose gel (60 V for 60 min) and the gels were stained with ethidium bromide for band visualisation. Expected band sizes were 2 Kb for the M-E insert and 1.8 Kb for the NS3
insert. Bands of the correct sizes were subsequently gel purified using the QiaexII Gel Extraction kit (Qiagen) to obtain purified WNV DNA.

For amplification of the DNA inserts, PCR products were cloned into the pCR-BluntII-TOPO vector (Invitrogen) and then transformed into *Escherichia coli*. Bacterial colonies were grown on selective agar plates; DNA was extracted from them and screened for the WNV insert by PCR using the same specific primers and conditions as above.

To obtain complementary sticky ends for ligation purposes, restriction enzyme digests of DNA for both of the WNV inserts and pDC316 were performed. Tubes were set up as follows: 1 µg of DNA (either M-E, NS3 or pDC316), 1 µl of both BglII and EcoRI, 2.5 µl 10× reaction buffer (ReAct buffer 3) with total volume made up to 25 µl with nanopure water. Reactions were left at 37 °C for 70 min and then purified using the QIAquick PCR purification kit. Two ligation reactions were set up to insert the WNV DNA into pDC316, one each for M-E and NS3: 4 µl 5x ligase buffer, 2 µl pDC316 DNA (vector), 3 µl either M-E or NS3 DNA (this was omitted from the vector only control tube), 0.5 µl T4 ligase and reaction volume was topped up to 20 µl with nanopure water. Ligation reactions were left at room temperature (approx 25°C) for 2 hours and were then used to transform *E.coli* for DNA amplification. Three µl of each ligation reaction was added to 100 µl of DH5α *E. coli* and incubated on ice for 30 min, cells were then heat shocked at 42°C for 30 sec and immediately placed back on ice for 2 min. 250 µl of room temperature SOC medium were added to each reaction and the tubes were shaken horizontally for 60 min at 37°C. Fifty µl and 100 µl of each transformation reaction were then spread onto pre-warmed LB plates with ampicillin and the plates placed upside down in a stationary incubator for approximately 30 hours. Several bacterial colonies from each plate were selected for screening for the presence of the
WNV insert DNA using colony PCR, using the same PCR primers and conditions as for creating the WNV insert DNA. The PCR products were run on a 1% agarose gel, at 60 V for 60 min and visualised with ethidium bromide. Positive colonies showed a band at the correct size for the inserted WNV DNA and two colonies for both M-E and NS3 were selected. As the WNV DNA was now in the pDC316 shuttle plasmid, the DNA was called E-316 and 3-316 respectively. The same colonies were re-selected again from the plates and used to further amplify the DNA by further amplification of the same bacterial colonies and extracting DNA using a miniprep kit (Qiagen).

To check that the WNV inserted DNA in the shuttle plasmid was correct, DNA for sequencing was made from the bacterial colonies transformed with either E-316 or 3-316 using a standard miniprep kit (Qiagen). Primers were designed that started just outside of the inserted region and were complementary to the shuttle plasmid so that the same primers (Fwd-GGTCTATATAAGCAGAGCTC, Rev-GCATTCATTTTATGTTTCAGG) could be used to sequence both E-316 and 3-316. DNA samples were diluted to 200 ng/µl and primers to 5 pmol/µl and were then taken to the NAPS (Nucleic Acid Protein Service) unit at UBC for sequencing using the 3730 DNA Analyzer (Applied Biosystems).

Co-transfection of 293 cells were undertaken, using Lipofectamine and Plus reagent (Invitrogen), including DNA from the BHG plasmid and either E-316 or 3-316 plasmids to create the recombinant adenovirus vaccines, rAdE or rAdNS3 respectively. The BHG plasmid was also co-transfected with pDC316 as an empty shuttle plasmid to create the empty vector control recombinant adenovirus, rAdMT. 293 cells for the co-transfections were set up in two 6-well tissue culture plates (DMEM, with 10% FBS, 1× pen/strep and 1× Hepes incubated at 37°C, 5% CO₂) to be approx. 50% confluent just prior to
co-transfection. Three wells were set up for E-316/BHG co-transfection, 3 wells for 3-316/BHG, 2 wells as a BHG only control, 2 wells with pFG140 DNA (an infectious adenovirus plasmid, supplied with the system as a positive transfection control), 1 well for a mock transfection control (reagents but no DNA) and 1 well was left as a cells only control. Reagents were mixed in 1.5 ml tubes, one per well of the 6-well plate, prior to addition of reagents to the cells. Two µg of relevant DNA (0.5 µg for pFG140) were diluted in 100 µl transfection medium (as for DMEM for 293 cells but serum free) and then 6 µl plus reagent were added, tubes were incubated at room temperature for 15 min. Four µl of lipofectamine were diluted in 100 µl transfection medium and then this was added to the tubes already containing the DNA. The tubes were again left at room temperature for 15 minutes. During this time the growth medium was removed from the 6-well plates and they were rinsed with 500 µl per well of transfection medium to remove all serum from the cells. The total volume of transfection reagents and DNA was then added to the respective wells of each 6-well plate, with transfection medium alone being added to the cells only well. Plates were incubated at 37 °C, 5% CO₂ for 3 hours then the transfection medium was removed and the cells were overlaid with an agarose overlay (0.5% seaplaque agarose in 293 DMEM with 2% FBS), plates were returned to 37 °C and checked regularly for plaque formation.

2.2.3 Selection and Purification of Viral Recombinants

To ensure that only a single clone for each recombinant virus was present in the final stocks of virus vaccine, viral plaques present after co-transfection were re-isolated twice more following initial selection and harvest. Virus stocks were then screened for the presence of either M-E or NS3 WNV DNA, to confirm recombination had occurred. Subsequently
recombinant virus stocks were further amplified to generate sufficient material for further testing.

The plates of co-transfected 293 cells were maintain at 37 °C and 5% CO₂. At 6 days post transfection an additional 2 ml of overlay were added to each well of both plates. At 14 day post transfection plaques were visible and large enough to harvest. Three plaques were harvested for rAdE but only 1 for rAdNS3 as it was the only plaque of sufficient size. To harvest the adenovirus from the plaques, a pipette tip was inserted through the agarose overlay and used to scrape off some of the virus containing cells, the tip was then rinsed in 1.5 ml 293 DMEM (2% FBS), in a 1.5 ml tube, to make a crude virus stock and the tip was then discarded. Any crude virus stocks not used for further purification processing were placed at -80 °C for use if required. For further purification, approximate 10-fold dilutions were made of one crude virus stock of each adenovirus. Dilutions were made by taking 100 µl of the crude virus stock and adding it to 1 ml of 2% 293 DMEM (2% FBS). This dilution was repeated twice more to obtain 3 dilutions of each adenovirus. Two 6-well plates of 293 cells, at 60% confluency, were then infected with the adenovirus dilutions. Medium was removed from the plates and the entire volume of diluted virus was added to one well per dilution per virus, thus 6 wells were used for the 2 different adenovirus dilutions and 3 wells were left as cell only controls, to which was added 1 ml 293 DMEM only. Both plates were returned to 37 °C, 5% CO₂ and the following day 2 ml of agarose overlay were added to each well as before. After approximately 1 week, when plaques were large enough to harvest, the entire purification process was repeated, namely harvesting of 3 plaques per adenovirus, dilution, infection and subsequent overlaying of new 293 cells. Approximately 1 week later the new round of plaques were considered large enough to harvest. Again 3 plaques per
adenovirus were harvested into 1.5 ml 293 DMEM and placed at -80 °C as purified crude virus stocks to await further testing.

To initially amplify purified virus stocks for both adenoviruses, 100 µl of stock from plaque 1 of the final round of purification were added to 1.4 ml of 293 DMEM (2% FBS) and this volume was then added to a T25 flask of 293 cells at 60% confluency, from which the medium had already been removed. A control flask with cells and medium alone was also maintained, the same as the infected cells, to ensure that the cells themselves were growing well. The infected flasks were incubated at 37 °C, 5% CO₂ for 1 hour and then a further 10 ml of 293 DMEM (2% FBS) were added to each flask and flasks were returned to the incubator. Four days post-infection 95% of the 293 cells in each flask showed cytopathic effects of infection, as demonstrated by the cells having a more rounded phenotype than usual. They were also likely to have detached from the flask and be floating in the medium. Both flasks were then placed at -20 °C for 30 minutes, until frozen, to release the adenoviruses from the cells by rupturing the cell membranes. For each adenovirus, 3 ml of this infected media were added to a T75 flask of 293 cells at 60% confluency, both flasks were incubated at 37 °C, 5% CO₂ for 1 hour, during this time the flasks were gently rocked several times to ensure that the entire cell monolayer was kept moist. An additional 12 ml of 293 DMEM (2% FBS) were then added to each flask and both flasks were returned to the incubator. Three days later 100% of cells exhibited cytopathic effects so the entire volume of medium from each flask was collected and aliquots were placed at -80 °C. Prior to these supernatants being used for further analysis, each aliquot was thawed at 37 °C and re-frozen at -80 °C, two more times to release viral particles from the cells. These aliquots were then termed crude cell lysates.
Adenoviral DNA was isolated for analysis as follows: 200 µl of crude cell lysate were placed in a 1.5 ml tube, 14 µl 10% SDS, 6 µl 0.5M EDTA and 40 µl of 20 mg/ml proteinase K were added and contents were mixed. Tubes were incubated for 3 hr at 55 °C and then 5 min at 95 °C to inactivate the proteinase K digestion of the viral capsid. The total volume per tube was increased to 400 µl by adding deionised water and then 200 µl of 7.5M ammonium acetate was added. The mixtures were extracted twice with 1:1 phenol-chloroform by adding 600 µl phenol-chloroform per tube, the tubes were then vortexed and centrifuged briefly in a microfuge to separate the aqueous and organic layers. The DNA in the top layer of each sample was removed and placed into a fresh 1.5 ml tube. Following both extractions, 1.2 ml 100% ethanol were added to each tube and the tubes were centrifuged in a microfuge for 10 min at maximum speed. Each DNA pellet was washed twice with 1 ml of 70% ethanol by resuspending the pellet by vortexing and spinning in the microfuge on maximum speed for 5 min after which the ethanol was carefully removed. The DNA was then dissolved in 25 µl nanopure water per tube by leaving tubes on the bench for 15 min after which time tubes were placed on ice for use and later frozen at -20 °C for storage.

Adenovirus DNA previously prepared was used to screen the selected and amplified recombinant viruses to ensure that the specific relevant WNV DNA was present in each of the recombinant adenovirus vectors. This was achieved by a PCR reaction using primers designed to amplify a small region internal to either the Env or the NS3 region of DNA (Env fwd - TTG GAA GGA GTG TCT GGA GCA ACA, Env rev - TTT CCG TGC GAC TCC ACA GTA GTT, NS3 fwd - AGT GCA GGG TGA AAG GAT GGA TGA, NS3 rev -TCACACTCTTCCGGCTGTCAATCA) in conjunction with a Taq polymerase. PCR reactions were set up in 0.2 ml PCR tubes and contained the following: 5 µl KCL buffer, 1 µl 10 mM dNTPs, 3.5 µl 25 mM MgCl₂, 1 µl
each of forward and reverse primers, approximately 1 ng relevant DNA, 0.5 µl Taq polymerase with the total volume made up to 50 µl with nanopure water. Cycler conditions were: 1 cycle of 3 min at 95°C, 35 cycles of 30 sec at 95°C, 30 sec at 70°C and 1 min at 72°C and finally 1 cycle of 10 min at 72°C. 2 µl of each PCR product were loaded onto a 1% agarose gel and run at 75 V for 1 hr to confirm the presence of a product band as well as to assess the size of the product.

Adenoviral DNA prepared as previously mentioned was also used as samples for DNA sequencing. Sequencing primers were designed that ran from the region just outside of the WNV DNA insert, in the adenovirus vector, so the same primers could be used for sequencing both recombinant adenoviruses (fwd TGGCACTCATTCTATTGG, rev GCAAAACAGATACAAAACCTA). DNA samples were diluted to 200 ng/µl and primers to 5 pmol/µl and were then taken to the NAPS unit at UBC for sequencing using the 3730 DNA Analyzer (Applied Biosystems).

2.2.4 Assisting mRNA Expression of the Adenovirus Vaccines

Following infection with either of the two recombinant adenoviruses, mRNA expression was demonstrated in two different cell lines; A549 cells, a human cell line known to highly express adenoviruses when infected, were used to show expression under optimal conditions and QT35, a quail fibroblast cell line, was used to confirm expression in avian cells. Both cells lines were infected with the adenovirus vaccines and the infected cell were harvested and RNA was isolated. Any pre-existing DNA ‘contamination’ was removed from the samples and de novo cDNA was created from the RNA. Samples were then used as templates to amplify any cDNA by PCR, using WNV specific primers. PCR products were run on a gel to confirm the presence of DNA bands.
A549 cells were grown in standard DMEM (10% FBS, 1× pen/strep and 1× HEPES) incubation conditions 37 °C, 5% CO2) in 6 cm diameter plates, until approximately 80% confluent. Medium on the plates was then changed to 2% FBS DMEM and crude cell lysates of either of the two recombinant adenoviruses, rAdE or rAdNS3 were added to 2 plates per virus, to infected the cells at a MOI of 20. Two plates of cells received medium only, these acted as the non-infected control cells. Plates were gently rocked to distribute the virus evenly over the plates and then returned to the incubator for 2 days. RNA was harvested from the infected cells using Trizol reagent, medium was removed from the cells and 2 ml reagent were added directly to each plate of cells and the mixture was pipetted up and down gently several times before being evenly divided between 2 separate 1.5 ml tubes per plate. Tubes were initially placed on ice until all samples were collected after which they were allowed 15 min at room temperature (15-30°C). 200 µl chloroform were added to each tube, tubes were shaken vigorously for 15 sec and left at room temperature for 2-3 min, before being centrifuged at 12,000 g for 15 min at 4 °C to separate the phases. RNA in the upper aqueous phase was carefully removed from each tube and placed into new tubes. To precipitate the RNA, 500 µl of isopropyl alcohol were added to each tube, they were incubated at room temperature for 10 min and then centrifuged at 12,000 g for 10 min at 4 °C. The RNA pellets were resuspended in 1 ml of 75% ethanol per tube by vortexing, some tubes were place at -80 °C while still in ethanol for storage, the rest were centrifuged at 7,500 g for 5 min at 4 °C. This procedure was repeated to provide a second ethanol wash to those samples that were going to be further processed. After the ethanol was removed RNA pellets were air dried for approx 30 min, to allow evaporation of all the ethanol. Each pellet was then re-dissolved in
50 µl of DEPC treated water by incubation at 55 °C for 10 min before being placed on ice. RNA in all samples was then quantified using a spectrophotometer.

Any cellular DNA contamination present in the samples was removed by a DNase digest using the Ambion DNA-free kit. Ten µg of total RNA per sample were placed into a fresh 1.5 ml tube, 7.5 µl of 10× DNase I buffer and 2 µl DNase were added to each tube and total volume was made up to 50 µl per tube with DEPC treated water. Tubes were incubated at 37°C for 30 min. DNase inactivation reagent was re-suspended by vortexing and 2 µl were added to each tube which was then mixed by flicking. Tubes were left at room temperature for approximately 2 min and then centrifuged at 10,000g for 1.5 min to remove the inactivation reagent. The RNA was again transferred to fresh 1.5 ml tubes.

Superscript III Reverse Transcriptase (Invitrogen) was used to create the first strand cDNA directly from the RNA. In fresh 1.5 ml tubes, 1 µl oligo dT primers (50 µM), 1 µl 10 mM dNTP mix and approximately 200 ng of each RNA sample were made up to 13 µl with distilled water. The tubes were then incubated at 65 °C for 5 min and then left on ice for at least 1min. Then 4 µl 5x First Strand Buffer, 1 µl DTT (0.1M), 1 µl RNase Inhibitor (40 U/µl) and 1 µl Superscript III Reverse Transcriptase (200 U/µl) were added and the contents were mixed gently by pipetting. Tubes were incubated at 50 °C for 60 min and then at 70 °C for 15 min to inactivate the reaction before being returned to ice.

A PCR reaction was used to check for the presence of specific cDNA in each sample. The primer sets with sequences internal to the two different WNV DNA inserts were used, the same sets as used to screen the rAd DNA for the presence of the insert DNA (section 2.2.3). The fully processed cDNA samples were used as the template in some reactions, others were set up using the RNA samples following DNase digest as templates. This was to check if any
contaminating DNA remained following the RNA isolation and purification process. Reactions were set up in thin-walled 0.2 ml PCR tubes as follows: 5 µl 10× buffer containing 500 mM KCl, 1 µl 10 mM dNTP mix, 3.5 µl 25 mM MgCl₂, 1 µl each of 0.1 mM forward and reverse primers, 2 µl cDNA or RNA as template or 1 µl DNA as template (for positive controls), 0.5 µl Fermentas Taq Polymerase (2.5U) and each tube was made up to 50 µl total volume with nanopure water. Three tubes were set up using the env primer sets and either cDNA or RNA from the rAdE infected cells or E-316 DNA as templates and 3 tubes were set up using the NS3 primer sets and either cDNA or RNA from the rAdNS3 infected cells or 3-316 DNA as templates. Negative control reactions were set up using both primer sets, 2 tubes also contained either cDNA or RNA from non-infected cells and one tube was set up with no template to check for possible contamination of reagents. After the first run, some optimisation was required to removed additional non-specific binding from the env reaction tubes. This was achieved by reducing the amount of 25 mM MgCl₂ from 3.5 µl to 2.5 µl in subsequent PCR reactions. PCR cycler conditions were as follows: 1 cycle of 95 °C for 3 min, 35 cycles of 95 °C for 30 sec, 70 °C for 30 sec and 72 °C for 1 min and then 1 cycle of 72 °C for 10 min. PCR reaction products were run on a 25 ml 10% agarose gel, containing 2 µl SYBR safe (10,000× conc.) in TAE buffer, for 45 min at 75 V and a photograph of the gel was taken under UV light.

To confirm mRNA expression in an avian cell line, QT35 cells were assessed as above: cells were infected and RNA harvested in Trizol reagent, DNA contaminates were digested, cDNA was synthesised from the RNA and finally a PCR reaction was performed to check for the presence or absence of cDNA. All steps were exactly as for the A549 cell samples, unless stated otherwise in the following paragraph. QT35 cells were maintained in F12
Ham’s medium (supplemented with 10% FBS, 5% tryptose phosphate broth, 1× HEPES and 1× Pen/Strep), grown in 10 cm diameter plates. One plate was infected with either rAdE or rAdNS3 crude cell lysate stocks at a MOI of 100, and 1 plate was left in media only as a non-infected control. Three days after infection, cells were harvested by removing the medium and adding 1.5 ml Trizol per plate. A cell scraper was then used to remove the cells from the plate into the Trizol. All volumes of reagents used subsequently, in the Trizol protocol, were increased by 1.5 times, although the final RNA pellets were still dissolved in 50 µl DEPC treated water. PCR reactions and conditions were the same as for the A549 samples, except that 3 µl of all templates and 1 µl Taq were added to each reaction tube.

### 2.2.5 Assessing Protein Expression of the Adenovirus Vaccines

Protein expression was demonstrated in two different cell lines following infection with either of the two recombinant adenoviruses; A549 cells, to show expression under optimal conditions and QT35 cells to confirm expression in avian cells. Both cell lines were infected with the adenoviruses at varying MOIs, cells were harvested into a lysis buffer and protein levels were assessed. The same amount of protein from each sample was then mixed with gel loading buffer to denature the proteins. The samples were loaded onto a polyacrylamide gel to separate the proteins based on size and the proteins were then transferred to a membrane. The membrane was incubated with a primary antibody to detect WNV proteins, an enzyme conjugated secondary antibody and subsequently a substrate were added to enable visualisation of the proteins on the membrane.

A549 cells were set up in 6 cm diameter plates and grown in DMEM (10% FBS and other supplements as previously stated) until approximately 80% confluent. Medium was changed to DMEM (2% FBS and other supplements as previously stated) and crude cell lysate of
either rAdE or rAdNS3 were added such that one plate for each virus was infected at 5, 10 or 100 MOI (6 plates total), 2 plates were left as non-infected controls. After 3 days cells were washed twice with PBS and then lysis buffer was added to each plate. After optimisation, different procedures were used for harvesting the cells depending on which virus had been used to infect them. rAdNS3 infected cells were scraped from the plates into 175 µl standard western lysis buffer (stock buffer – 1.5 ml 1M Tris (pH7.5), 3 ml 10% NP-40, 3 ml glycerol, 20 ml distilled water, 0.9 ml 5M NaCl, 0.3 ml 0.5M EDTA). One Roche Complete Protease Inhibitor Tablet was dissolved into 10 ml of stock buffer just prior to use. This mixture was placed into 1.5 ml tubes. rAdE infected cells were scraped from the plates into 175 µl of NP-40 lysis buffer (20 mM Tris (pH 8.8), 1% NP-40, 2 mM EDTA and 150 mM NaCl). One Roche Complete Protease Inhibitor tablet was dissolved into 10 ml of stock just prior to use. This mixture was placed into 1.5 ml tubes. All tubes were left on ice for at least 10 min to allow lysis of the cells to occur. As WNV NS3 protein is intracellular but WNV env protein has a transmembrane region, samples were processed differently for the next step. For rAdNS3 infected cells samples were centrifuged at maximum speed in a microfuge for 4 min, to remove the cellular debris and the supernatant was used in subsequent steps. For rAdE infected cells, where the env protein was potentially still within the membranes of the cells the samples were vortexed and/or pipetted, as required, to ensure that any particulate matter present was as even distributed throughout the sample as possible, before use in any subsequent steps.

Ten µl of each sample were transferred to one well of a 96-well flat bottom plate for use in a Pierce BCA Protein Assay (ThermoScientific) to quantify the amount of protein present. Assay reagents A and B were mixed in a 50:1 ratio and 200 µl of this mixture were added to
wells of the 96-well plate containing either the unknown samples or 6 BSA standards (ThermoScientific) of known protein concentration (4, 2, 1, 0.5, 0.25 and 0.125 mg/ml). The plate was placed at 37 °C for 30 min to allow the colourmetric assay to develop and then the absorbance values of the plate were read at 562 nm in a plate reader. Concentration of protein in the samples was calculated based on the standard curve created from the BSA standards.

Samples in the lysis buffers were then denatured and reduced by placing 9 volumes of sample into 1 volume of a 10× reducing sample buffer (200 µl of an 18% SDS in tris and 0.05M DTT solution was added to 1 ml of non-reducing sample buffer (250 mM Tris (pH 6.8), 0.05% bromphenol blue, 50% glycerol and 10% (w/v) SDS) samples were incubated at 100 °C for 5 min. Based on the protein concentrations for each sample, calculated from the BCA assay, different volumes of each of these samples were loaded on to the gel for protein separation, but always all samples on the same gel had the same amount of protein loaded.

Protein separation was achieved using a standard polyacrylamide gel technique. A 10% running gel was used (required volume taken from, 2 ml nanopure water, 1.6 ml 30% acrylamide, 1 ml 2 M Tris (ph 8.8) 25 µl 20% SDS and 50 µl 10% APS with 20 µl TEMED added just before pouring) on top of which, where the wells were located, was a stacking gel (required volume taken from, 885 µl water, 335 µl 30% acrylamide, 75 µl 0.5M Tris (pH 6.8), 10 µl 20% SDS and 20 µl 10% APS with 2 µl TEMED added just before pouring). Gels were submerged in a running buffer (diluted to 1× concentration from a 10× stock (30 g Tris base, 144 g glycine, 10 g SDS, to 1 litre with nanopure water)) prior to loading the samples, in the sample buffer, into the wells of the gel. At least one well per gel contained a standard protein marker (Invitrogen) to provide bands of a known size, this was to enable estimation of the size of the bands of the sample protein. The gel running apparatus was connected to a
power pack and 100 V was applied to the gel until the blue dye in the sample buffer could be seen near the bottom of the gel, usually after approximately 1.5 hr.

To facilitate subsequent steps for indentifying the proteins, the proteins were transferred from the gel to a nitrocellulose membrane, which is more robust for handling. This was achieved by sandwiching the running gel region against the membrane between layers of blotting paper and mesh sponges. The assembled gel carrying case was placed in a transfer tank and orientated so that current would flow correctly to move the proteins from the gel to the membrane (not from the gel to the blotting paper!). The gel holding tank was filled with a 1× transfer buffer (700 ml nanopure water, 200 ml 100% methanol, 100 ml 10× transfer buffer stock (30.3 g Tris base, 144.1 g glycine made to 1 litre with nanopure water)) and the entire apparatus was placed at 4°C, a charge of 70 V was applied for 80 min to facilitate protein transfer to the membrane.

The membrane, containing the proteins, was placed in a small plastic tray and stained with approximately 10 ml of Ponceau S solution (0.1% (w/v) Ponceau S in 5% acetic acid) for 5-10 min on a rocker, until protein bands were visible. The staining solution was removed and the membrane rinsed once in approximately 5 ml of blocking buffer (5% BSA in PBS) to remove excess stain. The location of samples on the membrane were noted and the membrane was trimmed, as required, to separate samples for rAdE infected cells from samples infected by rAdNS3, as different specific antibodies would be used to visualise the different proteins. The membrane sections were placed in separate trays and amply covered with blocking buffer, the trays were sealed with Saran wrap and were left overnight at room temperature on a rocker. This was to block any potential non-specific antibody binding sites on the membranes.
The monoclonal antibodies (mAbs) used to visualise protein bands were diluted in 10 ml of blocking buffer. For rAdE infected samples, the mAb against the WNV env protein, mAb 3A3 (BioReliance, concentration 0.5 mg/ml) was used at a dilution of 1:500 and for rAdNS3 infected samples, the mAb against a recombinant WNV NS2b/NS3 protein (R&D Systems, MAB2907, concentration 0.5 mg/ml) was used at a dilution of 1:1,000. The blocking buffer was removed from the trays and the diluted mAbs were added to the relevant tray. The trays were left on a rocker at room temperature for 2 hr to allow the mAbs to bind to the proteins. The mAbs solutions were removed from the trays and the membranes were washed 4 times, 1 wash step constituting adding 10 ml fresh blocking buffer per tray and leaving the trays on the rocker at room temperature for 15 min. The secondary antibody for both types of samples was a goat anti-mouse IgG polyclonal antibody peroxidise conjugate (ThermoScientific-Pierce, 31430, concentration 0.8 mg/ml) which was diluted 1:10,000 in blocking buffer for use. 10 ml diluted secondary antibody were added to the trays which were left on the rocker at room temperature for 1 hr. The trays were washed 4 times with blocking buffer as previously.

The chemiluminescent peroxidise enzyme substrate Lumigen-PS3 (Amersham) was used to visualise the protein bands. Reagents A:B were mixed 40:1 just prior to use, for a total volume of 1 ml per membrane, and allowed to come to room temperature before being pipetted gently over each membrane for 1 min. The edge of each membrane was then touched to a tissue to absorb excess substrate and then wrapped carefully in Saran wrap and placed immediately in a light proof cassette. In a dark room light sensitive film (Amersham, ECL film) was placed inside the cassette, and thus exposed to the light from the substrate, for a measured length of time. The film was then developed (Kodak Developer) and re-aligned.
with the membrane to enable the film to be marked with the original position of the membrane and the location of the standard marker bands. This was so that the sample bands, visible only on the film, could be aligned with the standard marker bands, visible only on the membrane, to establish the sizes of the sample protein bands.

2.2.6 Expansion and Purification of Viral Clones into Working Vaccine Stocks

Following the positive results obtained after initial testing of the crude cell lysates for both recombinant adenoviruses, lysates had to be further amplified and purified to provide stocks suitable for use as vaccines. Cultures of 293 cells were expanded and infected with crude cell lysate stocks for each adenovirus. Following infection, cells were harvested and lysed to release viral particles, and centrifuged in a caesium chloride gradient to separate the viral particles from cell debris and other contaminants. The virus samples were then dialysed to remove the caesium chloride and to transfer the samples to an adenovirus specific buffer. Samples were assayed to establish infectivity of each batch of vaccine and were then stored at -80 °C until required.

293 cells were used for adenoviral amplification as they are stably transfected with the adenoviral E1 gene and thus enable replication in the otherwise replication-incompetent recombinant adenoviruses. Cells were initially grown in static cultures in progressively larger flasks up to T175 (all incubations at 37 °C, 5% CO₂ in DMEM (2% or 10% FBS and other supplements as previously stated)) before the cells were transferred to roller bottle cultures (Corning, #430699, 1750 cm² surface area) for infection. Roller bottles were used to maximise the surface area available for cells whilst keeping medium volumes and incubator space usage to a minimum. The roller bottles work as a closed system so the lids are solid, not vented, and an appropriate air mixture (10% CO₂, 90% air, Praxair Inc.) is introduced.
into the bottles from a gas cylinder via a sterile pipette, while the bottles are still in the tissue culture hood. The usual culture temperature of 37 °C is achieved in a normal warm room, the roller bottles are placed horizontally on a series of rotating rollers that turn the bottles, so that all of the internal surface area is under the medium at some time. The bottles were rotated at 1 rpm for culturing and infection. For sub-culturing of 293 cells, flasks of cells were rinsed with PBS (volume used relative to size of flask) to remove traces of serum, sufficient TrypLE™ Express (Invitrogen) was added to just cover the cells and the flasks were returned to 37 °C for 10 min. Flasks were gently tapped to dislodge the loose cells which were re-suspended in fresh DMEM, cells were then divided into flasks for general sub-culturing or several flasks of cells were accumulated for seeding a roller bottle. Cultures in roller bottles were never sub-cultured. Cells were generally re-seeded at 10% in static cultures and 40% for roller bottle cultures, unless cells were otherwise required for specific purposes. Prior to seeding the roller bottles 280 ml of medium was added to each bottle, the gas mixture was introduced and the bottles were placed on the roller apparatus in the 37 °C warm room for a minimum of several hours. This was to allow the medium to come to temperature and to coat the plastic with proteins from the medium. Cells, in roller bottle cultures, were infected with the crude cell lysate of either adenovirus, rAdE, rAdNS3 or rAdMT at an approximate MOI of 1 (approximate due to the difficulty of estimating the number of cells in such a large culture vessel).

Cells were generally harvested 3-5 days following infection, or when the majority of cells were floating rather than attached, although infected cultures were not left longer than 5 days. If all infected cells were not floating, bottles would be gently shaken to dislodge all cells, all medium containing cells was collected from the bottles and placed into 250 ml conical
centrifuge tubes (Corning, #430776). Tubes were centrifuged at 1,500 rpm in a bench top centrifuge for 5 min to pellet the cells. Cell pellets from the same roller bottle were re-suspended into the same 8 ml of sterile PBS which was then transferred to a 50 ml conical tube, with a total volume of approximately 9 ml and placed on ice. Once all cells from that batch had been harvested all 50 ml tubes were placed horizontally, to decrease time to freeze, at -80 °C, for at least 30 min until frozen all the way through. Tubes were removed and placed directly into a 37 °C water bath to thaw, this generally took 5-10 min, but tubes were constantly monitored after 5 min and shaken often, to ensure that the contents thawed thoroughly but did not warm up excessively. This freeze/thaw process was repeated two more times to ensure that all cells were lysed and all possible viral particles released. Tubes were centrifuged at 2,000 rpm in a bench top centrifuge for 10 min to pellet the cellular debris. The cleared virus supernatant from each tube was transferred to a fresh 50 ml conical tube containing 4.4 g of caesium chloride and the tubes were vortexed vigorously to dissolve the caesium. The entire volume of each tube, approximately 10 ml, was transferred to a 13.2 ml ultracentrifuge tube (Coulter Beckman, #331372, thin walled polyallomer). Tubes were balanced by weight, using mineral oil to adjust the weight, and placed into the buckets of the SW41 Ti rotor. The buckets were loaded onto the rotor and placed in the Ultracentrifuge (Beckman Coulter, floor centrifuge, room 378, Michael Smith Labs) and centrifuged at 176,000 g/32,000 rpm for 18-24 hr at 10 °C, after which tubes were carefully removed from the buckets, so as to not disturb the gradient. The denser white bands of infectious viral particles were collecting using a 3 ml syringe and 18 g 1.5 inch needle. The volume collected was kept to a minimum whilst trying to ensure the entire band had been collected; volumes were generally between 0.5 and 1.5 ml.
To remove the caesium chloride, virus samples were placed directly from the syringes into slide-a-lyser cassettes (ThermoScientific, 20K MWCO, 3 ml, #66003), these were then suspended, using the supplied floats, in 500 ml of A195 buffer, a buffer specifically prepared for adenovirus storage (10 mM tris, 75 mM NaCl, 5% sucrose, 0.02% PS-80, 1 mM MgCl$_2$, 100 µM EDTA, 0.5% EtOH and 10 mM histidine, pH 7.4) [68] and placed at 4 °C. The 500 ml of A195 was twice changed for fresh buffer after approximately 2 hr each time and then left in the final change of buffer overnight at 4 °C. Virus samples, now considered purified vaccine stocks, were again removed from the cassettes with a needle and syringe and aliquoted into 1.5 ml tubes and placed at -80 °C for storage until required.

To quantify the infectivity of the different batches of vaccines produced the Adeno-X Rapid Titre Kit (Clontech, #632250) was used. 293 cells were seeded into wells of a 24-well plate at 100% confluency in 1ml per well DMEM (10% FBS) and were infected at the same time. Vaccine stocks to be tested were diluted in DMEM (10% FBS) by 10-fold dilutions down to $10^{-8}$; for each vaccine a single well was used for a dilution of $10^{-4}$ and duplicate wells for dilutions from $10^{-5}$ to $10^{-8}$, 50 µl of the relevant dilution were added per well and one well per plate was left with DMEM alone as a non-infected control well. Plates were placed in the incubator (37 °C, 5% CO$_2$) for 48 hr, after which they were placed in the tissue culture hood, the medium removed and the cells allowed to dry for 10 min. Cells were fixed by adding 500 µl ice cold 100% methanol per well and plates were incubated at -20 °C for 10 min. Cells were then washed with 500 µl wash buffer (1% BSA in PBS) per well being added and then aspirated off. The wash step was repeated a total of 3 times. Antibodies were used to identify adenovirus infected cells, the primary antibody, mouse anti-adenovirus hexon protein, was diluted 1:1,000 in wash buffer and 250 µl were added to each well. Plates were returned to
the incubator for 1 hr. The primary antibody solution was aspirated from the wells and the plates were washed three times as before. The secondary antibody, rat anti-mouse IgG HRP conjugate, was diluted in wash buffer 1:500 and 250 µl were added to each well of the plates, which were again returned to the incubator for 1 hr. DAB substrate was mixed (1:9, 10× DAB substrate to 1× peroxidase buffer) and allowed to come to room temperature. The secondary antibody solution was removed from the wells and the plates washed 3 times as before. 250 µl DAB substrate were added to each well to activate the HRP which would generate a brown precipitate in all adenovirus infected cells. The substrate was removed after 10 min on the cells at room temperature and 250 µl PBS were added to each well. The number of brown stained cells positive for infection was enumerated, by counting at least 4 fields of view under a light microscope, at two consecutive dilutions of virus. Values were used in the following equation to calculate viral titre as measured in infectious units per ml (ifu/ml). The number of fields of view per well is taken from a table given in the protocol and is based on magnification and number of wells of the plate used.

\[
\text{ifu per ml} = \frac{\text{(# infected cells)} \times \text{# fields per well}}{\text{volume virus added (ml)} \times \text{dilution factor}}
\]

2.3 Assessing the Immune Response to the Vaccine in Japanese Quail

As previously stated, Japanese quail were selected as the avian species for use in testing the vaccines. Throughout the entire project, 2 groups of male quail were purchased from the Pacific-Agri Food Research Centre in Agassiz, B.C.. Birds were bred from the wild-type quail line, in two groups, hatched during November 2009 and November 2010. Male birds were separated from females and kept at the facility in Agassiz until collected for
transportation to UBC during the December following hatch. Birds were subsequently housed at the South Campus facility at UBC, initially in the Small Mammal Unit of the Zoology Department, but from July 2011 in the Annex of the Main Animal Care Facility. Floor pens of approximately 6 ft by 4 ft by 1.5 ft high, with plastic netting as lids, were used to house the birds in groups of no more than 22, with deep litter pine or aspen chips as flooring/bedding and randomly placed cardboard boxes provided for cover. During reorganisation at the Zoology Small Mammals Unit, for several months during the summer of 2010 birds not being used for experiments were housed in a open aviary area. Approximately 100 birds were in an area approximately 12 ft by 20 ft by 8 ft high, with a concrete floor and 3 ft high walls, with the rest of the area enclosed by supported wire mesh. The entire area was roofed to provide cover from rain, however the roof was raised by several feet above the wire mesh to provide daylight and ventilation. The same deep-litter flooring, as used in the floor pens, was provided as were a suitable number of boxes to allow the birds to hide. Lighting conditions were normal daylight for the location and time of year. After several months birds were moved back inside to conditions as previously stated. Birds were always fed *ad libitum* with turkey starter crumble and water and were monitored daily. Inside the Zoology Unit birds were maintained under artificial lighting conditions with 9 hr of light per day, this was to try and reduce any possible aggression by restricting the birds from going into breeding behaviour. However this timing regime was not maintained once birds were transferred to the Animal Care Facility Annex. Only male birds were purchased, mainly because if the sex-ratio is not right then male quails can become aggressive with each other in the pursuit of gaining a female which can also become dangerous for any females that are present (Dr. D. Bennett, private communication). Also having only one sex of birds used in
the trials means that any potential differences due to sex are eliminated. In addition males are not in such demand at the Agassiz facility as the females.

There were 2 main types of study performed to assess the immune response of the quail to the vaccines; a dose response trial and a time course trial. The dose response trial aimed to test for differences in immune responses in birds receiving different doses of vaccine. The time course trial consisted of all birds receiving the same dose of the different vaccines and samples were collected over time to assess the birds’ immune responses at different times post vaccination.

For both assays used to assess the response to the vaccines in quail, a WNV antigen from suckling mice was used. Sucrose solution was added to the brains of mice infected with WNV, the mixture homogenised, extracting twice with acetone and lyophilised. The antigen was supplied by Ms. A. Dilbernardo of the National Microbiology Lab (NML) in Winnipeg.

2.3.1 Dose Response

The dose response trail consisted of a larger study with 3 different vaccine doses, of $1 \times 10^8$ IFU per bird (low), $5 \times 10^9$ IFU per bird (medium) or $2.5 \times 10^{10}$ IFU per bird (high) and a smaller scale study using a dose of $5 \times 10^{10}$ IFU per bird. In the larger study all birds received the stated dose with an adjuvant followed by a boost of the same dose, with the same adjuvant. The adjuvant was Montanide 201(SEPPIC), it is a water-in-oil-in-water (W/O/W) emulsion based on specific enriched light mineral oil. One dose consisted of the relevant IFU of adenovirus vaccine, diluted into 101 µl total volume with A195 buffer and then combined 1:1 (w/w) with the adjuvant, resulting in a total volume of 194 µl; this volume was administered to each bird via an intramuscular injection into the left breast muscle, using a 1 ml insulin syringe and 29g needle (from BD Bioscience, #309311 (discontinued)). The low
dose of each vaccine was administered to the relevant birds on day 1 of the trial, the medium dose on day 8 and the high dose on day 15, in order to stagger the timing of sample collection. The low and medium dose groups received their boost injections 28 days post initial vaccination but the high dose boost was delayed until 33 days post initial vaccination. All samples were collected at various time points after boost vaccination. In the smaller study only one dose of vaccine was given and no adjuvant was used. For both dose response studies the assays used to assess the immune response were an intracellular interferon-gamma (IFN-γ) assay, to assess the T cell response, and a serum ELISA assay to assess the antibody response.

For the larger study, birds were divided between four floor pens, 24 birds per pen, one for each of the four different vaccination treatments being used. The treatments were a PBS injection for the negative control group with the other three groups being vaccinated and receiving a single dose of either rAdMT, rAdE or rAdNS3. Within each pen for the same treatment were eight birds that received each of the different doses of that particular vaccine, although all PBS control birds received exactly the same volume of PBS. For the small scale study, numbers were somewhat restricted by the amount of vaccine available. It involved two birds per group, a PBS control group, and groups vaccinated with either rAdE or rAdNS3. For the large study within each dose, spleens of 2 birds per group were collected at three different time points for the T cell IFN-γ assay. Blood samples for serum ELISA analysis were collected from all remaining birds per group at three different time points.

2.3.1.1 IFN-γ Production in T Cells

Once activated during an immune response one role of T cells is to produce IFN-γ. To what extent the vaccines were inducing T cells to produce IFN-γ was determined by quantifying
the number of cells positive for T cell markers (CD4 or CD8) that were also positive for intracellular IFN-γ, following *ex vivo* re-stimulation of the cells.

Birds were euthanised by an isoflurane overdose, spleens were then harvested and placed in PBS on ice until being returned to the lab for processing. Spleens were disaggregated into single cell suspensions by pressing them through 70 µm nylon cell strainers, into 3 ml of RPMI 1640 medium. Cells were placed on ice as processed, once all samples were in suspension cells were counted, using a haemocytometer, to obtain a count of cells with a lymphocyte type morphology. To set up the IFN-γ assay for lymphocyte re-stimulation, splenocytes from each sample were set up in round bottomed 96-well tissue culture plates. Re-stimulation treatments were set up in duplicate or even triplicate if enough cells, and space on a plate, were available. For each sample, the appropriate volume of cells, in RPMI 1640 medium, to contain 1x10⁶ cells was added to relevant wells of each plate, and plates were centrifuged at 1,500 rpm in a bench top centrifuge for 5 min at 4 °C. The medium was removed by flicking the plate and cells were re-suspended in fresh RPMI 1640 medium, with or without other components, to set up the following re-stimulation conditions:

- **No Re-stimulation (negative control)** – 200 µl RPMI 1640 medium alone were added to each of the relevant wells.

- **MT Re-stimulation** – 200 µl RPMI 1640 medium containing 1x10⁵ IFU of rAdMT were added to each of the relevant wells.

- **E/3 Re-stimulation** – both rAdE and rAdNS3 vaccines were diluted in RPMI 1640 medium to 1x10⁵ IFU virus in 200 µl. For samples from birds that received either the negative control injection or the rAdMT vaccine, 100 µl of diluted rAdE (5x10⁴ IFU) and 100 µl of diluted rAdNS3 (5x10⁴ IFU) were added to each of the relevant wells.
For samples from birds that received either rAdE or rAdNS3 vaccines, 200 µl ($1 \times 10^5$ IFU) of only the same vaccine as the bird was injected with were added to each of the relevant wells.

- WN Re-stimulation – this was only used in the final run of the assay for each dose. WN antigen (NML) was diluted to 140 µg/ml in RPMI 1640 medium and then 100 µl were added to each of the relevant wells.

To prepare the WN antigen (NML) for re-stimulation use, one vial was reconstituted in 1 ml nanopure water and protein content quantified using a BCA assay (see section 2.2.5 for details). The antigen was diluted to 1.4 mg/ml in RPMI 1640 medium, and sonicated on ice at 30 watts for 15 sec, left for a 2 min rest and then received another sonic burst at 60 watts for 30 sec.

Two 96-well plates were set up with identical conditions, one to be used to identify CD8 cells and one for CD4 cells. The plates were incubated at 39 °C, 5% CO$_2$ for 2 days to allow any cells that recognise the relevant stimulant to be activated. Golgistop™ (from BD Bioscience) was added to the plates to prevent any cellular products from being secreted from the cells. It was decided to leave the stimulate containing medium on the cells, so Golgistop™ was diluted to a concentration of 7.5 µl/ml in RPMI 1640 medium and then 50 µl were added to all wells, which already contained 200 µl medium giving a final concentration of 1.5 µl/ml. It was discovered, part way through the trial that the final concentration should have been 0.67 µl/ml, but to enable comparisons between each dose sample it was decided to continue using the incorrect concentration for all remaining assays in the trial. Plates were gently shaken laterally, to mix, and were returned to the incubator for 6 hr.
For antibody staining the Cytofix/Cytoperm™ Plus Kit (BD Bioscience) was used to fix and permeabilise the cells in conjunction with specific antibodies. For all doses the CD8 specific antibody Du CD8-1 mAb (Abcam, #ab41327), this antibody was unconjugated so a secondary PE conjugated antibody (mouse IgG, Abcam, #ab97041) was also used. For all doses, the same CD4 specific antibody, CT-4 mAb, PE conjugated was used, and the same IFN-γ specific anti-chicken IFN-γ pAb (Genway, #GWB-374ABE) antibody was used. The IFN-γ antibody was conjugated in house (AbLab, Biomedical Research Centre, UBC). For the first two runs through the assay for each dose, a FITC conjugate was added and for subsequent runs an AlexaFluor® 488 dye was added. All antibodies were used at 0.2 µg per well, each well containing 1x10^6 cells. Depending on which combination of antibodies were used slightly different staining controls were set up. There were always two unstained control wells, two wells each stained with CD8, CD4 or IFN-γ antibodies alone and if secondary antibodies were used two wells received the secondary only with no primary antibody. For the intracellular IFN-γ antibody an isotype control was included to ensure antibody specificity. The splenocytes used to set up the control wells came from the sample with the highest cell count per ml.

Plates were removed from the incubator and centrifuged in a bench top centrifuge at 1,500 rpm for 5 min and the plates were flicked to removed the medium, and were then washed once with 200 µl FACS buffer (2% FBS in PBS; FB) per well. For cell surface staining of CD markers, for wells that needed a secondary antibody, unconjugated primary antibody was added in 100 µl FB per well, if no secondary antibody was used that specific well received 100 µl FB only, and plates were left on ice for 30 min (if none of the samples required a secondary antibody this step was omitted). Plates were washed once with 200 µl
FB per well. To complete surface staining, if a secondary antibody was required, or for the conjugated primary antibodies, whichever was relevant was added in 100 µl FB per well and the plates were left on ice for 30 min and then washed once as before. Cells were then fixed and permeabilised by re-suspending the cells in 100 µl per well of fixation/permeabilisation solution, from the Cytofix/Cytoperm™ kit, and plates were placed at 4 °C for 20 min. Plates were then washed twice in 250 µl per well of 1× perm/wash buffer also from the kit, buffer was removed by spinning and flicking the plates as before. To stain any intracellular IFN-γ, the IFN-γ antibody was diluted in 1× perm/wash buffer and added to all wells, except relevant control wells, in a volume of 100 µl per well, plates were then left on ice for 30 min under foil. Plates were washed twice with 1× perm/wash buffer and once with FB and cells were then transferred to tubes for processing by flow cytometry (BD FACS Calibur).

Results were initially assessed using Flowjo software, to establish relevant populations of cells and assess levels of positive staining. The number of positive cells were then analysed using a specially written script in the R program, that used a generalised linear mixed model, using Poisson regression and taking into account both fixed and random effects to assess the data.

### 2.3.1.2 Serum ELISA

A direct serum ELISA was used to assess the level of antibody production after each dose of vaccine. WNV antigen was bound to a flat bottom 96-well plate and then serum was added to allow any WNV antibodies in the serum time to bind to the antigens on the plate. After washing the plate, to detect any WNV specific antibodies a second, enzyme conjugated, antibody was added. Substrate for the enzyme was added and colour allowed to development was measured by reading the absorbance.
Blood, for serum preparation, was collected from all birds 1 or 4 days prior to the vaccination of the low dose group and then again at 16 and 35 days post boost for all birds. Approximately 200 µl of blood were collected from the brachial wing vein of each bird, the vein was punctured with a 26g needle and blood from the oozing droplet was collected into the capillary end of serum Microvette CB300 tubes (Sarstedt, #16.440). Tubes of blood were fully capped and held at room temperature for at least 30 min to allow clotting to occur. The tubes were then centrifuged at 5,500 rpm in a microfuge for 10 min. The serum was carefully removed and transferred into fresh 1.5 ml tubes and placed at -80°C until testing was undertaken.

One vial of WNV antigen (NML) was reconstituted in 1 ml nanopure water, aliquoted and stored at -20 °C until required. The antigen was assayed using a BCA assay (see section 2.2.5 for details) to quantify the amount of protein present in each batch of antigen. To set up a plate to use for an ELISA, WNV antigen was diluted in 0.05 M carbonate buffer (pH 9.6) to a concentration of 60 µg/ml and 50 µl (3 µg) were added to relevant wells of a flat bottom 96-well ELISA plate (Immuron 4HBX). Lids were place on the plates which were sealed in a humid plastic bag, placed at 4 °C and used within 7 days of the antigen being added.

Plates of antigen were taken from 4 °C and washed by adding 200 µl per well wash buffer (0.1% tween-20 in PBS) and removing it by flicking the plate and tapping the plate flat on a kimwipe to remove residual buffer from the plate, this wash step was repeated 5 times. 200 µl of blocking buffer (BB) (5% milk powder in PBS) were added to each well, to block any non-specific binding of subsequent antibodies, and plates were placed in the humid plastic bag at 37 °C for 40 min, followed by another 5 washes. Two-fold serial dilutions of serum, from 1:20 to 1:160, in BB were set up going across the plate by adding 50 µl BB to
each well for the 1:40 to 1:160 dilutions and 95 µl BB for the 1:20 dilution. Five µl of each serum sample were then added directly to make the 1:20 dilution, serum and BB were mixed by pipetting up and down and then 50 µl were transferred to the 1:40 dilutions. This was repeated until all wells had received the appropriate concentration of serum. A positive control well was set up, in duplicate, with serum from a WNV infected goose (supplied by the National Microbiology Lab) at 1:200 dilution and 2 negative control/background wells were included that contained BB only. Plates were again sealed in the humid plastic bag and placed at 37 °C for 2 hr, followed by 5 washes as before. An anti-bird IgG HRP conjugated antibody (Benthyl Labs, #A140-110P, concentration 1mg/ml) was diluted 1:600 in BB and then 100 µl was added to all wells, the plate, in the humid bag, was incubated for 1hr as before and then washed 5 times. Sufficient SureBlue Reserve Peroxidase Substrate (KPL, #53-00-01) for 100 µl per well was warmed to room temperature prior to use. This was added to all wells and left until the colour was deemed suitable dark, usually 5 min. The reaction was stopped by adding 100 µl of 1N sulphuric acid per well and the plate was then read at 450 nm on a plate reader, within 15 min. A paired-T test analysis of the results was undertaken using Minitab v16 Statistical Software (Minitab Inc.).

2.3.2 Time Course

The main aim of the time course trial was to see if a peak response time could be identified for T cell activation to produce IFN-γ, in response to a single dose of the vaccines, as well as to monitor early antibody responses.

Vaccine was in limited supply and so the trial consisted of 30 quail, 2 groups of 15 birds, one control group received 200 µl of A195 buffer alone and the other group received the rAdE vaccine, at a single dose of 5x10^{10} IFU per bird, in a 200 µl volume with A195 buffer.
Vaccine was injected intramuscularly into the left breast muscle with a 1 ml insulin syringe and 29g needle. The intracellular IFN-γ assay and serum ELISA were again used to assess any vaccine responses and samples were collected on days 5, 7, 10, 13 and 15 post vaccination. See section 2.3 for general housing conditions for the quail. During these experiments lighting conditions provided between 8-12 hr of light for the birds, but were a little unpredictable due to re-organisation of animal housing at South Campus.

2.3.2.1 IFN-γ Production in T Cells

The same method for detecting intracellular IFN-γ was used as for the dose response IFN-γ assay (see section 2.3.1.1) with some alterations. Spleen samples for the IFN-γ assay were collected on days 5, 7, 10, 13 and 15 post vaccination, when 3 birds per group were euthanised. Spleen samples were treated as before, mashed, counted and set up on 96-well round bottom plates for re-stimulation treatments in RPMI 1640 medium. The following treatments were included:

- No Re-stimulation – 200 µl RPMI 1640 medium only was added to each of the relevant wells
- MT Re-stimulation – 200 µl RPMI 1640 medium containing 1x10⁵ IFU of rAdMT was added to each of the relevant wells.
- Env Re-stimulation – 1x10⁵ IFU rAdE in 200 µl RPMI 1640 medium was added to each of the relevant wells.
- WN Re-stimulation – WN antigen (NML) was diluted in RPMI 1640 medium to a final concentration of 0.1 mg/ml and 200 µl were added to each of the relevant wells.
Both adenoviruses used for re-stimulation were heat inactivated before use by heating to 70 °C for 25 min and then placing on ice to cool. They were diluted into medium on day 4 of the study and kept at 4 °C until ready to use. The WN antigen was first diluted to 0.5 mg/ml in RPMI 1640 medium and then sonicated on ice at 60 W for 1.5 min, aliquots were stored at -20 °C and diluted just before use.

Plates for re-stimulation treatments were incubated as before, but for four days instead of two, before adding Golgistop™ for 6 hr, then fixing and staining the cells as previously described. The antibodies used were the same as before but this time Du CD8-1 was used consistently for CD8 recognition, and a new unconjugated version of the same CD4 specific CT-4 antibody (Abcam, ab25345) was used. Both antibodies were conjugated in house with the APC fluorophore so no secondary antibody was required. The amount of antibody used was the same for both the CD4 and the IFN-γ antibodies, 0.2 µg per 1×10^6 cells, but this time the Du CD8-1 level was reduced to 0.1 µg per 1×10^6 cells. After staining was completed the amount of fluorescence was again measured using the FACS Calibur flowcytometer. Data analysis was the same as for the analysis of the IFN-γ assay for the dose response trials.

**2.3.2.2 Serum ELISA**

Brachial blood samples, for serum preparation, were collected from all birds the day before vaccination as for the dose response trial (see section 2.3.1.2). At the 5 different time points trunk blood was collected directly into 4 ml serum microtainer tubes (BD Bioscience, #367812) following isoflurane overdose and decapitation of the birds used for the IFN-γ assay. On the same day, brachial wing vein samples were also taken from 6 additional birds, those scheduled for spleen collection at the following time point. Both types of blood
samples were centrifuged as before and serum was isolated and stored at -80 °C until required.

WN antigen was set up on the same ELISA plates as before and was used the following day in the ELISA assay, which was run exactly as for the dose response trial (see section 2.3.1.2). Statistical analysis, using a paired T-test, was undertaken using Minitab Statistical Software (Minitab Inc.).

2.4 Artificial Infection of House Sparrows with West Nile Virus

HOSP were used as the representative avian species for the challenge assay (see section 1.5.1 for more details). Initially wild HOSP were caught and subsequently infected with WNV to establish baseline measurements and to ensure a sufficient dose of virus was used. After this, a second batch of birds was caught and vaccinated with the recombinant vaccines; 4 weeks later birds were challenged with the dose of WNV established from the first round of infections. Outcomes were measured by extracting RNA from blood and tissue samples, then using the RNA to make cDNA to act as a template in a qRT-PCR reaction to identify and quantify any WNV present in the sample. Due to the combination of working with a level 3 virus, such as WNV, and with live birds, the NML in Winnipeg was the only place able to accommodate both requirements. As biosafety level 3 work was required, the full ‘application’ process to enable to work to be undertaken was a long and arduous one, extending for over 18 months. Necessary paperwork and other processes included, but was not limited to, completion of forms for animal usage and confirmation of acceptable animal protocols, a form to authorise access to and use of WNV, a full physical medical exam and submission of an extensive form completed by the doctor, vaccinations relevant to the work.
being undertaken were required, blood samples were taken at the start and end of the work and full security clearance, to secret level, was required for me to be authorised. This included a search for personal criminal records.

HOSP were captured using mist nets, over several days, in the the North End suburb of Winnipeg, prior to transportation to the biocontainment level 3 facility of the NML, which is housed in the Canadian Science Centre for Human and Animal Health. Limits on the number and sex ratio of birds dictated that experiments be conducted in groups of 9, each with 4 females and 5 males and thus slightly male biased. In the level 3 facility, birds were housed in metal cages (63cm×56cm×43cm high), paper covered the floor and perches and cuttlefish and food and water were provided. Food consisted of a premium wild bird seed, which was fed ad libitum and was supplemented, every 1-2 weeks, with a ‘High Potency Super Fine’ bird feed (Harrison’s Bird Foods) a complete bird food, bread was also occasionally provided; clean fresh water was also provided on a daily basis. To reduce the stress on the birds caused by the presence of people in the room, sheeting was suspended in front of each cage to restrict the visual field from each cage. Following capture, the birds were allowed at least one week to acclimatize to captivity during which they were not removed from the cages and were disturbed as little as possible. Over several weeks of working directly with the birds, either to remove them from the cage for sampling or to be weighed or just for daily changing of food and water, it was found that the least stress was caused to the birds by doing all work in the dark. Initially such work was undertaken under red light but this was later refined to use of infrared night vision goggles. When the birds had to be removed from the cage, the light in the lab was turned off, the birds were individually captured by hand and placed into a pillowcase, secured by a rubber band. The pillowcase was then moved to a
bench and covered with more sheeting to maintain the birds at a lower light level. The light in the lab was then turned on and the birds were removed from the pillowcase one at a time for processing. After processing each bird was returned directly to their relevant cage, being released into the cage by touch as the sheeting was in place over the front of the cage. All birds were weighed regularly to monitor general health.

Prior to any experiments being undertaken using the HOSP, all birds were screened for any previous WNV infection using a blocking ELISA assay. WNV antigen was bound to an ELISA plate, serum samples were added so that any WNV specific antibodies present could bind, an anti-WNV antibody and HRP conjugated secondary antibody were then used to measure the amount of WNV specific binding that had not been previously blocked by the serum. A blood sample was collected from all birds by pricking the brachial vein with a 27g needle and collecting the blood into a 50 µl capillary tube. The bleeding from the vein was stopped and the bird was returned to its cage. Finally the blood was expelled into a 500 µl EDTA microtainer tube (BD Bioscience). Once all samples were collected the tubes were centrifuged at maximum speed in a microfuge for 10 min and the serum was collected and placed in fresh 200 µl screw-top tubes. Serum samples were heat-inactivated, to inactivate any active WNV that may be present, by heating to 56 ºC for 1 hr after which samples could be removed from the level 3 containment area. For the blocking assay itself, standard WNV antigen (NML) was used as well as negative antigen (prepared in the same way as the WNV antigen but from mice that had not been infected with WNV). WNV antigen and negative antigen were diluted in carbonate buffer (0.05M, pH 9.6) to 1:100 and 1:250 dilutions respectively, 100 µl of antigen were added to relevant wells of an ELISA plate (Immuron 4HBX), one WNV antigen and one negative antigen well per sample. The plate was sealed
with saran wrap and placed in a humid chamber at 4 °C overnight. Just prior to use the plate was washed 5 times using an automated plate washer (BioTek, Winooski, U.S.), for each wash 300 µl wash buffer (0.1% tween-20 in PBS) were added to each well and then removed by the plate washer. After the fifth wash the plate was removed from the washer and blotted on absorbent paper to remove any remaining solution. 200 µl of blocking buffer (5% milk powder in PBS) were added to each well of the plate, the plate was covered with a plastic lid and placed at 37 °C for 40 min in a humid chamber. During this time 11 µl of each serum sample were added individually to wells of a standard 96-well plate containing 99 µl of blocking buffer. These 1:10 dilutions of serum were then ready for direct transfer to the ELISA plate. At least 2 additional samples were included, one was a previously tested positive serum and one or more samples of serum that had previously tested negative in this assay. The ELISA plate was washed 5 times as before and 50 µl of each diluted serum sample were transferred to two wells of the plate, one coated with the WNV antigen and one with the negative antigen. The lidded plate was returned to the humid chamber and placed at 37 °C for 2 hr, followed by 5 washes. A WNV specific monoclonal antibody, 7H2 (BioReliance, Rockwell, U.S.) was used to detect any unbound antigen, the antibody was diluted 1:2,000 in blocking buffer and 50 µl were added to each sample well and the plate was returned to 37 °C as before for 1 hr. The secondary antibody, goat anti-mouse IgG peroxidase conjugated (ThermoScientific) was diluted 1:1,250 in blocking buffer. The ELISA plate was again washed 5 times and 50 µl of the secondary antibody were added to all sample wells and the plate again incubated at 37 °C for 1 hr. After a final 5 washes, 75 µl of TMB substrate (SureBlue Reserve, KPL Inc.) were added to each sample well of the plate and left in the dark, usually after 5 min colour development was sufficient. 50 µl per well of
1M H$_2$SO$_4$ were added to each well to stop the reaction and the optical density of the wells was measured in a plate reader at 450 nm. For each sample the percentage by which the serum had inhibited binding of the WNV specific antibody was calculated as follows:

\[
\% \text{ Inhibition} = 100 - \left( \frac{\text{TS} - \text{TB}}{\text{CS} - \text{CB}} \right) \times 100
\]

where TS = optical density of the test serum sample in the WNV antigen well, TB = optical density of the test serum sample in the negative antigen well, CS = optical density of the negative serum sample in the WNV antigen well and CB = optical density of the negative serum sample in the negative antigen well. A percentage inhibition of 30% or less indicates no previous exposure to WNV. Only birds found to be negative were used in subsequent experiments. Calculations were performed using Microsoft Excel.

For both the infection response trials and the challenge assay the ongoing form of sample collection, to quantify the level of WNV infection, was cloacal swabs and blood samples. Cloacal swabs were collected by pre-moistening the swabs with PBS and then gently inserting the swab into the bird’s cloaca. On removal the swab was placed directly into 400 µl of RLT buffer (RNeasy 96 kit, Qiagen). Once the bird had been returned to the cage, the handle was cut off of the swab and the tip left in the RLT buffer. Tubes were placed at -80 °C once all samples for that day had been collected. For the blood samples, approximately 50 µl of blood were collected by pricking the brachial vein of the bird and collecting the blood into 50 µl capillary tubes as it bled from the vein. Blood was left in the capillary tubes while the bird's bleeding was stopped and the bird returned to its cage; blood was then expelled from the capillary tube into 50 µl of BA diluent (M199 Hanks Salts, 1% bovine serum albumin, 0.05M Tris (pH7.6), 0.35mg/l sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 µg/ml fungizone), flicked gently to mix and 50 µl of diluted
blood was then transferred to 1 ml of TriPure® isolation regent (Boehringer Manheim) which was mixed ready for RNA extraction. Once all samples had been collected for the day, the blood samples, both in TriPure® reagent and in BA diluent, were placed at -80°C. Due to sampling restrictions, blood samples could only be collected from a sub-set of each group per day, which also restricts the statistical use of the blood viremia data. As swab samples could be collected daily from all birds, with larger numbers of samples for every day, it was considered that swab viremia would provide a more statistically robust indication of the performance of the vaccines, than the restricted blood samples.

To establish the full extent of infection and as a final measurement of viremia for both types of study, 14 days after infection blood and organs were collected from all surviving birds. On day 14 birds were euthanised with an overdose of isoflurane; just prior to death blood samples were collected from the jugular vein, using a 3 ml syringe and 27g ½inch needle, and directly afterwards, various organs were harvested to assess the level of WNV viremia present throughout the body. Blood was aliquoted into 500 µl EDTA microtainer tubes (BD Bioscience) and left at room temperature until all samples were collected. Tubes were then centrifuged at maximum speed in a microfuge for 5 min, serum was carefully collected and placed into fresh 500 µl tubes for storage at -80°C. The organs harvested were brains, hearts, kidneys and spleens. Organs were placed into 2 ml screw-top tubes, individually weighed and a volume of BA diluent was added as soon as possible once all organs were harvested from a single bird. Hearts and brains were placed in a volume of BA equivalent to 100 µl per 100 mg of tissue, for spleens and kidneys the volume of BA added to each tube was equivalent to 300 µl per 100 mg of tissue; a 4.5 mm copper clad BB pellet was added to each tube and the tops were screwed on tightly. Samples were homogenised in a Qiagen tissue
grinder for 1.5 min at 30 cycles per second and then centrifuged at maximum speed in a microfuge for 10 min to pellet the cellular debris. One hundred µl of cleared supernatant were added to tubes containing 400 µl RLT buffer, 1 tube per organ type, 4 tubes per bird and samples were placed at -80 °C until ready to be processed to extract RNA.

Any birds that died during the course of an experiment were placed at -80 °C as soon as discovered. On day 14 of the experiment frozen birds were thawed at room temperature and the same organs were harvested and treated in the same manner, as birds that survived until the end of the experiment.

For both the infection response trials and the challenge assay, RNA extraction from thawed organ samples was undertaken using the RNeasy 96 kit (Qiagen) and for the thawed blood samples TriPure® isolation reagent was used. For RNA extraction using the RNeasy kit, 400 µl of 70% ethanol was added to each tube and the tubes were inverted 5-7 times to mix. The full volume of each sample was then loaded into a single well of the spin column plate (96 well format), which was sat on top of a block for collection of flow through. The plate was sealed with a tape sheet (as it was for each spin) and the plate and block were then centrifuged at 5,600 g for 4 min at room temperature to bind the samples to the filters. The samples were then washed once with 800 µl per well of RW1 buffer and centrifuged as before, and the blocks for collecting the flow through were replaced with new empty blocks. Samples were then washed twice with 800 µl per well of RPE buffer, the first spin as before but with the second spin lasting for 10 min to remove all traces of ethanol. The spin column plate was then transferred to the top of an elution microtube (strips of 1.2 ml tubes) rack and 50 µl of RNase-free water were added to each sample well. The plate was left to stand at room temperature for approximately 1 min before being centrifuged as before for 4 min. To
ensure full recovery of all RNA from the column filters a further 50 µl RNase-free water were added to each well, the tape sheet seal was left off of the plate for the final spin which was as for the first elution spin. Strips of lids were used to seal the sample tubes. Usually the qRT-PCR reaction was set up directly following extraction, in which case the tubes were held on ice until used, but for longer term storage tubes were placed at -20°C. For RNA extraction of blood samples from the TriPure® reagent, after thawing samples, 200 µl of chloroform were added to each tube and tubes were shaken vigorously for 15 sec then left at room temperature for 10 min. Tubes were centrifuged at 12,000 g for 15 min at 4 °C to separate the samples into three phases, with the RNA being in the upper clear aqueous phase. The upper phase of each sample was transferred to fresh 1.5 ml tubes, and the RNA was precipitated by adding 500 µl of isopropanol to each sample. Tubes were inverted 5-7 times to mix, incubated at room temperature for 10 min and then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was removed from each tube by pipetting, taking care to avoid the position of any pellet present but not visible. One ml of 75% ethanol was added to each tube, to wash the RNA, tubes were vortexed and then centrifuged at 7,500 g for 5 min at 4 °C to re-pellet the RNA. Excess ethanol was removed by pipetting and tubes were left open in a biosafety cabinet until practically all of any remaining ethanol had evaporated. RNA was re-suspended in 50 µl RNase-free water (from the RNeasy kit) by adding the water to the tubes and gently flicking them. Tubes were then incubated at 55 °C for 10 min to allow the RNA to dissolve. If the qRT-PCR reaction was being set up directly following extraction, tubes were held on ice until used, otherwise they were placed at -20 °C for longer term storage.

To most accurately quantify any WNV RNA in the samples, standards were set up that included the same amounts of tissue homogenate or blood as the samples. This was achieved
by using blood and tissue homogenates from non-infected birds, as for the harvested test
samples, and then spiking these samples with known amounts of WNV. These spiked
samples were run through the same extraction process and qRT-PCR conditions as their
respective unknown samples. Following the qRT-PCR the spiked samples were used to
create a standard curve, for each tissue type or blood, for quantification of the unknown
samples of that same type.

For the qRT-PCR reactions to quantify the amount of any WNV RNA in the samples, the
same set of primers and probes were used for all reactions; they were specific for the
envelope region of WNV (fwd - TCA GCG ATC TCT CCA CCA AAG, reverse - GGG TCA GCA
CGT TTG TCA TTG, probe - TGC CCG ACC ATG GGA GAA GCT C).

Following several test reactions, different qRT-PCR reagents were found to work better with
different samples. For the TriPure® extracted blood samples the QuantiTect® Probe RT-PCR
kit (Qiagen) was used; the following volumes of reagents were added to a single master mix
tube, each volume multiplied by the number of reactions required: 25 µl 2x master mix
buffer, 18.7 µl RNase-free water, 0.2 µl of both the forward and reverse primers, 0.4 µl probe
and 0.5 µl QuantiTect® mix. For the RNeasy 96 extracted RNA samples the Taqman®
One-Step RT-PCR Master Mix (Applied Biosystems) kit was used, again a master mix was
made up for the number of reactions required, volumes per reaction were: 25 µl 2x reaction
buffer, 17.7 µl RNase-free water, 0.5 µl of both the forward and reverse primers, 0.3 µl probe
and 1 µl Taqman® enzyme. Forty-five µl of either of the master mixes were then aliquotted to
relevant wells of a MicroAmp® optical 96-well reaction plate. Prior to addition of the RNA
samples to the qRT-PCR plate, all samples were assessed using a Nanodrop
(ThermoScientific, Wilmington, U.S.) quantifier, to quantify the amount of RNA per sample.
The sample with the lowest RNA concentration was used to calculate the maximum amount of RNA to be added to each reaction in a 10 µl template volume; samples with higher concentrations were rounded to the nearest 50 ng/µl and the volume of sample used was reduced as required to keep the amount of RNA present in each reaction tube approximately the same. Between 3.3 µl and 10 µl of each RNA sample was added to individual wells of the plate containing master mix. For both sets of reagents the RT-PCR reaction conditions were exactly the same, using a 7900HT Fast RT-PCR System (Applied Biosystems), with the standard reaction setting for a 50 µl reaction volume, cycling conditions were set to: stage 1, 50 °C for 30 min; stage 2, 95 °C for 10 min; stage 3, 95 °C for 15 sec then 60 °C for 1min, stage 3 was repeated 40 times. Results were initially analysed using the SDS software (Applied Biosystems) supplied with the RT-PCR system, using absolute quantification methods with comparisons to standard curves for each sample type.

2.4.1 Infection Response

To establish a baseline for measurable responses in HOSP to a WNV infection, two infection response trials were run, using the 22 birds caught in the first batch. The first trial involved two groups of 9 HOSP, one group was left as a non-infected control group; the other group was infected subcutaneously with a dose of $3.5 \times 10^3$ PFU of WNV per bird, diluted in PBS, with 200 µl volume per dose. The additional 4 ‘spare’ birds were left alone as much as possible, other than having to change food and water, to act as a control for any stress and/or mortality caused purely by handling and collecting samples from the birds. Cloacal swabs and blood samples were collected, from the 2 groups of 9 birds, on days 1,2,3,4,6 and 8 post-infection. Due to technical difficulties with the collection and processing of the samples, no WNV could be recovered from the samples following RNA extraction and qRT-PCR.
analysis, issues which were resolved for the second trial. However much lower mortality rates than expected were also seen, which lead us to believe that the dose of WNV used was also not sufficient. The second infection response trial was run using the same batch of birds and so there was some restrictions on sample collection.

For the second infection response trial the 9 birds that had been the non-infected controls for the first trial were used as the infected group and 4 additional birds that had been caught as ‘spares’ were used as the non-infected control group. The birds to be infected each received $3.5 \times 10^4$ PFU of WNV diluted in 200 µl of PBS, injected subcutaneously into the inguinal fold of their leg. For the infected group blood samples were taken from 3 birds on days 1, 2, 3, 4, 5 and 7 post-infection and for the control group blood samples from 2 birds were taken on days 1, 3, 5 and 7. Cloacal swab samples were also taken on the same days as blood samples, for each group, but were taken from all birds in the group. Fourteen days after infection all birds were euthanised as previously described and organs harvested for processing. All samples were placed at -80 °C until removed for RNA extraction for RT-qPCR analysis. Standards for quantification of WNV RNA were set up using additional tissue homogenates and blood samples collected from the 4 non-infected birds from the second trial.

Results from the SDS software of the qRT-PCR machine were further analysed and plotted using Microsoft Excel.

2.4.2 Challenge Assay

For the challenge assay a second batch of 40 HOSP were caught and transported into the biocontainment level 3 facility as previously described. Birds were place into 4 cages in groups of 10, and as before, left for at least 7 days to acclimatise and then screened for
previous WNV infections using the competitive ELISA. As soon as possible after screening 1 bird was removed from each cage and the birds were euthanised with isoflurane. Blood and tissues (brain, heart, kidney and spleen) were harvested from them for use to set up the spiked samples to use to quantify the unknown samples. The remaining 9 birds in each cage were assigned to one of four different treatment groups with regards to vaccination, with each bird per group receiving:

1. Negative Control (Neg) –50 µl A195 buffer (adenovirus storage buffer) only.

2. NS3 –1.25x10⁹ IFU of rAdNS3 vaccine and 1.25x10⁹ IFU of rAdMT, mixed and diluted in 50 µl A195 buffer.

3. Env – 1.25x10⁹ IFU of rAdE vaccine and 1.25x10⁹ IFU of rAdMT, mixed and diluted in 50 µl A195 buffer.

4. E/3 – 1.25x10⁹ IFU of rAdE vaccine and 1.25x10⁹ IFU of rAdNS3, mixed and diluted in 50 µl A195 buffer.

The birds were injected intramuscularly into the left breast muscle using 1 ml insulin syringes. All birds were returned to their cages and checked daily for the following 28 days, to allow time for an immune response to the vaccines to be raised. After this time birds were infected with WNV as per the second infection response trial, with 3.5x10⁴ PFU of WNV each. Samples were collected daily from day 1 to day 8 post-infection, consisting of cloacal swab samples from all birds in all groups and blood samples from 3 birds per group. Samples were collected as previously described and stored at -80 °C until ready to process for RNA extraction and qRT-RCP analysis, again as previously described. Any birds found dead were placed, intact, at -80 °C as soon as possible after discovery. Moribund birds were euthanised
with an overdose of isoflurane and then frozen whole. On day 14 post-infection, all remaining birds were euthanised and tissues harvested as previously described and all birds that died during the study were thawed and the same tissues were harvested. Due to technical difficulties, possibly during transport from Winnipeg to Vancouver, the swab samples for days seven and eight did not produce high enough output values, when analysed, to enable quantification of viremia, and so were omitted from further analyses.

Results from the SDS software of the qRT-PCR machine were further analysed and plotted using Microsoft Excel. Additional analysis and plots were also undertaken using either Minitab software or Prism (GraphPad) software.
Chapter 3: Identifying Reagents for Assessing the Avian Immune System

3.1 Rationale for Experiments

Chickens are the main bird model for studying the avian immune system, and thus it is not surprising that most of the reagents used, such as antibodies, relate to the chicken. It was hoped that the vaccines would be able to be used in other avian species, which are more susceptible to a WNV infection, so it was necessary to assess which available reagents would work in different species. This was achieved using different fluorescent antibody reagents for flow cytometry analysis of cells from different avian species. These reagents were subsequently used for assays to analyse the performance of the vaccines, as well as to make an assessment of the quail immune system.

3.2 Antibody Screens for Flow Cytometry Analysis

The various avian antibodies (see Table 2.1) which have reactivity for either chicken, turkey or duck, and are specific for one of the T cell markers, either CD4 or CD8, were used to test direct binding to live cells freshly harvested from quail spleens or blood. The splenocytes from HOSP and WCSP were as fresh as possible taking into account the different collection protocols that had to be used. These experiments were done to test for any cross reactivity with the species that cells were harvested from.

3.2.1 Results – Avian Antibodies Suitable for Flow Cytometry

For all species tested, the population of cells initially examined were those still alive after processing. This was achieved by selecting cells based on their size (forward scatter (FSC)) and granularity (side scatter (SSC)) using a positive selection gate on the FSC/SSC plot. If
possible this population of cells was then refined by removing autofluorescent cells by exclusion gating; these are cells that naturally produce a high fluorescence signal, so the signal specific to the fluorescent antibody cannot be reliably measured. The remaining cells were then displayed on a graph displaying the number of cells against the level of fluorescence recorded for each cell. Negative control samples of cells not stained with any antibodies were included in each assay. See Appendix B for some examples of the gating strategies.

Japanese quail were the first avian species screened. Figure 3.1 shows that all sample fluorescence profiles were similar to the negative control profile. This indicates that for quail, neither of the chicken antibodies, CT-8 or the 3-298, recognised the quail CD8 T cell marker in either splenocytes or PBMCs (peripheral blood mononucleated cells).

![Graph showing fluorescence profiles](image)

**Figure 3.1 CD8 Molecules on Quail Cells are Not Recognised by Antibodies 3-298 and CT8.**
Screen of quail splenocytes (Sp) and PBMCs (Bl) cells with two CD8 antibodies, 3-298 (298) and CT8. FL2-H indicates the level of antibody fluorescence recorded and count is the number of cells at each level of fluorescence. Sp Neg is the unstained control sample.

Another chicken antibody for CD8 called EP72 was tested at concentrations of 0.2, 0.4 or 0.6 µg/10⁶ cells, to see if concentration would affect antibody binding (Figure 3.2). A ‘standard’ antibody concentration to use is 0.2 µg/10⁶ cells and at this concentration a small number of cells can be seen with increased levels of fluorescence, as indicated by the trailing right hand edge of the profile extending to the 10³ mfu mark on the graph. The extreme end of the profile does form a small peak but its small size and lack of complete separation from the negative stained population on the left side of the graph, meant it was only considered to be an indication of potential antibody binding. For this reason the two higher concentrations were tested to see if a more distinctive positively stained population would result. However, although the higher concentrations of antibody did result in a shift of the entire cell sample to increased levels of fluorescence it did nothing to further distinguish a separate population of positive cells (Figure 3.2). Due to this inconclusive result this antibody was considered negative for cross-reactivity and was not used in further experiments.
Figure 3.2 CD8 Molecules on Quail Splenocytes are Not Recognised by Different Concentrations of Antibody EP72.

All samples are of quail splenocytes stained with different concentrations of EP72, 0.2, 0.4 or 0.6 µg/10^6 cells (0.2 3, 0.4 3 and 0.6 3 respectively). FL4-H indicates the level of antibody fluorescence recorded and count is the number of cells at each level of fluorescence. Ctrl no stain is the unstained control sample.

The chicken CD4 antibody EP96 was tested at three different concentrations (see Figure 3.3) to see if it would recognise quail CD4. Although the increasing amounts of antibody did shift the fluorescence profile to the right, as for the EP72 CD8 antibody, this did not result in a clear separate population of positively stained cells; it was concluded that this antibody did not recognise quail CD4.
Figure 3.3 CD4 Molecules on Quail Splenocytes are Not Recognised by Different Concentrations of Antibody EP96.

All samples are of quail splenocytes different concentrations of EP96, 0.2, 0.4 or 0.6 µg/10^6 cells (0.2 5, 0.4 5 and 0.6 5 respectively). FL4-H indicates the level of antibody fluorescence recorded and count is the number of cells at each level of fluorescence. Ctrl no stain is the unstained control sample.

Figure 3.4 demonstrates that the chicken CD4 antibody CT4, does bind to the quail CD4 molecule. For both splenocytes and PBMCs from blood samples there is a positively stained population of cells indicated by the peaks on the right hand side of the graph. There is a clear separation between the negative population and the stained cells for both cell types, with the dip in numbers between the two populations reducing to almost zero, although the level of fluorescence for the splenocytes population is generally higher than for the PBMCs.
Figure 3.4 CD4 Molecules on Quail Cells are Recognised by Antibody CT4.

Screen of quail splenocytes (Sp) and PBMCs (Bl) cells with the CD4 antibodies, CT4. FL2-H indicates the level of antibody fluorescence recorded and count is the number of cells at each level of fluorescence. Sp Neg and Bl Neg are the unstained control sample for splenocytes and PBMCs respectively.

The fluorescence graphs for quail splenocytes stained with the duck specific antibodies for both CD8 and CD4 are shown in Figure 3.5. The fluorescence staining profiles are unusual as the positively stained peaks are similar in size, if not larger, than the negative stained peaks with both antibodies. The positive peaks are quite distinctive but the separation is not clearly defined, with a quite high cell count occurring in the dip between the two populations.
Figure 3.5 CD4 and CD8 Molecules on Quail Cells are Both Recognised by Duck Reactive Antibodies.

Screen of quail splenocytes (Sp) with both CD4 (Du-CD4-1) and CD8 (Du-CD8-1) antibodies. FL1-H indicates the level of antibody fluorescence recorded and count is the number of cells at each level of fluorescence. Sp Neg is the unstained control sample.

Although the duck specific CD8 antibody was the only one that gave a positive result with quail cells, it displayed an unusual fluorescence profile so further testing was undertaken to see if this could be improved. Different concentrations of antibody were used when staining cells, either 0.1 µg/10^6 cells or 0.2 µg/10^6 cells, to see if concentration would affect the staining profile. Figure 3.6 shows more usual profiles for staining in splenocytes than the first time this antibody was used, with a more distinct separation between the positively stained cells on the right and the unstained cells on the left. It shows little difference between the two different concentrations of antibody, although median fluorescence for 0.1 µg antibody is 1152 mfu and for 0.2 µg is 1439 mfu. The frequency of CD8 positively stained cells to the
total number of cells is 3.89% and 3.23 % for the lower and higher antibody concentrations respectively, although that same positive population compared to live cells is 8.63% and 7.39% respectively.

Figure 3.6 For Measurements of CD8 Molecules on Quail Cells, Recognition is Not Affected by Concentration of Antibody.

Staining of quail splenocytes with two different concentrations of duck specific CD8 antibody, 0.1 µg/10⁶ cells (0.1B) and 0.2 µg/10⁶ cells (0.2B). FL2-H indicates the level of antibody fluorescence recorded and count is the number of cells at each level of fluorescence. ‘CD8 titre no stain’ is the unstained control sample.

HOSP splenocytes generally produced quite different profiles from the quail cells when stained with the different antibodies. Figure 3.7 shows that unstained cells (the red line) produce a similar negative profile to quail cells but once samples have been treated with antibody the profile curves all move to the right indicating higher levels of fluorescence. A difference between the profiles of the two different individual HOSP tested is also evident,
independent of the antibody used. For the HOSP samples stained with the CD8 antibodies CT8 or EP72, however there is no distinction between a negative and positive stained population of cells for either HOSP sample or for either antibody. This shift to higher fluorescence does not indicate that either antibody recognizes the HOSP CD8 cell marker. The same effect can be seen in Figure 3.8 when the duck antibody for CD8 is used with HOSP splenocytes samples and again, although the level of fluorescence increases it does not indicate that the antibody recognizes HOSP CD8.

**Figure 3.7 CD8 Molecules on HOSP Splenocytes are Not Recognised by the Antibodies CT8 or EP72.**

Splenocytes from two different HOSP (Sp2 and Sp4) were tested, FL4-H indicates the level of antibody fluorescence recorded and count is the number of cells at each level of fluorescence. ‘ctrl Sp2 No stain’ is the unstained control sample from HOSP Sp2.
Figure 3.8 CD8 Molecules on HOSP Splenocytes are Not Recognised by the Duck Antibody Du-CD8-1.

Splenocytes from two different HOSP (Sp2 and Sp4) were tested, FL4-H indicates the level of antibody fluorescence recorded and count is the number of cells at each level of fluorescence. ‘ctrl Sp2 no stain’ is the unstained control sample from HOSP Sp2.

When the CD8 antibody 3-298 was used with HOSP samples (Figure 3.9) a small positive population is evident as a small peak, to the right of, and quite separate from, the negatively stained population for one of the birds tested. The peak is not as highly fluorescent or as well separated from the negative population as is apparent for the chicken splenocyte sample, included as a positive control; however the result indicates that at least some of the HOSP splenocytes are being bound by the 3-298 antibody.
Figure 3.9 CD8 Molecules on HOSP Splenocytes are Recognised by Antibody 3-298.

Splenocytes from two different HOSP (Sp2 and Sp4) were tested and chicken splenocytes (ctrl chk 3-298 PE) were included as a positive control. FL2-H indicates the level of antibody fluorescence recorded and count is the number of cells at each level of fluorescence. ‘ctrl Sp 2 no stain’ is the unstained control sample from HOSP Sp2 and ‘ctrl chk no stain’ is the unstained chicken sample.

The two CD4 antibodies tested with the HOSP splenocytes, EP96 and CT4, are both specific for the chicken CD4 cell marker. For both antibodies, staining the cells results in a shift to the right of the entire population of cells and an increase in fluorescence, although again as there is no separated population of cells demonstrating positive staining, neither of the CD4 antibodies would be said to recognize the HOSP CD4 T cell marker (see Figure 3.10 and Figure 3.11, respectively). For the CT4 antibody (Figure 3.11) a chicken splenocytes sample was included as a positive control, which show a second clear peak to the right of the peak of the negatively stained cells, even though the number of fluorescent cells in the dip between the two populations does not entirely drop to zero.
Figure 3.10 CD4 Molecules on HOSP Splenocytes are Not Recognised by Antibody EP96.

Splenocytes from two different HOSP (Sp2 and Sp4) were tested. FL4-H indicates the level of antibody fluorescence recorded and count is the number of cells at each level of fluorescence. ‘ctrl Sp2 no stain’ is the unstained control sample from HOSP Sp2.

Figure 3.11 CD4 Molecules HOSP Splenocytes are Not Recognised by Antibody CT4.
Splenocytes from two different HOSP (Sp2 and Sp4) were tested and chicken splenocytes (ctrl chk CT4 APC) were included as a positive control. FL2-H indicates the level of antibody fluorescence recorded and count is the number of cells at each level of fluorescence. ‘ctrl Sp2 no stain’ is the unstained control sample from HOSP Sp2 and ‘ctrl chk no stain’ is the unstained chicken sample.

White Crowned Sparrows (WCSP) were also screened for cross-reactivity with CD4 and CD8 T cell marker specific antibodies. As a limited number of samples were available only over a short period of time, a combination of different antibodies and different antibody concentrations all had to be attempted at the same time to see what, if any, combinations would work. Figure 3.12 shows that neither the CD8 antibody 3-298 nor the CD4 antibody CT4 recognized the corresponding cell marker in the single WCSP tested, as the fluorescence profiles are all very similar to the unstained sample. The duck antibody for CD4, Du-CD4-1, also shows a negative response to WCSP CD4 as all profiles from the three different birds tested are all similar to the unstained negative control (see Figure 3.13).

Figure 3.12 CD8 Molecules and CD4 on WCSP Splenocytes are Not Recognised by Antibodies 298 and CT4 Respectively.

Splenocytes from a single WCSP were tested against the chicken antibodies 3-298 for CD8 (298) and CT4 for CD4, at three different concentrations for each antibody. Concentrations were 0.2, 0.1 and 0.05 µg/10^6
cells (2, 1 and 05 respectively). FL2-H indicates the level of antibody fluorescence recorded and count is the number of cells at each level of fluorescence. ‘No stain’ is the unstained control sample.

Figure 3.13 CD4 Molecules on WCSP Splenocytes are Not Recognised by the Duck Antibody Du-CD4-1.

Splenocytes from three different WCSP (numbered 10, 52 and 67) were tested against the duck antibody for CD4, Du-CD4-1. FL4-H indicates the level of antibody fluorescence recorded and count is the number of cells at each level of fluorescence. ‘negative’ is the unstained control sample.

When the antibody against duck CD8 was tested against WCSP splenocytes, small but positively stained populations were evident for all three birds tested (see Figure 3.14). The effect was best seen in bird #52, as the dip separating the two populations of cells was lower than for the other two birds. However the possible positive populations were very small in number and as such were not considered conclusive. Further testing with different concentrations of the antibody would be required for confirmation. Further samples from WCSP were unavailable so no further additional testing could be undertaken.
Figure 3.14 CD8 Molecules on WCSP Splenocytes are Partially Recognised by the Duck Antibody Du:CD8:1.

Splenocytes from three different WCSP (numbered 10, 52 and 67) were tested against the duck antibody for CD8, Du-CD8-1. FL4-H indicates the level of antibody fluorescence recorded and count is the number of cells at each level of fluorescence. ‘Negative’ is the unstained control sample.

For a summary of antibody binding to samples from the different species of birds used, see Table 3.1. Antibodies deemed not to recognize their specific cell marker in any of the species tested are not included.
<table>
<thead>
<tr>
<th>Antibody ID/Species</th>
<th>Quail</th>
<th>HOSP</th>
<th>WCSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chk CD8 – EP72</td>
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<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Chk/Tky CD4 – CT4</td>
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<td>Negative</td>
<td>Negative</td>
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<tr>
<td>Duck CD4 – Du-CD4-1</td>
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<td>Negative</td>
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<tr>
<td>Duck CD8 – Du-CD8-1</td>
<td>Positive</td>
<td>Negative</td>
<td>Likely</td>
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<tr>
<td>Chk CD8 – 3-298</td>
<td>Negative</td>
<td>Likely</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Table 3.1 Summary of CD8/CD4 Antibody Binding Results.

Antibody binding definitions – possible means some cells demonstrated higher fluorescence but with no positive population being apparent; likely means at least a small positive population was seen but further testing would be required for confirmation; positive means the antibody did bind to its specific molecule and negative means it did not. Antibody ID consists of species reactivity (abbreviations: chk – chicken, tky – turkey), CD marker specificity and antibody name.

3.2.2 Discussion – Avian Antibodies Suitable for Flow Cytometry

When quail cells were initially screened with the duck reactive antibodies for both CD4 and CD8 (Figure 3.5), fluorescence profiles were somewhat unusual when compared to the ideal profile specified in Figure 2.1 (page 38). In Figure 3.5 the positively stained populations were similar in size to the negatively stained ones. Whilst this in itself does not invalidate the results a lower level of positive cells was expected. The proportion of CD4 and CD8 positive cells in chicken splenocytes is 5-7% and 53-55% respectively [69], but this was measured in seven week old chickens. For chickens the levels of T cells fall as the bird ages. The quail were over twelve months of age when used for antibody screening. The quail cells were held on ice for a substantial length of time before they could be analysed by flow cytometry, which may have contributed to any difference. After this time the dead cells were removed from analysis by exclusion gating, so the plot in Figure 3.5 shows the number of cells positive for either CD4.
or CD8 compared to remaining live cells. The numbers may be skewed due to more CD4 or CD8 negative cells dying resulting in the positively stained cells being over-represented in each individual sample. This idea was confirmed when the Du-CD8-1 antibody was re-tested, and the samples analysed soon after staining, the profiles showed a much more usual pattern with the positive population representing a much smaller percentage of the live cell population (Figure 3.6).

After the initial screen of antibodies in quail, only the duck reactive antibody Du-CD8-1 gave a positive response, this is why further investigations using different antibody concentrations was undertaken. The second screen using different concentrations of Du-CD8-1 gave improved profiles, although again some anomalies were apparent regarding concentration. The two concentrations used were 0.1 and 0.2 µg/ml per 10^6 cells, and as expected with the higher amount of antibody the cells showed an increased median fluorescence; values were 1152 mfu for 0.1 µg/ml and 1439 mfu for 0.2 µg/ml. However it was the lower concentration that resulted in more positively stained cells, with 3.89% of total cells and 8.63% of live cells in the positive population for 0.1 µg/ml, whilst these values were 3.23% and 7.39% respectively for the 0.2 µg/ml concentration of antibody. As there was a slightly larger number of cells in the sample for 0.2 µg/ml (14,042 verses 13,557 for 0.1), with more cells gated out as dead, this would result in very similar numbers of positive cells being expressed as a higher percentage. It was deemed that this was the case, so for all subsequent experiments, using quail, the CD8 specific antibody, Du-CD8-1 was used at a concentration of 0.1 µg/ml per 10^6 cells. For CD4 in quail, although the initial screen for the Du-CD4-1 antibody (Figure 3.5) gave a similar profile as the Du-CD8-1 antibody, and as such was considered to potentially recognise quail CD4, the CD4 antibody called CT4 provided a more
clearly defined profile (Figure 3.4), so no further testing of the Du-CD4-1 antibody was undertaken.

Most of the CD8 or CD4 antibodies tested using HOSP splenocytes were considered not to recognise the equivalent HOSP CD8 or CD4 molecule, except for the CD8 antibody 3-298, which did show a small positive population. All of the negative antibodies did however show quite a large difference between the unstained sample and the stained samples (see Figure 3.7, Figure 3.8, Figure 3.10 and Figure 3.11). This is indicative of an increase in the overall background fluorescence of the cells, to varying degrees following incubation with the antibodies; it may be due to random and/or non-specific interaction between individual cells and the antibody. HOSP samples had been held on ice for significantly longer than the quail samples due to transport times, thus this additional handling may have contributed to the more unusual results seen in the fluorescent profiles for most of the HOSP samples.

Although the 3-298 antibody did reveal a small positive population, further testing would need to be done to confirm results are consistent and reproducible, especially in light of the fact that one of the birds tested in the initial screen in Figure 3.9 did not produce a positive population. However no further HOSP samples were available at the time.

For the WCSP samples only the Du-CD8-1 antibody gives any positive results, but even this would require further testing, including utilising different antibody concentrations, to confirm active positive binding to the WCSP CD8 molecule.

Closely related bird species would, by definition, have more closely related DNA, thus closely related species may have more cross-reactivity, with regards to antibody recognition of specific cell markers, than would more distantly related species. The results summarised in Table 3.1 do show a slight tendency towards this. Chickens, turkeys and quail are in different
subfamilies of the same family: Phasianidae (class: Aves, order: Galliforms) and two of the
chicken/turkey antibodies tested on quail gave positive results. HOSP and WCSP are in the
order Passeriformes and so are more distant from chickens and turkeys than quail, which is
reflected in the fact that only one of the antibodies tested positive against both species of
sparrows. Ducks are in an order, different from any previously mentioned, called
Anseriforms. For the duck antibodies, as for chicken/turkey antibodies, two duck reactive
antibodies gave positive results in quail and only one antibody in the sparrows. However this
may be because some antibodies may recognise regions that are more conserved across
different species of birds which would reduce the relevance of relatedness.

3.3 Antibody Screens and Optimisations for West Nile Virus ELISA

The testing and optimisation for the ELISA assay was to ensure that the detecting
(secondary) antibodies would bind to any avian related antibodies present and that any WNV
antibodies present would bind to the WNV antigen. Optimisation was also performed to
ensure a concentration of detecting antibody that would work best with likely concentrations
of WNV antibodies present in serum samples to be tested. Binding also needed to be
established for both avian species for which it was planned to use the ELISA.

3.3.1 Results - Avian Antibodies and Concentrations Suitable for ELISA

The absorbance readings obtained from the checkerboard titration of different dilutions of the
detecting (secondary) antibody when bound to different dilutions of quail IgY can be seen in
Figure 3.15. At the two highest concentrations of IgY there is little drop in OD values,
probably due to the IgY binding capacity of the plate itself still being saturated at the lower
of the two concentrations. At the two highest concentrations of detecting antibody, namely
1:400 and 1:800 dilutions, there is a slight increase in OD values as the IgY concentration falls. This is likely due to non-specific binding of the detecting antibody, as it is present in excess amounts, when the levels of IgY are also high. However for lower levels of IgY, all concentrations of detecting antibody showed reducing OD values as the level of detecting antibody falls. Also, as expected, as the concentration of detecting antibody falls, the sensitivity of IgY detection also falls, with the OD value of 0.2 being reached at IgY concentrations of 40, 20 and 10 ng/ml respectively for the 1:1,600, 1:800 and 1:400 dilutions of detecting antibody. Overall, a detecting antibody concentration of between 1:800 and 1:1,600 would be considered optimal for quail serum samples.

Figure 3.15 Checkerboard Titration Established Suitable Concentrations of Quail IgY and Anti-Bird IgG Antibody.

Serial dilutions of quail IgY were quantified to give ng/ml units. The Ab Dilutions are for the anti-bird IgG detecting antibody.
To confirm the detecting antibody concentration suggested from the checkerboard titration and test the assay with actual samples, sera from vaccinated quail were tested at three different concentrations of detecting antibody. Sera were collected from birds vaccinated with either rAdE, rAdNS3, WN antigen or PBS as a negative control group. For the levels of WNV specific antibodies present in actual samples, the best dilution of the detecting antibody to use was 1:600 (Figure 3.16). This is because this dilution has similar OD values to the higher (1:800) concentration but with slightly better separation between the positive and negative samples, yet provided higher OD values than the lower (1:400) concentration, which may help to improve sensitivity.

Figure 3.16 Quail Serum ELISA, 1:600 Dilution of Detecting Antibody is Optimal.
Each bird was injected with either: rAdE, rAdNS3, WNV antigen or buffered saline, defined as E, NS3, WN Ag and PBS respectively (see legend). The numbers in brackets are the individual bird identification numbers. Bkgrd/Ave Bkgrd is the OD value for background control wells, that had antigen and all relevant antibodies but no serum added.

The results regarding the effectiveness of the vaccines themselves indicated that while the WN antigen did appear to be effective in inducing antibody production in the quail, the vaccines themselves were not; although there were very slightly higher OD values for the rAdE vaccinated quail than for the rAdNS3 vaccinated birds or the control group (Figure 3.16). The birds vaccinated with the WN antigen would have raised antibodies to all proteins present in the antigen, this would include all WNV structural and non-structural proteins, as the antigen is prepared from WNV infected tissue. However, birds receiving either of the two vaccines, rAdE or rAdNS3, would only have antibodies relevant to the single WNV protein created from the vaccine. As the full WN antigen is used to capture antibodies during the ELISA assay, the small amount of any single WN protein in the antigen and thus available for binding by any WN antibodies relevant to that protein, would severely reduce the sensitivity of the assay if only a single relevant antibody was present in the sample. A further ELISA was run that used more specific proteins as antigen.

The specific antigen ELISA assay was set up using purified proteins for either the env or NS3 protein as the antigen on the plate, to enable higher levels of these specific antibodies to be bound to the plate thus increasing the sensitivity of the assay for those specific antibodies (see methods 2.1.2.1, page 41, for methods). There was only a limited amount of protein available to use as antigen, so only two different dilutions of serum could be used. Although a full range of dilutions was used for the plate using the full WN antigen, only the relevant
two are shown for comparison (Figure 3.17). The positive control sample was serum from a goose infected with WNV and as can be seen there is a considerable drop in OD values for this positive sample from just over 1.6, down to approximately 0.5 when the more specific antigen is used. For the other samples there is a general increase in OD values when using the more specific antigen; for the 1:10 dilution all of the samples from the four vaccinated quail are greater than 0.4 whereas with the full WN antigen only one test sample gives a value of greater than 0.4. The two negative control vaccinated birds always showed the lowest OD values which ever antigen was used.

![Figure 3.17](image)

**Figure 3.17** In a Serum ELISA, Env/NS3 Protein as Antigen Provides Higher Values Than WN Antigen.

Serum samples are from quail vaccinated with either rAdE (Env), rAdNS3 (NS3) or PBS (Neg), the numbers identify the individual birds. `Bkgrd` is the background level for wells with no serum added and `Positive` contained a sample of serum from a goose infected with WNV.

Serum samples from HOSP were also tested for compatibility with the serum ELISA assay by directly binding the serum to the plate at the start of the assay. Figure 3.18 shows that the four HOSP samples all gave similar OD values and although actual values in this particular
The assay were fairly low, the values for the HOSP did fall between the values obtained for the chicken and the quail samples. This indicates that the ELISA assay should work for HOSP serum samples.

![Serum ELISA](image)

Figure 3.18 Anti-Bird IgG Antibody is Suitable for an ELISA to Assess HOSP Serum.

The numbers after 'HOSP' identify the four different individual HOSP used. Serum from a quail and a chicken were included as positive controls. 'bkgrd' is the OD values from wells where no serum was added.

### 3.3.2 Discussion – Avian Antibodies and Concentrations Suitable for ELISA

The two most important components in the ELISA are the antigen bound to the plate, for capture of the WN antibodies in test samples, and the detection antibody used to measure the captured WN antibodies; both components of the assay were assessed.

To evaluate a concentration of detecting antibody, first quail IgY was bound to an ELISA plate and the antibody was titered against it. This suggested that dilutions of between 1:800 and 1:1,600 would work (Figure 3.15), as they both gave sufficiently high OD values and
were quite sensitive, sensitivity being assessed by the dilution of the antibody when the OD value was at 0.2 as this is considered a maximal OD value for background readings.

However, when avian serum samples were used, these dilutions were found to be too high and a dilution of only 1:600 was found to achieve similar results (Figure 3.16). This may be due to the lower levels of specific antibodies bound to the plate, once the assay was run in conjunction with the WN antigen, as opposed to the higher amount of antibody that would have been present when directly binding the IgY to the plate, as in the initial experiment.

Different binding levels were seen when serum samples from chicken, HOSP and quail were detected using the anti-bird IgG antibody (Figure 3.18), where quail samples resulted in lower binding than HOSP or chicken samples. The sparrows referred to in the literature for the antibody are likely HOSP and the IgG from the different species was purportedly present in the goat vaccination in equal proportion. However neither of these statements may be true; this would explain why the binding of the antibody to chicken IgG appeared higher that for the HOSP samples. Also the affinity of binding may be different between the species; both possibilities indicate that it would not be valid to try and make a direct comparison between species, even if samples were run as part of the same ELISA. It also explains the fairly low OD levels seen in the WN specific ELISA assays for quail samples (Figure 3.18), indicating that the detecting antibody only bound to quail IgY at low levels, or with lower affinity.

The other main component of the ELISA that needs to be assessed is the antigen used for capture of WN specific antibodies. The graphs in Figure 3.17 are from assays run with either the full WN antigen or with an antigen made up of the WN proteins (env and NS3) relevant to the vaccines. Direct comparisons of ELISAs run at different times are not considered accurate on a statistically significant level but inferences may be drawn. With the same
samples, OD values are greater when the more specific antigen is used and as the background value in the protein antigen assay is lower than for the WN antigen assay, increased background binding is unlikely to be the cause of this overall increase in values. The same amount of overall protein, 3 µg, was added to each well of the plate for both assays but as the WN antigen consisted of a wider variety of proteins the amount of env and/or NS3 present, and actually bound to the plate, was likely less than for the specific protein antigen plate. The relative higher level of the two specific antigens on the plate would enable more env or NS3 specific antibodies to bind compared to the WN antigen plate, which is the likely cause of the higher OD values for the specific antigen plate. However as all samples did not show a similar level of increase in OD values when the more specific antigen was used, simply the amount of antigen present is not the complete answer.

The differences in OD values in Figure 3.17 may relate to the specificity of binding between the antigen and the antibodies present in the sera. The serum from bird #4 vaccinated with rAdNS3 showed the greatest increase, when the specific antigen was used. This may be due to the antigen being in a form more compatible to induce binding with the NS3 serum antibodies. Bird #4 may have responded well to the vaccine and had high levels of NS3 antibodies which saturated the amount of NS3 specific antigen in the WN antigen plate but more were able to bind to the higher levels of NS3 antigen present with the more specific antigen. However the OD values for bird#3, also vaccinated with rAdNS3, hardly seem to change at all whichever antigen is used, indicating that this bird did not respond very well to the vaccine and consequently did not produce very high levels of NS3 antibodies. Both rAdE vaccinated birds showed similar increases in OD values with the specific antigen implying that both had env antibody levels high enough to reflect the additional binding to the higher
amount of antigen, although again, it could also involve a higher level of specificity acting to increase binding. Both birds from the negative control group showed similar OD readings for both antigen variants, which is expected as no WN related antibody should be present.

Although the ELISA was deemed to be suitable for HOSP serum samples it was never run using them, because during the investigations involving the HOSP no spare blood samples were available for use in an ELISA. Samples collecting during the first week post-infection were used for viremia analysis and samples collected later were not considered likely to be helpful, as they were taken long enough after infection that all birds, vaccinated or not, should have had antibodies present at similar levels due to infection rather than vaccination.

3.4 Assessment of CD4 and CD8 in Japanese Quail

Once it had been established which avian related CD4 and CD8 antibodies would work with quail lymphocyte samples, samples were repeatedly assessed using different individual birds to establish average population sizes for both CD4 and CD8 cells in quail, from both spleen and blood samples.

3.4.1 Results – CD4 and CD8 Positive Cell Population Proportions in Quail

Cells were stained with CD4 or CD8 antibodies and analysed by flow cytometry, enabling different populations of the entire cell sample to be included or omitted by using different selection gates. Figure 3.19 shows an example of this type of gating. In a.) the cells are selected based on size (FSC-H) and cellular granularity (SSC-H) to include all live cells, here this is 24% of all cells in the sample. In b.) the live cells are displayed on axes for FL1-H and FL3-H. These display fluorescence at different wavelengths but neither correspond to the fluorescent markers used on the CD identifying antibodies, instead they are used to measure
the level of background fluorescence and only cells with low levels are selected. The cells selected in b.) are then assessed, in c.), for their level of fluorescence corresponding to the fluorophore used to label the CD identifying antibodies. The lower population is considered to be negative for the specific cell marker and the upper population, in this case 33.3%, is considered positive for the marker, in this example for CD8. This same gating strategy was applied to all splenocyte samples for both CD4 and CD8 assessment.

The gating strategy for PBMC samples was very similar to that for splenocytes (see Figure 3.20), with live cells selected. Subsequently, cells with high levels of background fluorescence were removed. For PBMCs however, background fluorescence was seen and excluded, based on two different wavelengths, displayed as FL2-H and FL3-H in b.); the gate used is an exclusion gate, which excludes cells within the gate, which is why although only a few cells are inside the gate the percentage of cells selected (97.8%) is high. Finally, cells were selected based on the fluorescence levels from the cell marker antibodies (FL4-H) and were assessed as high levels being positive for the marker, in this example 12.3% of cells were positive for CD8.

Figure 3.19  Gating Strategy for Assessment of Splenocytes for CD4 or CD8.
The three graphs demonstrate cell selection for CD8 positive cells: a.) shows the entire sample population of cells and selection of those considered to be alive based on physical properties of the cells; b.) shows the selection of cells in which fluorescence can be measured by exclusion of cells with high levels of background fluorescence in a single wavelength and c.) show the final cross gate on the remaining cells to assess whether or not they are considered positive for CD8 (FL4-H) based on level of fluorescence. Legends below each graph show the sample name, the gate name containing the cells displayed (or ungated if no gate applies) and the number of cells displayed.

Figure 3.20 Gating Strategy for Assessment of PBMCs for CD4 or CD8.

The three graphs demonstrate cell selection for CD8 positive cells: a.) shows the entire sample population of cells and selection of those considered to be alive based on physical properties of the cells; b.) shows the selection of cells in which fluorescence can be measured by exclusion of cells with high levels of background fluorescence in two different wavelengths (this gate is an exclusion gate which is why the cells not required are selected) and c.) show the final cross gate on the remaining cells to assess whether or not they are considered positive for CD8 based on level of fluorescence. Legends below each graph show the sample name, the gate name containing the cells displayed (or ungated if no gate applies) and the number of cells displayed.

Samples of either splenocytes or PBMCs were assessed in the manner described above and some final cross gate images from an example of each combination of cell type and cell marker can be seen in Figure 3.21. Examples of the percentages of positive cells for each combination can be seen as 4.74% for CD4 splenocytes, 28.1% for CD8 splenocytes, 1.57% for CD4 PBMCs and 4.15% for CD8 PBMCs.
Figure 3.21 Cross Gating Example Results for CD4 and CD8 in Splenocytes and PBMCs.

The top row is splenocytes samples and the bottom row PBMCs, with staining for CD4 on the left and CD8 on the right. High levels of fluorescence in the FL4-H wavelength indicate high levels of cell marker, hence the positive population of cells for each cell type/cell marker combination is in the Q1 section of each cross gated graph. Legends below each graph show the sample name (made up of cell marker and cell type), the gate name containing the cells displayed and the number of cells displayed.
Table 3.2 shows results from 4 experiments run to assess the overall percentages of cells for both CD4 and CD8 cells markers in samples collected from spleen and blood of quail. Unfortunately useable blood samples were not available for every experiment. The average percentages of CD8 positive cells are 31% and 12.5% in spleen and blood respectively and CD4 positive cells are 3.7% and 0.85% respectively. As more samples from spleen were available, additional statistical analysis was performed on these samples providing the additional details shown at the bottom of Table 3.2.

<table>
<thead>
<tr>
<th>Date</th>
<th>Cell Marker &amp; Type</th>
<th>CD4 PBMC</th>
<th>CD4 Spleen</th>
<th>CD8 PBMC</th>
<th>CD8 Spleen</th>
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</thead>
<tbody>
<tr>
<td>15/09/2011</td>
<td>N/D</td>
<td>6.9</td>
<td>N/D</td>
<td>33.7</td>
<td></td>
</tr>
<tr>
<td>28/09/2011</td>
<td>0.9</td>
<td>3.3</td>
<td>7.1</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>29/09/2011</td>
<td>N/D</td>
<td>3.1</td>
<td>N/D</td>
<td>24.8</td>
<td></td>
</tr>
<tr>
<td>14/10/2011</td>
<td>0.8</td>
<td>1.4</td>
<td>17.8</td>
<td>36.3</td>
<td></td>
</tr>
<tr>
<td><strong>Overall Average (mean)</strong></td>
<td><strong>0.85</strong></td>
<td><strong>3.7</strong></td>
<td><strong>12.45</strong></td>
<td><strong>31.0</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Range of Percentages</strong></td>
<td><strong>0.147 - 1.83</strong></td>
<td><strong>0.237 - 8.39</strong></td>
<td><strong>2.9 - 26.4</strong></td>
<td><strong>15.0 - 46.2</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Standard Deviation</strong></td>
<td><strong>N/D</strong></td>
<td><strong>2.313</strong></td>
<td><strong>N/D</strong></td>
<td><strong>5.093</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Coefficient of Variation</strong></td>
<td><strong>N/D</strong></td>
<td><strong>62.9%</strong></td>
<td><strong>N/D</strong></td>
<td><strong>16.5%</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 Means of Percentages of CD4+ and CD8+ Cells from Quail Spleens and PBMCs.

Values are means of the percentages of positive cells, from all individual birds, from each day’s experiment and overall average is the mean of those means. Range of Percentages is the highest and lowest percentages recorded from an individual quail for the given cell type/cell marker combination from all experiments. PBMC stands for peripheral blood mononucleated cells. Dates indicate the date the experiment was run, format is DD/MM/YYYY.
During set up and running of the experiments to measure the CD4 and CD8 populations in quail, some of the experiments showed some unusual results, whereby cells positive for CD8 displayed two different levels of fluorescence. Figure 3.22 shows an example of the gating strategy used to identify these cells, with selection on physical size and exclusion of cells with high background, as for the other experiments. In these experiments, however, when the final cross gating was done there appeared to be two different populations of CD8+ cells, one with a high level and one with a lower level of fluorescence. In the example, which was typical of the experiments in which this phenomenon was seen, of the 29.3% of cells considered positive for CD8, 19.8% demonstrated high fluorescence and 9.5% low fluorescence.

Figure 3.22 CD8 High and Low Positive Cells Discovered in Quail Spleen Samples.

The three graphs demonstrate cell selection for CD8 high and low positive cells: a.) shows the entire sample population of cells and selection of those considered to be alive based on physical properties of the cells; b.) shows the selection of cells by exclusion of cells with high levels of background fluorescence in two different wavelength (denoted by FL2-H and FL3-H) and c.) show the final cross gate on the remaining cells to assess to what extent they are considered positive for CD8 based on level of fluorescence.
Additional experiments were run using samples from quail spleens to further investigate the CD8+low cells, to see if they were also positive for CD4, by including antibodies for both CD4 and CD8 in the same sample for staining, Figure 3.23 shows an example of analysis. In a.) the gate is used to select all live cells, including both a population of larger cells (on the right hand side) and smaller sized cells (on the left hand side), with dead cells on the extreme left being excluded. As shown previously, these cells underwent further selection to exclude cells with high background levels of fluorescence (not shown here) before the final gating, shown in b.) to indentify positively stained cells. The FL4 axis relates to CD8 and the FL2 axis to CD4, so Q1 contains CD8+ cells, Q3 contains CD4+ cells, Q4 contains CD4-CD8- cells and Q2 contains CD4+CD8+ cells. Nearly all of the double positive cells in Q2 demonstrate the lower level of fluorescence for CD8. For the three individual quail assessed in this experiment mean averages for each population were calculated: 39% were CD8+ only, 1.71% were CD4+ only and 2.6% were CD4+CD8+ double positive cells, giving an overall CD4+ of 4.31% and CD8+ of 41.6%.

Figure 3.23 Cells Double Positive for CD4 and CD8 Discovered in Quail Splenocyte Populations.
The initial gating on physical properties of the cells (FSC-SSC in a.) includes 2 different populations of living cells as indicated by the gate. The final gating is shown in b.) with FL4-H showing fluorescence relating to CD8 and FL2-H relating to CD4 antibody staining and section Q2 relating to CD4+CD8+ cells.

3.4.2 Discussion– CD4 and CD8 Population Proportions in Quail

For quail there appears to be many fewer CD4+ cells than CD8+ cells, in both spleen and PBMC samples. CD4+ cells comprise between 0.1 and 1.8% of PBMCs while CD8+ cells account for between approximately 3 and 26% of the same population; for splenocytes 0.2 to 8.4% of cells are CD4+ while 15 to 46% of cells are CD8+ cells. However, five studies to quantify T cells in peripheral blood from chickens came up with a variety of different answers; Table 3.3 shows that three of the five showed there to be substantially more CD4+ cells than CD8+ cells, one showed the numbers to be very similar and one showed almost eight fold more CD8+ cells than CD4+ cells. The studies referenced in Table 3.3 were all done using naïve chickens from various lines and at several different ages, both of which were shown to affect the numbers and proportions of T cells present in the blood. Overall, for chickens, there are more CD4+ cells than CD8+ cells in peripheral blood, which is in opposition to the measurements made in quail. The work conducted in quail was also done using naïve quail from the same breeding line, all of which were approximately twelve months old. The effects of age on the number and proportion of T cells in quail will not be quantifiable until data from quail of other ages are available for comparison. The low proportion of CD4+ cells seen in quail may be due to to properties of the CD4 antibody used. It is possible that it does not bind to all cells positive for the marker and thus underestimates the final number. However if the cell numbers for quail, as presented in Table 3.2 are correct
they would be in concordance with the results for chickens seen in Table 3.3, [70], which also shows CD8+ cells in greater numbers than CD4+ cells in the blood.

<table>
<thead>
<tr>
<th>%CD4 in PBMC</th>
<th>%CD4 in Spleen</th>
<th>%CD8 in PBMC</th>
<th>%CD8 in Spleen</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 – 32</td>
<td>5 – 7</td>
<td>18 – 19</td>
<td>53 – 55</td>
<td>naïve, 7wks old</td>
<td>[69]</td>
</tr>
<tr>
<td>1.6 – 3.4</td>
<td>ND</td>
<td>11 - 19</td>
<td>ND</td>
<td>naïve, 2 different breeding lines</td>
<td>[70]</td>
</tr>
<tr>
<td>7 – 14</td>
<td>ND</td>
<td>7 - 12</td>
<td>ND</td>
<td>naïve, 7 different breeding lines</td>
<td>[71]</td>
</tr>
<tr>
<td>4 – 31</td>
<td>ND</td>
<td>1 - 10</td>
<td>ND</td>
<td>naïve</td>
<td>[72]</td>
</tr>
<tr>
<td>41</td>
<td>ND</td>
<td>14 alpha 5 beta</td>
<td>ND</td>
<td>naïve, 6 wks old, numbers decrease with age</td>
<td>[73]</td>
</tr>
</tbody>
</table>

Table 3.3 Summary of Percentages of CD4 and CD8 Populations in Chickens.

The average percentage or range of percentage for both CD4 and CD8 populations in chickens are shown. The details column contains information on the chickens used for each study. ‘alpha’ and ‘beta’ denotes the different CD8 molecules.

T cell numbers and proportions of CD4/CD8 cells from spleen samples, appear to be similar between naïve quail and naïve chickens, although only one study could be found involving splenocytes from chickens (Table 3.3 Summary of Percentages of CD4 and CD8 Populations in Chickens.[69]). Spleen samples from chickens show median values of 6% for CD4+ and 54% for CD8+ cells compared with a mean average of 4% and 31% respectively for quail. The percentages for quail are likely correct, as they are similar to known values for another avian species, however the variation seen in the populations of the same cells in blood samples from different lines of the same species i.e. chickens, renders the interspecies comparison a less compelling, although still valid, indicator of accuracy. Repeatability, as measured by variation within the cell population percentages, may provide better values to judge accuracy on. For splenocytes, Table 3.2 shows that for CD4, although two individual
results were very close, 3.1% and 3.3%, the means covered a range of 5.5% and a standard deviation of 2.313 was calculated; for CD8 the range of means was 11.5%, although values were much higher than for CD4 cells, and the standard deviation was 5.093. For comparison of variation between populations with large differences in mean, the coefficient of variation provides a better value than using standard deviation directly, as it is calculated as a ratio of the mean itself. For CD4 cells this value was 62.9% and for CD8 cells it was 16.5%. This indicates that variation was less between measurements of CD8 populations than they were for CD4 populations, indicating those measurements were likely more accurate for CD8.

The phenomenon of CD8+ populations of cells that display high and low levels of the CD8 cell marker, as seen in the quail experiments (Figure 3.22), has also been observed in chickens as well as in pigs and monkeys [74]. For chickens, it was shown that the population of cells with low levels of CD8 expression were in fact CD4+ cells, that had reacquired the ability to express the CD8α molecule after leaving the thymus, and therefore could express CD8αα dimers, albeit at low levels, but not CD8αβ heterodimers. In one chicken line that demonstrated particularly high numbers of these double positive cells, for spleen samples levels of this cell type where as high as 10-20% and the cells were shown to have the phenotype of memory CD4+ cells [75]. The CD8+low population in quail may be similar to the CD4+CD8+low population seen in chickens. Although the CD8+low population was seen in several CD8 single staining experiments, only one experiment was run when CD4 and CD8 double staining was undertaken, gave notable results, see Figure 3.23. However these results were considerable when taking into account the low level of CD4 binding seen in the single CD4 staining experiments. Further experiments would need to be undertaken to
confirm the double staining results, preferably with a different CD4 antibody, ideally one raised specifically against quail CD4 molecules.

3.5 Chapter Conclusions - Suitable Avian Reagents

This chapter pertains to the use of antibodies as reagents; antibodies made using markers specific to birds from species other than quail. During interpretation, it should be noted that results are correct for the specific antibodies used, even though they may not accurately reflect the actual numbers of cells, or even proportions of cell populations, for quail. This applies to all results including those that render quail CD4/CD8 cell proportions similar to chickens, such in splenocyte samples, as well as those that are dissimilar, such as the blood samples. Any differences between results obtained, with the anti-chicken and anti-duck antibodies, and the actual numbers for quail, will only be known when results are obtained for antibodies specific for quail CD4 and CD8.

Having accepted that results are accurate for the antibodies used, it is likely that the same results also indicate that the CD4 antibody used, may not have been binding to all CD4+ cells, despite being the best antibody screened. This is inferred from the low numbers of CD4+ cells in proportion to CD8+ cells recognised for quail in all experiments when compared with chickens. CD4+ cells typically predominate CD8+ cells in chickens and in mammals, such as mice and humans, making it unlikely that quail are an exception to this rule.

Other issues that may be affecting the number or proportion of T cell types, is the age and immunological status of the quail used. All chicken experiments were run using young birds (maximum 7 weeks old) which were specifically bred for research and were considered
pathogen free. Quail were from a similar source as the chickens but were approximately
twelve months old when the experiments were run. Also, although the quail were pathogen
free on purchase they had been housed with direct access to the external environment, so
although they were never intentionally exposed to any antigens this may have occurred
‘naturally’ from the environment. It follows from this that some of the quail’s T cells might
have been activated, by random antigens, and that as activated T cells are larger than truly
naïve T cells [76] this may have resulted in two different populations of T cells being
present. It is possible that these are the populations seen in some of the flow cytometry
graphs with an FSC axis that indicates size, such as Figure 3.22a.), where the population on
the right was considered to be live cells, when in fact they may have been just the larger
activated T cells. The smaller cells in the population nearer the centre of the FSC axis were
excluded because they were considered likely to be dead due to their proximity to the very
small dead cells (on the extreme left) but they may have been the smaller truly naïve T cells.

Despite the many combinations, of antibody and cells from different avian species, that did
not provide usable results from flow cytometry, enough antibody reagents were found to
allow assessment of the immune system of Japanese quail to be completed.
Chapter 4: Generation and Testing of Recombinant Adenovirus Vaccines

4.1 Rationale for Experiments

The most important objective in this entire project could be considered to be the actual creation of the recombinant adenoviruses that are designed to be tested as vaccines. After creation, the screening and functional testing of the initial recombinants are also important steps to ensure the adenoviruses perform as expected. Specifically there was a need to demonstrate that the recombinant adenoviruses express both mRNA and protein from the inserted WNV DNA. The initial infection testing and all expression experiments were first performed in the human cell line A549, as this line is known to be highly susceptible to adenovirus infection and able to express recombinant proteins at high levels. The vaccines were subsequently tested in avian cell lines.

4.2 Adenovirus Infection in Avian Cell Lines

Before starting construction of the adenovirus vaccines, it had to be confirmed that recombinant human adenoviruses would be able to infect avian cells and to express proteins. A pre-existing recombinant adenovirus that expresses the enhanced green fluorescent protein (eGFP) was used to infect different avian and human cells, the latter as controls. The level of eGFP in each cell was then measured using flow cytometry analysis.

4.2.1 Results – Adenovirus Infects Avian Cells

The human cell line A549 was used as a positive control and chicken cells (CEF) were tested for adenovirus infection. Following selection of live cells, from the flow cytometry analysis, histogram plots of level of fluorescence were constructed for each cell line. Samples
included: cells infected with rAdeGFP, non-infected control cells and cells infected with an empty (non-fluorescent) recombinant adenovirus vector (Figure 4.1). Although several dilutions of virus were used to infect the cells, only the single dilution shown gave positive results in either cell line, as indicated by higher levels of fluorescence. Cells infected with the empty recombinant adenovirus showed very similar levels of fluorescence to the non-infected control cells.

![Figure 4.1 Recombinant Adenoviruses Can Infected Chicken CEF cells.](image)

The above shows fluorescence histogram graphs, Chicken CEF cells on the left are denoted by C in the sample name, and human A549 cells, shown on the right, by an A. Sample names also indicate infection status: MT – infected by adenovirus vector only, 2 – infected by a $10^{-2}$ dilution of rAdeGFP and 0 – non-infected controls. FL1-H is the fluorescence axis for the GFP and ‘count’ is the number of cells demonstrating that particular level of fluorescence.

The quail cell line, QT35, was tested later after the rAdeGFP stock had been quantified, so cells could be infected at specific MOIs rather than just at different dilutions of virus. For this experiment rAdNS3 infected cells were included as non-fluorescently stained controls and non-infected cells were also included. Again, following live cell selection, graphs showing levels of fluorescence for the different levels of infections were plotted (Figure 4.2). Cells
with higher levels of infection showed higher levels of fluorescence and cells infected with a non-fluorescent adenovirus showed similar fluorescence levels as non-infected cells.

4.2.2 Discussion – Adenovirus Infects Avian Cells

Unfortunately, the two experiments to test human adenovirus infection in chicken and quail cells were separated by a considerable period of time, as the quail cells were not available at the start of the project, so direct comparisons between the two experiments is limited. However, the ability of a human adenovirus to infect avian cells is clearly confirmed in both experiments.

Figure 4.2 Recombinant Adenoviruses Can Infect Quail QT35 Cells.

The fluorescence histogram graph shows QT35 cells infected at 3 different MOI, 1, 5 and 100 (eGFP 1, 5 and 100 samples respectively). For controls, cells were infected with rAdNS3 (NS3) or were left not infected (NI). FL1-H is the fluorescence axis for the GFP and ‘count’ is the number of cells demonstrating that particular level of fluorescence.
Practically all of the human cells within a sample became infected when virus was added (Figure 4.1), as indicated by the shift of the main peak to the right. Very few cells showed fluorescence at the same level as the non-infected cells. For the chicken cells the main peak was likely still negative, for infection and increased fluorescence, as it has only shifted very slightly to the right. The same amount of adenovirus infects fewer avian cells than human cells, indicated by the height of the peaks. However, the level of protein expression in those cells that are infected is higher in chicken cells than in human cells, as the peak of infected cells for CEF is just above $10^3$ mfu, while for human cells it is just below $10^2$ mfu. Although there are some human cells showing the same level of fluorescence as the infected CEF the peak is much lower.

For quail cells (Figure 4.2) nearly all cells become infected, as the peaks are all of similar height as the peak for the non-infected control cells, and the amount of fluorescence increases with increasing MOI. It follows that at the higher MOIs more than one adenovirus particle has infected any single QT35 cell yielding the higher amount of fluorescence.

### 4.3 Generation of Plasmids and Their Recombination

Many DNA manipulations are required to prepare the plasmids for the recombination reaction that will create the new vaccines, with assays required to ensure that the product from each step is as it should be. These manipulations include:

- Conversion of WNV RNA to DNA.
- PCR of WNV DNA to create the insert DNA for the shuttle plasmid, containing appropriate restriction enzyme sites and transcription codons as well as the WNV sequence.
• Amplification and purification of the insert DNA by cloning into *E.coli*, and screening for colonies positive for the WNV DNA.
• Digestion of cloned WNV insert DNA and the shuttle plasmid with appropriate restriction enzymes to enable the subsequent ligation reaction to occur to place the WNV DNA into the shuttle plasmid.
• Diagnostic digests of the shuttle plasmids to confirm inserted DNA is present and that they are of the correct size.
• Amplification and purification of the shuttle plasmid with the different WNV DNA by cloning into *E.coli*, and screening for colonies positive for the WNV DNA.
• Amplification and purification of the adenovirus genomic plasmid, and the empty shuttle plasmid, by cloning into *E.coli* and restriction enzyme digests to confirm the plasmid produced band sizes as expected.

### 4.3.1 Results – Generation of Plasmids

Following the PCR to create the DNA to be inserted into the shuttle plasmids, reaction products were run on an agarose gel to confirm the products were of the expected size. Taking into account the size of the WNV specific region as well as the additional bases required, the env insert should have been 2 Kb and the NS3 insert 1.8 Kb. Figure 4.3 shows the results of two of the PCR reactions run to create the insert DNA. The DNA bands can be seen as single bands of the appropriate size for each insert. Several PCR runs were required to generate sufficient DNA for subsequent steps, but all runs were screened and produced results similar to those in Figure 4.3.
Once the insert DNA had been ligated into the shuttle plasmid, and the ligation mixture was used to transform bacteria for DNA amplification, colonies were screened for the presence of the WNV DNA. This was done by PCR with the same primers used when creating the inserts, so the expected band sizes were the same as for the inserts. Figure 4.4a.) shows bright bands for two colonies out of three indicating the presence of the env insert DNA - the 2 Kb size is correct. In b.) all four colonies appear to be positive for the NS3 insert DNA, although the colony on the left has the brightest band and was selected for subsequent experiments. The size of the bands for the NS3 insert appeared to be slightly smaller than the 1.8 Kb expected, but plasmids were also sequenced to ensure that inserted DNA was correct.
Figure 4.4 WNV Insert DNA Present in Some Bacterial Colonies Following Screening.

In a.) E-316 colonies were screened using env insert primers, three lanes are shown which contain two positive colonies and one negative colony. The bands are very bright indicating that lots of DNA is present, which is also why the bands appear as a smear extending down to 1Kb in size. In b.) four 3-316 colonies are screened using NS3 insert primers and all four appear positive to varying degrees. The numbers on the marker ladders are the size of the bands in Kb.

After plasmid DNA was sequenced, as the original samples of WN RNA were from an avian source, the sequences were compared to WNV taken from a flamingo infected with WN in 1999 in New York (NCBI Accession # AF196835.2). For E-316 the WNV region showed 99% identity to the reference DNA over bases 466-1453 for the forward primer and 1496-2469 for the reverse primer. For 3-316 the WNV region showed 99% identity to the reference DNA, over bases 4612-5672 for the forward primer and 5420-6469 for the reverse primer. See Appendix C for details of sequence alignments.

4.3.2 Discussion – Generation of Plasmids

It had been decided to use the E and NS3 ‘genes’ from WNV as they were highly immunogenic, strongly inducing the humoral response or the cellular response, respectively. However, as previously stated, to enable proper folding of the E protein, the M protein was also required to be present [64]. This is why sequences for the pre-membrane and membrane
proteins were included with the sequences for the env protein itself, when primers were designed to amplify specific WNV regions for use in the vaccine constructs.

The exact source of the original WNV RNA samples could not be established, other than the fact that it was from a bird infected with the NY-99 strain of WNV. It was decided that, for all DNA molecular work, sequence comparisons should be to the WNV isolated from an infected flamingo (AF196835.2). As mentioned in the results section, this flamingo source was also avian and the same strain of WNV; however the sequence of the original source may not be 100% that of the flamingo sample. By using the WNV sequence AF196835.2 as a reference, base numbers 466-2469 were included for the env constructs (consisting of pre-membrane 466-741, membrane 742-966 and env 967-2469) and base numbers 4612-6468 for the NS3 construct.

When the sequence of the plasmids was checked it was noted that a 41-base pair region, between bases 1453 and 1496 of the env insert, had not been covered by the sequencing reaction.

4.4 Selection and Purification of Viral Recombinants as Vaccine Candidates

After recombination of the genomic and shuttle plasmids when the recombinant adenoviruses were created, the first step is to purify each colony, that forms a single plaque, to ensure that a single clone virus is going to be selected. After selection and initial amplification each clone of vaccine virus needs to be screened for the presence of the WN insert DNA to ensure it is a recombinant virus, as well as sequencing the WNV relevant region, before any subsequent testing can be undertaken.
4.4.1 Results – Vaccine Creation

Screening of each vaccine clone is undertaken using a PCR reaction with primers designed to amplify a small region internal to either the Env or the NS3 region of inserted WNV DNA. Using these internal primers as specified in section 2.2.3 (page 55), PCR product band size should be 400 bp if Env DNA is present or 800 bp if NS3 DNA is present. This was to allow distinction between the two different DNA inserts and to test for any cross contamination between the two, either prior to or during co-transfection and recombination reactions. An example of one batch of screening, containing all controls and two positive samples, can be seen in Figure 4.5. The lanes one and two contain reactions run with DNA from each adenovirus, rAdE or rAdNS3, but with primers specific for the other one. This was done to check for any cross contamination. Lanes three and four contain reactions using DNA from uninfected cells as a template to check that the cell lines were not contaminated with either adenovirus. Lane five is a negative control, with no DNA template, to check for PCR reagent contamination. Lanes six and seven contained the test samples; the presence of a band indicates the presence of the specific WN DNA within each adenovirus.

At this stage in vaccine production DNA from each clone was also sequenced to confirm the WNV DNA was still correct. As for the sequencing from the shuttle plasmids (see section 4.3.1) sequences were again compared to the AF196835.2 sequence. For rAdE the WNV region showed 97% identity to the reference DNA over bases 466-1248 for the forward primer and 1681-2469 for the reverse primer. For rAdNS3 the WNV region showed 99% identity to the reference DNA over bases 4612-5499 for the forward primer and 98% identity
to the reference DNA over bases 5429-6239 for the reverse primer. See Appendix D for details of sequence alignments.

Figure 4.5 WNV Insert DNA in PCR Products from Screen of rAd DNA, on an Agarose Gel.

Numbered lanes contain: 1- rAdE DNA with NS3 primers, 2 - rAdNS3 DNA with Env primers, 3&4 - DNA from non-infected 293 cells with Env and NS3 primers, 5 - no DNA template with Env and NS3 primers (neg ctrl), 6 – rAdE DNA with Env primers and 7 – rAdNS3 with NS3 primers. The two outside lanes contain a 1kB marker ladder, the numbers on the right are the size of the bands in Kb.

4.4.2 Discussion – Vaccine Creation

Sequencing of the recombinant adenoviruses was never fully completed to demonstrate 100% matching with the AF196835.2 WNV DNA sequence. The entire region of WNV E DNA, within rAdE, was not fully covered during initial sequencing and no further DNA, from either vaccine, was subsequently submitted for sequencing. This should have been done to ensured that the vaccines were producing an accurate reflection of the relevant WNV protein and thus were inducing a fully appropriate immune response. For WNV NS3 even a single amino acid change can have a significant effect on viral virulence and interactions
with cellular proteins [77]. With such noticeable differences it implies that the secondary structure of the protein must also have changed. Any immune response raised against such ‘incorrect’ protein variants, especially antibody related responses, may not interact in the same way, if at all, with the native form of the WNV protein following infection. T cell responses may also be affected if vaccine proteins and therefore antigens are incorrect. When the vaccine antigens are processed for presentation to T cells, if the antigen is not identical to native WNV antigens, a different subset of T cells would be activated by the vaccines than would be activated during an infection. Thus the T cells activated by the vaccines may not provide protection following an infection of WNV.

4.5 Assessing mRNA Expression of the Adenovirus Vaccines

It was necessary to confirm that both of the vaccines would be able to induce expression of the WNV DNA once avian cells were infected, the first step of which is mRNA expression. This was done by isolating total RNA from infected cells, making cDNA from it and then running a PCR experiment to assess what specific DNA was present in the samples.

4.5.1 Results – mRNA Expression from Vaccines Confirmed

Samples from each stage of the experiment were kept to check for contamination between each step and all samples were then run on a gel at the end of the experiment. Figure 4.6 shows the results from the gel for A549 infected cells and Figure 4.7 those from infected QT35 cells, both are very similar. The ‘DNA’ included is from a standard DNA preparation from the same adenovirus and is included as a positive control to show the PCR is working and thus should produce a band on the gel. Each RNA sample is taken after the DNA digest has been completed on the original RNA sample, so if there is no original DNA
contamination left in the sample the RNA containing reaction should not produce a band. The FScDNA samples are taken from the final step and as the RNA has been converted back into DNA and each sample should produce a band on the gel, but only if the RNA was present originally. For the primers used in these experiments, any bands for rAdE should be 400 bp and for rAdNS3 800 bp.

Figure 4.6 mRNA Expression Confirmed from Both Recombinant Adenoviruses in Human A549 Cells. rAdE samples are in a.), lane details: 1. – FScDNA, 2. – RNA, 3. – DNA and 4. – no DNA (neg ctrl). rAdNS3 samples are in b.), lane details: 1. – FScDNA, 2. – RNA and 3. – DNA. Other lanes: 4. – FScDNA non-infected cells, 5. RNA from non-infected cells and lane 6 is the no template reaction (neg ctrl). The outside lanes in both a.) and b.) contain 1Kb ladders, the numbers by the ladders are the sizes of the bands in Kb. FScDNA – first strand copy DNA.
For both cells lines, all the lanes for FScDNA and DNA reactions show bands of the correct size for each relevant rAd and the RNA containing reactions show no bands, which is as expected. As the primers are specific for WNV DNA, any samples from non-infected cells should not produce any bands from the PCR, and this is also seen to be the case (Figure 4.6 and Figure 4.7).

4.5.2 Discussion – mRNA Expression from Vaccines Confirmed

At the stage of processing when the samples designated ‘RNA’ were collected, the fact that no PCR bands were produced, demonstrates no contaminating DNA was present at this stage of processing. It follows that the DNA present after first strand creation, demonstrated by the presence of PCR bands in all the FScDNA samples, must have come from expressed RNA. Also as the PCR primers were specific to WNV DNA only, with different size bands distinguishing between the two different forms of the adenoviruses, the presence of the FScDNA bands also confirms that the RNA must be WNV specific for either env or NS3.

Most of the samples show a single band in each lane, as would be expected. However when rAdE was expressed in QT35 cells there were two bands, at 1 Kb and 600 bp in addition to the expected one at 400 bp (Figure 4.7a. lane 1.). These same additional bands had also been observed in the reactions for A549 infected samples (data not shown) but had disappeared when the MgCl₂ content in the PCR reaction was reduced to 2.5 µl per reaction, to improve
the specificity of the PCR reaction. For the rAdE infected QT35 cells, even with the same reduced level of MgCl$_2$ in the PCR reactions the bands still remained. There may be some differences in mRNA processing of WNV env DNA in bird cells compared to human cells, such as different splicing events, resulting in the bands of different sizes. Under the same PCR conditions, the additional bands are not seen in the reaction that had directly prepared DNA as a template, whereas the two additional bands are still seen in the sample with a template of DNA prepared from RNA (Figure 4.7). If the bands occurred as a result of non-specific binding due to PCR conditions they should have disappeared from both reactions, as conditions were the same.

4.6 Assessing Protein Expression of the Adenovirus Vaccines

The final step to demonstrate functionality of the vaccines is their ability to produce the final protein from each WNV ‘gene’, so the proteins may stimulate an appropriate immune response. Protein expression was investigated by running samples of cells infected with each recombinant adenovirus through standard western blot procedures followed by detection with antibodies specific for Env and NS3 of WNV. As with mRNA, protein expression was first tested by infecting human cells, to ensure all reagents were working and that proteins could be expressed under favourable conditions before running the same experiments in quail cells.

4.6.1 Results – Protein Expression from Vaccines Confirmed

Protein sizes were calculated to be 73 kDa for the env/membrane polyprotein, 54 kDa for the env protein alone and 69 kDa for the NS3 protein. Images of the western blot films, of samples from human A549 cells infected either rAdE or rAdNS3, can be seen in Figure 4.8. For the env protein, seen in a.), bands can be seen just below the 55 and 72 kDa marker
bands at both MOIs used. For NS3 protein, in b.), a large band can be seen in the MOI 100 lane below the 72 kDa marker band, but no bands are visible in any of the lower MOIs tested.

Figure 4.8 WNV Protein Expression Confirmed from Both Recombinant Adenoviruses in Infected Human A549 Cells.

Samples from rAdE infected cells are in a.) and those from rAdNS3 infected cells in b.). Relevant lanes are labelled with the different MOIs used to infect each batch of cells and ‘non-inf’ is the non-infected A549 cells used as negative controls. The numbered bands are drawn from the position of bands from the protein marker ladder, band sizes are in kDa.

Once protein expression had been confirmed in human cells, the quail cell line QT35 was infected with the adenoviruses and harvested for protein extraction. Figure 4.9 shows images of the western blot films from 3 experimental runs, one looking for the env protein (a.) and two the NS3 protein (b. and c.).
Figure 4.9 WNV Protein Expression Confirmed from Both Recombinant Adenoviruses in Infected Quail QT35 Cells.

Samples from rAdE infected cells are in a.) relevant bands are visible just below the 55 kDa size. Samples from rAdNS3 infected cells in b.) just above 72 kDa as indicated by the arrow and in c.) one single band of approximately 72 kDa in the MOI200 lane. Relevant lanes are labelled with the different MOIs used to infect each batch of cells and ‘non-inf’ is the non-infected QT35 cells used as negative controls. The numbered bands are drawn from the position of bands from the protein marker ladder, band sizes are in kDa.

The bands for env are seen in all infected cell samples at just below the 55 kDa marker band, with the intensity of the bands increasing with increasing MOI infection levels. For NS3, bands are only seen in the highest MOI used for each experiment, in b.) the relevant band is visible in the MOI200 lane, just above the height of the 72 kDa marker band, as indicated by the arrow (additional non-specific bands are also visible across all samples), in c.) the NS3 specific band in the MOI200 lane is the only one visible but it was quite faint even on the
original film and became even less clear when the image was digitised. The apparent large size of the NS3 band in b.) is considered to be due to misaligning the position of the membrane when the marker bands were drawn in, compared to the original position of the membrane, the correct protein band size is shown in c.).

4.6.2 Discussion – Protein Expression from Vaccines Confirmed

The usual protocol for obtaining samples for protein extraction, is completed by centrifuging the samples, to remove cellular debris, and then running the protein containing supernatant on a gel for further analysis. However, initial western blots, where samples were centrifuged as usual, showed no env proteins when imaged. The env protein of WNV contains a transmembrane region which may have been ‘trapping’ the protein within membranes of infected cells. Once the samples were vortexed, to resuspend all particulate matter, instead of being centrifuged to remove it, the env protein was then shown to be in the samples. This indicates that the transmembrane region of the protein was functional in attaching the protein to membranes, either on the surface or to secretory vacuoles or other membranes within the infected cells.

Although the expression of env appears to be dose dependent, the same was not true for the NS3 protein. Env showed higher levels of protein expression from the samples infected with the higher levels of virus. This was especially evident in the quail cells. However, protein bands for NS3 only appeared in highly infected cells indicating that there may be a threshold under which no protein can be seen , although whether this is caused by a limit in detection or by a drop off in expression could not be determined. More testing with MOIs of closer concentrations e.g. MOIs 25, 50, 75 and 100 would need to be tested further to define if there
was such a threshold or if a dose dependent expression would be seen across more similar doses.

rAdE was constructed to include DNA that codes for WNV pre-membrane and membrane proteins, as well as that for the env protein itself. This was to enable proper folding of the env protein when the DNA was expressed. It is interesting to note the apparent differences in the processing of the polyprotein between human and avian cells. In human cells, proteins are present that correspond to the sizes of both the membrane-env polyprotein as well as to the env protein alone; however in bird cells, all the env specific protein present is of the env only size and has been cleaved from the membrane protein.

4.7 Expansion and Purification of Viral Clones into Working Vaccine Stocks

After basic functional testing of the adenoviruses had been completed, all viruses had to be expanded and purified ready for assessment of their ability to function as vaccines. Before this could be undertaken, quantification of the amount of viable virus present in each batch of vaccine produced was required, in order to enable calculation of the volume to be administered for each set dose of vaccine.

4.7.1 Results – Attainment of Vaccine Stocks

Adenovirus quantification was undertaken using the Adeno-X Rapid Titre Kit, with cells stained brown, by the action of the HRP enzyme on the provided substrate, being counted as infected. Dilutions that had between 25 and 75 positive cells were counted as this provided the most accurate counts for calculations; one dilution higher was always counted as well to ensure the number of positive cells was approximately 10 fold less, as a check that dilutions were done accurately. Dilutions were set up, and counted, in duplicate. Counts were used to
calculate the final titre of each batch of virus produced, using the equation from section 2.2.6.

The following is an example calculation:

\[
\text{Mean Positive Cells} = \frac{\text{Total # of Positive Cells Counted}}{\text{# of Fields Counted}} = 35
\]

Number of Fields Per Well = 79 (for 24 well plate at 100× magnification)

Amount of Virus Dilution Added Per Well (ml) = 0.05 ml

Virus Dilution of Well Counted = \(10^{-5}\)

\[
\frac{35 \times 79}{0.05 \times 10^{-3}} = 5.53 \times 10^9 \text{ ifu per ml}
\]

4.7.2 Discussion – Attainment of Vaccine Stocks

As previously described, recombinant adenoviruses, such as those created for this project, are replication incompetent as they are missing the E1 gene which is essential for replication. They can however replicate in 293 cells because the cells have been stably transfected with the adenoviral E1 gene, which provides the packaging ability for the virus. However a random recombination event could occur, by chance, between the adenovirus and the complementary adenoviral gene within the cell line, which would serve to convert the adenovirus back to the wild-type, replication competent form. The chances of such a recombination event happening is increased with the number of times that any single replication incompetent viral particle is allowed to replicate within the 293 cells. To keep this chance to a minimum, after mRNA and protein expression had been confirmed in the basic virus stocks, a single round of amplification for each virus was undertaken. Cleared cell lysates from these amplifications were frozen at -80 °C to establish the main stock for each
adenovirus construct. For all later rounds of infections, to amplify stocks sufficiently for all subsequent vaccine testing in birds, the virus for infection was taken directly from these main stocks.

4.8 Chapter Conclusions - Constructing the Vaccines

The main aim of the experiments with rAdeGFP in section 4.2 was to ensure that the recombinant adenoviruses could infect avian as well as human cells. However, even the basic infection experiments seemed to show interesting differences between infection of human cells and avian cells. Different levels of infection and recombinant protein expression was apparent, especially in the chicken cells. Unfortunately no human cells were infected for comparisons when quail cells were tested for infection. Although infection of avian cells was confirmed, no quantification was attempted or relevant specific comparisons made, all of which would be necessary to assess any difference in adenovirus infection between human and avian cells.

There also appears to be some differences in expression of the WNV env DNA in human and avian cells at both mRNA and protein levels. The additional bands, not present in human cells but seen in the mRNA PCR from quail cells (Figure 4.7), may represent avian related alternate spliceoforms of the env transcript, although the bands would need to be isolated and sequenced to confirm this. For the env protein, the band corresponding to the env itself was seen at around 55 kDa in both cell types and the band at approximately 72 kDa was seen in infected human cells only, (Figure 4.8). With the larger band likely representing the intact membrane/env polyprotein, it may indicate that the polyprotein is less efficiently processed in human cells than in avian cells. Such a difference may relate to the difference in WNV
susceptibility shown between humans and birds, although WNV env protein expression and subsequent protein interactions would need to be investigated further to confirm whether or not this is the case.

Overall, the adenovirus vaccines were successfully constructed, based on a recombinant adenovirus platform and were shown to be able to express both mRNA and proteins from either WNV ‘gene’ that had been included in the two different forms of the vaccines.
Chapter 5: Assessing Responses to the Vaccines in Japanese Quail

Japanese quail were selected for the main effort of testing the immune responses to the vaccines in birds. With any vaccine testing there are two main details that need to be established: what would be a suitable dose of vaccine to use and when should samples be taken to measure the peak of the immune response. These can be decided by a dose response, when different doses are administered to different groups, and a time course experiment, when samples are taken at different time points, respectively. Although it is somewhat debatable as to which type of study should be done first, for the recombinant adenoviruses it was decided to do a dose response study first.

5.1 Rationale for Dose Response Experiments

To establish a suitable dose of the vaccines that would raise a measurable immune response in quail, three different doses of each vaccine construct were tested in different groups of quail, low dose of $1 \times 10^8$ IFU per bird, medium dose of $5 \times 10^9$ IFU per bird or a high dose of $2.5 \times 10^{10}$ IFU per bird, in conjunction with an adjuvant and a booster injection. To enable further comparison of the different doses, samples were also collected at a number of different time points following the boost vaccination. Immune responses, such as T cell activation and WNV specific antibody responses, were measured and compared to see if one dose would induce a higher level of immune response compared to the other doses.

5.1.1 Dose Response - IFN-γ Production in T Cells

To assess the T cell response to the vaccines, intracellular IFN-γ was measured in either CD4+ or CD8+ cells, following ex-vivo re-stimulation of the cells after harvesting from
vaccinated quail. The in-vivo vaccination of the quail should trigger the T cells to recognise specific antigens within the vaccine. When the cells are later re-stimulated with various antigens, only those T cells that receive an antigen corresponding to the original trigger antigen, should start to produce IFN-γ. Groups of quail received either of the vaccines rAdE or rAdNS3 and control groups received either a PBS injection or the rAdMT empty vector. Once T cells were harvested they were re-stimulated for two days, with rAdMT, combined rAdE and rAdNS3 or a WNV antigen. A sample from each bird was also left un-stimulated for comparison. Cells were then stained with antibodies for either CD4 or CD8 and intracellularly for the presence of IFN-γ and data were collected using the BD FACSCalibur flowcytometer to measure the different levels of fluorescence.

5.1.1.1 Results –IFN-γ Produced in T Cells

Data were analysed using Flowjo software, to select live populations of cells and then to assess levels of fluorescence relevant to either CD4/CD8 or to IFN-γ. This analysis was done with a gating strategy set up using single stained controls and then applying the same gates to all samples. Figure 5.1 shows an example of single stained control samples used for setting up the selection gates for a CD4 experiment. During the full experiment, staining for both CD4 and CD8 was analysed using the FL2-H axis (only CD4 is shown in the figure) and IFN-γ on the FL1-H axis. Differentiation between CD4 and CD8 was performed by recording which antibody was added to the sample initially, as both antibodies are not used together in the same sample and results are analysed separately, this does not affect results if they are displayed on the same axis.
Figure 5.1 Examples of Controls Used to Set Up the Gating Strategy for IFN-γ Assay.

FSC-H/SSC-H plots display cells by size (FSC) and granulation (SSC) and the gates contain the population of live cells selected for further analysis. FL1-H/FL2-H plots display the fluorescence from the antibodies for CD4 (FL2-H) or IFN-γ (FL1-H). The bottom left gate contains negatively stained cells. For the CD4 stained sample, positive cells are in the top left gate and for the IFN-γ stained sample positive cells are in the bottom right gate. Numbers in the corners are the percentage of cells within each gate, 16.61% are IFN-γ+ and 39.46% are CD4+. 
After initial selection gating, the same cross gate was applied to all control samples and adjusted until the relevant cells were selected in all controls (even if this may mean it is not optimal in all controls). This same gate is then applied to all samples. Figure 5.2 shows an example of a sample from a rAdE vaccinated bird, stained for CD8 and intracellular IFN-γ. Graphs for each of the three different re-stimulation treatments provide a percentage of cells positive for both CD8 and IFN-γ, with 3.28% for no re-stimulation, 6.93% for rAdMT re-stimulation and 7.92% for rAdE/rAdNS3 re-stimulation.

Figure 5.2 Sample from an rAdE Vaccinated Quail Stained for CD8 and Intracellular IFN-γ.

FSC-H/SSC-H plots display cells by size (FSC) and granulation (SSC) and the gate contains the population of live cells selected for further analysis. FL1-H/FL2-H plots display the fluorescence from the antibodies for CD8 (FL2-H) or IFN-γ (FL1-H). The top right hand gate in each plot shows the double
positive stained cells, with percentages. The three FL1-H/FL2-H plots are for samples re-stimulated with either rAdMT (MT), a combination of rAdE and rAdNS3 (E3) or with nothing (negative control – NS). This sample does show an unusually high level of IFN-γ positive cells.

Once the numbers of double positive cells had been established for each experiment numbers were analysed using a specially written script in R; the script made use of a generalised linear mixed (GLM) model, using Poisson regression and took into account both fixed and random effects to assess the data (see Appendix E for the script). CD4 and CD8 were analysed separately and the script can be edited to include all cells positive for either CD marker, or just those cells positive for IFN-γ as well as for the CD marker. A weighted average number for both CD4+ and CD8+ cells was calculated per sample, from the duplicate or triplicate samples assayed. This weighting accommodates the differences in the final number of cells selected following gating of each sample. These data were then fitted into the GLM by taking the effects of the vaccine and the re-stimulation treatments as fixed effects and the genetic differences between the individual birds, as random effects. The output is in the form of a graph plotting the number of cells for the various combinations of vaccine and treatment and a table showing measures of significance difference between each re-stimulation treatment for each vaccine group when compare to the no re-stimulation treatment for the same group.

An example of the results is shown in Figure 5.3. The figure shows assessment of CD4+ IFN-γ+ cells, with the upper panel showing analysis for the random effects, with the numbers on the right being equivalent to P values and thus indicate that any differences in the responses between the individual birds does not have a significant effect. The bottom panel shows the fixed effects of vaccine and re-stimulation treatment. The single effect lines of calculation have little meaning in themselves as neither the vaccinations or the
re-stimulations occurred without the other also being present, but they are required for
calculation purposes of the vaccine: re-stimulation interaction lines. To assess significance,
each vaccine/re-stimulation interaction value is compared to the no re-stimulation treatment
for the same group of birds. In the example the rAdE/WN re-stimulation sample is highly
significantly different (Pr < 0.001) from the rAdE/no re-stimulation sample and the
rAdNS3/WN re-stimulation is significantly different (Pr < 0.05) from the rAdNS3/no
re-stimulation sample.

Figure 5.3 Example of a Significance Table Produced by the R Script Analysis for IFN-γ Data.
The analysis is for cells that are double positive for CD4 and IFN-γ (TR denotes T cells Responding to
stimulation). In the random effects analysis the two columns on the right are the P values. For the fixed
effects the vaccine:restim interactions are most relevant. Significance markers are in the right hand
column and the key in the last line indicates the Pr value cut-off associated with the different levels of
significance e.g. Pr < 0.001 = ***.
The graphs plotted by the R script show the numbers of positive cells, either all cells positive for either CD4 or CD8 or restricted to those cells also positive for IFN-γ staining. As examples the graphs from the IFN-γ assay for the medium dose of vaccine with samples taken on day 43 post boost are shown in Figure 5.4. and for the high dose sampled on day 51 post boost in Figure 5.5, the graphs also include the significance markers taken from the corresponding tables. The birds injected with the active vaccines have generally higher levels of activated (IFN-γ+) T cells as well as higher numbers of T cells overall, especially following re-stimulation of the samples compared to the no re-stimulation treatment. However, although the increase shows a definite trend, the numbers of significant differences is much reduced. Graphs from all other experiments can be found in Appendix F.
Figure 5.4 Graphs Showing a Trend of Higher Numbers of IFN-γ Producing Cells in Quail Vaccinated with $5 \times 10^9$ IFU of Either Vaccine.

The above graphs show results from a medium dose experiment, for samples from 43 days post boost (dpb). Each line of the x axis is for one specific vaccine/re-stimulation combination and each dot is the average number of cells from a single bird. A.) shows CD4+ IFN-γ+ cells, b.) CD8+ IFN-γ+ cells, c.) all CD4+ cells and d.) all CD8+ cells. Significance markers are as for the output table.
Figure 5.5 Graphs Showing a Trend of Higher Numbers of IFN-γ Producing Cells in Quail Vaccinated with $2.5 \times 10^{10}$ IFU of Either Vaccine.

The above graphs show results from a high dose experiment, for samples from 51 days post boost (dpb). Each line of the x axis is for one specific vaccine/re-stimulation combination and each dot is the average number of cells from a single bird. A.) shows CD4+ and IFN-γ+ cells, b.) CD8+ and IFN-γ+ cells, c.) all CD4+ cells and d.) all CD8+ cells. Significance markers are as for the output table.
To further clarify any differences between the different injection doses, a summary table of results was collated from the tables and graphs for all experiments run (Table 5.1). The re-stimulation with rAdMT was added as a control, to see if the vaccines were inducing a response to the adenovirus itself. For the birds injected with rAdMT itself for all different T cell groups, only a single result which was from a medium dose trial, showed a positive response on re-stimulation with rAdMT. However more response to rAdMT re-stimulation was seen in the birds receiving the WNV vaccines. For all T cell groups, from birds vaccinated with rAdE, the table shows 1 positive result for the low dose, 3 positive results for the medium dose and 2 positive results for the high dose when re-stimulated with rAdMT. In rAdNS3 vaccinated birds for all T cell groups, there were no positives for the low dose, 4 for the medium dose and 5 for the high dose, that were positive for a response when re-stimulated with rAdMT. rAdMT vaccinated birds also showed some positive responses when re-stimulated with either of the WNV specific treatments (rAdE/NS3 combo or WN antigen), although for the vaccine re-stimulation treatments it could be the adenoviral vector rather than the inserted WNV DNA causing the re-stimulation. For all T cell groups, a medium dose of rAdMT resulted in 2 positives out of 14 possible and a high injected dose with 3 positive results out of 16 possible, with WNV specific re-stimulation.
### Table 5.1 Overall Analysis of Significant Results from Dose Response Data Indicate that the 2.5×10^10 IFU Dose is Preferable for IFN-γ Production.

The table shows data from all IFN-γ assays for all doses and all timepoints (no CD8 data for D8 Med and no early timepoint for Low), DXX = day post boost samples collected and Low, Med or High is the dose.
For each assay the different combinations of vaccine and re-stimulation are shown and results are sub-divided into the different T cell types and activated (IFN-γ+) cells are shown separately (in the top two sub-tables). Results key: ‘·’ = no difference, ‘o’ = P<0.1, ‘*’ = P<0.05, ‘**’ = P<0.01, ‘***’ = P<0.001 and ‘ND’ = not done. Underlined results indicate a positive difference to the negative control, otherwise results are lower than the negative control. Table cells with bold outlines are those that should give a positive result if either vaccine induces a specific T cell response i.e. those where the birds were vaccinated by either rAdE or rAdNS3 and then re-stimulated with either E3 or the WN antigen.

Also in Table 5.1, the boldly outlined cells (groups vaccinated with either rAdE or rAdNS3 and re-stimulated with either E3 or WN antigen) of each sub-table contain results particularly relevant to vaccine performance, as these should contain positive results if either vaccine was inducing a WNV specific response in the T cells. Looking at the boldly outline regions for activated T cells (IFN-γ+), for both CD4 and CD8 only 2 of the 14 possible show positive results for the medium dose, 1 for rAdE and 1 for rAdNS3, and 3 of 16 possible are positive for the high dose, 2 for rAdE and 1 for rAdNS3. As far as total T cell numbers were concerned, from the boldly outlined areas of the tables, results with P<0.1, show the low dose with 3 positive out of 12 possible, 2 for rAdE and 1 for rAdNS3, for the medium dose 7 positive out of 14 possible, 3 for rAdE and 4 for rAdNS3 and for the high dose 7 out of 16 possible were positive, 2 for rAdE and 5 for rAdNS3. A summary to highlight these specific results is shown in Table 5.2.

<table>
<thead>
<tr>
<th></th>
<th>rAdE IFN-γ+ Ts</th>
<th>rAdE All Ts</th>
<th>rAdNS3 IFN-γ+ Ts</th>
<th>rAdNS3 All Ts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Dose</td>
<td>0/6 (0%)</td>
<td>2/6 (33%)</td>
<td>0/6 (0%)</td>
<td>1/6 (17%)</td>
</tr>
<tr>
<td>Med Dose</td>
<td>1/7 (14%)</td>
<td>3/7 (43%)</td>
<td>1/7 (14%)</td>
<td>4/7 (57%)</td>
</tr>
<tr>
<td>High Dose</td>
<td>2/8 (25%)</td>
<td>2/8 (25%)</td>
<td>1/8 (13%)</td>
<td>5/8 (63%)</td>
</tr>
</tbody>
</table>

Table 5.2 Summary of WNV Relevant Results of Dose Response IFN-γ Assays.

A summary of results from Table 5.1 that should all be positive if the doses of vaccine were inducing T cells to respond when re-stimulated. Results here are shown as fractions, being the number of cells in the
previous table that were positive out of those that could have been, if all possible timepoint and re-stimulation combinations had worked. For ease of comparison, each values is also expressed as a percentage, in brackets.

5.1.1.2 Discussion –IFN-γ Produced in T Cells

Due to the different re-stimulation treatments that are applied to cell samples ex-vivo the assay is assessing the ability of the T cells to activate when they see the same antigen for a second time, the first encounter with antigen being when the bird was vaccinated. As T cells are activated when antigenic peptides are presented in the context of a MHC molecule, for re-stimulation the whole antigen does not need to be the same as the original, as long as some of the peptides correspond to the original stimulant. For the final IFN-γ assay for each dose, a WN antigen re-stimulation treatment was included to ensure that all possible WNV peptides were present for cells to process and present as antigen to activated T cells.

The timing of sample collection following vaccination would access different T cell populations. Within approximately the first 2-3 weeks of vaccination, samples would more likely contain T cells that were currently active in responding to the vaccine and after that time it is more likely that the cells would be vaccine specific memory T cells, or at least a higher ratio of memory T cells to active T cells, depending on the timing of sample collection. I could find no references that detailed specifically when memory T cells would start to be seen in quail samples. From the timing of samples for the dose response experiment, samples from days 8 and 11 post boost were likely currently activated T cells and samples from all other days were likely to contain more memory T cells. The differences between the two types may mean different re-stimulation times would be required to achieve optimal production levels of IFN-γ. However, for all assay runs, re-stimulation treatments...
were incubated for two days. It was not realised until part way through the study that a longer period for re-stimulation may have provided better results. However it was considered necessary to keep using the same timings for the whole study to enable proper comparisons.

Only 2 birds were harvested per time point for each of the four vaccination group, giving a total of 8 birds. As the assay set up was fairly complex and time consuming, 8 was the maximum number of birds that could be processed on any given day. However for statistical analysis to be considered robust it would have been preferable for a minimum of 3 per group to be done.

The rAdMT vaccination group, and re-stimulation treatment, were included to see if any responses raised following vaccination were specific for the adenovirus backbone of the vaccine rather than to the WNV specific portion of the virus vaccine. If a response was being raised against the adenovirus region of the vaccine, it would tend to reduce the level of the immune response to the WNV region of the vaccines, as the immune system would have more adenovirus related proteins to target for destruction thus destroying more vaccine infected cells which would reduce the amount of WNV proteins produced. For the rAdMT re-stimulation treatment, lower responses were seen in the birds had been vaccinated with rAdMT when compared to those that received either full form of the vaccine. With the single rAdMT vaccination positive response observed only with the medium dose, six rAdE positive responses were seen, one for the low dose, three for the medium dose and two for the high dose. For rAdNS3 a total of 9 positive responses were seen, four for the medium dose and five for the high dose. The inclusion of the WNV regions within the adenovirus vector appears to also increase the response to the vector part of the vaccine. This may be
because the full vaccines trigger an overall higher immune response than the vector alone, thus increasing the response to all components of the vaccines.

In contrast, for the birds vaccinated with rAdMT, which of any group would be most likely to have responded to the rAdMT as a re-stimulation, only one positive response was seen, from the medium dose group. But when rAdE/NS3 restimulation was applied to samples from the same birds, four showed positive results, 1 from the medium dose and 3 from the high dose. Also one positive for the medium dose was present when WN re-stimulation was used, even though no WN specific antigen should have been present in the original vaccinations. Even though the E3 re-stimulation treatment would have included the adenovirus region of the vaccine and thus some of the same peptides as in the rAdMT vaccine, the results still imply that the re-stimulation treatments themselves were causing some level of response. When the adenoviruses were being used for re-stimulation they were simply diluted in cell medium prior to being added to the cells, so the viruses were still able to actively infect cells during the incubation period. If this happened these newly infected cells would respond to the re-stimulation as if it were an infection, perhaps by way of co-stimulatory molecules which would present new antigens to activate naïve T cells. So responses to antigens that T cells had not previously been exposed to could occur. Thus it would be preferable for the adenovirus to be used as a re-stimulant to be inactivated in some way. This explanation cannot however, account for the rAdMT vaccinated birds that responded to the WN antigen which is already in the form of inactivated proteins and to which they should not have had prior exposure. At some time the birds may have been accidentally exposed to additional antigens, possibly some that were mouse related, as the birds were housed in a facility which also contained mice. The WN antigen was prepared from mice infected with WNV and as
such would contain mouse proteins as well as the WNV ones. By chance the birds that were
vaccinated with rAdMT may have already encountered some mouse related antigens and that
these same antigens were also present in the WNV antigen preparation.

The technical intricacies of accurately repeating the experiment each time, coupled with the
unrealized complexity of the analysis of results may have contributed to the lack of clearly
conclusive results. Another confounding factor that may apparently be reducing the number
of activated cells is the use of the chicken IFN-γ antibody when detecting cells that are
producing IFN-γ. Although homology studies indicate that there would be cross-reactivity
between chicken and quail IFN-γ [78] this has not been confirmed and initial testing of the
antibody, undertaken during this project, were inconclusive (data not shown).

There is little difference between the medium and high doses of rAdE, with both giving 4
positive results (Table 5.2). However it appears that the high dose was able to activate more
T cells than then medium dose. If looking at the reactivity to the vector alone re-stimulation
treatment, the higher dose shows lower levels of response, which is preferable, than the
medium dose. So although the difference is only slight, the high dose, $2.5 \times 10^{10}$ per bird, is
the best dose for rAdE. For rAdNS3 the results for the medium, five positive results, and
high doses, six positive results are very similar (Table 5.2). The extra positive result for the
high dose came from increased T cell numbers rather than from activated T cells. Although
the response to the vector alone treatment was also slightly higher in the high dose, overall
the high dose, $2.5 \times 10^{10}$ per bird, would be the best for the rAdNS3 vaccine.

5.1.2 Dose Response – Serum ELISA

Only the four birds per dose/vaccination group that were available for blood collection at all
timepoints, namely days 0, 16 and 35 post boost, were used for analysis of serum ELISA
data. Samples were frozen at -80 °C on the day of collection and analysed at a later date. For the assay itself, WN antigen was bound to 96 well plates and then incubated with serial dilutions of serum samples; any serum antibodies that bound the antigen were subsequently detected using an anti-bird IgG antibody and visualized with horseradish peroxidase and substrate; OD readings were taken of all plates.

5.1.2.1 Results –ELISA, Antibodies Found in Serum

OD readings were initially manipulated using Microsoft Excel and then paired-T tests were run using Minitab software. Raw data and the full analysis from each T-test can be found in Appendix G. Two paired-T tests were run for each of the higher doses for day 16 and day 35, but only one for the low dose for day 35. The T tests compared OD readings for samples from day 0, to those from samples taken on the other specified day, and assessed any differences between the two days. The readings for each individual bird/dilution combination were entered into Minitab based on day number (day 0 paired with either day 16 or day 35), and the possible effects of both vaccination group and serum dilution were assessed. This was done by first calculating differences between all paired values and then calculating the mean of those differences. Statistical calculation based on these values were then used to calculate T and P values. As can be seen in Table 5.3 using a cut off value of P<0.01 for significance, all comparisons for the medium and high doses were significant for both days tested. This applies to all vaccination groups even the two control groups. This indicates an increase in WNV antibody levels, in all groups, over time. T values are used to calculate P values and are related inversely to P such that larger T values equate to smaller P values; in this instance, when P values are too small to be useful T values could be used to make further inferences on significance.
<table>
<thead>
<tr>
<th>Dose</th>
<th>Day cf to Day 0</th>
<th>Vaccination Group</th>
<th>Mean of Diffs</th>
<th>Rank of Mean Diff</th>
<th>T Value</th>
<th>P Value</th>
</tr>
</thead>
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<tr>
<td>Low</td>
<td>35</td>
<td>MT</td>
<td>0.0181</td>
<td>1</td>
<td>1.08</td>
<td>0.298</td>
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<tr>
<td>Low</td>
<td>35</td>
<td>Env</td>
<td>-0.01111</td>
<td>-</td>
<td>-2.84</td>
<td>0.012</td>
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<tr>
<td>Low</td>
<td>35</td>
<td>Neg</td>
<td>-0.00258</td>
<td>-</td>
<td>-0.79</td>
<td>0.443</td>
</tr>
<tr>
<td>Low</td>
<td>35</td>
<td>NS3</td>
<td>-0.00150</td>
<td>-</td>
<td>-0.30</td>
<td>0.771</td>
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<td>Medium</td>
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<td>Env</td>
<td>0.1371</td>
<td>1</td>
<td>3.36</td>
<td>0.004</td>
</tr>
<tr>
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<td>MT</td>
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<td>2</td>
<td>5.07</td>
<td>0.000</td>
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<td>NS3</td>
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<td>3</td>
<td>3.77</td>
<td>0.002</td>
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<tr>
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<td>3.18</td>
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<td>Env</td>
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<td>5.12</td>
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<td>NS3</td>
<td>0.01864</td>
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<td>4.65</td>
<td>0.000</td>
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<td>3.54</td>
<td>0.003</td>
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<td>4.64</td>
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<td>1</td>
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<td>Env</td>
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<tr>
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<td>Env</td>
<td>0.01836</td>
<td>4</td>
<td>7.87</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 5.3 Summary of Paired-T Test Results for Serum Samples from All Doses Indicates that the 5×10^9 IFU Dose is Preferable for Antibody Production.

Day cf to Day 0 contains the number of the other day results from day 0 were compared with; Mean of Diffs is the mean of the differences between paired values of all OD values, between day 0 and either day 16 or day 35, for each dose and vaccination group specified; Rank Of Mean Diff places the diff of means in order with the largest value as 1; T Value is a value used to calculate the P value; P values of < 0.01 are
considered significant and values of $P = 0.000$ are in fact smaller than that but the 3 decimal place cut off means the numbers are not shown.

For the medium dose, by day 16 the rAdE vaccination group showed the largest difference from day 0 followed by the rAdMT group then the rAdNS3 group (Table 5.3). By day 35 post boost the rAdE vaccinated group still showed the greatest difference with the rAdNS3 vaccinated group the second. Still for the medium dose, looking at the T values, on day 16 rAdMT had the largest value followed by rAdNS3 then rAdE and on day 35 the highest T values if for rAdE followed by rAdNS3 then rAdMT. For the highest dose, at both day 16 and day 35 post boost it can be seen that the negative control group had the largest difference from day 0, followed by rAdE then rAdNS3 on day 16 and by rAdNS3 then rAdMT on day 35. If T values are used, on day 16 the negative control group still shows the largest values followed by rAdNS3 then rAdE, although on day 35 the order, from largest T value down is rAdE, rAdNS3 and then the negative control group.

5.1.2.2 Discussion –ELISA, Antibodies Found in Serum

For all groups tested, there were significant differences between serum antibody levels on day 0 and day 16. This includes the two control groups, neither of which received any WNV specific antigens when vaccinated. These results could indicate several possibilities. Firstly the ELISA assay itself is not as specific for WNV as it should be and that other non-WNV specific antibodies were being quantified. As the WNV antigen was made from the whole brains of mice infected with WNV it would include mouse related proteins as well as WNV proteins. As the quail were being housed in a mouse facility they may have been exposed to mouse related materials that would have triggered a mouse specific immune response, the
antibodies from which could then bind to the mouse related antigens within the WN antigen preparation. The only group not showing an increase in all vaccination groups was the low dose group, in which only the rAdMT group showed a slight, but not significant, increase by day 35. The possible exposure to the mouse related antigen must have occurred sometime prior to vaccination and served to increase the background levels of antibodies detected by the ELISA, and the vaccinations then served to boost levels of antibodies detected in a more specific and time related manner.

Generally by day 35 the differences in OD readings had fallen relative to day 16 for all vaccination groups within the medium and high dose groups, although all were still significantly higher than at day one. This is likely to be a time dependent response to the vaccines; one previous study used live and attenuated avian adenoviruses to vaccinate chickens. By 21 days post vaccination, levels of antibodies were beginning to fall compared to day zero [79]. This would also explain why the low dose groups showed no differences, as even with the increased background, by day 35 antibody levels were no longer detectable as the low dose induced low antibody production. An assay run on day 16 for the low dose groups may have detected the cumulative effect of the background and the more time specific increase in WN specific antibodies.

Judging by the OD values seen for the medium dose, especially on day 16, it is apparent that for the ELISA, for both vaccines, the medium dose gave better antibody responses, although the highest levels of antibody were seen for the rAdE vaccine compared to the rAdNS3 vaccine.
5.2 Rationale for Time Course Experiments

The time course experiment was undertaken to demonstrate on which day after vaccination the vaccines would produce a maximal immune response. One of the main assays for assessing the immune response to the vaccines, used in this project, was testing for IFN-γ production in T cells. Timepoints for the time course trial were selected based around a time for peak primary production of IFN-γ from T cells in chickens, which was found to be between eight and ten days post-infection [80]. For this reason time course samples were collected on days 5, 7, 10, 13 and 15 post vaccination for both the IFN-γ assay, to establish the day when IFN-γ production peaked. Serum samples were also collected for ELISA analysis, to look for early antibody responses.

The time course experiment occurred after the birds had been re-housed to the UBC Animal Care Annex. The floor bins of birds were not labelled sufficiently, thus during the move two different populations of quail may have been mixed for the time course trial. This would have meant that some of the groups may have been of different ages to the other groups, although which particular groups cannot be specified. Any age difference would be of approximately one year and immune responses, specifically T cell responses, in birds are known to decrease with increasing age [81].

5.2.1 Time Course - IFN-γ Production in T Cells

Most of the details for the IFN-γ assay for the time course trial were the same as for the dose response trial, with samples being collected and data analysed in the same manner, but only over the course of ten days and only one of the vaccines, rAdE, was used.
5.2.1.1 Results – IFN-γ Produced in T Cells

As for the dose response, the data were first selected using Flowjo software, manipulated in Excel and analysed with a specially written R script, which produced a final output of tables showing significance and graphs. Graphs for day ten (Figure 5.6) and day thirteen (Figure 5.7) can be seen in this section, all other graphs can be found in Appendix H. By day seven post vaccination there was already a trend for more T cells from the vaccinated birds. By day ten there were significantly more active T cells, both CD4 and CD8, for the E3 re-stimulation and for CD4 for the WN antigen re-stimulation, although there was also a significant response to the rAdMT re-stimulation for both T cell types (Figure 5.6). Unfortunately, for day thirteen, data for only one bird per group was available for CD4 T cells, which has made all data points for the vaccinated group show as significant. Overall, for both CD4 and CD8 cells, much higher numbers of activated T cells and all T cells can be seen for the vaccinated group of birds. However for the vaccinated group, numbers for the no re-stimulation treatment were also quite high although not quite as high as for the E3 re-stimulation treatment. For the non-vaccinated group the same can be seen for CD8 cells but not for CD4 cells.
Figure 5.6 Graphs from Day 10 PV of Time Course Indicate Higher Levels of IFN-γ Production in Vaccinated Quail.

As stated on each graph, either CD4 or CD8 T cells and either just active (IFN-γ+) or all T cells within that cell type are shown. The vaccination group and re-stimulation treatments are on the x-axis. Each dot represents the cell count from a single bird.
Figure 5.7 Graphs from Day 13 PV of Time Course Indicate Higher Levels of IFN-γ Production in Vaccinated Quail.

As stated on each graph, either CD4 or CD8 T cells and either active (IFN-γ+) or all T cells for either cell type are shown. The vaccination group and re-stimulation treatments are on the x-axis. Each dot represents the cell count from a single bird.
Below is a table showing a summary of significant results for all days for the vaccinated group, indicating which T cell groups are responding on the different days (Table 5.4). More groups of cells are responding on day ten compared to earlier days. Although results from day thirteen indicate higher T cell numbers for the vaccinated group (Figure 5.7) this is not reflected by significance of results; ‘significance’ of the CD4 results may be an artefact of analysis as data from only one bird per group was available for analysis. For day fifteen, the graphs (Appendix H) show a trend for larger numbers of T cells, especially for the IFN-γ+ groups, for the samples from the vaccinated group, but this trend is not significant.

<table>
<thead>
<tr>
<th>Re-Stimulation</th>
<th>IFN-γ CD4</th>
<th>All CD4+</th>
<th>IFN-γ CD8+</th>
<th>All CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MT</td>
<td>E3</td>
<td>WN</td>
<td>MT</td>
</tr>
<tr>
<td>Day 5</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
<td>o</td>
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<td>-</td>
</tr>
<tr>
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<td>*</td>
<td>**</td>
<td>o</td>
<td>-</td>
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<tr>
<td>Day 15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.4 Summary of Significant IFN-γ Assay Results for the Vaccinated Group of the Time Course.

Each column shows significant results for that particular cell type and re-stimulation treatment combination for rAdE vaccinated birds compared to the no re-stimulation treatment for the same cell type. Key: ‘-o’ = negative result $P<0.01$, ‘-‘ = no significant difference, (all other results are positive), ‘o’ = $P<0.1$, ‘*’ = $P<0.05$, ‘**’ = $P<0.01$, ‘***’ = $P<0.001$. 
5.2.1.2 Discussion – IFN-γ Produced in T Cells

To avoid the potential difficulties of an active virus being able to infect naive cells when used as a re-stimulation treatment, all the adenoviruses used as re-stimulation treatments in the time course experiment were heat inactivated before use. This means that the viruses should only enter cells when they are taken up for peptide processing by antigen presenting cells present in the sample. Once peptides are being presented, T cells previously been exposed to the antigen should respond in a more timely manner than T cells not previously exposed to it and in the incubation time frame naïve T cells should not be triggered to respond.

From the significant responses seen in Table 5.4, day ten shows peak numbers of activated T cells of all the days tested. Although other results are not significant, the graphs demonstrate a noticeable trend to increased T cell numbers extending until day fifteen post vaccination.

5.2.2 Time Course – Serum ELISA

The serum ELISA for the time course was run in the same manner as for the dose response trial, with WN antigen being fixed to a plate, serial dilutions of serum samples added and antibodies detected by anti-bird IgG antibodies and read by colour developed by the conjugated enzyme.

5.2.2.1 Results – ELISA, Antibodies Found in Serum

At all time points analysed for the ELISA, serum samples from six birds were used, except for day 15 when only three birds per group remained. Results were measured as OD values, data were then manipulated, and initial graphs produced, in Microsoft Excel and then finally analysed by a paired-T test using Minitab software. For the paired-T test, data from either day 7,10, 13 or 15 were always paired to data from day 0 for the same individual bird and for
each dilution. For each day the control group and the vaccinated group were analysed separately.

Mean OD values were calculated for all serum dilutions for all days and graphs showing the difference between the means for all subsequent days and their respective day 0 were plotted by group (Figure 5.8). For all days shown, there is a larger difference between mean OD values for each specified day and day 0, for birds from the vaccinated group compared to those from the control group. This means that for the control group, the OD values for all subsequent days are more similar to day 0.

Table 5.5 shows a summary of results from the paired-T test between day 0 and the days specified in the table and includes values for the differences in mean ODs calculated by including all serum dilutions. For both the control group and the vaccinated group there is a significant increase in OD between day 0 and day 7 and day 0 and 10 day, however from day 13 onwards the difference to day 0 is still only significant for the vaccinated group. This shows that mean OD difference from day 0 extended over a longer period of time for the vaccinated group. For the control group the difference in mean OD values from day 0 is highest by day 7 and then decreases from there, ending up being less than day 0 values by day 15. However, for the vaccinated group the difference in means from day 0 is highest by day 7, falls by days 10 and again by day 13 but then increases by day 15.
Figure 5.8 Graphs Show Higher Mean Differences in Antibody Levels, for all Days Compared to Day Zero (0), in Vaccinated Quail Compared to Unvaccinated Control Group Quail.

As indicated by each graph title, each shows the difference in mean OD value, for each serum dilution, for each day minus the mean OD values for day 0. Data is grouped for unvaccinated control group (blue lines) or vaccinated group (red lines).

<table>
<thead>
<tr>
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<td>P Value</td>
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<td>P Value</td>
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</table>

Table 5.5 Summary of Paired-T Test Analysis of Time Course Serum ELISA Data.
The mean diff is the difference of the mean OD values for all birds and all dilutions per group between each subsequent day and day 0. T values is used to calculate P value and has an inverse relation to it, * indicates significant differences between the means with P<0.05.

To illustrate the differences in OD values for one day’s comparisons, additional graphs for day 15 can be seen in Figure 5.9, which shows OD values for all serum dilutions, for each bird in both groups. OD values have increased substantially between day 0 and day 15 for two out of the three vaccinated birds and none of the non-vaccinated birds. Two birds from the control group also give high OD values, both on day 0 and day 15, importantly, these birds do not demonstrate any increase over time, as can be seen in the lower of the three graphs.

**5.2.2.2 Discussion – ELISA, Antibodies Found in Serum**

The purpose of the serum ELISA assay is to quantify the amount of WN specific antibodies present in the blood samples collected from the birds at the different time points, in relation to any amounts detectable at the start of the assay. By comparing the difference between the OD reading from each subsequent day and day 0 for each bird, it would measure the increase, or decrease, in antibodies present over time.

The summary in Table 5.5 shows that for the vaccinated group the increase from day 0 is still significant on day 15 whereas for the control group the significant increase has disappeared between day 10 and day 13. The mean difference values also show that the vaccinated group has higher increases in the level of antibody compared to the control group, having approximately twice the increase by day 7. This higher difference is maintained over all days tested. The observations from the summary table can be seen to be true across all dilutions of
Figure 5.9 Graphs for All Birds Assessed on Day 15 Demonstrate that 2 Out of 3 Vaccinated Birds Showed An Increase in Antibody Levels.

Graphs show all birds assessed on Day 15. Individual birds are numbered 13 to 15 with C being the control group and V being the vaccinated group, these same birds were sampled on day 0 (top left hand graph) and day 15 (top right hand graph). Bkgrd is the OD values for wells with no serum added. The lower graph plots the differences between the OD values between day 15 and day 0 for all points serum tested, as seen in Figure 5.8, with the differences between the groups being smallest on day 13 and largest on days 7 and 15.

The observation of the increased levels of antibodies seen initially in the control group is not expected, nor are the high OD values for birds from the control group, as seen in Figure 5.9. However, as discussed for the dose response experiment, both observations may relate to exposure to mouse related antigens. For further discussion of this point, see the next section.

For the time course experiment, vaccine availability was limited, which meant the same birds were being used for both assay. The IFN-γ assay involved harvesting 3 birds per group per
time point, which meant that for the ELISA different birds were used for blood collection at each time point. Thus comparisons within each day are valid, as the same birds are being used for day 0 and the subsequent days readings, but comparisons between days may not be so reliable. Although taking the difference in OD values between day 0 and subsequent days was an attempt to minimise this effect, it cannot take into account the difference antibody responses, in both level of antibody and timing of production, that may vary between individual birds. This may be the reason for the apparent fall in OD differences seen on day 13, when instead of an actual reduction it may have been because the birds tested on that day had either not responded very well to the vaccine or had early peak antibody production that had already fallen by day 13.

Peak antibody levels may have occurred before the first time point for which data was available, day 7, however it can be seen that for the vaccinated group, by day 7 levels of antibody are significantly higher than day 0, as well as being higher than those of the non-vaccinated group. As the samples were collected from different birds each time, it is likely that the vaccinated group antibody levels were falling steadily from day 10 onwards.

5.3 Chapter Conclusions - Immune Responses in Japanese Quail

One of the most important factors to play a role in the work done with the quail, may have been the way in which they were housed and handled. Although they were initially obtained from a clean, enclosed environment, for several months, following transport to UBC, birds were housed free-range, in a single room that was enclosed and sheltered but with access to the outside. Birds selected for a certain trial were randomly selected from the large free-range group and placed in floor bins in an inside room, where the Biosafety level 2 adenoviruses
could be used. During experiments all floor bins were covered with plastic netting, to keep the birds in the bins, but the individual bins were not ‘self-contained’ in any other way. The negative control group was always in the same room as the vaccinated groups, although they were the first group handled for any given procedure, such as taking blood samples. Partly for practical reasons, this was considered to be sufficient and the chance of contamination from outside or between groups, once trials began, was considered to be suitably small. With hindsight, and in the light of some of the results, it is possible that some exposure to undesired antigenic material, occurred from external sources or between experimental groups, and triggered additional immune responses in some quail.

Regarding the unexpected results from the ELISA experiments, some of the responses seen in control groups may have been due to exposure to mouse related antigens. One way to test this would be to run the samples again but using WN env and NS3 proteins as the more specific antigen. As seen in Figure 3.17 (page119) this is likely to provide higher OD values and better definition between samples and it would not bind any mouse related antigens that were present, thus providing more specific results. One reason the more specific antigen was not used was that the apparently low level of response to the vaccines, seen in ELISA results from the initial runs of this assay (Figure 3.16, page 117), were considered to be due to factors other than the type of antigen used. Causes such as too small a vaccine dose or interference by adjuvant were possible, as was the fact that the initial results were not clear as there were only a very small number of birds in each group. Even if the importance of the antigen had been recognised sooner, it would have been necessary to produce the proteins in the lab, as the proteins were quite expensive to purchase and a fairly large amount would have been required to run all assays.
In the ELISA assays done with the quail, there were also large variations in OD values between individual birds regardless of which treatment group they were in. The analysis was run as a paired-T test, to remove the variation per bird by calculating the difference between samples taken earlier and those taken at a later date from the same bird. However, a considerable amount of variation within each treatment group was also seen, which would affect the mean per group used in the T test analysis. This variation may be due to different responses by the individual birds, as unlike mice, the birds are not all genetically identical, however for the ELISA it may also relate to the specificity of the antigen being used and the possibility of exposure of the birds to murine antigens.

Regarding the IFN-γ assay work done with the quail, the complex experimental plan and statistical analysis, especially for the dose response study, may have served to reduce the clarity of the final results. As was seen in the ELISA assay, there was quite a large variation between the responses measured in the IFN-γ assay for each bird, even within each vaccination group. Again this may be due to different levels and timing of responses by the genetically dissimilar birds.

The number of birds processed for each experiment was very small, statistically, but they were the largest that one person could practically deal with, based on the complexity of experimental set up and processing. For the dose response IFN-γ assay there were only two birds per vaccination group, which is below the basic minimum for calculation of a mean which is considered to be three. Although three birds were used for the IFN-γ assay for the time course, so calculation for that analysis meets the minimum value.

Most of the caveats mentioned for the work done in quail simply serve to highlight the difficulties of working with an animal model that is not considered a standard model for the
type of assays being undertaken. As such a considerable amount of testing of the new reagents and refinement of the new assay protocols was required, none of which would have been necessary if the vaccines were tested in mice, as these assays and many more are already tried and tested in such standard models of the immune response. Despite this the WNV vaccines show some significant results and an overall trend in inducing both T cell activation and antibody production in vaccinated quail.
Chapter 6: Artificial Infection of House Sparrows with West Nile Virus

To further assess the efficacy of the vaccines, a challenge assay experiment was undertaken, whereby birds were first vaccinated, subsequently infected with WNV and viremia was measured. Viremia was used to assess the level of protection that the vaccines provided from the deleterious effects of a WNV infection. House Sparrows (HOSPs) were selected as the species of choice because HOSPs were shown to be much more susceptible to WNV infection than quails [62].

To infect birds with WNV, some studies allow infectious mosquitoes to feed on the birds for a certain length of time [62]. During feeding, mosquitoes can expectorate between 6 and 3,777 plaque forming units of WNV particles into the bird’s bloodstream [82]. To avoid this variability, we exposed birds to a single fixed dose of WNV via subcutaneous injection. Based on the amount of virus transmitted by a mosquito bite [82], an initial dose of \(3.5 \times 10^3\) PFU of WNV per bird was considered a suitable starting dose.

Prior to running the challenge assay, it was necessary to confirm that the suggested dose of WNV would create suitably measurable results; an infection response study was undertaken, in which birds were directly infected with WNV without vaccination.

6.1 Rationale for the Infection Response Trial

Although HOSP had previously been shown to be susceptible to WNV infection [62], it was necessary to run an infection response study, to confirm the level of susceptibility in our hands. It would also highlight any differences that may occur, for infections run under conditions mentioned in methods (section 2.4.1), compared to those used in the previously
mentioned study. As mentioned in the methods section, an initial infection response trial was run using the dose of $3.5 \times 10^3$ PFU of WNV per bird; however, due to technical issues with sample collection and processing, no detailed data were available. Only one bird, out of the nine infected, died post-infection, giving a mortality rate of 11% for HOSP; which was considerably less than the 50% mortality expected [62]. Due to the lack of data, other than mortality, it was difficult to ascertain the full cause of the apparently reduced effects of infection, although a higher dose of WNV would likely be required to observe the same effects as previously seen in HOSP. Therefore, a dose of $3.5 \times 10^4$ PFU of WNV per bird was used in the second infection response. Sampling and sample processing protocols were amended to correct for the difficulties encountered in the first trial, and the infection response study was run a second time.

Group size and sample collection were somewhat restricted, as some of the same birds from the first run of the infection response study were also used in the second run. One group of nine HOSP were infected with WNV, these were the non-infected control group from the first trial. The non-infected control group for the second run consisted of only four birds. Blood samples were collected daily for the first eight days post-infection, from three different birds per day from the infected group; but only on alternate days, from two different birds in the non-infected group. Samples from four different types of tissue were collected from all birds on day fourteen post-infection when the study was concluded.

### 6.1.1 Results – Infection Response, Baseline Viremia Established

Sera from birds were screened for previous exposure to WNV. Table 6.1 shows that all birds tested had levels of less than 30% inhibiting antibodies, therefore all birds were suitable to be used in the infection response study.
Table 6.1. Infection Response Study, All Birds Tested Negative for Prior WNV Exposure.

Percent Inhibition Frequencies for WNV Screen for Infection Response Trial in HOSP. ‘% Inhibition’ shows the value or range for the calculated % inhibition for serum from all birds and ‘No. of Birds’ indicates the number of birds in each group.

The only mortality was a single bird, from the group of nine infected, which was found dead on day four of the trial, thus the mortality rate was calculated to be 11%. This death was considered to be from the effects of the WNV infection rather than from any effects of capture and handling of the birds, as no birds died in the non-infected group.

All samples, including blood, swabs and four tissue types, were assessed for viremia by first extracting RNA, which was then used as a template for assessment by qRT-PCR. Blood samples provide a picture of viremia during the first week of infection and the tissue samples provide a snapshot of the spread of the virus fourteen days after infection. Figure 6.1 shows the levels of viremia from blood samples as the log of mean WNV PFU per ml blood per day; all results shown are from the infected group, as none of the samples collected from the

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Table: Frequency Values for % Inhibition
control group showed any detectable viremia, as expected. From the figure, a peak viremia of just over $1 \times 10^5$ PFU/ml can be seen on day two post-infection, with levels falling but still present by day eight post-infection. However, the apparent increase on day eight is likely an artefact of the sampling schedule, as different birds were sampled on consecutive days; it is unlikely that viremia levels actually increased at any time following the single peak reached on day two.

![WNV Blood Viremia](image)

**Figure 6.1 Infection Response Study, WNV Blood Viremia Profile in HOSP.**

All results shown are from the WNV infected group. Values indicated are the log of the mean WNV PFU per ml of blood for the three birds sampled each day. The re-appearance of viremia on day 8 is considered to be an artefact of the sampling schedule.

Tissue samples were assessed at fourteen days post-infection, apart from the samples from the single bird which was found dead on day four post-infection, which were therefore assessed at that time-point. For all tissues, viremia was much higher on day 4 than on day 10, although the day 4 samples were from a fatal infection (Figure 6.2). The measurement
showing viremia in a spleen in the control group is from a single bird in the group, and is likely due to contamination of the reaction during the set up of the RT-qPCR reaction; no viremia was seen in any of the other tissues for the same bird nor in any of the blood samples collected from the same bird during the course of the study.

Figure 6.2 Infection Response Study, WNV Viremia Levels in HOSP Tissues.

Results shown are from the infected (red bars) and control (green bar) groups, all samples are from day fourteen post-infection apart from the ‘Dy4 Sample’ (blue bars) which were taken from the bird found dead on day four post-infection. Specific tissues sampled are detailed along the lower axis. Viremia was calculated as WNV PFU per 100 mg of each tissue and results are shown as log viremia. For the ‘WNV Inf’ group the log viremia is shown as a mean for the whole group, ‘Dy4 Sample’ and ‘Ctrl’ are both values from a single bird.
6.1.2 Discussion – Infection Response, Baseline Viremia Established

Blood samples can only be physically collected from birds that are still alive, so by definition any viremia high enough to cause fatality may not be measured, as no sample would be available on the day of death. Due to the sampling schedule, samples from fatally infected birds might not be available for up to two days prior to death, as in this case, when the bird that died on day four was previously sampled on day one. Thus the fatally infected bird only had one blood sample taken, but even at that stage it showed markedly higher levels of viremia than other birds sampled on the same day. For day one, the bird that died showed 4.17 log PFU/ml viremia whereas the mean value for the other two birds sampled was only 2.3 log PFU/ml, a value approximately 100 fold less. Overall results for blood viremia may be lower than they would be if all birds, including fatally infected birds, could be sampled daily. However, as long as all groups are sampled in the same manner, the values should still be useful for comparison between groups, which is important when assessing the effects of vaccines used in the different groups.

The viremia seen in tissue samples (Figure 6.2) demonstrate the high levels of viremia that can be reached during fatal infections, as the day four sample shows much higher viremia than the day 14 samples. However, as all other tissue samples were collected 10 days after the ones from the bird that died, the reduced level of viremia may be due to timing of sampling rather than severity of infection. Comparison of the level of viremia between tissue types collected on the same day may not be accurate either, due to the nature of the qRT-PCR experiment. Different tissues sampled may naturally contain enzymes, or other molecules, that would act as inhibitors to a PCR reaction, also any inhibitors that are present, would likely be in differing amounts in different tissues [83]. This problem was negated, as far as
possible, for quantification of viremia for each tissue type; standard curves were created that also contained the same amount of tissue, corresponding to the samples being quantified. Further details and discussion on this point can be found in this chapter’s conclusion, section 6.3. Tissue viremia for the infection response study show that WNV can permeate quite a range of tissues, including penetration into the immunologically privileged site of the brain, which clearly demonstrates the neurotrophic behaviour of WNV.

Even with the higher dose of WNV, used in the second run of the infection response study, results were lower than expected. Expected results included mortality of 50% and a peak viremia of up to 10.3 log PFU/ml, with peak viremia occurring on day three and four post-infection; with viremia lasting for six days, although with a considerable drop after day five [62]. However, results obtained in our study were 11% mortality with a peak viremia of 5.1 log PFU/ml occurring on day two, with viremia likely lasting for five days (see next paragraph regarding length of viremia). As a much higher dose than the previous study [62] was likely used, another cause for the 1,000 fold reduction in viremia had to be found; in the previous study they had taken blood samples, of 100 µl, from all birds in the group every day, for the first seven days following infection. For our study, a limit was set of 50 µl of blood, taken every third day from any given individual bird, for the first week following infection. This was to conform with animal care protocols in place at the facility in Winnipeg. As the volumes and timings of the blood samples collected were much lower for our study in comparison to the previous one [62], it suggests that additional stress from blood sampling itself may have contributed to results in the referenced study. A subsequent study confirmed this possibility, they infected two groups of HOSP, and took blood samples from one group daily but not the other group [84]; they obtained mortality of 27.8% for the
sampled group but only 8.4% in the group not sampled. This indicates that HOSP are not naturally as susceptible to WNV infection as initially supposed for this study.

The usual viremia profile during infection would be a bell shaped curve skewed towards the left, with peak viremia occurring relatively soon after infection and then gradually reducing over the following days. Results obtained from blood samples likely follow this pattern, and the apparent gap in the curve, seen on days six and seven, is because not all birds in the group could be sampled every day. A single bird with a very high level of viremia was sampled on days 2, 5 and 8 which boosted the mean viremia for those days. Without that single high viremia bird, levels of viremia would likely have been lower on day 5 and non-existent for day 8. Thus, the positive result seen on day eight, which consists only of viremia from that same highly infected bird, is not representative of the mean for the infected group as a whole. Overall blood viremia was considered likely to fit the standard skewed bell curve pattern of infection, with levels peaking on day two and then falling consistently, being lower on day five than on day four.

Lower mortality than expected meant that mortality would likely not be a useful indicator of infection levels, however, it was considered that viremia levels, in both blood and tissues, would still be an applicable measure to use to assess the effectiveness of the vaccines. Therefore it was decided to continue with the challenge assay, using HOSP as a model species.

6.2 Rationale for the Challenge Assay

To fully assess the level of protection that vaccination could provide to birds following infection with WNV, it was necessary to undertake an *in vivo* challenge assay. Results from
vaccinated groups of birds would be compared to an unvaccinated group to establish the effects of vaccination. For this, a second group of HOSP were captured, and four cages of nine birds were set up. After acclimatising the birds for seven days, vaccinated groups were intramuscularly injected with a total dose of $2.5 \times 10^9$ IFU adenovirus per bird, in a volume of 50 µl. The effects of each single vaccine, plus the possibility of any synergistic effect if they were used in combination, was investigated.

The control group received an injection of an equivalent volume of vaccine buffer only; the rAdE group received combined half doses of both rAdE and rAdMT; the rAdNS3 group received combined half doses of both rAdNS3 and rAdMT, and the both group received a combination of half doses of both rAdE and rAdNS3. Twenty-eight days after vaccination, all birds were challenged with $3.5 \times 10^4$ PFU per bird of WNV. Blood samples were collected daily from three birds per group for seven days, and tissues were harvested from all remaining birds fourteen days after infection. All birds that died during the study were frozen as soon as they were discovered and subsequently thawed, fourteen days after infection, and their tissues harvested.

### 6.2.1 Results – Challenge Assay, Vaccinated Birds Show Reduced Viremia

Before the start of the challenge assay, birds were screened for prior exposure to WNV. Table 6.2 shows that all of the birds demonstrated percentage inhibition values of less than 30%, thus, all birds were considered suitable for use in the challenge assay.
Percent Inhibition Frequencies for WNV Screen for the Challenge Assay in HOSP. ‘% Inhibition’ shows the range for the calculated inhibition for serum from all birds and ‘No. of Birds’ indicates the number of birds in each group.

Birds were monitored daily following vaccination, but before WNV infection, and there were no apparent adverse reactions to any of the vaccine combinations as administered. However, a low level gradual loss of weight was noticed, that occurred over the entire course of the trial and in all groups. For individual birds, this weight loss was less than 4% of the starting body weight, between any two weigh points, and less than 20% of the starting weight, over the whole period.

One of the main differences between the infection response study and the main challenge assay is the number of birds that died during the fourteen days following infection. Table 6.3 shows that mortality rates in all groups were between two and six times the level of mortality seen during the infection response study.

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Table 6.3 Challenge Assay Shows Increased Mortality Rates Compared to the Infection Response Study.
Details of when birds died, in each group during the fourteen days following infection, can be seen in the survivorship curve in Figure 6.3. The first birds were found dead on day four post-infection, with two birds dying in both the rAdNS3 vaccinated group (NS3) and the unvaccinated control group (Ctrl) and the final bird, one from the control group, was found dead on day nine post-infection. Two birds died in the rAdE vaccinated group (Env) and six birds died in the group that received the combination of both vaccines (Both).

![Survivorship Curve](image)

**Figure 6.3. Survivorship was Highest in the rAdE Vaccinated Group.**

Survivorship Curves for 14 Days Following WNV Infection of HOSP. The left hand axis indicates the number of birds still surviving for each day per group. Birds were counted as having died on the day they were found dead, birds were monitored daily between approximately 10am and 3pm for the first seven days post-infection and daily between 10am and 12noon for the second seven days. Legend: ‘Ctrl’ – unvaccinated group, ‘NS3’ – rAdNS3 vaccinated group, ‘Env’ – rAdE vaccinated group and ‘Both’ – group vaccinated with both rAdE and rAdNS3.

However, when the number of birds that died were analysed, using two different variations of the Chi-squared test (Minitab V16) (Table 6.4), it was determined that the number of birds that died, in any group, was not significantly dependent on vaccination; none of the groups
showed numbers that died as significantly different from the expected number of deaths (P values = 0.159 and 0.164). The small group size may have contributed to this result, as the analysis is considered more robust if expected values are greater than 5.

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<td>13.000</td>
<td>36.000</td>
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Table 6.4 Chi-Square Analysis of Mortality Data Indicates No Significant Differences to Expected Values, for any Vaccination Groups.

Columns within each cell show: data relating to those birds that survived to the end ‘Lived’; that died during the trial ‘Died’; and total birds per group ‘All’ (from left to right). Rows within each cell show: name of group (All for total birds in all groups) and bird count; % contribution to row total for that specific count; % contribution to column total for that specific count (for these % the ‘All’ cell contains contribution made by all groups); and expected count values (from top to bottom). The bottom right cell shows the chi-squared values from two variations of the analysis and P values for each analysis, both P values are > 0.05 thus the null hypothesis of no dependency between death and vaccination group must be accepted. Note the warning produced by the analysis software concerning the low values for the expected counts in all vaccination group.
Figure 6.4 shows blood viremia levels, from blood samples collected during the seven days following infection. Values are displayed as the log of the median viremia per vaccination group per day; the survivorship curve corresponding to the same days is included in the figure, to facilitate comparison. There were no significant differences between viremia for any of the vaccination groups on any individual day. Taking an overview of each viremia profile, they generally fit the ideal profile, with a peak relatively soon after infection and then subsequently decreasing. However, there are some discrepancies to this model: viremia for the ‘both’ group seems to fall on day three when it should not have, and the control group, as well as the NS3 group, shows increased viremia on day seven, after falling to zero by day five. Blood viremia levels are similar for all groups, although the peak for the control group, on day three, is approximately 1,000 fold greater than the other groups, and the peak for the ‘Both’ group, on day five, is approximately 10,000 fold more. When compared to the control group the Env group has lower viremia levels for 4 of the 7 days, the NS3 group for 3 of 7 and the ‘Both’ group for only 2 of the 7 days assessed. For the first four days following infection, both of the singly vaccinated groups show lower viremia than either the control or the ‘Both’ group. However, for the Env vaccinated group, viremia is at a higher level than the other groups on days five and six. To enable an overall comparison of viremia over the 7 days of the experiment, the sum of each days’ difference in viremia from the control group was calculated for each vaccinated group. Overall the NS3 group had viremia of 3.54 log PFU/ml (3467 PFU/ml) less than the control group. The other vaccinated groups were very similar to the control group, over the 7 day period. Overall viremia for the Env group was 0.52 log PFU/ml less than the control group and viremia for the ‘Both’ group was 0.24 log PFU/ml more than the control group.
When comparing viremia levels to mortality (Figure 6.4) there appears to be strong correlation for the control group, with peak viremia levels on day three tying in with birds being found dead on day four. Similarly for the ‘Both’ vaccinated group, when an apparent second peak of viremia, on day four, resulted in birds being found dead on day five, however birds kept dying, even though viremia was below detectable levels by day five. For the NS3 group, viremia peaks at day two and falls steadily until day five but the first birds do not die until day four. For the Env group, viremia is the lowest for all groups, until day four, and is then followed by an apparent increase on day six; if correct, this increase would appear to tie in with birds starting to die from day six.
Figure 6.4. Blood Viremia Tends to be Lower in Singly Vaccinated Groups.

Blood viremia and survivorship graph for 8 days post-infection with WNV in HOSP. As labelled the top graph shows the log of the median values for daily blood viremia, given in WNV PFU per ml of blood, for the four different vaccination groups. It shows a trend for lower viremia in the two single vaccine groups, rAdE and rAdNS3. The lower graph indicates the number of birds still surviving per group for the first eight days post-infection. Ctrl – Buffer only group, NS3 – rAdNS3 vaccinated group, Env – rAdE vaccinated group and Both – combined rAdE/rAdNS3 group.
As previously mentioned, due to animal care protocols, blood sampling had to be restricted to every third day for any individual bird, although this did have to be adjusted somewhat due to mortality; however, all sampling was still within the overall limit of five 50 µl samples per bird per four week period. The sampling restriction meant that any given bird may only have been sampled two or three times during the seven days; because of this it was decided to look at the viremia levels for each individual bird, per group per day, to see if this might provide more insight than the averaged results. Figure 6.5 shows daily blood viremia levels as log PFU/ml, displayed by individual bird within each group, for the seven days post-infection. In agreement with the median calculations (Figure 6.4), viremia is apparent from day one post-infection, generally higher on days two and three post-infection, apart from the ‘both’ group which appears higher on day four, and low for most birds from day five onwards. The viremia for individual birds, which appear to be noticeably different from others for the same group and same day are: on day two, bird NS3-6 had viremia over 100 times greater than the next lowest bird, NS3-6 died on day four; on day two, bird E6 had viremia approximately 100 times greater than the next lowest bird, viremia for E6 remained high, with similar values seen on day five and it died on day six, it may be that this individual did not raise a protective immune response following vaccination; on day three, bird NS3-9 and bird B8 both had viremia just over 1,000 times greater than the next lowest bird in their group, NS3-9 died on day four and B8 on day five; and on day four, bird E3 had viremia approximately 1,000 time greater than the next lowest bird, E3 died on day seven. Several of the birds
Figure 6.5. Individual Blood Viremia Show Substantial Variation Within Vaccination Groups.
Blood viremia challenge assay individual birds by day. The graph shows log of blood viremia for each of the seven days post-infection for each individual bird in each group (in group order: control, rAdNS3, rAdE and both). Colour links each group: red – control (C), green – rAdNS3 (NS3), purple – rAdE (E) and blue – Both (B). The x-axis shows Bird ID, to identify individual birds, as the abbreviated group name (shown in brackets in previous sentence) and the number, one to nine, for each individual bird tested that day. If no bar is shown it indicates that the viremia for the specific bird tested was below detectible levels on that day.

that showed low or no viremia on day five or six, had higher viremia when sampled again later (Figure 6.5).

The maximum peak viremia, within each group, was also ascertained for individual birds, with the caveat that birds may not be sampled on the day of their personal peak viremia. Peak viremia for the Env group was 10 times lower than the other groups (Table 6.5), although if the bird that likely did not respond to the vaccine, E6, is removed from analysis, then the peak for the Env group is 100 time lower than for the other groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Bird ID</th>
<th>Peak Viremia (log PFU/ml)</th>
<th>Day Sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>C9</td>
<td>8.6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>C5</td>
<td>8.4</td>
<td>3</td>
</tr>
<tr>
<td>NS3 (rAdNS3)</td>
<td>NS3-6</td>
<td>8.6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>NS3-9</td>
<td>8.2</td>
<td>3</td>
</tr>
<tr>
<td>E (rAdE)</td>
<td>E6</td>
<td>7.3</td>
<td>2</td>
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<tr>
<td></td>
<td>E6</td>
<td>7.1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>*E3</td>
<td>6.2</td>
<td>4</td>
</tr>
<tr>
<td>Both (rAdNS3/E)</td>
<td>B3</td>
<td>8.7</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>B8</td>
<td>8.3</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 6.5 Lowest Peak Blood Viremia Seen in the rAdE Vaccinated Group.
Challenge assay, individual birds with the top two peak viremia per group. The table shows the two highest viremia for individual birds per group, the bird ID is the abbreviated group name and the individual bird number. * for the Env group the highest two viremia were from the same bird sampled on different days, so the third highest value for viremia was also included.

Figure 6.6 shows results for swabs sample viremia for days one to six. The data point are linked by fairly smooth curves and fit the skewed bell shape curve pattern usual for post-infection viremia; however, there is still an apparent slight dip for the Env group on day four. As for blood viremia, swab viremia confirms that the Both group and the Control group have higher viremia than either single vaccine group; although viremia for the Env group seems to be maintained at a higher level, relative to the other groups, for longer following infection, as was also seen in the blood viremia graph (Figure 6.4). There is a peak of viral shedding between 3 and 4 days post-infection and levels of virus shed fall gradually from then on (Figure 6.6).
Swab viremia for 6 days post WNV infection of HOSP. Viremia from swab samples are shown as median viremia, in log PFU/ml, per vaccination group per day. Vaccination groups are detailed in the legend on the right.

Tissue samples were also assessed for the amount of any virus present in either the spleens, hearts, kidneys or brains of the infected HOSP. Birds that died during the trial were frozen whole, as soon as possible after discovery, and tissues were sampled when the trial finished; all other birds were euthanised on day 14 post-infection, when the trial finished, and tissues were harvested directly. Due to the different days on which individual birds died, for those birds that died during the trial, to facilitate comparisons, tissue viremia has been plotted for each individual bird (Figure 6.7).
Figure 6.7 HOSP Tissue Viremia (Spleen, Heart, Kidney and Brain) for Days 4 to 9 Post WNV Infection.

Separate graphs are shown for each tissue type, as detailed above each graph. Viremia in the different tissues is shown as log PFU per 100 mg of tissue as vertical bars and group is highlighted by the colour of the bars (control – red, NS3 – green, Env – purple, Both – blue). The individual bird’s ID is on the x axis and consists of group name and bird number. The day the samples were collected is indicated by the staggered line above the bars that shows the day number post-infection: 4 birds died on each of days 4 and 5, 2 on days 6 and 7 and 1 bird on both day 8 and day 9. For the spleen sample, bird ‘Both5’ provided an ‘undetermined’ result and so was not included in the graph.
Overall, birds showed similar levels of viremia, for each day and tissue type, regardless of vaccination group. Within day, comparisons further showed: for day 4, ‘Neg9’ shows viremia, for all tissue types, the same or higher than other birds that died on the same day; for day 5, at least one of the birds from the Both group shows higher viremia than ‘NS3-9’, apart from in the spleen; for days 6 and 7, the two birds that died from the Env group show slightly higher viremia than the birds from the Both group that died on the same day, apart from day 6 kidney samples; and on days 8 and 9, only a single bird died, so it is hard to make comparisons, but generally, although they still succumbed to the infection, these birds appear to have a lower level of viremia than seen in tissue of birds that died earlier.

For tissue samples collected from birds on day 14 post-infection, as they were all collected on the same day, median viremia values were calculated per group per tissue type (Prism software); graphs were plotted for each tissue type, and included median with interquartile ranges and data points for individual birds (Figure 6.8). When compared with viremia on all previous days, viremia on day 14 for all tissues is substantially lower. Kidney and brain show the largest ranges of values on day 14, from zero up to as high as $5 \times 10^8$, and indicate falls in viremia, of approximate between $10^3$-$10^5$ and $10^2$-$10^4$, respectively, from earlier samples. For heart tissue, the drop in viremia is approximately $10^3$, and the range of values seen on day 14 is much less. The largest decrease in viremia is seen in the spleen, where viremia falls from the approximate $10^{10}$ seen on previous days to less than 20 for most birds. Regarding differences in median tissue viremia between vaccination groups, the highest values for both heart and brain samples are seen in the NS3 group, although the difference between this group and the others is small. The most noticeable difference for median viremia values is for the control group kidney samples, these show much higher median viremia compared to the
other vaccination groups. However, as indicated by the overlapping interquartile ranges, none of the differences in median viremia between groups on day 14 post-infection are significant.

Figure 6.8 HOSP Tissue Viremia Show No Significant Differences Between Vaccination Groups at Day 14 Post-Infection.

Tissue viremia for spleen, heart, kidney and brain. Within each tissue type and vaccination group the median viremia is represented as the thin horizontal line and the interquartile range by the vertical lines. Due to the large range of values, there are no significant differences, as seen by the overlapping interquartile ranges. All graphs show viremia as PFU per 100mg of tissue, but values of the y axes are different for the different tissues and that three of the four graphs have a segmented y axis due to the large range of values present. The control group is shown here as ‘Neg’, otherwise group names are as previously used.

6.2.2 Discussion – Challenge Assay, Viremia Reduced in Vaccinated Birds

From the graphs of blood viremia for the individual birds (Figure 6.5), following day five some birds show a relative increase in viremia levels, when viremia should be falling
steadily. Of the eight birds sampled on day five that were subsequently re-sampled: one showed increased viremia on day seven, from a low value on day five (C4); three confirmed negative at subsequent testing on day seven (C6, NS3-7 and B7) and four showed increased viremia, compared to a negative viremia on day five (NS3-5, NS3-8, E4 on day seven and B1 on day six). Loss of sample during processing may be responsible for an inaccurate negative result, as seven birds, out of the eight later re-tested, showed negative for viremia on day five. Therefore, it is likely that an error occurred during sample collection, or storage, that resulted in reduced viremia being detected for day five. Even the three birds confirmed negative when tested again on day seven may not actually have been negative on day five. That an error occurred, in sampling or sample processing, on day five, is reinforced by the observation that the levels of overall viremia seen on day four, would otherwise have been unlikely to have dropped to undetectable levels in one day. Two birds from day six were also re-sampled on days seven, bird B2 showed higher viremia on day seven than on day six and bird B9 was negative on both days; it is quite likely that B9 was actually negative for viremia on day six, as its viremia was already low on day three. However, no conclusions can be drawn regarding the anomalous data for B2. Only one bird, from the Env group, was tested on day five and subsequently re-tested, so comparisons cannot be drawn for the other two Env group birds, however, as there appears to be some problems with sample processing on day five, it is possible that the viremia values for the Env group would not be the highest, if the other groups had be analysed correctly.

Blood viremia between the infection response study and the challenge assay were compared (Figure 6.9). Average levels were generally lower during the infection response than during the challenge assay, although the same dose of WNV was used in both cases. During the
challenge assay, the peak levels of viremia in the control group, and likely the ‘Both’ group (allowing for sampling differences), are similar to levels seen by Komar et al. [62], the results of which, as previously described, were likely due to additional stresses on the HOSP, rather than direct consequences of WNV. Thus additional stresses may be increasing the effect of the infection during the challenge assay.

Figure 6.9 Average Blood Viremia from Both Studies Indicate Lower Viremia in the Infection Response Study.

Includes data from Figure 6.1 and Figure 6.4, showing the average blood viremia from the infection response study in comparison to those from the challenge assay. Legend: Inf Resp – group infected for infection response study, other labels as for Figure 6.4

Additional stresses on the birds could have been the cause of both the increased viremia, as well as the increase mortality rates, seen in the challenge assay, and also may be contributing factors to reduced immune responses to vaccination itself. Increased mortality, as seen in the challenge assay, may not be directly linked to higher viremia, if other causes of death are
possible. Reasons why the effects of the infection seem more widespread during the challenge assay, than during the infection response study may include:

- Adverse side effects of the vaccines themselves, although this would not affect the control group, unless the effect was due to a component or contaminant within the vaccine buffer that was used in all groups.
- Although the bird’s diet of seed was being occasionally supplemented, this may not have been sufficient to provide a full nutritional spectrum for the birds, so nutritional deficiency resulting in stress may have occurred. All birds lost weight during the entire course of the study.
- Possible concurrent infection may have been present in some of the birds when captured and spread to other birds due to the confinement of captivity. There was some evidence of a possible Salmonella infection.
- Other general stresses related to being held in captivity for an extended period of time, especially as birds for the challenge assay were held for an additional four weeks, compared to those used for the infection response study, to allow time for the vaccines to take effect.
- Towards the end of the challenge assay, but before infection, some of the birds were losing more feathers than before, which may be an indicator of stress or nutritional deficiencies.

Viremia levels seen in collected swab samples (Figure 6.6) reflect those seen in the blood, with generally higher levels in the ‘Both’ and control groups and lower levels in the single vaccine groups, for some days. However, compared to blood viremia (Figure 6.4) values remain relatively high until day six, the last day for which results are available. Swab viremia
would appear to provide a more accurate viremia profile, as for blood viremia there are anomalies that are likely due the sampling schedule. Swab viremia may also provide a more accurate picture of relative viremia levels as more birds could be sampled every day. However, although swab viremia was equated to a PFU/ml standard, each sample is from a single swab and as such the amount of actual sample material present may vary and is essentially unquantifiable, this may increase variability in results.

Levels of swab viremia confirm that the birds were shedding additional virus following infection. Initially uninfected cage mates of WNV infected birds can demonstrate levels of viremia similar to those birds directly infected [62]. Although this phenomenon has not been seen in HOSP before, some birds may have been picking up additional virus from faeces, which would alter the effective inoculation amount of WNV and could change their viremia profile, as well as reducing any protection provided by the vaccines.

Levels of tissue viremia at day 14 post-infection do not show a clear difference between vaccination groups when all tissues are taken into account as a whole, as for individual tissues the highest median viremia is seen in different vaccination groups. It does indicate, however, that virus can persist in tissues after it is no longer detectable in the blood. Blood viremia had fallen to under 1,000 PFU/ml for all groups by day seven and would likely be undetectable by day 14. Viral persistence in tissue has been shown to occur in HOSP following infection with WNV, with infectious virus detectable until 43 days post-infection and viral RNA still present until day 65 post-infection [85].

The reason the ‘Both’ group was included was to see if any synergistic effect would occur if both vaccines (rAdE and rAdNS3) were used together, this meant injecting a set dose of both vaccines into each bird for that group. To keep the total dose of adenovirus the same for the
birds receiving only a single vaccine, the same dose of the empty vector adenovirus (rAdMT) was included with the set dose of the WNV specific vaccine. Any synergistic effect of the combined vaccines, could be said to be detrimental, as it resulted in both higher levels of viremia in blood and swabs and higher mortality rates in the ‘Both’ group. However, the increased mortality seen in the ‘Both’ group was not significantly different from that expected, given the number of birds that also died in the other groups (Table 6.4). If possible, the cause of the apparent difference between the effects of the single vaccines and the combined vaccine should be established. The vaccine combination may be over stimulating the immune response, leading to excessive levels of cytokines and other immune modulators following infection, resulting in excessive inflammation, such as that seen during an avian influenza infection [86].

The dose of vaccine used was based on the work done in quail, suggested that a dose of 5x10^9 IFU vaccine per quail was preferable; this dose was then adjusted to allow for the reduced size of the HOSP compared to the quail. Resources were not available to practically assess whether or not the calculated vaccine dose would induce satisfactory immune responses in HOSP, before the commencement of the challenge assay. If the dose was too low, insufficient immune responses may have been induced in the birds; it has been shown for flaviviruses, that sub-neutralising levels of antibody can bind to the virus and actually enhance infection, by facilitating uptake of the virus through Fc-receptor bearing cells [87]. Possibly the dose was too high, which may have exacerbated the adverse effects seen in the Both vaccinated group by overloading the immune system; or, as seen in the quail, it may have reduced the immune response due to increased reaction to the adenovirus itself, thus reducing expression of the WNV DNA in the vaccines.
To help understand the higher mortality and viremia seen in the challenge assay compared to the infection response, an additional control group should have been included in the challenge assay, consisting of nine birds that were not infected with WNV. These could have been monitored solely for any affects on longevity, other than the direct effects of infection. Such information could have helped to settle the issue of additional deaths and therefore maybe also the cause of the higher viremia levels compared to the infection response study. Such a group was not included as four cages of birds was the maximum number that the facility could realistically house and that could be processed by a single person and including the ‘both’ treatment was hoped to have provided more insight than including another control group.

6.3 Chapter Conclusions - Infections in House Sparrows

For all samples collected during the first week post-infection, including blood, swabs and the tissues from the birds that died, there was a consistent, although not significant, trend for the control group to demonstrate higher levels of viremia than for either of the single vaccine groups. The group that received the combined vaccines tended to show higher viremia than the control group, where comparisons were possible. When the vaccines were administered separately, they acted to somewhat reduce viremia following WNV infection.

Both the infection response and the challenge assay, assessed viral load in blood, swabs and tissue using a PCR based technique. Although very fast and potentially accurate, there are inherent limitations of using such techniques, especially in relation to viral quantification, in that only viral RNA is being detected rather than active viral particles. A subsequent test of
the samples using a plaque neutralisation assay would be required to make any assessment on
the level of active virus that may be present in any of the samples.

The PCR reactions, used in this specific series of experiments, were based on the use of a
single set of primers within the WNV env region, so detection was further restricted to that
single mRNA. It is likely that primers targeted to different regions of the WNV RNA would
have each produced slightly different measures of viremia, for comparisons sake using the
same primers for all reactions should be sufficient. With hindsight, however, it may have
been preferable to avoid PCR primers directed to either of the WNV ‘genes’ used for the
vaccines. This would avoid any complications due to RNA from vaccine expression being
present, and also to negate the fact that the specific response raised by either vaccine, may
have a larger effect on the WNV ‘gene’/protein corresponding to the vaccine, than on any of
the other WNV ‘genes’/proteins. One of the other concerns when using qRT-PCR techniques
for quantification in general, is the necessity for consistency between reactions to enable
proper comparisons. Many factors have been found to affect the efficiency of the PCR
reaction itself, that could in turn affect the quantification results, including the amount of
RNA template present at the start of the reaction and the presence of any reaction inhibitors
[83]. To try and negate the first of these, RNA extraction samples were first quantified using
a nanodrop and template volumes were adjusted so that each reaction had approximately
500ng of total RNA at the start of the qRT-PCR reaction. For quantification purposes, a
standard curve was created for each tissue type, by spiking a known amount of WNV into the
same amount of the same tissue. This enabled heart tissue samples to be compared to
standards that also contained the same amount of heart tissue, so any inhibitors present in
heart tissue would be in samples and standards, therefore the effect would be negated within
each tissue type. However, for more accurate quantification, and to allow true comparisons between tissue types, it would have been necessary to include internal control primers and RNA in with each sample reaction. Quantification from sample reactions containing the control reagents, could then be compared to reactions run with the control primers and RNA but no sample; this would enable quantification of any inhibition of reaction caused by any inhibitors from the samples themselves. Unfortunately, such internal controls were not available when these experiments were conducted.

For both the infection response and the challenge assay, as with the studies involving quail, the number of birds that could be processed for experiments was relatively small for each group, which meant it was very restricting with regards to numbers for statistical analysis. All blood viremia measurements were based on an average of three birds a day, which is a minimum number for calculation of an average. It is likely that because the number of birds sampled daily was small, coupled with the fact that not all birds were allowed to be sampled every day, discrepancies were seen between the profiles obtained and those expected of viremia following infection. Such variations make analysis difficult as well as clouding any conclusions that can be drawn from the data. One solution would be to increase the number of birds that can be processed during each experiment, however, this would require more than one person to be working on the experiment. The problem of small group size was resolved to some extent, when sample numbers could be increased, as for the swab viremia, this resulted in smoother curves and better viremia profiles, indicating that larger groups size is preferable.

Viremia levels for both blood and swab samples for the challenge assay, do not provide clear evidence of the performance of either vaccine, as higher viremia can be seen in different
groups, including the vaccinated groups, on different days. The vaccines reduce the levels of blood viremia, in the two singly vaccinated groups, when taken as a whole over the 7 days post-infection. This effect is especially noticeable in the NS3 vaccinated group which has an overall viremia of over 3,000 PFU/ml lower than the control group. The Env vaccinated group shows viremia lower than the control group, for both blood and swab samples, for the majority of the time; the same applies to the NS3 vaccinated group, although viremia is lower or the same as the control group. Overall, the vaccines have been shown to induced an immune response specific for WNV, but only to a level sufficient to provide partial protection from WNV viremia.
Chapter  7: Concluding Comments

7.1 Experimental Conclusions

The main aim of this thesis was the development and testing of WNV vaccines for use in birds, and although vaccine efficacy results were not as definitive as they might have been, significant progress regarding vaccine development was still achieved. Heldens et.al. report that there are six stages in the development of a veterinary vaccine; documentation, feasibility, pre-development, development, registration and commercialisation [88]. Checking against their criteria, the first three stages were included in work done for this thesis, this included antigen selection, vaccine formulation and proof of concept, from the feasibility stage. From the pre-development stage, production processes and safety and efficacy testing were undertaken, as well as completion of product profiles forming the documentation stage. Both vaccines have shown indications of efficacy, as well as demonstrating safety, in both quail and HOSP, as neither species displayed any adverse reactions to either vaccine. Both vaccines have also fully fulfilled the feasibility stage of development, as well as demonstrating viable basic production techniques, thus the vaccines should now be considered candidates for the next stage in development, which would include field trials.

As well as vaccine development, two other areas of related research were included: that of assessing avian antibody reagents for cross-reactivity between bird species and use in assays, as well as the analysis of T cell populations in Japanese quail (Chapter  3:).

Although there is a certain level of antibody cross-reaction between avian species, seen during the assessment of reagents demonstrated, it occurs only in a limited way. Antibody reagents were shown to bind to cells from avian species other than the one the antibodies
were specific for. However, although such reagents cannot be used for quantitative assessments, they can still be useful for comparative studies, within any given species, as the proportion of cells that the antibodies bound to should be similar for all individual birds from the same species.

Proportions of both CD4+ and CD8+ cells were determined for quail using the antibodies specified, as well as identification of the population of T cells that are double positive for both CD4 and CD8. This is the first time, to our knowledge, that a CD4/CD8 double positive population has been identified in Japanese quail.

Overall, experiments demonstrated that for both avian models used, the single vaccines did induce both the humoral and cellular immune responses. These effects on the immune system were measured directly in quail, and were shown to provide a certain level of protection, from the effects of WNV infection in HOSP, by reducing levels of viremia. Unfortunately, many of these results were not significant.

7.1.1 Hypotheses

The main hypotheses were related to the vaccines and were defined in section 1.7. The first one stated that mRNA and protein expression from the vaccines would be detectable. Expression of both mRNA and protein was confirmed at several different concentrations of the both vaccines, although no attempt to quantify expression was undertaken. The hypothesis was accepted.

The second hypothesis stated that vaccines would trigger a significant immune response specific to WNV. This was tested in quail and it was confirmed that the vaccines did induce an immune response, as measured by levels of WNV specific antibodies and by T cell
activation, although for most results the outcome was not significantly different. This means that the hypothesis, as it stands, had to be rejected.

The final hypothesis stated that significant protection from WNV infection would be provided by vaccination with either vaccine and this hypothesis was tested in HOSP. The experiment also included assessment of objective vi.) whereby any synergistic effect of using the vaccines together was tested. Viremia was reduced in WNV infected birds, following vaccination with either vaccine but not to a significant extent. The hypothesis therefore had to be rejected. Any synergy achieved by using the vaccines in combination was shown to have a detrimental effect when tested in HOSP, compared to the use of either vaccine alone, although again results were not significant.

### 7.1.2 Strengths and Limitations of Experiments

The main limitation to the experiments conducted for this project was that non-standard models were used; by using Japanese quail and HOSP this was also true even by avian standards, as the chicken is the avian model mostly used. This meant that no previously tried and tested assays were available for use, and that essentially all assays used had to be developed or refined for the specific application required here. In the initial stages of testing there were some problems even with finding appropriate material to use as positive controls for testing the assays. This was particularly in evidence for the testing of the chicken IFN-γ antibody, whereby not only the antibody itself, but different concentrations of different substances to stimulate IFN-γ production in quail cells, all had to be assessed at the same time. This was because although chicken IFN-γ was available, quail IFN-γ was not, so could not be used as a positive control for testing the antibody, prior to testing it directly on stimulated cells.
Another difficulty with using non-standard models for testing the immune response is the likely increase in levels of genetic variation, that could result in a wider range of responses. A wide range of responses were seen in many results from this project, which contributed to unclear results. The gold standard for a genetically similar model must be the mouse, whereby for many strains 100% of mice are considered to be homozygous at all loci [60]. However, for avian models it is somewhat different. Chickens are the main avian model; in a study looking at genetic similarity between five chicken breeding lines, they found within line similarities of 81 – 96% and between line similarities of 78 – 95% [89]. For Japanese quail, a similar study was undertaken, which showed a 75 – 82% similarity within lines and 76 – 81% similarity between lines [90]. So even the best avian model showed considerably more variation than the best, and most commonly used model for vaccine testing, the mammalian model. In addition quail showed even more variation than chickens. No genetically related data were available for HOSP, but as all birds used in this study were from wild populations it is likely that genetic similarity was low. For Zebra Finches (Taeniopygia guttata), a species which had been bred in captivity for 200 years, birds from a captive population showed a decrease of 39% in the number of alleles per locus when compared to a wild population. This indicates the increase of similarity caused by any form of captive breeding, which would be entirely lacking in the HOSP used for the challenge assay.

Despite the problems caused by using non-standard animal models, their use also provided some interesting aspects to this thesis. Using wild and non-standard species as models for vaccine testing, a task more usually fulfilled by standard animal models such as mice, the work relates to the relatively new field of ‘wild immunology’. It should be considered a
strength of this study that it may contribute to a greater interest and understanding of this innovative area of research, by utilizing unusual assays and models to strive for a more holistic approach to immunological studies. ‘Wild immunology’ attempts to use knowledge from laboratory based studies of the immune system and apply them to the study of animals in wild populations. It also includes expanding laboratory based research to include studies on the genetic, parasitic and environmental variants, that can affect an animal’s immune system [91]. The overall aim of ‘wild immunology’ is to gain insight into the effects on immunity as seen in the ‘real world’ [91] and to link immunological studies to overall host fitness for life in a natural environment [92]. By assessing vaccine effectiveness in both a genetically diverse population of quail, as well as in a wild population of HOSP, this study attempted to contribute towards the overall aims of ‘wild immunology’.

7.2 Application and Significance of Research Findings

It would have been ideal if two vaccines with proven functionality had been the outcome of these experiments. Although this was not the case, the recombinant adenoviruses were constructed and tested positive for protein expression. The vaccines also showed a trend towards positive function. Further testing of the vaccines, in a more defined model species, such as mice, may provide a clearer picture of their potential functionality. Whether or not they are used as vaccines, the adenovirus constructs themselves could be useful, for in vitro experiments to assess the roles or functions of the WNV env or NS3 proteins. They could be used to assess effects of gene expression, by infecting different cell types, or to produce the WNV proteins for further study, by infecting cell lines (such as the A549 line) that are able to express high levels of adenovirus.
This research also included the further development of assays not generally used in birds, in particular, the assay to assess the level of intracellular IFN-γ for T cells. For them to be fully useful to assay any particular species of bird, they would need to be run with antibodies directly related to the species being tested. However, proof of principle that the assay can work for comparative purposes, for species that show some antibody cross-reactivity, has been demonstrated in this thesis.

That CD4/CD8 double positive populations of T cells exist in Japanese quail is also a useful contribution to the overall knowledge of this fairly recently discovered cell type. The range of species in which these cells are found may prompt further study which could provide details of the cells’ function.

It is in the context of ‘wild immunological’ studies, mentioned in the previous section, that the work undertaken with the HOSP may prove most valuable. This would be especially true if existing samples could be re-analysed to provide information on other factors that may have been affecting the birds immune responses. One such re-analysis could involve establishing the presence, and possibly even quantification, of any Salmonella infection that may have been present in the birds prior to commencement of the challenge assay. Synthesis of DNA PCR primers and probe specific for Salmonella would enable this re-assessment of existing RNA samples, to be done using the qRT-PCR technique already mentioned. If there were any positive results, it would then be possible to link levels of pre-existing infections with either, responses to vaccination or to WNV infection, depending on which groups any Salmonella positive birds were in.
To further develop the vaccines constructed during my thesis research to the next stage, it would be necessary to undertake a field trial of each vaccine. This would involve using a larger test population than used in the current studies and over a longer period of time. Initiation and length of immunity would also need to be established over a period of up to two years in order to assess longevity of the response to a single dose and to assess the effectiveness of any ‘annual’ booster vaccination [88]. The effectiveness of these type of studies is generally confirmed using a challenge assay situation [88]. For the WNV vaccines initially assessed in this thesis, such an assay would include another switch in test species to one which was much more susceptible to the effects of a WNV infection, as mortality rates would be the easiest, and more relevant, indicator to use to assess such large numbers of test subjects.

Although the HOSP, used in the challenge assay, may have provided interesting information regarding WNV infection of birds from a wild population, they were not a truly suitable model organism for WNV, mainly due to the apparent lack of effects following WNV infection. A preferred avian model would be a species in which the effects of WNV are readily apparent, for example in measurements such as mortality as well as viremia. As corvids are highly susceptible to WNV infections they make likely candidates. Within the corvid family, crows are severely affected by WNV [93], they are however fairly large birds and highly intelligent, thus making capture and care of the birds a problematic task. Magpies, a group of smaller sized corvids, have also been shown to be highly susceptible to WNV infections [94]. With some magpie species being common, further infection response studies
could be undertaken to see if they would make a more ideal species for studies of WNV avian infections.

Further work to investigate the CD4+/CD8+ T cell population in quail would provide additional information on the immune system of this species. Work could include assessment of the proportion of T cell populations in birds that had been immunologically stimulated, for comparison with naive birds. It would be ideal if quail specific antibodies for CD4 and CD8 could be created, which would enable accurate quantification of T cell numbers. This would also enable confirmation of the presence, as well as quantification of the double positive CD4/CD8 population of cells, using the quail specific antibodies.

Re-analyse of the vaccines’ performance using a standard model, such as the mouse, may serve to provide clearer results. There are many standard murine specific assays available to assess immune responses, assays that have been previously tried and tested and confirmed to work consistently.

Further investigation of the apparent detrimental effects of administering the two vaccines in combination could also provide useful insights into WNV toxicity specifically in relation to the two WNV proteins used. As vaccination itself showed no apparent adverse effects, the immune response to the combined vaccine may have produced an adverse response to the WNV, when the birds were challenged. Excessive inflammation may have been the cause of increased viremia, however further investigation would be necessary to confirm this.

Although the vaccines were developed with the idea of being utilised in birds, it was not targeted to birds in any specific way. The vaccines could be modified to include DNA that would target them more specifically to birds. For two DNA vaccines used in birds, by including a bird specific promoter, and optimising DNA codons for the specific species of
bird vaccinated, responses to the vaccines were greatly enhanced [95]. Unfortunately the paper [95] was published after the recombinant vaccines used in this thesis had been constructed. However there is no apparent reason why this idea should not work for a recombinant adenovirus, as the adenovirus is also linear DNA as was the DNA used in the previous study, so it would be worth pursuing. If specific ‘bird’ related DNA could be included in the recombinant vaccines it may induce an increased immune responses to them, which could only serve to improve their overall effectiveness.
References


Appendices

Appendix A Admax™ (Microbix) Plasmid Maps.

Figure A.1 Map of the Admax™ Adenoviral Genomic Plasmid pBHGlox(∆)E1,3Cre.

Plasmid map of pBHGloxΔE1,3Cre, which is the replication-deficient recombinant adenovirus genomic plasmid. The ability to replicate has been terminated due to the removal of both the E1 and the E3 gene from the plasmid. Note the presence of a loxP site, where this plasmid will recombine with the shuttle plasmid and the presence of the Cre gene which produces the cre that facilitates recombination between the plasmids, but which will be excised following recombination.
Figure A.2 Map of the Admax™ Shuttle Plasmid pDC316.

Plasmid map of the shuttle plasmid pDC316, note the multiple cloning site for insertion of the WNV ‘genes’, the presence of the MCMV promoter, the location of the inverted terminal repeat (ITR) sequences and the presence of the loxP site, where this plasmid will recombine with the pBHGIoxΔE1,3Cre plasmid.

The DNA of the final recombinant adenoviruses starts from the left pointing ITR region in pDC316, this contains the adenovirus packaging sequence followed by the MCMV promoter and the inserted WNV DNA and runs to the loxP site. As the loxP sites from pDC316 and pBHGIoxΔE1,3Cre are merged during recombination, the final adenovirus DNA then continues from the loxP site in pBHGIoxΔE1,3Cre includes the pIX gene and other adenoviral genes and finishes with the SV40 AN region.
Figure A.3 Map of the Admax™ Adenoviral Positive Control Plasmid pFG140.

Plasmid map of the positive control plasmid pFG140, which is an infectious adenovirus plasmid used as a positive control during co-transfections.

Figure B.1 Gating Strategy for the Avian Antibody Flow Cytometry Screens.

The above graphs show an example of the gating strategy used to assess cells for avian T cell markers, CD4 or CD8, antibody binding. In the graphs on the far left the entire population of cells samples are seen, the axes are based on physical properties of the cells (FSC-H represents cell size and SSC-H is cell granularity) and those considered to be alive are selected in the ‘Pop’ gate. These cells are then displayed using the FL2 and FL3 fluorescent channels on the axes, this is to check for and exclude, by means of the ‘Autoflur’ gate, any cells that are showing high autofluorescence. In the graphs on the far right hand side, the final population of cells remaining, those alive and with low autofluorescence are display as a histogram to show the fluorescence of the antibody specific for the T cell marker. The top graphs shows a CD8 antibody (Du-CD8-1) and the bottom one a CD4 antibody (CT4).
Appendix C  Sequence Checks of Plasmid DNA Against WNV DNA.

Plasmid DNA, from the shuttle plasmid pDC316 containing either the env ‘gene’ or the NS3 ‘gene’ from WNV was extracted and aligned to the NY99 stain of WNV isolated from a flamingo (NCBI Accession No. AF196835).

E-316 Forward Primer.

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| Sbjct: 466 | gttacctctctacacctccaggggaagtgtatatagtacgtcacac 525 |
| Query: 114 | gatgtcatcagattcacaagctgctgatggaagaaacactatatttcagacagtggat 173 |
| Sbjct: 526 | gatgtcatcagattcacaagctgctgatggaagaaacactatatttcagacagtggat 585 |
| Query: 174 | gttgggtataatgtcagtacgtactatcacttctagatggtcagctggtgtggtat 233 |
| Sbjct: 586 | gttgggtataatgtcagtacgtactatcacttctagatggtcagctggtgtggtat 645 |
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| Sbjct: 646 | gatccagaagacatcgactgttggtgcacaaagtcagca 705 |
| Query: 294 | tgccaccaagacacatcgacttgggtgcacaaagtcagca 353 |
| Sbjct: 706 | tgccaccaagacacatcgacttgggtgcacaaagtcagca 765 |
| Query: 354 | gaaagcaacatctacattccagatagctggtgtatggtggtggtggtgtgtgtatggatgcctggtccagctggtctggtccagc 413 |
| Sbjct: 766 | gaaagcaacatctacattccagatagctggtgtatggtggtggtggtgtgtatggatgcctggtccagctggtctggtccagc 825 |
| Query: 414 | ttggatataaagcagatctacattccagatagctggtgtatggtggtggtggtgtgtatggatgcctggtccagctggtctggtccagc 473 |
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| Query: 474 | attttccaaaacagatcatctggtatcattccagatagctggtgtatggtggtggtggtgtgtatggatgcctggtccagctggtctggtccagc 533 |
| Sbjct: 886 | attttccaaaacagatcatctggtatcattccagatagctggtgtatggtggtggtggtgtgtatggatgcctggtccagctggtctggtccagc 945 |
| Query: 534 | ttggtaggagggcaacacatccagatagctggtgtatggtggtggtggtgtgtatggatgcctggtccagctggtctggtccagc 593 |
| Sbjct: 946 | ttggtaggagggcaacacatccagatagctggtgtatggtggtggtggtgtgtatggatgcctggtccagctggtctggtccagc 1005 |
| Query: 594 | ggagttgcgtctggagcaacatccagatagctggtgtatggtggtggtggtgtgtatggatgcctggtccagctggtctggtccagc 653 |
| Sbjct: 1006 | ggagttgcgtctggagcaacatccagatagctggtgtatggtggtggtggtgtgtatggatgcctggtccagctggtctggtccagc 1065 |
| Query: 654 | atgtcaagggacaagctccccatctcagatagctggtgtatggtggtggtggtgtgtatggatgcctggtccagctggtctggtccagc 713 |
| Sbjct: 1066 | atgtcaagggacaagctccccatctcagatagctggtgtatggtggtggtggtgtgtatggatgcctggtccagctggtctggtccagc 1125 |
E-316 Reverse Primer

gi|11597239|gb|AF196835.2|AF196835 D West Nile virus strain NY99-flamingo382-99, complete genome, Length = 11029, Score = 1897 bits (957), Expect = 0.0 Identities = 970/974 (99%), Gaps = 1/974 (0%), Strand = Plus / Minus

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Query: 834  caaggagtggtggacaggggctggggcaacggctgcggactatttggcaaaggaagcatt 893
Sbjct: 1246 caaggagtggtggacaggggctggggcaacggctgcggactatttggcaaaggaagcatt 1305

Query: 894  gacacatgcgccaaatttgcctgctctaccaaggcaataggaagaaccatcttgaaagag 953
Sbjct: 1306 gacacatgcgccaaatttgcctgctctaccaaggcaataggaagaaccatcttgaaagag 1365

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Sbjct: 1509  tccaagctttagtg 1496

3-316 Forward Primer

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|Identities = 1056/1062 (99%), Gaps = 2/1062 (0%), Strand = Plus / Plus |

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Sbjct: 4731  gatggttgaaggtggtttttcccacaccctttggcatacaacaaaaagagagccgctttgtgag 4790
3-316 Reverse Primer

gi|11597239|gb|AP196835.2|AP196835

West Nile virus strain NY99-flamingo382-99, complete genome, Length = 11029, Score = 2076 bits (1047), Expect = 0.0
Identities = 1049/1050 (99%), Strand = Plus / Minus

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Sbjct: 6469 aacgtttcgcagcgaatccttgtgactagcctgttttagtgggttgtcgtataaccc 6410

Query: 113  tgggtgcaatcagctcggctcgcaacattgtgaaacctgcagttgcgtacatcag 172

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Sbjct: 6349 cttcgtgttgtctctcaaatgggctttttgcggcgtggtatc 6290

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Sbjct: 6289 cttcgtgttgtctctcaaatgggctttttgcggcgtggtatc 6230

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Query: 353  tcctcaacagctccgagcagttgcgtggtatcctccgatcatcag 412

Sbjct: 6169 tcctcaacagctccgagcagttgcgtggtatcctccgatcatcag 6110

Query: 413  tgatgttgtccagcattgtgactagcctgttctcctcttcctctcctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctc
Query: 893  tctcagtctgtaagtcggaaattggtgaattggactctgggaatggatctgaagtgcctg 952
Sbjct: 5629  tctcagtctgtaaagcggaaattggtgaattggactctgggaatggatctgaagtgcctg 5570
Query: 953  gtggggtggctgtcatgaatattgccgccgcctcccctagctcgacctttgtggaaatgt 1012
Sbjct: 5569  gtggggtggctgtcatgaatattgccgccgcctcccctagctcgacctttgtggaaatgt 5510
Query: 1013  aacctcttgctgcaatgctagctgggtcggtgaaatgagcctcatccatcacgaacaggt 1072
Sbjct: 5509  aacctcttgctgcaatgctagctgggtcggtgaaatgagcctcatccatcacgaacaggt 5450
Query: 1073  tgtagttcggcaccctgtgaggagacatca 1102
Sbjct: 5449  tgtagttcggcaccctgtgaggagacatca 5420
Appendix D  Sequence Checks of Recombinant Adenoviruses DNA Against WNV DNA.

Viral DNA was extracted from the finalised recombinant adenoviruses rAdE and rAdNS3 and the region of DNA containing the WNV env ‘gene’ or the NS3 ‘gene’ were aligned with a sequence from the NY99 strain of WNV isolated from a flamingo (NCBI Accession No. AF196835).

**rAdE fwd** – Blast search 97% identity (over 88% of the query) with AF196835 (blasted 1/1/09)

<table>
<thead>
<tr>
<th>Query</th>
<th>Sbjct</th>
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<tbody>
<tr>
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<td>466</td>
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<td>158</td>
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<td>586</td>
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<td>278</td>
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<td>398</td>
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<tr>
<td>578</td>
<td>946</td>
</tr>
<tr>
<td>638</td>
<td>1006</td>
</tr>
</tbody>
</table>

From Chromatogram 760/784 (97%), Identities = 729/784 (92%), Gaps = 1/784 (0%)
**rAdE rev** – Blast search 97% identity (over 70% of the query) with AF196835 (blasted 1/1/09)

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>757</td>
<td>1125</td>
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<tr>
<td>817</td>
<td>1185</td>
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<tr>
<td>877</td>
<td>1244</td>
</tr>
<tr>
<td>881</td>
<td>1248</td>
</tr>
</tbody>
</table>

AF196835.2|AF196835  West Nile virus strain NY99-flamingo382-99, complete genome
Length=11029, Score = 1158 bits (627), Expect = 0.0
From Chromatogram 772/800 (97%), Identities = 734/800 (91%), Gaps = 18/800 (2%)
Strand=Plus/Minus
rAd3 fwd – Blast search 99% identity (over 78% of the query) with AF196835 (blasted 1/1/09)

**AF196835.2** | **AF196835**
--- | ---
West Nile virus strain NY99-flamingo382-99, complete genome
Length=11029, Score = 1559 bits (844), Expect = 0.0
From Chromotogram 886/888 (99%), Identities = 868/888 (97%), Gaps = 0/888 (0%)
Strand=Plus/Plus

Query | 95
--- | ---
GGAGGCCGGTTGTTGGGACACTCCCTCACCAAAGGAGTACAAAAAGGGAACACGCCACC | 154
Sbjct | 4612
GGAGGCCGGTTGTTGGGACACTCCCTCACCAAAGGAGTACAAAAAGGGAACACGCCACC | 4671

Query | 155
--- | ---
GCGTAGGATTGGTTGGGACACTCCCTCACCAAAGGAGTACAAAAAGGGAACACGCCACC | 214
Sbjct | 4672
GCGTAGGATTGGTTGGGACACTCCCTCACCAAAGGAGTACAAAAAGGGAACACGCCACC | 4731

Query | 215
--- | ---
ATTGTATGGGCTTTCCACTACACTCGGCTGACTGGGAGGACAGGATGACGTTGGGAC | 274
Sbjct | 4732
ATTGTATGGGCTTTCCACTACACTCGGCTGACTGGGAGGACAGGATGACGTTGGGAC | 4791

Query | 275
--- | ---
GGAGGCCGGTTGTTGGGACACTCCCTCACCAAAGGAGTACAAAAAGGGAACACGCCACC | 334
Sbjct | 4792
GGAGGCCGGTTGTTGGGACACTCCCTCACCAAAGGAGTACAAAAAGGGAACACGCCACC | 4851

Query | 335
--- | ---
GGACCTGGCAAGAACGTTAAGAACGTCCAGACGAAACCAGGGGTTCAAAACACCTGGA | 394
Sbjct | 4852
GGACCTGGCAAGAACGTTAAGAACGTCCAGACGAAACCAGGGGTTCAAAACACCTGGA | 4911

Query | 395
--- | ---
GAACCTGGCAAGAACGTTAAGAACGTCCAGACGAAACCAGGGGTTCAAAACACCTGGA | 454
Sbjct | 4912
GAACCTGGCAAGAACGTTAAGAACGTCCAGACGAAACCAGGGGTTCAAAACACCTGGA | 4971

Query | 455
--- | ---
GGAGAAAATCGGGGCCGTGACTTTTGGAACACTCCCACCTGAACGAGGATGACGTTGGG | 514
Sbjct | 4972
GGAGAAAATCGGGGCCGTGACTTTTGGAACACTCCCACCTGAACGAGGATGACGTTGGG | 5031

Query | 515
--- | ---
GACAAAAACGTGATGATTGGGCTTTGGAACACTCCCACCTGAACGAGGATGACGTTGGG | 574
Sbjct | 5032
GACAAAAACGTGATGATTGGGCTTTGGAACACTCCCACCTGAACGAGGATGACGTTGGG | 5091

250
rAd3 rev – Blast search 98% identity (over 72% of the query) with AF196835 (blasted 1/1/09)

AF196835.2|AF196835
West Nile virus strain NY99-flamingo382-99, complete genome
Length=11029, Score = 1349 bits (730),  Expect = 0.0
From Chromatogram 803/817 (98%), Identities = 783/817 (95%), Gaps = 9/817 (1%)
Strand=Plus/Minus
Little letters etc above query sequence are from the check against the chromatogram for the bases that seem to have errors on them,

* = probable error, base = probable base (may or may not be wrong) and base = likely base (may or may not be wrong).
Appendix E  R Script for IFN-γ Assay Analysis.

```r
library(ggplot2)
library(lme4)

# Load dataset
# Original dataset

# Axis limits
yaxis <- c(0, 1700)

# Read in new data
CD4 <- read.csv('dataCD4.csv')
CD8 <- read.csv('dataCD8.csv')

# Set the levels

CD4$Restim <- factor(CD4$Restim, c('NS', 'MT', 'E3', 'WN'))  # Four Restimulations
CD4$Restim <- factor(CD4$Restim, c('NS', 'MT', 'E3'))  # 3 Restimulations
CD4$Vaccine <- factor(CD4$Vaccine, c('NS', 'MT', 'E', 'NS3'))  # 4 vaccines
CD4$Vaccine <- factor(CD4$Vaccine, c('NS', 'E', 'NS3'))  # 3 vaccines

CD8$Restim <- factor(CD8$Restim, c('NS', 'MT', 'E3', 'WN'))  # Four Restimulations
CD8$Restim <- factor(CD8$Restim, c('NS', 'MT', 'E3'))  # 3 Restimulations
CD8$Vaccine <- factor(CD8$Vaccine, c('NS', 'MT', 'E', 'NS3'))  # 4 vaccines
CD8$Vaccine <- factor(CD8$Vaccine, c('NS', 'E', 'NS3'))  # 3 vaccines

# Remove the columns we are not using
keep <- c('Bird', 'Vaccine', 'Restim', 'TR', 'TNR', 'Total', 'Duplicate')
CD4 <- CD4[keep]

# Average out duplicates
weightedAverage <- function(data){
  data$TR.add <- data$TR * data$Total
  data$TNR.add <- data$TNR * data$Total
  data.avg <- data[FALSE, keep]
  for(i in 1:nrow(data)){
    if(data$Duplicate[i] == 1){
      TR.acc <- data$TR[i]
      TNR.acc <- data$TNR[i]
    } else{
      TR.acc <- TR.acc + data$TR.add[i]
      TNR.acc <- TNR.acc + data$TNR.add[i]
      print()
      if(i == nrow(data) & data$Duplicate[i+1] == 1){
        newrow <- data[i,]
        newrow$TR <- TR.acc / data$Total[i]
        newrow$TNR <- TNR.acc / data$Total[i]
        data.avg <- rbind(data.avg, newrow)
      }
    }
  }
}

CD4.avg <- weightedAverage(CD4)
CD8.avg <- weightedAverage(CD8)

# Fit the data
CD4.lme <- glmer(TR + TNR ~ Vaccine*Restim + (1+Restim|Bird), family=poisson, data = CD4)
CD4.lme <- glmer(TR ~ Vaccine*Restim + (1+Restim|Bird), family=poisson, data = CD4)
summary(CD4.lme)
CD8.lme <- glmer(TR + TNR ~ Vaccine*Restim + (1+Restim|Bird), family=poisson, data = CD8)
CD8.lme <- glmer(TR ~ Vaccine*Restim + (1+Restim|Bird), family=poisson, data = CD8)
summary(CD8.lme)

# Plot the data
if(save){
  jpeg('CD4.jpg'
}
ggplot(data=CD4) + geom_point(aes(Vaccine, Restim, size=TR)) + scale_size_continuous('TR')
if(save){
  dev.off()
}

if(save){
  jpeg('CD8.jpg'
}ggplot(data=CD8) + geom_point(aes(Vaccine, Restim, size=TR)) + scale_size_continuous('TR')
if(save){
  dev.off()
}
```

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Appendix F  Output from IFN-γ Assay Analysis, Dose Response.
The graphs in this appendix show the results from each run of the R script for data collected from the IFNg assay, following initial screening of the data using Flowjo software, for all doses and all time points within the dose response trial for which data was available.

The tabulated output is for all time points for the medium dose only, as an illustration of this type of analysis output.
Tabulated Output from the R Script for IFN-γ Assay

Medium Dose CD4 Activated T Cells – 8 Days Post Infection
Generalized linear mixed model fit by the Laplace approximation
Formula: TR ~ Vaccine * ReStim + (1 + ReStim | Bird)
Data: CD4

AIC   BIC logLik deviance
240.4 274.1 -102.2    204.4

Random effects:
Groups Name        Variance Std.Dev. Corr
Bird  (Intercept) 0.049433 0.22234
      ReStimMT 0.036221 0.19032 -0.462
      ReStimE3 0.159666 0.39958 -0.569  0.992
Number of obs: 48, groups: Bird, 8

Fixed effects:      Estimate Std. Error z value Pr(>|z|)
(Intercept)          2.55290    0.20998 12.158  < 2e-16 ***
VaccineMT            0.37129    0.28670   1.295    0.195
VaccineE             1.67441    0.26919  6.220 4.96e-10 ***
VaccineNS3           1.67441    0.26919  6.220 4.96e-10 ***
ReStimMT             0.21808    0.22932   0.951    0.342
ReStimE3             0.34953    0.33700   1.037    0.300
VaccineMT:ReStimMT  -0.05698    0.30883  -0.185    0.854
VaccineE:ReStimMT   -0.08348    0.27850  -0.300    0.764
VaccineNS3:ReStimMT -0.08348    0.27850  -0.300    0.764
VaccineMT:ReStimE3  -0.26469    0.46800  -0.566    0.572
VaccineE:ReStimE3   -0.19502    0.44749  -0.436    0.663
VaccineNS3:ReStimE3 -0.33603    0.45815  -0.733    0.463

---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
(Intr) VccnMT VaccnE VccNS3 RStmMT RStmE3 VMT:RSM VE:RSM
VNS3:RSM
VaccineMT -0.732
VaccineE -0.780  0.571
VaccineNS3 -0.756  0.554  0.590
ReStimMT -0.603  0.442  0.471  0.456
ReStimE3 -0.632  0.463  0.493  0.478  0.745
VccnMT:RStmMT  0.448 -0.589 -0.349 -0.339 -0.743 -0.553
VccnE:RStmMT  0.497 -0.364 -0.567 -0.376 -0.823 -0.614  0.611
VccNS3:RStmMT  0.468 -0.343 -0.365 -0.572 -0.776 -0.578  0.576  0.639
VccnMT:RStE3  0.455 -0.622 -0.355 -0.344 -0.537 -0.720  0.752  0.442  0.416
VccnE:RStE3  0.476 -0.349 -0.611 -0.360 -0.561 -0.753  0.417  0.794  0.435
VccNS3:RStE3  0.465 -0.341 -0.363 -0.616 -0.548 -0.736  0.407  0.451  0.765
VMT:RSE VE:RSE
VaccineMT
VaccineE
VaccineNS3
ReStimMT
ReStimE3
VccnMT:RStMT
VccnE:RStMT
VccNS3:RStMT
VccnMT:RStE3
VaccineMT
VaccineE
VaccineNS3
ReStimMT
ReStimE3
VccnMT:RStMT
VccnE:RStMT
VccNS3:RStMT
VccnMT:RStE3

261
Medium Dose CD4 All T Cells - 8 Days Post Infection
Generalized linear mixed model fit by the Laplace approximation
Formula: TR + TNR ~ Vaccine * ReStim + (1 + ReStim | Bird)
Data: CD4
AIC  BIC logLik deviance
452 485.7   -208      416

Random effects:
Groups  Name        Variance  Std.Dev.  Corr
  Bird   (Intercept) 0.040192 0.20048
         ReStimMT  0.065627 0.25618  -0.210
         ReStimE3  0.070183 0.26492  -0.438   0.935
Number of obs: 48, groups: Bird, 8

Fixed effects:
                    Estimate Std. Error t value  Pr(>|t|)
(Intercept)          5.73014    0.14476   39.58 < 2e-16 ***
VaccineMT           -0.02287    0.20466  -0.11   0.91102
VaccineE            -0.07945    0.20477  -0.39   0.69802
VaccineNS3          -0.43696    0.20570  -2.12   0.03365 *
ReStimMT            -0.76147    0.18828  -4.04 5.25e-05 ***
ReStimE3            -0.54944    0.19338  -2.84   0.00449 **
VaccineMT:ReStimMT   0.77418    0.26445   2.93   0.00342 **
VaccineE:ReStimMT   -0.74558    0.26467  -2.82   0.00485 **
VaccineNS3:ReStimMT  0.64382    0.26636   2.42   0.01564 *
VaccineMT:ReStimE3   0.67804    0.27123   2.49   0.01272 *
VaccineE:ReStimE3    0.46468    0.27266   1.70   0.08834 .
VaccineNS3:ReStimE3  0.53060    0.27392   1.94   0.05274 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
                (Intr) VccnMT VccnE VccNS3 RStmMT RStmE3 VMT:RSM VE:RSM
VccnMT:RStmMT  0.163 -0.230 -0.115 -0.115 -0.712 -0.638
VccnE:RStmMT  0.163 -0.218 -0.231 -0.115 -0.711 -0.637  0.506
VccNS3:RStmMT 0.162 -0.114 -0.114 -0.235 -0.707 -0.633  0.503  0.503
VccnMT:RStmE3 0.317 -0.448 -0.224 -0.223 -0.637 -0.711  0.906  0.453 0.450
VccnE:RStmE3  0.317 -0.224 -0.448 -0.223 -0.635 -0.709  0.452  0.904 0.449
VccNS3:RStmE3 0.315 -0.223 -0.223 -0.451 -0.633 -0.706  0.450  0.450 0.899
VMT:RSM:VE:RSM
VaccineMT
VaccineE
VaccineNS3
ReStimMT
ReStimE3
VccnMT:RStmMT
VccnE:RStmMT
VccNS3:RStmMT
Medium Dose CD4 Activated T Cells - 43 Days Post Infection

Generalized linear mixed model fit by the Laplace approximation

Formula: TR ~ Vaccine * ReStim + (1 + ReStim | Bird)

Data: CD4

AIC  BIC  logLik  deviance
7387  7428  -3676     7351

Random effects:

Groups Name       Variance Std.Dev. Corr
Bird  (Intercept) 0.096651 0.31089
       ReStimMT    0.241924 0.49186  -0.891
       ReStimE3    0.075180 0.27419   0.209 0.254

Number of obs: 72, groups: Bird, 8

Fixed effects:

                      Estimate Std. Error z value Pr(>|z|)
(Intercept)         5.600827   0.221210 25.319  < 2e-16 ***
VaccineMT           0.546631   0.312423  1.750  0.08018 .
VaccineE            0.635471   0.312374  2.034  0.04192 *
VaccineNS3          0.175174   0.312666  0.560  0.57530
ReStimMT            0.001282   0.349565  0.004  0.99707
ReStimE3            0.018046   0.197044  0.092  0.92703
VaccineMT:ReStimMT  0.074532   0.493800  0.151  0.88003
VaccineE:ReStimMT   0.156090   0.493727  0.316  0.75189
VaccineNS3:ReStimMT 1.275457   0.493749  2.583  0.00979 **
VaccineMT:ReStimE3  0.308513   0.277553  1.112  0.26633
VaccineE:ReStimE3   0.127878   0.277517  0.461  0.64501
VaccineNS3:ReStimE3 0.263122   0.277981  0.947  0.34387

---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:

                      (Intr) VccnMT VaccnE VccnE:RStE VccnMT:RStE3 VMT:RSM VE:RSM
VccnMT:RStE3  -0.708
VaccnE:RStE3  -0.708  0.501
VaccnE         -0.707  0.501  0.501
ReStimMT       -0.889  0.630  0.630  0.629
ReStimE3       -0.190 -0.135 -0.135 -0.134  0.258
VccnMT:RStMT   0.630 -0.890 -0.446 -0.445 -0.708 -0.182
VccnE:RStMT    0.630 -0.446 -0.890 -0.445 -0.708 -0.182  0.501
VccnMT:RStE3   0.630 -0.446 -0.446 -0.890 -0.708 -0.182  0.501  0.501
VaccnE:RStE3   -0.135  0.194  0.096  0.095 -0.183 -0.710  0.257  0.130  0.130
VccnE:RStE3    -0.135  0.095  0.194  0.095 -0.183 -0.710  0.129  0.257  0.130  0.130
VccnMT:RStE3   -0.135  0.095  0.095  0.192 -0.183 -0.709  0.129  0.129  0.257  0.257
VMT:RSM VE:RSM

VaccnE
VaccnE:RStMT
VaccnE:RStE3
VaccnMT:RStMT
VaccnMT:RStE3
VaccnMT:RStE3
VaccnMT:RStMT
### Medium Dose CD8 Activated T Cells - 43 Days Post Infection

**Generalized linear mixed model fit by the Laplace approximation**

**Formula:** \( TR \sim \text{Vaccine} \times \text{ReStim} + (1 + \text{ReStim} | \text{Bird}) \)

**Data:** CD8

<table>
<thead>
<tr>
<th>AIC</th>
<th>BIC</th>
<th>logLik</th>
<th>deviance</th>
</tr>
</thead>
<tbody>
<tr>
<td>258</td>
<td>298.7</td>
<td>-111</td>
<td>222</td>
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</table>

**Random effects:**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Name</th>
<th>Variance</th>
<th>Std.Dev.</th>
<th>Corr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bird</td>
<td>(Intercept)</td>
<td>0.027233</td>
<td>0.16503</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ReStimMT</td>
<td>0.047454</td>
<td>0.21784</td>
<td>0.372</td>
</tr>
<tr>
<td></td>
<td>ReStimE3</td>
<td>0.032156</td>
<td>0.17932</td>
<td>-0.267  0.796</td>
</tr>
</tbody>
</table>

Number of obs: 71, groups: Bird, 8

**Fixed effects:**

|                         | Estimate  | Std. Error | z value | Pr(>|z|) |
|-------------------------|-----------|------------|---------|----------|
| **(Intercept)**         | 5.037116  | 0.121269   | 41.54   | < 2e-16 *** |
| VaccineMT               | -0.041980 | 0.171611   | -0.24   | 0.80675  |
| VaccineE                | 0.313262  | 0.170660   | 1.84    | 0.06642  |
| VaccineNS3              | -0.432148 | 0.173173   | -2.50   | 0.01258  |
| ReStimMT                | 0.008632  | 0.160840   | 0.05    | 0.95720  |
| ReStimE3                | 0.296150  | 0.134089   | 2.21    | 0.02720  *|
| VaccineMT:ReStimMT      | 0.113543  | 0.227425   | 0.50    | 0.61760  |
| VaccineE:ReStimMT       | 0.457989  | 0.225550   | 2.03    | 0.04230  *|
| VaccineNS3:ReStimMT     | 0.689085  | 0.228284   | 3.02    | 0.00254  **|
| VaccineMT:ReStimE3      | -0.142485 | 0.190139   | -0.75   | 0.45363  |
| VaccineE:ReStimE3       | 0.232483  | 0.187995   | 1.24    | 0.21622  |
| VaccineNS3:ReStimE3     | 0.480160  | 0.191487   | 2.51    | 0.01216  *|

---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

**Correlation of Fixed Effects:**

<table>
<thead>
<tr>
<th>VNS3:RSM</th>
<th>VaccineMT</th>
<th>VaccnE</th>
<th>VccnMT:RSTMT</th>
<th>RStmMT</th>
<th>RStmE3</th>
<th>VMT:RSM</th>
<th>VE:RSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>VaccineMT</td>
<td>-0.707</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>VaccineE</td>
<td>-0.711</td>
<td>0.502</td>
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<tr>
<td>VaccineNS3</td>
<td>-0.700</td>
<td>0.495</td>
<td>0.498</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>ReStimMT</td>
<td>0.287</td>
<td>-0.203</td>
<td>-0.204</td>
<td>-0.201</td>
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<td></td>
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<tr>
<td>ReStimE3</td>
<td>-0.310</td>
<td>0.219</td>
<td>0.220</td>
<td>0.217</td>
<td>0.771</td>
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<td></td>
</tr>
<tr>
<td>VccnMT:RSM</td>
<td>-0.203</td>
<td>0.286</td>
<td>0.144</td>
<td>0.142</td>
<td>-0.707</td>
<td>-0.545</td>
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</tr>
<tr>
<td>VccnE:RStMT</td>
<td>-0.205</td>
<td>0.145</td>
<td>0.299</td>
<td>0.143</td>
<td>-0.713</td>
<td>-0.550</td>
<td>0.504</td>
</tr>
<tr>
<td>VccnNS3:RStMT</td>
<td>-0.202</td>
<td>0.143</td>
<td>0.144</td>
<td>0.269</td>
<td>-0.705</td>
<td>-0.543</td>
<td>0.498</td>
</tr>
<tr>
<td>VccnMT:RSE3</td>
<td>0.218</td>
<td>-0.310</td>
<td>-0.155</td>
<td>-0.153</td>
<td>-0.544</td>
<td>-0.705</td>
<td>0.770</td>
</tr>
<tr>
<td>VccnE:RStE3</td>
<td>0.221</td>
<td>-0.156</td>
<td>-0.305</td>
<td>-0.155</td>
<td>-0.550</td>
<td>-0.713</td>
<td>0.389</td>
</tr>
<tr>
<td>VccnNS3:RSE3</td>
<td>0.217</td>
<td>-0.153</td>
<td>-0.154</td>
<td>-0.321</td>
<td>-0.540</td>
<td>-0.700</td>
<td>0.382</td>
</tr>
<tr>
<td>VMT:RSE</td>
<td>VE:RSE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

VaccineMT
VaccineE
VaccineNS3
ReStimMT
ReStimE3
Medium Dose CD4 All T Cells - 43 Days Post Infection

Generalized linear mixed model fit by the Laplace approximation

Formula: TR + TNR ~ Vaccine * ReStim + (1 + ReStim | Bird)

Data: CD4

AIC  BIC logLik deviance
6749 6790  -3356     6713

Random effects:

Groups Name     Variance Std.Dev. Corr
Bird  (Intercept) 0.093804 0.30627
      ReStimMT 0.169468 0.41166 -0.901
      ReStimE3 0.037656 0.19405  0.342  0.072 -0.901

Number of obs: 72, groups: Bird, 8

Fixed effects:

                                   Estimate Std. Error z value Pr(>|z|)
(Intercept)                       5.72822    0.21782 26.297 < 2e-16 ***
VaccineMT                        0.72401    0.30760  2.354  0.01859 *
VaccineE                         0.70366    0.30760  2.288  0.02216 *
VaccineNS3                       0.21538    0.30798  0.699  0.48435
ReStimMT                         0.04161    0.29294  0.142  0.88705
ReStimE3                         0.02357    0.14119  0.167  0.86740
VaccineMT:ReStimMT              0.10318    0.41357  0.249  0.80299
VaccineE:ReStimMT               0.36128    0.41352  0.874  0.38229
VaccineNS3:ReStimMT             1.23402    0.41373  2.983  0.00286 **
VaccineMT:ReStimE3              0.38973    0.19800  1.968  0.04903 *
VaccineE:ReStimE3               0.38178    0.19803  1.928  0.05387 .
VaccineNS3:ReStimE3             0.47771    0.19889  2.402  0.01631 *

---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:

                                (Intr) VccnMT VaccnE VccnNS3 RStmMT RStmE3 VMT:RSM VE:RSM
VccnMT:RSM  -0.708              -0.501     -0.501     -0.501     -0.243     -0.243
VccnE:RStmMT -0.501              -0.708     -0.707     -0.707     -0.243     -0.243
VccnNS3:RStmMT -0.501     -0.708              -0.707     -0.707     -0.243     -0.243
VccnMT:RStmMT -0.501     -0.708     -0.707              -0.707     -0.243     -0.243
VccnE:RStmE3  -0.243     -0.243     -0.243     -0.243              -0.243     -0.243
VccnNS3:RStmE3 -0.243     -0.243     -0.243     -0.243     -0.243              -0.243
VaccineMT    VccnMT VaccnE VaccnNS3
VaccineE
Medium Dose CD8 All T Cells – 43 Days Post Infection
Generalized linear mixed model fit by the Laplace approximation
Formula: TR + TNR ~ Vaccine * ReStim + (1 + ReStim | Bird)
Data: CD8
AIC  BIC logLik deviance
363.5 404.2 -163.8    327.5
Random effects:
Groups  Name        Variance Std.Dev. Corr
Bird   (Intercept) 0.043362 0.20824
       ReStimMT  0.038116 0.19523   0.142
       ReStimE3  0.033844 0.18397  -0.572  0.730
Number of obs: 71, groups: Bird, 8
Fixed effects:
                     Estimate Std. Error t value  Pr(>|z|)
(Intercept)          5.165990   0.150471   34.33  < 2e-16 ***
VaccineMT           -0.005855   0.212770   -0.03 0.978048
VaccineE            -0.436876   0.212044   -2.06 0.039369 *
VaccineNS3          -0.351840   0.213717   -1.65 0.099703 
ReStimMT            0.007717   0.144741   -0.05 0.957478
ReStimE3            0.318499   0.136307    2.34 0.019459 *
VaccineMT:ReStimMT  0.094212   0.204470    0.46 0.644968
VaccineE:ReStimMT   0.352860   0.202620    1.74 0.081598
VaccineNS3:ReStimMT 0.751462   0.204967    3.67 0.000246 ***
VaccineMT:ReStimE3  -0.174585   0.193054   -0.90 0.365821
VaccineE:ReStimE3   -0.146357   0.191166   -0.77 0.443913
VaccineNS3:ReStimE3 0.456172   0.193947    2.35 0.018670 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Correlation of Fixed Effects:
   (Intr) VccnMT VaccnE VccNS3 RStmMT RStmE3 VMT:RSM VE:RSM
VNS3:RSM          0.707
VaccineMT        -0.707 0.502
VaccineE         -0.704 0.498 0.500
VaccineNS3       -0.704 0.498 0.500
ReStimMT         0.089 -0.063 -0.063 -0.063
ReStimE3         0.581 0.411 0.413 0.409 0.713
VccnMT:RStM      -0.063 0.089 0.045 0.044 -0.708 -0.505
VccnE:RStM       -0.064 0.045 0.098 0.045 -0.714 -0.510 0.506
VccNS3:RStM      -0.063 0.045 0.045 0.080 -0.706 -0.504 0.500 -0.504
VccnMT:RStE3     0.411 -0.580 -0.291 -0.289 -0.504 -0.706 0.713 0.360 0.356
VccnE:RStE3      0.415 -0.293 -0.581 -0.292 -0.509 -0.713 0.360 0.719 0.359
VccNS3:RStE3     0.409 -0.289 -0.290 -0.585 -0.501 -0.703 0.355 0.358 0.718
VMT:RSM VE:RSM
Medium Dose CD4 Activated T Cells - 93 Days Post Infection

Generalized linear mixed model fit by the Laplace approximation

Formula: TR ~ Vaccine * ReStim + (1 + ReStim | Bird)

Data: CD4

AIC  BIC logLik deviance
164.4 220.5 -56.18 112.4

Random effects:

Groups Name     Variance Std.Dev. Corr
Bird  (Intercept) 0.094895 0.30805
       ReStimMT 0.101883 0.31919 -0.127
       ReStimE3 0.240855 0.49077 -0.036 0.996
       ReStimWN 0.085657 0.29267 -0.436 0.948 0.915

Number of obs: 64, groups: Bird, 8

Fixed effects:

             Estimate Std. Error z value Pr(>|z|)
(Intercept) 2.14904  0.27629   7.778 7.35e-15 ***
VaccineMT  0.21105  0.38398   0.550 0.582568
VaccineE  0.61401  0.37380   1.643 0.100469
VaccineNS3 1.07717  0.36562   2.946 0.003218 **
ReStimMT -0.00517  0.32856  -0.016 0.987446
ReStimE3  0.12026  0.41850   0.287 0.773461
ReStimWN -0.13456  0.32262  -0.417 0.676624
VaccineMT:ReStimMT -0.27908  0.45804  -0.609 0.542331
VaccineE:ReStimMT  0.49760  0.42944   1.159 0.246575
VaccineNS3:ReStimMT -0.20309  0.42451  -0.478 0.632361
VaccineMT:ReStimE3 -0.46369  0.59316  -0.782 0.434374
VaccineE:ReStimE3  0.14924  0.56871   0.262 0.792997
VaccineNS3:ReStimE3 -0.22803  0.56254  -0.405 0.685216
VaccineMT:ReStimWN  0.13794  0.43857   0.315 0.753128
VaccineE:ReStimWN  1.39031  0.40908   3.399 0.000677 ***
VaccineNS3:ReStimWN  0.94507  0.40140   2.354 0.018550 *

---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:

(Intr) VccnMT VaccnE VccnNS3 RStmMT RStmE3 RStmWN VMT:RSM VE:RSM
VaccineMT  -0.720
VaccineE   -0.739  0.532
VaccineNS3 -0.756  0.544  0.559
ReStimMT    -0.385  0.277  0.285  0.291
ReStimE3    -0.273  0.197  0.202  0.206  0.785
ReStimWN   -0.544  0.392  0.402  0.411  0.692  0.706
VccnMT:RStm  0.277 -0.365 -0.204 -0.209 -0.717 -0.563 -0.497
Medium Dose CD8 Activated T Cells - 93 Days Post Infection

Generalized linear mixed model fit by the Laplace approximation

Formula: TR ~ Vaccine * ReStim + (1 + ReStim | Bird)

Data: CD8

AIC  BIC logLik deviance
151.7 207.9 -49.87    99.74

Random effects:

Groups   Name        Variance Std.Dev. Corr
Bird     (Intercept) 0.18939  0.43519
         ReStimMT    0.14000  0.37417  -0.564
         ReStimE3    0.38991  0.62443  -0.816  0.634
         ReStimWN    0.19555  0.44221  -0.913  0.839  0.888

Number of obs: 64, groups: Bird, 8

Fixed effects:

                  Estimate Std. Error    z value  Pr(>|z|)   
(Intercept)      0.98818    0.43057    2.295   0.0217 *
VaccineMT       -0.67881    0.67579   -1.004   0.3152
VaccineE        1.24104    0.55485     2.237   0.0253 *
VaccineNS3      0.22792    0.59393     0.384   0.7012
ReStimMT        -0.36156    0.53499    -0.676   0.4992
ReStimE3        0.58453    0.57952     1.009   0.3131
ReStimWN        0.87564    0.47632     1.838   0.0660 .
VaccineMT:ReStimMT 1.11690    0.78534     1.422   0.1550
VaccineE:ReStimMT -0.18978    0.65417    -0.290   0.7717
VaccineNS3:ReStimMT 0.94297    0.68487     1.377   0.1686
VaccineMT:ReStimE3 0.35238    0.88135     0.400   0.6893
VaccineE:ReStimE3  -0.92033    0.77227    -1.192   0.2334
VaccineNS3:ReStimE3 -0.05995    0.80436    -0.074   0.9406
VaccineMT:ReStimWN -0.09720    0.76453    -0.127   0.8988
Medium Dose CD4 All T Cells – 93 Days Post Infection

Generalized linear mixed model fit by the Laplace approximation

Formula: TR + TNR ~ Vaccine * ReStim + (1 + ReStim | Bird)

Data: CD4

<table>
<thead>
<tr>
<th></th>
<th>AIC</th>
<th>BIC</th>
<th>logLik</th>
<th>deviance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>267.2</td>
<td>323.4</td>
<td>-107.6</td>
<td>215.2</td>
</tr>
</tbody>
</table>

Random effects:

Groups Name        Variance  Std.Dev. Corr
Bird   (Intercept) 0.0657216 0.256362
            ReStimMT 0.0134325 0.115898 0.759
            ReStimE3 0.0107505 0.103685 0.754 0.904
            ReStimWN 0.0028042 0.052955 0.721 0.801 0.505

Number of obs: 64, groups: Bird, 8

Fixed effects:

|             | Estimate | Std. Error | z value | Pr(>|z|) |
|-------------|----------|------------|---------|----------|
| VaccineMT   | -0.637   |            |         |          |
| VaccineE    | -0.776   | 0.494      |         |          |
| VaccineNS3  | -0.725   | 0.462      | 0.563   |          |
| ReStimMT    | -0.586   | 0.373      | 0.455   | 0.425    |
| ReStimE3    | -0.805   | 0.513      | 0.625   | 0.584    | 0.524    |
| ReStimWN    | -0.871   | 0.555      | 0.676   | 0.631    | 0.622    | 0.770    |
| VccnMT:RSMT | 0.399    | -0.673     | -0.310  | -0.289   | -0.681   | -0.357   | -0.424   |
| VccnE:RStMT | 0.479    | -0.305     | -0.572  | -0.347   | -0.818   | -0.428   | -0.509   | 0.557    |
| VccnMT:RStMT | 0.353    | -0.819     | -0.411  | -0.384   | -0.344   | -0.658   | -0.507   | 0.595    | 0.282    |
| VccnE:RStE3 | 0.604    | -0.385     | -0.793  | -0.438   | -0.393   | -0.750   | -0.578   | 0.268    | 0.522    |
| VccnMT:RStE3 | 0.580    | -0.370     | -0.450  | -0.805   | -0.377   | -0.720   | -0.555   | 0.257    | 0.308    |
| VccnMT:RSWN | 0.542    | -0.857     | -0.421  | -0.393   | -0.387   | -0.480   | -0.623   | 0.673    | 0.317    |
| VccnE:RStWN | 0.676    | -0.431     | -0.865  | -0.490   | -0.483   | -0.599   | -0.777   | 0.329    | 0.636    |
| VccnNS3:RSWN | 0.641    | -0.408     | -0.497  | -0.883   | -0.458   | -0.567   | -0.736   | 0.312    | 0.375    |
| VccnMT:RSMT | 0.593    | -0.327     | -0.507  | -0.450   | -0.377   | -0.720   | -0.555   | 0.257    | 0.308    |
| VccnE:RStMT | 0.641    | -0.379     | -0.683  | -0.483   | -0.567   | -0.736   | 0.312    | 0.375    |          |
| VccnMT:RStMT | 0.303    | 0.759      | 0.360   | 0.346    |          |          |          |          |
| VccnNS3:RStMT | 0.378    | 0.394      | 0.768   | 0.431    | 0.484    |          |          |          |
| VccnMT:RSWN | 0.561    | 0.493      | 0.943   |          |          |          |          |          |
| VccnE:RStE3 | 0.561    | 0.493      | 0.943   |          |          |          |          |          |
| VccnNS3:RStMT | 0.561    | 0.493      | 0.943   |          |          |          |          |          |
| VccnNS3:RSWN | 0.561    | 0.493      | 0.943   |          |          |          |          |          |

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
(Intercept)          6.59213    0.18222   36.18  < 2e-16 ***  
VaccineMT           -0.25159    0.25789   -0.98  0.32928  
VaccineE            0.17852    0.25759    0.69  0.48828  
VaccineNS3          0.37135    0.25749    1.44  0.14925  
ReStimMT            -0.01544    0.08607   -0.18  0.85758  
ReStimE3            0.03502    0.07778    0.45  0.65251  
ReStimWN            -0.23812    0.04669   -5.10 3.39e-07 ***  
VaccineMT:ReStimMT   0.09323    0.12254    0.76  0.44677  
VaccineE:ReStimMT   0.04463    0.12122    0.37  0.71276  
VaccineNS3:ReStimMT -0.14326    0.12103   -1.18  0.23654  
VaccineMT:ReStimE3  0.06440    0.11073    0.58  0.56085  
VaccineE:ReStimE3   0.08583    0.10939    0.78  0.43269  
VaccineNS3:ReStimE3 -0.09309    0.10914   -0.85  0.39371  
VaccineMT:ReStimWN  0.17027    0.06670    2.55  0.01069 *  
VaccineE:ReStimWN   0.17339    0.06458    2.68  0.00726 **  
VaccineNS3:ReStimWN 0.12997    0.06382    2.04  0.04171 *  
---  
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1  

Correlation of Fixed Effects:  

|                | VaccineMT | VaccineE | VaccineNS3 | ReStimMT | ReStimE3 | ReStimWN | VaccineMT:ReStimMT | VaccineE:ReStimMT | VaccineNS3:ReStimMT | VaccineMT:ReStimE3 | VaccineE:ReStimE3 | VaccineNS3:ReStimE3 | VaccineMT:ReStimWN | VaccineE:ReStimWN | VaccineNS3:ReStimWN |
|----------------|-----------|----------|------------|----------|----------|----------|-------------------|-------------------|---------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|                      |
| VaccineMT      | -0.707    |          |            |          |          |          |                   |                   |                     |                   |                   |                   |                   |                   |                   |
| VaccineE       | -0.707    | 0.500    |            |          |          |          |                   |                   |                     |                   |                   |                   |                   |                   |                   |
| VaccineNS3     | -0.708    | 0.500    | 0.501      |          |          |          |                   |                   |                     |                   |                   |                   |                   |                   |                   |
| ReStimMT       | 0.697     | -0.493   | -0.493     | -0.493   |          |          |                   |                   |                     |                   |                   |                   |                   |                   |                   |
| ReStimE3       | 0.682     | -0.482   | -0.483     | -0.483   | 0.863    |          |                   |                   |                     |                   |                   |                   |                   |                   |                   |
| ReStimWN       | 0.535     | -0.378   | -0.379     | -0.379   | 0.697    | 0.476    |                   |                   |                     |                   |                   |                   |                   |                   |                   |
| VaccineMT:ReStimMT | -0.490   | 0.689    | 0.346      | 0.347    | -0.702   | -0.606   | -0.489            |                   |                     |                   |                   |                   |                   |                   |                   |
| VaccineE:ReStimMT | -0.495   | 0.350    | 0.702      | 0.350    | -0.710   | -0.613   | -0.495            | 0.499             |                     |                   |                   |                   |                   |                   |                   |
| VaccineNS3:ReStimMT | -0.496   | 0.350    | 0.351      | 0.705    | -0.711   | -0.614   | -0.495            | 0.499             | 0.505                |                   |                   |                   |                   |                   |                   |
| VaccineMT:ReStimE3 | -0.479   | 0.675    | 0.339      | 0.339    | -0.606   | -0.702   | -0.334            | 0.861             | 0.430                |                   |                   |                   |                   |                   |                   |
| VaccineE:ReStimE3 | -0.485   | 0.343    | 0.689      | 0.343    | -0.614   | -0.711   | -0.338            | 0.431             | 0.867                |                   |                   |                   |                   |                   |                   |
| VaccineNS3:ReStimE3 | -0.486   | 0.344    | 0.344      | 0.692    | -0.615   | -0.713   | -0.339            | 0.432             | 0.437                |                   |                   |                   |                   |                   |                   |
| VaccineMT:ReStimWN | -0.375   | 0.525    | 0.265      | 0.265    | -0.488   | -0.333   | -0.700            | 0.698             | 0.346                |                   |                   |                   |                   |                   |                   |
| VaccineE:ReStimWN | -0.387   | 0.273    | 0.551      | 0.274    | -0.504   | -0.344   | -0.723            | 0.354             | 0.708                |                   |                   |                   |                   |                   |                   |
| VaccineNS3:ReStimWN | -0.392   | 0.277    | 0.277      | 0.561    | -0.510   | -0.348   | -0.732            | 0.358             | 0.362                |                   |                   |                   |                   |                   |                   |

Medium Dose CD8 All T Cells - 93 Days Post Infection  
Generalized linear mixed model fit by the Laplace approximation  
Formula: TR + TNR ~ Vaccine * ReStim + (1 + ReStim | Bird)
Data: CD8
AIC  BIC logLik deviance
341.4 397.6 -144.7 289.4

Random effects:

<table>
<thead>
<tr>
<th>Groups</th>
<th>Name</th>
<th>Variance</th>
<th>Std.Dev.</th>
<th>Corr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bird</td>
<td>(Intercept)</td>
<td>0.043639</td>
<td>0.20890</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ReStimMT</td>
<td>0.024482</td>
<td>0.15647</td>
<td>-0.898</td>
</tr>
<tr>
<td></td>
<td>ReStimE3</td>
<td>0.017610</td>
<td>0.13270</td>
<td>-0.9450.992</td>
</tr>
<tr>
<td></td>
<td>ReStimWN</td>
<td>0.013115</td>
<td>0.11452</td>
<td>-0.6170.6620.664</td>
</tr>
</tbody>
</table>

Number of obs: 64, groups: Bird, 8

Fixed effects:

|                         | Estimate | Std. Error | z value | Pr(>|z|) |
|-------------------------|----------|------------|---------|----------|
| (Intercept)             | 5.864406 | 0.150100   | 39.07   | < 2e-16  ***|
| VaccineMT               | 0.056436 | 0.212193   | 0.27    | 0.79026  |
| VaccineE                | 0.535209 | 0.211614   | 2.53    | 0.01143 * |
| VaccineNS3              | 0.304293 | 0.211853   | 1.44    | 0.15091  |
| ReStimMT                | 0.165791 | 0.116416   | 1.42    | 0.15441  |
| ReStimE3                | 0.289608 | 0.100234   | 2.89    | 0.00386 **|
| ReStimWN                | 0.266350 | 0.088386   | 3.01    | 0.00258 **|
| VaccineMT:ReStimMT      | 0.064715 | 0.164325   | 0.39    | 0.69371  |
| VaccineE:ReStimMT       | 0.073066 | 0.162961   | 0.45    | 0.65389  |
| VaccineNS3:ReStimMT     | -0.078063| 0.163726   | -0.48   | 0.63351  |
| ReStimMT:ReStimE3       | 0.019440 | 0.141490   | 0.14    | 0.89072  |
| ReStimE3:ReStimM        | -0.089226| 0.140053   | -0.64   | 0.52407  |
| ReStimWN:ReStimE3       | -0.055049| 0.140699   | -0.39   | 0.69561  |
| VaccineMT:ReStimWN      | 0.009811 | 0.124722   | 0.08    | 0.93730  |
| VaccineE:ReStimWN       | -0.045217| 0.123043   | -0.37   | 0.71325  |
| VaccineNS3:ReStimWN     | -0.120011| 0.123933   | -0.97   | 0.33287  |

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 . ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:

<table>
<thead>
<tr>
<th>VaccineMT</th>
<th>VaccineE</th>
<th>VaccineNS3</th>
<th>ReStimMT</th>
<th>ReStimE3</th>
<th>ReStimWN</th>
<th>VNT:RSM</th>
<th>VE:RSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>VaccineMT</td>
<td>-0.707</td>
<td></td>
<td>-0.709</td>
<td>-0.709</td>
<td>-0.610</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VaccineE</td>
<td>-0.709</td>
<td>0.502</td>
<td></td>
<td></td>
<td>-0.624</td>
<td>-0.624</td>
<td>-0.624</td>
</tr>
<tr>
<td>VaccineNS3</td>
<td>-0.709</td>
<td>0.501</td>
<td>0.503</td>
<td></td>
<td>-0.880</td>
<td>0.623</td>
<td>0.624</td>
</tr>
<tr>
<td>ReStimMT</td>
<td>-0.918</td>
<td>0.649</td>
<td>0.651</td>
<td>0.650</td>
<td>0.944</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ReStimE3</td>
<td>-0.610</td>
<td>-0.610</td>
<td>0.431</td>
<td>0.433</td>
<td>-0.601</td>
<td>-0.432</td>
<td>0.646</td>
</tr>
<tr>
<td>ReStimWN</td>
<td>-0.624</td>
<td>-0.445</td>
<td>-0.884</td>
<td>-0.446</td>
<td>-0.610</td>
<td>-0.624</td>
<td>-0.624</td>
</tr>
<tr>
<td></td>
<td>VccnMT:RSMT</td>
<td>VccnE:RStMT</td>
<td>VccNS3:RSMT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>------------</td>
<td>-------------</td>
<td>-------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VccnMT:RSE3</td>
<td>0.475</td>
<td>0.507</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VccnE:RSTE3</td>
<td>0.480</td>
<td>0.505</td>
<td>0.510</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VccNS3:RSE3</td>
<td>0.948</td>
<td>0.505</td>
<td>0.510</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VccnMT:RSWN</td>
<td>0.325</td>
<td>0.650</td>
<td>0.329</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VccnE:RStWN</td>
<td>0.330</td>
<td>0.651</td>
<td>0.332</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VccNS3:RSWN</td>
<td>0.646</td>
<td>0.328</td>
<td>0.650</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

272
Appendix G  Dose Response Serum ELISA Paired-T Test Full Results.

Results from paired-T test to compare day 1 post boost with day 16 post boost for the low dose group and from 16 and 35 days post boost for the medium and high dose groups.

Low Dose
Paired T-Test and CI: Day 35, Day 1
Neg Group
Paired T for Day 35 - Day 1

<table>
<thead>
<tr>
<th>Day 35</th>
<th>N</th>
<th>Mean</th>
<th>StdDev</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 35</td>
<td>10</td>
<td>0.0134</td>
<td>0.0440</td>
<td>0.0110</td>
</tr>
</tbody>
</table>

Paired T-Test and CI: Day 35, Day 1
Day 1

| Day 1 | 10 | 0.0180 | 0.0452 | 0.0113 |

Difference 16 -0.00238 0.01307 0.00327

95% CI for mean difference: (-0.00856, 0.00439)
T-test of mean difference = 0 (vs not = 0): T-value = -0.70  P-value = 0.473

Low Dose
Paired T-Test and CI: Day 35, Day 1
HT Group
Paired T for Day 35 - Day 1

<table>
<thead>
<tr>
<th>Day 35</th>
<th>N</th>
<th>Mean</th>
<th>StdDev</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 35</td>
<td>10</td>
<td>0.0683</td>
<td>0.0718</td>
<td>0.0180</td>
</tr>
</tbody>
</table>

Paired T-Test and CI: Day 35, Day 1
Day 1

| Day 1 | 10 | 0.0584 | 0.0323 | 0.0081 |

Difference 16 0.0098 0.0671 0.0168

95% CI for mean difference: (-0.0877, 0.0538)
T-test of mean difference = 0 (vs not = 0): T-value = 1.08  P-value = 0.298

Low Dose
Paired T-Test and CI: Day 35, Day 1
Env Group
Paired T for Day 35 - Day 1

<table>
<thead>
<tr>
<th>Day 35</th>
<th>N</th>
<th>Mean</th>
<th>StdDev</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 35</td>
<td>10</td>
<td>0.0219</td>
<td>0.0233</td>
<td>0.0050</td>
</tr>
</tbody>
</table>

Paired T-Test and CI: Day 35, Day 1
Day 1

| Day 1 | 10 | 0.0449 | 0.0421 | 0.0105 |

Difference 16 -0.0111 0.0152 0.00391

95% CI for mean difference: (-0.0848, -0.00278)
T-test of mean difference = 0 (vs not = 0): T-value = -2.84  P-value = 0.002

Low Dose
Paired T-Test and CI: Day 35, Day 1
HL Group
Paired T for Day 35 - Day 1

<table>
<thead>
<tr>
<th>Day 35</th>
<th>N</th>
<th>Mean</th>
<th>StdDev</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 35</td>
<td>16</td>
<td>0.00113</td>
<td>0.00146</td>
<td>0.00081</td>
</tr>
</tbody>
</table>

Paired T-Test and CI: Day 35, Day 1
Day 1

| Day 1 | 16 | 0.00202 | 0.00788 | 0.00042 |

Difference 16 -0.00150 0.00203 0.000506

95% CI for mean difference: (-0.01228, 0.00928)
T-test of mean difference = 0 (vs not = 0): T-value = -0.30  P-value = 0.771
### Medium Dose

**Paired T-Test and CI: Day 16, Day 1**

#### N Group

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 16</td>
<td>16</td>
<td>0.034</td>
<td>0.027</td>
<td>0.012</td>
</tr>
<tr>
<td>Day 1</td>
<td>16</td>
<td>0.017</td>
<td>0.013</td>
<td>0.008</td>
</tr>
</tbody>
</table>

**Difference** | 16 | 0.017 | 0.013 | 0.008 |

95% CI for mean difference: (0.012, 0.022)

T-Test of mean difference = 0 (vs not = 0): T-value = 3.18  P-value = 0.006

### Medium Dose

**Paired T-Test and CI: Day 16, Day 1**

#### MT Group

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 16</td>
<td>16</td>
<td>0.069</td>
<td>0.026</td>
<td>0.013</td>
</tr>
<tr>
<td>Day 1</td>
<td>16</td>
<td>0.042</td>
<td>0.006</td>
<td>0.004</td>
</tr>
</tbody>
</table>

**Difference** | 16 | 0.027 | 0.019 | 0.010 |

95% CI for mean difference: (0.012, 0.076)

T-Test of mean difference = 0 (vs not = 0): T-value = 5.07  P-value = 0.000

### Medium Dose

**Paired T-Test and CI: Day 16, Day 1**

#### Env Group

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 16</td>
<td>16</td>
<td>0.133</td>
<td>0.132</td>
<td>0.042</td>
</tr>
<tr>
<td>Day 1</td>
<td>16</td>
<td>0.163</td>
<td>0.014</td>
<td>0.008</td>
</tr>
</tbody>
</table>

**Difference** | 16 | 0.030 | 0.048 | 0.004 |

95% CI for mean difference: (0.050, 0.224)

T-Test of mean difference = 0 (vs not = 0): T-value = 3.38  P-value = 0.004

### Medium Dose

**Paired T-Test and CI: Day 16, Day 1**

#### NS1 Group

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 16</td>
<td>16</td>
<td>0.085</td>
<td>0.027</td>
<td>0.019</td>
</tr>
<tr>
<td>Day 1</td>
<td>16</td>
<td>0.047</td>
<td>0.012</td>
<td>0.008</td>
</tr>
</tbody>
</table>

**Difference** | 16 | 0.038 | 0.015 | 0.010 |

95% CI for mean difference: (0.021, 0.075)

T-Test of mean difference = 0 (vs not = 0): T-value = 3.77  P-value = 0.002

### Medium Dose

**Paired T-Test and CI: Day 35, Day 1**

#### N Group

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 35</td>
<td>16</td>
<td>0.035</td>
<td>0.024</td>
<td>0.007</td>
</tr>
<tr>
<td>Day 1</td>
<td>16</td>
<td>0.017</td>
<td>0.007</td>
<td>0.003</td>
</tr>
</tbody>
</table>

**Difference** | 16 | 0.019 | 0.017 | 0.004 |

95% CI for mean difference: (0.004, 0.023)

T-Test of mean difference = 0 (vs not = 0): T-value = 3.14  P-value = 0.003

### Medium Dose

**Paired T-Test and CI: Day 35, Day 1**

#### MT Group

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 35</td>
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<td>0.011</td>
<td>0.008</td>
<td>0.005</td>
</tr>
<tr>
<td>Day 1</td>
<td>16</td>
<td>0.008</td>
<td>0.004</td>
<td>0.003</td>
</tr>
</tbody>
</table>

**Difference** | 16 | 0.003 | 0.004 | 0.002 |

95% CI for mean difference: (0.009, 0.024)

T-Test of mean difference = 0 (vs not = 0): T-value = 4.64  P-value = 0.000

### Medium Dose

**Paired T-Test and CI: Day 35, Day 1**

#### Env Group

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 35</td>
<td>16</td>
<td>0.072</td>
<td>0.031</td>
<td>0.018</td>
</tr>
<tr>
<td>Day 1</td>
<td>16</td>
<td>0.043</td>
<td>0.022</td>
<td>0.013</td>
</tr>
</tbody>
</table>

**Difference** | 16 | 0.029 | 0.009 | 0.006 |

95% CI for mean difference: (0.000, 0.027)

T-Test of mean difference = 0 (vs not = 0): T-value = 4.65  P-value = 0.000

### Medium Dose

**Paired T-Test and CI: Day 35, Day 1**

#### NS1 Group

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 35</td>
<td>16</td>
<td>0.071</td>
<td>0.031</td>
<td>0.018</td>
</tr>
<tr>
<td>Day 1</td>
<td>16</td>
<td>0.043</td>
<td>0.022</td>
<td>0.013</td>
</tr>
</tbody>
</table>

**Difference** | 16 | 0.029 | 0.009 | 0.006 |

95% CI for mean difference: (0.000, 0.027)

T-Test of mean difference = 0 (vs not = 0): T-value = 4.65  P-value = 0.000
<table>
<thead>
<tr>
<th>Group</th>
<th>T-Test for Mean Difference</th>
<th>T-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Dose (Day 16 vs Day 1)</td>
<td>0.04076, 0.01138</td>
<td>5.20</td>
<td>0.000</td>
</tr>
<tr>
<td>High Dose (Day 16 vs Day 1)</td>
<td>0.0263, 0.0772</td>
<td>4.33</td>
<td>0.001</td>
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<tr>
<td>High Dose (Day 16 vs Day 1)</td>
<td>0.0416, 0.1049</td>
<td>4.94</td>
<td>0.000</td>
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<tr>
<td>High Dose (Day 16 vs Day 1)</td>
<td>0.0420, 0.1020</td>
<td>5.07</td>
<td>0.000</td>
</tr>
<tr>
<td>High Dose (Day 35 vs Day 1)</td>
<td>0.01188, 0.03116</td>
<td>5.34</td>
<td>0.000</td>
</tr>
<tr>
<td>High Dose (Day 35 vs Day 1)</td>
<td>0.01052, 0.02803</td>
<td>4.89</td>
<td>0.000</td>
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<tr>
<td>High Dose (Day 35 vs Day 1)</td>
<td>0.02000, 0.07790</td>
<td>7.33</td>
<td>0.000</td>
</tr>
<tr>
<td>High Dose (Day 35 vs Day 1)</td>
<td>0.01139, 0.03334</td>
<td>7.87</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Appendix H  Output from IFN-γ Assay Analysis, Time Course.

The graphs in this appendix show the results from each run of the R script for data collected from the IFN-γ assay, following initial screening of the data using Flowjo software, for all time points for which data was available. Graphs from day 10 and day 13 in main text.
CD4 Activated T Cells – 10 Days Post Infection
Generalized linear mixed model fit by the Laplace approximation
Formula: TR ~ Vaccine * ReStim + (1 + ReStim | Bird)
Data: CD4
AIC  BIC logLik deviance
299.1 329.5 -131.6 263.1
Random effects:
  Groups Name        Variance Std.Dev. Corr

Bird (Intercept) 1.408809 1.18693
ReStimMT 0.067968 0.26071 -1.000
ReStimE3 0.149928 0.38721 -0.508 0.508
ReStimWN 0.441261 0.66427 -0.230 0.230 0.955

Number of obs: 40, groups: Bird, 5

Fixed effects:

| Estimate | Std. Error | z value | Pr(>|z|) |
|----------|------------|---------|----------|
| (Intercept) | 2.4346 | 0.6958 | 3.499 | 0.000467 *** |
| VaccineE | 1.4941 | 1.0932 | 1.367 | 0.171711 |
| ReStimMT | -0.2211 | 0.2312 | -0.956 | 0.338847 |
| ReStimE3 | 0.5110 | 0.2706 | 1.888 | 0.058988 . |
| ReStimWN | 0.3994 | 0.4144 | 0.964 | 0.335122 |
| VaccineE:ReStimMT | 0.6805 | 0.3014 | 2.258 | 0.023945 * |
| VaccineE:ReStimE3 | 1.0849 | 0.3947 | 2.748 | 0.005987 ** |
| VaccineE:ReStimWN | -1.2584 | 0.6450 | -1.951 | 0.051054 . |

---

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:

<table>
<thead>
<tr>
<th></th>
<th>VaccineE</th>
<th>ReStimMT</th>
<th>ReStimE3</th>
<th>ReStimWN</th>
</tr>
</thead>
<tbody>
<tr>
<td>VaccineE</td>
<td>-0.636</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ReStimMT</td>
<td>-0.727</td>
<td>0.463</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ReStimE3</td>
<td>-0.491</td>
<td>0.312</td>
<td>0.495</td>
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<tr>
<td>ReStimWN</td>
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<td>0.165</td>
<td>0.283</td>
<td>0.862</td>
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<tr>
<td>VaccineE:ReStimMT</td>
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<td>-0.834</td>
<td>-0.767</td>
<td>-0.380</td>
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<tr>
<td>VaccineE:ReStimE3</td>
<td>0.336</td>
<td>-0.499</td>
<td>-0.339</td>
<td>-0.686</td>
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<td>VaccineE:ReStimWN</td>
<td>0.167</td>
<td>-0.248</td>
<td>-0.182</td>
<td>-0.554</td>
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</table>

CD8 Activated T Cells – 10 Days Post Infection

Generalized linear mixed model fit by the Laplace approximation

Formula: TR ~ Vaccine * ReStim + (1 + ReStim | Bird)

Data: CD8

AIC 378.7  BIC 412.4  logLik -171.3  deviance 342.7

Random effects:

<table>
<thead>
<tr>
<th>Groups</th>
<th>Name</th>
<th>Variance</th>
<th>Std.Dev.</th>
<th>Corr</th>
</tr>
</thead>
<tbody>
<tr>
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<td>ReStimMT</td>
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<td>ReStimE3</td>
<td>0.849840</td>
<td>0.92187</td>
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<tr>
<td></td>
<td>ReStimWN</td>
<td>0.567312</td>
<td>0.75320</td>
<td>-0.921</td>
</tr>
</tbody>
</table>

Number of obs: 48, groups: Bird, 6

Fixed effects:

| Estimate | Std. Error | z value | Pr(>|z|) |
|----------|------------|---------|----------|
| (Intercept) | 2.0342 | 0.6107 | 3.331 | 0.000865 *** |
| VaccineE | 0.1374 | 0.8620 | 0.159 | 0.873341 |
| ReStimMT | -0.5831 | 0.2850 | -2.046 | 0.040773 * |
| ReStimE3 | 0.1859 | 0.5702 | 0.326 | 0.744475 |
| ReStimWN | 1.0971 | 0.4691 | 2.339 | 0.019358 * |
| VaccineE:ReStimMT | 1.2873 | 0.3621 | 3.555 | 0.000378 *** |
| VaccineE:ReStimE3 | 1.3906 | 0.7983 | 1.742 | 0.081493 . |
| VaccineE:ReStimWN | -0.1938 | 0.6628 | -0.292 | 0.769999 |

---

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Correlation of Fixed Effects:

<table>
<thead>
<tr>
<th></th>
<th>VaccineE</th>
<th>ReStimMT</th>
<th>ReStimE3</th>
<th>ReStimWN</th>
<th>VaccineE:ReStimMT</th>
<th>VaccineE:ReStimE3</th>
<th>VaccineE:ReStimWN</th>
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<td></td>
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<td>ReStimWN</td>
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<td>0.644</td>
<td>0.499</td>
<td>0.618</td>
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<tr>
<td>VaccineE:ReStimMT</td>
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<td>-0.787</td>
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<td>VaccineE:ReStimE3</td>
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<td>-0.714</td>
<td>-0.441</td>
<td>0.103</td>
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<tr>
<td>VaccineE:ReStimWN</td>
<td>0.643</td>
<td>-0.908</td>
<td>-0.353</td>
<td>-0.437</td>
<td>-0.708</td>
<td>0.544</td>
<td>0.621</td>
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</table>

CD4 All T Cells – 10 Days Post Infection

Generalized linear mixed model fit by the Laplace approximation

Formula: TR + TNR ~ Vaccine * ReStim + (1 + ReStim | Bird)

Data: CD4

AIC  BIC logLik deviance
5821 5852  -2893     5785

Random effects:

<table>
<thead>
<tr>
<th>Groups</th>
<th>Name</th>
<th>Variance</th>
<th>Std.Dev.</th>
<th>Corr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bird</td>
<td>(Intercept)</td>
<td>0.894925</td>
<td>0.94600</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ReStimMT</td>
<td>0.231848</td>
<td>0.48151</td>
<td>-0.113</td>
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<tr>
<td></td>
<td>ReStimE3</td>
<td>0.041652</td>
<td>0.20409</td>
<td>-0.736 -0.365</td>
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<tr>
<td></td>
<td>ReStimWN</td>
<td>0.123705</td>
<td>0.35172</td>
<td>-0.600 -0.636 0.949</td>
</tr>
</tbody>
</table>

Number of obs: 40, groups: Bird, 5

Fixed effects:

| Estimate | Std. Error | z value | Pr(>|z|) |
|----------|------------|---------|----------|
| (Intercept) | 7.17990 | 0.54630 | 13.143 | <2e-16 *** |
| VaccineE | 0.19913 | 0.86385 | 0.231 | 0.8177 |
| ReStimMT | -0.41265 | 0.27871 | -1.481 | 0.1387 |
| ReStimE3 | 0.09668 | 0.11888 | 0.813 | 0.4160 |
| ReStimWN | 0.37562 | 0.20361 | 1.845 | 0.0651 |

VaccineE:ReStimMT | 0.69079 | 0.44061 | 1.569 | 0.1169 |
VaccineE:ReStimE3 | 0.42432 | 0.18811 | 2.256 | 0.0241 * |
VaccineE:ReStimWN | -0.35573 | 0.32221 | -1.104 | 0.2696 |

---

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:

<table>
<thead>
<tr>
<th></th>
<th>VaccineE</th>
<th>ReStimMT</th>
<th>ReStimE3</th>
<th>ReStimWN</th>
<th>VaccineE:ReStimMT</th>
<th>VaccineE:ReStimE3</th>
<th>VaccineE:ReStimWN</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>ReStimWN</td>
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<td>-0.630</td>
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<td>VaccineE:ReStimMT</td>
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<td>-0.114</td>
<td>-0.633</td>
<td>0.226</td>
<td>0.399</td>
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<tr>
<td>VaccineE:ReStimE3</td>
<td>0.462</td>
<td>-0.732</td>
<td>0.226</td>
<td>-0.632</td>
<td>-0.596</td>
<td>-0.355</td>
<td></td>
</tr>
<tr>
<td>VaccineE:ReStimWN</td>
<td>0.379</td>
<td>-0.600</td>
<td>0.398</td>
<td>-0.596</td>
<td>-0.632</td>
<td>-0.629</td>
<td>0.945</td>
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</tbody>
</table>

CD8 All T Cells – 10 Days Post Infection

Generalized linear mixed model fit by the Laplace approximation

Formula: TR + TNR ~ Vaccine * ReStim + (1 + ReStim | Bird)

Data: CD8

AIC  BIC logLik deviance
6193 6227  -3079     6157
Random effects:

<table>
<thead>
<tr>
<th>Groups</th>
<th>Name</th>
<th>Variance</th>
<th>Std.Dev.</th>
<th>Corr</th>
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</thead>
<tbody>
<tr>
<td>Bird</td>
<td>(Intercept)</td>
<td>0.354042</td>
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<tr>
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<td>ReStimMT</td>
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<td>0.039747</td>
<td>0.19937</td>
<td>-0.257</td>
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Number of obs: 48, groups: Bird, 6

Fixed effects:

|                         | Estimate | Std. Error | z value | Pr(>|z|) |
|-------------------------|----------|------------|---------|----------|
| (Intercept)             | 6.19750  | 0.34408    | 18.012  | < 2e-16 *** |
| VaccineE                | 0.50906  | 0.48646    | 1.046   | 0.295    |
| ReStimMT                | -0.02338 | 0.25970    | -0.090  | 0.928    |
| ReStimE3                | 0.34121  | 0.30939    | 1.103   | 0.270    |
| ReStimWN                | 0.86884  | 0.11737    | 7.402   | 1.34e-13 *** |
| VaccineE:ReStimMT       | 0.35580  | 0.36683    | 0.970   | 0.332    |
| VaccineE:ReStimE3       | 0.69713  | 0.43723    | 1.594   | 0.111    |
| VaccineE:ReStimWN       | -0.27220 | 0.16552    | -1.645  | 0.100    |

---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:

<table>
<thead>
<tr>
<th></th>
<th>(Intr)</th>
<th>VaccineE</th>
<th>ReStimMT</th>
<th>ReStimE3</th>
<th>ReStimWN</th>
<th>VaccineE:ReStimMT</th>
<th>VaccineE:ReStimE3</th>
<th>VaccineE:ReStimWN</th>
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</thead>
<tbody>
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<td>0.092</td>
<td>-0.159</td>
<td>-0.708</td>
<td>-0.607</td>
<td>0.224</td>
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<tr>
<td>VaccineE:ReStimWN</td>
<td>0.185</td>
<td>-0.260</td>
<td>0.002</td>
<td>-0.608</td>
<td>-0.709</td>
<td>-0.006</td>
<td>0.859</td>
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